Improved diagnosis of prosthetic joint infection using a marine nuclease

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Abstract

Prosthetic joint infection is devastating for the patient and costly to the healthcare provider. Key to the treatment of prosthetic joint infection is the identification of the causative organism. This can be challenging since biofilm encapsulated bacteria can establish themselves on the implant surface avoiding standard microbiological detection. Focus, therefore has fallen on increasing the yield of bacteria from the explanted implant surface to make a diagnosis. The marine nuclease NucB is a novel enzyme capable of digesting eDNA a component of the biofilm matrix, utilised by causal organisms in prosthetic joint infection. Use of NucB results in the release of bacterial cells from the biofilm, allowing them to be brought into planktonic suspension, thereby potentially aiding in their identification and the diagnosis of infection. To evaluate the potential of NucB to improve the diagnosis of prosthetic joint infection, a biofilm model was established initially using a microtiter plate system, then subsequently a series of simulated prosthetic implant surfaces using isolates of *Staphylococcus epidermidis* and *Staphylococcus aureus*. Finally, the ability of NucB to release bacteria from biofilms formed on explanted joint replacements from patients undergoing revision arthroplasty surgery was evaluated. Results demonstrated that NucB produced a significant increase in the number of bacteria released when compared to controls and was, for the majority of isolates evaluated, comparable to sonication, another technique used to increase the yield of bacteria from prosthetic implant surfaces. Results from the evaluation of the explanted joint replacements demonstrated that NucB was comparable to sonication and also compatible with standard microbiological processing systems within the UK National Health Service. This has work demonstrated that NucB is effective at increasing the bacterial yield from biofilm encapsulated bacteria and has the potential to improve the accuracy of diagnosis in prosthetic joint infection, thus improving patient care.

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Abbreviations

ASA	American society of anesthesiologists
AO	Arbeitsgemeinschaft für osteosynthesefragen
CRP	C-reactive protein
CT	Calf thymus
CDC	Centers for disease control and infection
CLSM	Confocal laser scanning microscopy
Co-Cr	Cobalt-chrome
CFU	Colony forming units
CV	Crystal violet
DAIR	Debridement, antibiotics and implant retention
rhDNase	recombinant human deoxyribonuclease
DMARDs	Disease modifying anti-rheumatic drugs
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
eDNA	Extracellular deoxyribonucleic acid
ESR	Erythrocyte sedimentation rate
EDTA	Ethylenediaminetetraacetic acid
GCP	Good clinical practice
HMWPE	High molecular weight polyethylene
ISDA	Infectious diseases society of America
MRSA	Methicillin-resistant *Staphylococcus aureus*
MIC	Minimal inhibitory concentration
MSIS	Musculoskeletal infection society
NAC	N-Acetylcysteine
NCTC	National collection of type cultures
NIH	National institutes of health
NETs	Neutrophil extracellular traps
NUPPA	Newcastle University Protein and Proteome Analysis
OD	Optical density
PROMS	Patient reported outcome measures
PBS	Phosphate buffer saline
PMMA	Polymethyl methacrylate

PMN%	Polymorphonuclear neutrophil percentage
PJI	Prosthetic joint infection
Ra	Average roughness
Rq	Root mean square roughness
MSSA	Methicillin sensitive *Staphylococcus aureus*
THR	Total hip replacement
TKR	Total knee replacement
TBS	Tryptic soy broth
SMI	Standards for microbiology investigations
UHWPE	Ultra-high molecular weight polyethylene
WBC	White blood cell
WHO	World health organisation

1. Chapter 1. Introduction

1.1. Osteoarthritis

'Life is movement, movement is life'
AO foundation

The ability to undertake pain free movement is seen as an entitlement to younger generations. However, in later years the development of joint disease can limit activity, reduce mobility and affect the quality of life. Thus, the Swiss Orthopaedic Arbeitsgemeinschaft für Osteosynthesefragen (AO) foundation in 1958 adopted the motto 'Life is movement, movement is life' underlining the importance of mobility. While there are many causes of limited mobility one of the most common is osteoarthritis.

Osteoarthritis is a debilitating and progressive disease affecting synovial musculoskeletal joints. The disease can affect almost all joints. However, those most commonly affected are hips, knees, ankles, hands and spine (Neogi and Zhang, 2013). The World Health Organisation (WHO) characterises osteoarthritis as loss of articular cartilage and progressive joint destruction. Although osteoarthritis primarily affects the joint articulation the disease pathology affects 9the surrounding tissues of the synovial joint including the synovium, ligaments, joint capsule and the muscles crossing the joint (Racine and Aaron, 2013). While the main trigger for the imbalance in the mechanical and biochemical properties within the joint that result in the development of osteoarthritis remain unclear, several risk factors for the development of the disease have been identified. These include age, although sex, obesity, previous trauma, genetics and biomechanical alignment have all been demonstrated to contribute a degree of risk (Blagojevic et al., 2010, Palazzo et al., 2016). The ageing population and increased number of risk factors particularly obesity and sedentary lifestyle suggest that the incidence of osteoarthritis will increase over the coming decades (Zhang and Jordan, 2010, Birtwhistle et al., 2015, Culliford et al., 2015)

The progressive nature of osteoarthritis means that as symptoms worsen patients seek referral to health care providers. Treatment options include non-operative management, including physiotherapy, with the aim of improving muscle function and stability across the joint, analgesia, titrated to pain following a recognised analgesic ladder. In addition, lifestyle modifications including altering roles at work and adaptations to accommodation such as grab rails and stair lifts. These changes are often able to

provide a period of symptomatic improvement meaning that surgery may be delayed or not required (Filardo et al., 2016). However, given the nature of osteoarthritis, a significant portion of patients will fail to achieve symptomatic control without surgery. Having exhausted conservative treatment options for patients with symptomatic osteoarthritis of the hips and knees a number of patients will progress to total joint arthroplasty.

1.2. Total joint replacement arthroplasty

Total joint replacement arthroplasty involves the surgical excision of the degenerative joint surface and replacement with a synthetic material normally modelled on the excised joint. The aim is to replace the degenerative joint providing pain relief and improving function (Hussain et al., 2016). Modern total joint arthroplasty provides an effective management tool for osteoarthritis of the hips and knees. Surgical management has advanced significantly with the current design of the total hip replacement (THR) developed by Professor Sir John Charnley in the 1970's remaining the 'gold standard' to which current joint replacement implants are compared (Charnley, 1970, Charnley and Feagin, 1973). While the majority of initial surgical interest focused on the total hip replacement, the development of the total knee replacement (TKR) followed a similar path. By the 1970s, designs were developed for the early cruciate retaining total knee replacements that resemble the modern TKR used today (Yamamoto, 1979).

Patient related outcome measures have shown that hip and knee replacements are effective at reducing pain and improving mobility (HSC, 2016). As a consequence, the number of total joint replacements has been increasing steadily. In 2017 there were around 67,000 total hip replacements and 66,000 total knee replacements in the UK (NJR, 2017) resulting from an ageing population, increased life expectancy with the greater expectation of pain-free mobility in later life. Associated with an increase in the prevalence of known risk factors such as obesity, the incidence of osteoarthritis and therefore the expected demand for total joint arthroplasty in the UK and the developing world is expected to increase in the decades to come (Neogi and Zhang, 2013, Johnson and Hunter, 2014, Pilz et al., 2018). Thankfully, following decades of development, surgical management with hip and knee total joint arthroplasty is an effective treatment for symptomatic osteoarthritis.

1.3 Prosthetic joint infection (PJI)

While the majority of patients will have an excellent outcome from joint replacement, a number of patients will experience complications, of these prosthetic joint infection (PJI) is one of the most devastating. Prosthetic joint infection is the clinical manifestation from the immune response due to microorganisms within the replaced joint (Zimmerli, 2014). The manifestation and development of PJI depends on a number of factors including the type of joint replaced, pathogenesis of the causal organism and systemic host factors of the patient (Tande and Patel, 2014).

Rates of PJI are difficult to measure primarily due to variations in the quality of data collected (Tanner et al., 2013). Infection rates remain low affecting around 1 % of THR and 2 % of TKRs (Pulido et al., 2008). This increases to 5-12% for revision surgery (Mortazavi et al., 2010). The greatest risk period for the development of infection is up to 2 years following the index surgery where around 60-70% of infections occur (Pulido et al., 2008, Kurtz et al., 2010). Several risk factors are recognised for the development of PJI, Zhu et al in a systematic review and meta-analysis of 14 studies identified 31 potential risk factors for PJI (Zhu et al., 2015). Of these diabetes mellitus and rheumatoid disease were two of the most strongly associated systemic conditions increasing the risk of PJI with a combined odds ratio of the studies of 1.26 and 1.41 respectively (95% Confidence interval). The effect of diabetes mellitus is multifactorial with established microvascular disease affecting wound healing leading to wound breakdown and subsequent PJI (Chun et al., 2014, Liu et al., 2017b). Secondarily poorly controlled perioperative hyperglycaemia has been demonstrated to dampen the immune response resulting in impaired immune function and an increase in the risk of PJI (Mraovic et al., 2011, Maradit Kremers et al., 2015). Rheumatoid arthritis is frequently managed with the use of disease modifying anti-rheumatic drugs (DMARDs) that modify the immune response to improve symptoms. As a consequence of the immune-modulation, there is an increased risk of PJI after joint arthroplasty (Kawakami et al., 2010). These systemic risk factors for PJI cannot be avoided, therefore focus has fallen on the optimisation of patient factors prior to surgery. For example, improving hyperglycaemic diabetic control prior to surgery (Agos et al., 2014, Yang et al., 2017). For rheumatoid patients, withholding of certain DMARDS prior to joint arthroplasty is recommended by the British Society for Rheumatology (Ding et al., 2010).

As well as systemic conditions there are several perioperative risk factors that have been demonstrated to increase the incidence of PJI. Perioperative transfusion via its immunomodulatory effect has been demonstrated to increase the risk of PJI (Munoz et

al., 2005) as well as increased length of surgery has been demonstrated to increase infection risk. This may be a direct effect from a prolonged exposure of the joint or a surrogate marker for the complexity caused from another known risk factor such as obesity. Due to these confounding factors, attempts have been undertaken to develop a composite risk score. The National Nosocomial Infection Surveillance System risk score aims to aggregate the number and the influence of multiple risk factors both systemic and perioperative (Berbari et al., 1998, Aslam et al., 2010). A score of two or more has been associated with an increased risk of PJI (Moran et al., 2010) however, it is not frequently utilised in the UK. While several attempts have been made to reduce infection rates, unfortunately some patients will still develop PJI following surgery and once established, it is helpful to further classify the type of PJI to guide management options.

1.4 Classification of prosthetic joint infection

To aid in the discussion, comparison and management of PJI several classification systems have been developed. The most simple but widely accepted of these was adopted by Zimmerli who divided infection into early, delayed and late categories in relation to the time of infection since surgery (Zimmerli et al., 2004).

Early	within 3 months of implantation
Delayed	3–12 months after implantation
Late	more than 12 months after implantation

Early infections are thought to start at the time of the procedure via direct inoculation into the joint by a virulent organism. These patients present with sudden onset joint pain, erythema and warmth at the implant site within the first few weeks of surgery. Delayed presentation is again related to direct inoculation but with a less virulent organism producing a lower grade infection with less systemic effects and with more subtle signs and symptoms such as persistent joint pain and intermittent joint swelling. Late infections occur via haematogenous spread from established bacterial infection distant from the joint. Haematogenous prosthetic joint infection is unrelated to the surgery and involves colonisation of the joint from another source of infection seen most commonly within 2 years of initial surgery (Bozic et al., 2010). Sources of infection commonly include chest, genitourinary tract or dental surgery (Chen et al., 2014).

Tsukayama *et al* divided PJI into four groups not solely based on the time since operation but also on the presumed mode of infection for hip and subsequently knee arthroplasty infections (Tsukayama et al., 1996, Tsukayama et al., 2003). Types 2-4 included similar time scales for infection being early, delayed or late as well as a type 1 infection relating to an unexpected positive culture result from a presumed non-infected revision due to aseptic loosening. A further classification system by McPherson *et al* included features about the host as well as the mode of infection (McPherson et al., 2002). Infections are graded as type I, II, or IV, grade 2-4 being early postoperative infection, haematogenous infection, and late chronic of the Tsukayama classification as well as the systemic host status graded as A – Uncompromised, B – Compromised and C - Significant compromise. The limb is also graded with regards the soft tissue envelope associated with the implant that can be; 1 – uncompromised , 2 - compromised, or 3 significantly compromised (McPherson et al., 1999, McPherson et al., 2002). Development of staging systems in PJI has helped both in the clinical management and in research, improving comparison between studies. These systems have improved clinical care resulting in a more individualised approach as well as the development of treatment strategies and prognostic information for these complex cases of PJI.

1.5 Management of Prosthetic joint infection

Successful management of PJI requires surgical intervention and antibiotic treatment and is best managed by a multidisciplinary team. Tande and Patel in their review article for prosthetic joint infection summarises effectively the goals of surgery (Tande and Patel, 2014) which are; “To eradicate the infection, restore pain free function of the infected joint, and minimise PJI related morbidity and mortality for the patient.” Treatment options vary and are often guided by the classification of the PJI. Debridement, antibiotics and implant retention (DAIR) is often attempted in the presence of early infection with well-fixed implants (Lora-Tamayo et al., 2013) (Bergkvist et al., 2016) The joint replacement is washed and debrided with exchange of the modular components that can easily be removed at the time of surgery. Subsequently the patient is treated with antibiotics for a period of time. This approach is often seen as favourable, being less destructive than a full revision. The outcomes from DAIR are dependent on several factors such as the antibiotic sensitivity of organism and host factors including liver cirrhosis predicting failure (Tornero et al., 2014a). Thorough debridement is crucial with novel techniques such as the use of acetic

acid being evaluated to improve outcomes (Williams et al., 2017). Nevertheless, the outcomes following DAIR are variable with inconsistencies in the techniques and indications. A recent retrospective review of 67 consecutive patients presenting with acute PJI following TKR demonstrated success of DAIR in 69 % of all patients. However when the resistant organisms were excluded such as methicillin-resistant *Staphylococcus aureus* (MRSA) and those with *Pseudomonas aeruginosa* the success rate improved to 85% (Duque et al., 2017). For certain patients with PJI DAIR is not an option and they often require revision of the joint. This involves the complete removal of the implant, debridement and implantation of a new joint. This can be undertaken as a single stage procedure or with a time interval between removal of the infected joint and implantation, also known as a two-stage procedure. A systematic review demonstrated no clear advantage over either approach (Beswick et al., 2012). However, a prospective randomised controlled trial currently recruiting will enable the comparison of both techniques and help guide future management (Strange et al., 2016). Occasionally complex PJI is not amenable to further revision arthroplasty, and salvage procedures such as knee fusion in selected patients has achieved acceptable function (Razii et al., 2016).

The complexity of management choices for surgical intervention is challenging and due to ongoing clinical research, is often evolving. This is summarised below in Figure one which shows the algorithm used within the Bone Infection Unit at Oxford BIUO– this provides insight into the approach undertaken within the UK (Moran et al., 2010).

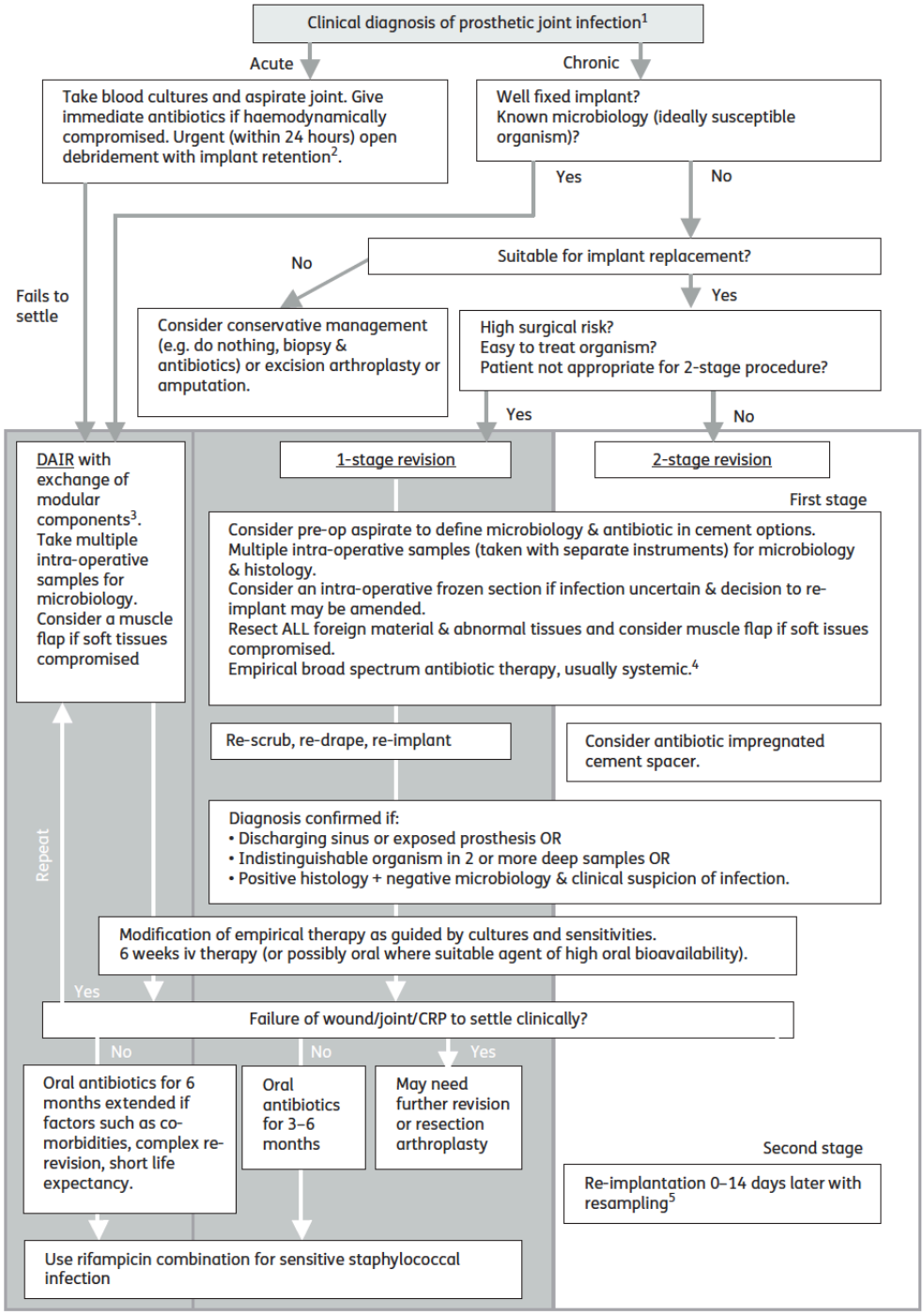


Figure 1. Flowchart summarising the selection of an appropriate management strategy for an infected prosthetic joint, Nuffield Orthopaedic Centre, Oxford, UK.

No matter which surgical strategy is undertaken the goals of surgical intervention remain the same. The debridement of infected tissue, explantation or exchange of modular components - depending upon the planned strategy - and postoperative antimicrobial therapy to target any remaining infection.

1.6 Increasing challenge and outcomes of prosthetic joint infection

While thankfully rare, the incidence of PJI is predicted to increase (Tande et al., 2014), this is related to a number of factors. Increasing demand for prosthetic joint replacement associated with an increasing life expectancy will play a significant role. The last 15 years have seen an increasing number of patients undergoing joint replacement and this trend is set to continue for hip and knee replacements with two studies predicting significant increases into 2030-5 in the UK and USA respectively (Kurtz et al., 2007, Culliford et al., 2015). Prosthetic replacements for other joints such as ankle replacements are less common. The National Joint Registry UK however, has demonstrated the practice is increasing rapidly (NJR, 2017). The life expectancy increase means that patients undergoing joint replacement will have increased residency of their joint. This increases the chance of haematogenous spread with late PJI set at 1% per year (Huotari et al., 2015). Increasing life expectancy will therefore correlate with increased incidence of joint infection. Antibiotic resistance is also increasing with WHO and the United Nations both identifying emerging resistance as one of the greatest challenges facing modern day healthcare (The Lancet Gastroenterology Hepatology, 2016, Castro-Sánchez et al., 2016). Obesity as mentioned previously is also a known risk factor for infection, nationally the rates of obesity are increasing (Arroyo-Johnson and Mincey, 2016). As a consequence, rates of infection secondary to obesity are set to increase. Finally, clinicians are getting better at diagnosing infection in part due to their increased awareness of the underlying microbiology and secondarily in recent years by the development of new biomarkers that may prove more sensitive and specific for PJI (Deirmengian et al., 2014). Overall these factors will likely result in the continued rise in prevalence of PJI as demonstrated by a recent analysis using National Joint Registry data by Lenguerrand et al. Using linked data from 2005-13 analysis demonstrated that the prevalence of revision due to PJI in the three months following primary hip arthroplasty increased 2.3 fold (95% CI 1.3 to 4.1) and 3.0-fold (95% CI 1.1 to 8.5) following revision hip arthroplasty (Lenguerrand et al., 2017).

Successfully managed patients with PJI and their clinicians face further challenges following the eradication of infection. Quality of Life measures are poorer compared to matched patients having undergone the same surgery without associated PJI (Helwig et al., 2014). Patients undergoing revision surgery for PJI have an increased rate of 90-day mortality compared to those revised for aseptic loosening (Zmistowski et al., 2013). PJI associated mortality is often underestimated with rates worse than cancer diagnosis

for prostate and breast. (Berend et al., 2013). As well as being devastating for the patient, the predicted increase in cases means PJI represents a costly and avoidable expense to healthcare providers. With patients requiring prolonged periods of inpatient hospital care, multiple hospital admissions and, if offered revision surgery, costly new revision implants. In the USA by 2020 the cost burden due to PJI is expected to be $1.62 billion per annum (Kurtz et al., 2012).

In summary, prosthetic joint infection is a problematic complication for the patient almost always requiring further surgery. Further surgery can be extensive, repetitive and is not without complications. Depending upon treatment options many patients have prolonged periods without a functioning joint and associated immobility. They may require long periods of antibiotic therapy in an attempt to clear infection. Incidence of PJI is increasing, costly and challenging problem for healthcare providers. For the patient PJI often represents a life-changing event associated with long term loss of expected function and increased risk to life. The aims of management are to provide a painless, functional and infection free joint replacement. Key to this is the diagnosis of infection and the identification of the causative organism.

1.7 Diagnosis of prosthetic joint infection

The approach to PJI diagnosis is to firstly confirm if the joint is infected, secondly identify the causal organism and subsequent antimicrobial sensitivity to guide further treatment. Currently there is no single diagnostic test that is 100% accurate for PJI diagnosis. Diagnosis requires a high index of clinical suspicion starting with initial history and examination, plain film radiographs, laboratory results from peripheral blood and synovial fluid from joint aspiration to attempt the isolation of the causative organism (Osmon et al., 2013a). Since there is no single test that is able to confirm PJI, several diagnostic criteria have been established. The Infectious Diseases Society of America (IDSA) and the Musculoskeletal Infection Society (MSIS) both based in the USA, have published criteria for the diagnosis of PJI (Parvizi et al., 2011b, Osmon et al., 2013a). Within England, Public Health England (PHE) collect data on the reporting of surgical site infection including PJI using the Centre for Disease Control (CDC) definition (Horan et al., 1992). This is non-specific for PJI and therefore the MSIS criteria have frequently been adopted. The IDSA divide their classification into major and minor (Della Valle et al., 2010, Osmon et al., 2013b). Major criteria for joint infection of which one or other is considered diagnostic are either:

- Two positive periprosthetic cultures with identical organisms
- A sinus tract communicating with the joint.

Minor criteria where three conditions have to be met to be considered an infection:

- Elevated serum C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)
- Elevated synovial fluid white blood cell (WBC) count OR ++change on leukocyte esterase test strip
- Elevated synovial fluid polymorphonuclear neutrophil percentage (PMN%) - Positive histological analysis of periprosthetic tissue
- A single positive culture

The MSIS also have major and minor criteria:

Major Criteria:

- Sinus tract communicating with the prosthesis
- An identical pathogen isolated by culture from at least two separate tissue or fluid samples obtained from the affected prosthetic joint

Minor Criteria:

- Elevated serum erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) concentration
- Elevated synovial fluid leukocyte count
- Elevated synovial fluid neutrophil percentage (PMN%)
- Presence of purulence in the affected joint
- Isolation of a microorganism in one culture of periprosthetic tissue or fluid
- Greater than five neutrophils per high-power field in five fields observed from histologic analysis of periprosthetic tissue at x400 magnification.

The development of these diagnostic criteria has aided both the clinical management and research into PJI. Although there are several classification systems available for use they are similar in their inclusion criteria, with work demonstrating high concordance in PJI diagnosis (Melendez et al., 2013). Whichever system is utilised both systems place heavy credence on intraoperative culture techniques. Using the above

guidance whichever criteria are used PJI can be easy to diagnose. This is more often the case in early infections or when the patient presents systemically unwell with fever. However, identification of the organism can often be problematic or impossible especially if the presentation is related to a chronic low-grade infection with no systemic symptoms or if antibiotics have been prescribed prior to investigations, since antibiotics kill the more easy to culture planktonic bacteria, reducing the likelihood of recovering viable bacterial cells from tissues samples or synovial fluid aspiration (Osmon et al., 2013a). These patients therefore represent a significant challenge. Incorrectly assuming that either the revision joint surgery is not infected or that the infection has been successfully treated as part of a two stage procedure meaning that patients may receive inadequate and suboptimal treatment, increasing their risk of subsequent failure and further surgery (Nelson et al., 2014, Jacobs et al., 2017) This group of patients are designated as having culture negative prosthetic joint infections (Berbari et al., 2007). When faced with the possibility of culture negative prosthetic joint infection by definition, the challenge of identification of the causal organism is increased or impossible. This phenomenon of a culture negative joint infection can be explained in the context of a biofilm-associated infection.

1.8 Biofilms and their role in prosthetic joint infection

Bacteria are known to exist in two distinct forms, planktonic and sessile (McDougald et al., 2012). Planktonic bacteria lead an individualised existence and are motile, fast dividing and usually promote a potent immune response (Hall-Stoodley et al., 2004). Planktonic bacteria subsequently received much of the early attention of microbiologists, being responsible for epidemics that drove the development of the modern antibiotic era. Conversely, sessile bacteria live within a community, share resources, communicate and are protected within a matrix known as a biofilm. William Costerton and colleagues were the first to recognise the association of chronic infections and biofilm encapsulated sessile bacteria (Costerton et al., 1978) describing biofilms as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface". Biofilms therefore are a community of bacteria and/or fungi surrounded by a slime like matrix produced by the organisms that offers a protective environment within which cells can resist antibiotic concentrations up to 1000 times the Minimal Inhibitory Concentration (MIC) (Donlan, 2000). Biofilms are found in around 65% of clinical infections in the developed world (Center for Disease Control). The development of biofilms can be divided into four

stages. Initial cell adhesion, aggregation, maturation and subsequent dispersal. Common surfaces used in joint replacement such as cobalt-chrome alloys, titanium, stainless steel, polymethyl-methacrylate (PMMA) cement and high molecular weight polyethylene (HMWPE) have all been shown to act as surfaces for biofilm formation (Rochford et al., 2012). This process occurs quickly with Gristina labelling the process as the "race to the surface" (Gristina, 1987). This represents the short period of time following the implantation of the new prosthetic joint replacement and the colonisation of the implant with either host cells or bacteria that may be present at the time of surgery. Key to understanding the formation of the biofilm is understanding its life cycle.

1.8.1 Life cycle of biofilms

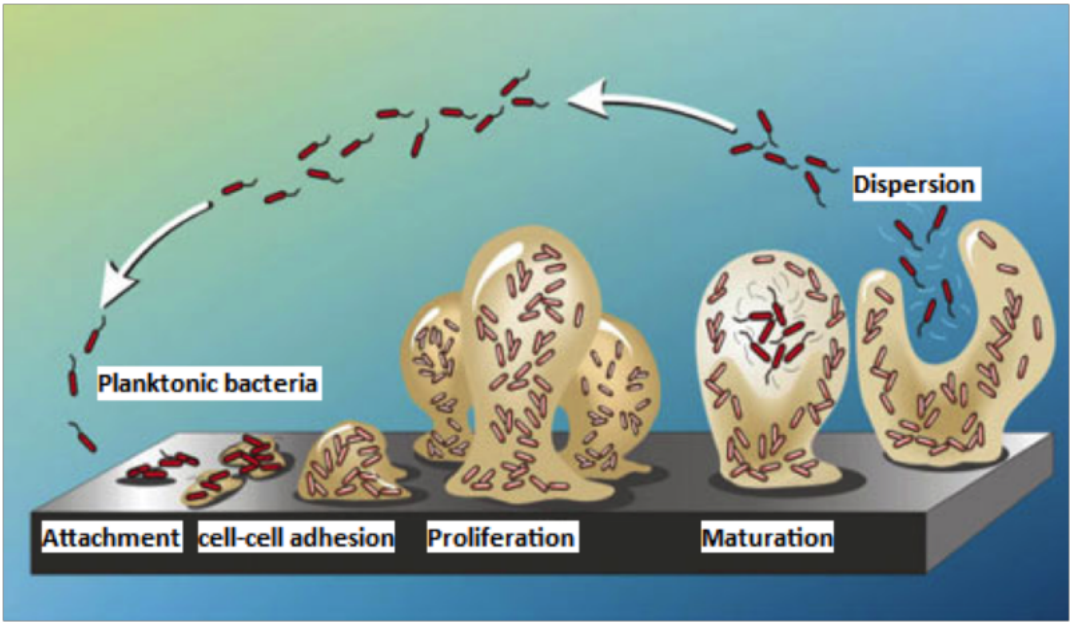


Figure 2. Classic understanding of biofilm formation P. Dirckx, Center for Biofilm Engineering, Montana State University, Bozeman

Individual planktonic bacteria are mobile and migrate to prosthetic surfaces via electrostatic and hydrostatic forces, cell adhesion starts within a few seconds of implantation of the prosthetic joint. Many bacteria are known to produce adherence proteins for initial attachment. The initial attachment is progressive and reversible (Garrett et al., 2008). Cellular aggregation follows with activation of genes prompting the proliferation of extra cellular matrix producing a recognised early biofilm that can colonise a surface (Costerton et al., 1999). Communication via quorum sensing allows the coordinated maturation of the biofilm (Yarwood et al., 2004). Maturation of the

biofilm allows stabilisation of the matrix which develops into a complex 3-D structure. Following maturation, the biofilm can release planktonic cells. These cells are motile and are able to travel to distance sites and adhere to new uncolonised surfaces, repeating the process (Costerton et al., 1999, Stoodley et al., 2002). Bacteria that are able to form biofilm-associated colonies have a number of advantages compared to their planktonic counterparts and it has been suggested that the more sessile nature could represent the preferred form of existence. (McDougald et al., 2012). The biofilm encapsulated organisms benefit from the defensive capabilities of the biofilm matrix with it primarily acting as a barrier protecting the organism from antimicrobial compounds, bactericidal agents, shear stress forces and phagocytic clearance by the host immune system (Jefferson, 2004). The extracellular matrix is also able to pool resources for the encapsulated organisms including carbon, nitrogen and phosphate, all key nutritional components for bacterial cell life (Beveridge et al., 1997). Antibiotic resistance provided by the bacteria is multifactorial. The biofilm matrix can act as a physical diffusion barrier preventing antibiotic penetration to the deeper embedded cells. While the altered environment including a lower pH has also been shown to reduce the effectiveness of certain antibiotics towards bacterial cells within the biofilm (Donlan, 2000). Some of the protection afforded to sessile biofilm encapsulated bacteria relates to its structure.

