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MOLECULAR DETECTION OF VIABLE BACTERIA IN PROSTHETIC JOINT INFECTION

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Abstract

Introduction

Diagnosis of periprosthetic joint infection (PJI) can be challenging and discrimination between septic and aseptic loosening might be difficult due to the high rate of false negative bacterial culture results particularly when patients have been on long term antibiotics. Quantitative polymerase chain reaction (qPCR) seems to be a promising technique with higher sensitivity than conventional microbiological methods in the diagnosis of PJI in such cases. However, this technique has proved to have a lower specificity due to, at least in part, detection of DNA from dead bacteria. Propidium Monoazide (PMA), a DNA binding reagent, can inhibit the DNA from membrane-compromised cells from being amplified during the PCR. In this study, we have tested the performance of qPCR of clinical samples with and without prior treatment with PMA as a diagnostic tool for PJI diagnosis and compared to the conventional microbiological culture.

Methods

208 periprosthetic tissues and/or explanted prosthesis samples were collected from 62 revision episodes from 60 patients undergoing revision arthroplasties due to either PJI or non-infective causes. Tissues were homogenized, and prostheses were sonicated. Homogenates and sonicates were used for aerobic and anaerobic cultures, qPCR, and PMA-qPCR. Our PCR assay included genus-specific primers for staphylococci and enterococci and species-specific primers for *Propionibacterium acnes* being

the commonest PJI causative organisms. Sample analysis was performed blindly without referral to patients' clinical data which was reviewed later.

Results

Among the 62 revision episodes, 15 satisfied the Musculoskeletal Infection Society (MSIS) criteria for diagnosing PJI and 46 did not. One patient who was indeterminate as not satisfying the MSIS criteria but with a high clinical suspicion was included in the PJI group for a more conservative data analysis. Two PJI episodes caused by organisms outside our qPCR panel were excluded. Sensitivity of culture, qPCR, and PMA-qPCR were 63%, 71%, and 79% respectively. Their specificities were 96%, 72%, and 89% respectively.

Conclusion

PMA-qPCR (viability PCR) has the potential to improve the diagnosis of PJI as it increases not only the specificity but also the sensitivity of qPCR with a higher overall accuracy. It could be used as a viability marker to confirm eradication of infection prior to reimplantation in the two-stage treatment protocol of PJI or in post septic arthritis patients before considering arthroplasty.

Clinical Significance

PMA-qPCR can improve the diagnosis and guide the treatment of PJI. It has the potential to improve the success rate of the two-stage treatment protocol of PJI.

Declaration

This is to certify that the work submitted in this thesis is the result of original work of the author except where specific reference has been made to other sources. It has not been submitted in whole, or in part, for any other degree or professional qualification. I have received the following assistance:

- The idea of using the PMA to enhance the specificity of PCR in the diagnosis of PJI was conceived by Professor Roger Bayston.
- The patients recruited in this study has been counselled for participation by their treating surgeons and consented by a nurse practitioner.
- The experiments of PMA optimisation were performed by an MSc student who has been working under Professor Roger Bayston's supervision and I was her bench supervisor. These experiments were designed by myself with the help of Professor Roger Bayston.
- The patients' clinical data was collected by an orthopaedic senior house officer.

Supervision of this work was undertaken by Professors Roger Bayston and Brigitte Scammell.

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Presentations

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Oral presentation: “2 minutes of impact” presentation in the Medicine &Health Science Faculty Postgraduate Research Forum 2016, on the 29th of June, 2016.

Oral presentation: “Egypt, UK, and Germany: a joint experience” Lunchtime seminar in Orthopaedics, Trauma & Sports Medicine Department, University of Nottingham on the 27th of June 2016.

Oral presentation: “Propidium Monoazide-Quantitative Polymerase Chain Reaction for Diagnosis of Periprosthetic Joint Infection; a step forward?” Lunchtime seminar in Orthopaedics, Trauma & Sports Medicine Department, University of Nottingham on the 5th of March 2018.

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Fellowship in hip & knee revision including one-stage septic exchange, ENDO-Klinik, Hamburg, Germany, in June 2016.

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Abbreviations

ABB	Anaerobic basal broth
API	analytical profile index
AUC	Area under the curve
bp	Base pair
BR-PCR	Broad range - Polymerase Chain Reaction
CI	Confidence interval
CN-PJI	Culture-negative – prosthetic joint infection
CoNS	Coagulase negative staphylococci
CRP	C-reactive protein
CT	Computed tomography
Ct	Cycle threshold
DAIR	Debridement, Antibiotics, and Implant Retention
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.coli	*Escherichia coli*
E.faecalis	*Enterococcus faecalis*
EBJIS	European bone and joint infection society
ED	Emergency Department
EMA	Ethidium monoazide
EMSON	East Midland Specialized Orthopaedic Network
ESR	Erythrocyte Sedimentation Rate
GADPH	glyceraldehyde-3-phosphate dehydrogenase

HPF	High Power Field
IL-6	Interleukin-6
LE	Leukocyte esterase
LIRC	London Implant Retrieval Centre
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of flight mass spectroscopy
MIC	Minimal Inhibitory Concentration
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSIS	Musculoskeletal Infection Society
NHSB	Nottingham Health Science Biobank
NICE	National Institute for Health and Care Excellence
NJR	National Joint Registry
NTC	Non Template Control
P.acnes	*Propionibacterium acnes*
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PHE	Public Health England
PICC	Peripherally Inserted Central Catheter
PJI	Prosthetic Joint Infection
PMA-qPCR	Propidium Monoazide - Quantitative Polymerase Chain Reaction
PMMA	Polymethylmethacrylate
qPCR	Quantitative Polymerase Chain Reaction

RNA	Ribonucleic acid
ROC	Receiver Operator Characteristic
ROM	Range of Motion
rRNA	Ribosomal Ribonucleic acid
RT-PCR	Real time–Polymerase Chain Reaction
S.agalactiae	*Streptococcus agalactiae*
S.aureus	*Staphylococcus aureus*
S.epidermidis	*Staphylococcus epidermidis*
S.lugdunensis	*Staphylococcus lugdunensis*
S.mutans	*Streptococcus mutans*
SCV	Small Colony Variant
SPECT	Single-photon emission computed tomography
SSI	Surgical Site Infection
TAE	Tris-acetate-EDTA
THR	Total Hip Replacement
TKR	Total Knee Replacement
Tm	Melting Temperature
tRNA	Transfer Ribonucleic acid
TSB	Tryptic soy broth
VAC	Vacuum assisted closure
VBNC	Viable but non-culturable
WBC	White blood cells

Chapter 1. Introduction

This chapter outlines the rationale of this PhD study. It starts with a summary of the basic knowledge about prosthetic joint infection (PJI) and shows the magnitude of the problem. Currently available diagnostic tests of PJI are also described with the main focus on the molecular methods. It then explains the principles of polymerase chain reaction (PCR) briefly. Finally, the research question and hypothesis are presented along with a list of the aims and objectives of the study.

1.1 Arthroplasty

Arthroplasty is the surgical procedure in which a diseased or damaged native joint is replaced by an artificial implant or prosthesis. It is one of the most common, life-enhancing, and successful operations worldwide.

Development of arthroplasty has been inextricably linked to innovation in materials and instruments, as well as operative and anaesthetic techniques. The success of arthroplasty cannot be referenced to a single breakthrough. The earliest recorded attempts of arthroplasty date back to as early as 1891 when Themistocles Gluck tried to use ivory implants (Wong et al., 2011). Trials continued afterwards with different materials inter-positioned between the articulating surfaces. However, total hip replacement (THR) was not known in its modern style until Sir John Charnley (1911-1982), a British orthopaedic surgeon, developed the low-friction arthroplasty. Charnley is known as the father of arthroplasty (Learmonth et al., 2007). He developed the ultra-high molecular weight polyethylene cups and introduced the use of polymethylmethacrylate (PMMA) bone cement to fix the metallic components of the implants in place.

Hips and knees are by far the most commonly replaced joints. However, shoulders, elbows, and ankle are also common. Figure 1-1 depicts the number of procedures performed in the UK from 2003 to 2014 according to the National Joint Registry (NJR) (National Joint Registry, 2015).

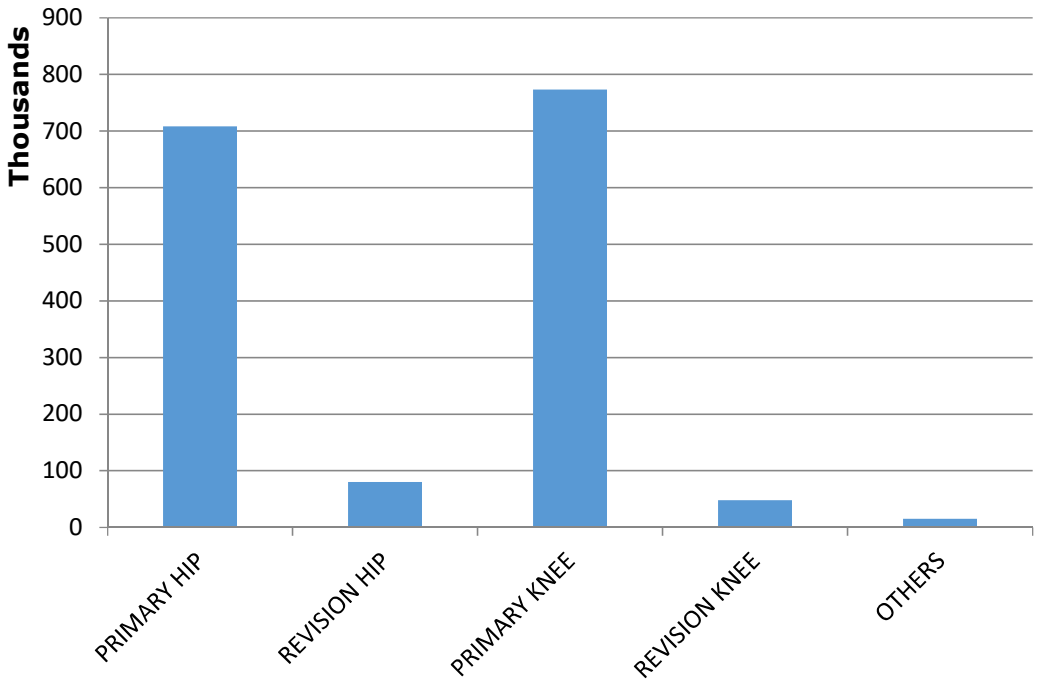


Figure 1-1 Joint Replacements in UK from 2003 to 2014. Others include shoulder, elbow, and ankle replacements. Data compiled from National Joint Registry 2015.

Hip and knee arthroplasties have greater than 95% survivorship at 10-year follow-up. Nowadays, about 800,000 joint arthroplasties per year are done in the UK and USA, with projections to greater than 4 million by 2030 (Kurtz et al., 2007, Patel et al., 2015).

1.2 Prosthetic Joint Infection

Despite the huge success achieved by arthroplasty procedures, complications still and will continue to occur. Amongst all the complications listed in literature, infection can be considered the most devastating joint complication leading to great morbidity and significant burden to health care systems. Prosthetic joint infection (PJI) is one of the most feared modes of failure of arthroplasty because of the difficulty in diagnosis and treatment.

1.2.1 **Definition**

Infections involving the implanted joint prosthesis and surrounding tissues are referred to as PJI.

Microbiologically, PJI can be defined as a microbial colonisation of the prosthetic components of the joint and the adjacent tissues.

1.2.2 **Epidemiology**

PJI incidence is about 1% for hip arthroplasties and ranges between 1% and 2% for knee arthroplasties (Dale et al., 2009, Kurtz et al., 2010, Blom et al., 2004). According to the Public Health England (PHE), the incidence of PJI is around 0.6% (Public Health England, 2016) Infection accounted for 14. 8% and 25.2% of revisions after hip and knee arthroplasty respectively and it was the most common cause of revision after knee replacements (Parvizi et al., 2010, Bozic et al., 2010)

It could be argued that the incidence of PJI has been declining over the years due to the implication of better prophylactic measures (Blom et al., 2004). However, the total number of PJI cases is definitely increasing due to the continuously increasing number of primary replacements and revisions. The absolute number of knee replacement revisions in England and Wales due to infection has risen by 2.8 fold from 2005 to 2014 (Lenguerrand et al., 2017).

1.2.3 **Economic and Health Impact**

Costs of revisions due to infection are substantial and several-fold higher than for other revision indications. The cost of the first revision of a septic hip is around £22,000 per patient (Vanhegan et al., 2012). The cost of re-

revision is about 61% higher (Merollini et al., 2013) making the total cost of a second revision around £57,000. Aside from the impact on patients, the extended length of stay, readmissions and reoperations, lengthened rehabilitation time, and extended use of antibiotics and analgesics translate into considerable excess costs to the health economy. In England and Wales, about 2200 prosthetic hip and knee joints are revised annually because of infection. In the USA alone, costs of infection exceeded US$900 million in 2012 and are expected to exceed $1.6 billion by 2020(Kurtz et al., 2012).

1.2.4 **Causative Organisms**

The most frequent PJI pathogens are *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS). *S.aureus* is one of the most virulent bacteria and it is well known as a nosocomial pathogen (Canvin et al., 1997). CoNS include at least fifteen different species. Some CoNS are well known as PJI causing pathogens such as *Staphylococcus epidermidis* (Hamad et al., 2015), *staphylococcus lugdunensis* (Sampathkumar et al., 2000) and *Staphylococcus capitis* (Tevell et al., 2016). Most of the publications about PJI do not specify individual organisms within the CoNS, hence there is uncertainty of the relative pathogenicity of each (Tande and Patel, 2014).

Enterococci have been reported to cause PJI both alone (El Helou et al., 2008) or in polymicrobial infection pattern (Bozhkova et al., 2016). Enterococci seem to cause more early than late-onset PJI.

Propionibacterium acnes (P.acnes), anaerobic Gram-positive bacterium, is commonly found in the sebaceous glands of the human skin, and is also a causative organisms of many PJIs especially of shoulder arthroplasties (Rienmuller and Borens, 2016, Pinder et al., 2016).

Other organisms are also isolated from PJIs but to a lesser extent such as streptococci, Gram-negative bacilli, or polymicrobial infections.

In England, more than half of the hip and knee PJIs in the three-year period between April 2010 and March 2013 were caused by Staphylococci. Interestingly, Methicillin-resistant *S.aureus* (MRSA) had a significantly higher incidence in the Midlands and East of England in comparison to other regions (London, North, and South of England) (Hickson et al., 2015).

1.2.5 **Pathogenesis**

The probability of surgical site infection (SSI) is directly correlated with the number of bacteria reaching the wound at the time of operation. There is no clear evidence for the most significant source for these bacteria, though the patient's own skin is a very important potential source (Parvizi et al., 2013). Lonner et al. (1999) hypothesized that the wound edges can be recolonized by bacteria within 30–180 minutes after skin preparation. Airborne infection is another possibility which has been addressed by a number of studies (Lidwell et al., 1983, Lidwell et al., 1982, Whyte et al., 1982).

Bacterial adherence on the surfaces of the implant is believed to be the key step in the development of prosthesis related infection. Many factors control the success of bacteria to colonise the cement mantle and the implant surfaces such as the prosthetic surface characteristics, presence of dead

bone fragments, and unsurprisingly, the number of bacteria succeeding to reach the implant and their genetic characteristics (Dunne, 2002).

“The race for the surface” theory proposed by Gristina et al. (1990) is a widely accepted scenario occurring shortly after the insertion of the implants. According to this concept, if the bacteria reach the implant surface first, they will multiply, expand, and protect themselves through production of extracellular proteins forming the “biofilm”. Biofilm (Figure 1-2) consists of bacterial as well as host parts that are created by different host cells such as neutrophils, histiocytes, fibroblasts and other constituents. According to Kiedrowski and Horswill (2011), biofilm could be defined as a mode of bacterial growth into sessile communities in which cells are attached to surfaces and embedded in extracellular matrix making them unsusceptible to host immunity system and antimicrobials.

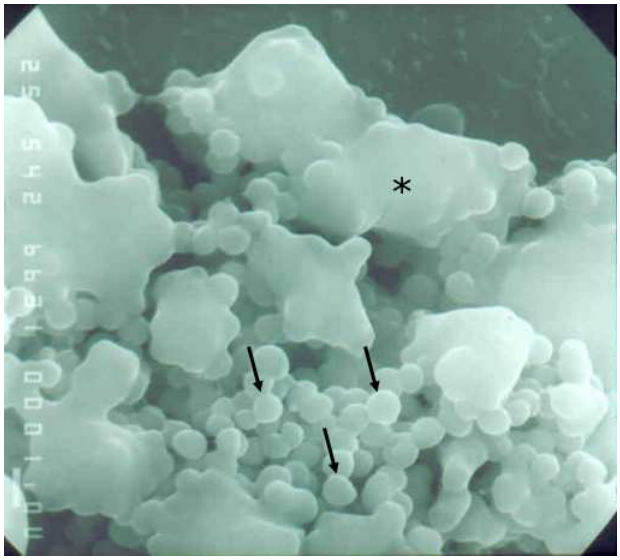


Figure 1-2 Scanning electron microscopy image of biofilm showing *S.epidermidis* (arrows) embedded in the extracellular matrix (asterisk) ; x5400 initial magnification. Courtesy of Professor Roger Bayston.

1.2.6 **Classification**

PJI can be classified in many different ways and these classifications are of crucial importance as they should guide the treatment. The most widely acceptable and widely used classification system is based on the timing of appearance of symptoms and the potential source of infection. According to this onset of infection classification scheme, infection can be early, delayed or late as illustrated in Figure 1-3 (Tande and Patel, 2014). Haematogenous infection is an infection occurring in a longstanding infection-free joint due to presence of a septic focus elsewhere (e.g. infected skin ulceration, infected gallbladder) (Tande and Patel, 2014).

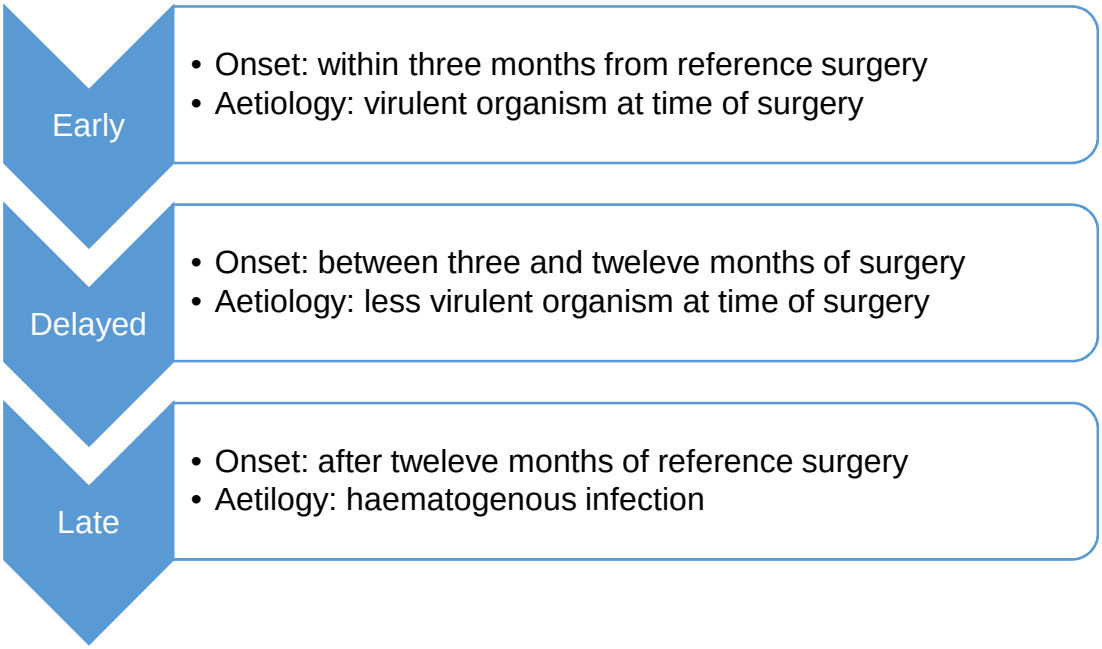


Figure 1-3 Classification of PJI.