1.8.2 Structure of biofilms

The structure and composition of the extra cellular matrix varies between bacterial species and within strains of the same species. Even within the same strain, environmental factors including availability of nutrients or shear stresses affect biofilm development (Branda et al., 2005). However, key structural components are similar and include polysaccharides, proteins and extracellular DNA (eDNA) (McDougald et al., 2012).

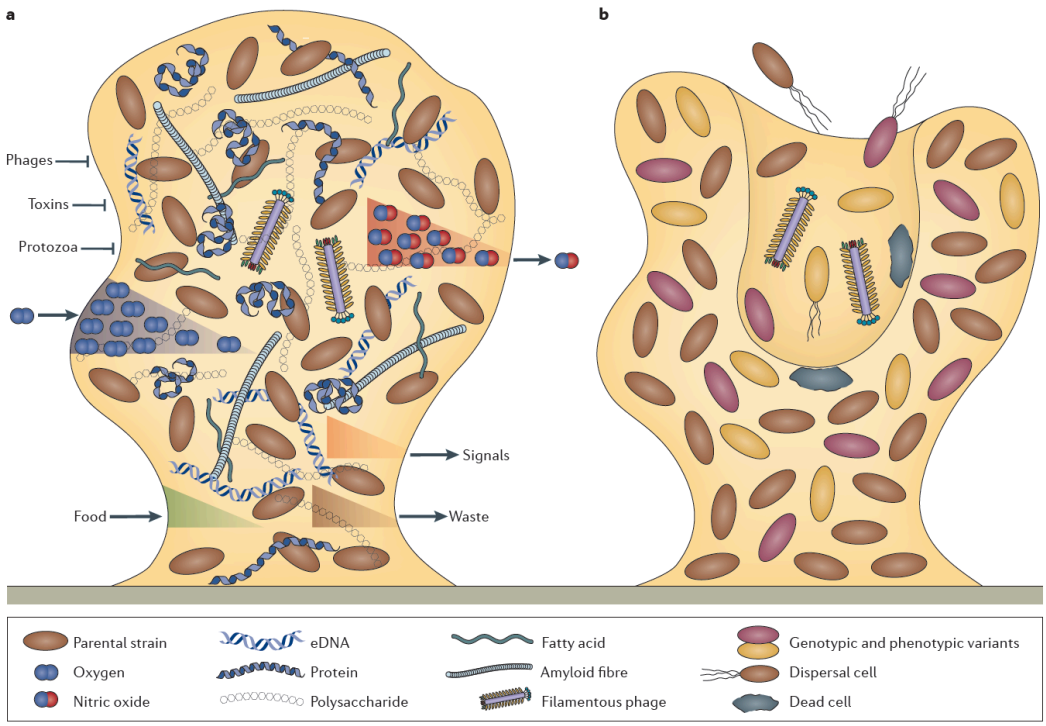


Figure 3. Complex structure of bacterial biofilm matrix demonstrating key components and metabolic activity (McDougald et al., 2012).

1.8.3. Extracellular DNA (eDNA) and its role within the biofilm matrix

The role of Extracellular DNA in biofilms was first noted by Catlin et al in 1956 (Catlin, 1956). This work initially received little attention and eDNA was not studied as much as other better known biofilm constituents such as proteins and polysaccharides. eDNA was thought to be irrelevant, a waste product of the biofilm. However, Whitchurch *et al* showed that DNase-1 was effective at dispersal of *Pseudomonas aeruginosa* biofilms (Whitchurch et al., 2002) and since then numerous papers have highlighted the important role of eDNA in biofilm stability (Montanaro et al., 2011, Jakubovics et al., 2013, Okshevsky and Meyer, 2014, Ibanez de Aldecoa et al., 2017). Varying mechanisms exist for the release of eDNA from the biofilms of different microorganisms. These include active secretion seen in *Pseudomonas aeruginosa* (Schooling et al., 2009) or by cell lysis, as seen in *Staphylococcus epidermidis* and *Staphylococcus aureus.* Work by Qin et al in 2007 noted that the release of *Staphylococcus epidermidis* eDNA was related to autolysin AtlE-mediated cell lysis. AtlE has an autolytic activity resulting in a subpopulation of bacteria lysing, releasing DNA and thus promoting biofilm formation for the remaining bacteria. The DNA released was identical to chromosomal DNA and inactivation of atlE reduced DNA release by more than 90% in the isolates tested (Qin et al., 2007). Other

Autolysins may play a role in cell lysis and the triggering of release is thought to be controlled by quorum sensing within the colony. This fratricidal cell death has been observed in other bacteria including *Staphylococcus aureus* mediated through the activity of murein hydrolases, encoded by the atl and lytM genes producing altruistic cell death (Thomas et al., 2009, Mann et al., 2009).

Within the biofilm eDNA has several important and distinguishable roles (Okshevsky and Meyer, 2015, Vorkapic et al., 2016). One of the primary roles is structural support providing a key component to the mechanical stability of the biofilm. DNA is well suited to this role being a relatively stable molecule with Peterson *et al* highlighting the role of eDNA in the viscoelasticity of the biofilm and the ability of the matrix to withstand mechanical deformation (Peterson et al., 2013). Since biofilm constituents are known to vary with environmental factors, it could be postulated that biofilms within these environments may have a higher eDNA component. Part of the structural role of eDNA is its ability to bind to itself and other constituents of the of extra cellular matrix thus increasing its stability. This includes DNA binding proteins released by bacteria including integration host factor a DNA-binding protein capable of linking eDNA strands (Brockson et al., 2014). EDNA is capable of interacting with polysaccharides, another component of the extra cellular biofilm matrix, increasing its stability (Okshevsky et al., 2014). As well as interacting with other constituents of the biofilm matrix, eDNA can also bind directly with the bacterial cell wall via surface proteins recently demonstrated within meningococcal biofilms (Arenas et al., 2013). As well as its important structural and mechanical properties of eDNA can act as a nutritional store, is a mechanism for genetic exchange and can add to antibiotic resistance by its ability to bind to positively charged antibiotics such as aminoglycosides (Das et al., 2013). eDNA importance has also been highlighted in mixed species biofilms particularly *Staphylococcus epidermidis* and *Candida albicans* (Pammi et al., 2013). Furthermore, bacterial strains with decreased levels of eDNA in their biofilm have been shown to be easily dispersed. Rice et al has demonstrated that a *Staphylococcus aureus* mutation in the cidA gene which codes for a murein hydrolase regulator. Plays an important role in cell lysis and subsequent eDNA release. In mutant strains biofilm had significantly reduced amounts of eDNA and demonstrated weaker biofilm morphology and adherence (Rice et al., 2007). The opposite affect was seen by modulation of the lrgAB operon by Mann et al promoting cell lysis and subsequent biofilm formation in *Staphylococcus aureus* (Mann et al., 2009).

Although recognised many years ago as being part of bacterial biofilms, only in recent years has eDNA been thought of as a potential target for therapeutic intervention. Since eDNA forms part of the biofilm produced by several bacteria commonly seen in prosthetic joint replacement, an anti-eDNA approach seems an attractive target for biofilm control (Montanaro et al., 2011, Okshevsky et al., 2014). This includes the use of nucleases to disrupt the eDNA component of the biofilm formed in PJI to aid in the diagnosis of infection from the implant surface as well as other techniques to increase yield.

1.9 Improving diagnostic microbiological tests for PJI

One of the most important techniques to improve the diagnosis of PJI is to increase the yield from explanted prosthetic implant surfaces by disrupting the biofilm matrix. These techniques have the advantage of providing a causal organism, which can be tested for antibiotic sensitivity and therefore guide antimicrobial management. Since biofilm encapsulated organisms are known to form freely on prosthetic implant surfaces (Costerton et al., 1978, Neut et al., 2003) which are often exchanged at the time of surgery. These implants if evaluated for the presence of biofilm encapsulate microbes can provide important addition information that is often overlooked.

1.10 Techniques to increase the yield of bacteria from Prosthetic implant surfaces

The use of sonication to aid the diagnosis of prosthetic joint infection was initiated by Trampuz *et al* (Trampuz et al., 2007). Sonication utilises ultrasonic energy which generates a rapid change in pressure on the surface of prosthetic joint replacements which dislodges attached microorganisms. The technique requires the explanted prosthesis to be transported from the operating theatre to the microbiology laboratory in a sterile fashion. Although techniques vary, a recognised technique uses a period of vortexing and then sonication for 5 minutes, followed by additional vortexing of the prosthesis. The resulting sonicated fluid is then processed using standard culture techniques (Trampuz et al., 2007).

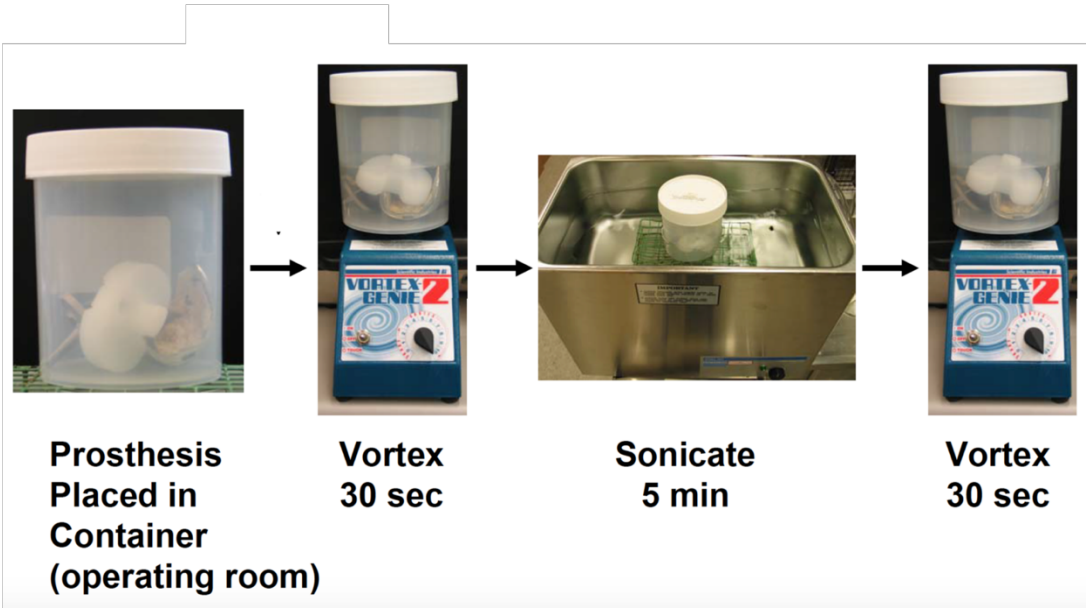


Figure 4. Sonication technique demonstrating the votexing and sonication steps required for processing samples (Trampuz et al., 2007).

Results of a meta-analysis of 12 papers have shown that sonication provided a combined sensitivity of 0.80 and specificity 0.95 (Zhai et al., 2014). The use of sonication was therefore more effective than standard tissue culture techniques within this analysis (Zhai et al., 2014). Sonication has been shown to be especially helpful when used for patients that have been on antibiotic therapy within 14 days of sampling (Trampuz et al., 2007). This supports the rationale that while planktonic bacteria may have been affected by antibiotics given around the time of revision, the bacteria present within a biofilm matrix established on the implant surface are protected due to the higher antibiotic concentrations required to kill them. These however, are not isolated by standard sampling techniques providing a false negative result. Sonication has been shown to provide faster culture and sensitivity results than standard culture methods (Zhai et al., 2014). Sonication has also been used to augment other diagnostic techniques such as polymerase chain reaction (PCR) and certain microcalorimetry techniques (Borens et al., 2013, Renz et al., 2018). However, sonication has a number of limitations that have prevented it being widely adopted outside of research centres. Explanted prostheses are large, difficult to handle and transport in a sterile fashion. Initial work with sonication was associated with contamination of the cultures providing false positive results (Trampuz et al., 2006). Some studies were unable to process all of the explanted prosthesis due to size especially revision implants or mega prothesis. Therefore, sonication could not be utilised for the most high-risk cases where the correct identification of causal organism is paramount. The samples require

specialist equipment and skills not available in most microbiology laboratories. The technique is time consuming, requiring several steps and processing in a laminar flow hood. Prolonged or high-energy sonication is also known to kill bacteria (Monsen et al., 2009). Although optimal levels of sonication have been identified for diagnostic analysis, the potential for inadvertent bactericidal effects remain, reducing the likelihood of a successful culture identification. Fungal joint infection is rare and often difficult to diagnose. To date, sonication has not be validated for fungal infections. Finally, there is a risk that sonication of antibiotic cement may cause release of antibiotics which kill the very cells that need to be cultured for diagnosis (Hendriks et al., 2003).

Therefore, the Infectious Diseases Society of America suggest that explanted prosthesis can be submitted for sonication recognising that ultrasonicate can improve the sensitivity of aerobic and anaerobic culture compared to traditional tissue culture (Osmon et al., 2013a). However complications have meant that sonication is not currently recommended for routine use by the International Consensus on Periprosthetic Joint Infection 2013 (Parvizi et al., 2013).

"We do not recommend routine sonication of explants. Its use should be limited to cases of suspected or proven PJI (based upon presentation and other testing) in which preoperative aspiration does not yield positive culture and antibiotics have been administered within the previous 2 weeks.

Delegate Vote: Agree: 84%, Disagree: 9%, Abstain: 7% (Strong Consensus)"

The Microbiology Investigations UK Standards guidance for the investigation of prosthetic joint infection samples is currently under review but recommends that sonication should be used only as a research tool and not for routine practice. (B44, 2017)

While sonication has proven effective and is most widely utilised to increase culture yield from the explanted implant surface there are other techniques available. These include the use of chemical agents such as Dithiothreitol (DTT). DTT is a strong reducing agent used in microbiology laboratories for liquefying specimens from the respiratory tract by cleaving disulfide bonds present within the mucus (Guiot et al., 2017) One laboratory and two clinical studies have shown that DTT is equivalent to sonication at identification of causal organisms from the implant surface of explanted joints (Drago et al., 2012, Drago et al., 2013). This technique is not widely practiced

but has recently been commercialised as MicroDTTect (Microdttect) with a recent independent evaluation in a clinical series of 232 patients undergoing revision joint surgery. Overall DTT treatment was able to demonstrate comparable results to sonication (Sambri et al., 2018). To date sonication seems the most effective method of increasing yield from prosthetic surfaces of explanted joint replacements. However, several reasons have led to the technique not being widely introduced. Potentially an enzymatic approach may provide a solution in releasing bacteria from the implant surfaces.

1.11. Nucleases and their potential role in releasing biofilm encapsulated bacteria via digestion of eDNA

Nucleases are a group of enzymes capable of the hydrolysis of phosphodiester bonds of nucleotide subunits seen in nucleic acids. Thus, nucleases are capable of digesting eDNA which is known to form part of the biofilm matrix. The use of nucleases in medicine has a long and successful history, most notably in the management of Cystic Fibrosis, a condition associated with chronic biofilm associated lung infections. Here once daily nebulised recombinant human deoxyribonuclease (rhDNase) is used as a mucolytic capable of reducing the viscoelasticity of sputum and enhancing the clearance of secretions (Suri, 2005). Although effective in Cystic Fibrosis rhDNase, which is currently produced in genetically engineered hamster ovarian cells is expensive primarily due to the low yields achieved, thus potentialy, limiting its wider usage outside of therapeutics (Ferrari et al., 1998, Okshevsky et al., 2014). However, nucleases are abundant in nature and several non-mammalian nucleases have been identified. Their secretion has been linked with the dispersal of several bacterial biofilms to assist in sporulation as well as to allow bacterial species to digest the eDNA component of the biofilm matrix as a nutritional store. (Mishra, 2002, Mann et al., 2009, Palchevskiy and Finkel, 2009, Vorkapic et al., 2016)

1.12 Marine Nuclease NucB

The action of deoxyribonuclease was first identified in 1978 by Akrigg and Madelstam in the marine organism *Bacillus subtilis* being most active during the late stages of sporulation (Akrigg and Mandelstam, 1978). Elevated levels stimulated by manganese release were shown to cause sporulation with self-induced degradation of the biofilm matrix to enable release of planktonic bacteria. Later Nijland *et al* recognised a

sporulation specific extra cellular nuclease while observing marine biofilms. The supernatant of *Bacillus licheniformis* EI-34-6 was capable of dispersing bacterial biofilms (Nijland et al., 2010). The enzyme subsequently was isolated and purified by the same group. Since the discovery of the marine nuclease NucB several advantages have been identified compared to other nucleases (Burgess et al., 2017). The production of large quantities of the secreted enzyme have been optimised by, Ragarajan *et al* allowing the scale NucB production providing cost-effective expression systems of NucB (Rajarajan et al., 2013). This production method is significantly cheaper especially if utilised within a commercial scale production *Bacillus* fermentations (de Souza Vandenberghe et al., 2016), when compared to those requiring a mammalian line cell. Further evaluation has demonstrated that compared to other nucleases NucB is a robust and relatively small protein (~12 KDa) around half the size of human DNase I that can potentially penetrate the biofilm matrix more efficiently for eDNA degradation (Basle et al., 2018). The same group demonstrated that NucB is a non-specific endonuclease capable of the digestion of several sites of both single and double stranded and DNA substrates rather than the more limited nuclease enzymes. In addition NucB demonstrated increased thermal stability compared to DNase I when increasing its commercial application.

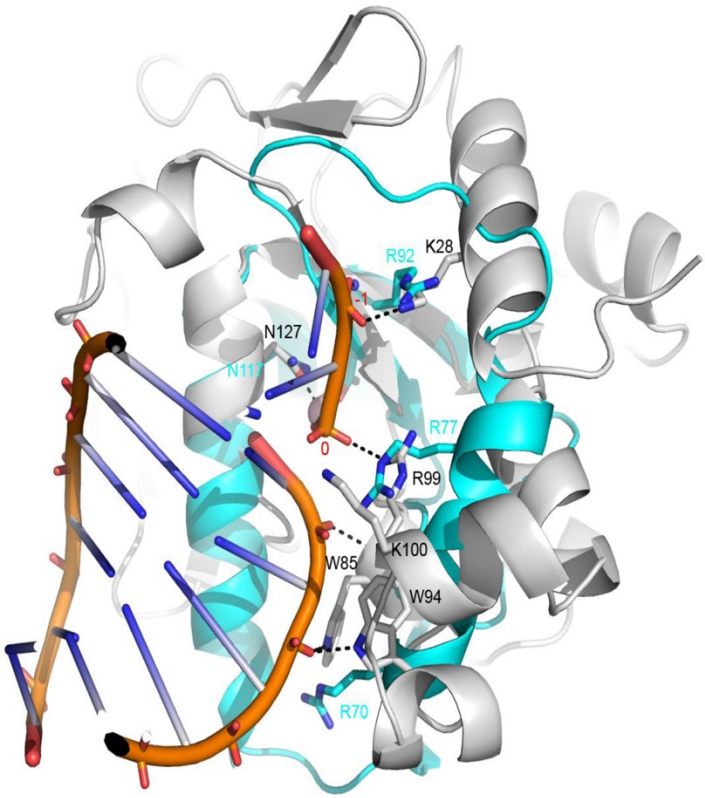


Figure 5. A model of the interaction of *Bacillus licheniformis* NucB with DNA (Basle et al., 2018)

Finally work undertaken by Nijland *et al* has demonstrated that NucB was better adapted for bacterial biofilm dispersal than bovine rhDNase I (Nijland et al., 2010). Following this Shakir *et al* utilising NucB demonstrated that it was able to disperse complex well-established mixed species biofilms from tracheoesophageal speech valves (Shakir et al., 2012). Mann *et el* demonstrated that nucleases were effective at the disruption of early biofilms established by *Staphylococcus aureus* however, less effective in more established biofilm colonies (Mann et al., 2009). Overall however, the nuclease degradation of biofilms has been evaluated on over 35 primarily bacterial species including both gram positive and negative bacteria as well as eukaryotic microbes including *Candida albicans* (Okshevsky et al., 2014), with several of these microbes being known causal agents in PJI (Aggarwal et al., 2014).

As a result of the increased understanding of the importance of biofilm associated PJI, with the unique properties of NucB and with the ubiquitous presence of eDNA in microbial biofilms. Associated with the developing recognition of the importance of eDNA in the structure of biofilms and the with limitations in existing techniques to disrupt the biofilm matrix form the implant surface. The potential of NucB to improve the diagnosis of prosthetic joint infection by releasing biofilm encapsulated bacteria via it action of eDNA is an appealing clinical target and will be evaluated in this work.

Aims and objectives

The overall aim of this work is to evaluate the use of NucB to assist in the diagnosis of prosthetic joint infection by disrupting biofilm encapsulated bacteria from the implant surfaces. Allowing the liberated less dormant planktonic bacteria to be identified by culture techniques improving the diagnosis of infection and the management of prosthetic joint infection.

The initial objective is to establish if NucB can effectively release biofilm encapsulated bacteria formed by reference strains and clinical isolates recruited from PJI in a microtiter plate model.

Subsequently, utilising a simulated implant surfaces model to more closely replicate a prosthetic joint infection. The effect of NucB will be evaluated on bacterial biofilms established on these implant surfaces and evaluated against sonication.

Finally, to establish if NucB can successfully increase the yield of microorganisms from the explanted prosthetic joint replacements recruited from patients undergoing revision surgery. Comparing both NucB and sonication techniques as well as evaluating the compatibility of NucB into the standard microbiology processing within the NHS.

2. Chapter 2. An *in vitro* model for assessing the efficacy of NucB in releasing bacteria from biofilms of clinically relevant bacteria

2.1. Introduction

Diagnosis of prosthetic joint infection is challenging, this in part is related to the ability of bacteria to produce a biofilm matrix on the implant surface, which protects the bacteria allowing them to avoid detection. Consequently, the implant, which is frequently explanted at the time of revision surgery, often has biofilm encapsulated bacteria colonising its surface. Once released from this biofilm, the bacteria could be cultured and identified confirming the diagnosis of infection and guiding patient management. The marine nuclease NucB is capable of digesting eDNA which is known to form part of the bacterial biofilm matrix. This enzyme has the potential to degrade the biofilm matrix established on the implant surface releasing the encapsulated bacteria and allowing them to be identified by standard culture techniques used within the NHS. To appraise the potential of NucB to assist in the diagnosis of prosthetic joint infection an *in-vitro* model plate system was utilised allowing initial evaluation. The first task was to acquire both clinical isolates and reference strains of bacteria for the *in-vitro* model. *Staphylococcus aureus* and *Staphylococcus epidermidis*, were recruited since combined they are the most common causative organisms isolated from prosthetic joint infections accounting for 50% to 60% of infected total joint arthroplasty (Pulido et al., 2008, Tande and Patel, 2014, Hickson et al., 2015). Following the recruitment of bacterial isolates their ability to form a biofilm on synthetic surfaces was evaluated. This is a prerequisite for their use in the biofilm model. This assessment was undertaken using confocal laser scanning microscopy (CLSM). CLSM is an established technique used to evaluate the formation of biofilm from the chosen isolates (Schlafer and Meyer, 2016). This technique provides an insight into the nature of biofilms without disturbing their complex structure. The availability of fluorescent dyes can also be utilised to evaluate the presence of eDNA. Several stains are capable of interacting with DNA however, in order to stain the eDNA component of the biofilm the stains need to be impermeable to the cell membrane to avoid staining the intracellular DNA which does not form part of the biofilm matrix. An evaluation of the eDNA fluorescent stains demonstrated that intercalating cyanide fluorescence dyes provided the best choice for evaluation of eDNA in the extra cellular matrix (Schlafer and Meyer, 2016). These cyanide dyes have high affinity to DNA and are cell-impermeable and develop over a thousand-fold increase in its green fluorescence when

bound to DNA (Larsson et al., 1994). The cyanide dye YO-YO-1 was employed to confirm the presence of eDNA in the bacterial biofilm confirming a target for nuclease activity on the biofilm matrix.

Having chosen the bacterial strains and confirmed their ability to form biofilm, the optimal growth media composition was established. This is important since the availability of nutrients can influence the growth rate of bacteria and subsequent biofilm production. Increased biofilm production has been demonstrated in low nutrient conditions, this phenomenon as a result of restricted growth media is known as the 'stringent response' and would encourage bacteria to form a protective biofilm community in a poor nutritional environment as a means of preservation (de la Fuente-Nunez et al., 2014, Strugeon et al., 2016). Evaluating and optimising the growth media concentration will ensure the abundant and constant biofilm for evaluation of NucB activity. The ability of the nuclease NucB to be used as a diagnostic tool requires certain enzyme characteristics. Firstly, the nuclease should be an effective and stable enzyme. Secondly, that the presence of NucB is non-cytotoxic or inhibit bacterial growth which is required for culture and identification. To determine this, the activity of NucB was evaluated and the ability to digest a quantifiable amount of calf-thymus DNA as a substrate. This was calculated both using gel-electrophoresis and mass-spectrometry. To determine the effect that the presence of NucB may have on bacterial cell growth, bacterial cultures were exposed to NucB and the rates of cell division determined by changes in optical density and the quantification of colony forming units over time.

Lastly the effectiveness of NucB on the release of biofilm encapsulated bacteria on the chosen isolates was evaluated. This was initially a simple in-vitro model using a microtiter polystyrene plate. While this synthetic surface is not used for prosthetic joint replacement it provides a reproducible and manageable surface. The release of bacterial cells was quantified by calculating the colony forming units from the supernatant and from the optical density staining of the solubilised residual biofilm stained with crystal violet (Peeters et al., 2008).

Overall the objectives of this chapter are

1. To obtain both clinical isolates of bacteria from NHS patients undergoing investigation or surgery for prosthetic joint infection and reference strains of the same species of bacteria from national collections.
2. To optimise their growth conditions in vitro and to evaluate their ability to form a biofilm in a reproducible static in-vitro model.
3. To determine if these isolates utilise eDNA as part of their biofilm and therefore have the potential for nuclease (NucB) degradation of the matrix.
4. To evaluate the effectiveness of NucB to digest biofilm eDNA and consequently to release biofilm encapsulated bacteria by disrupting the biofilm matrix.

2.2. Materials and Methods

2.2.1. Isolation and identification of clinical bacterial isolates utilised in in-vitro biofilm model.

Clinical isolates were sourced from the Newcastle upon Tyne Hospitals NHS Foundation Trust. The clinical microbiology department from the trust were contacted and supplied clinical isolates from implant associated infections. Once identified by MALDI-ToF mass spectrometry (Gaudreau et al., 2018) at Newcastle upon Tyne Hospitals NHS Foundation Trust, samples were established on agar slopes for transfer to Newcastle University. Samples were inoculated into tryptic soy broth (TSB) and incubated overnight at 37^0C. Samples were then centrifuged to make a pellet before resuspension in TSA and creation of a glycerol stock for storage at -80^0c.

2.2.2. Sourcing of bacterial reference strains utilised in in-vitro biofilm model.

Reference strains were sourced from the National Collection of Type Cultures (NCTC). *Staphylococcus aureus* strain 6571 was deposited in the NCTC in 1944 and remains the clinical isolate used for the evaluation of clinical diagnostic microbiology laboratories serving as a reference strain for antimicrobial susceptibility evaluation (Heatley, 1944). The reference strain *Staphylococcus epidermidis* NCTC 11047 reference strain was isolated from the nasal cavity. It is used for evaluation of antibiotic sensitivity in research laboratories (Piddock and Zhu, 1991, McLaws et al., 2008).

2.2.3. Optimisation of growth media for bacterial biofilm formation.

To identify the optimal concentration of TSB (Sigma-Aldrich, Dorset, UK) the following experiments were undertaken. Each clinical isolate and reference strain was established from glycerol stocks on to TSA plates and statically incubated over night at 37 ^{0}C. Representative colonies were chosen and inoculated into 5mls of sterile TSB and again incubated over night at 37 ^{0}C. TSB media was reconstituted as per manufactures instructions and was diluted as required with distilled water to produce final concentrations of 100%, 50%, 20% and 10% before autoclave sterilisation. The overnight TSB samples were centrifuged at 14,000 rpm for 10 minutes and then suspended in 5ml of 10% TSB. Using a spectrophotometer, the absorbance at 600 nm each media concentration was then inoculated with the re-suspended culture to create an optical density of 0.01 using unconditioned media of each concentration to measure background absorbance. Having established each media concentration with set inoculum, 180µL were added in triplicate wells to a sterile 96-well plate (Greiner Bio-One). Appropriate blanks of media for each concentration were included. Samples were incubated statically overnight at 37^{0}C. To quantify biofilm formation, liquid medium was aspirated from the plate and each well was washed with sterile isotonic phosphate buffer (PBS) three times to remove non-adherent cells and samples were allowed to dry. Biofilm formation was quantified by staining with 200µL 0.1 % (w/v) crystal violet (per well). After incubating for 15 minutes at room temperature (20-25°C) the wells were washed gentle three times with PBS and allowed to dry. The residual stained biomass was solubilised in 200µL 7 % acetic acid (v/v) and the *A*570 was read in a microplate reader (Synergy HT). The un-inoculated media acting as blanks were stained and washed in a similar fashion and the reading subtracted from sample absorbance values (Christensen et al., 1985).

2.2.4. Characterisation of biofilm formation with Confocal Laser Scanning Microscopy (CLSM)

To evaluate and characterise the ability of the clinical isolates and reference strains to form biofilms containing eDNA each isolate was recovered from glycerol stocks on to TSA plates and statically incubated over night at 37 ^{0}C. Representative colonies were chosen and inoculated into 5mls of sterile TSB and incubated over night at 37 ^{0}c. The resultant stock culture was used to create an inoculum of 0.01 (O.D. A600) using sterile TSB. Sterilised 13mm glass cover slips were placed into a sterile six well tissue culture plates and covered with 3 mls of inoculum wrapped in para-film. The plates were then incubated statically at 37 ^{0}C for 24 hours. Following incubation, the liquid medium was aspirated from each well and the coverslips stained prior to microscopy. Both stains Y0-Y0-1 and Nile Red (Sigma-Aldrich, Sigma-Aldrich Company Ltd, Dorset UK) were reconstituted as per manufacturing instruction. The slides were covered with YO-Y 1 for 15 minutes at room temperature in the dark. Following this the cover slips were stained with Nile Red Sigma-Aldrich, Sigma-Aldrich Company Ltd, Dorset UK) and again incubated in room temperature for 30 minutes. Excess stains were removed, and the slides transferred from the six well plates and mounted on microscope slide using a prepared well filled with PBS. Samples were kept in the dark for transfer for CLSM. Imaging was undertaken using the Nikon AR1 CLSM (Newcastle University Bio-imaging Unit) with visualisation of Nile Red (excitation 549 nm, emission 428 nm), and Y0Y0-1 (excitation 491 nm, emission 509 nm) (Barnes et al., 2012). The acquired stacks of image were further analysed using 3D imaging software (Imaris, Bitplane)

2.2.5. Sourcing and characterisation of NucB nuclease utilised for biofilm disruption.

NucB was supplied by the Newcastle University Protein and Proteome Analysis department (NUPPA) and stored locally at -80^{0}C. To minimise the effect of freeze thawing the enzyme was divided into aliquots required for individual experiments in 2ml eppendorfs. To evaluate the enzyme activity and stability a series of experiments were conducted to compare the variation of activity between enzyme batch and effect of storage over time. Calf thymus DNA (Sigma D1501 - 1G) was utilised as substrate with effect of NucB being quantified by enzyme digestion. The early part of the reaction was evaluated using agarose gel electrophoresis. This technique was employed since perchloric acid used for the solubilisation of DNA is unable to solubilise larger base pair units. Once DNA digestion is established the later part of the reaction was

quantified spectrophotometrically. Standard assay conditions used for the evaluation of enzyme activity were as follows: 125µL of Tris Buffer 50mM Tris (pH8.0), 12.5 µL 5mM $MnSO_4$. (Stock 100mM) and 95.65 µL Sterile distilled water was reconstituted and added to 125µg Calf thymus (CT) DNA and 5 ng NucB creating a final reaction volume of 250µl. Having created the reaction mixture from stock solutions 125µg of calf thymus (CT) DNA from a 2mg/ml stock solution was reconstituted and stored as per manufacturing guidelines. These were mixed and pre-equilibrated at 37ºC for 10 minutes in a sterilised 2ml eppendorf. The reaction started by the addition and of 1.25 µL NucB of a Stock 1µg/ml followed by further incubation at 37ºC. To act as a control a no enzyme assay was included with the volume was made up with buffer solution. Incubation time intervals of 15 minutes, 30 minutes and 60 minutes were taken. For analysis by agarose gel (0.8% w/v) electrophoresis 50µl of the reaction was halted by the addition and mixing of 50 µl of phenol/chloroform/isoamyl alcohol mix for both enzyme and control. The mixture was shaken forming emulsion and centrifuged at 13k rpm at 4ºC for 3 mins in a benchtop microfuge forming two layers. For electrophoresis the DNA was taken from the upper layer and stored in the fridge until all time intervals were processed.

DNA was separated and visualised on 0.8 % molecular biology grade agarose (Melford). 100mls 1 x TAE buffer (40 mM Tris, 20 mM glacial acetic acid (Fisher Scientific), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich), pH 8.0) was transferred to a 250ml conical flask 0.8g agarose was added to buffer and dissolved by gently heating in a microwave. After the solution had cooled but remained liquid 5µl of Gel Red was added and swirled to mix. The agarose solution was then poured into gel trays, with gel combs, and allowed to solidify. Set gels were placed into a gel tank and immersed in 1x TAE buffer. 5µl of Lambda DNA digested with HinDIII ladder (Thermo-fisher Scientific) was added to first well. Samples containing DNA were mixed with 5x DNA loading dye (1:5) (Bioline). 5µl of each interval DNA was mixed with DNA loading buffer (Bioline) and loaded onto Gel Gels were run at 100V for up to 90 minutes. DNA bands were visualised with an ultraviolet source (G:Box, Syngene).

The later part of the reaction was quantified by spectroscopic measurement. Separate reaction volumes including controls were used to for evaluation. The reaction was initiated as previously described and incubated for 60 mins at 37ºC. The reaction was halted with by the addition of 250µl of cold (4ºC) 4% (v/v) perchloric acid. After

mixing, the reaction mixture was incubated on ice for 40 minutes and then centrifuged at 13k rpm at 4ºC in a Bench-top centrifuge (MiniSpin Eppendorf) to precipitate high molecular weight DNA. 250µl of the supernatant was then diluted to 1ml with Tris buffer and the amount of low molecular weight DNA generated measured by the absorbance at 260 nm using NanoDrop (ND-1000 NanoDrop) spectrophotometer. After appropriate cleaning and blanking of the Nano Drop 2 µL volumes of each sample were loaded onto the device and their values recorded (Nestle and Roberts, 1969)

2.2.6. Evaluation of potential NucB toxicity to bacterial cells.

To evaluate the effect of the presence of NucB on the growth rate and viability of bacterial cells, a series of experiments were undertaken. As previously described isolates were grown from glycerol stocks on to TSA plates and statically incubated over night at 37 ^{0}c. Representative colonies were chosen and inoculated into 5mls of sterile TSB and again incubated over night at 37 ^{0}C. From this overnight TSB inoculum, a standardised OD 600 of 0.01 was obtained by dilution with fresh TSB. 10mls of the subsequent standardised cultures were incubated with and without the presence of NucB at a concentration of 1000 ng/ml in the presence of 5mM $MnS0_4$ in falcon tubes. Growth of the cultures was recorded by optical density and assessment of cell numbers by measuring colony forming unit calculated following serial dilutions taken at hourly time intervals and plated onto TSA plates incubated at 37^{0}c overnight.

2.2.7. Evaluation of the effect of NucB on preformed bacteria biofilms in a 96 well plate.

To evaluate the initial effectiveness of NucB on the release of biofilm encapsulated bacteria, an experimental set up was adapted from Christensen *et al* (Christensen et al., 1985). Isolates from both reference strains and clinical isolates were evaluated. Samples maintained in a glyverol stock and stored at -80^{0}C were recovered onto TSA plate and incubated overnight at 37°C. Representative colonies were chosen and inoculated into 5mls of TSB and incubated overnight at 37°C, 200 rpm. The following morning the culture was diluted to produce an OD600 = 0.1, This culture was then used to create a biofilm formed on synthetic surface on a 96 well microtitre plate. To form bacterial biofilms 180 µl were placed into each well covered and incubated statically at 37°C for 24hours. Each experiment evaluated with each isolate in triplicate with sterile TSB included to act as controls. Following incubation, the liquid medium was aspirated, and

the plates were gently washed with sterile isotonic phosphate buffer (PBS; 10mM, pH 7.4) to remove non-adherent cell. The residual biofilm was then exposed to either control or NucB. A NucB aliquot was removed from storage and brought to room temperature prior to utilisation. The reaction volume was pre-warmed to 37^0C and contained 50mM Tris-HCl, 5mM $MnSO_4$.H2O (pH 8.0) plus NucB at a concentration of 1µg/ml which was added and mixed just prior to exposure to the biofilm. The control containing no NucB was included with the volume made up of sterile distilled water. 200 µl of either NucB or control solution was pipetted into each well and incubated statically at 37^0C. After incubation all the supernatant from each well was removed and placed into a new sterile 96 well plate for serial dilution. The residual biofilm was stained with 200 µl of 0.1% crystal violet (CV) (Sigma-Aldrich, Dorset, UK) for 15 minutes including blank wells that had solely contained sterile TSB. Excessive stain was removed by three serial washes with sterile distilled water. The wells were dried at room temperature for 30 minutes. The amount of residual biomass was quantitated by solubilizing the biofilms for 15 minutes with 200 µl of 33% (v/v) acetic acid as previously described by Merritt et al (Merritt et al., 2005). The absorbance of the CV solution at 595 nm were measured with a Fluostar Optima plate reader (BMG Labtech, Bucks, HP19 8DP, UK), using the MARS software package (BMG Labtech). The removed supinated underwent serial dilution and calculation of CFU following the Miles and Misra method (Miles et al., 1938). Utilising the new 96 well plate a serial dilution was undertaken utilising sterile PBS. In-vitro microtiter plate model using 20 µl of suspension with 180 µl of diluent. A TSA plate was divided into 8 equal sections and labelled with the appropriate dilutions and dried within a microbiological safety cabinet. In each sector, 3 x 20 µl of the appropriate dilution is dropped onto the surface of the agar and the drop allowed to spread naturally. The plates are left upright in the microbiological safety cabinet to dry for 15 minutes before inversion and incubation at 37 °C for 18 – 24 hours until the CFU could be counted. The following day the dilution with the number of colonies between 10-100 were counted. An average of the three samples for each isolated were obtained and the final CFU/ml calculated based on the dilution counted. To assist in the quantification of the number of CFUs the open source Java image processing programme Image J was utilised if required (Schneider et al., 2012). Experiments was under taken in triplicate.

2.3. Results

In order to establish an in vitro model of biofilm growth, 4 clinical isolates and 2 reference strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* were obtained. These are summarised below.

2.3.1. Isolation of clinically relevant strains of bacteria

Table 1. Clinical isolates evaluated for their ability to form biofilm and sensitivity to NucB

Organism	Strain	Source	Site
Staphylococcus aureus	76901	Newcastle upon Tyne Hospitals NHS Foundation Trust	Prosthetic joint infection
	518F	Newcastle upon Tyne Hospitals NHS Foundation Trust	Prosthetic joint infection
Staphylococcus epidermidis	76933	Newcastle upon Tyne Hospitals NHS Foundation Trust	Prosthetic joint infection
	096R	Newcastle upon Tyne Hospitals NHS Foundation Trust	Prosthetic joint infection

Table 2. Reference strains evaluated for their ability to form biofilm and sensitivity to NucB

Organism	Strain	Source	Site
Staphylococcus aureus	6571*	National Collection of Type Cultures	nasal cavity
Staphylococcus epidermidis	11047**	National Collection of Type Cultures	nasal cavity

* also known as American Type Culture Collection ATCC 9144
** also known as American Type Culture Collection ATCC 14990

The above clinical isolates strains were sourced from patients undergoing revision surgery for implant associated infection from regional hospitals in the North-East of the

UK. Clinical isolates were obtained from the National Culture Collection. These isolates were used for the subsequent use in the biofilm in-vitro model.

2.3.2. Visualisation of biofilm formation by CLSM

Having obtained the clinical isolates and reference strains the ability of these organisms to form a biofilm was established by CLSM microscopy. The presence of eDNA was evaluated with the use of YOYO-1. These results are presented in figures 6 and 7

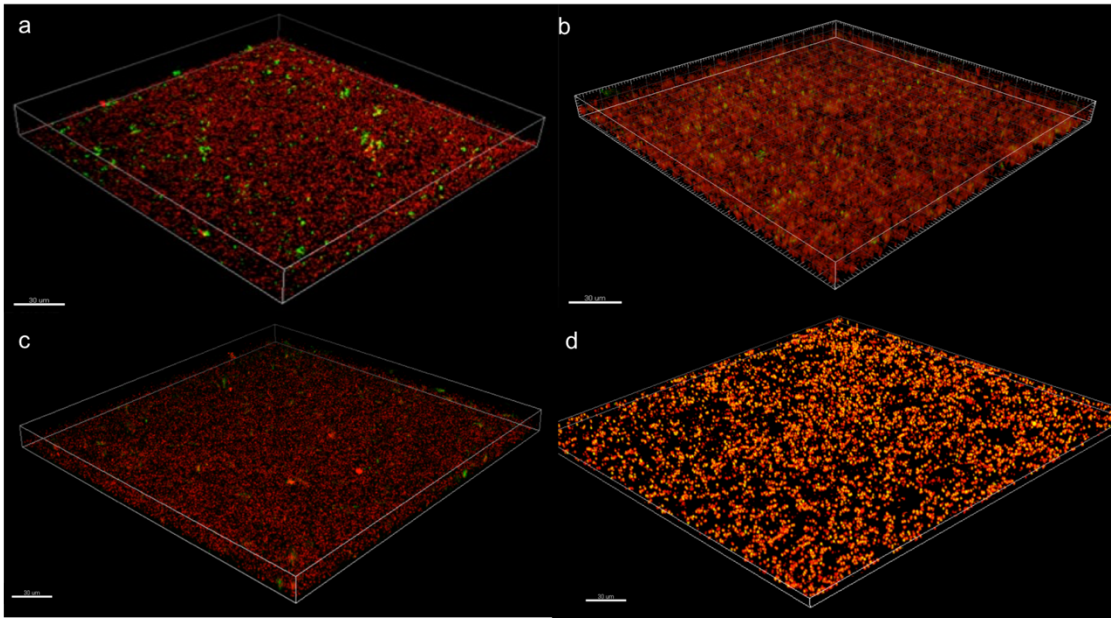


Figure 6. CLSM images of biofilm of a) *Staphylococcus epidermidis* 096R b) *Staphylococcus epidermidis* 76933 c) *Staphylococcus aureus* 518F d) *Staphylococcus aureus* 76901. Established on glass cover slips stained with Nile Red = Membrane Dye and YOYO-1 = eDNA Dye visualised with CLSM. Scale bar = 30µm

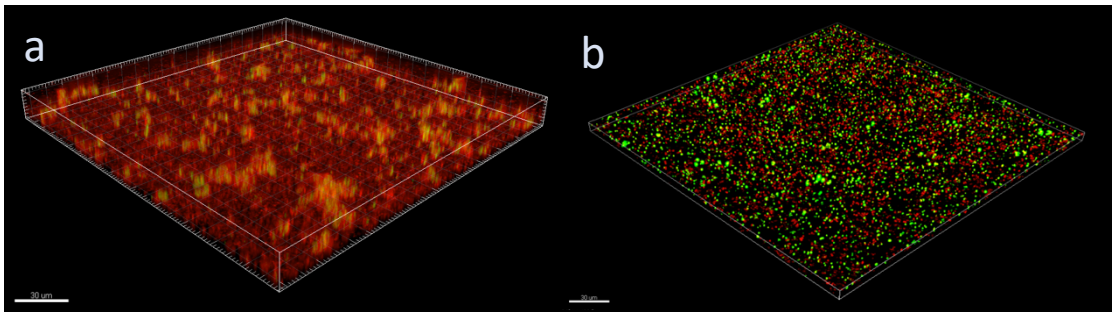


Figure 7. CLSM images of biofilm of a) *Staphylococcus aureus* 6571 NCTC b) *Staphylococcus epidermidis* 11047 NCTC established on glass cover slips stained with Nile Red = Membrane Dye and YOYO-1 = eDNA Dye visualised with CLSM Scale bar = 30µm

CLSM has the ability to demonstrate the complex and variable structure of the biofilm matrix.

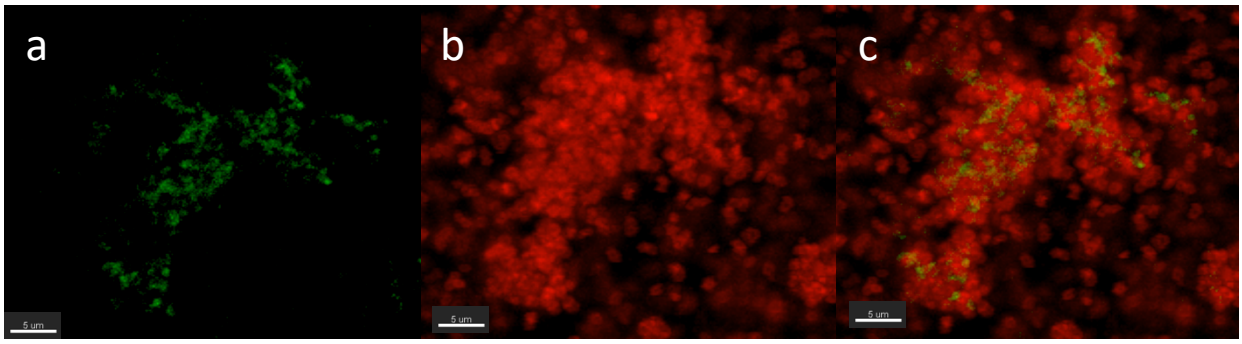


Figure 8. CLSM image of focused view of *Staphylococcus aureus* biofilm established on glass cover slips stained with Nile Red = Membrane Dye and YOYO-1 = eDNA Dye visualised with CLSM. a) YOYO-1 b) Nile Red c) combined Scale bar = 5µm

Figure six and seven show biofilm formation by clinical isolates and reference strains established on glass cover slip. All clinical isolates demonstrated the ability to form biofilm. The use of YOYO-1 to stain extracellular DNA confirms that all clinical isolates and reference strains have eDNA as part of the biofilm matrix. Figure 8 demonstrates the immediate relationship between biofilm encapsulated bacteria and eDNA indicating a fundamental role for eDNA in the biofilm matrix.

2.3.3. Effect of variation in TSB concentration on biofilm formation

In order to establish the growth conditions which provided the most abundant and reproducible biofilm, a series of experiments were undertaken using increasing concentration of tryptic soy broth (TSB). The bacterial cell growth was quantified by change in absorbance for four different concentrations of growth media, these are summarised below.

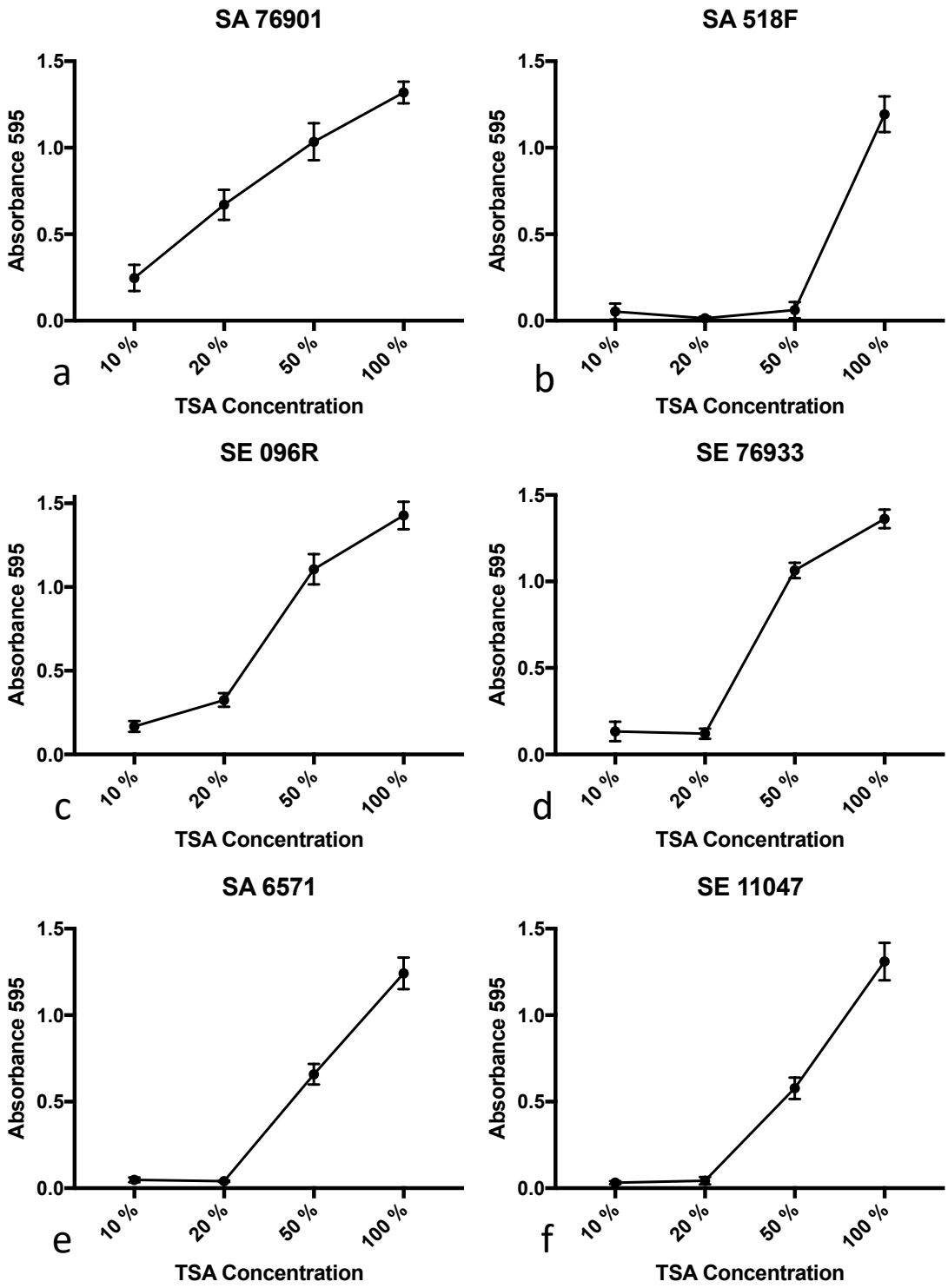
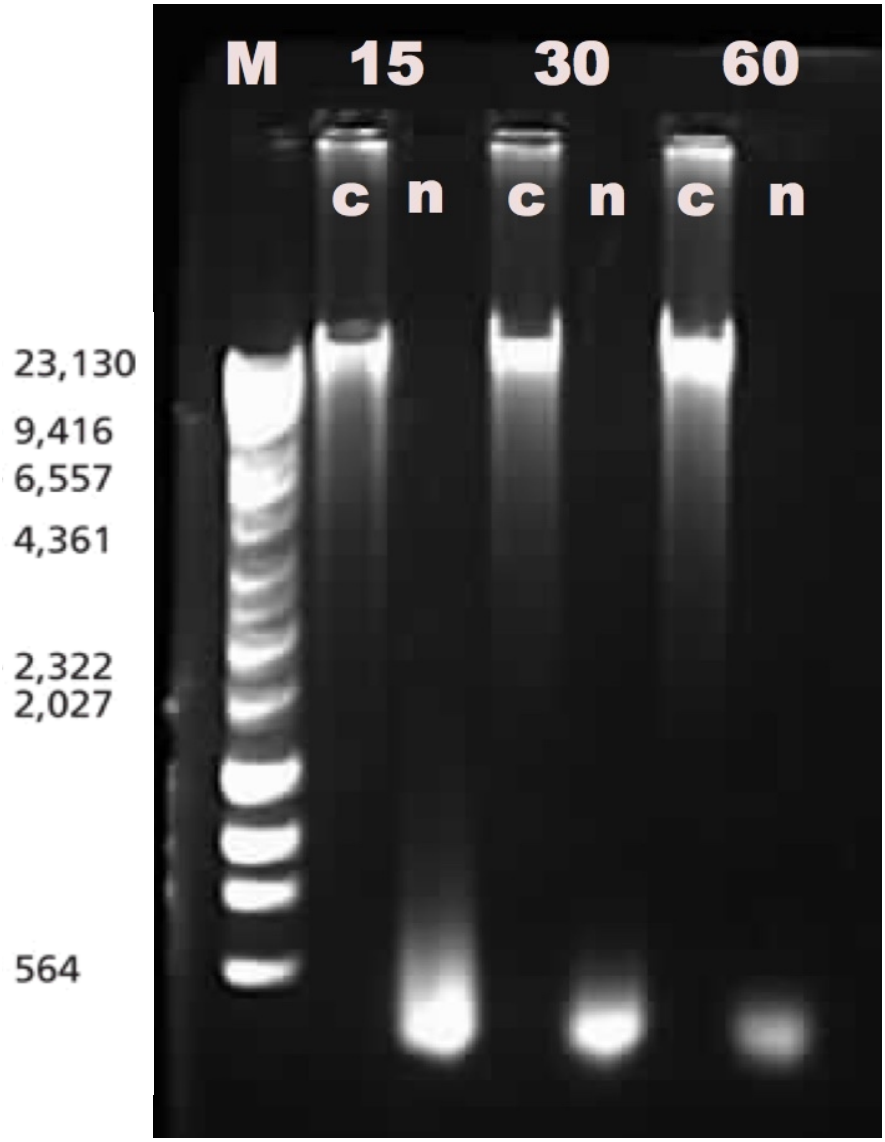


Figure 9. Effect of varying concentrations of TSB media on biofilm formation of a) SA 79601 b) SA 518F c) SE 096R d) SE 76933 e) SA 6571 f) SE11047 clinical isolates and reference strains established in 96 microtiter plates. Measured by staining with crystal violet and determining the attenuance at 595 nm (A595) each experiment was repeated in triplicate (n=3). The graph is presented as the mean and standard deviation.

Figure nine summarises the effects of TSB concentration on the biofilm formation. In all cases the most abundant biofilm formation was seen in 100% TSB concentration, with biofilm formation falling comparatively as concentration of TSB decreased. Apart from SA 76901 little biofilm was formed in 10% and 20% concentrations. Biomass produced at all TSB concentrations showed little variation between experimental replicates. The 100% TSB concentration provided the most consistent biomass for all isolates.

2.3.4. Assessment of enzyme activity

In order to be used as part of a diagnostic tool, NucB should display stable and reproducible enzyme characteristics. To ensure consistency of experimental conditions a series of experiments were devised to evaluate the reproducibly and efficacy of the enzyme NucB at DNA digestion. To test the efficacy of the nuclease activity two separate experiments were undertaken to quantify the digestion the calf thymus DNA substrate. The early reaction was evaluated by gel electrophoresis. Figure ten demonstrates that after exposure of NucB to Calf thymus DNA significant digestion has occurred after 15 minutes when compared no enzyme control. Further time points of thirty and sixty minutes demonstrates further digestion. The later part of the reaction was evaluated using quantifiable spectroscopic analysis. Figure eleven shows the absorbance levels obtained from calf thymus DNA following exposure to three different NucB preparations following 60min exposure. NucB preparation A and B showed a comparable level of activity with no significant difference $P = 0.942$, with NucB preparation C demonstrating a significant decrease in active compare to preparation A and B ($P = 0.001$). To ensure that the enzyme activity did not deteriorate while stored over the experimental period. NucB preparation A was evaluated over a 60-day time period figure 12 There was no significant variation in the enzyme activity over the 60 days.



M = molecular weight markers (Lambda DNA digested with *Hin*DIII)
Time intervals 15 mins, 30 mins,60 mins, c = no NucB, n= NucB)

Figure 10. Image of gel electrophoresis from NucB digestion of DNA Result of digesting 125 micrograms of calf thymus DNA with 5ng NucB in Tris buffer for 15, 30 and 60 minutes. Samples of the digestion products were separated by agarose (0.8% w/v) gel electrophoresis. C = NucB control N = NucB

2.3.5. Assessment of the later enzyme digestion of DNA

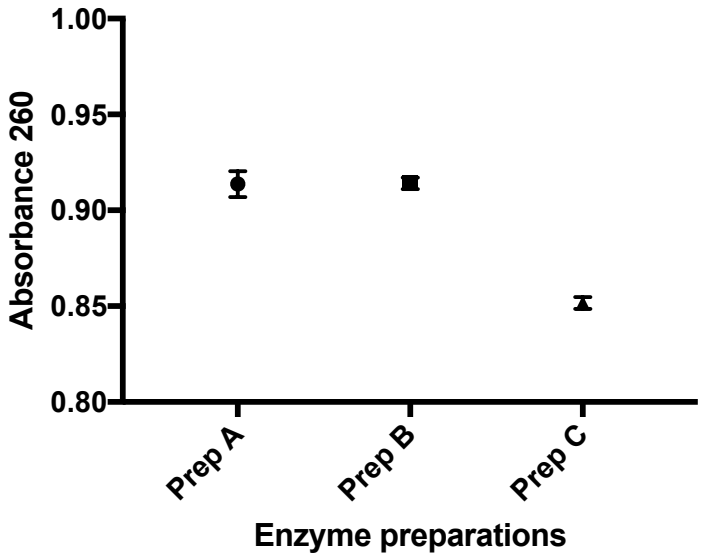


Figure 11. Evaluation of the activity of varying preparations of the enzyme NucB Results of assays with NucB from three independent preparations (A-C). In each assay 10ng of NucB was used to digest 125 micrograms of CT DNA in Tris buffer at 37ºC for 60 minutes. repeated in triplicate (n=3) and presented as the mean and standard deviation

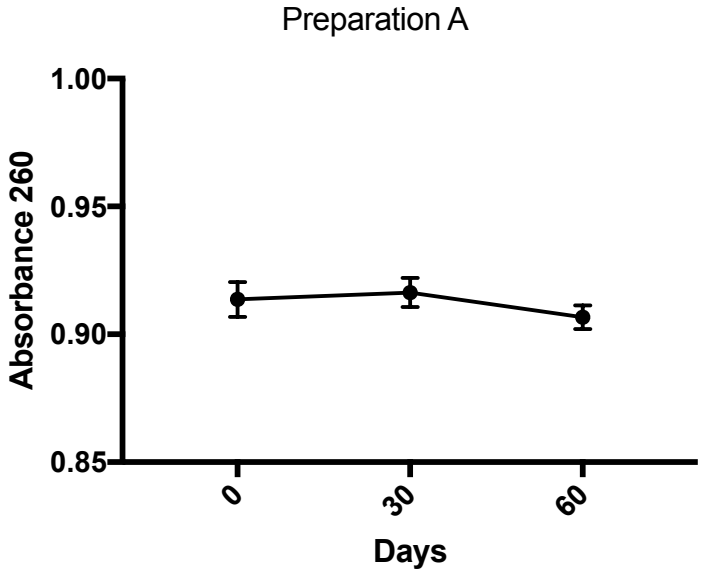


Figure 12. Results of the variation in activity for NucB Preparation A overtime (days) In each assay 10ng of NucB was used to digest 125 micrograms of CT DNA in Tris buffer at 37ºC for 60 minutes each analysis was repeated in triplicate (n=3) Graph presented as mean and standard deviation from mean

2.3.6. Assessment of the presence of NucB on bacterial cell growth

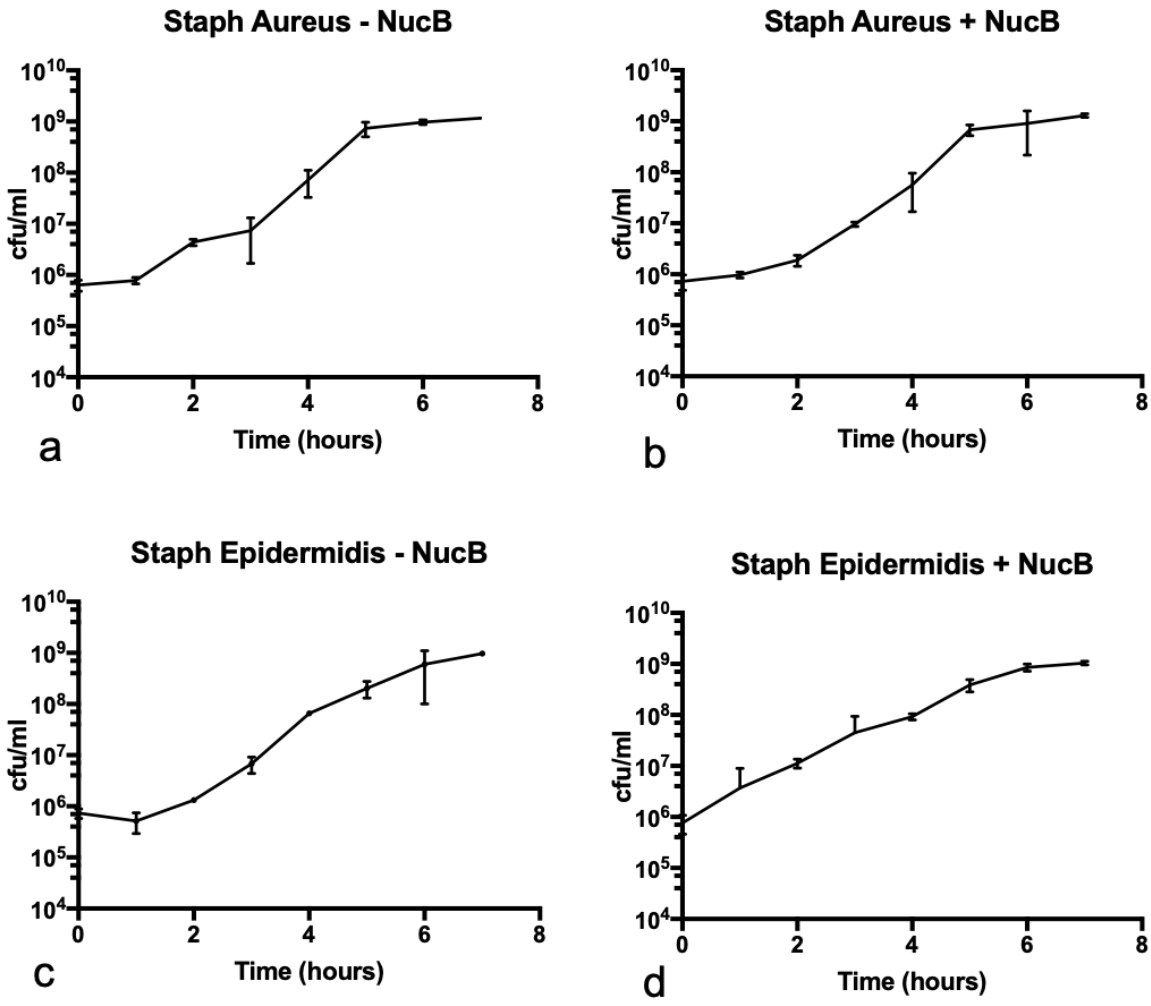


Figure 13. Effect of the presence of NucB on the growth of clinical isolates of *Staphylococcus aureus* (a + b) and *Staphylococcus epidermidis* (c + d) CFU/ml in TSB culture incubated statically at 37 ^{0}c over time. Demonstrated by the calculation of CFU/ml repeated in triplicate (n=3) and presented as the mean and standard deviation.