However, there is a large debate between authors regarding the time frame for defining early versus late and acute versus chronic PJI. McPherson et al. (2002) consider the infection which is symptomatic for at least four weeks to

be chronic. Infection occurring more than four weeks after surgery is a late chronic infection according to Garvin and Hanssen (1995).

1.2.7 **Diagnosis**

PJI poses a huge burden on surgeons and microbiologists due to the inherently challenging nature of its diagnosis. In fact, some of the currently reported “aseptic loosening” cases might be caused by less virulent or difficult to detect organisms (Kurtz et al., 2007, Sharkey et al., 2014). Culture-negative PJI (CN-PJI) is an example of a situation in which there is lack of a clear microbiological evidence of infection despite a strong clinical suspicion and hence lack of guidance and uncertainty during treatment (Berbari et al., 2007). In spite of the presence of a battery of diagnostic tests for PJI, there is still an unmet need for a gold standard accurate and sufficiently informative test. Currently, there is no such a stand-alone test (Della Valle et al., 2004).

Effective management of PJI is largely dependent on a rapid and accurate diagnosis. In early infection, diagnosis within the first 2 weeks can lead to successful treatment before the development of resistant biofilms (Brammen et al., 2015). While acute infections are clinically evident, chronic infections can be more challenging to diagnose. Pain may be incorrectly attributed to aseptic loosening, or mechanical causes. That is why, some infections might remain undiagnosed until late in the disease, which not only leads to deterioration of the patient’s condition, but also makes the eradication of infection much more difficult (Gundtoft et al., 2016).

The Musculoskeletal Infection Society (MSIS) guidelines for the diagnosis of PJI were developed by experts who acknowledged the possibility of presence of PJI even if the proposed criteria were not fully met (Parvizi et al., 2011). Unless there is a pus-draining sinus, diagnosis of PJI requires a combination of clinical, microbiological, serum markers, and histopathological evidence (Parvizi et al., 2011).

According to the Musculoskeletal Infection Society (MSIS) (Parvizi et al., 2011), Prosthetic joint infection (PJI) can be diagnosed in the presence of one major or four minor criteria. Major criteria include presence of a sinus tract communicating the prosthesis to the skin and isolation of the same pathogen from at least two separate samples (tissue or fluid) obtained from the suspected prosthetic joint. Minor criteria include (1) isolation of the pathogen from only one sample (tissue or fluid) obtained from the suspected prosthetic joint; (2) elevated levels of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in the serum; (3) elevated leucocyte count in the synovial fluid; (4) elevated percentage of neutrophils in the synovial fluid; (5) purulence around the prosthetic joint; (6) histopathological examination of periprosthetic tissue showing more than five neutrophils in high power field (HPF) in five fields.

More recently, the international consensus meeting on periprosthetic joint infection held in Philadelphia 2013 (Parvizi et al., 2013), has agreed to exclude the intra-articular purulence from the minor criteria list as it can present a form of adverse local tissue reaction (Mikhael et al., 2009).

1.2.7.1 Clinical Diagnosis

Suspicion of early postoperative infection should be aroused if the wound is showing redness, heat, swelling, dehiscence, or if the patient has unexplained fever, or complaining of acute pain at the wound site. Haematogenous infection mimics acute postoperative infection in presentation in addition to presence of a septic focus.

Symptoms and signs in patients with delayed infection are usually subtle and infection has to be ruled out if there is persistent pain after a discomfort-free period. Delayed infection could be difficult to discriminate from aseptic loosening.

While presence of a sinus tract is a definite sign of infection, the percentage of PJI patients presenting with a sinus was reported to be around 5%, compared to 42% of them presenting with pain (Peel et al., 2012).

1.2.7.2 Radiology

Conventional X-ray is inconclusive especially in early PJI cases. However in late cases, X-ray can be suggestive of infection if there is evidence of loosening of a previously well fixed prosthesis, bone resorption near the prosthesis, or signs of chronic osteomyelitis (e.g. sinus, sequestrum, etc.) In such circumstances, serial X-rays can be helpful to track changes over a period of time (Tigges et al., 1994).

Other imaging modalities may be of more significance as bone scintigraphy and single-photon emission computed tomography (SPECT). However, there are no current studies addressing their cost-effectiveness. The artifact created by the prosthesis limits the use of magnetic resonance imaging

(MRI) in diagnosing PJI(Love et al., 2001). A study evaluating the usability of computed tomography (CT) in diagnosing hip PJI concluded that soft tissue changes can be used as markers of infection with a sensitivity and specificity of 94% and 89% respectively (Cyteval et al., 2002).

1.2.7.3 Histopathology and Gram Staining of Periprosthetic Tissues

One of the MSIS minor criteria for diagnosing PJI is a high-power field that contains at least 5 neutrophils on histopathology. Biopsies have a reasonable accuracy in diagnosing PJI (Fink et al., 2013).

Intraoperative frozen sections can be used for rapid diagnosis of PJI. However, the sensitivity of frozen sections is lower than permanent sections. In addition, experience of the histopathologist and the inter-observer differences will definitely affect the accuracy of the decision (Widmer, 2001).

Morawietz et al. (2006) classified the histopathological findings of periprosthetic interface membranes into four types. Type I is the “wear-particle-induced” type in which macrophages and multinucleated giant cells predominate and is characterized by polyethylene and bone cement particles. Neutrophilic infiltration characterizes type II which is the infective type. Type III is combination of both of the previous two types while type IV has indeterminate histology. Interestingly, the correlation between the histopathology and microbiology results in their study was as high as 89.7% with good inter-observer reproducibility.

Gram staining of periprosthetic tissues has been repeatedly reported to have very low sensitivity (Morgan et al., 2009, Chimento et al., 1996, Spangehl et al., 1999). While Della Valle et al. (2004) recommended restriction of the use

of Gram staining to aid in the empirical antibiotic treatment in cases with gross purulence, Johnson et al. (2010) believe that it should no longer be used.

1.2.7.4 Biomarkers

Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) have been ubiquitously used for diagnosing PJI. However, the sensitivity and specificity of both are questionable (Ghanem et al., 2009a). Berbari et al. (2010) conducted a meta-analysis to determine the pooled sensitivity and specificity of each. Their results are presented in Table 1-1. In the acute stages, CRP, but not ESR, should be used to diagnose PJI (Bedair et al., 2011).

Table 1-1 Characteristics of CRP and ESR in a meta-analysis by Berbari et al. (2010)

	CRP	ESR
Pooled sensitivity	88%	75%
Pooled specificity	74%	70%
No. of studies	23	25
No. of patients	3,225	3,370
Commonest cutoff	10 mg/litre	30 mm/h

Elevated peripheral white cell count is suspicious of infection. One possible cause of high neutrophil count with shift to the left is bacterial infection. Interleukin 6 (IL-6) has proved to have a relatively high specificity but the high cost is still limiting its routine clinical use (Deirmengian et al., 2010).

1.2.7.5 Synovial Fluid Markers

Alpha-defensin is a product of activated neutrophils with antimicrobial activity against bacteria, viruses, and fungi (Lehrer and Ganz, 1992). Over the past few years, there has been growing interest in both clinical use of alpha-defensin and reporting its performance in the literature. Alpha-defensin has outperformed other diagnostic methods because of its simplicity, short turnaround time, and high accuracy which is affected by neither prior antibiotic treatment (Shahi et al., 2016) nor arthroplasty site (Sigmund et al., 2017, Frangiamore et al., 2015). Table 1-2 shows the results of three recent meta-analyses for evaluation of alpha-defensin performance in PJI diagnosis However, the major drawback of its use is the high cost in addition to the lack of identification of infective organisms and susceptibility to antimicrobials.

Table 1-2 Alpha-defensin performance in three meta-analyses.

	(Yuan et al., 2017)	**(Xie et al., 2017)**	**(Li et al., 2017)**
Pooled sensitivity	96%	96%	98%
Pooled specificity	95%	95%	97%
No. of studies	11	6	4
No. of patients	2,715	2,321	421

Leucocyte esterase (LE) is another synovial fluid marker that can be used for PJI diagnosis which is commercially available as a colorimetric strip. It was originally developed to be used in urine (Wenz and Lampasso, 1989) and has been used in synovial fluid more recently (Wetters et al., 2012). Variation in chemical and physical properties between urine and synovial fluid could be

behind the lower performance of LE in PJI diagnosis in comparison to alpha-defensin (Deirmengian et al., 2015).

1.2.7.6 Microbiological Culture

Microbiological cultures are the reference standard in diagnosing PJI. Periprosthetic tissue, joint aspirate, retrieved implant sonicate, and swabs from a sinus tract can all serve as a sample for cultures with different significances of each.

Multiple tissue samples are required to avoid the potential confusion and uncertainty about the source of the growing organism from a single tissue sample. The optimal number of samples for diagnosis of PJI has been addressed by a number of studies. Atkins et al. (1998), Bemer et al. (2016), and Peel et al. (2017) recommended five to six, four, and three to four samples respectively. They processed tissue samples by manual bead milling, automated bead milling and homogenization respectively. Different tissue processing could be one possible explanation for the variability of recommendations. Atkins et al. (1998) evaluated 297 revision arthroplasty cases. They collected a standard set of five samples per case. Antibiotics were ceased for at least two weeks preoperatively, no prophylactic antibiotics were given until samples were taken, and each sample was collected with a new sterile set of instruments and transported in a separate sterile container. They concluded that isolation of pathogens from three or more independent samples is highly predictive of infection with a sensitivity and specificity of 65% and 99.6% respectively. Bemer et al. (2016) conducted a multicentre study and recommended four samples to be collected and cultured on three different media for an accurate cost-effective diagnosis of PJI. Peel et al.

(2016) found that culturing of tissue samples in blood culture bottles can enhance the sensitivity of PJI diagnosis without compromising the specificity.

Tissue cultures should be incubated for a sufficient time for the best detection of growing colonies. Aerobic cultures are traditionally incubated for 4 days (Tande and Patel, 2014). Small colony variants (SCVs), with low growth rate, need a longer incubation time than normal variants (NV) to be detected (Tande et al., 2014). In shoulder arthroplasty, where *P. acnes* is a common causative organism, the anaerobic culture incubation time should extend to 14 days (Butler-Wu et al., 2011).

Sonication has been used to dislodge the bacteria from the retrieved implants. In this technique, ultrasound waves of low frequency are transmitted through the surrounding fluid to the prosthesis creating compressions and rarefactions (i.e. areas of low and high pressure). Microscopic bubbles are created in a process known as “cavitation” during the stage of low pressure. These bubbles collapse in a process known as “implosion” during the stage of high pressure. As a result, energy is released on the implant’s surface leading to bacterial liberation to the surrounding fluid which can be cultured subsequently (Trampuz et al., 2003).

Cultures of sonicates have drawn much attention due to higher sensitivity in comparison to tissue cultures (Trampuz et al., 2007). In a meta-analysis including 1,637 patients in 12 articles, the pooled sensitivity and specificity of sonicate culture was 80 and 95% respectively (Zhai et al., 2014).

Tetreault et al. (2013) conducted a study to assess the significance of a sinus swab culture in PJI diagnosis. They found that the results of swab

culture were concordant with culture results of deep tissues harvested at operation time in less than half of the studied cases (47.3%). They recommended against the use of superficial swabs cultures in PJI diagnosis, as other authors did (Aggarwal et al., 2013).

1.2.7.7 Molecular Methods

Since they have been developed and evaluated for the first time by a team in Thomas Jefferson University in the USA (Levine et al., 1995), molecular methods have been gaining popularity aiming to overcome the limitations of the conventional bacterial isolation methods.

Polymerase Chain Reaction (PCR) is a culture-independent molecular technique in which the genetic material of interest is amplified in a doubling fashion during repetitive cycles until reaching a detectable limit. The target of amplification in the PCR could be either bacterial deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). See heading 1.3 below for more details about the principles of PCR.

Most studies reported PCR as a promising and powerful tool due to excellent sensitivity and speed (Bauer et al., 2003, Trampuz et al., 2003). On the other hand, some authors are sceptical about the beneficial advances that can be achieved by PCR in diagnosing PJI (Hischebeth et al., 2016).

Use of PCR in PJI diagnosis poses several advantages over conventional culture. Because of its high sensitivity, PCR has the ability to detect organisms in negative cultures either because of prior antimicrobial therapy, fastidious organisms, or unmet culture requirements (Wolk et al., 2001). In addition, PCR can shorten the time needed for a diagnosis to few hours

while cultures can take up to two weeks in the case of slow-growing organisms (Morgenstern et al., 2017).

On the other hand, several drawbacks should be borne in mind when considering this molecular technique. Firstly, there are false positive results due to a very high sensitivity. As PCR is capable of producing more than 10^7 copies of a single DNA template, it is inherently prone to these false positive results due to contamination at any stage of sample harvesting, transporting, or processing. This possibility is much higher in broad range (BR) and conventional PCR in comparison to specific and real time PCR (See heading 1.3.2 and 1.3.3 below). What makes this issue of false positive results more complicated especially for BR-PCR is the possibility of the reagents themselves being contaminated as the DNA polymerase enzyme is manufactured by recombinant technology in *Escherichia coli* (Corless et al., 2000, Hoeffel et al., 1999). Techniques such as ultraviolet irradiation or prior enzymatic treatment (e.g. DNase) of the reagents aiming at removal of the extraneous DNA, have been shown to negatively affect the polymerase enzyme activity, hence lower the PCR sensitivity (Corless et al., 2000). Although reducing the number of cycles during PCR will reduce its sensitivity, it is a simple way of avoiding false positive results caused by “low-level” contamination (Mitchell et al., 2017).

Given that the false positive results are a continuing problem limiting the use of PCR application in clinical situations to aid the diagnosis of PJI, multiple studies have been conducted to overcome this obstacle. Bjerkan et al. (2012) tried to reduce the risk of these false positive PCR results by minimising the risk of contamination as much as possible by working in

aseptic conditions. Although this protocol appears to be cumbersome, they managed to reduce the false positive results down to two out of 216 negative samples. However, the primers used were broad-range targeting segments of the 16S rRNA gene, which are universal to all bacteria, including contaminants, and not limited to the pathogenic ones alone.

Hoeffel et al. (1999) performed a two-stage study in which they developed genus-specific primers to reduce false positives resulting from irrelevant DNA amplification. Firstly, they tested 21 samples from ten PJI patients and they reported their results as 91% sensitivity and 100% specificity for the PCR. Unfortunately, when they subsequently evaluated 108 samples from 63 THR revisions amongst whom there were only 11 infected cases, they got discouraging results as PCR was surprisingly positive in 102 samples giving sensitivity and specificity of just 71% and 49% respectively on this occasion. According to the authors, contaminants had caused this high rate of false positive results. However, subclinical infection was considered as another potential cause. Interestingly, they did not comment on the possibility of amplification of remnants of dead bacterial DNA. These results led them to recommend using PCR as a confirmatory test of infection rather than a screening tool for PJI.

Detection of dead bacterial DNA is an important probable source, at least in part, of these false positive results. Cazanave et al. (2013) have developed a genus/group specific PCR assay panel in an attempt to shorten the time needed to diagnose PJI. This panel targets the common causative bacteria of PJI. They studied 434 revision arthroplasty cases. According to the criteria they used to define PJI, there were 144 PJI cases. However, PCR results

were positive in 6 cases where there was no previously diagnosed infection. This was explained by the possibility that positive PCR results might be reflecting killed rather than live bacterial DNA product.

There have been some studies aimed at restricting the amplification during PCR to the viable bacterial genetic material alone (Bergin et al., 2010, Lee et al., 2013, Kobayashi et al., 2009). They targeted either bacterial ribosomal RNA (rRNA) or messenger RNA (mRNA) which are produced only by viable metabolically active bacteria and their results were promising. Yet, two major drawbacks are hindering this technique. Firstly, it is detecting metabolically active rather than viable bacteria. Ability to detect bacteria in suppressed metabolic state such as SCVs (Johns et al., 2015, Tande et al., 2014) and viable non-culturable bacteria (VNCB) (Li et al., 2014b) by this technique is questionable. Secondly, there is no clear idea about the persistence of RNA after bacterial death and all of the available studies in that area so far are in-vitro.

False negative results because of PCR inhibitors is another limitation of PCR. Clinical samples are notorious for containing or producing PCR inhibitors. However, inadequate bacterial DNA extraction or purification can cause false negative results as well.

Problems in identifying certain bacterial species (unless using species-specific primers or post-amplification sequencing) is a third drawback for the PCR. In case of BR-PCR, it could be challenging to interpret multiple products from polymicrobial PJI.

Lastly, lack of susceptibility data which is crucial for treatment can limit the usefulness of the use of PCR in PJI diagnosis. However, with more understanding of antibiotic resistance, it becomes possible to study the susceptibility of organisms to antibiotics by molecular methods. This is based on the identification of genes responsible for drug resistance (Huletsky et al., 2004) and the development of specific primers to detect these “resistance” genes. An example of such detectable antibiotic resistance is the *ermB* gene which is responsible for erythromycin resistance (Jasir et al., 2001, Martineau et al., 2000), *vanA* and *vanB* which are responsible for vancomycin resistance (Carlesse et al., 2016). On the other hand, this cannot be applied to all antibiotic resistance e.g. clarithromycin resistance which is associated with mutation in 23S rRNA gene rather than being linked to a specific PCR-detectable gene (Maeda et al., 2000). In addition, the problem of unknown expression of resistance genes (i.e. the genes might be present but not expressed i.e. the bacterium is still susceptible) may also limit the molecular judgement on antimicrobial susceptibility.

Both conventional culture methods and molecular techniques are affected by potential sampling errors and type of sample used. Omar et al. (2016) reported significantly low PCR results compared to tissue culture when they used swab samples. Suda et al. (2017) used unprocessed periprosthetic tissue samples for their PCR studies and the sensitivity of their assay was 30.8% compared to 84.6% for conventional microbiological methods. They admitted that these results could have been improved if they had pre-treated the samples with dithiothreitol (DTT) or if they used implant sonicates instead. Most of the studies so far have used either joint aspirates or

sonicates of retrieved implants as samples for molecular testing because of the relatively easier DNA extraction from these samples. Periprosthetic tissue samples have been used to a less extent. In fact, they have been shown to be of lower sensitivity than sonicates (Rak et al., 2016).

Table 1-3 summarizes the main features of some studies using PCR for PJI diagnosis. It is noteworthy that these studies are not directly comparable because of lack of standardization. Studies differ in type of PCR, type of samples, prior processing of samples, target genes, gold standards against which PCR is evaluated, number of patients studied, study design (prospective vs retrospective), condition of the samples (fresh vs frozen) and so on (Qu et al., 2013).

Table 1-3 Examples of studies using PCR for PJI diagnosis.

No.	Study	Type of PCR	Type of samples	No. of pts	Diagnostic criteria of PJI	Results/ conclusion	
						SN %	SP %
1	(Ryu et al., 2014)	Multiplex	tissues	95	Their own criteria	16	97
2	(Portillo et al., 2012)	Multiplex	Sonicates	86	Their own criteria	96	100
3	(Gomez et al., 2012)	BR-PCR	Sonicates	366	Their own criteria	70	98
4	(Gallo et al., 2008)	BR-PCR	Synovial fluid	101	Their own criteria	71	97
5	(Miyamae et al., 2013)	BR-PCR MRS-specific PCR	tissues	63	Not available	90	45
6	(Mariaux et al., 2017)	BR-PCR Multiplex Staph-specific	Sonicates of cement spacers	30	Their own criteria	PCR is not helpful in detecting bacteria	
7	(Lausmann et al., 2017)	Multiplex	Sonicates	60	MSIS	PCR is reliable in acute PJI	

8	(Renz et al., 2017)	Multiplex	Sonicates	111	EBJIS	PCR and culture are comparable	
9	(Sambri et al., 2017)*	Multiplex	Eswab	70	MSIS	87	100

Abbreviations: No., number; No. of pts, number of patients; SN, sensitivity; SP, specificity; BR-PCR, broad range PCR; MSIS, Musculoskeletal Infection Society; EBJIS, European Bone and Joint Infection Society.

* In this study, authors excluded cases caused by bacteria outside their PCR assay panel.

This current study aims to develop and evaluate a new PCR technique that overcomes, at least in a part, the problem of false positive results. This technique will distinguish between live and dead bacteria by a modification which will prevent DNA from dead bacteria taking part in the PCR. The principle of this technique is the ability of propidium monoazide (PMA), which is a DNA-intercalating dye, to penetrate only membrane compromised bacterial cells and bind with their DNA preventing them from taking part in the PCR reactions (Figure 1-9).

The technique has been evaluated in the laboratory and shown to be promising (Kobayashi et al., 2010). Now, it is time for the crossover of this technique towards clinical application to determine its usefulness in PJI diagnosis. See 1.3.6 below for in-detail PMA characteristics.

1.2.8 **Treatment**

Eradication of infection is the ultimate goal of treatment of PJI, as well as regaining joint function and the range of motion (ROM), alongside pain alleviation.

Various treatment protocols have been used in PJI. Each has indications, contraindications, pros, and cons. These protocols include surgical debridement, antibiotics, and implant retention (DAIR), single and two-stage revision, explantation of the implant without re-implantation (Girdlestone), arthrodesis, and long term suppressive antibiotic therapy.

1.2.8.1 Debridement, Antibiotics, and Implant Retention (DAIR)

DAIR is a conservative surgical strategy aiming at control of infection in its early stage (Silva et al., 2002). This procedure includes a thorough debridement of any infected or necrotic tissues, evacuation of any haematoma or purulence, and exchange of polyethylene liners, followed by a copious irrigation. Importantly, DAIR should be followed by a period of antibiotics. However, this antibiotic duration is quite variable and debatable between health carers (Byren et al., 2009, Puhto et al., 2012, Moran et al., 2007)

It is noteworthy that infection with methicillin resistant *S.aureus* (MRSA) has been shown to carry a higher risk of DAIR failure in multiple studies (Triantafyllopoulos et al., 2015, Lora-Tamayo et al., 2013, Ferry et al., 2010). Non-exchange of the liner has been reported as a risk factor for treatment failure by Choi et al. (2011).

Appropriate selection of patients who are optimum candidates for DAIR is a crucial determining factor for its success. For a potentially successful procedure, patients should not be immunocompromised (Qasim et al., 2017), soft tissue should be in a good condition with no sinuses (Segawa et al., 1999, Marculescu et al., 2006), and symptoms should not be existing for more than two weeks (Silva et al., 2002). In case of DAIR failure, more aggressive and eradicative approach (such as two-stage revision) should be implemented instead of repeat DAIR (Martel-Laferriere et al., 2013, Vilchez et al., 2011a). Table 1-4 shows the success rate of the DAIR procedure according to different studies.

Table 1-4 Success rate of DAIR procedure in different studies.

No.	Study	No. of pts.	Mean follow up (months)	Success rate (%)
1	(Triantafyllopoulos et al., 2015)	78	79.7	55
2	(Koyonos et al., 2011)	136	54	31, 44, and 28 in acute, acute delayed, and chronic infections respectively
3	(Chung et al., 2014)	16	70	62.5 *
4	(Puhto et al., 2012)	86	Not available	89.5
5	(Segawa et al., 1999)	81	48	85
	* For arthroscopic DAIR.			

1.2.8.2 Single-stage Revision

In this procedure, a new prosthesis is implanted in the same procedure after an aggressive debridement has been carried out. Advantages of this treatment protocol over the two-stage revision include reduced costs, faster return to normal life style and avoiding further surgeries. Buchholz et al. (1981) were the first to describe the single stage revision approach which is now gaining popularity in many European centres (Singer et al., 2012).

With appropriate patient selection, success rates are comparable to the gold standard two-stage protocol (Table 1-5). George et al. (2015) published their surgical technique for single stage revision. They paid much attention to patient selection criteria as a key factor in the success of the procedure.

Table 1-5 Success rates of single stage revision in different studies

No.	Study	No. of pts.	Mean follow-up (months)	Success rate (%)
1	(Parkinson et al., 2011)	12	24	100
2	(Buechel, 2004)	22	132	90.9
3	(Singer et al., 2012)	63	36	95
4	(Antony et al., 2015)	57	11	89

1.2.8.3 Two-stage Revision

Two stage revision is the “gold standard” treatment of PJI. This protocol entails a first stage of an aggressive and meticulous surgical debridement, explantation of all hardware and bone cement, and insertion of an antibiotic-loaded spacer. This is followed by a period of intravenous antibiotic treatment which usually extends for about 4-6 weeks. An antibiotic free period in which the patient is evaluated for any signs of persistent infection is crucial to decide if the second stage should be deferred with or without repeat first stage (Bejon et al., 2010). A second stage, when infection is confirmed to be eradicated, then follows in which a new prosthesis is implanted.

Antibiotic-loaded polymethylmethacrylate (PMMA) spacers could be articulating (dynamic) or non-articulating (static), prefabricated or hand-made intraoperatively (Mazzucchelli et al., 2015). The main two functions of these spacers are mechanical support and local antibiotic delivery. Providing mechanical support in between the first and second stage prevents soft tissue contracture and facilitates the re-implantation. Eluted antibiotics from the spacer ensure excellent local delivery with very low systemic concentration or significant toxicity (Sterling et al., 2003).

Antibiotics used in spacers have to be thermo-stable and water soluble to withstand the exothermic reaction of PMMA polymerization and ensure good diffusion into the surrounding tissues respectively (Cui et al., 2007). Porosity of the cement affects the elution of antibiotics. As the porosity decreased with vacuum mixing, the antibiotic elution also decreases (Neut et al., 2003). On the contrary, dextran increases both the cement porosity and antibiotic release (Kuechle et al., 1991). Frew et al. (2017) found that the elution of gentamicin and vancomycin from “home-made” bone cement was twofold and fivefold higher than “ready-to-use” cement respectively. They prepared the “home-made” cement by manually adding vancomycin powder to gentamicin-containing PMMA cement which increases the probability of vancomycin clumping within the cement. These findings confirm the increased elution of antibiotics with increasing the cement porosity.

It is as yet unclear for how long the antibiotic-loaded spacers will continue to achieve effective local concentrations which are greater than the minimal inhibitory concentration (MIC) for a pathogen. Masri et al. (1998) reported that effective concentrations of tobramycin and vancomycin remained for four

months postoperatively. Fink et al. (2011) found that the antibiotics concentrations, six weeks after surgery, were sufficient for PJI treatment.

The two most debatable issues with the two-stage revision strategy are the timing of re-implantation and the ideal antibiotic duration in between stages. In fact, both are derived from a common question about timing of infection eradication. The recommended antibiotic therapy duration ranges between six and twelve weeks (Parvizi et al., 2013). Puhto et al. (2014) achieved over 90% success rate with six-week antibiotic therapy duration. Interestingly, a group in Sheffield, UK, has published their protocol of treatment of 114 patients with only one intra-operative and two postoperative parenteral doses of broad spectrum antibiotics and they have achieved an 87% success rate (Stockley et al., 2008). These results highlighted the importance of radical debridement and local antibiotics using spacers or beads in the management of PJI and undermined the presumed necessity of a long postoperative antibiotic course. This not only saves costs but also reduces antibiotic induced complications (e.g. resistance, pseudomembranous colitis, and toxicity).

Improvement of the clinical condition, along with decline of the biomarkers (ESR and CRP), have been used as surrogate indicators for control of infection (Kusuma et al., 2011, Shukla et al., 2010). However, there are no clear cutoff values for these markers to guide the proper timing for antibiotic discontinuation or for re-implantation (Ghanem et al., 2009a).

The role of culture at time of re-implantation remains controversial. Puhto et al. (2014) found no association between the culture results and the treatment

outcome as they reported only one out of five patients with positive cultures at time of re-implantation whose treatment failed. On the other hand, a positive culture of sonication of spacers retrieved at time of surgery has been associated with a poor outcome. Hence, it should prompt a more aggressive antibiotic regimen after re-implantation with a closer follow-up (Mariconda et al., 2013, Nelson et al., 2014).

A systematic review by Lange et al. (2012) has shown that the success rate of the two-stage revision strategy in infected hip replacements in 929 patients was 89%. In another systematic review by Jamsen et al. (2009), success rate of this protocol, for infected knee replacements, was between 82 and 100%.

Table 1-6 and Table 1-7 summarize the results of this protocol in infected hip and knee arthroplasty respectively in different published studies.

Table 1-6 Success rates of two-stage revision hip replacement in different studies.

No.	Study	No. of pts.	Mean follow-up (months)	Success rate (%)
1	(Ibrahim et al., 2014)	125	100	96
2	(Berend et al., 2013)	186	53	83
3	(Klouche et al., 2012)	46	>24	97.8
4	(Leung et al., 2011)	38	58	79
5	(Oussedik et al., 2010)	39	80	95
6	(Fink et al., 2009)	36	35	100
7	(Masri et al., 2007)	29	>24	90

Table 1-7 Success rates of two-stage revision knee replacement in different studies.

No.	Study	No. of pts.	Mean follow-up (months)	Success rate (%)
1	(Pelt et al., 2014)	58	38	66
2	(Mahmud et al., 2012)	253	48	93
3	(Hart and Jones, 2006)	48	49	88
4	(Pitto et al., 2005)	19	24	100
5	(Meek et al., 2003)	54	41	96

The outcome of the two-stage procedure is dependent on many factors. Firstly, surgeon's and microbiologist's experience, good debridement and proper antibiotic choice. Secondly, patient-related factors including underlying medical condition, bone stock, and soft tissue status. Last but not least, organism-related factors such as virulence and resistance. Infection after a previous two-stage revision arthroplasty could be either recurrent or persistent (Zmistowski et al., 2013). As the success rate of each subsequent two-stage revision is lower than its previous attempt (Fehring et al., 2017, Kalra et al., 2010), treatment of patients with failed two-stage revision is challenging and amputation may be mandatory to save the patient's life, in case of overwhelming sepsis. In addition, from a financial point of view, a second two-stage revision is 61% more costly than the first trial (see 1.2.3 above for the economic impact of PJI).

Given the aforementioned data, it is crucial to determine the proper timing for the re-implantation in order to lessen the risk of recurrence of infection to the minimum and enhance the patient opportunity to get cured the first time.

1.3 Basic Principles of Polymerase Chain Reaction

In the mid-1980s, Kary Mullis invented the technique of in vitro amplification of specific regions of DNA by which easily detectable large number of copies could be produced in a short time (Mullis, 1990). In fact, this technique mimics what is happening in living cells for DNA replication before mitotic division. Many advances and modifications have been made to the technique of PCR to match the purpose that it is designed for.

In addition to polymerase, the key enzyme in PCR, the assay requires primers, DNA template, and free nucleotides which are the building blocks of the new DNA copies. Primers are artificially designed in pairs (forward and reverse) as short DNA fragments with a complementary sequence to the target DNA of interest. These components are mixed up and placed in a reaction tube in a PCR machine. This machine is basically a thermocycler that elevates and lowers the temperature of the tube contents in a pre-programmed manner for a certain number of cycles (Weier and Gray, 1988). At the end of the reaction (or during the reaction in a real-time PCR), potential products can be identified.

1.3.1 **Steps of PCR and Thermal Profile.**

PCR involves three repetitive steps in each cycle; denaturation followed by annealing, and finally extension (Figure 1-4). Upon heating the reaction tube components above a certain limit (the melting point), the two DNA strands

are separated from each other in a process known as denaturation. Then, for the annealing step in which the primers will bind to their specific complementary pieces in the target DNA, the temperature has to be lowered again. In the last step, the polymerase will extend the primers by adding more nucleotides to the growing strand. These steps occur at different temperatures and this fluctuating temperature is known as the thermal profile (Figure 1-5).

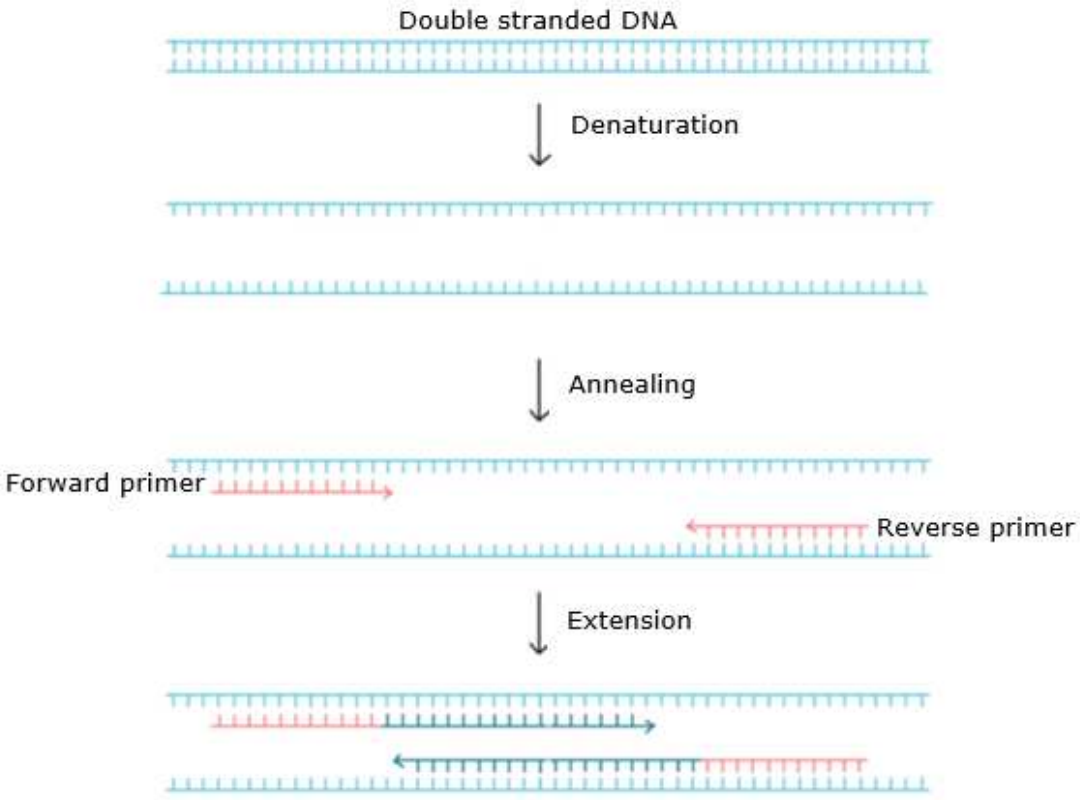


Figure 1-4 Schematic diagram of PCR steps.

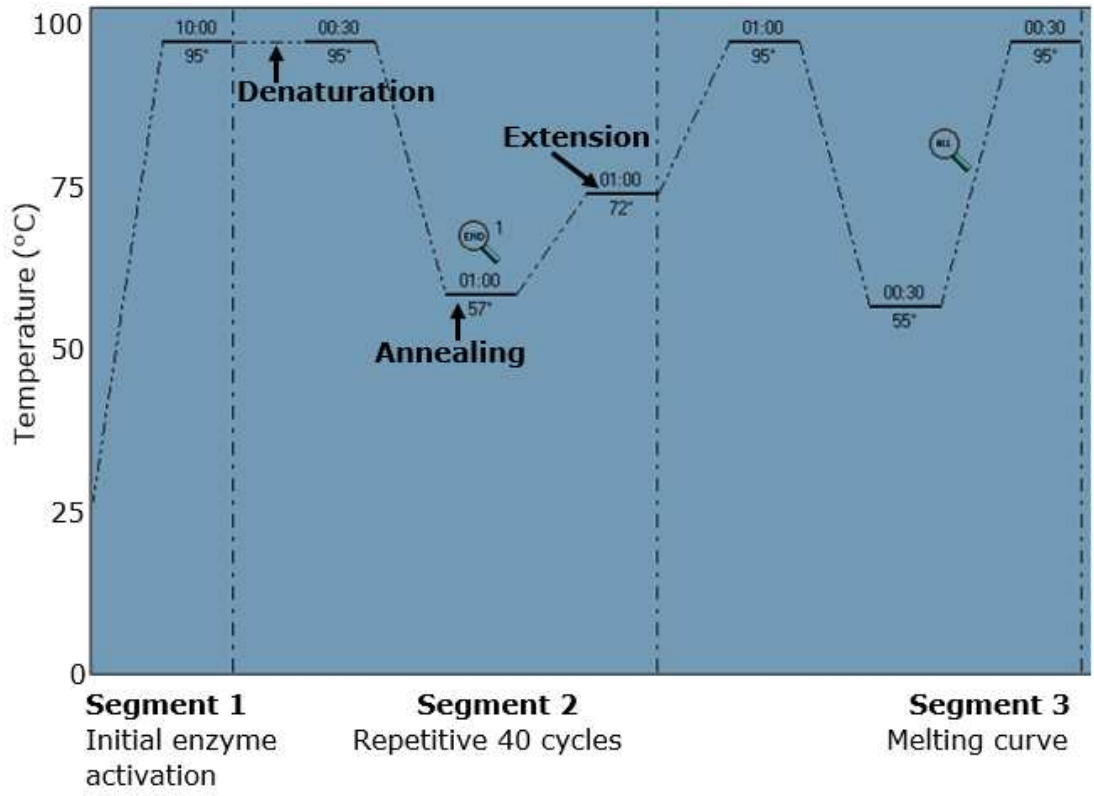


Figure 1-5 Thermal profile of PCR cycles followed by post-amplification melting curve.

1.3.2 **Real-Time versus Endpoint PCR**

Conventional PCR is a two-step technique in which the template is amplified in a thermocycler followed by post-amplification detection of the amplicon (e.g. by gel electrophoresis). The drawback of this post-amplification processing is the prolonged time and the increased risk of contamination. On the contrary, real-time PCR (RT-PCR) is a closed system with simultaneous amplification, detection, and quantification of the products. One method for detecting the products in RT-PCR is the use of fluorescent dye that binds to any double-stranded DNA non-specifically. Fluorescence can be measured after each cycle permitting product detection and quantification in a real-time fashion (Singer et al., 1997).

1.3.3 **Broad-range versus Specific PCR**

When the PCR assay uses primers that will anneal to universal DNA targets shared by most bacteria, it is known as broad-range PCR (BR-PCR). The most commonly used universal bacterial target is 16S rDNA (Kolbert and Persing, 1999). 16S rDNA contains alternating conserved and heterogeneous regions. Conserved regions can be used as a universal bacterial target while the heterogeneous regions allow for post-amplification sequencing to identify specific bacteria (McCabe et al., 1999). 16S rDNA is specific for bacterial identification as it is absent in humans, viruses, and fungi (Trampuz et al., 2003). Its presence in multiple copies in bacteria increases the sensitivity of PCR assay in clinical samples (Sloan et al., 2002). On the contrary, primers can be designed to be specific (unique) for a single organism or a group/genus of organisms.