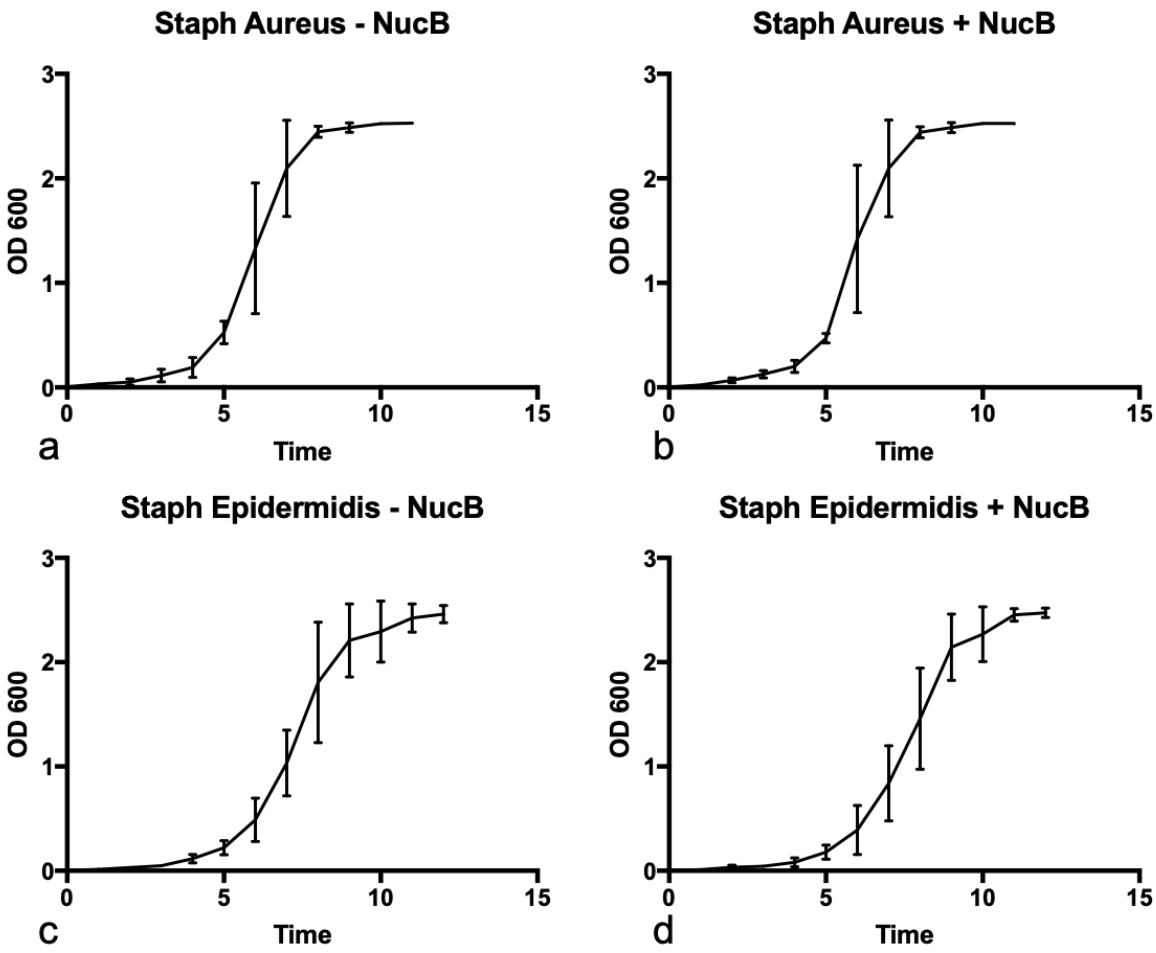


Figure 14 Effect of the presence of NucB on the growth of clinical isolates of *Staphylococcus aureus* (a + b) and *Staphylococcus epidermidis* (c + d) in TSB culture incubated statically at 37 ^{0}C over time. Expressed as change in optical density and repeated in triplicate (n=3) and presented as the mean and standard deviation

These experiments were undertaken to evaluate the potential toxicity of the nuclease NucB on bacterial cell growth. There was no demonstrable effect on the presence on NucB on *Staphylococcus aureus* and *Staphylococcus epidermidis* growth seen in figure thirteen and fourteen. Both strains were able to demonstrate standard growth curves with the *Staphylococcus aureus* isolate exhibiting slightly faster growth as expected. Having established the efficacy of NucB, its effect on the release of preformed bacterial biofilms was evaluated.

2.3.7. Effect of NucB on release of biofilm encapsulated bacteria from clinical isolates

Having isolated and evaluated clinical isolates and reference strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*, optimised their growth conditions and analysed their ability to form biofilm containing eDNA. The ability of NucB to release biofilm encapsulated bacteria formed in microtiter plates were subsequently evaluated. this was quantified by CV assay and supernatant CFU/ml calculations.

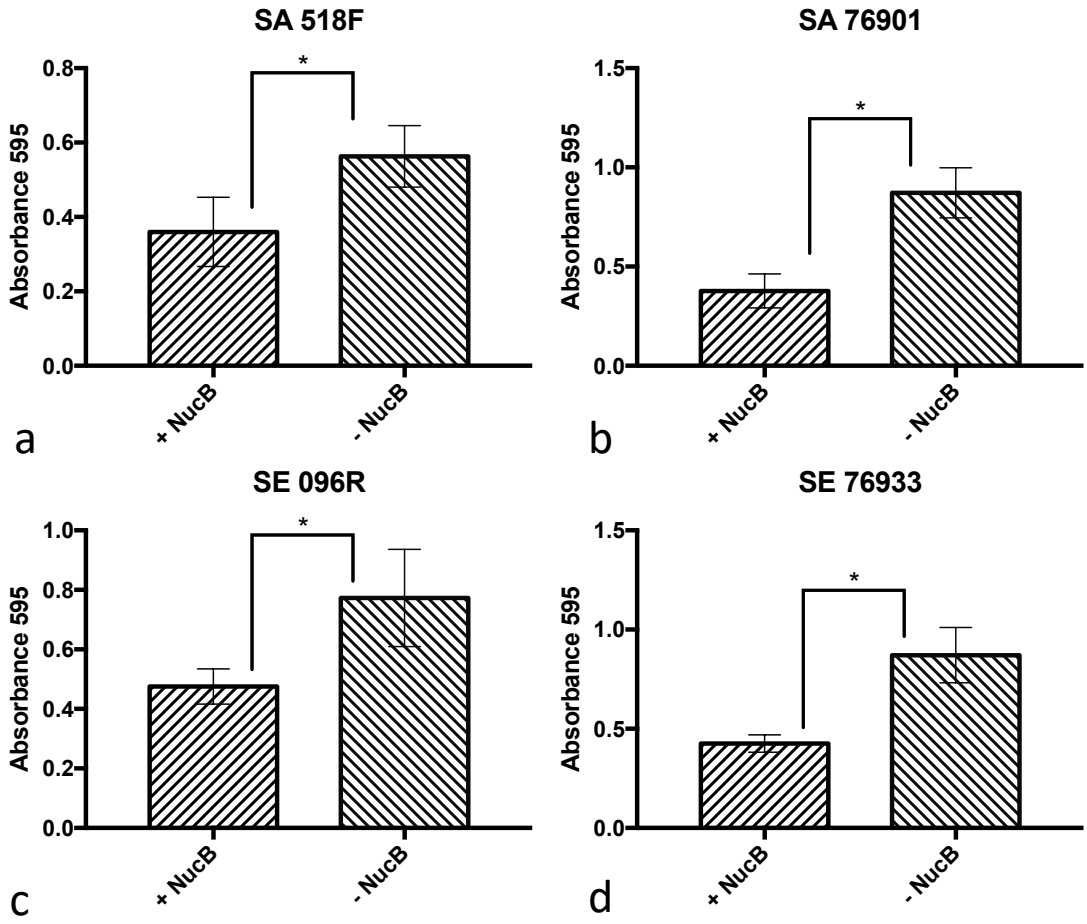


Figure 15. Effect of NucB on preformed *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms measured by determining the attenuance of the residual biomass at 595nm repeated in triplicate (n=3) and presented as mean and standard deviation. * = statistical significance un-paired student t-test ($P < 0.05$)

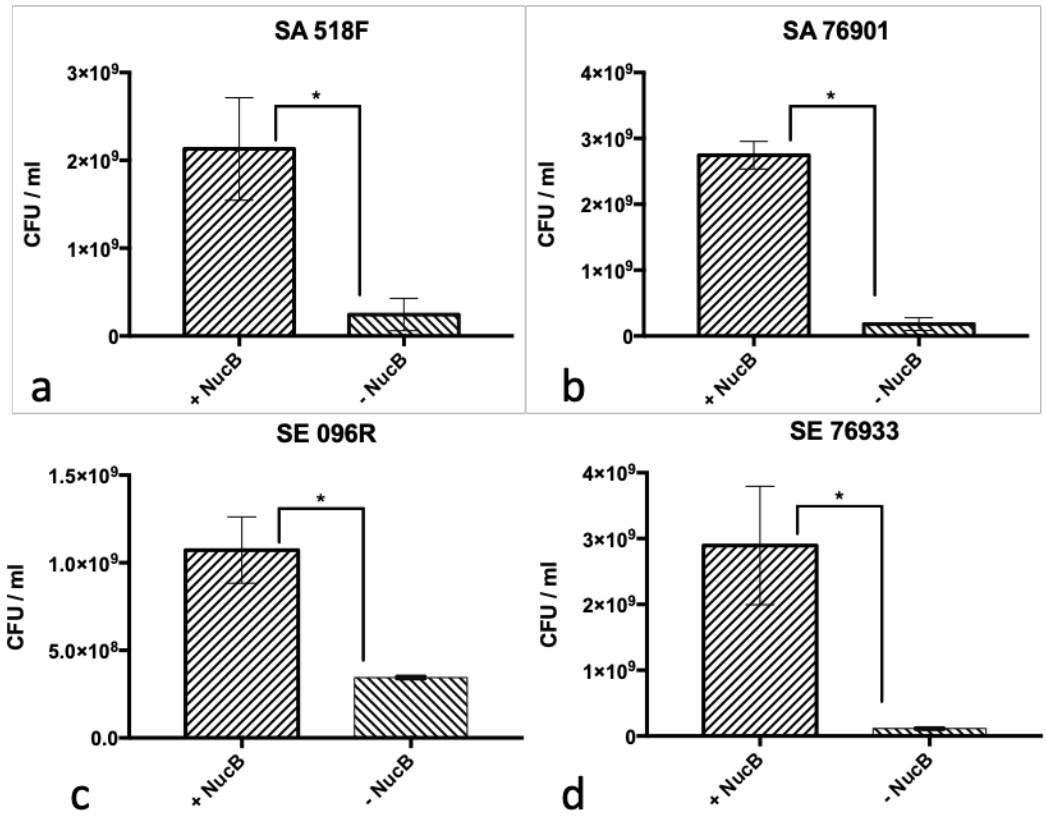


Figure 16. Effect of NucB on the preformed biofilms from clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* (CFU/ml) repeated in triplicate (n=3) and presented as the mean and standard deviation. * = statistical significance un-paired student t-test ($P < 0.05$)

2.3.8. Effect of NucB in biofilm dispersal on reference strains

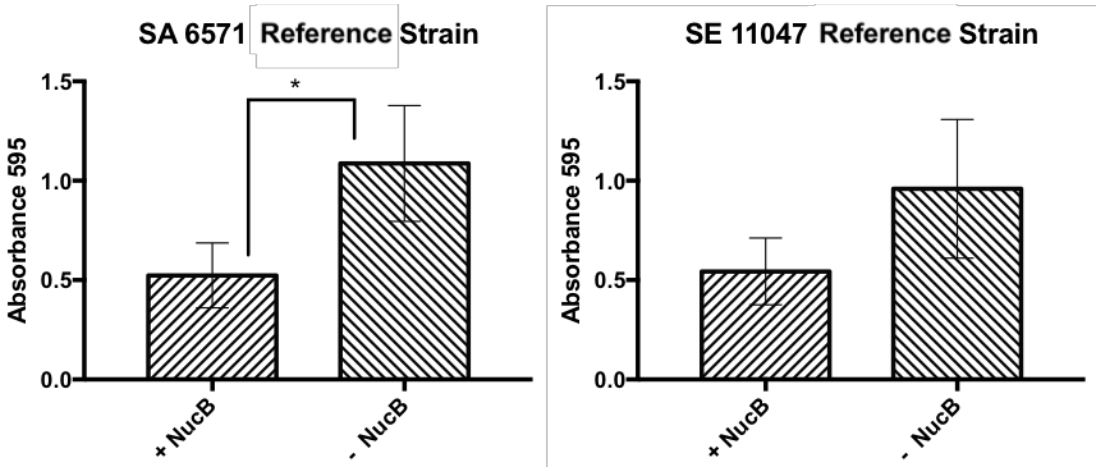


Figure 17. Effect of NucB on the preformed biofilms of *Staphylococcus aureus* 6571 and *Staphylococcus epidermidis* 11047 reference strains measured by determining the attenuance of the residual biomass at 595nm repeated in triplicate (n=3) and presented as mean and standard deviation * p = 0.0432 un-paired student t-test (P <0.05)

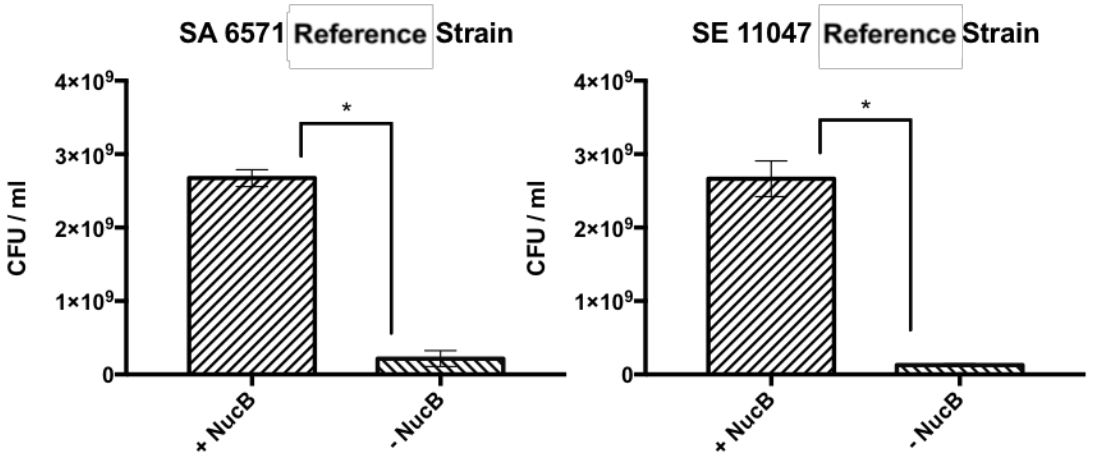


Figure 18. Effect of NucB on preformed biofilms of isolates of *Staphylococcus aureus* 6571 *Staphylococcus epidermidis* 11047 reference strains CFU/ml) repeated in triplicate (n=3) and presented as the mean and standard deviation. * = significance un-paired student t-test (P <0.05)

Figure 17 and 18 summarises these results. NucB showed a significant reduction in biomass by crystal violet quantification from all clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis.* The reference strain of *Staphylococcus aureus* reference strain, demonstrated a statistically significant reduction with a p 0.0432, while

Staphylococcus epidermidis reference strain did not reach a statistical reduction p 0.1365. The clinical strains demonstrated a similar reduction in biomass when comparing *Staphylococcus aureus* and *Staphylococcus epidermidis.* The number of viable cells released (measured as CFU/ml) from the biofilm by addition of NucB was in all cases statistically greater than the control. Again, similar numbers of viable cells were released from both *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates.

2.4. Discussion

2.4.1. Rationale for the choice of bacterial isolates selected for evaluation

The initial aim of this chapter was to obtain and characterise clinical isolates and reference strains of bacteria commonly isolated from prosthetic joint infections. Six isolates were obtained, two clinical isolates and one reference strain for both *Staphylococcus aureus* and *Staphylococcus epidermidis* - table 1 and 2. The reference strains used were not isolated from the prosthetic joint infection but rather the nasal cavity. They are routinely used in *in-vitro* biofilm models (Liu et al., 1997, Fey and Olson, 2010, Jeyaseelan and Jashothan, 2012) and are well characterised having been used for many years. These reference strains, considered representative for their species, are widely available, standardised and allow comparisons between varying interventions. There are however, disadvantages to solely using reference strains. Firstly, it is recognised that reference strains originally isolated from clinical setting will over time become less virulent (Fux et al., 2005). An example being the successful production of the BCG vaccination (Luca and Mihaescu, 2013) where successive sub culturing resulted in a non-pathogenic organism able to prime the immune response providing host immunity without active infection. The successive sub-culturing of reference strains will often select for faster growing planktonic bacterial phenotype. For example, successive culturing of *Escherichia coli* in such conditions results in altered gene expression for the phenotype required for flagella formation. Flagella are not essential for rapid division but are key component of bacterial adhesion and subsequent virulence (Sendi et al., 2010). Therefore, to provide a comparison for evaluation four clinical isolates were obtained. The clinical isolates were sourced from patients undergoing surgery for implant associated infection. These isolates have demonstrated a known pathogenicity being able to colonise the synthetic joint *in-vivo* causing infection. Contextualising the relevance of this study, the production of eDNA has recently been evaluated by Zatorska *et al.* This work compared the production of eDNA from clinical isolates from prosthetic joint infection and those from healthy volunteers as well as reference strains (Zatorska et al., 2017). This research demonstrated that pathogenic isolates produced a greater quantity of eDNA in their biofilms compared to those isolated from health volunteers as well as the reference strains used. While these strains have not been previously utilised in an *in-vitro* biofilm model they have demonstrated their causality for the disease model being simulated. Generally bacterial biofilm studies have utilised reference strains and clinical isolates

when evaluating a novel antibiofilm approaches (Bjerkan et al., 2009, Francolini and Donelli, 2010, Drago et al., 2012). Therefore, to provide a breath of knowledge the effect of NucB was initially evaluated on both clinical isolates and reference strains. This tests the ability of enzymatic activity against the most aggressive clinical bacterial infections from the target environment as well as providing a comparison with a standardised isolate.

2.4.2. Biofilm forming potential of isolates chosen for evaluation

Having isolated and identified the bacterial isolates for study a series of experiments were undertaken to evaluate the ability of the chosen organisms to form biofilms. Results from figure six demonstrated that all clinical isolates possessed the ability to form biofilm. Several studies have established that *Staphylococcus aureus* and *Staphylococcus epidermidis* demonstrate the ability to readily form biofilms on synthetic surfaces (Thomas and Hancock, 2009, Fey and Olson, 2010). Both of the reference strains were also able to establish biofilms (figure 7). The ability of reference strains to form biofilms is more variable and it is recognised that reference isolates may demonstrate a lesser ability to form biofilms than their pathogenic counterparts (Fux et al., 2005). This is as a consequence of the sequential selection of planktonic broth cultures excluding bacteria, which have preferentially formed adherent biofilm. Subsequent analysis of the preferential planktonic bacterial sub-culture may lack the necessary phenotypes that enable the formation for biofilm seen *in-vivo*. This phenomenon has been identified in *Pseudomonas aeruginosa* when comparing clinical isolates to reference strains isolated from cystic fibrosis patients, where the clinical isolates more readily formed biofilm compared to the reference strains (Head and Yu, 2003). This phenomenon was however not seen from the reference strains selected for use within this study with both strains being able to develop a biofilm. This has been demonstrated by several other studies utilising reference strains in clinical biofilm experiments (Sendi et al., 2010, Drago et al., 2012, Zatorska et al., 2017).

Having established that all isolates were able to form a biofilm on a synthetic surface the same technique of confocal microscopy was utilised to demonstrate the presence of eDNA with Y0-YO-1 cyanide dye. From the same figures 6 and 7 all clinical isolates and reference strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* utilised eDNA as part of their matrix. This finding is consistent with previous earlier work confirming the presence and importance of eDNA in these bacterial biofilms (Biswas

et al., 2006, Thomas et al., 2009, Fey and Olson, 2010, Montanaro et al., 2011, Sadykov and Bayles, 2012). Utilising the ability of the confocal microscopy to evaluate hydrated biomass in-situ, figure 8 shows that eDNA is integral to the biofilm matrix being present in-between the bacterial cells allowing them to aggregate forming a complex community. eDNA has been known to form an important structural and adhesion role in bacteria biofilms (Peterson et al., 2013), this similar close relation has been demonstrated by Schlafer et al utilising the another of the cyanide fluorescent stains (Schlafer and Meyer, 2016). The use of confocal imaging has confirmed the presence of eDNA in all bacterial biofilms - this being the necessary substrate for nuclease activity and indicates that NucB may be able to disrupt the biofilm matrix of these strains.

2.4.3. Optimisation of the biofilm model and NucB toxicity

The next stage in the development of this *in-vitro* model was to evaluate the optimal growth media concentration that provided the most abundant and consistent biofilm. In all cases the largest biomass was produced in the highest concentration of TSB (figure 9). This is most likely related to availability of nutrients allowing rapid bacterial division and subsequent biofilm formation. The 'stringent effect' was not evident in this model suggesting that the formation of biofilm can be related to a stress response to limited resources including nutrient deprivation (Jefferson, 2004). Therefore, it could be expected that the lower nutrient levels of media could have produced greater amounts of biofilm. Since the formation of biofilm provides several other advantages including community habitation, defence and diversity, a genetically homogenous population when exposed to a potential colonisation surface has been shown to express a diverse response. This therefore allows a subset of the population to colonise the surface forming a more stable and protected biofilm encapsulated state, while other members of the population continue in the more motile planktonic form. The availability of nutrients allows greater cell division allowing the community to adopt a diverse response optimising the organisms chance of survival.

The use of confocal microscopy was able to confirm that all isolates were able to form biofilm. This ability has been corroborated with the crystal violet providing a quantitative assessment of biomass. This demonstrated no difference in the total biomass formed between reference strains and clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* (figure 9) The literature has suggested that

clinical isolates can produce greater biofilm mass compared to reference strains (Sanchez et al., 2013). Once again, this is related to virulence and was not evident from the bacterial isolates utilised. The clinical isolates of both bacteria produced a similar total biomass to the reference strains in most media concentrations suggesting that while the reference strains may have been to subject to recurrent sub culturing they have maintained their ability to form comparable biofilms to the clinical strains. As well as the volume of biomass produced the consistency of biofilm is important to provide a reproducible assay for evaluation. The amount of biofilm produced at increasing media concentrations was consistent between replicates seen for all isolates. This was apart from SA 76901 at the 20% concentration. When comparing the *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, both were able to produce equivalent biomass, even though the growth rate of *Staphylococcus aureus* is known to exceed that of *Staphylococcus epidermidis (Mason, 1935).* The final biomass production seen is comparable at 24 hours incubation again allowing direct comparison. This series of experiments has demonstrated that the microtiter plate model has the ability to provide a stable reproducible biofilm for the evaluation of NucB. Given that the largest and most consistent biofilms from all isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* were produced from 100% media concentration, this was the media used for the subsequent 96 well titre-plate model and later synthetic implant surface the model.

Following the development of a successful biofilm model, evaluation of the NucB enzyme was undertaken to quantify its activity and evaluate potential bacterial toxicity. The series of experiments (figure 13 and 14) demonstrated that NucB is able to digest DNA and maintain enzyme activity over time with little variation between preparations. NucB has been well characterised and has been used over many years. It is known to be a small 12kDa enzyme (Nijland et al., 2010) which is heat stable being able to refold its structure after heating (Basle et al., 2018). This stability and effectiveness of the enzyme demonstrates that NucB would have appropriate characteristics to utilise as a diagnostic agent. For evaluation preparation A was utilised to assess degradation of the eDNA component of the biofilm. The presence of NucB did not inhibit bacterial cell growth as demonstrated in figures thirteen and fourteen. This correlates with previous work with by Shakir et al demonstrating that the presence of a NucB is did not affect bacterial growth of the bacterial communities established on tracheoesophageal speech valves (Shakir et al., 2012) and from bacteria isolates commonly seen in chronic rhinosinusitis (Shields et al., 2013). Secondly the common pathogens in prosthetic joint

infection such as *Staphylococcus aureus* and *Staphylococcus epidermidis* are not naïve to nuclease exposure. *Staphylococcus aureus* produces two nucleases, one of which NUC1 is actively secreted to digest neutrophil extracellular traps (NETs). These NETs are secreted by host neutrophils to aid in phagocytosis (Berends et al., 2010, Thammavongsa et al., 2013). Nevertheless, the presence of an exogenous nuclease specifically NucB, has not be evaluated on clinical isolates and reference strains of *Staphylococcus aureus* and *Staphylococcus epidermidis.* This therefore confirms that the presence of NucB did not influence the growth of bacterial isolates from prosthetic joint infection. This is fundamental to its potential use in diagnosis of infection via a culture method.

2.4.4. Evaluation of the effect of NucB on biofilm disruption.

Having established a reproducible model of biofilm formation and evaluated the activity of NucB, its effectiveness on the release of biofilm encapsulated bacteria was evaluated. The nuclease NucB is effective at releasing biofilm encapsulated bacteria of *Staphylococcus aureus* and *Staphylococcus epidermidis* (figures 16 and 18). This is key to the successful use of NucB as a potential agent in biofilm associated infection which relies on the ability of the enzyme to digest DNA and dismantle the biofilm. This effectiveness of NucB has been echoed in early studies in bacteria biofilms from isolates not related to prosthetic joint infection. Work undertaken by the Nijland *et al* team who initially isolated NucB identified the effectiveness of NucB on both Gram-positive and Gram-negative bacterial isolates (Nijland et al., 2010). These bacterial biofilms were environmental isolates, sequenced type strains and non-pathogen laboratory clones, as well as clinical isolates seen in chronic rhinosinusitis (Shields et al., 2013). The results of this work correlate with published literature on other nucleases that have been utilised for biofilm including DNase. A study by Tetz *et al* evaluated the effect of bovine DNase on biofilm matrix and a synergistic effect with antibiotics. This study utilised primarily reference strains including *Staphylococcus aureus* and *Escherichia coli* both known to cause PJI and other clinical isolates from patients in the Russian healthcare system. While the main focus was on a synergistic response to antibiotic treatment. The presence of the nuclease did demonstrate a significant reduction in biomass formed in 24 hours in 96 well microtiter plates from in both reference strains of *Staphylococcus aureus* and *Escherichia coli* (Tetz et al., 2009). While Tang *et al* adopting an alternative approach evaluated *Staphylococcus aureus* nuclease Nuc-1. Initially demonstrating a knock out to the nuc1 gene has reduced

ability to form a biofilm and secondly that recombinant NUC1 protein could degrade the biofilm of *Pseudomonas aeruginosa, Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis* (Tang et al., 2011). This however, is the first time that nucleases have been evaluated on the bacterial biofilms of clinical isolates from prosthetic joint infection focusing of the release of viable organisms.

Overall when comparing the CFU release to the reduction in biomass measured via the attenuance of crystal violet a greater effect has been demonstrated with the release of CFU. This is particularly evident with the reference strain of *Staphylococcus epidermidis* not reaching a statistically significant difference (figure 17). This effect may be related to the underlying mechanism of NucB in sporulation and its own endogenous role within the bacterial biofilm. Bacterial biofilms are known to be complex structures with relatively few bacteria compared to the total biomass the biofilm (Periasamy et al., 2012, Otto, 2013). The specific action of NucB on biofilm release could preferentially release bacteria from the biofilm seen in the significant difference in CFU but a lesser reduction in residual biomass demonstrated by the crystal violet assay. When reviewed in the larger context the response to the presence of NucB seen from bacterial biofilms isolated from prosthetic joint infection mimics the presence of endogenous nuclease release from many bacterial organisms. Since the CFU release is equivalent bacterial release and essential to the microbiological diagnosis of infection this method was taken forward for assessment on the simulated explant surface model.

2.5. Conclusions

This work has identified and characterised clinical isolates and reference strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* confirming and optimising their biofilm formation in an in-vitro microtiter plate model. Nuclease digestion of eDNA by NucB has been quantified and subsequent confocal microscopy has confirmed the presence of eDNA in the biofilm matrix of the chosen organisms. Evaluation of the ability of NucB to digest eDNA within the biofilm and subsequently degrade the biofilm matrix was evaluated on both clinical isolates and reference strains. NucB was able to significantly increase the number of bacterial cells released from biofilms for all isolates with an average of a 3-4 fold increase in CFU/ml, as well as

leaving a significantly reduced biomass on the synthetic surface for the majority isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*.

3. Chapter 3. Release of biofilm encapsulated bacteria from simulated prosthetic implant surfaces by the nuclease NucB

3.1. Introduction

Having identified suitable clinical isolates and reference strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*, optimised their growth conditions for biofilm formation and evaluated the effect of NucB on the release of biofilm encapsulated bacteria, the aims of this chapter were to develop an *in-vivo* model of prosthetic joint infection using simulated implant surfaces, rather than the microtiter plates used in the previous chapter. While the polystyrene microtiter plates used in the previous chapter provided a reproducible and stable surface for biofilm formation, this material is not routinely used in orthopaedic implants (Sonntag et al., 2012). The substrate composition is known to effect biofilm formation (Teughels et al., 2006, Rochford et al., 2012), therefore simulated implant surfaces were obtained in order to more accurately model PJI. The first stage of this process was to review the materials commonly utilised in prosthetic joint replacement. The National Institutes of Health (NIH) in America estimated that around 8- 10% of people have a medical device implanted (Brandt, 2000), these devices are made from biomaterials. An excepted definition of a biomaterial is a material that is able to function in intimate contact with living tissue, with minimal adverse reaction or rejection by the body (Agrawal, 1998). The desirable qualities of a biomaterial depend on its use within the human body. This will differ significantly for example, when comparing a cardiac pacemaker to a total hip replacement. Desirable characteristics of a joint replacements include; a biocompatible composition to avoid adverse tissue reactions, resistance to degradation, mechanical strength to sustain cyclic loading and high wear resistance to minimise debris generation (International and Davis, 2003). As well as mechanical properties the biomaterials have to adhere to manufacturing guidelines including the method of manufacture, the quality of raw materials, the consistency of material and importantly, regarding biofilms is the final surface finish and smoothness of the product. Materials currently used in joint replacement fall roughly into three groups, those of metals, polymers and ceramics (Navarro et al., 2008). Due to its advantageous mechanical properties almost all joint replacements have a metal component either as part of the body of the prosthesis or as a part of the articulating surface. Stainless steel was one of the first metals used in the manufacture of total joint replacements (Charnley, 1961)

and is still currently in use. There are several formulations available, however the most abundantly used is austenitic stainless steel 316L (Cahoon and Paxton, 1970). Stainless steel is a cheap material, with a long history of use in joint replacement. However, stainless steel has reduced wear characteristics when compared with other tougher alloys such as cobalt- chrome (Co-Cr). Cobalt-chrome alloys such as Co-Cr-Mo ASTM 75 have been used in total hip replacements since the mid 1970's (McKee and Watson-Farrar, 1966). Co-Cr alloys have greater corrosion resistance compared to stainless steel with greater mechanical properties including a high elastic modulus which is greater than bone (Marti, 2000). These properties are useful when constructing joint replacements. A side effect of the mechanical strength is unwanted stress shielding of the adjacent bone stock causing reabsorption of bone around the implant over time (Bauer and Schils, 1999). Titanium alloys first used in aeronautics have demonstrated a lower elastic modulus and the potential for bone ingrowth known as osseointegration (Escalas et al., 1976). This allows direct integration of the bone to the joint without the need of cement to fix the prosthesis. Titanium is now frequently used in the tibial base plate of total knee replacements since its elastic modulus is closer to that of bone unlike some of the stiffer metals alloys used for the joint surface itself (Long and Rack, 1998).