Another type of PCR is the multiplex PCR, in which multiple specific primers are used and multiple PCR assays are simultaneously run. In fact, this technique is technically demanding and its optimisation is challenging because of the interference between the different primer sets in the same PCR run.

1.3.4 **Threshold Cycle (Ct) and Melting (Dissociation) Curve.**

At the early cycles of PCR, fluorescence level might fluctuate as the reaction medium is changing. This fluctuating fluorescence generates “background signal”. Threshold is a calculated level above this background noise. With each cycle of PCR, the amount of DNA is doubled and the fluorescence is measured. The cycle at which the fluorescence level of a certain PCR well

crosses the threshold, is known as threshold cycle (Ct) Figure 1-6 shows the fluorescence threshold and the Ct.

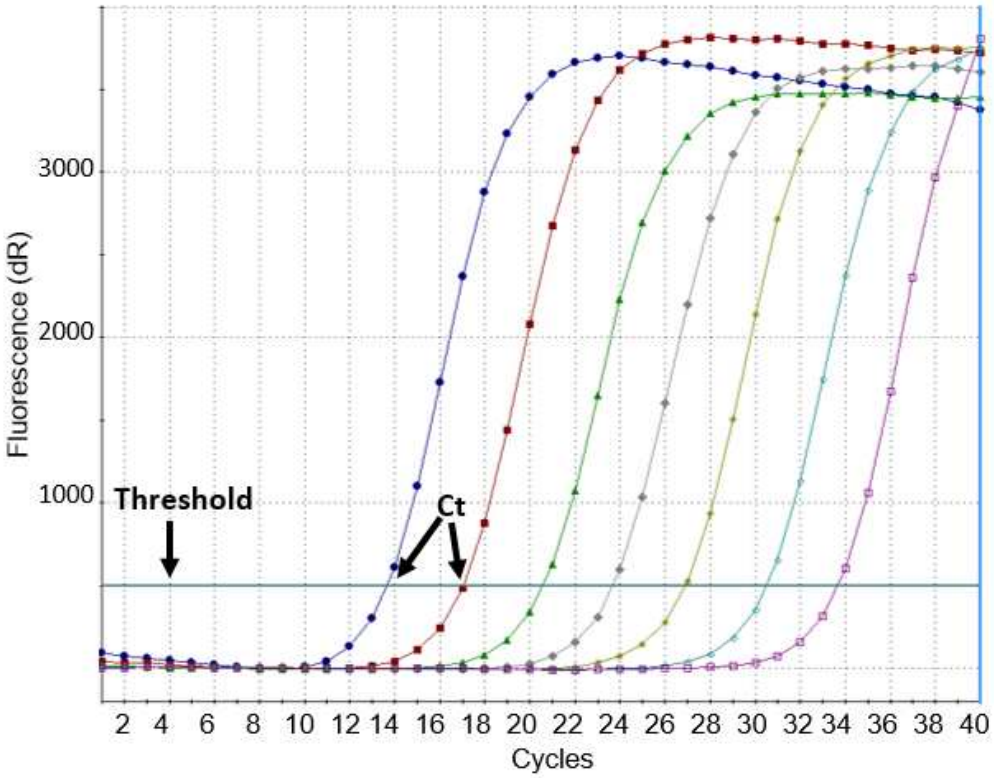


Figure 1-6 PCR amplification plots showing fluorescence threshold and threshold cycle (Ct).

Post amplification melting curve (Figure 1-5) is a useful way to judge the specificity of the PCR products and check for primer-dimer formation. Primer-dimer is generated when the forward and reverse primers are hybridized to each other. Different PCR products have different melting temperatures because this melting temperature is dependent on, among other factors, the length of the product. Primer dimer, being shorter, usually have lower melting temperature than the significant product of interest (Figure 1-7).

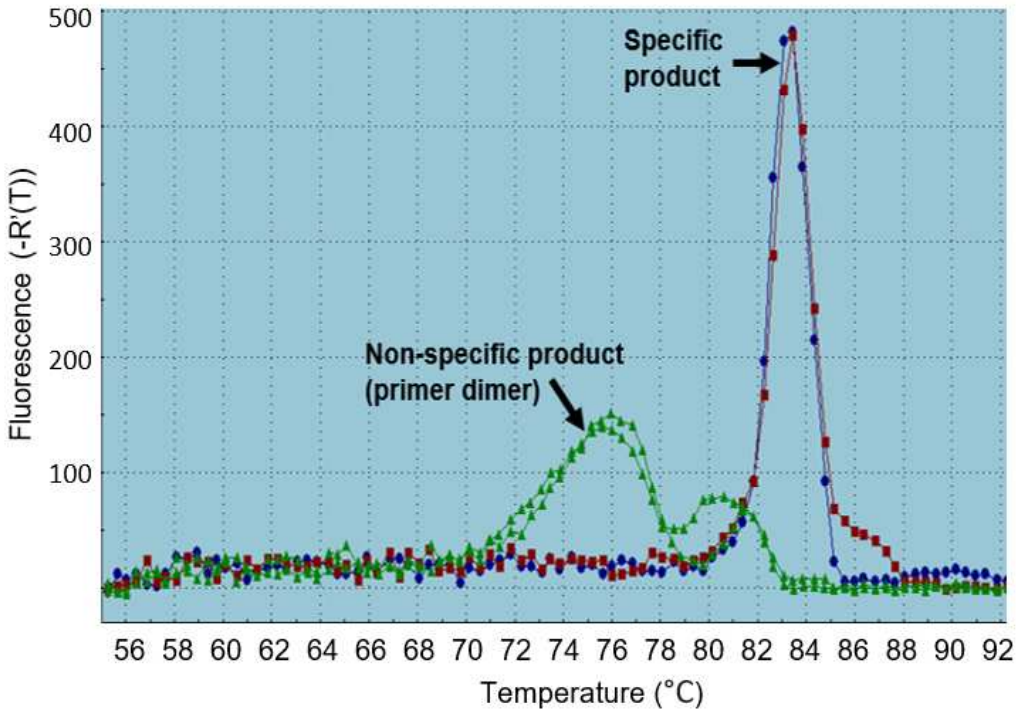


Figure 1-7 Graph showing melting curve for differentiation of specific staphylococci product and non-specific primer-dimer.

1.3.5 **Standard Curve for qPCR**

For absolute quantification of DNA template by RT-PCR, a standard curve has to be generated. It can be generated with the Ct values of a serial dilutions of known DNA quantity (Figure 1-8). Once generated, an unknown sample could be quantified by comparing its Ct against the standard curve. There is an inverse relationship between the amount of DNA and the Ct value. Furthermore, a standard curve could be used to determine the PCR efficiency and detection limit. PCR efficiency is determined by the slope of the standard curve and should ideally be between 90-110% (Eischeid and Kasko, 2015). In an ideal 100% PCR assay, the amount of DNA template is precisely doubled on each cycle.

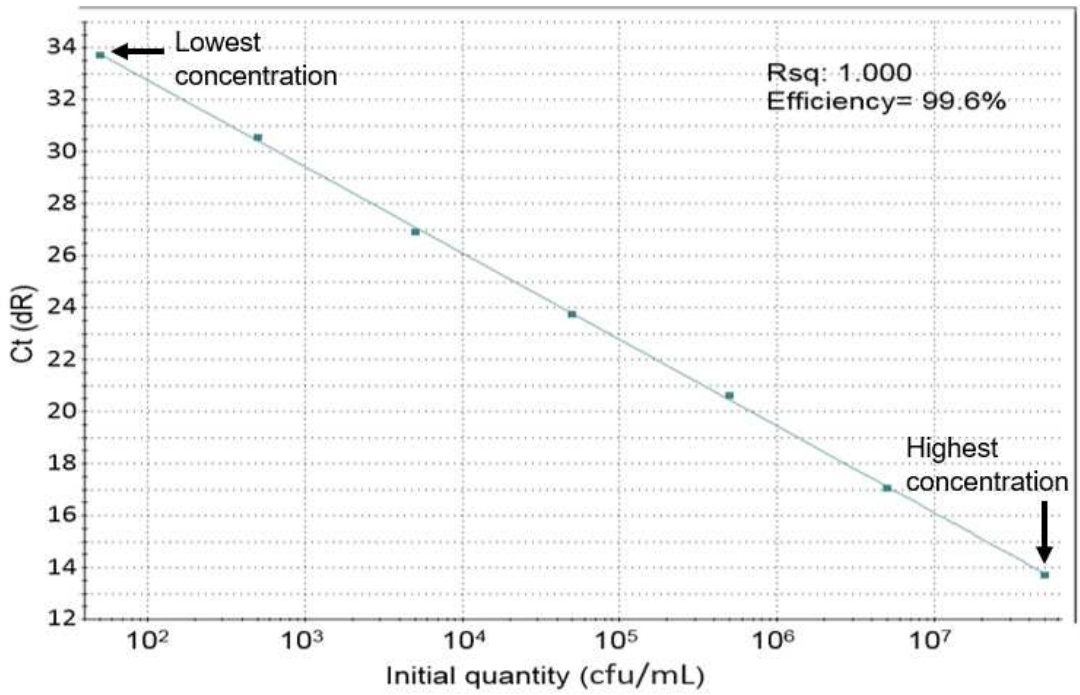


Figure 1-8 Standard curve generated by RT-PCR of seven ten-fold serial dilutions of Enterococcal DNA, initial DNA quantity (X-axis) is plotted versus Ct (Y-axis), PCR efficiency is 99.6%.

1.3.6 **PMA.**

Differentiation between viable and dead bacteria is crucial in diagnostic microbiology as the potential pathogenicity of a bacterial population is limited to its live population. In fact, this is important not only in diagnosis of infectious diseases in humans but also in environmental samples and dairy products testing (Scaturro et al., 2016, Erkus et al., 2016, Nocker et al., 2007). DNA from dead bacteria can persist for months in clinical samples after achievement of a clinical cure (Branger et al., 2003, Bemer et al., 2014). The PCR, as a DNA-based technique, will overestimate the number of viable bacteria as the DNA from both live and dead bacterial sources will be amplified indiscriminately. One method for selectively limiting the PCR to the viable bacteria is the use of a DNA-binding dye that is capable of penetrating

only membrane-compromised (i.e. dead or dying) bacteria. Ethidium monoazide (EMA) and PMA are examples of DNA-binding dyes.

EMA and PMA are azide derivatives of ethidium bromide and propidium iodide respectively. The addition of this azide group lets the molecule bind covalently to the DNA upon light exposure (Nocker et al., 2006). Both molecules can selectively enter only membrane-compromised cells as intact cell membranes are impermeable to them (Chang et al., 2010). However, the higher charge of PMA (two versus one positive charge for EMA) makes it more specific for non-viable cells than EMA (Nocker et al., 2006). In addition, EMA has been shown to be toxic to some bacteria with a potential underestimation of viable cells (Nocker and Camper, 2006).

When PMA is added to a mixture of dead/live bacteria, it will selectively enter the cell-compromised cells. Upon exposure to light, nitrene is produced from the azide group creating covalent links between the base pairs of exposed DNA from dead cells. This process is known as “intercalation” and the PMA is called DNA-intercalating dye. This cross linking makes the DNA non-accessible to the polymerase elongation because of structural changes. In addition, DNA becomes water insoluble leading to its loss during the DNA extraction process. The remaining unbound PMA is simultaneously deactivated upon light exposure and becomes no longer capable of binding to the DNA that will be released by extraction (Taylor et al., 2014). Figure 1-9 illustrates the mechanism of action of PMA.

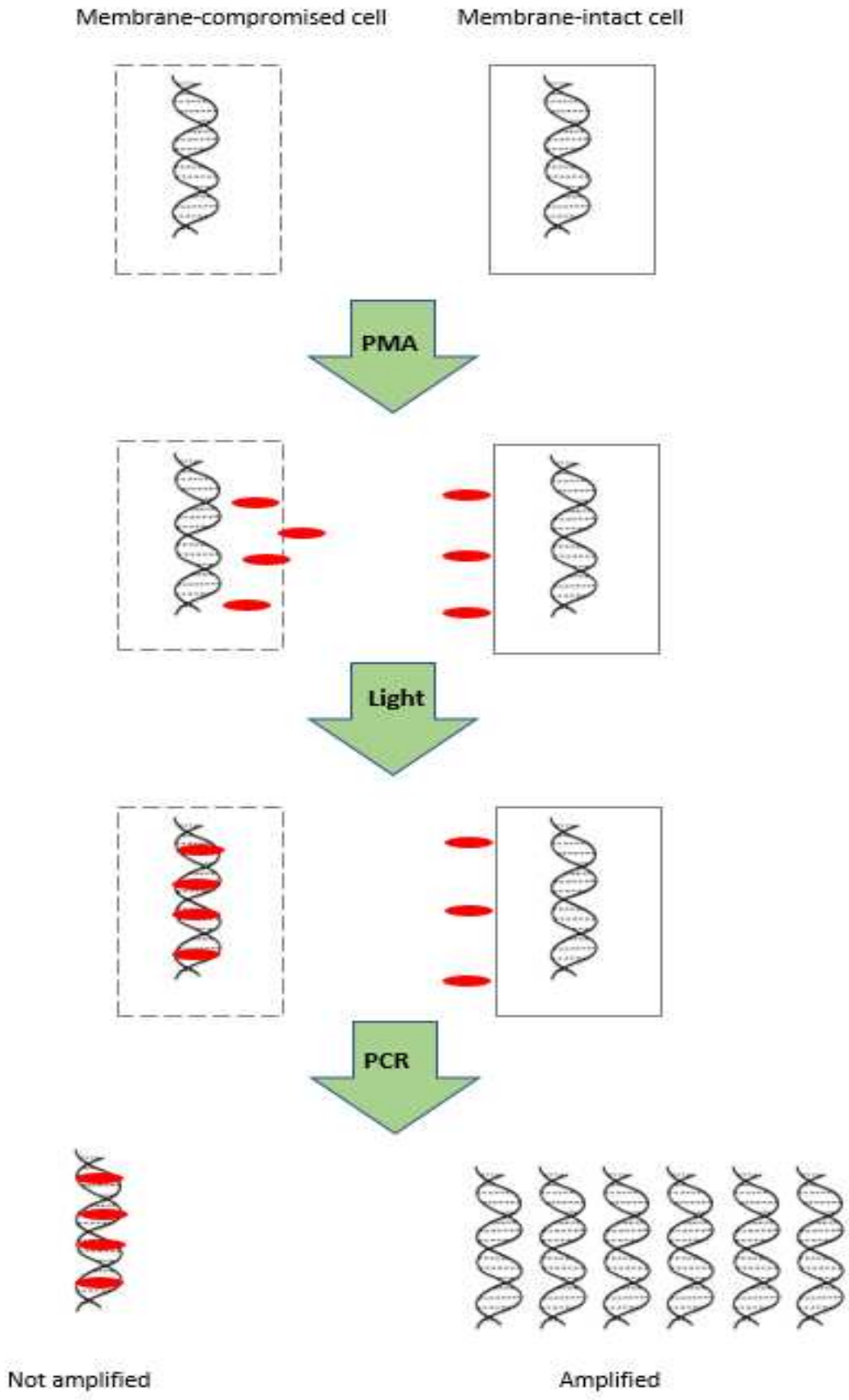


Figure 1-9 Mechanism of action of propidium monoazide.

1.4 Research Aim and Hypothesis

1.4.1 Overall Aim

The principal aim of this study is to investigate if the prior treatment of the clinical samples with PMA can enhance the specificity of PCR in PJI diagnosis. This method aims to distinguish between live and dead bacteria.

1.4.2 Objectives

1. To review the current guidelines for diagnosis of PJI.
2. To determine the sensitivity and specificity of the newly developed PCR method of diagnosis of PJI.
3. To compare the performance of PCR of periprosthetic tissue samples and sonicates.
4. To differentiate between PJI and aseptic loosening.
5. To properly assess the completeness of bacterial eradication from tissues prior to re-implantation in two-stage revision treatment protocol of PJI.
6. To help identification and characterization of certain causative organisms of PJI.
7. To judge the effectiveness of the first stage (debridement) and compare different treatment protocols from different institutions.
8. To determine the length of the required duration of inter-stage antibiotic treatment to eradicate the infection.
9. With clinical follow-up of the patients, and correlation of the incidence of infection recurrence, this method can be used to predict the outcome of the re-implantation. At a later stage, it could be used to determine the appropriate timing of re-implantation.

10. To solve some complicated clinical scenarios e.g. when arthroplasty could be safely considered in a post-septic arthritis joint.

1.4.3 **Research Question**

The study compares the performance of PCR with and without prior PMA treatment of the samples (viability and conventional PCR respectively) in the diagnosis of PJI.

The research questions posed are:

1. Will the new tested technique reduce the rate of false positive results of conventional PCR (i.e. enhance the specificity)?
2. Is the modified molecular technique capable of detecting bacteria in culture-negative PJI (i.e. improved sensitivity)?
3. Is it possible to distinguish between live and dead bacteria in clinical samples?
4. Which samples have higher sensitivity for diagnosing PJI with PCR; periprosthetic tissues or the retrieved implant prosthesis?

1.4.4 **Research Hypothesis**

The hypothesis is that this new technique will increase the specificity of PCR in PJI diagnosis. The null hypothesis (H_0) is “There is no significant difference between the results of PCR with and without PMA treatment” and the alternative hypothesis (H_1) is “There is a significant difference between the results of PCR with and without PMA treatment”.

Chapter 2. Method Development

In this chapter, optimisation of different steps in the workflow is discussed. These optimisations were essential to achieve the aims of this thesis which is the discrimination between viable and dead bacteria in clinical samples in PJI. The chapter begins with the optimisation of the tissue samples processing to maximise the release of bacteria without compromising their viability. It then explains the optimisation of the PMA treatment of the homogenized tissue samples. Finally, the optimisations of primers and master mix of the PCR assay are described.

2.1 Tissue Processing Optimization.

A critical step in this research was to develop reproducible, efficient, accurate and swift extraction of bacteria from the tissue samples. As the main objective of the research is to discriminate between viable and dead bacteria, it was crucial to ensure the survival of live bacteria in tissues during processing. In addition, the accuracy of every subsequent step is dependent on the quality of the initial tissue processing which should ensure maximum possible release of bacteria from samples.

Homogenization has been reported to be mainly used for disruption of cells to release the intracellular components (Simpson, 2010). Bead beating was used for the same purpose by Plain et al. (2015). A study by Redanz et al. (2015) tested automated tissue homogenization as a preparatory step prior to microbiological diagnostics. Their study was indirectly concerned with the viability of organisms as the endpoint was the quantitative recovery of these organisms by direct plating. However, the authors did not use a control group to measure the percentage of reduction of viable bacteria after processing. Roux et al. (2011) recommended the use of a beadmill for prior preparation of soft tissue samples to improve the outcome of the subsequent microbiological tests. In their results, 77 out of 92 PJI cases were correctly identified using a protocol which included bead beating at 1800 cpm for 210 seconds. They found that the beadmill was an effective method for bacterial recovery from tissue samples in PJI diagnosis.

2.1.1 **Methods**

For testing the effect of each tissue processing method on bacterial viability, bacterial suspensions were prepared from laboratory-isolated organisms.

Suspension of *Staphylococcus aureus* (as a Gram positive organism), *and Escherichia coli* (as a Gram negative organism) were prepared by suspending in 3mL tryptic soy broth (TSB, Oxoid, Basingstoke, UK) and the suspension was left overnight in the incubator at 37±0.5°C.

The concentration used, 10^5 cfu/mL, was controlled by absorbance calibration using a Jenway 6705 UV/Vis spectrophotometer (Fisher Scientific, Leicestershire, UK), and plating of serial dilutions. Fresh bacterial suspensions were prepared every time. 100 µL of each suspension was added to 3 mL of TSB for preparation of the next day's suspensions. Ten independent experiments were carried out for each bacterium.

2.1.1.1 Homogenization versus Bead Beating.

100 µL of each of the previously prepared suspension were added to 1mL of phosphate-buffered saline (PBS; NaCl [137 mmol/L], KCl [2.7 mmol/L], Na2HPO4 +2H2O [10 mmol/L], KH2PO4 [2.0 mmol/L]) in 2 mL sterile tightly capped tubes (Sarstedt, Nümbrecht, Germany). Three tubes were prepared from each suspension; one control, one to be homogenized for two cycles, and the third for four cycles. For bead beating, 0.1 mm tubes containing glass beads were used. Likewise, two tubes were prepared; one to be bead beaten for two cycles and the other for four cycles.

For both homogenizing and bead beating the samples, Roche magNA Lyser homogenizer (Roche, Basel, Switzerland) was used. Each cycle ran for 45 seconds at a speed of 4500 rpm. Each sample was homogenized for two or four cycles as labelled. After each cycle, the lid of the homogenizer was left

open to ensure that temperature of tubes did not rise. Time in between cycles was standardized to 45 seconds.

100µL of each of processed and control (unprocessed) bacterial suspension was spread on blood agar plates (Oxoid). Colonies were counted the next morning, and results recorded in a Microsoft excel spreadsheet.

2.1.1.2 Mechanical Processing of Artificially Inoculated Pork Meat

Pork boneless meat was obtained from a local grocery store. Equal pieces weighing 7.5g were prepared. Each piece was thoroughly and repeatedly washed in sterile water and further divided into equal thirds (2.5 g each), and stored in -20°C freezer for future use.

100µL of 10^5 cfu/mL concentration was used for inoculation at the centre of the meat piece. Inoculation was done with 1 mL plastic syringe (Becton Dickinson, Drogheda, Ireland) and 16 mm, 5/8" needle (Becton Dickinson).

A pork meat piece was inoculated with either *S.aureus* or *E.coli*, while the third was left “blank” without inoculation to report on possibility of contamination.

After inoculation, samples were left on the bench for two hours then processed by either homogenization, bead beating, or vortexing. Aliquots of 0.5 g of each piece were transferred to 2 mL tubes along with 1 ml of PBS. For bead beating, tubes contained 0.1 mm glass beads. Four cycles of homogenization and bead beating were run, 45 seconds each at 4500 with 45 seconds in between. Vortexing was used to simulate the routine tissue processing in many microbiology laboratories. This was the same as the

tissue homogenization tube but used for vortexing for 20 seconds instead of homogenization.

100µL of each product was plated out and left overnight. Colonies were counted and registered. For the results to be considered valid, the blank piece plates should have neither *S.aureus* nor *E.coli*, though they may have other irrelevant bacteria despite the prior extensive rinse.

2.1.1.3 Dithiothreitol versus Homogenization.

Suspensions of *S.aureus* and *E.coli* were prepared as previously described. 100µL of bacterial suspensions were added to 0.1% w/v dithiothreitol (DTT, Thermo Fisher Scientific, Vilnius, Lithuania) in sterile water for 5 minutes before plating to be compared to homogenized samples for four cycles as previously described.

In a second step, fourteen infected tissue samples were collected from either diabetic feet or prosthetic joint infections with pus-draining sinuses and were processed by either homogenization or DDT.

2.1.1.4 Results Validation in Known Infected Human Tissue Samples.

Twenty-six tissue samples were collected under the ethical approval of Nottingham Health Science Biobank (NHSB) as discussed later in the Methods Chapter. Tissue samples were collected from 6 patients diagnosed with PJI according to the MSIS criteria. 0.5 gm of each sample was added to 1 mL of PBS and processed by either homogenization, bead beating, vortexing, sonication, DTT, or Proteinase K treatment. Homogenization, bead beating, vortexing, and DDT were run as described above. For Proteinase K (Roche, Mannheim, Germany), 20µL were added 10 minutes before plating.

One tube was sonicated for 5 minutes at 50 Hz. The fluid product of each processing was cultured on blood agar plates, and incubated aerobically and anaerobically for 72 hours and 14 days respectively.

GraphPad Prism 7 was used to analyse data and produce charts. P values <0.05 were considered significant.

2.1.2 **Results**

2.1.2.1 Homogenization versus Bead Beating.

Quantitative bacterial recovery from either homogenized or bead beaten suspension was significantly lower than from the control group. Table 2-1 shows the percentage of reduction in processed samples in comparison with the unprocessed control suspension. Bacterial recovery from bead beaten samples was lower than from homogenized samples. Difference in reduction between two and four cycles of homogenization was statistically significant for *S.aureus* but not for *E.coli* (Figure 2-1and Figure 2-2).

Table 2-1 Percentages of reduction in bacterial count after different processings.

Organism		**Processing**			
		Homogenization		Bead beating	
	Variable	2 cycles	4 cycles	2 cycles	4 cycles
S.aureus	Median	14	35	48	80
	interquartile range	2-24	24-43	33-60	70-84
	P-value (compared to control)	0.04	<0.01	<0.01	<0.01
E.coli	Median	10	13	67	92
	interquartile range	0.5-20	5-20	54-76	83-96
	P-value (compared to control)	0.01	0.03	<0.01	<0.01

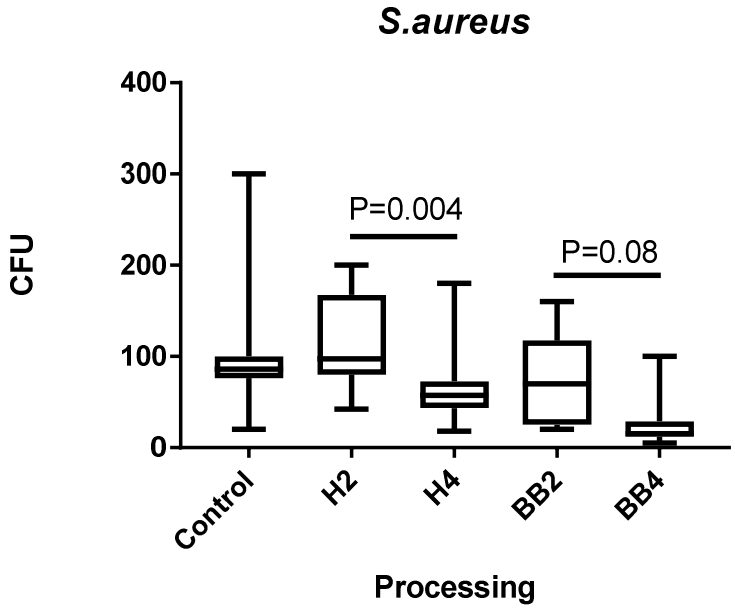


Figure 2-1 Box-whisker plot displaying bacterial recovery from 100µL of *S.aureus* suspension after homogenization and bead beating; H2 and H4: two and four cycles of homogenization respectively, BB2 and BB4: two and four cycles of bead beating respectively (Paired t-test was used).

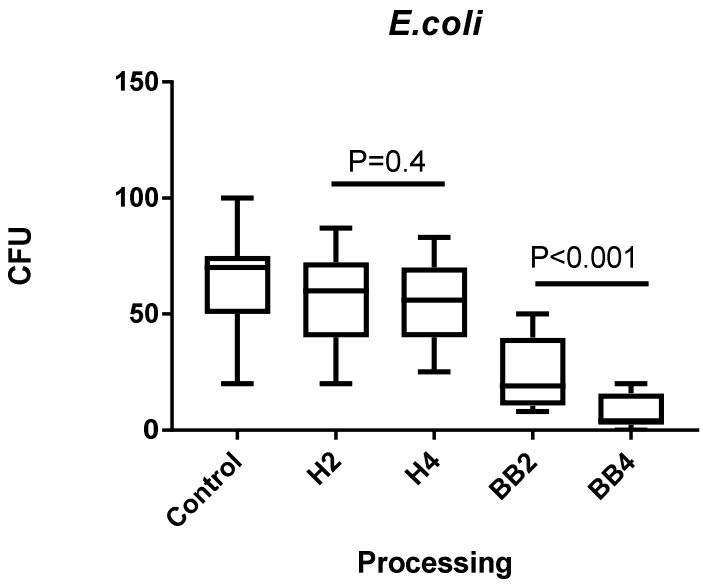


Figure 2-2 Box-whisker plot displaying bacterial recovery from 100µL of *E.coli* suspension after homogenization and bead beating; H2 and H4: two and four cycles of homogenization respectively, BB2 and BB4: two and four cycles of bead beating respectively (Paired t-test was used).

2.1.2.2 Mechanical Processing of Artificially Inoculated Pork Meat

Quantitative bacterial recovery from artificially inoculated pork samples was significantly different among samples either homogenized or bead beaten relative to vortexed samples. For *S.aureus*, means of recovered bacteria by homogenization, bead beating, and vortexing were 394, 36, and 136 cfu/mL respectively. For *E.coli*, means were 448, 70, and 166 cfu/mL respectively. Results are shown in Figure 2-3 and Figure 2-4.

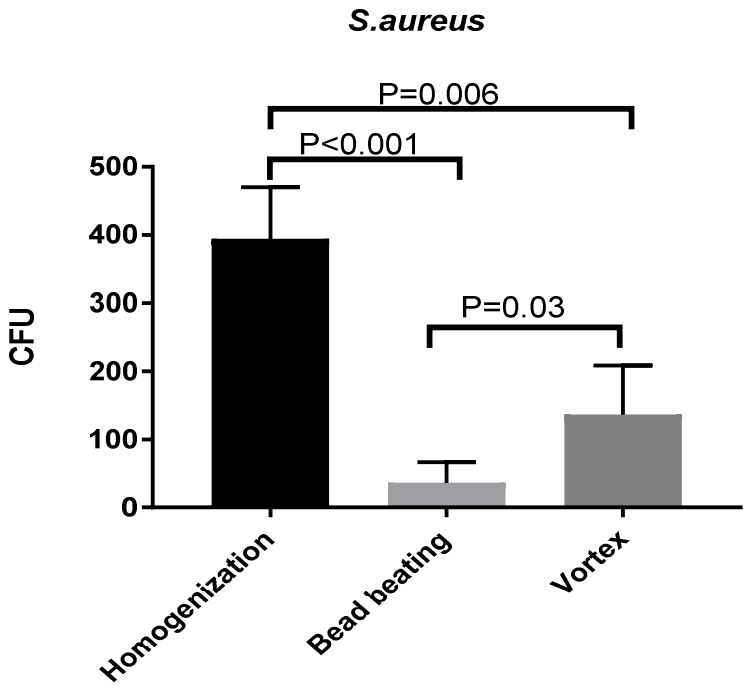


Figure 2-3 Quantitative recovery of *S.aureus* from inoculated pork samples by homogenization, bead beating, and vortexing (Paired t-test was used).

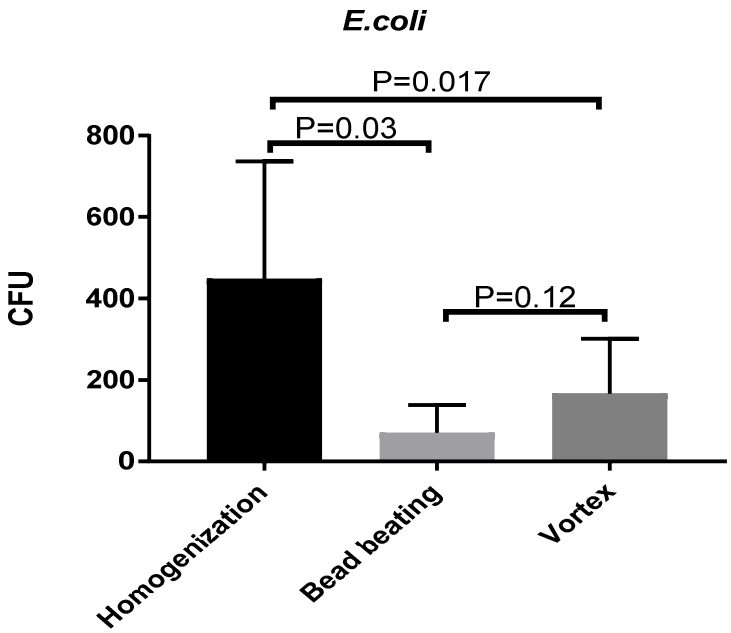


Figure 2-4 Quantitative recovery of *E.coli* from inoculated pork samples by homogenization, bead beating, and vortexing (Paired t-test was used).

2.1.2.3 Dithiothreitol versus Homogenization.

There was no statistically significant difference between bacterial suspension samples treated by either homogenization or DTT as shown in Figure 2-5 and Figure 2-6. However, bacterial recovery from infected tissues was significantly higher with homogenization than DDT (Figure 2-7). Table 2-2 summarizes the results of homogenization versus DTT.

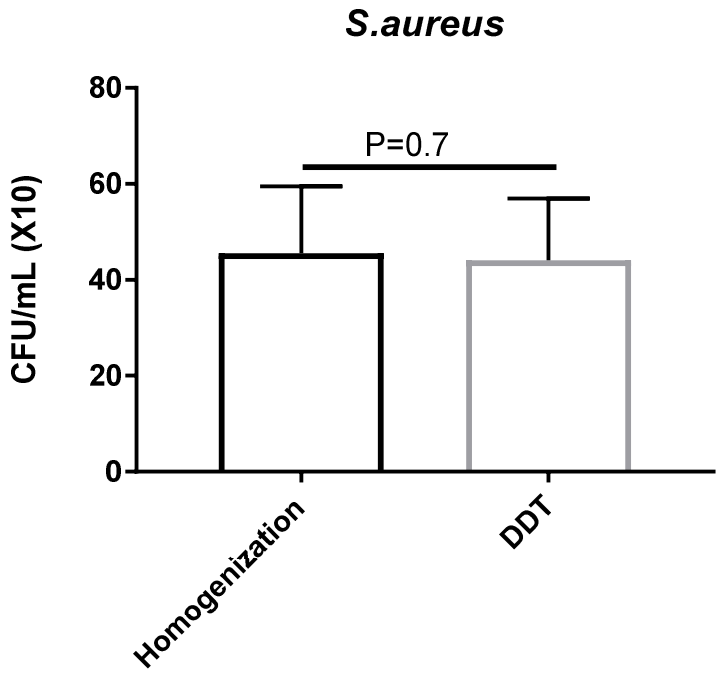


Figure 2-5 Bacterial recovery from *S.aureus* suspension after homogenization versus DTT (Paired t-test was used).

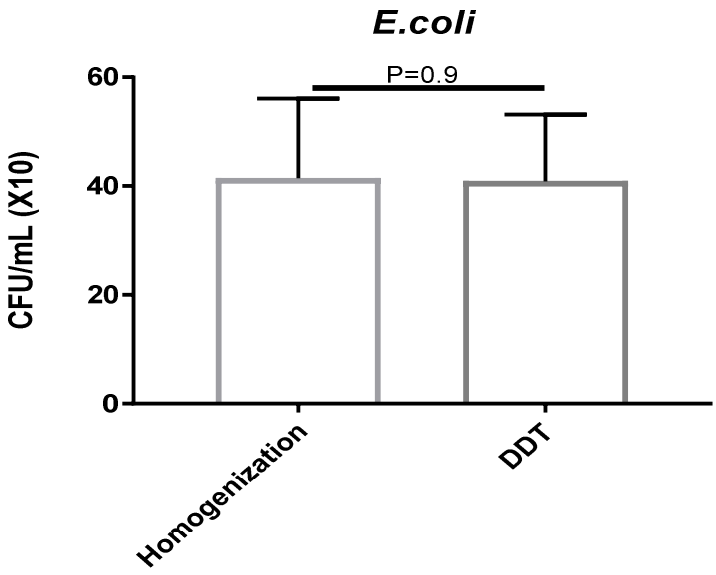


Figure 2-6 Bacterial recovery from *E.coli* suspension after homogenization versus DTT (Paired t-test was used).

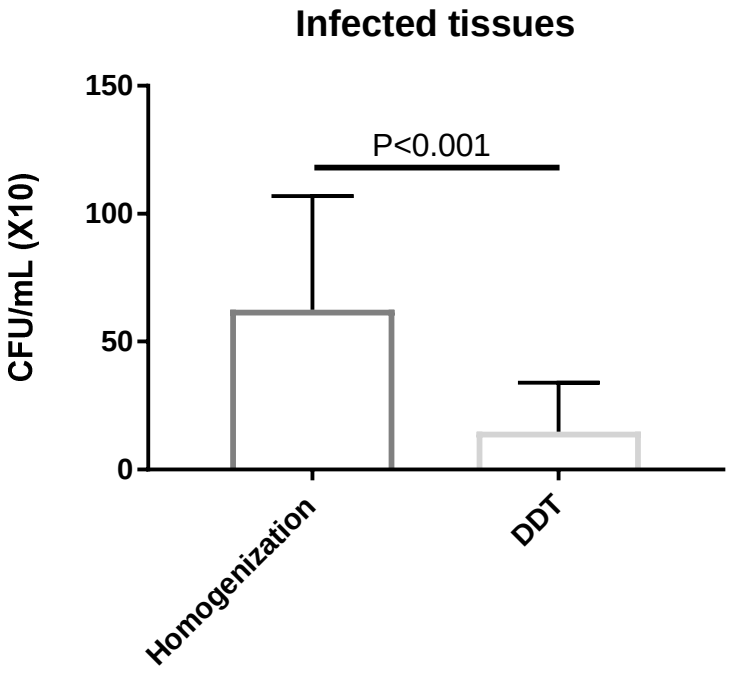


Figure 2-7 Bacterial recovery from infected tissue samples processed by homogenization versus DTT (Paired t-test was used).

Table 2-2 Bacterial recovery from infected tissue samples processed by homogenization versus DTT (cfu/100µL).

Sample	Infection	Organism	Homogenization	DTT
1	Diabetic feet	*S.epidermidis* *Corynebacterium pseudotuberculosis*	53	4
2	Diabetic feet	*S.aureus*	45	2
3	Diabetic feet	*Streptococcus dysgalactiae*	128	12
4	Diabetic feet	*Serratia marcescens* *Staphylococcus capitis*	140	13
5	PJI	*Staphylococcus epidermidis*	3	1
6	PJI	*Enterococcus faecalis*	125	74
7	PJI	*Enterococcus faecalis*	56	16
8	PJI	*Enterococcus faecalis*	78	7
9	PJI	*Enterococcus faecalis*	35	32
10	PJI	*Enterococcus faecalis*	2	0

11	PJI	*Enterococcus faecalis* *S.aureus*	12	0
12	PJI	*Enterococcus faecalis* *S.aureus*	58	17
13	PJI	*Enterococcus faecalis* *S.aureus*	73	13
14	PJI	*Enterococcus faecalis* *S.aureus*	68	16

2.1.2.4 Results Validation in Known Infected Human Tissue Samples.

Homogeneity of the product of tissue processing varied according to the processing method used (Figure 2-8). Nine out of the twenty-six tissue samples were positive by at least one processing method. Only one patient with PJI had negative culture results with all processing methods. Quantitative bacterial recovery from infected human tissues was significantly higher in homogenized than every other sample (Figure 2-9 and Table 2-3).