Common polymers used in orthopaedics implant surgery include polymethyl-methacrylate (PMMA) bone cement and polyethylene (PE) (Kurtz et al., 1999). Of these, polyethylene is commonly used as an implant surface in total hip and knee replacements. It is employed to either form the lining of the acetabular component or as the insert for the tibial component. High molecular weight polyethylene (HWPE) is most often used and again has a long history of use within orthopaedic implants. This is related to its low friction, abrasion resistance and high impact compression strength (Atienza and Maloney, 2008). However, wear and resultant debris production remain the main limitation of use (Massin and Achour, 2016).

Ceramics are also frequently used in joint replacement, their development was driven due to the potential wear characteristics of HWPE. Ceramics when used as part of the acetabular cup of total hip replacements demonstrate improved wear characteristics while maintaining the low friction properties required (Hench and Wilson, 1993) however, the ceramic was brittle and early designs suffered from fractures (Hamadouche and Sedel, 2000). Later developments of more resilient ceramic bearing surfaces have been utilised more recently. This suggests a growing confidence in the material especially with its use in high demand for the younger patient population,

where longevity of the implant is fundamental (D'Antonio and Sutton, 2009, Atrey et al., 2017). Part of the initial work of this chapter was therefore to identify and recruit a range of surfaces constructed from materials commonly used in prosthetic joint replacement.

Whilst mechanical properties primarily influence the choice of material for prosthetic joint replacement, in recent years there has been increasing recognition of the importance of biofilm infection. This has shifted the focus of material choice to potential anti biofilm properties of the implant materials. Factors influencing bacterial adherence to a biomaterial surface include chemical composition of the material, surface charge, hydrophobicity and surface roughness (Katsikogianni and Missirlis, 2004). Surface roughness is a two-dimensional parameter that is specific to each material. It can be evaluated in several ways; (Poon and Bhushan, 1995) with the most frequent use of average roughness (Ra) and root mean square roughness (Rq). Generally, the smoother the surface the more resistant to bacterial adhesion (Scheuerman et al., 1998, Teughels et al., 2006). This is primarily related to roughness on a nanoscale where the rougher surface can provide a greater number of contact points allowing bacterial adhesion (Truong et al., 2010). Surface roughness can vary between replicates of each material, the surfaces once obtained were optimised for use in a biofilm model primarily in terms of their surface roughness.

Having obtained, characterised and optimised the chosen surfaces each were evaluated in its ability to host a biofilm from the clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* utilised in chapter three. These surfaces were then utilised to evaluate both the efficacy of the nuclease NucB as well as sonication to offer a comparison to NucB. Sonication is the process of exposing the biofilm which is formed on the synthetic surface to ultrasonic energy. This creates pressure changes at the interface dislodging the bacteria allowing them to be isolated and identified (Trampuz et al., 2007). As noted above sonication has proved effective in assisting with the diagnosis of biofilm associated prosthetic joint infection. However, it has a number of limitations which means its use has not been widely adopted. Nevertheless, it is the most established technique to assist in the release of biofilm encapsulated bacteria and therefore would provide a valid comparison to the effectiveness of NucB.

Therefore, the aim of chapter three is to more closely simulate an infected prosthetic implant surface model with the use of biomaterials commonly utilised in total joint

replacement. In order to more closely simulate the PJI infection model the clinical isolates were utilised to evaluate the use of NucB in releasing biofilm encapsulated bacteria to assist in the diagnosis of infection with comparisons to the more established technique of sonication.

3.2. Materials and Methods

3.2.1. Sourcing of and preparation of preparation of simulated implant surfaces

Simulated implant surfaces were sourced from companies supplying the commercial manufacturers of prosthetic joint replacements. The Titanium and Cobalt-Chrome were provided in rod form and were cut and polished into discs either within Newcastle University (School of Mechanical Engineering) or at a specialist polishing company (Tecomet, Inc. Sheffield UK). The polyethylene pre-cut rough discs were processed within Newcastle University where polishing was undertaken progressively using silicon carbide fine grit discs on a rotary pregrinder (metasev UK) with water cooling. Following polishing, measurement of their surface topography and size were recorded. The surface roughness of all discs was evaluated using a calibrated stylus contact optical surface profiler (Alpha-Step D 500 KLA Tencour USA) at room temperature and mean roughness (Ra) recorded (Wennerberg and Albrektsson, 2000). Disc height and circumference measurements were obtained for each disc using a micrometer.

3.2.2. Optimisation of methods used to quantify release of biofilm encapsulated cells from simulated implant surfaces

To evaluate the effectiveness of NucB and sonication to release biofilm encapsulated bacteria from a simulated implant surface the previous model was adapted from the 96 well plate. Adaptation of the method required optimisation of a washing stage following inoculation and culture to remove the non-adherent bacterial cells. This then provides a substrate on which to evaluate the action of NucB and sonication. To evaluate the number of washing steps required the following experiments were undertaken. As previously described, isolates were maintained in a glycerol stock and stored at -80^{0}C before being recovered onto a TSA plate and incubated overnight at 37°C. Representative colonies were chosen and inoculated into 5mls of TSB and incubated overnight at 37°C, 200 rpm. The following morning the culture was diluted to produce an OD600 = 0.1. This culture was then used to create a biofilm formed on the simulated implant surfaces.

The simulated implant surfaces Cobalt-Chrome, Titanium and Polyethylene discs were washed in 1% Virkon followed by 70% ethanol and then rinsed with sterile distilled water. The discs were autoclaved in a sealable container, heat dried and then allowed to return to room temperature. Standard checks were made to ensure the effectiveness

of the sterilisation process. Using sterilised forceps, discs were transferred to a separate sterile 50ml Falcon tubes (Thermo Fisher Scientific). 5mls of the inoculum was then added to each tube and incubated statically at 37°C for 24 hours. To ensure the sterility of the discs a separate disc was incubated with sterile media and checked to ensure no growth at the 24 hours. If contamination had occurred the experiment was abandoned. To establish the number of washing steps required to remove non-adherent bacteria, ten washing baths of 15mls sterile PBS were created for each disc. Individually each disc was then dipped into the subsequent sequential baths, from each of the 10 baths. A sample from each of the sequential baths underwent serial dilution and calculated of CFU following the Miles and Misra method (Miles et al., 1938) utilising a 96 well plate as previously described. Following serial dilution, the samples were then plated on TSB plates again in a similar fashion as previously utilised. The plates were then incubated overnight at 37 °C for 18 – 24 hours and then CFU counted allowing an extrapolation to quantify the number of bacteria removed from the disc with each serial washing step. To ensure that the residual discs had viable biofilm encapsulated bacteria, one of discs from each of the material was further inoculated into sterile TSB and cultured statically for 24 hours at 37 °C. Following this, the OD was measured with comparisons made to sterile TSB which was incubated over the same time frame. To further confirm the amount of biomass on the inoculated discs crystal violet assay was undertaken on the remaining discs and with comparisons made to the control non-inoculated discs. To achieve this following the washing step the discs were allowed to dry and then transferred to a sterile polystyrene 6-multiwell plate and stained with 5ml of 0.1% crystal violet (CV) for 20 minutes. After washing with sterile water, the discs were transferred to sterile polystyrene 6-multiwell plate and the crystal violet dye was solubilized in 5 ml of 33% acetic acid for 30 minutes. 200µl of the solution of each well were transferred to a 96 well microtiter plate and the CV absorbance was recorded at 595nm as previously described. Each of the discs were evaluated separately and experiments were repeated in triplicate.

3.2.3. Evaluation of sonication as a method for the release of biofilm encapsulated bacteria

To evaluate the sonication energy produced from the sonication bath (Engisonic EngisLtd, Kent, UK), a calibrated hydrophone visualised on a digital storage

oscilloscope (tektronix 2024C North America) within Newcastle University (School of Electrical and Electronic Engineering) was utilised. The sonication bath was filled with water at room temperature and hydrophone submerged. The energy produced was recorded in terms of frequency and intensity. (Trampuz et al., 2007)

3.2.4. Evaluation of the effect of NucB on bacterial biofilms established on simulated implant surfaces compared to sonication

This experimental model was adapted from previous work evaluating the disruption of biofilms established of disc surfaces (Drago et al., 2012). Each simulated surface was evaluated in individual experiments using each of the chosen bacterial isolates. Three discs were used for each treatment arm those being NucB, sonication, NucB control and where enough discs were available a combination of NucB and Sonication. Blank discs were also used to ensure no contamination throughout the experimental process.

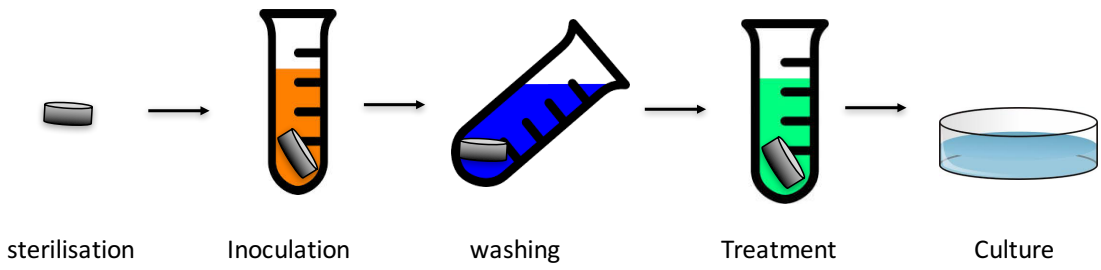


Figure 19. Pictorial representation of the experimental process showing initial inoculation washing and subsequent intervention.

Representative colonies were chosen and inoculated into 5mls of sterile TSB and again incubated over night at 37 ^{0}c. Using a spectrophotometer the absorbance at 600 nm each media concentration was then inoculated with the re-suspended culture to create an optical density of 0.01. Having standardised the inoculum 10-13 sterilised discs were transferred into eight sterile 50ml falcon tubes. 5mls of inoculum was added to nine of the tubes and one with sterile TBS used to reconstitute the inoculum to ensure the sterility of the discs and media. These were then incubated over night at 37 ^{0}C. The following morning checks were made to ensure that the control media remained clear, if contamination had occurred the experiment was abandoned. Following this the residual cultured media was removed and individual discs were immersed sequential six times to remove the non-adherent bacteria cells and then left to dry in a sterile container. Depending on requirements 9-12 discs were used and divided into three

treatment groups for further evaluation, occasionally one of the discs was dropped and therefore discarded with the spare disc substituted.

The reaction mixture for the NucB and NucB control were constituted in a single 50ml falcon before being divided between the discs for treatment. Pre-aliquoted 150 µl stock solutions of 100 µl/ml NucB enzyme were removed from -80^{0}c storage and allowed to thaw prior to use. The following reaction mixture was then created vortex and then brought to room temperature. 0.75 mL tris buffer pH8.0, 6.6 mL of $MnSO_4$ and 7.5 mL Sterile distilled water, creating a final volume of 15ml The NucB solution 150 µL of NucB (100 µl/ml) was then added before mixing again and then divided into 5 ml aliquots before exposing to the discs assigned for NucB treatment. The solution for NucB control was constituted in the same replacing the NucB volume with sterile distilled water. Both the NucB and NucB controls samples were then transferred to tilt table at 10 revolutions per minute (Heidolph polymax 1040 Heidolph UK – Radleys Shire Hill Saffron Walden Essex, CB11 3AZ) at room temperature for one hour. The sonication method was adapted from Trampuz et al (Trampuz et al., 2007). Discs with established biofilm were transferred to sterile containers with 5ml PBS solution. The falcon tubes were initially vortexed for 30 seconds using a Vortex-Genie 2 (Scientific Industries) before being transferred to the sonication bath (engisonic EngisLtd, Kent, UK) for 5 minutes, followed by subsequent vortexing for 30 seconds. To evaluate a synergistic effect of the combination of NucB and Sonication on the recovery of bacteria Polyethylene and Co-Cr discs were exposed initially to sonication and then subsequent NucB Treatment following the above methods.

Having undergone either treatment by sonication, NucB, NucB control or combined treatment the 200 µl from each sample was then removed and transferred to a sterile 96 well plate where a serial dilution was undertaken before pipetting onto TSA plates as previously described. These plates were then incubated over night before CFU units were recorded. Knowing the surface area of the discs the CFU/mm2 for each of the discs were recorded for each of the bacterial isolates tested. Results were analysed using un paired student t-tests and presented using Prism 7.0 (Graph-pad Software incorporated USA). Statistical significance was considered at a value of $p \leq 0.05$.

3.3. Results

In order to create a simulated prosthetic joint infection model discs of material commonly used in joint replacements were first recruited. These are summarised in the table below.

3.3.1. Simulated prosthetic implant surfaces

Table 3. Summary of simulated prosthetic implant surfaces used for evaluation of biofilm dispersal

Titanium	Titanium 6AL4V ELI ASTM F136 Grade 23
	Source: Ti industries Birmingham UK
Cobalt-Chrome	Cobalt-chrome-Mo Alloy ASTM 75
	Source: Acnis international Villeurbanne France
Polyethylene	High molecular weight polyethylene (HMPE)
	Source: Biosurface Technologies Corporation Montana USA

Table three summarises the materials that were obtained to stimulate a prosthetic joint replacement. All of the surfaces were obtained from companies that supply the manufactures of prosthetic implants therefore, meeting the requirements for use as medical devices.

3.3.2. Preparation of simulated implant surfaces

In order to ensure there was no variation between the size and surface roughness of the discs obtained following optimisation the results are summarised in the table below.

Table 4. Summary of size and surface area of simulated implant surfaces

	Size		
Surface	Average depth mm	Average radius mm	Surface area mm^2
Titanium	3.95	8.97	727.85 +/- 1.88
Cobalt-Chrome	5.08	8.99	794.65 +/- 3.18
Polyethylene	5.81	10.6	1092.60 +/- 1.38

Table 5. Summary of the surface roughness of simulated implant discs

	Surface roughness	
Surface	Ra	Rq
Titanium	0.551 +/- 0.172	0.698 +/- 0.059
Cobalt-Chrome	0.196 +/- 0.017	0.255 +/- 0.028
Polyethylene	0.669 +/- 0.100	0.789 +/- 0.059

Table 4 and 5 summarise the surface characteristics of the simulated implant surfaces used to evaluate biofilm disruption, including standard deviations seen between the 3 discs. Ra = Average roughness for a given sample length. Rq = Root mean square roughness for given sample length. The discs available were evaluated to ensure conformity allowing direct comparison between the interventions. The variation seen between discs was minimal in terms of their surface topography and size. The Ra and Rq values identified that the Co-Cr surface was the smoothest surface followed by Titanium and Polyethylene.

3.3.3. Optimisation of biofilm formation on simulated implant surfaces.

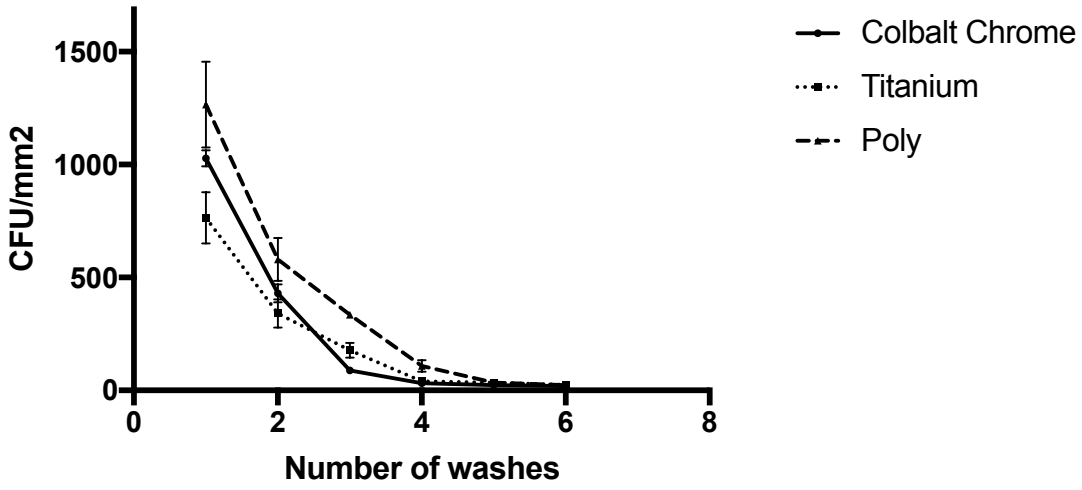


Figure 20. Demonstration of the effect of washing on the number of non-adherent bacteria removed from the simulated implant surface evaluated by CFU/mm^2. Repeated in triplicate (n=3) and presented as the mean and standard deviation

As expected high numbers of CFU were initially washed from the discs in the early washes 1 and 2 from all of the surfaces utilised. Having completed the 6th wash there was no significant further reduction in the CFU released for all of the surfaces. Six washes were therefore chosen for the simulated prosthetic implant surface model.

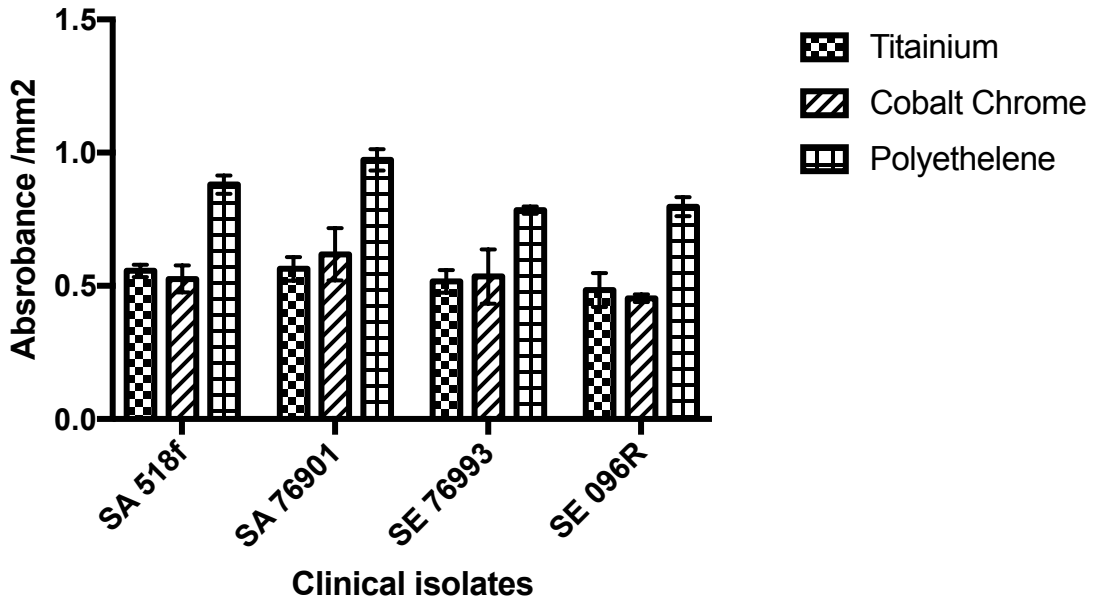


Figure 21. Biomass remaining following washing from each of the isolates utilised on simulated implant surfaces Absorbance/mm^2 Repeated in triplicate (n=3) and presented as the mean and standard deviation

Absorbance levels demonstrate that the polyethylene surface had the highest biomass following the washing stages with cobalt chrome and titanium demonstrating similar readings. Over all *Staphylococcus aureus* and *Staphylococcus epidermidis* biomass was comparable over all of the surfaces utilised.

3.3.4. Calibration of sonication technique for simulated implant surfaces

Energy calculations for the sonication machine were undertaken using a calibrated hydrophone at the School of Electrical and Electronic Engineering at Newcastle University. A frequency of 40 kHz and intensity of intensity 62 w/m2 was demonstrated from the sonication device utilised during the experimental process.

3.3.5. Effect of NucB on bacterial biofilms established on simulated implant surfaces compared to sonication

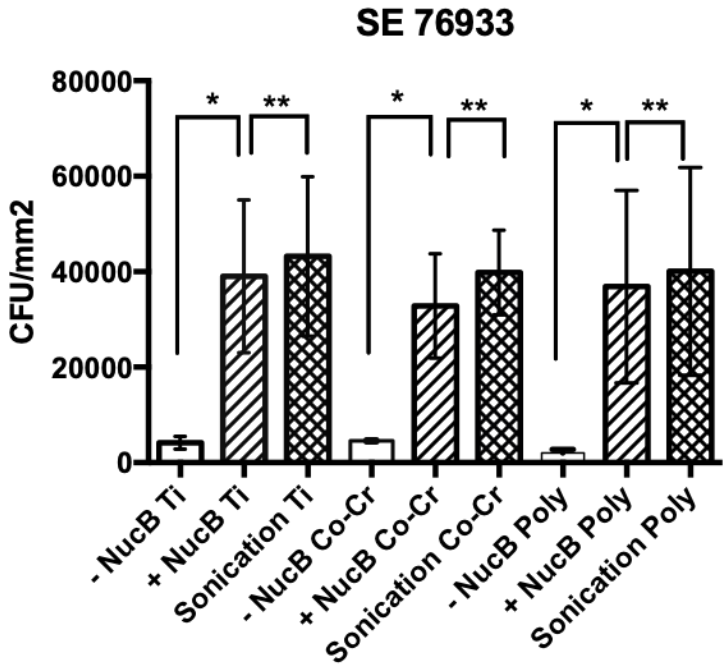


Figure 22. Effect of the presence of NucB on the release of *Staphylococcus epidermidis* 76933 from biofilms grown on simulated implant surfaces compared to sonication. Repeated in triplicate (n=3) and presented as the mean and standard deviation

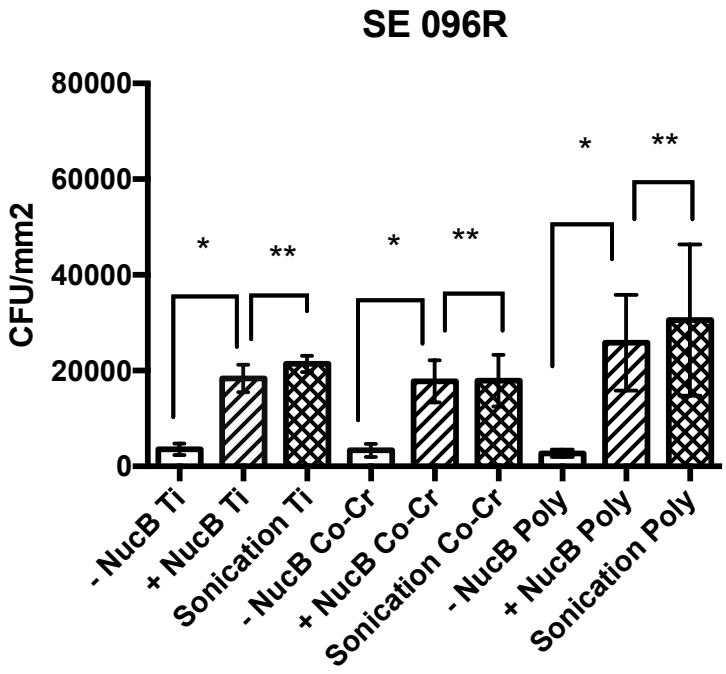


Figure 23. Effect of the presence of NucB in release of *Staphylococcus epidermidis* 096R on biofilm encapsulated bacteria on simulated implant surfaces compared to sonication Repeated in triplicate (n=3) and presented as the mean and standard deviation

Figure 22 and 23 demonstrate the effect of the presence of NucB in release of SE 096R and SE 76933 (clinical isolates) biofilm encapsulated bacteria on simulated implant surfaces compared to sonication. * indicates a significant difference ($P < 0.05$) un-paired t-test ** indicates no significant difference ($P > 0.05$) un-paired t-test Graph plotted with mean and standard deviation

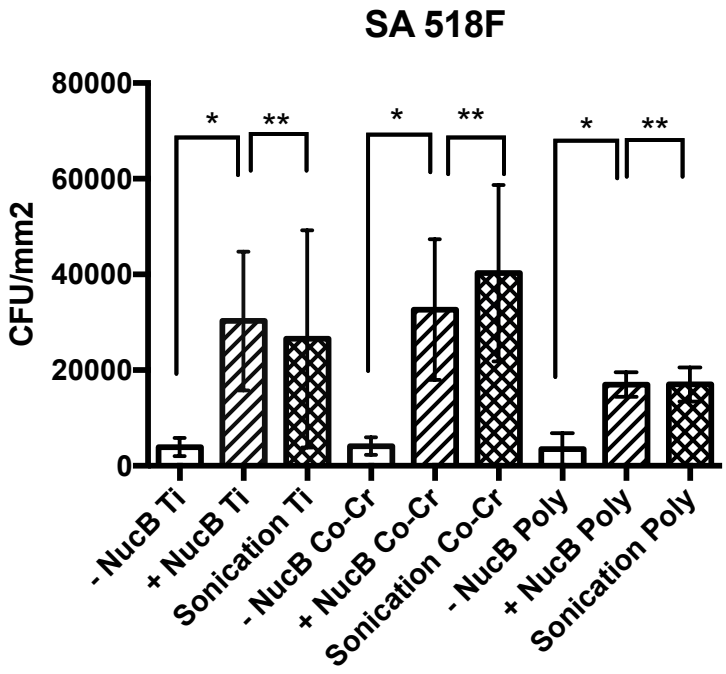


Figure 24. effect of the presence of NucB in release of *Staphylococcus aureus* 518F biofilm encapsulated bacteria on simulated implant surfaces compared to sonication * indicates a significant difference ($P < 0.05$) un-paired t-test ** indicates no significant difference ($P > 0.05$) un-paired t-test. Repeated in triplicate (n=3), graph plotted with mean and standard deviation

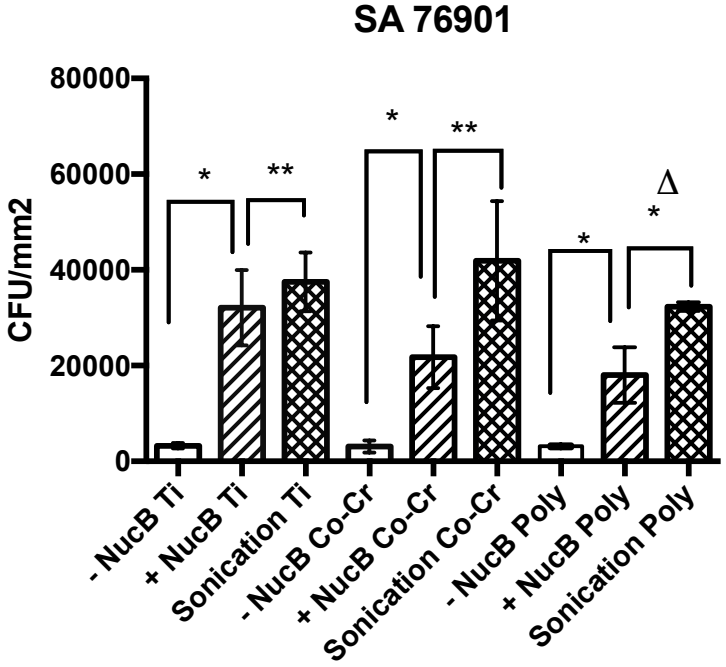


Figure 25. Effect of the presence of NucB in release of *Staphylococcus aureus* 76901 biofilm encapsulated bacteria on simulated implant surfaces compared to sonication. * indicates a significant difference ($P < 0.05$) un-paired t-test ** indicates no significant difference ($P > 0.05$) un-paired t-test. Repeated in triplicate (n=3) , graph plotted with mean and standard. Δ No significant difference on un-paired t-test between NucB and Sonication

The above graphs summarise the effect of NucB on the release of biofilm encapsulated bacteria from simulated implant surfaces compared to a NucB control and sonication. NucB was significantly more effective than NucB control in all isolates and on all surfaces evaluated. NucB was comparable to sonication with no significant differences for both isolates of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolate SA 518F this was evident on all three surfaces. When evaluating SA 76901 on polyethylene surface sonication demonstrated a significant difference compared to NucB treatment (P = 0.014). The other surfaces of Co-Cr and Titanium evaluated with the *Staphylococcus aureus* isolate SA 76901 demonstrated comparable results with NucB and sonication treatment.

3.3.6. Effect of combination NucB and sonication on release of biofilm encapsulated bacteria from prosthetic implant surfaces.