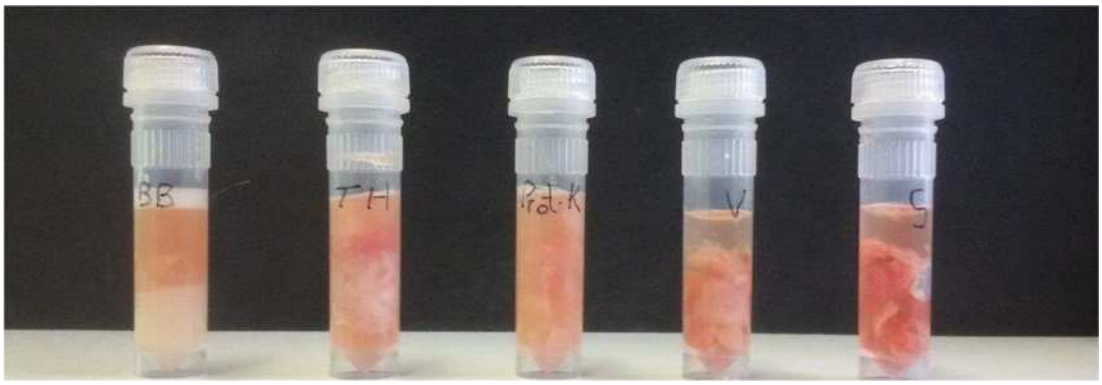


Figure 2-8 Homogeneity of products of tissues processing by different methods: from left to right: bead beaten, homogenized, Proteinase K treated, vortexed, and Sonicated

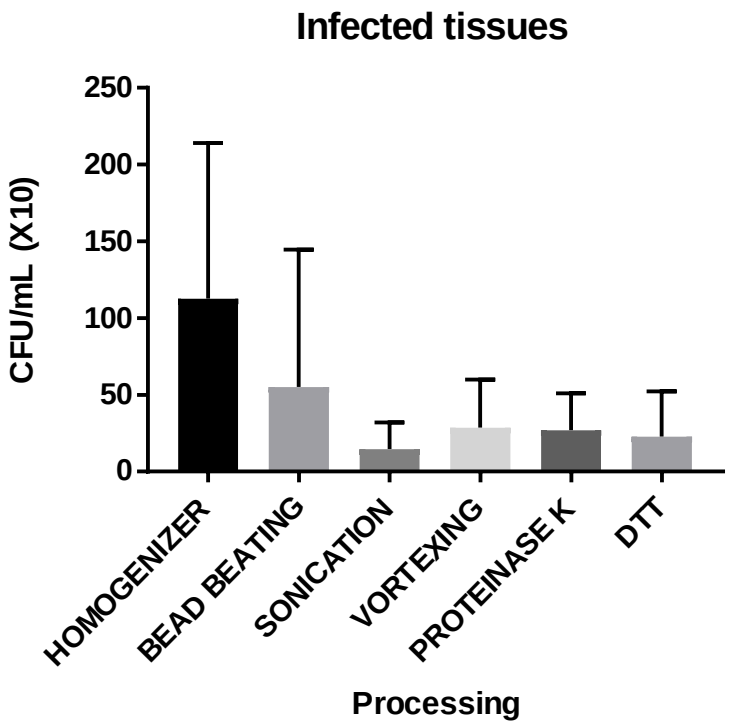


Figure 2-9 Quantitative recovery from infected tissue samples processed by different methods.

Table 2-3 Bacterial recovery in cfu/100µL from infected tissue samples processed by different methods (H: homogenization; BB: bead beating; S: sonication; V: vortexing; PK: proteinase K; DTT: Dithiothreitol).

Sample	Organism	H	BB	S	V	PK	DTT
1	*Staphylococcus epidermidis*	5	0	0	0	1	0
2	*Enterococcus faecalis*	180	68	15	92	40	91
3	*Enterococcus faecalis*	139	68	19	21	44	22
4	*Enterococcus faecalis*	174	60	48	32	32	36
5	*Enterococcus faecalis*	300	280	16	59	71	26
6	*Staphylococcus epidermidis*	4	0	0	1	1	0
7	*Enterococcus faecalis*	5	4	0	0	3	0
8	*Enterococcus faecalis*	66	10	35	41	38	31
9	*Staphylococcus epidermidis*	142	8	0	13	14	2

2.1.3 **Discussion and Conclusion.**

The first steps of tissue samples processing have a great impact on the downstream steps in the microbiological workflow. For diagnosis of PJI, in which recovery of bacteria from infected tissues is a gold standard, the ability to release the bacteria from tissues is crucial. As the majority of bacteria are not expected to be on the outside surfaces of collected tissue samples, it is not sufficient just to streak tissues on agar plates. Prior steps of releasing bacteria should be carried out first to enhance the sensitivity of the microbiological tests.

There are two prerequisites for an ideal processing method: to release as many bacteria as possible and not to adversely affect the bacterial viability. Here, a “tissue processing paradox” is hypothesized in which the higher the force delivered to tissues for processing, the more homogeneous the end product, apparently the more bacterial release, but the lower bacterial survival (Figure 2-10). A balance should be achieved to ensure both maximal release and preservation of the viability of bacteria. As quantitative rather than qualitative estimation of bacteria in tissues is crucial for guiding the treatment, initial tissue processing is a vital step.

The ideal tissue processing method should have the highest possible bacterial release and survival. In other words, it should not be too harsh for the bacteria to survive. In addition, it should be less liable to contamination and not over-dependent on worker’s skills.

Microbial distribution and loads within the potentially infected tissues can vary widely. Depending on whether the dissection and harvest planes will

cross a focus of bacterial collection, some microbes could be located on the surface of the tissues and might be retrieved by direct streaking or swabbing of the tissues. However, in such cases, the superficial microbes will not reflect the real load within the tissues.

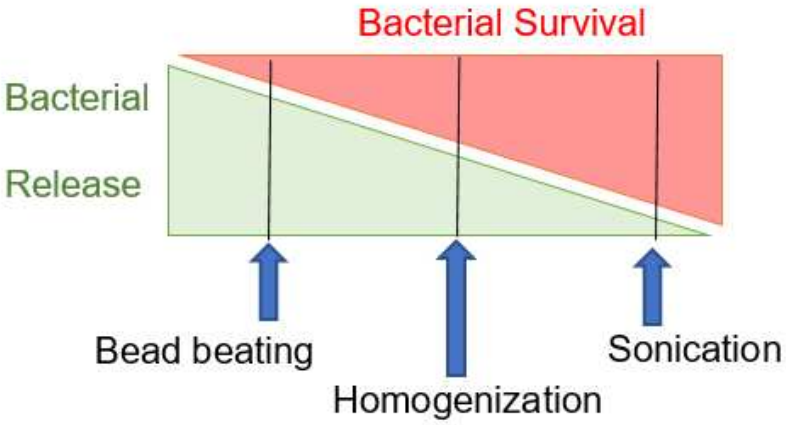


Figure 2-10 Tissue processing paradox; the higher the force of processing, the higher is the bacterial release from tissues and the lower the bacterial survival. Each processing method can be placed somewhere on that scale.

In these experiments, the ability of the processing method to keep the bacteria alive and cultivable was firstly addressed by processing bacterial suspensions and comparing them to the control non- processed group. Homogenization showed the highest bacterial survival.

Common causative organisms of PJI were chosen to be tested. The effect of processing can vary among different bacterial species and strains, most probably because of variability in their cell envelope structure and hence resistance to mechanical stresses. Two and four cycles of homogenization and bead beating were tested. As toughness of different tissues is variable, the amount and duration of the force that needs to be delivered to obtain a

homogeneous mixture can vary. Tissue processing should always be kept to the minimum required as the longer the processing, the lower the viability of the released bacteria.

In order to investigate if this conclusion held true in case of infected tissues, artificially inoculated pork tissues were processed differently, and the same result was obtained. Pork was chosen because of presumptive similarity with human tissues (Swindle et al., 2012). In an attempt to eliminate potential confusion by contaminating microflora, samples were washed as thoroughly and repeatedly as possible.

DTT has been recommended for tissue processing because of its high sensitivity, specificity, practicability, and relatively low cost (Drago et al., 2013, De Vecchi et al., 2016). In this study, DTT was found to be as safe as homogenization in terms of bacterial viability. However, when they were compared in terms of bacterial release from known infected tissue samples, homogenization outperformed DTT significantly. Lack of this comparative study was admitted as a limitation by the authors recommending the use of DTT (De Vecchi et al., 2016). Nevertheless, DTT could be considered as an alternative in case of unavailability of homogenizers because of its preservation of bacterial viability.

As a final step, tissue samples collected from PJI patients according to the MSIS criteria were used to compare homogenization, bead beating, sonication, vortexing, Proteinase K, and DTT treatment. Homogenization showed significantly higher bacterial release. The enhanced quantitative and qualitative microbial yield using homogenization has been previously

reported (Redanz et al., 2015). Manual and automated bead beating have also been described in PJI diagnosis (Atkins et al., 1998, Bemer et al., 2016, Roux et al., 2011). In our study, bead beating, in spite of a very homogeneous product, seems to be highly destructive to the released bacteria with low bacterial count end result. Sonication has a good reputation in releasing bacteria from biofilms from the surfaces of the implants (Janz et al., 2015, Trampuz et al., 2007, Renz et al., 2015, Portillo et al., 2013) but this method neither gives a homogeneous product in the case of tissues nor releases the bacteria from tissues effectively.

These experiments confirm the results of Redanz et al. (2015) but give a clearer view of the superiority of homogenization firstly by giving an estimate of loss of bacterial viability using different processing methods. Secondly, this is a comparative study between different published mechanical and chemical methods. The study by Redanz et al. (2015) compared homogenization to direct plating which is not a suitable alternative because of lack of actual processing.

In conclusion, homogenization offers the most effective method for retrieval of bacteria from tissue and retains their viability. It has been decided to utilize homogenization for tissue processing in this study.

2.2 PMA Optimisation

PMA, outperforming EMA (see heading1.3.6 above), has been widely used for dead/live bacterial discrimination in environmental samples (Nocker et al., 2007, Scaturro et al., 2016, Jager et al., 2018), dairy and meat products

(Erkus et al., 2016, Scariot et al., 2018, Zhang et al., 2015), and to a lesser extent in clinical samples (Exterkate et al., 2015, Nikolayevskyy et al., 2015). However, PMA treatment conditions varied significantly among different studies making their optimisation for the current study crucial.

Given the mechanism of action of PMA previously described in heading 1.3.6 above (Figure 1-9), at least three treatment conditions can affect its live dead discrimination ability. These conditions are the PMA concentration, dark incubation, and photo-activation time periods. Each one of these parameters was tested separately while the other two variables were kept constant. As a first stage, these conditions were tested on laboratory prepared live and dead bacterial suspensions. In a second stage, to mimic a clinical situation, the optimised conditions were tested against artificially spiked human tissue homogenates for validation.

Suspensions of *S.aureus*, being the commonest PJI causing pathogen, were used for PMA optimisation. Suspensions were freshly prepared every day as described in 2.1.1 above. The bacterial suspension was divided in half; live and dead.

For bacterial killing, an easily controllable DNA non-damaging method was needed. Heat inactivation was selected as DNA is exposed to different temperatures during the PCR without damage. Different temperatures were used for different durations to determine the lowest temperature and shortest duration needed for apparent complete bacterial killing without over-heating. A heating dry block was used with spread plating of the bacterial suspension

on blood agar plates to judge the completeness of bacterial killing. Heating at 95°C for 10 minutes was finally chosen for bacterial killing.

Each of the live and dead portions was further divided into two halves; one was treated with PMA (Biotium, Fermont, USA) and the other half left untreated. Dark incubation was done at 37°C in a shaking incubator for adequate mixing. PMA-Lite™ LED Photolysis Device (Biotium) was used for the photo-activation of the PMA. Unlike commercial halogen lamps, this device with its three LED lights next to each tube ensures providing illumination from all sides of the tube obviating the need for frequent flipping. In addition, an internal fan is fixed to prevent samples from overheating avoiding the need of the samples to be placed on ice. Lastly, with a timer incorporated in this device, timing errors could be eliminated.

Bacterial DNA was extracted and PCR was run as described later in the Methods Chapter in 3.7.1 and 3.7.2 respectively. Each sample was run in duplicate in the same PCR assay and each test was repeated three times for statistically significant conclusions. For each of the live and dead samples, ΔCt (difference in Ct) was calculated, where; ΔCt viable is the difference between the Ct value of viable bacteria with and without PMA treatment and ΔCt dead is the difference between the Ct value of dead bacteria with and without PMA treatment. The ideal PMA treatment conditions should have the highest ΔCt dead as PMA should inhibit as much as possible the amplification of DNA of dead bacteria and lowest ΔCt viable as it should not affect the amplification of DNA of viable bacteria (Desneux et al., 2015).

Experiments were performed in triplicates and the data shown in relevant figures are their mean values with standard error bars.

GraphPad Prism 7 was used to analyse data and produce charts. P values <0.05 were considered significant.

2.2.1 **PMA Concentration Optimisation.**

PMA concentration varied widely in literature from as low as 2 µM (Bae and Wuertz, 2009) to as high as 300 µM (Varma et al., 2009). Four different PMA concentrations were tested; 10, 20, 50 and 100 µM. After addition of the PMA, samples were incubated in the dark for 5 minutes and then photo-activated for 20 minutes.

As shown in Figure 2-11, ΔCt viable increased with the increase of PMA concentration from 20 to 100 µM. ΔCt dead increased with the increase in PMA concentration from 10 to 50 µM. Both 20 and 50 µM concentration gave the same ΔCt dead (statistically non-significant difference). However, ΔCt viable varied significantly between these two concentrations. 20 µM was chosen to be used as it is the lowest concentration providing good live/dead discrimination.

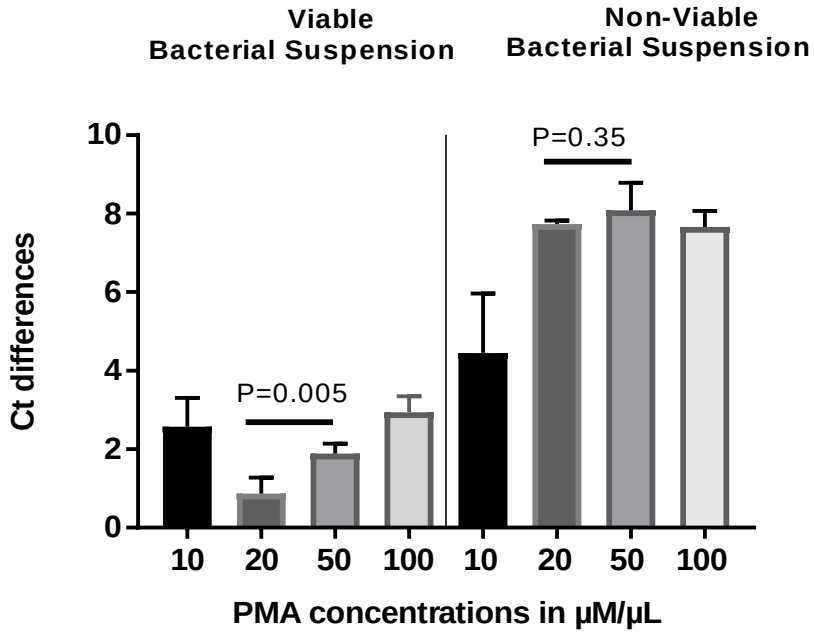


Figure 2-11: Effect of PMA concentration on its live / dead discrimination ability; ΔCt viable and ΔCt dead are shown on left and right respectively (Paired t-test was used).

2.2.2 **Dark Incubation Optimisation**

Five and ten minutes of dark incubation are the commonest used duration as reported in the literature (Varma et al., 2009, Bae and Wuertz, 2009, Wagner et al., 2008, Taskin et al., 2011).Building upon the previous step, a concentration of 20 µM was used to test the effect of different dark incubation times on the live/dead discrimination ability of PMA. Five, ten, and thirty minutes were tested with the same photo-activation time of 20 minutes.

Differences in either ΔCt viable or ΔCt dead for different dark incubation times were not statistically significant (Figure 2-12). The shortest tested time (5 minutes) was chosen for time-saving.

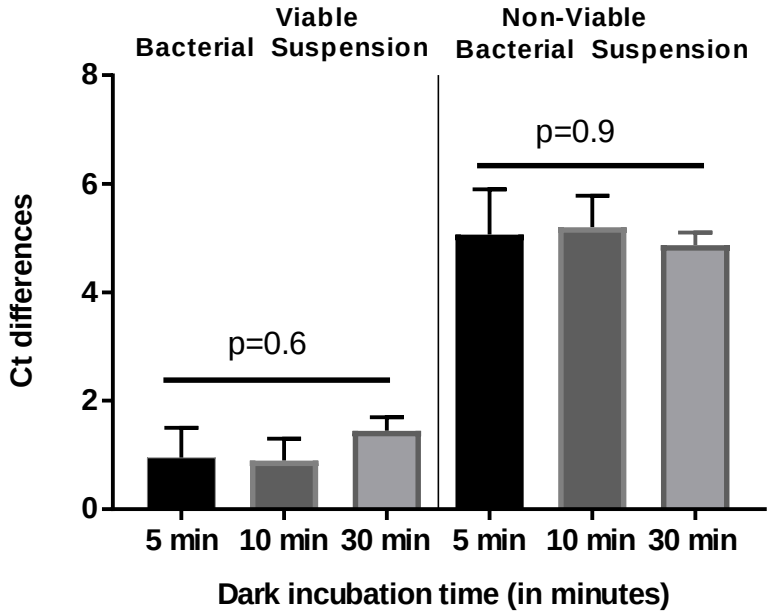


Figure 2-12: Effect of different incubation times on live / dead discrimination ability of PMA; ΔCt viable and ΔCt dead are shown on left and right respectively (ANOVA test was used).

2.2.3 **Photo-activation Optimisation**

Photo-activation time varied among literature from one to twenty minutes (Bae and Wuertz, 2009, Wagner et al., 2008, Li et al., 2014a). Ten, twenty, and thirty minutes of photo-activation were tested. Building upon the two previous steps, a concentration of 20 μM and a five-minute dark incubation were used.

ΔCt viable, but not ΔCt dead, varied significantly between different photo-activation times (Figure 2-13). As the ten-minute duration showed the lowest ΔCt viable, it was chosen to be the optimal photo-activation time.

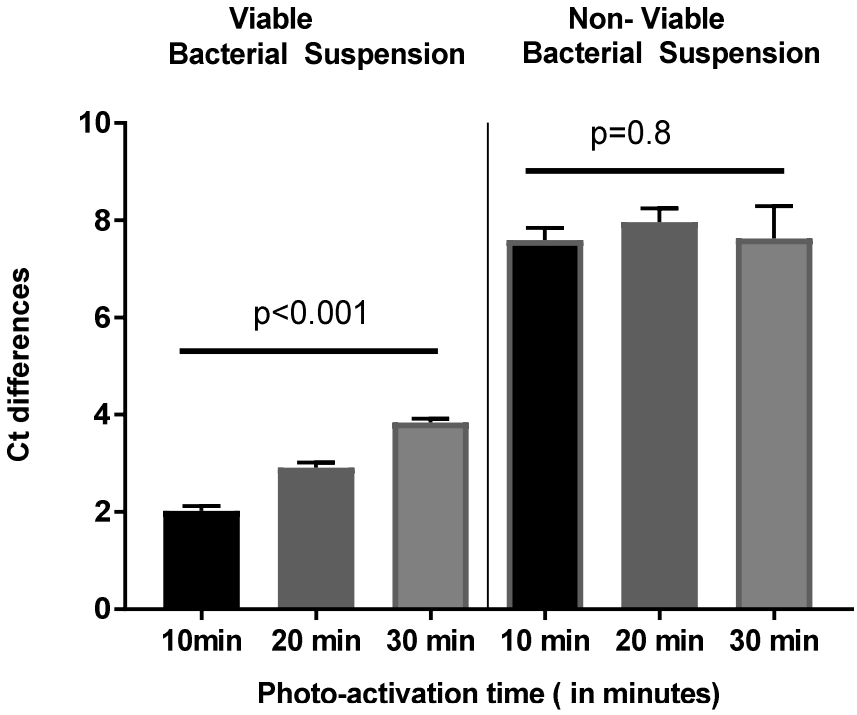


Figure 2-13: Effect of different photo-activation times on live / dead discrimination ability of PMA; ΔCt viable and ΔCt dead are shown on left and right respectively (ANOVA test was used).

Figure 2-14 is an amplification plot of live and dead bacterial suspensions with/without PMA treatment with the following methodological parameter; PMA concentration of 20 μM, dark incubation and photo-activation of five and ten minutes respectively. ΔCt viable in these conditions was below 2 cycles and ΔCt dead was above 7 cycles.

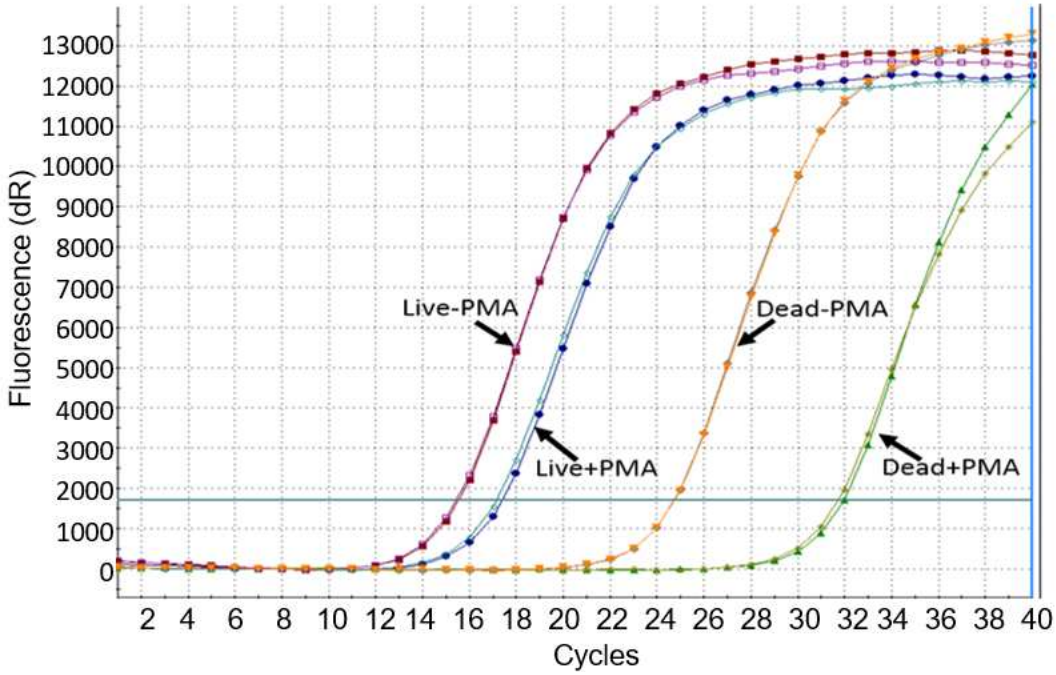


Figure 2-14 Amplification plot of live and dead bacterial suspensions with/without PMA treatment.

2.2.4 **Validation of PMA Optimised Conditions in Human Tissue Samples.**

The next step in PMA optimisation was to find out if the previously decided in vitro parameters will hold true in ex vivo conditions.

For this purpose, a known non-infected human tissue sample was homogenized as described later in the methods chapter. Homogenates were spiked with live and dead *S.aureus* suspensions prepared as previously described in this chapter. Spiked homogenates were divided into halves; one of which was treated with PMA prior to DNA extraction followed by PCR.

ΔCt viable and ΔCt dead were calculated and compared to in vitro results (Figure 2-15). Interestingly, ΔCt viable was significantly lower in ex vivo than in vitro (P=0.02).

Table 2-4 shows the differences in the means of Ct values of live and dead bacterial suspensions with and without PMA treatment in both in vitro and ex vivo samples.

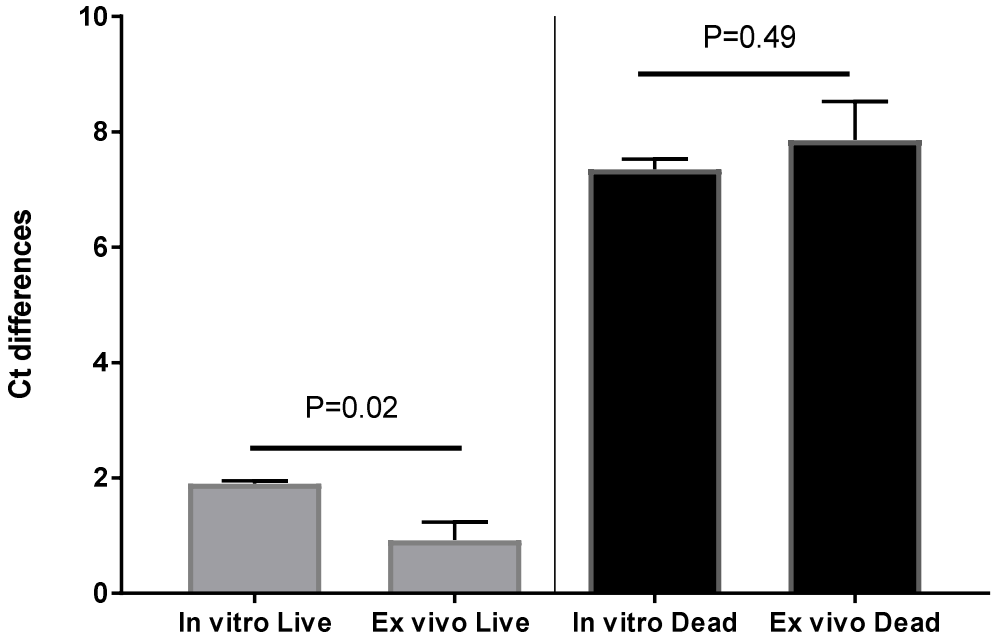


Figure 2-15 Comparison of in vitro and ex vivo ΔCt viable and ΔCt dead (Paired t-test was used).

Table 2-4 Means of Ct values of live and dead bacterial suspension with and without PMA treatment both in vitro and ex vivo.

		In vitro	Ex vivo (spiked tissues)	Delay in amplification (in cycles)
Live bacteria	With PMA	15.5	16.9	1.4
	Without PMA	13.8	16.5	2.7
Dead bacteria	With PMA	30.2	29.1	-1.1*
	Without PMA	23	23.2	0.2

*In this case, amplification of dead bacteria treated with PMA of the ex vivo sample was earlier than the in vitro sample.

2.2.5 **Discussion and Conclusion**

Over the recent years, PMA has been widely used to restrict the qPCR detection to live cells. However, unlike the broth cultures prepared in microbiology laboratories, clinical as well as environmental samples pose a complex nature that could be negatively reflected on the PMA performance. Some studies have addressed the effect of turbidity (Varma et al., 2009, Bae and Wuertz, 2009) and organic material contents (Fittipaldi et al., 2011) of the environmental samples on the PMA performance. Nevertheless, performance of PMA in clinical samples has not met a parallel interest.

Given the fact that PMA has the ability to react not only with DNA from different sources but also with various inorganic and organic molecules

(Taylor et al., 2014), clinical samples can have a deleterious effect on PMA. Hence, the importance of PMA optimisation for these samples.

In this study, different methodological factors have been optimised for PMA treatment on bacterial suspension and then, were examined on simulated infected clinical samples. The three principal factors that were tested are the concentration of the dye and the duration of both dark incubation and photo-activation.

The results of these experiments have shown that the 20 µM PMA concentration was optimal for live/dead bacterial discrimination. Higher concentrations did not inhibit the dead bacterial DNA amplification any further. On the contrary, amplification of the live bacterial DNA started to be inhibited (as evidenced by increasing ΔCt viable). In other words, with higher PMA concentration, its specificity to dead cells might be compromised as the dye might start penetrating the viable cells.

Furthermore, the PMA performance could be affected by the duration of dark incubation and photo-activation. These time frames should be sufficient for the dye to flow inside the membrane-compromised cells and get intercalated with the freely floating DNA. In this experiment, instead of a halogen lamp, a photolysis device that delivers a light at a close wavelength to the maximum PMA absorption (456nm) was used (Vesper et al., 2008). Our results suggested that five and ten minutes of dark incubation and photo-activation respectively had the highest live/dead discrimination.

Upon applying the above mentioned conditions to simulated infected human tissue samples (i.e. ex vivo), this live/dead discrimination was not

compromised. On the contrary, ΔCt viable was significantly lower which is desirable. On further scrutinizing these results, it was noted that the amplification of the live bacterial samples was delayed when spiked in tissues in comparison to pure bacterial suspension which could be explained by probable PCR inhibition by tissue products. This delay in amplification was less in samples treated with PMA than untreated samples which raises a possibility that PMA could be inhibiting the production of some PCR inhibitors. Although it is unclear how the PMA could inhibit the PCR inhibitors, its ability to react with various inorganic and organic materials might be an answer. In the case of dead bacteria (with much lower bacterial DNA available for amplification), there was even an earlier amplification for samples treated with PMA, a finding that strongly argued for desirable impact of PMA on the sensitivity PCR assay. This possibility has been further explored in the Methods and Results Chapters to study the effect of PMA on PCR assay sensitivity.

2.3 Molecular Method Development

2.3.1 Primer Optimisation

In this project, three pairs of primers targeting the commonest PJI causative organisms were used as shown in Table 2-5.

Table 2-5 Primer pairs used in the current study.

Bacteria	Gene	Amplicon size in base pair (bp)	Reference
Staphylococci	*tuf* gene	462	(Sakai et al., 2004)
Enterococci	23S rRNA	92	(Ryu et al., 2013)
P.acnes	16S rDNA	586	(Sfanos and Isaacs, 2008)

As a part of the optimisation of the whole PCR assay, different primer concentrations should be assessed. For each pair, different combinations of the forward and reverse primers were tested along with non-template control (NTC) to test its specificity. The ideal primer pair should yield the lowest Ct value with a single melting curve and negative NTC result. Once an ideal primer pair was chosen, an assay of a whole range of serial dilutions was carried out to test its performance through a wide range of template concentrations.

For staphylococcal primers, combinations of pairs tested are shown in Figure 2-16. Results are shown in Figure 2-17. Equimolar primer pairs of 250 nM concentration of both the forward and reverse primers gave the lowest Ct value and performed well on a wide range of different template concentrations. Enterococcal and *P.acnes* primers were tested similarly and the same concentrations performed well.

		Forward Primer (nM)			
Reverse Primer (nM)		125	250	375	500
	125				
	250				
	375				
	500				

Figure 2-16 Chess board diagram of the different staphylococcal primer pair concentrations tested.

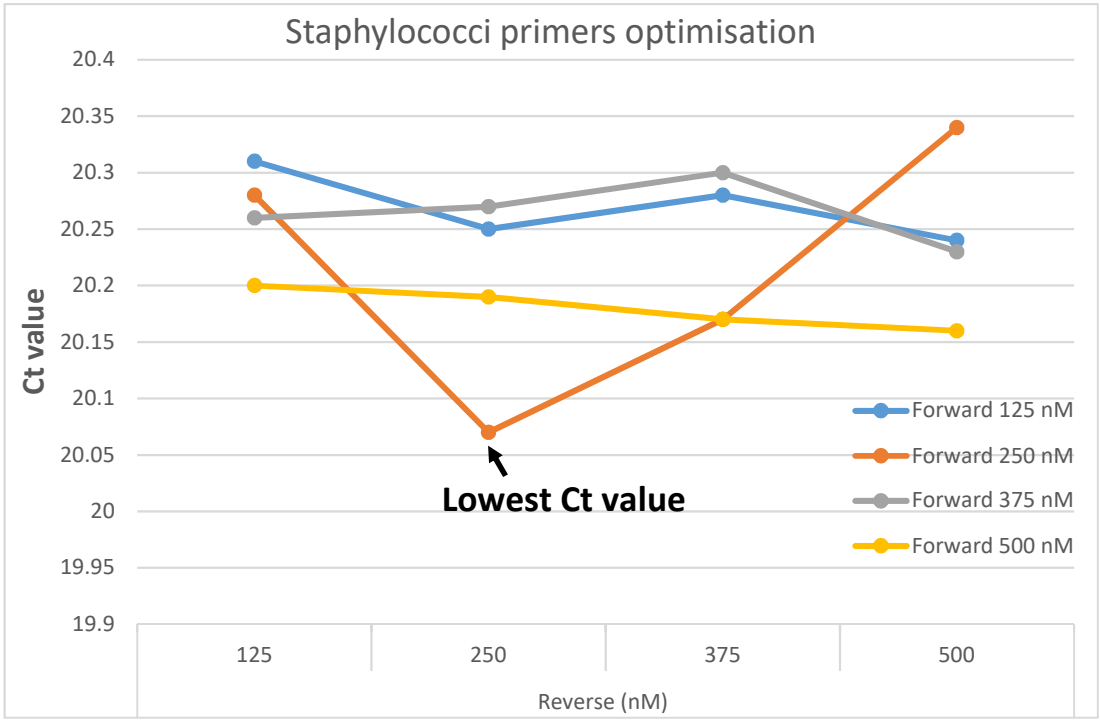


Figure 2-17 Ct values using different staphylococcal primers concentrations.

2.3.2 **Master Mixes Comparison**

As there are currently many real-time master mixes available in the market, such comparative evaluation would be helpful to increase the overall performance of PCR assay. Two commercially available kits were chosen and compared. These kits are Brilliant II SYBR® Green QPCR Master Mix (Agilent, California, USA) and SensiFAST™ SYBR® Lo-ROX Kit (Bioline, London, UK).

Serial dilutions of *S.aureus* DNA extraction were made as described later in standard curve preparation in the Methods Chapter. Two identical PCR assays were run with a different master mix used in each. Samples were run in duplicates and the mean Ct value for each concentration was calculated. A standard curve was generated for each kit and efficiency and Rsq (slope of the curve) were calculated.

Table 2-6 shows the Ct values for the serial concentration in the different master mix kits assays. The difference in Ct values was statistically significant (p=0.004). Figure 2-18 and Figure 2-19 are standard curves generated from Bioline and Agilent kits respectively and show the efficiency and Rsq of each. Figure 2-20 is an amplification plot of the serial concentration with the two kits showing the difference in the fluorescence data collected at the reaction endpoint for each of the kits.

Table 2-6 Ct values of different DNA template concentration by two different master mix kits PCR assays.

Concentration (cfu/mL)	Agilent	Bioline
10^8	14.07	12.98
10^7	17.83	16.63
10^6	23.38	20.1
10^5	27.83	23.58
10^4	31.14	27.84
10^3	32.9	30.4
10^2	Not detected	32.96

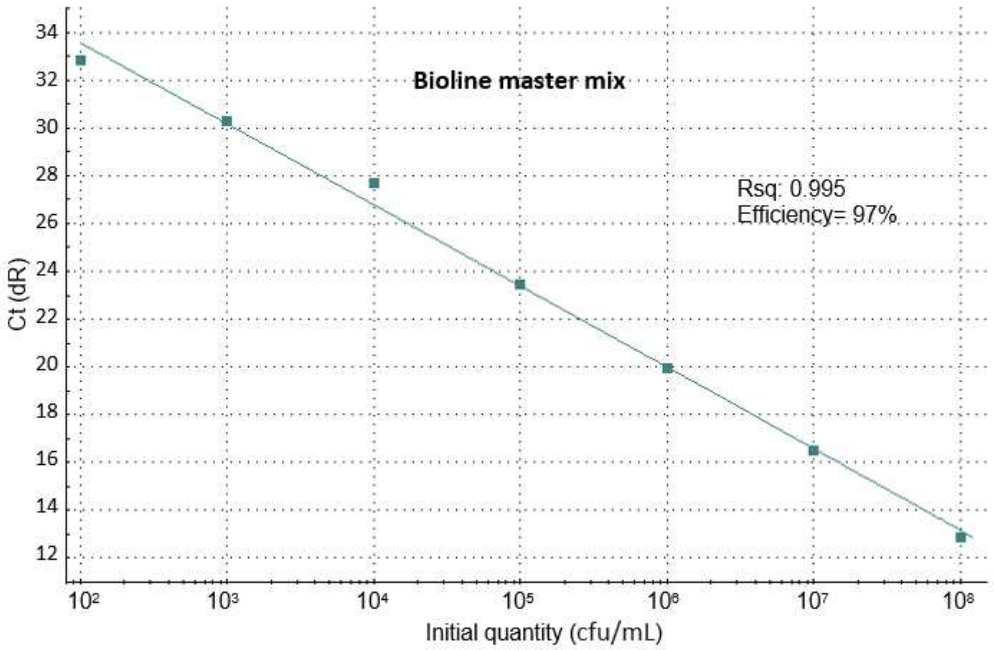


Figure 2-18 Standard curve of serial dilutions of *S.aureus* DNA templates in a Bioline master mix PCR assay with an efficiency of 97% and Rsq of 0.995.

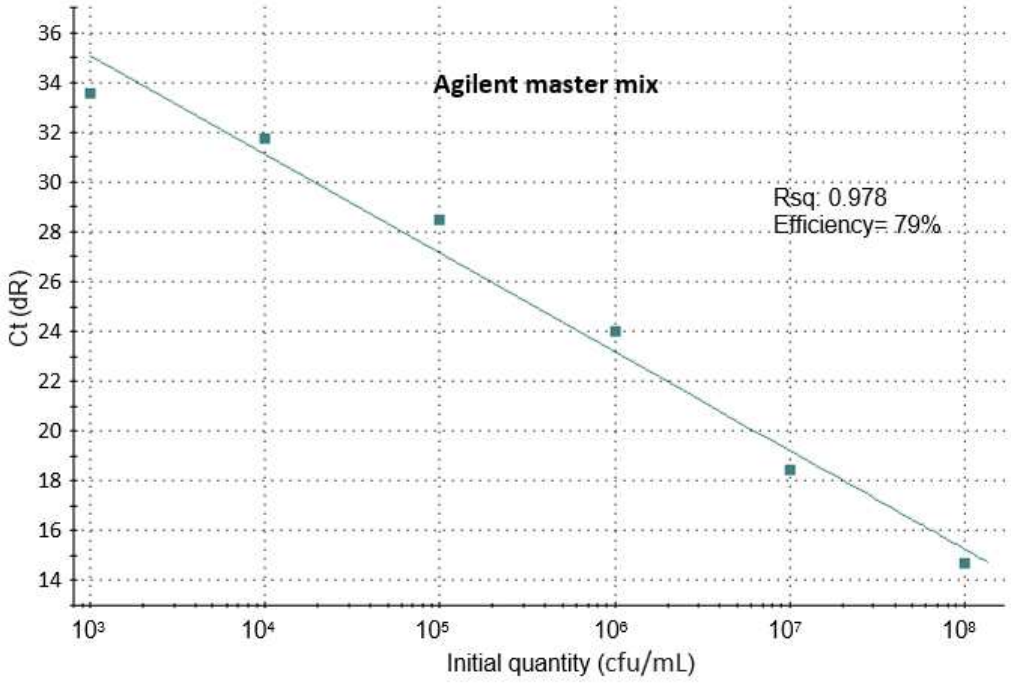


Figure 2-19 Standard curve of serial dilutions of *S.aureus* DNA templates in a Agilent master mix PCR assay with an efficiency of 79% and Rsq of 0.978.