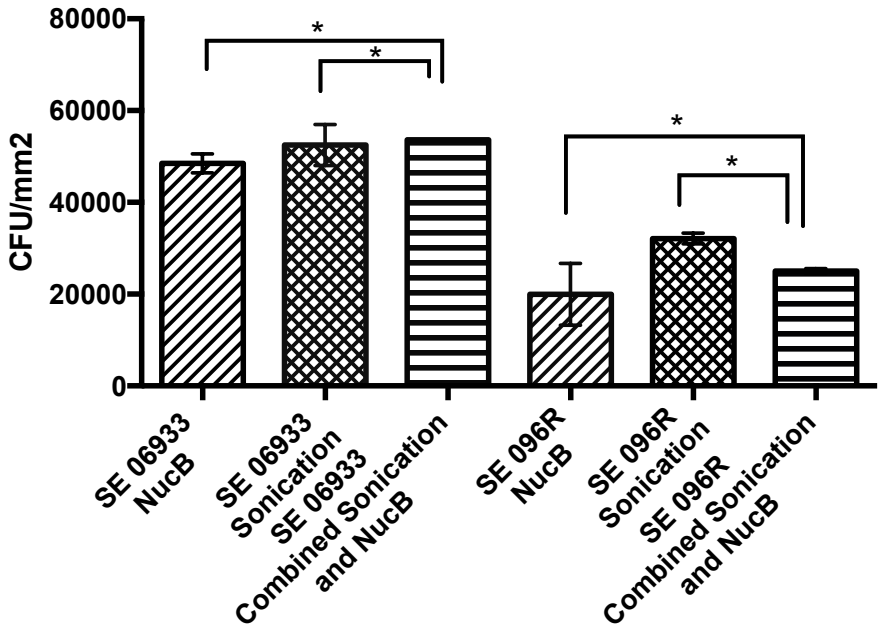


Figure 26. Effect of the combination of NucB and sonication on *Staphylococcus epidermidis* biofilms formed on polyethylene surfaces ** indicates a significant difference (P < 0.05) un-paired t-test * indicates no significant difference (P > 0.05) un-paired t-test. Repeated in triplicate (n=3), graph plotted with mean and standard deviation

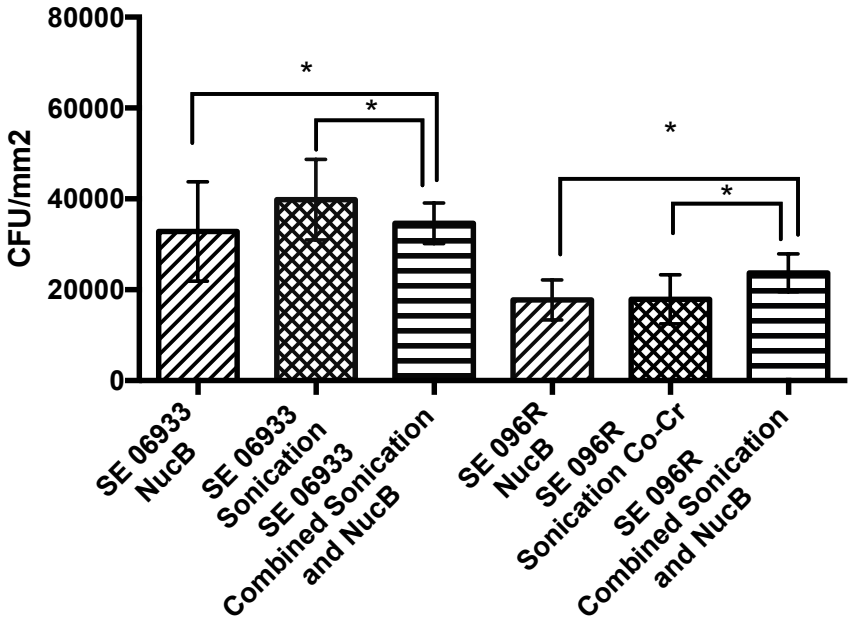


Figure 27. Effect of the combination of NucB and sonication on *Staphylococcus epidermidis* biofilms formed on cobalt-chrome surfaces ** indicates a significant difference ($P < 0.05$) un-paired t-test * indicates no significant difference ($P > 0.05$) un-paired t-test. Repeated in triplicate, graph plotted with mean and standard deviation

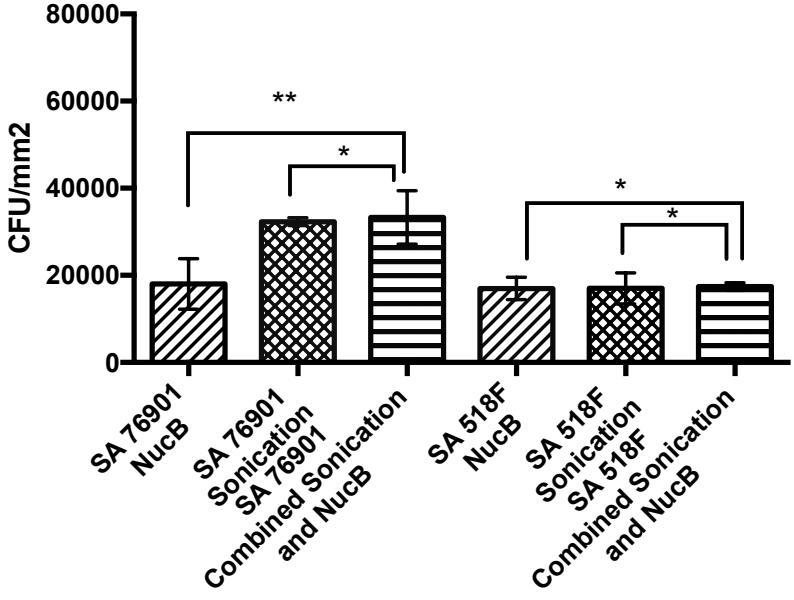


Figure 28. Effect of the combination NucB and sonication on *Staphylococcus aureus* biofilms formed on polyethylene surfaces ** indicates a significant difference ($P < 0.05$) un-paired t-test * indicates no significant difference ($P > 0.05$) un-paired t-test. Repeated in triplicate (n=), graph plotted with mean and standard deviation

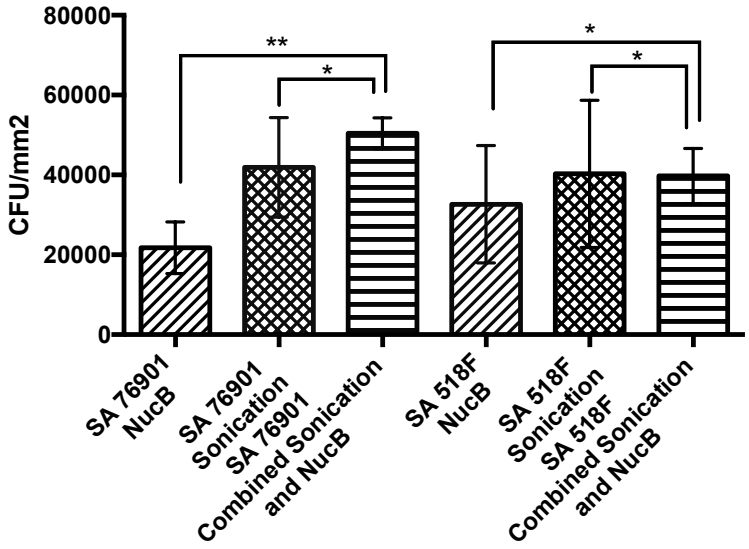


Figure 29. Effect of the combination NucB and sonication on *Staphylococcus aureus* biofilms formed on cobalt-chrome surfaces ** indicates a significant difference ($P < 0.05$) un-paired t-test * indicates no significant difference ($P > 0.05$) un-paired t-test. Repeated in triplicate (n=3) , graph plotted with mean and standard deviation

Combining the NucB and sonication treatment did not significantly increase the yield from the isolates of both *Staphylococcus epidermidis* isolates and *Staphylococcus aureus* isolate SA 518F. There were no significant differences seen between the release of colony forming units when comparing combined NucB and sonication treatment with either standalone treatment with NucB or sonication. *Staphylococcus aureus* isolate SA76901 demonstrated a significant increase in the CFU release when comparing NucB treatment to combined treatment on the surfaces of polyethylene ($P = 0.0352$) and Co-Cr ($P = 0.0027$). No significant differences were seen when comparing sonication and a combination of NucB and sonication for SA 76901 on these surfaces.

3.4. Discussion

3.4.1. The biofilm model chosen for initial evaluation of the action of NucB

In vitro biofilm assays are a crucial first step in the assessment of the therapeutic effectiveness of novel anti- biofilm agents such as the nuclease NucB. The disc model utilised aimed to simulate more accurately a prosthetic joint surface. Similar models have been used to evaluate and compare several anti-biofilm agents in prosthetic joint infection (Bjerkan et al., 2009, Drago et al., 2013, Chen et al., 2016, Prieto-Borja et al., 2017) as well as the evaluation of several other biofilm systems including oral dental health and food production (Kadkhoda et al., 2016, Madeira et al., 2016). However, this model has not previously been used for the evaluation using nuclease activity of biofilm disruption. The disc model chosen ensured the evaluation of the unique prosthetic implant material utilised in clinical practice was reproducible, consistent as well as allowing the comparison with the sonication technique. Currently no in vitro model exists that can simulate the biomechanical environment within a prosthetic joint replacement (Drago et al., 2013). Other models do exist to evaluate the biofilm formation. These however would not have allowed the elevation of the specific implant surface used in hip and knee arthroplasty nor would they have more closely simulated the replaced implant (Coenye and Nelis, 2010, Coenye and Bjarnsholt, 2016).

The disc biofilm model utilised three surfaces. Titanium, Cobalt-Chrome and Polyethylene. These surfaces are commonly used in prosthetic joint replacement as previously discussed (Ribeiro et al., 2012). Stainless steel while still routinely used was difficult to source for evaluation. Several steel formulations were available however, 316L used for prosthetic joint replacement was only available in industrial quantities and was therefore not evaluated in this study. All of the other materials sourced were from suppliers that supplied the implant manufactures. Evaluation of the surfaces (table 4 and 5) demonstrated high concordance and tolerance in terms of variation of disc size and surface topography. Several techniques are available for the evaluation of surface topography the Ra and Rq values are accurate and frequently used to evaluate surface characteristics in orthopaedic implant surfaces (Munir et al., 2015). The surface roughness measured as Ra and Rq values achieved were comparable to the surface roughness of explanted joints and other studies utilising disc models for PJI (Bjerkan et al., 2009, Sorrentino et al., 2018). Surface roughness is one of the key components of bacterial biofilm initial adhesion and subsequent biofilm attachment

(Bazaka et al., 2011, Rochford et al., 2012). One advantage of this disc model was the high fidelity of the surface topography and disc material compared to actual joint prosthesis which other studies have not achieved (Drago et al., 2012). These subtle but important changes in material use as discussed can have a dramatic influence on the biofilm characteristics and therefore the subsequent interpretation results.

3.4.2. Optimisation of the disc model for biofilm evaluation

Having sourced the disc surfaces, optimisation of the biofilm disc model was undertaken. Of particular interest was the number of washing steps required to remove non-biofilm encapsulated bacteria. A review of several studies demonstrated variation in the numbers of washing steps using a comparable model. Drago et al using similar clinical isolates advocated three washes (Drago et al., 2012) while Bjerkan et al utilised six (Bjerkan et al., 2009). Optimisation of the washing steps (summarised in figure 20) demonstrated that six washes removed non-adherent bacteria. Several published studies using a disc model did not optimise the number of washing steps. Failure to remove the non-biofilm associated bacteria will lead to a subsequent over estimation of the effectiveness on any intervention when evaluating the retrieval of colony forming units. Examination of the subsequent biomass formation following the washing steps (summarised in figure 21) demonstrated that per mm^2 the polyethylene surface was the most heavily colonised in all isolates. Current literature would support this finding; the polyethylene surface was the roughest surface and surface roughness has previously been described as one of the strongest factors for bacterial biomass formation. (Ribeiro et al., 2012). The hydrophilicity of polyethylene is important in the initial bacterial attraction to the implant surface and is another factor in increasing bacterial biofilm formation for this surface (Banche et al., 2014).

3.4.3. Sonication technique adopted for evaluation of biofilm disruption

Significant variation exists in the sonication techniques utilised within the published literature (Monsen et al., 2009). Several variables exist including the sonication energy that the implant or discs are exposed to. For this study, the original technique reported by Trampuz et al was adopted (Trampuz et al., 2007). A review by Monsen *et al* demonstrated that the optimal sonication energy to maintain bacterial viability was 40 kHz (Monsen et al., 2009). Other studies have adopted a lower sonication energy of 30 kHz (Drago et al., 2012, Bjerkan et al., 2009). Sonication itself is bactericidal and this effect has historically been used in the control of bacterial biofilms in manufacturing and food industry (Burleson et al., 1975). It is also utilised in the sterilisation of medical instruments (Jatzwauk et al., 2001). Monsen *et al* demonstrated that ultrasonic energy had a potential inhibitory effect on bacteria. Erriu *et al* demonstrated that Gram-negative bacteria such as *Escherichia coli* which are known to cause prosthetic joint infection, are particularly sensitive with short exposures proving bactericidal (Erriu et al., 2014).

3.4.4. Effect of NucB on bacterial cell release and comparison to sonication

Having established a reliable model, the main focus of this chapter was to evaluate the effectiveness of NucB on the release of encapsulated bacteria. Overall, (summarised in figures 22 – 25) NucB demonstrated a statistically greater release of bacteria when compared to a non-NucB control. The non-NucB control group did demonstrate a detectable release of CFU from the discs but to a lesser extent. This may be related to the release of residual bacteria that were not biofilm encapsulated and were not removed during the washing step or the mechanical effect of the tilt table that the NucB and NucB control experiments were exposure to could be responsible for the back ground CFU released. Nevertheless, the natural cycle of the biofilm is of dispersal and flocculation. As previously mentioned the biofilm community is known to degrade its own biofilm to allow colonisation of new environments (Stoodley et al., 2002) This forms and essential part of the bacterial biofilm community (Costerton et al., 1999, Hall-Stoodley et al., 2004). Several studies have used a comparable model but have not included a control group for the intervention they are evaluating. Instead their intervention is compared to another technique for removal of biofilm such as scraping, sonication, DTT or NAC (Bjerkan et al., 2009, Drago et al., 2012). Bjerkan and Drago *et al* both demonstrated that sonication was effective at removal of biofilm encapsulated

bacteria from discs of a simulated implant surface. Differences in methodology prevent direct comparison, however the effectiveness of sonication was again demonstrated in this study.

Sonication was more effective in the release of biofilm encapsulated bacteria of the clinical isolate of *Staphylococcus aureus* SA 76901 on the surface of polyethylene. On the remaining surfaces of titanium and Co-Cr NucB and sonication demonstrated no significant difference for the same isolate. This would suggest that the SA 76901 isolate forming of polyethylene discs were more resistant to the effect of the nuclease. This may relate to the composition of the biofilm forming on polyethylene and its dependence on eDNA. The composition of the biofilm matrix is known to vary relating to several factors including the host surface (Costerton et al., 1999, Biswas et al., 2006, Arciola et al., 2012). This is related to the expression of adhesion molecules which are up or down regulated by the same isolate depending on environmental pressures. The nuclease NucB however, was for this isolate, on all surfaces more effective than the NucB control. The combination of sonication and NucB as expected therefore showed a slightly greater effect on the isolate *Staphylococcus aureus* SA 76901 (figure 28) on polyethylene but also for the same isolate on Co-Cr. This would suggest that overall biofilms of this isolate may be more resistant to nuclease mediated degradation, while still producing a significantly greater response overall compare to the NucB control. Sonication seemed to demonstrate less variation on the isolates investigated. This could be expected due to the mechanical nature of its action. However, as discussed, this mechanical action may be bactericidal reducing the number of CFU identified. Sonication could therefore demonstrate a degree of variability in viable CFU especially in gram negative bacteria (Erriu et al., 2014). The combination of NucB and sonication therefore may have resulted in a reduction of CFU recovered since the sonication energy may have killed bacteria already released from the biofilm by the action of the nuclease. However, this effect was not demonstrated.

3.5. Conclusions

This chapter has developed from the initial evaluation of NucB in chapter two. Having developed and evaluated the surface model of PJI, NucB was able to demonstrate a statistically greater number of CFU released when compared to controls. Overall, NucB was able to release comparable CFU to sonication from the majority of clinical surfaces evaluated. The combination of sonication and NucB improved the release of one isolated of *Staphylococcus aureus* from the surfaces evaluated when compared to NucB treatment alone. The results from chapters two and three have demonstrated for the first time the effectiveness of the nuclease NucB on the release of biofilm encapsulated bacteria from isolates commonly seen PJI joint infection. The demonstration that nuclease remains effective at bacterial release when biofilms have been established on the more complex surfaces of Chrome, Titanium and Polyethylene provide confidence in the further development of the use of NucB in the diagnosis of infection in PJI. This potential will be further evaluated in Chapter four.

4. Chapter 4. Release of biofilm encapsulated bacteria from explanted joints: a comparison of NucB and sonication

4.1 Introduction

Having established an in-vitro model of an infected prosthesis using simulated implant surfaces, a process was developed for evaluation of NucB on explanted joint prostheses. Chapters 2 and 3 evaluated the effect of NucB on the clinical isolates and reference strains for the most common causative organisms in PJI. Furthermore, the use of the simulated joint surfaces allowed the comparison of the NucB technique to sonication as well as creating a higher fidelity model of PJI. Within these experimental setup NucB was able to demonstrate its effectiveness. The subsequent challenge therefore is the evaluation of NucB on the actual explanted joint prosthesis from patients undergoing revision surgery. This will exposure the NucB technique to much wider selection of causative organisms and the actual clinical entity for which it may have a potential role in the diagnosis of infection. An initial review and assessment was undertaken into the standard processing of microbiology samples for PJI within the NHS and in particularly Northumbria Healthcare NHS Foundation Trust. Prospective ethical approval was obtained to recruit a cohort of patients undergoing revision surgery for joint arthroplasty of the hip and knee. The implants recovered were assigned to either NucB, non-NucB control or sonication treatments and the CFU from each technique recorded. Key to the potential use of NucB to assist in the diagnosis of PJI within the NHS is the ability of the NucB treated samples to be incorporated into automated microbiological culturing system Bactec ® system. The compatibility of NucB and the Bactec ® system was evaluated and an initial review into how NucB maybe combined with the current microbiological samples taken to evaluate PJI.

4.2 Material and Methods

4.2.1 Ethical approval for use of explanted joint prostheses

Ethical approval was granted for a prospective cohort study via a substantial amendment evaluating a potential biomarker for prosthetic joint infection (IRAS project ID: 133171) This was granted by NRES Committee North East - Tyne & Wear South REC reference: 13/NE/0270. Ethical approval was obtained for a prospective study to investigate the potential of a novel diagnostic technique on explanted joints recovered at the time of revision surgery Recruitment commenced on the 7th June 2015 with the study closing on the 30th June 2016.

4.2.1 Evaluation of current standard practice for the identification of prosthetic joint infection within Northumbria Healthcare NHS Foundation Trust.

Prior to planned revision surgery for joint replacement a standardised assessment of the patient was undertaken. This practice is based upon guidance provided by the UK Standards for Microbiology Investigations (SMIs) (SMI-B44, 2017). Patients underwent clinical evaluation in terms of history and clinical examination. Plain film radiographs and baseline blood tests including inflammatory markers were obtained. Routinely all patients were discussed at a multidisciplinary meeting involving orthopaedic surgeons, infectious disease doctors, microbiologists and pharmacists. The surgical plan and perioperative antibiotic treatment plan was decided based upon this discussion.

Within Northumbria Healthcare NHS Foundation Trust the majority of the planned revision patients undergo diagnostic synovial fluid aspiration using an effective and dedicated theatre list aspiration service undertaken in sterile conditions (Tingle et al., 2016). Subsequent microbiological culture was undertaken to identify any evidence of prosthetic joint infection prior to revision surgery. To optimise identification of infection, antibiotics were withheld for at least two weeks prior to aspiration. Routinely a combination of multiple deep soft tissue samples and synovial fluid were sent for microscopy, culture and testing for antibiotic sensitivity. Samples were sent separately in sterile universal containers. The samples transferred to the microbiology laboratory for processing.

4.2.2 Development of standard operating procedures for collection and transportation of clinical samples

Following the identification of a suitable patient by the clinical team and successful recruited by the research nurse a member of the laboratory research team was informed. A unique study patient ID was assigned to each patient recruited, and this was used to identify the implant for the purposes of research. The nominated member of the research team arrived at the operating theatre with a pre-sterilised and dried Pathopax container (Pathopax DGP Intelsius Ltd 1 Harrier Court, Airfield Business Park, Elvington. York UK) which was used to collect and transport the samples. These were wrapped in two layers inner green and outer blue Suradrape (Westfield Medical Limited Second Avenue, Westfield Trading Estate, Midsomer Norton, Radstock, BA3 4DP) with autoclave marking tape. The Pathopax containers were autoclaved within Newcastle University; heat dried and allowed to return to room temperature before use. The outer blue layer was removed ensuring positive colour change from indicator tape and ensuring no holes in the inner green layer. Sterile gloves were applied, and the inner green layer opened just prior to transfer of the explanted joint prosthesis. The researcher stood outside of the laminar flow surgical field. Once explanted from the patient the implant was kept within the sterile operative field. As soon as appropriate it was passed out by the scrub nurse in a sterile fashion to the researcher outside of the surgical field, but within the laminar flow. The lid was closed and the sample was further wrapped and labelled with the Study ID before being placed in a transport bag for transportation to Newcastle University. These steps are summarised in figure 30.

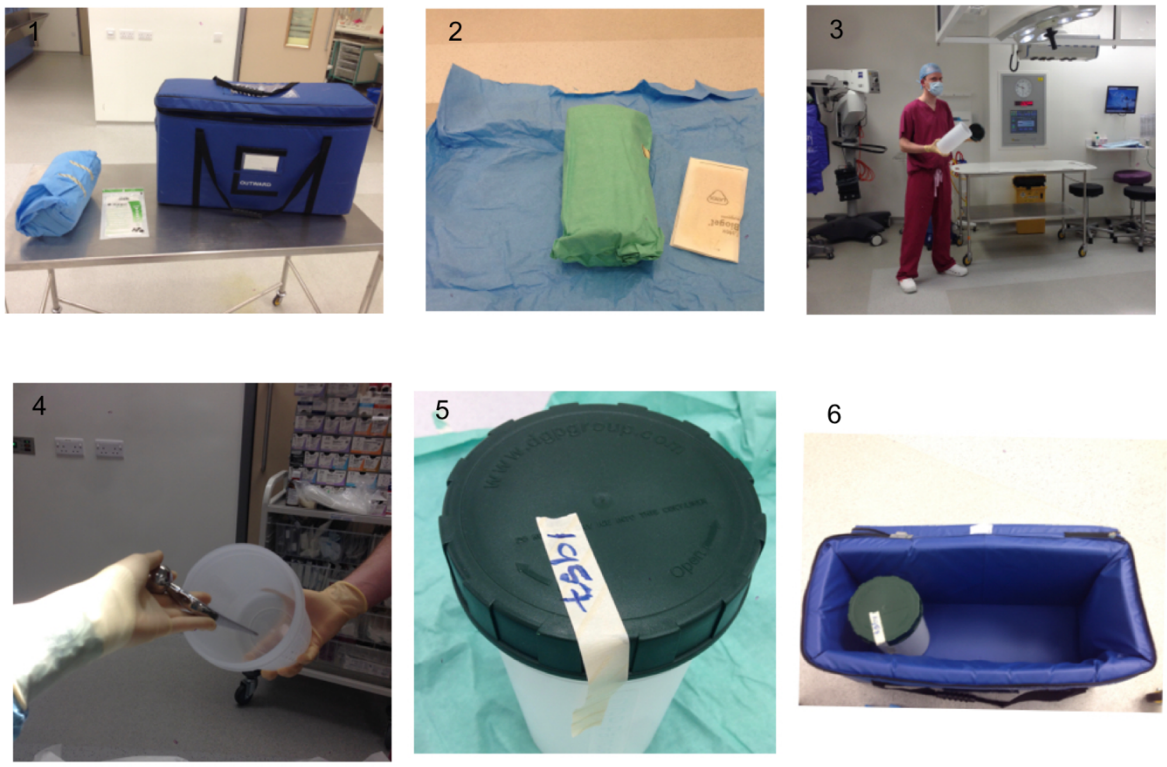


Figure 30. Summary steps in the collection of the explanted prosthesis to ensure sterile transfer for subsequent processing.1) equipment required. 2) preparation for receiving implant and ensuring container sterility. 3) positioning of researcher for implant transfers. 4) transfer of implant from surgical team into sterile plastic container. 5) labelling of sample. 6) packing of sample for transfer.

In order to ensure safe transportation, the UK Health & Safety Executive guidance was adopted primarily legislation UN 3373 (HSE-3373, 2016). The Pathopax containers utilised complied with UN 3373 covering the packaging and transportation of diagnostic specimens. The containers utilised also where CE marked confirming they were leak proof and complied with the EU in vitro Diagnostic Medical Devices Directive 98/79/EC Annex 1 B 2.1 (EU-DMDD, 2016). Once packaged the samples were transferred to Oral Biology Laboratory within the School of Dental Sciences at Newcastle University for further evaluation.

4.2.3 Processing of samples within the NHS.

Within Northumbria Healthcare NHS Foundation Trust, standard operating procedures exist for the evaluation of prosthetic joint infection at the time of surgery. Routine practice for any revision procedure requires microbiological evaluation of the periprosthetic soft tissue lying close to the revised implant. Samples of soft tissue are harvested using a sampling set with separate instruments for each sample to avoid contamination, and are taken from discrete areas around the joint. Separate fluid samples are sent if present and often inoculated into blood culture bottles (BD Bactec®). About 5-7 samples were sent from each case with an average size of $1cm^3$. Samples were placed into separate sterile universal containers labelled with patient identifying labels and then transferred via the internal porter staff to the clinical microbiology laboratory.

Within the laboratory, samples were processed within a class two cabinet and using sterile instruments used to prevent contamination. The soft tissue samples were processed for routine culture and sensitivity with prior homogenisation. Within the sterile cabinet, large pieces of tissue were placed in a sterile petri dish and cut into smaller pieces. These were aseptically added to a universal container containing 1ml of sterile saline and glass beads using sterile disposable forceps. The sample was then vortexed for 15 seconds. Following this, using single individually wrapped sterile pipettes, the resultant fluid was drawn up and used to inoculate each agar plate (LP-MIC-FLU-271, 2017). The agars used are summarised below and including an enrichment broth in the following order, and then spread with sterile loops.

Robertson's cooked meat broth	5 pipette drops (if sufficient)
Chocolate blood agar	1 pipette drop
Blood agar	1 pipette drop
FAA + 5µg metronidazole disc	1 pipette drop

Summary of the agar growth media used for prosthetic joint sample culture within the NHS

Following inoculation, the plates were placed into either aerobic or anaerobic incubators culture for 14 days at $37\ ^0C$. If culture positive single colonies were isolated for identification using the automated VITEK 2 system (bioMérieux, Marcy l'Etoile, France). Fluid samples that have been pre-inoculated into blood culture bottles in theatre were processed using the semi-automated BD Bactec® FX system (Becton Dickinson and Company, 1 Becton Drive Franklin Lakes, NJ USA). Samples arriving

to the laboratory were loaded into the BACTEC machine which is incubated at 36 ^{0}C and agitated for maximum recovery or organisms. The presence of microorganisms was detected via an automated photo-detector which monitors a colour change to a CO_2 sensitive maker in the bottom of the BACTEC bottle. Metabolic activity of the isolated organisms drives the formation of CO_2 prompting the detectable colour change. Positive cultures are flagged to the Biomedical staff who undertake the confirmation, isolation and identification of the organisms via VITEK 2 system.

4.2.4 Processing of samples at Newcastle University

Samples were transported to the Oral Biology Laboratory within Newcastle University for processing. Microbiological analysis was carried out in a class II cabinet, using aseptic technique to reduce the risk of contamination of the sample. Prior to analysis the cabinet was cleaned according to existing laboratory protocols. The explanted prostheses were processed with either NucB treatment or Non-NucB control. If a third sample was available this was evaluated with sonication.

4.2.5 Nuclease NucB treatment of explanted prosthetic joints

The reaction mixture for the NucB was prepared prior to the exposure to the explanted joint. Pre aliquoted 150 µl stock solutions of 100 µl/ml NucB enzyme was removed from -80^{0}c storage and allowed to thaw at room temperature. Prior to use 0.75 mL tris Buffer pH8.0 and 6.6 mL $MnSO_4$. (Stock 100mM) and 7.5 ml sterile distilled water were then combined, vortexed and then brought to room temperature. The final step was addition of 150 µL NucB (100 µl/ml), and gently mixed giving a final reaction volume of 15ml. The final reaction volume equated to 5mM $MnSO_4$ concentration required for for optimal NucB activity.

The explanted joint prosthesis was transferred into a sterile sampling bag (Fisher Scientific Twirl 'EM' sampling bag UK Fisher Scientific Ltd Bishop Meadow Road Loughborough LE11 5RG) with the use of sterile gloves within the class II cabinet. The NucB solution was then added to the bag with excess air removed prior to sealing the bag. The bag containing the explanted joint was then transferred to a tilt table at room temperature at 10 tilts per minute (Heidolph polymax 1040 Heidolph UK – Radleys Shire Hill Saffron Walden Essex, CB11 3AZ) and left for one hour.

The component of the explant joint replacement assigned to the non-NucB treatment was processed in an identical fashion using the reaction mixture listed above without the active addition of NucB with the residual volume reconstituted with sterile water. Once reconstituted, the implant was placed on the tilt table in an identical fashion.

4.2.6 Sonication treatment of explanted prosthetic joints

Sonication treatment was only used if there was a third component available for processing. This was undertaken using a modified technique based on Trampuz *et al* (2007) 400mls of pre-aliquoted and sterilised PBS solution was placed into a sterilised open mouthed 'Pathopax' container. The container was then vortex for 30 seconds using a Vortex-Genie 2 (Scientific Industries) before being transferred to the sonication bath (Engisonic Engis Ltd, Kent, UK) for 5 minutes, followed by subsequent vortexing for 30 seconds. Resultant supernatants from all processes were then processed in a similar fashion. Then 0.5 ml aliquots were taken from each of the processed samples, inoculated and spread onto tryptic soy agar, blood agar, chocolate agar plates and fastidious anaerobic Agar (EU-DMDD, 2016). A further 1 ml from each of the sample fluids was inoculated into 6mls sterile TSB, enrichment and anaerobic media. Plates and liquid cultures were then labelled and sealed before being transferred for aerobic or anaerobic incubation (Baker Ruskinn Bugbox Plus Ruskinn Technology, Ltd. 8 & 9 York Park Bridgend Industrial Estate, Bridgend South Wales) at 37 ^{o}C. Plates were checked on a daily basis for culture growth. If the cultures were positive the number of colony forming units were recorded and the plates reviewed by visual inspection for the presence of poly microbial infection. Three representative colonies were inoculated into the same sterile growth media as that the isolated were identified on and cultured for 24 hours prior to the creation of a glycerol stock which when was then stored at -80 ^{0}C. The liquid cultures were reviewed daily for evidence of organism growth. If growth was suspected, 0.5ml from the liquid media was inoculated onto agar plates and following further incubation, representative colonies were chosen in a similar fashion to create a glycerol stock. Following 14 days of culture, 0.5mls of all the liquid media samples were inoculated onto sterile media plates and cultured for a further 24 hours. If there was no growth at this stage the samples were discarded. All organisms isolated were reconstituted onto agar slopes and transferred to Newcastle upon Tyne Hospitals NHS Foundation Trust Microbiology Department at Freeman Hospital for identification by MALDI-TOF (Carbonnelle et al., 2011).

4.2.7 Method to evaluate compatibility with automated NHS culture and presence of NucB.

To evaluate the effect of NucB on the automated culture systems commonly used within the NHS (Minassian et al., 2014), the following experiments were undertaken using the Bactec® FX system (Becton Dickinson and Company, 1 Becton Drive Franklin Lakes, NJ USA). Clinical samples of *Staphylococcus epidermidis (SE 096R)* and *Staphylococcus aureus* (*SA 518F)* were utilised. A comparable method was used from chapter two (2.2.6) evaluating the effect of the presence on NucB on bacterial growth. The clinical isolates were established from glycerol stocks on to TSA plates and statically incubated overnight at 37 ^{0}C. Representative colonies were chosen and inoculated into 5mls of sterile TSB and again incubated overnight at 37 ^{0}C. Using a spectrophotometer the absorbance at 600 nm each media concentration was then inoculated with the re-suspended culture to create an optical density of 0.01. Having standardised the inoculum, 2.5 ml were transferred into sterile falcon tubes and incubated for 20 minutes at room temperature with and without the presence of NucB at a concentration of 1000 ng/ml in the presence of 5mM of $MnSO_4$. A separate sample containing sterile TSB solution used for the dilution of the culture stocks was included with NucB processing. The final samples were inoculated into Bactec ® bottles. Culture bottles were pre-labelled with unique research identifiers. The cover caps were removed, and the injection portal cleaned with pre-impregnated ethanol wipes. Using a sterile needle and syringe, 5mls of the culture media was inoculated into each pre-labelled culture bottle. Samples were gently mixed and packaged. The samples were transferred to Newcastle upon Tyne Hospitals NHS Foundation Trust microbiology department for processing according to standard NHS SMI protocols (SMI-B44, 2017).

4.2.8 Development of a randomisation strategy for treatment of explanted joints

To offer a randomised approach to the allocation of the explanted implants a randomisation schedule was established for both hip and knee joint replacements. This was developed following discussion and assistance from Mr Keith Gray a clinical statistician from the Research and Development department within Northumbria Healthcare NHS Foundation Trust. If one sample was retrieved this was processed with NucB treatment. If two samples were retrieved the samples were allocated according

to the group randomisation table corresponding to the number of the research samples. If three samples were retrieved the sample that remained was allocated to sonication treatment. This formed a group or block randomisation strategy with the aim of dividing the samples available randomly while trying to provide a balance of implants allocated treatment protocols (Moher et al., 2012).

4.2.9 Methods used for the identification and recruitment of participants.

Patients were identified on a weekly basis if undergoing planned revision surgery for either a hip or knee joint arthroplasty. Potential patients were identified from the scheduled theatre lists by the clinical team and approached prior to surgery by a dedicated research nurse. Patients presenting acutely and requiring revision surgery were identified by the on call surgical teams during the daily trauma meetings. These patients were either approached by the research nursing team or a member of the surgical team to discuss potential participation. Patient information sheets were provided and patients were recruited according to Good Clinical Practice (GCP) guidelines (MHRA, 2017). Patients were included in the study if they were aged over 18, were able to provide informed consent to participate and if the planned surgery required either exchange or removal of part of a joint arthroplasty from the hip or knee.

4.3 Results

4.3.1 Current UK practice for diagnosis of prosthetic joint infection

The current practice for the collection, transportation and processing of clinical samples retrieved for the diagnosis of prosthetic joint infection is covered by the UK Standards for Microbiology Investigations - Investigation of orthopaedic implant associated infections B44 produced by Public Health England (B44, 2017). These documents were updated during the time period of the MD project, however no significant changes were made to the recommended culture methods used. Northumbria Healthcare NHS Foundation Trust are compliant with the recommendations in sample processing. Methods used within the Trust were reviewed with the microbiology team within Northumbria Healthcare NHS Foundation Trust and discussed with the Consultant microbiology lead. Whist not identical, both sample processing with the NHS and the research laboratory were deemed comparable.

4.3.2 Recruited participants and subsequent treatment analysis

A total of 24 patients were recruited into the study enabling 66 separate implants to be evaluated. This flow diagram summarises the recruited patients and samples.

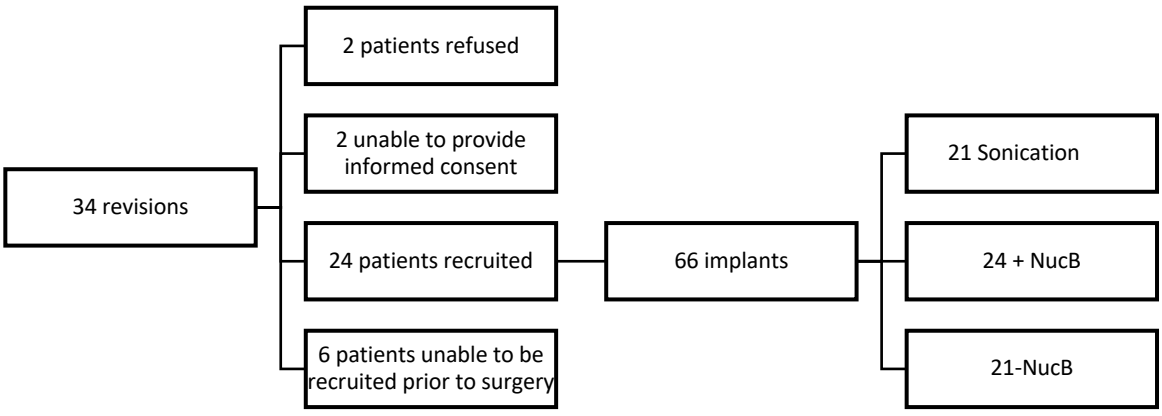


Figure 31. Flow diagram summarising the patients screened during the study period and allocation to treatment.

During the study period a total of 34 patients were screened. Two patients refused their consent to participate in the study, two were unable to provide informed consent and six patients were unable to be approached in a timely fashion prior to their surgery. Twenty four patients were therefore recruited allowing the processing of 66 implants during the study and these were divided between NucB and a non-NucB control and with sonication treatment if a third component of the explant joint was available.

4.3.3 Recruited participant demographics

At total of 24 patients were recruited into the study. This patient group was composed of 14 males and 20 females. The average age at the time of revision surgery was 79 with a range of 54-94. The American Society of Anesthesiologists (ASA) (Doyle, 2017) grade for each patient is presented below giving an appreciation of general health of the patients undergoing revision surgery. Of the patients recruited in to the study 5 were diabetic, 1 patient had a diagnosis of rheumatoid arthritis and three patients were active smokers.

Table 6. Summary of ASA grades of patients undergoing revision surgery.

(ASA)	Definition	Number of patients
1	Normal Healthy patient	0
2	Mild systemic disease	7
3	Systemic disease not incapacitating	15
4	Incapacitating disease that is a threat to life	1
5	Moribund patient unlikely to survive > 24 hours	0

Table 6 summarises the patient anaesthetic related score - American Society of Anesthesiologists (ASA). This provides a validated assessment of the patient's general health at the time of surgery.

4.3.4 Summary of prosthetic joints recovered and indication for revision

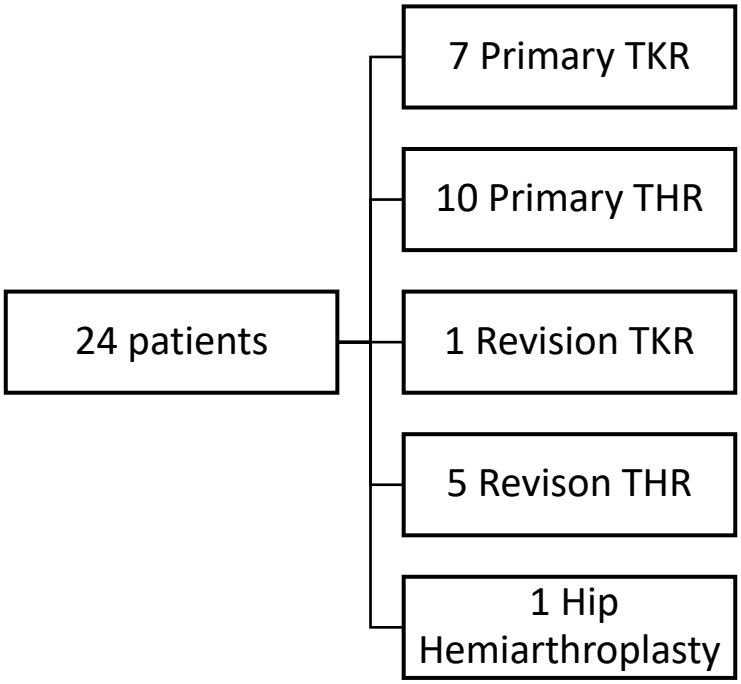


Figure 32. Summary of the prosthetic joints available from the 24 patients recruited

Figure 32 summarises the 24 patients that provided explanted joints for evaluation. The majority of joint replacements were either primary hip total replacements or primary total knee replacements. i.e. those patients who have only had one prosthetic joint replacement to the index joint prior to their revision surgery. The remainder were either revision joint replacement surgery i.e. further surgery on a joint that has been previously revised or hip hemiarthroplasty which is a monoblock replacement of the femoral head with no prosthetic replacement to the acetabulum. These implants are frequently used for patients that have fractured their femoral neck following a fall. The most common surgical intervention for the planned revision was a single stage revision. The numbers of each surgery are summarised below.

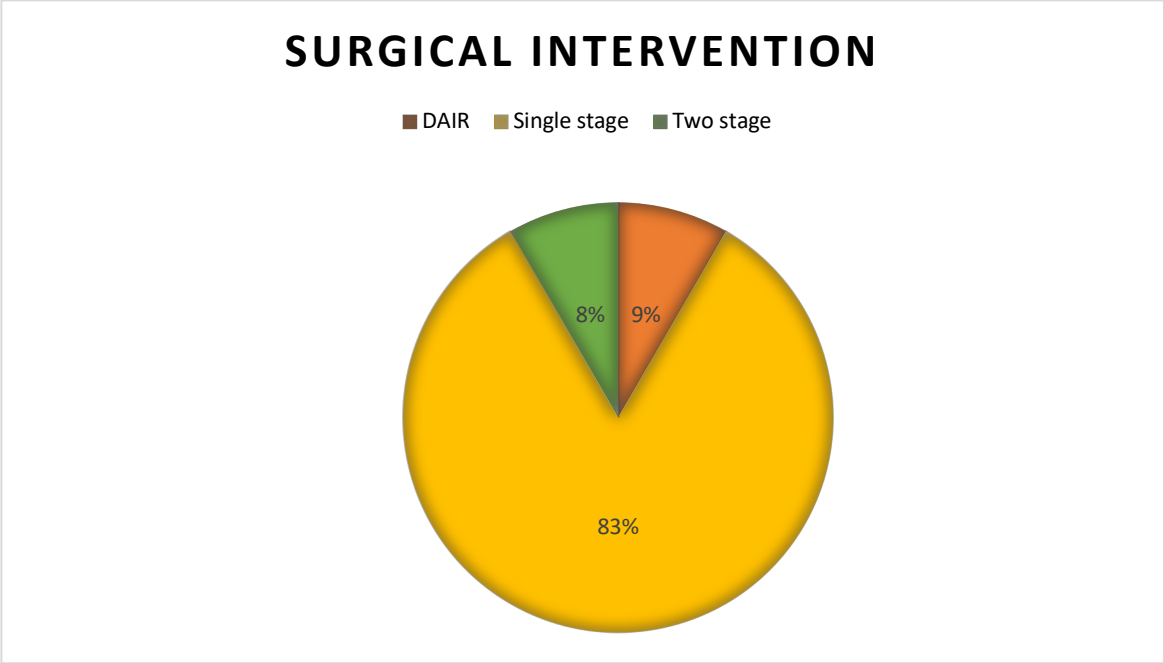


Figure 33. Summary of the interventions undertaken at the time of revision surgery

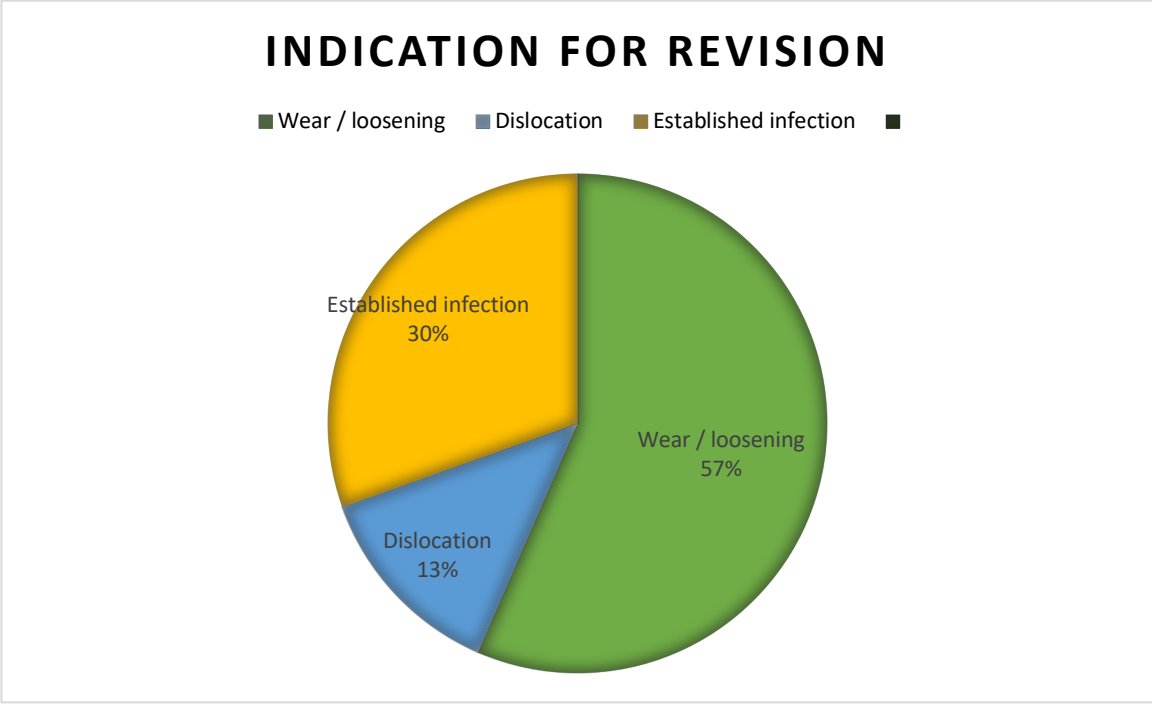


Figure 34. Summary of the indication for revision surgery of recruited patients.

Table 7. Summary of patients recruited into the study identifying their reason for revision presentation type, if the patients were on antibiotics prior to the time of their revision and the preoperative CRP

Reason for revision	Presentation	Antibiotics two weeks prior	CRP (mg/l)
Infection	Acute	no	130
Infection	Acute	no	101
Infection	Acute	yes*	106
Infection	Chronic	no	60
Infection	Chronic	no	27
Infection	Chronic	no	47
Infection	Chronic	no	55
Wear	Chronic	no	<5
Wear	Chronic	no	<5
Wear	Chronic	no	6
Wear	Chronic	no	<5
Wear	Chronic	no	<5
Wear	Chronic	no	<5
Wear	Chronic	no	<5
Wear	Chronic	no	7
Wear	Chronic	no	<5
Wear	Chronic	no	6
Wear	Chronic	no	<5
Wear	Chronic	no	<5
Wear	Chronic	no	not available
Wear	Chronic	no	<5
Dislocation	Chronic	no	<5
Dislocation	Chronic	no	<5
Dislocation	Chronic	no	not available

One of the patients recruited into the study was on antibiotic therapy at the time of their revision surgery. This patient underwent debridement and exchange of mobile components for a total knee replacement (DAIR). This was processed using NucB exposure and gave a positive culture of *Staphylococcus epidermidis*

In table 7 it is demonstrated that the majority of patients undergoing revision presented with a chronic duration with their symptoms and / or date of the index surgery being greater than six weeks after their revision. All patients that underwent a planned revision procedure for established infection met the MSIS major criteria for infection. The majority of patients did not have an elevated C-reactive protein (CRP). In the case of 2 patients recruited into the study a CRP value was unavailable. All patients that

underwent a revision surgical procedure for suspected infection had a significantly elevated CRP. While the remaining were mildly elevated or normal CRP levels below the MSIS minor diagnostic criteria threshold of >10 mg/l (Deirmengian et al., 2014).

4.3.5 Summary of clinical isolates recovered

Table 8. Summary of the clinical organism identified from the research samples

Isolates recovered	Number of isolates
Bacteria (gram positive)	
Staphylococcus warneri	1
Streptococcus dysgalactiae	1
Staphylococcus epidermidis	4*
S. aureus, methicillin - sensitive (MSSA)	1
Enterococcus faecalis	1
Fungal	
Candida albicans	1

* A total of 8 infected cases were identified however, one case was a polymicrobial infection of *Candida albicans* and *Staphylococcus epidermidis*

Table 8 summaries the organisms that were obtained from the research samples. The majority of these were *Staphylococcus epidermidis.* No gram-negative organism were identified. One sample was identified as a fungal organism.

4.3.6 Comparison of NucB compared to sonication for identification of biofilm encapsulated bacteria from explanted prosthetic implant surfaces

Of the 24 patients that were available for evaluation it was possible to isolate organisms from 8 patients using either + NucB, – NucB or sonication. The remaining 18 patients explanted implant surfaces were negative on all culture techniques matching the standard NHS results. When comparing the standard NHS techniques for the processing of infected samples within Northumbria NHS Foundation Trust and the NucB or sonication techniques used within this study, there were no samples with a positive culture from the NHS diagnosis that were not detected by either NucB or sonication treatment.

4.3.7 Review of the culture positive cases comparing NHS and Research samples

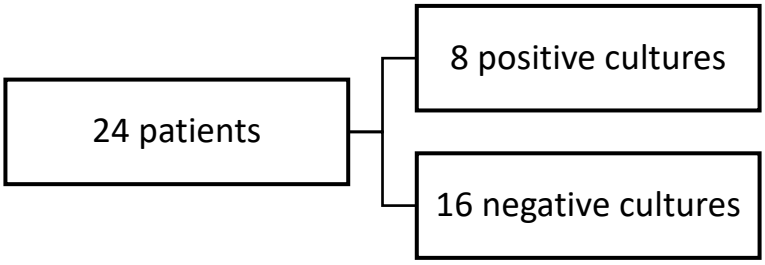


Figure 35. Summary of the number of culture positive cases recruited into the study

4.3.8 Comparison of clinical isolates identified from explanted joints prosthesis to NHS clinical data.

Table 9. Comparison of the research and the NHS positive culture results obtained from explanted joints

Infected sample	Research result NucB	Research result Sonication	Time to result	NHS Result	Time to Result
1	*Streptococcus dysgalactiae*	*Streptococcus dysgalactiae*	< 24	*Streptococcus dysgalactiae*	72
2	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	< 24	*Staphylococcus epidermidis*	48
3	*Staphylococcus warneri*	*Staphylococcus warneri*	24-48	**Not isolated**	**n/a**
4	*Candida*	*Candida*	< 24	*Candida*	48
4	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	< 24	**Not isolated**	**n/a**
5	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	< 24	*Staphylococcus epidermidis*	72
6	*Staphylococcus aureus, methicillin - sensitive (MSSA)*	*Staphylococcus aureus, methicillin - sensitive (MSSA)*	< 24	*Staphylococcus aureus, methicillin - sensitive (MSSA)*	48
7	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	< 24	*Staphylococcus epidermidis*	72
8	*Enterococcus faecalis*	*Enterococcus faecalis*	<24	*Enterococcus faecalis*	*24-48*

Table 9 summarises the bacteria identified from the explanted joints using NucB and sonication treatment compared to the NHS sampling techniques. Overall there was high concordance from the research results compared to the NHS samples. The majority of cases only a single organism was identified in both the clinical and laboratory investigations. Sample 4 appeared to be a polymicrobial infection with both *Candida albicans* and *Staphylococcus epidermidis* present. Sample 3 was found to contain *Staphylococcus warneri, which was* not detected using traditional NHS diagnostic processes. No other variations were identified.

Table 10. Summary of the organisms identified and the CFU/ml from each technique.

Infected sample	Causative organism identified in the laboratory	NucB cfu/ml	NucB control cfu/ml	Sonication cfu/ml	Antibiotics	CRP	Presentation
1	*Streptococcus dysgalactiae*	414	n/a	n/a	N	130	acute
2	*Staphylococcus epidermidis*	624	90	444	N	27	chronic
3	*Staphylococcus Warneri*	54	0	58	N	7	chronic
4	*Candida albicans*	n/a	n/a	n/a	N	101	acute
4	*Staphylococcus epidermidis*	92	n/a	n/a	N	101	acute
5	*Staphylococcus epidermidis*	212	n/a	n/a	Y	106	acute
6	*Staphylococcus aureus, methicillin - sensitive (MSSA)*	286	0	204	N	60	chronic
7	*Staphylococcus epidermidis*	730	90	810	N	47	chronic
8	*Enterococcus faecalis*	909	74	610	N	55	chronic

Where an implant was not available for analysis N/A was recorded. In sample 4, CFU could not be counted for the fungal infection.

Table 10 summaries the results from the infected samples comparing the CFU/ml obtained. When multiple plates of different culture media were positive for the same

sample the plate with the largest number of colonies were recorded for comparison. This was always the same plate culture medium for each prosthetic joint replacement, independent of which media was utilised. Sonication samples and NucB positive samples results were comparable for all prosthetic components. Samples 7 and 8 recorded positive results from the NucB control. Sample 3 and 4 revealed organisms from sonication and NucB but not from the NucB control. No variations were seen between the ultimate culture media or technique utilised and the final organism identified. In general, where sonication treatment was possible, NucB treatment released the most bacterial cells as indicated by cfu data. Although in some samples sonication gave slightly higher colony counts. In all cases, the NucB controls where no enzyme was used gave significantly lower colony counts indicating the efficacy of NucB in releasing viable cells from clinical samples.

Table 11 Summary of discordant results obtained from NHS processed samples and laboratory processed samples

Infected sample	Research result NucB	Research result Sonication	Reason for revision	Implant revised	CRP	Presentation type
3	*Staphylococcus warneri*	*Staphylococcus warneri*	Wear	*Primary THR*	*6*	*Chronic*
4	*Candida albicans*	*Candida albicans*	Infection	*Revision THR*	*101*	acute
4	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	Infection	*Revision THR*	*101*	acute

Discordant results summarised in the above table were identified on two occasions. Sample 3 was a planned revision for acetabular wear and lysis from a total hip replacement. Preoperatively there were no concerns regarding infection based on the standardised preoperative evaluation of combined clinical assessment and joint aspiration. However, both sonication and NucB mediated processing of separate explanted components identified *Staphylococcus warneri* as being present This finding would have influenced patient care in terms of follow up and possible use of antibiotic therapy as the revision was undertaken as a single stage due to the absence in the suspicion for infection. Sample 4 demonstrated a poly- microbial infection as identified by the NucB and sonication treated samples. The standard culture techniques identified

an isolated *Candida albicans* infection. This case was recognised clinically as an infected case and underwent a two stage revision. The presence of *Staphylococcus epidermidis was* not detected using the standard NHS protocols. This again would have influenced patient management in terms of antibiotic therapy. The results given above, while carried out on relatively small numbers of samples, provide an early indication that a NucB mediated process appears to help in the diagnosis of infection and the identification of the causative organism in a manner which is better than current microbiological practice. Therefore, the ease of incorporation of NucB mediated diagnostic protocols into NHS standard practice was investigated.

4.3.9 Potential integration of NucB protocols to aid in the diagnosis of infection from explanted joint prosthesis within UK current practice.

To evaluate the potential for NucB to be incorporated into the automated BD Bactec® FX system routinely used for the processing of PJI microbiological specimens a simulated sample with a known inoculum and causative organism was utilised with appropriate control. This is summarised in the table below.

Table 9. Summarised culture results from the automated culture system used within the NHS with and without the presence of NucB.

Sample	Culture report	Provisional report available	Final report
***Staphylococcus epidermidis* - NucB**	*Staphylococcus epidermidis*	< 24 hours	*Staphylococcus epidermidis*
***Staphylococcus epidermidis* + NucB**	*Staphylococcus epidermidis*	< 24 hours	*Staphylococcus epidermidis*
Sterile TSB + NucB	No growth	< 24 hours	No growth
Staphylococcus aureus - NucB	*Staphylococcus aureus*	< 24 hours	*Staphylococcus aureus*
***Staphylococcus aureus* + NucB**	*Staphylococcus aureus*	< 24 hours	*Staphylococcus aureus*
Sterile TSB + NucB	No growth	< 24 hours	No growth

The results of the experiment to evaluate the effect of the presence of NucB on the automated BD Bactec ® FX system. In all cases for both isolates the inoculated organism matched the final cultured organism. There was no difference in the timing of results obtained. Cultures results for negative inoculation were negative for growth as expected. While the presence of the Nuclease NucB would not be expected to restrict the processing of the culture sample within the automated system. The CO_2 sensitive disc at the base of the culture bottle may have been susceptible to the novel enzyme NucB, this however was not demonstrated.

4.3.10 Proposed clinical application of a closed sampling system incorporating NucB

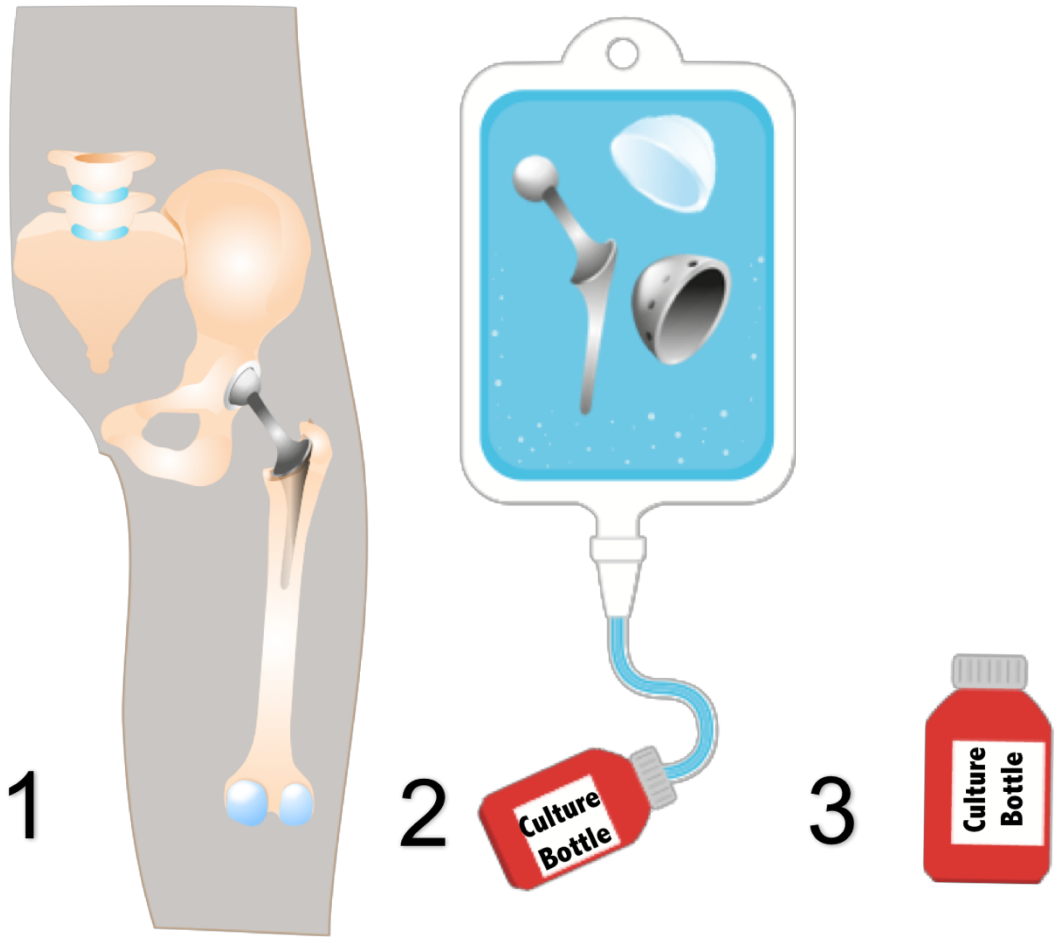


Figure 36. diagram of proposed intra operative sampling of explanted joint prosthesis and incorporation into NHS standard microbiology processing 1) joint explanted from patient 2) explanted joint placed into sealed bag containing NucB solution 3) following a period of exposure, fluid is transferred to culture bottle and sent to lab for standard processing.

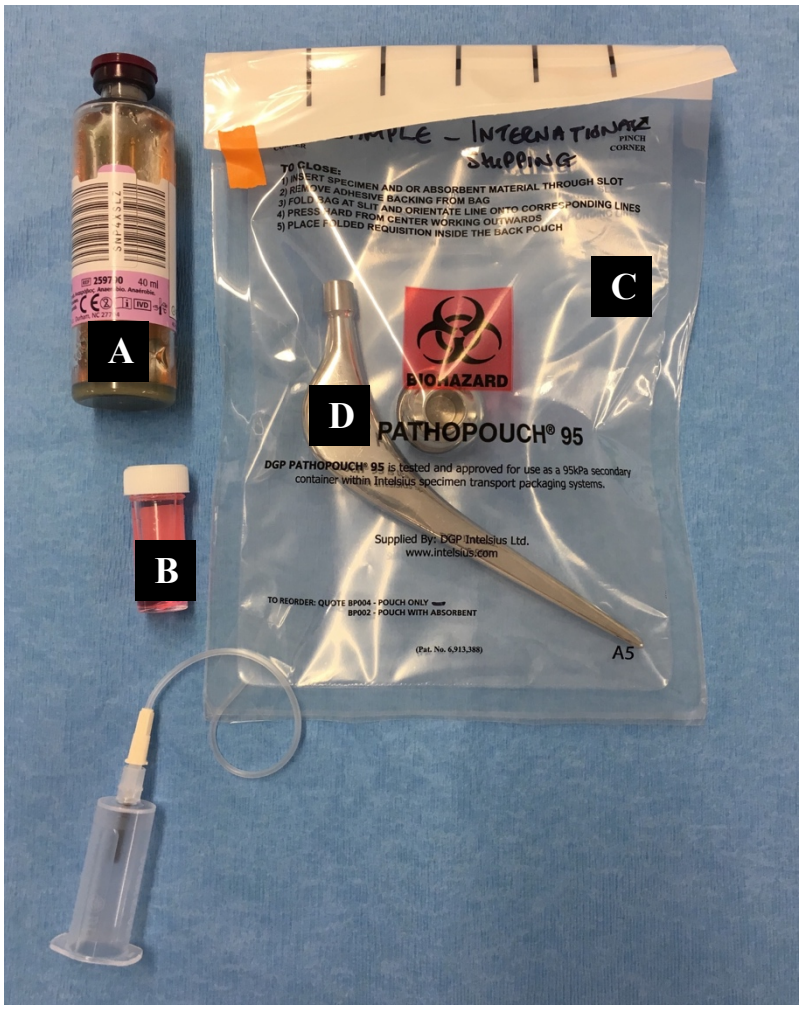


Figure 37. Proposed sampling system including sampling bag, example of explanted hip prosthesis and NucB solution with culture bottle. A) Culture Bactec ® bottle. B) NucB solution. C) Sterile processing bag with connection for culture bottle. D) explanted joint prosthesis.

Figures 36 and 37 summarise the proposed use of NucB in the clinical setting. This was not evaluated as part of the study but provides a foundation for future work incorporating NucB treatment into the NHS standard clinical diagnostic procedures summarised in Figure 38 below.

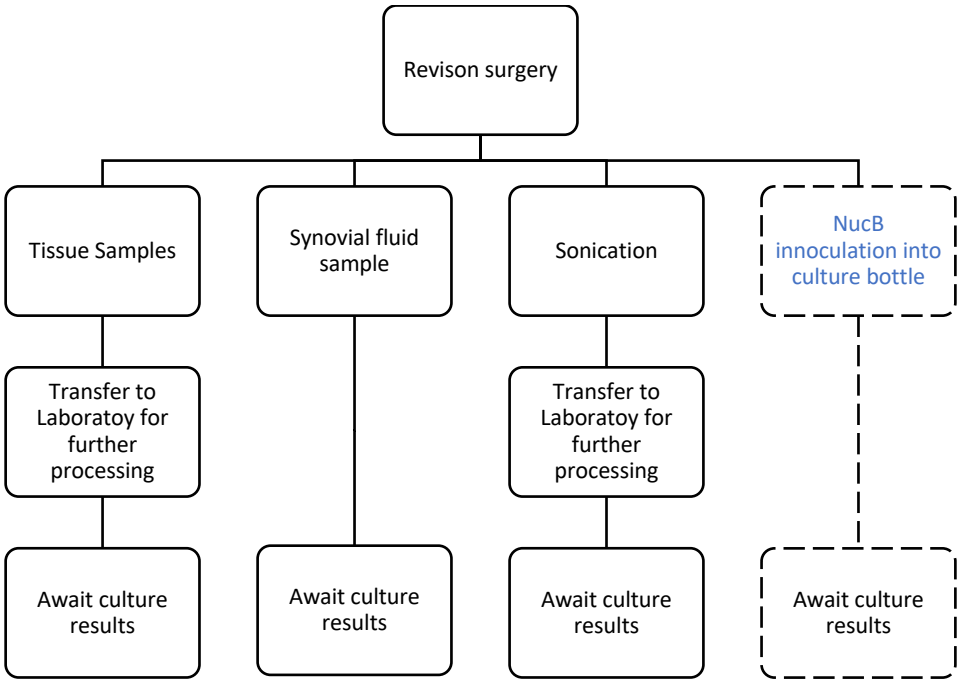


Figure 38. Flow diagram demonstration the standard NHS processing (SMI-B44, 2017) and the possible incorporation of NucB solution on the explanted joint.

These results indicate that NucB can be usefully applied to increase the release of viable bacterial cells from biofilm attached the explanted joint and that this appears to provide some advantage over standard practice in the diagnosis of PJI. Standard tissue samples and synovial fluid sampling undertaken at the time of surgery may be augmented by evaluation of the explanted joint which is currently often discarded. Sonication requires further processing and often awkward or time consuming steps. Use of a NucB solution within a receiving bag lowers the risk of contamination while utilising the explanted joint as a source of further information.

4.5 Discussion

4.1.1. Techniques in the diagnosis of PJI

Having evaluated the use of NucB in the removal of biofilm encapsulated bacteria on simulated prosthetic joint surfaces in Chapter 3, the clinical evaluation and pilot study into the potential effectiveness of NucB on explanted joint prosthesis from clinical patients undergoing revision surgery was undertaken. Initial work aimed to evaluate and compare the current practices and standards set out for the evaluation of microbiology samples sent at the time of revision arthroplasty surgery within the NHS and in particular Northumbria Healthcare NHS Foundation Trust. Site visits were undertaken to the microbiology department to evaluate the current practice and compare these to the proposed methods of processing explanted joints. Northumbria Healthcare NHS Foundation Trust like all accredited Trusts follow guidance which is summarised within the methods and results section. The microbiology facilities at Newcastle University while not accredited by Public Health England followed similar practices when handling clinical samples, making results comparable between the two laboratories during study period. Even with the guidance on offer to UK Hospitals via the UK Standards for Microbiology Investigations (SMIs) variation in actual protocols used does exist (Arciola et al., 2011, Hansen et al., 2012, Larsen et al., 2012). This is related to local skill mix and availability of equipment and is driven by the need to target harder to culture organisms such as *Propionibacterium acnes* (Butler-Wu et al., 2011*)*. All Trusts nevertheless, would be expected to provide standard culture procedures and determination of antibiotic sensitivities of bacteria isolated from tissue and fluid from around the joint. However, there is provision within the standards for further evaluation to be undertaken within the microbiology department. For example synovial white cell counts (Ghanem et al., 2008, Dinneen et al., 2013), MALDI-TOF mass spectrometry (Carbonnelle et al., 2011) or PCR (Lambert et al., 1996, Espy et al., 2006) to provide additional information. Outside the United Kingdom, further challenges exist in the processing of samples and subsequent comparisons (Societe de Pathologie Infectieuse de Langue et al., 2010, Osmon et al., 2013b) For the purposes of this clinical study comparison was made between the results from the standard culture processing undertaken with Northumbria Healthcare NHS Foundation Trust and the processing carried out within the Oral Biology Laboratory within the School of Dental Sciences at Newcastle University

4.1.2. Challenges with transportation and compliance with regulations

The recommended culture techniques and the requirements for collection, transportation and processing of clinical samples were guided by stringent regulations. This forms part of the compliance and governance stipulations for the transportation of clinical samples within the UK:

"European Parliament. UK Standards for Microbiology Investigations (SMIs) use the term "CE marked leak proof container" to describe containers bearing the CE marking used for the collection and transport of clinical specimens. The requirements for specimen containers are given in the EU via Diagnostic Medical Devices Directive (98/79/EC Annex 1 B 2.1) which states: "The design must allow easy handling and, where necessary, reduce as far as possible contamination of, and leakage from, the device during use and, in the case of specimen receptacles, the risk of contamination of the specimen. The manufacturing processes must be appropriate for these purposes".

In order to follow this legislation, the subsequent sourcing and validation of an acceptable container for transport of an explanted prosthesis proved to be a significant challenge. The containers not only had to meet the standards set out by several UK governing bodies but had to be suitable in size and shape in order to adequately accommodate the prosthesis. To avoid excessive cost, they were required to be autoclaved for reuse. Following discussion with microbiology departments around Europe regarding their choice of bottles for sonication there seemed to be no consensus (M Marsh data not shown). The published literature often does not mention which containers are used (Sebastian et al., 2018) or have used "lock and lock" containers more commonly used in food storage and are not CE marked for clinical samples or documented leak proof. Some centres have been using sterile bags for the transportation and processing of samples. The bags often have wider mouths and so can accommodate implants without the limitations often seen in bottles (Esteban et al., 2008). However, this practice is now concerning due to the potential leakage of bags and a subsequent risk of contamination, especially since the large volume of water used for the sonication has previously been a concern regarding potential water contamination within the microbiology department and the production of aerosols during sonication treatment. This risk was first highlighted by Trampuz *et al,* one of the early adopters of sonication of the explanted implant (Trampuz et al., 2006).

To overcome the challenges posed by the processing and transportation of clinical samples, studies often exclude implants that they cannot transport because they are too large (Van Diek et al., 2017, Erivan et al., 2018). This practice was evident throughout

the literature, however, was not encountered during this study. This is a significant limitation of any technique that requires the transportation of the implant with larger implants very often being excluded from evaluation. Larger implants those extending into the diaphyseal region of long bones are often encountered in revision surgery (Barry et al., 2017) or with endoprosthetic replacement used for bone malignancy (Hardes et al., 2010). Patients who are immunocompromised as a result of treatment for malignancy or patients undergoing multiple revision surgeries have increased susceptibility to infection and consequently, have an even greater need for the timely and accurate diagnosis of existing infection. However, due to the size of their explanted joint they may not be put forward for evaluation and therefore may not benefit from analysis of the explanted joint. This clinical study did not have to exclude any samples due to their size related to transportation or processing. Some larger implants including a longer stem femoral component were recruited into the study but no endoprosthetic replacements.

4.1.3. Review of patients recruited into the study

Two patients were unable to provide informed consent to participate and could therefore not be included in the study (figure 31). This is a limitation of the study design based upon ethical approval requirements. Patients without capacity are more likely to be trauma patients requiring revision surgery for a peri-prosthetic fracture which is a common injury seen following a fall. Impairment of capacity can also be caused by co-existing delirium as a direct consequence of acute sepsis from prosthetic joint infection. These patients are often at higher risk of complications, including infection, due to their unplanned surgery and lack of preoperative medical optimisation (Zmistowski et al., 2013). A presumed consent model including consultation with an appropriate consultee would have allowed inclusion of these patients into the study. This model already has a precedent within orthopaedic trials (Sims et al., 2016). However, this model is more suited to larger randomised studies rather than the initial evaluation of a novel concept such as the role of NucB in the diagnosis of PJI. Six patients were not approached in a timely fashion prior to surgery to enable inclusion in the study. This was a consequence of limited research support and availability to facilitate the recruitment and consent of participants. This patient group may represent patients requiring more urgent revision surgery rather than planned revision surgery and will again cause a degree of bias in the patients recruited. Due to the inclusion criteria only, hip and knee joint replacements were eligible for recruitment. These joints represent the greatest volume

of revision joint replacements on the National Joint Registry (NJR, 2017), there was a mix of both primary and revision joint replacements for evaluation. Revision surgical procedures are at greater risk of infection a consequence of the indicative number of procedures previously to the joint and effects on the surrounding soft tissue envelope health (Mortazavi et al., 2010). The inclusion of both primary and revision procedures in this cohort strengthens the results and reflects the case mix presented by the National Joint Registry as well as other clinical studies evaluating the effectiveness of other techniques to increase the bacterial yield from explanted joint prosthesis (Drago et al., 2012, Liu et al., 2017a)

The patient demographics followed a similar pattern to those presented within the data provided by the National Joint Registry. The average age for revision for hip or knee replacement surgery nationally for any cause was 71 years of age. There was a slight predominance of female to male of 56% to 44% nationally. The majority of patients from the national data were ASA grade 2 or 3 at the time of surgery (NJR, 2017). The recruited cohort of patients into this study had a similar female predominance as the national data, with a slightly higher average age of 79 and ASA. Nevertheless, this smaller cohort matched the national spread relating to patients requiring revision surgery. Indication for revision surgery was summarised in figure 33 and 34 the majority of cases were for wear or loosening, with the next largest group for established infection. The National Joint Registry again publishes the indication for revision surgery (NJR, 2017). This is separated into hip and knee revisions with further subdivision of the indication for revision. Infection as the indication for hip revision was 14% of single stage revision and 81% of two stage revisions. Loosening of the joint accounted for 60 % of single stage revisions and around 18% for two stage revisions. There were slightly higher numbers of revisions for infection for total knee replacements within the national data. There were no revisions for pain within this cohort. Depending on the joint revised this equates to around 12 % of revision surgery according to the National Joint Registry. Overall the small cohort of patients recruited into this study reflected the national trend well, in the indication for revision and planned surgical procedures suggesting there was no significant bias in the type of patients recruited.

Table 7 summaries the presentation type in terms of chronicity and the presence of antibiotics within the last two weeks as well as the patient's inflammatory markers. Elevation of peripheral blood C – reactive protein (CRP) is a marker of infection

(Berbari et al., 2010) however, CRP is also raised in any pro inflammatory condition (Lindqvist et al., 2005). It forms part of the standard assessment of patients suspected of prosthetic joint infection as well as one of the diagnostic criteria of infection (Parvizi et al., 2011a, Osmon et al., 2013a, Parvizi et al., 2014). C- reactive protein is highly sensitive but not specific for prosthetic joint infection. A value > 10 mg/l has been associated with a 96% sensitivity, a 92% specificity, a 74% positive predictive value and a 99% negative predictive value (Berbari et al., 2010). It can however be close to normal limits in chronic low grade prosthetic joint infection (Spangehl, 2016). C - reactive protein can be elevated with infection elsewhere and also in patients with non-infected inflammatory conditions such as rheumatoid arthritis. The majority of patients within this study with confirmed infection were those presenting acutely and had elevated C-reactive protein levels just prior to revision. In this situation, there is less of a clinical dilemma regarding the diagnosis of infection and identification of the causative organism is less of a diagnostic challenge (Della Valle et al., 2010). This however, can be confounded by the presence of antibiotics given close to the time of revision. The early evaluation of sonication by Trampuz *et al* demonstrated that sonication of the explanted implant was most beneficial for patients that had been on antibiotics within two weeks of the revision (Trampuz et al., 2007) when comparing tissue culture to sonication. Sonicate fluid culture was more sensitive than tissue culture 75% vs. 45%, P<0.001 respectively when antimicrobial agents were discontinued within 14 days of surgery. This finding demonstrated the benefit of sonication within this subset. The positive conclusion of this early work resulted in a change in standard practice within the UK and an education programme for general practitioners and hospital doctors regarding the effect of giving antibiotics in suspected prosthetic joint infection cases, if the patient is clinically well (Osmon et al., 2013a). The resultant change to standard practice ensured an antibiotic free window prior to aspiration. This was also standard practice for the Northumbria Healthcare NHS Foundation Trust and as a result only one patient was on antibiotics at the time of surgery (table 7). This patient presented clinically unwell with systemic signs of infection with antibiotics given prior to revision surgery to ensure timely and necessary treatment for a septic patient. In this case there was no diagnostic challenge in identifying the causative organism most likely due to the high bacterial load and the short interval between the commencement of antibiotics and surgery (less than 24 hours). The increasing awareness of sepsis via a World Health Organization initiative and developed by the Department of Health, may result in a justified shift back to the prescribing of antibiotics to potentially septic patients prior to aspiration (Reinhart et al., 2017). This

may result in an increase in the number of patients that have been started on antibiotics prior to their revision surgery thus making subsequent identification of the causative organism more challenging. This step would mean that a technique that can improve diagnosis of an infected explanted joint would be increasingly beneficial.

4.1.4. Organisms isolated from the explanted joint prosthesis

The most frequently isolated causative organism was Staphylococcus – most commonly *Staphylococcus epidermidis* and *Staphylococcus aureus* (Table 8). These causative organisms are recognised at the most common for implant associated infections (Trampuz and Zimmerli, 2005, Moran et al., 2007, Peel et al., 2012) and therefore formed the majority of the work undertaken on the pre-clinical evaluation of NucB in the previous chapters. *Enterococcus faecalis* was isolated as a causative organism for one case of prosthetic joint infection. Although rarer, *Enterococcus faecalis* represents around 3% of all infections and yearly rates have been increasing (Tornero et al., 2014b). These cases are more challenging to treat due to their greater antibiotic resistance (Tornero and Soriano, 2015, Lopez-Sanchez et al., 2016). *Enterococcus faecalis* is known to use eDNA as a fundamental element in its biofilm matrix (Thomas et al., 2008). In a similar manner to *Staphylococcus aureus* studies have demonstrated that *Enterococcus faecalis* uses a fratricidal signalling pathway for the release of eDNA in the formation of its biofilm (Thomas et al., 2009). *Enterococcus faecalis* biofilm sensitivity to NucB was not evaluated in the simulated model of prosthetic joint infection but the reliance of eDNA in the biofilm matrix would suggest that NucB would have a strong theoretical target on which to act.

In one case a *Candida albicans* infection was identified. Fungal infections are known causal agents in prosthetic joint infection and again rarer than other organisms more commonly encountered (Cuellar-Cruz et al., 2012). *Candida albicans* PJI forms a significant challenge requiring a long duration of antifungal medication, which is often poorly tolerated by the patient (Phelan et al., 2002). Additionally, revision surgery for *Candida albicans* infection has a higher failure rate (Reddy et al., 2013). eDNA also, plays an important role in *Candida albicans* biofilm formation (Martins et al., 2010, Costa-Orlandi et al., 2017). No gram-negative bacteria were isolated during the study period. These organisms are a rarer cause of prosthetic joint infection and make up between 9-25 percent of all infections (Hsieh et al., 2009). The more common gram-negative infections are due to *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia* are also known to utilise eDNA as an important component of

their biofilms (Whitchurch et al., 2002, Zhao et al., 2013, Alcantar-Curiel et al., 2013) with *Pseudomonas aeruginosa* being one of the first organisms with an identified susceptibility to nuclease action (Allesen-Holm et al., 2006) and therefore have a likely predisposition to nuclease degradation. While the use of NucB or sonication may not have been required to identify the causal agent in all of the explanted components (table 10) all of the isolates that were recovered have demonstrated have the potential for nuclease degradation via the presence of eDNA within the biofilm matrix.

4.1.5. Comparison between NHS results and results from experimental samples

When comparing the infection positive results obtained from the research samples and the standard NHS sampling there was a high level of concordance. This is similar to the majority of study results reviewing outcomes from sonication, overall the majority of samples correlated to the clinical results (Zhai et al., 2014). An early study with sonication recruited 404 patients comparing sonication and standard culture techniques - of these 12 patients had discordant results (Trampuz and Zimmerli, 2005). Within the cohort evaluated in the work of this thesis, 2 patients had discordant results from the 24 patients recruited (table 11). These discordant positive results between the standard NHS healthcare setting and the research results indicate the potential value of examining the explanted joint to aid diagnosis of infection. No discordance was seen however, between the samples processed by NucB and Sonication (table 9) demonstrating that within this cohort NucB was just as successful, as a new technique, at detecting a causative organism. Given the low total numbers of participants, calculations of the sensitivity and specificity between sonication and NucB would be unreliable. However, if the findings within this cohort are correct, evaluation of the explanted joint would have resulted in a change to the further management of 2 patients. In some instances, NucB was no more effective than the non-NucB control in releasing CFU (table 10, samples 2,7 and 8. These cases were seen in acute infections where there was likely a high bacterial count (Martínez-Pastor et al., 2009). The mechanical effect of the tilt table in samples with a control solution not containing NucB would likely have dislodged bacteria physically coating the explant, though possibly not necessarily encapsulated on the implant surface. This could be considered a similar process to scraping the implant surface to release bacteria. This technique is not routinely undertaken given its historical low yield (Bjerkan et al., 2009). This was seen in the non-NucB control in 2 samples and could be similar to the lower 'background'

readings seen in the non-NucB samples in chapter 3. Comparing the CFU/ml in table 10 provides an indication of the effectiveness of each diagnostic approach to the explanted joint. The variability in the size of implants, the different materials used and different surface roughnesses of the implants, all of which have been shown to affect bacterial biofilm adhesion (Teughels et al., 2006, Rochford et al., 2012, Veerachamy et al., 2014) makes direct comparison challenging. Nevertheless, dividing the samples between techniques allowed approximate comparison of the techniques used. There were 5 patients where 3 components were available. Here NucB and sonication produced comparable CFU while the non-NucB control produced fewer CFU suggesting that NucB and sonication were both cable of increasing the yield of bacteria from the explanted joints.

Reviewing the discordant results summarised in table 11 *Staphylococcus warneri* was identified from the research samples using NucB and sonication to dislodge cells but not from the standard NHS microbiologcal methods. This species is a coagulase-negative staphylococcus and is a normal skin commensal (Nagase et al., 2002). While not the most common cause, *Staphylococcus warneri* has demonstrated pathogenicity as has been shown to be the causal agent in prosthetic joint infection (Arciola et al., 2005). *Staphylococcus warneri* infection similar to *Staphylococcus epidermidis* infection, is considered a less virulent organism but is known to be able to form biofilms on prosthetic implant surfaces (Campoccia et al., 2010). The presence of poly microbial infection in PJI is increasing and provides a significant surgical challenge with greater rates of failure following revision (Wimmer et al., 2015). This is related to increased antibiotic resistance with a synergistic increase in biofilm formation being demonstrated by both of the causal organims (Pammi et al., 2013). Pammi et al evaluated the importance of eDNA in the synergistic formation of *Staphylococcus epidermidis* and *Candida albicans* biofilms in implant associated infection. This combination was also identified within this work with both NucB treatment and sonication (table 11)

Sonication has been demonstrated to increase the detection of polymicromial infections (Janz et al., 2015). Janz *et al* in a series of 109 patients undergoing revision surgery for TKR identified a polymicrobial infection in 29% of cases however, 50% of the polymicrobial isolations were only diagnosed using the sonicated fluid. This outcome maybe related to the presence of a dominant species within the polymicrobial infection and a more sessile species that forms a polymicrobial biofilm community on the prosthetic implant surface. Standard tissue culture or aspiration may therefore fail to

readily detect the second causative organism that remains dormant on the implant surface. Thus it can also be postulated that the use of NucB provides an additional technique with which to reveal polymicrobial infections that are not readily observed using traditional microbiological diagnostics.

4.1.6. Comparison between techniques available to increase the yield of viable bacteria from infected surfaces

Comparison between techniques undertaken to evaluate the explanted joint remain difficult. The largest controversy affecting the comparison of techniques remains the sonication method and the accepted cut-off for the CFU count which is accepted as representing confirmed infection. The original cuff off was 5 cfu/ml/plate based on 0.5 ml inoculation with 400 mls of fluid vortexing and no concentration step by centrifugation (Trampuz et al., 2007). However, a large variation remains principally surrounding the amount of fluid used and the inclusion of the vortexing step. Some techniques advocating covering the implant in sonication buffer fluid (Vergidis et al., 2011). Depending on the size of the implant this can introduce large variations. This also further depends on the implant and container used. A meta-analysis of 12 studies evaluating sonication suggested from subgroup analyses that the use of centrifugation or vortexing and 400–500 mL sonication solution may improve sensitivity and or specificity respectively with the best cut-off of ≥5 colony-forming units (Zhai et al., 2014). The meta-analysis did not evaluate the sonication energy levels or the containers used and it was also unable to review the cases that were excluded from all studies due to the size of the implant or contamination. These are all important factors that would have influenced results. These attempts to generate a cut-off of colony forming units are aimed at reducing false positive results, which may occur most likely from contamination during processing.

The cohort of patient samples used in this work showed a wide variation in colony forming units/ml. Using the established cut off 5 CFU/plate for sonication there were no patients that would have been classed below the level of possible contamination (Due to the volume of inoculant 10 cfu/ml equates to 5 cfu/plate used by Trampuz *et al*). Given the challenges with sonication and a validated CFU number, several studies have now adopted the use of culture bottle evaluation of the sonicated fluid (Larsen et al., 2012, Portillo et al., 2015). This moves away from the CFU number with blood bottle culture and theoretically reduces the risk of contamination. However, this

technique may increase the false positive rate as it is not possible to quantify the initial bacterial inoculation and thus provide a cut off.
Since the NucB technique is novel there is currently no guidance available on to establish a cut-off, recruitment of further patients would provide this further information to guide a cut off level.

Direct comparison regarding the time to positive culture between the NHS and research samples cannot be performed since they were processed by varying methods. Nearly all the samples showed positive growth under 24 hours on plates. The majority of samples within the NHS were available within 2-7 days. These are only released once formally approved by the microbiology department and therefore the results are susceptible to possible delay. There was no difference in time to culture between the components exposed to either sonication or NucB. Whilst formal comparison cannot be undertaken theoretically a technique that releases biofilm encapsulated bacteria and stimulates the conversion from a dormant slowly dividing cell to a more active planktonic form is recognised by Costerton et al (Costerton et al., 1978). These faster diving cells should allow for the earlier detection by microbiological culture-based methods. Earlier detection would improve patient care by commencing antibiotic treatment or following identification allowing their earlier rationalisation to optimise treatment (Osmon et al., 2013b).

4.1.7. Integration of NucB techniques into standard NHS diagnostics practice

The final studies were undertaken to determine if NucB processing of explanted joints could potentially be incorporated effectively into current NHS practice (table 12). Evaluation of the use of automated culture bottle techniques demonstrated compatibility. Such techniques have already been evaluated with the use of sonication fluid from explanted joints in two studies (Shen et al., 2015, Portillo et al., 2015). In both studies sonication fluid inoculated into culture bottles demonstrated higher sensitivity and specificity than standard tissue culture techniques. With Patel et al demonstrating improved sensitivities when comparing synovial fluid cultures in Bactec ® bottles and sonication fluid cultured in Bactec ® bottles with sensitivities of 64% and 88%, respectively (P = 0.009). The use of the Bactec ® bottles in the UK for the evaluation of prosthetic joint infection has recently been recognised as an accepted culture method (B44, 2017). However, as previously discussed the sonication technique

requires the transportation and processing of the explanted joint within a laboratory. The process requires the handling and transfer as well risk of fluid leak and contamination. This has been recognised with several studies demonstrating low levels of <5 CFU/plate being routinely cultured and designated as contamination (Trampuz et al., 2007, Puig-Verdie et al., 2013). To eliminate the need for transfer and allow prompt processing of the implant NucB solution could be used in theatre as soon as the implant is explanted (figures 36 and 37) This limits the risk of contamination due to minimal changes of receptacle and allows processing to be undertaken in the sterile environment of theatre. Further advantages are the minimal time delay in the processing of the sample before inoculation into culture medium (Van Cauter et al., 2018). This is of significant benefit with low grade infections caused by fastidious bacteria such as *Propionibacterium acnes* and has the potential to improve identification of causal organism (Shah et al., 2015). This technique for the processing of samples within a "closed" system has subsequently been developed using DTT and is currently undergoing clinical evaluation (Drago et al., 2012, Sambri et al., 2018). The use of DTT was not evaluated in this work but is the subject of future studies by this research team.

4.6 Conclusions

Overall this study has demonstrated that the treatment of samples with the nuclease NucB has shown high concordance in terms of identification of causative organisms compared to standard microbiological methods, suggesting a low false positive rate. NucB treatment was equally as effective as sonication and both methods identified the presence of pathogens not revealed by standard methods that would likely have influenced patient care. This underlines the importance for diagnosis of dislodging bacteria from biofilms prior to diagnosis. NucB demonstrated the ability to release causal organisms from a greater number of species including *Candida albicans* not evaluated in the first two chapters confirming the wider potential of NucB in the diagnosis of PJI. No benefit was observed in the diagnosis of acute infections where standard culture techniques were able to confirm PJI. The use of NucB appeared to be compatible with current NHS culture techniques and therefore has the potential for seamless incorporation into standard practice. However, this research study is only an initial validation of the effectiveness of NucB in the diagnosis of prosthetic joint infection. It has been undertaken with small numbers of patients and further recruitment

of patients in larger numbers will be required in order to confirm the suggested increase in sensitivity and specificity of this method.

4 Chapter 5 Overall discussion and conclusions

The solution to the diagnosis of orthopaedic implant associated infection remains elusive and may never be fully realised. Examination of the explanted joint has the potential to provide additional information to assist in the diagnosis of PJI. eDNA is now recognised as an essential part of the biofilm matrix. The nuclease NucB via its action on eDNA has proven a novel and effective method to release encapsulated bacteria, thereby aiding in the diagnosis of infection. The effect of NucB on clinical isolates has been shown to be comparable to its effect on reference strains and clinical isolates of the most commonly encountered causal bacteria. These results are largely comparable to sonication on a simulated implant model and to the clinically explanted joint replacements recruited into this study. Within the clinical series presented, the exposure of the explanted joint to either NucB or sonication improved detection of causal agents by the identification of a causal organism, which was not simultaneously detected using standard NHS culture techniques. Furthermore, NucB enabled identification of a second less dominant organism in a poly-microbial infection; that was also not detected by standard techniques. The true merit of NucB in future clinical practice, remains to be fully realised, due the varying processing techniques involved and a need for more samples. Utilisation of this enzyme could facilitate the diagnostic processing of samples from explanted joints which may provide a faster culture diagnosis. In addition to this, a timely diagnosis would enable earlier rationalisation of antibiotics and an overall improvement in patient care.

The study of bacterial biofilms, remains challenging due to inherent variances in the models utilised between different experimental techniques and clinical isolates used. In essence this means direct comparison between techniques is impossible. (Carli et al., 2016). An additional complexity is the lack of standardised definition of infection to which diagnostic tests are compared. A universal consensus regarding this definition would aid in the comparison of clinical research. The recommendation for the use of clinical isolates having been recruited from prosthetic joint infection improves the model methodology however, makes further comparison between studies more challenging (Fux et al., 2005). The creation of reference strains for clinical orthopaedic infection models and the standardisation of a model in which to evaluate novel agents may in the future improve comparison and aid in future research.

This work focused on the exogenous use of NucB to release biofilm encapsulated bacteria. However, it should be recognised that a similar action could be achieved by

the up-regulation and release of endogenous nucleases present within the bacterial biofilm. Such approaches have been investigated and the production of nucleases has been demonstrated to be tightly regulated and related to virulence (Olson et al., 2013) and appears to be under control of several distinct pathways. For example, for *Staphylococcus aureus,* sigma factor B has been identified as a potential trigger for nuclease release (Kiedrowski et al., 2011, Schulthess et al., 2011). Whilst increasing expression of endogenous nuclease may prove effective it is likely that it will only provide a solution for its own biofilm disruption and not those of other bacteria. This therefore may require a tailored target for each bacterium and would not provide a solution as a diagnostic tool. This approach would also be limited by the significant cost implications. Currently the most effective way to utilise the action of nucleases is via an exogenous source. NucB is effective at releasing viable cells from an encapsulated biomass and its use in prosthetic joint infection is comparable to the natural role this nuclease has evolved over time. Utilisation of this is natural biofilm dispersing ability of NucB is both promising and inviting. The initial experiments undertaken in this study have demonstrated its efficacy and suitability to provide a promising adjunct to the diagnosis of prosthetic joint infection.

However, evaluation of the effectiveness of NucB within this piece of research has a number of limitations. The initial in-vitro studies undertaken in chapters two and three utilise an accepted model previously employed for biofilm dispersal. However, these models could not reproduce the clinical and mechanical environment of the joint or the effect of a host immune response. Both of these factors would influence biofilm formation and is a recognised weakness of in-vitro models (Roberts et al., 2015). Animal models would have provided a closer simulation and may prove helpful in the further evaluation of the effectiveness of NucB in future studies. Whilst the pilot clinical study made use of the actual clinical challenge for which NucB effectiveness was evaluated, variation between the explanted joints and between patients made comparison more challenging.

Dividing the implants between NucB, NucB control and sonication meant significant variation existed between the surface area and implant material that could not be resolved. Undertaking this division did confirm that occasionally for the non NucB ie the control sample the causal organism could be identified. This incorporation of a

control is rarely included in other clinical studies where comparisons are made solely with the standard techniques, commonly tissue cultures specimens (Drago et al., 2012, Erivan et al., 2018). These patients often had acute infections where the evaluation of the explanted joint by any means would not have been beneficial since standard culture techniques were able to identify the causal organism anyway. This similar outcome been seen demonstrated in other studies evaluating sonication (Puig-Verdie et al., 2013). Another limitation of this study is the number of patient samples analysed; recruitment of a greater number of patients would have improved the ability to compare results. However, fortunately infection is a relatively rare event. Additionally, the exclusion of patients that were unable to provide informed consent meant that they could not be incorporated into the study. It is likely that these patients would have benefited to a great extent from the evaluation of the explanted joint since these patients often presented to hospital acutely as a result of infection and may have already been given antibiotics. In future research, this patient group could be included by adopting a presumed consent model which is now more frequently used in clinical research. This recruitment model would include this interesting patient group and subsequently have the potential to provide an insight into the effectiveness of NucB in these more challenging cases.

Another challenge is the complexity associated with using enzyme technology. The marine nuclease NucB whilst effective within the laboratory setting has the disadvantages common to the use of enzyme based technology in clinical practice. This is primarily a formulation issue of the enzyme, there are several examples, of enzymes being utilised commercially to reduce biofilm formation (Augustin et al., 2004, Lequette et al., 2010). Other techniques to increase the yield of explanted joints would not have similar issues (Drago et al., 2012). However, the marine nuclease NucB is currently the most suitable nuclease available in terms of effectiveness, ease of production and cost (Nijland et al., 2010, Basle et al., 2018).

NucB processing of the explanted joint was evaluated for incorporation into the standard NHS diagnostic pathway. Like other techniques such as sonication (Liu et al., 2017a) and DTT (Drago et al., 2013) NucB was comparable. However, like all of these techniques the interpretation of the results remains the greatest challenge. The emphasis placed on a positive culture result from the explanted joint should likely form part of the assessments available to diagnose PJI. The mainstay of diagnosis will rest with

clinical examination and investigations as part of a multi-disciplinary approach. Incorporating all culture results from the explanted joint treated with NucB into an algorithm taking all clinical information into consideration will be needed to provide a positive predictive calculation to improve the accuracy of diagnosis. This approach is currently being evaluated by Gallazzi *et al*, who are developing a combined diagnostic tool (Gallazzi et al., 2017).

In summary microbiological examination of the explanted joint for the presence of infection is a valuable tool in the diagnosis of infection. Since infectious agents are not always revealed by traditional methods which has significant implications for surgical decisions and the NHS as a whole. This work for the first time has evaluated a novel enzymatic approach to assist in the diagnosis of PJI. NucB appears to be effective at releasing biofilm encapsulated bacteria under both laboratory and clinical settings and has demonstrated comparable efficacy with sonication without many of the recognised disadvantages. These initial promising findings should be more thoroughly explored with a larger clinical trial to develop and evaluate further the effectiveness of NucB as a diagnostic adjunct, thus improving the diagnosis of PJI, which currently remains one of the most challenging complications in orthopaedic surgery.

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