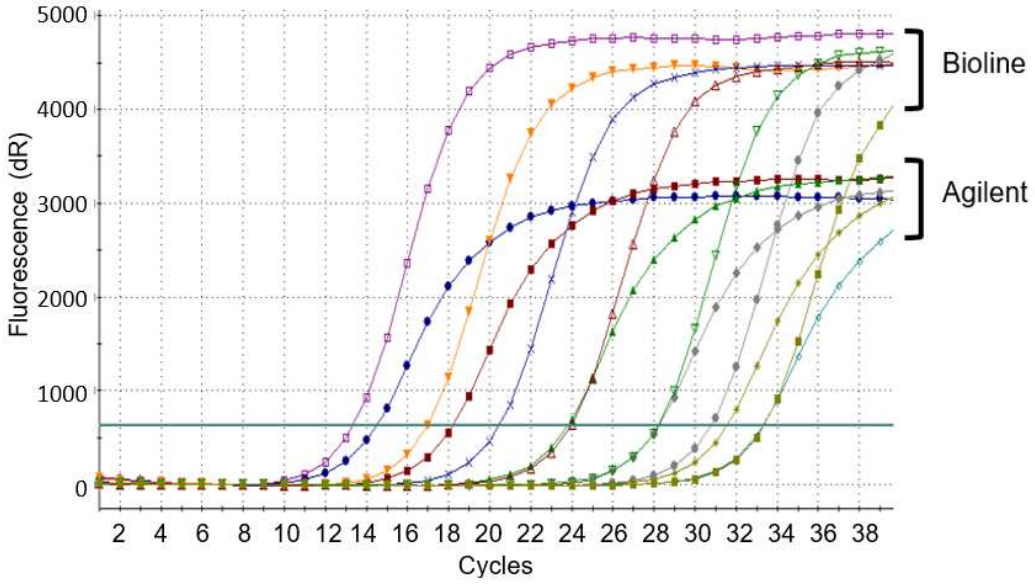


Figure 2-20 Amplification plots of ten-fold serial dilutions of *S.aureus* DNA templates using two different master mix kits for comparison; note the higher fluorescence of end reaction products with Bioline master mix.

Bioline master mix was chosen to be used as it outperformed Agilent for the following reasons; lower Ct value for the same DNA concentration, lower detection limit, higher efficiency and Rsq values, and higher fluorescence at the reaction endpoint. The lower fluorescence at the reaction endpoint in case of Agilent master mix means either the reagents have been consumed or inhibitors have accumulated at an earlier stage, and either or both of which can reduce the PCR reaction efficiency.

2.3.3 Gradient PCR and a Trial of Annealing Temperature Optimisation.

A pair of *Enterococcus faecalis* specific primers targeting its 16S rDNA gene with an amplicon of 143 bp (base pair) size was intended to be used (Ryu et al., 2013). Optimisation of its forward and reverse concentrations was carried out as described above for staphylococcal primers and results are shown in

Figure 2-21. On the melting curve analysis, double peaked curves were noted. Higher concentration (500 nM) of both forward and reverse primers were necessary to get a significant single peaked curve. Figure 2-22 shows the melting curves using low (250 nM) and high (500 nM) primer concentrations. Concentrations above 500 nM were associated with a lot of primer dimer formations.

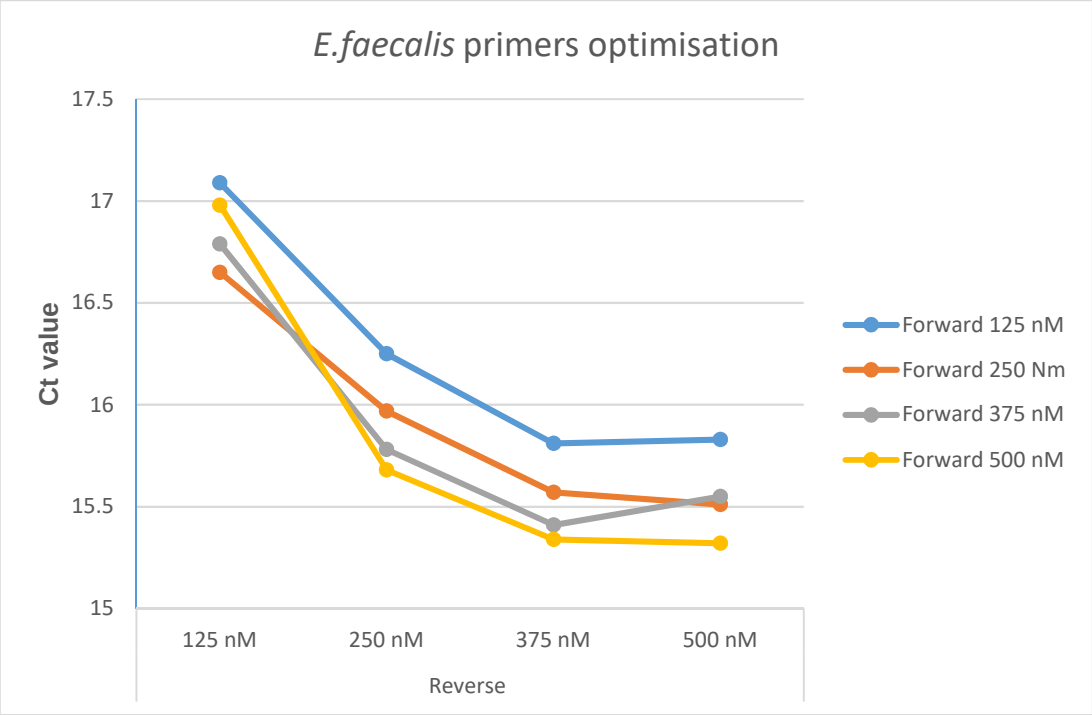


Figure 2-21 Ct values using different *E.faecalis* primers concentrations.

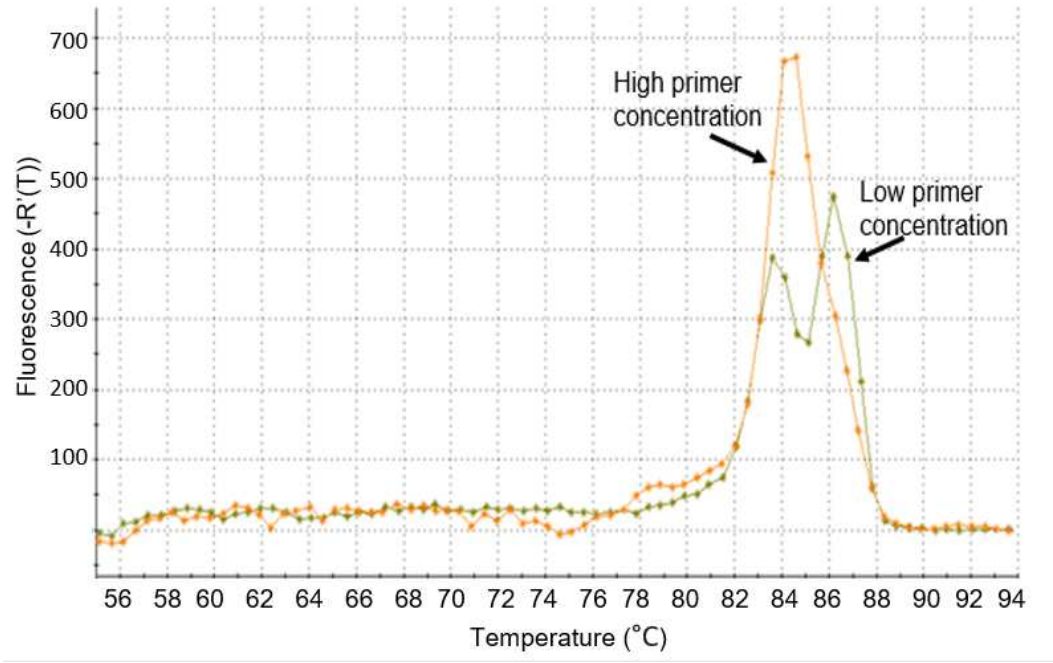


Figure 2-22 Melting curve analysis of PCR products using high (500 nM) and low (250 nM) concentration of *E.faecalis* primers.

However, upon applying this primer pair (equimolar primer pair of 500 nM concentration of both the forward and reverse primers) in clinical samples, a lot of insignificant amplifications were noticed in the background with insignificant melting curves. Furthermore, the sensitivity of the assay was compromised by getting false negative results from known infected tissue samples with *E.faecalis* (isolated by conventional bacterial culture).

Further optimisation of the annealing temperature was then tried by using a gradient PCR. Manufacturers theoretically calculate the melting temperature (Tm) of primers depending on different factors including their length and sequence. However, this calculated Tm is just given for guidance and can widely vary according to the method used for calculation. If a too low annealing temperature is used, non-specific products can be produced as in our case. On the other hand, too high annealing temperature can negatively

affect the whole PCR yield. The calculated Tm of the forward and reverse primers according to the manufacturer were 58.8°C and 56°C respectively, so a gradient scale of 52°C to 63°C was tried using ten identical reactions.

In a single gradient PCR reaction, different empirical annealing temperatures can be simultaneously tested. After running the gradient PCR reaction in MJ Research Tetrad PTC-225 Thermal Cycler (GMI, Ramsey, USA), products were analysed for the melting curve in the Mx3005P QPCR (Agilent, California, USA). In addition, they were run on agarose gel as described in Methods Chapter (3.7.6 below). Results are shown in Figure 2-23Figure 2-24.

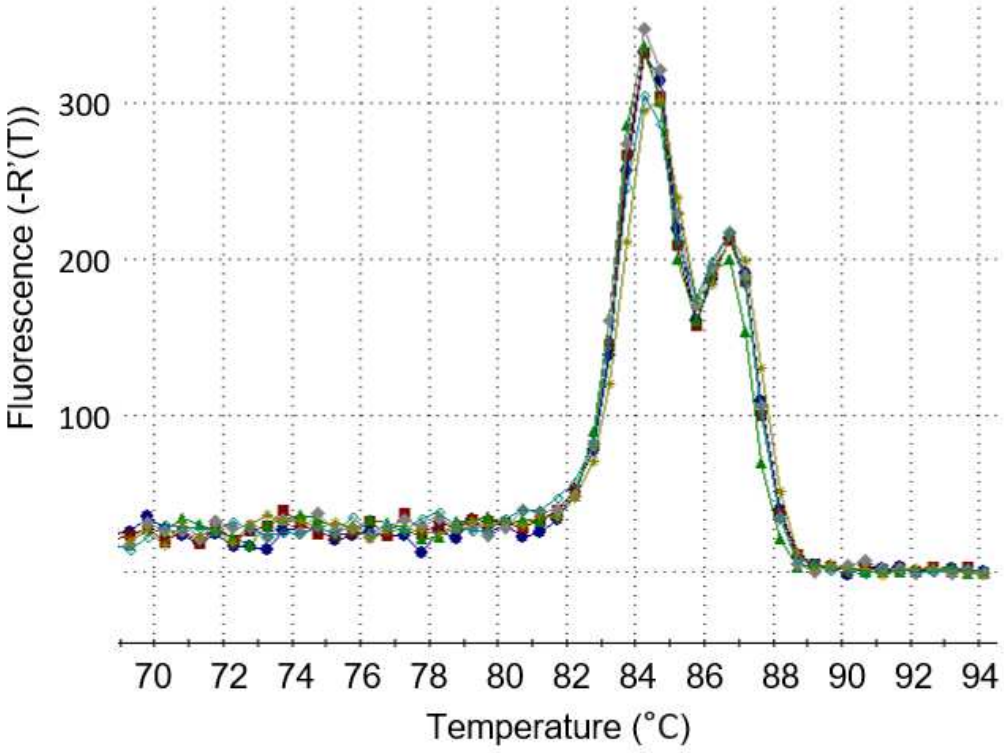


Figure 2-23 Melting curves of products of different annealing temperatures with no significant difference between them.

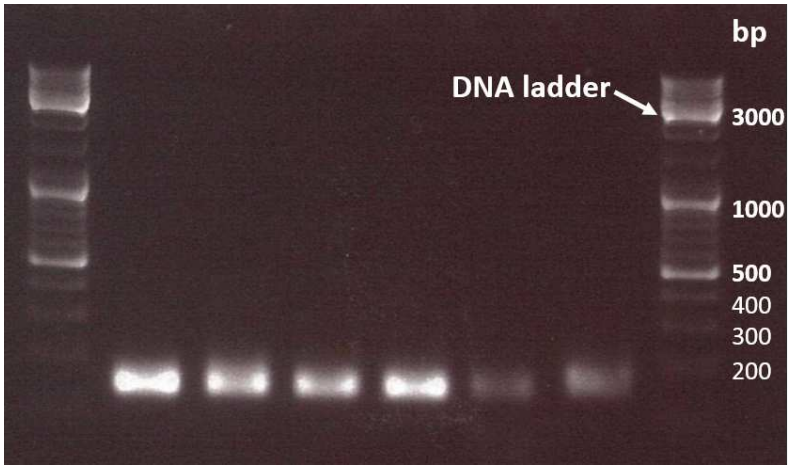


Figure 2-24 Agarose gel electrophoresis of products of different annealing temperatures; first and last lanes are DNA ladders, and the middle six lanes are six different products with no significant difference between them.

Finally, to compare between performance of these primers in simulated clinical and laboratory (pure bacteria suspension) samples, two sets of samples were prepared. Ten-fold serial dilutions of extracted *E.faecalis* DNA were suspended in either nuclease free water or in DNA extract of human tissues. PCR was run for both sets in the same assay.

In laboratory samples, primers were properly working regardless of the template concentration. On the contrary, in simulated clinical samples, with higher bacterial template concentration, it seems that primers react with and correctly amplify the gene of interest despite the presence of human DNA. However, in lower bacterial DNA comparative to human DNA, primers might be preferentially amplifying a human gene with early consumption of the reagents leading to false negative results. Melting temperature of the specific bacterial gene of interest and the probable amplified human gene was approximately 84°C and 87°C respectively.

As a result, it has been decided to abandon the use of these primers and replace them with an Enterococci genus-specific primers (see 2.3.1 above).

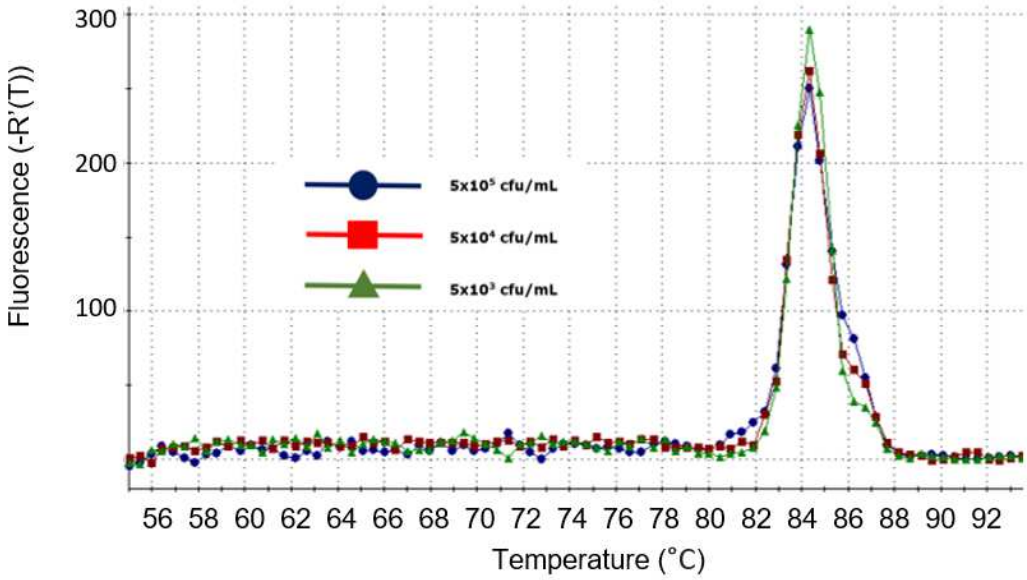


Figure 2-25 Melting curves of human genome-free laboratory bacterial samples. Performance of primers is not affected by template concentration; blue, red, and green curves are $5x10^5$, $5x10^4$, and $5x10^3$ cfu/mL respectively.

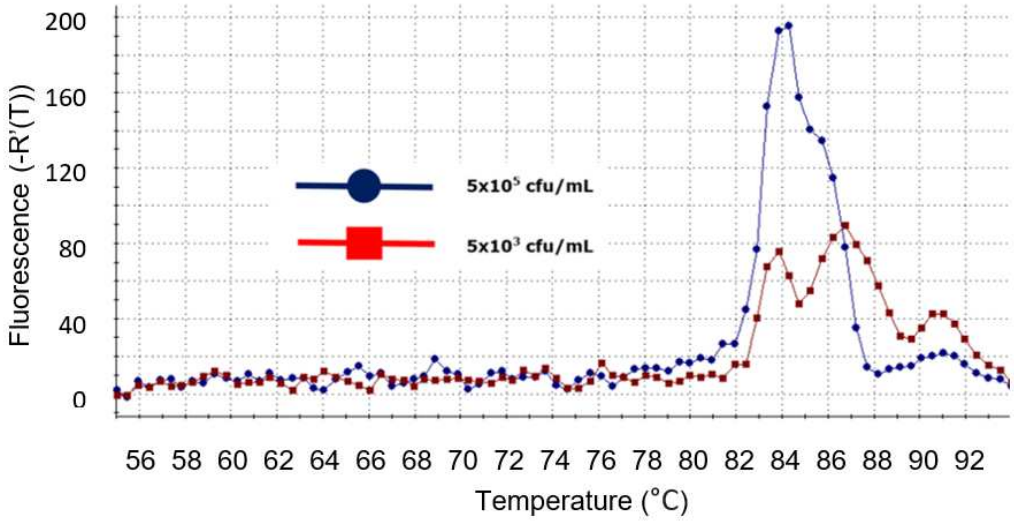


Figure 2-26 Melting curves of simulated clinical (containing human genome) bacterial samples. Performance of primers is affected by template concentration; blue and red lines are $5x10^5$ and $5x10^3$ cfu/mL respectively.

Chapter 3. Methods

Building upon the results of the previous chapter, methods employed in this project are described in this chapter. It begins with a brief description of the study design and approval followed by recruitment of patients and collection of samples. The workflow is then described in order, starting with the primary sample processing (homogenization of tissues and sonication of prostheses), followed by PMA treatment, and ending with PCR running and interpretation of results. Finally, the chapter describes the statistical methods used in this study.

3.1 Study Design

This is a prospective study designed to evaluate the performance of a new diagnostic test in line with the common study design of diagnostic tests evaluation according to Guyatt et al. (1986). For this kind of study, there has to be a “gold standard” test, the positive results of which are decided to be accepted as true (Bossuyt et al., 2003). In a study for a diagnostic test accuracy evaluation, like this study, the new test is applied to a series of patients. Then, the results obtained should be compared to the results of the gold standard in the same series. Comparing both results, the new test can then be evaluated in terms of how much its results are matching the results of the gold standard. This matching could be measured by pairs of sensitivities and specificities or by measuring the area under the curve (AUC) in a receiver operator characteristic curve (ROC) (Griner et al., 1981). ROC curve is a statistical graph generated to depict the effectiveness of a diagnostic test discrimination between diseased and non-diseased groups (Perkins and Schisterman, 2005). The larger the AUC, the more accurate is the diagnostic test. Graphically, the optimal cutoff value which has the largest sum of sensitivity and specificity is at the inflection point of the curve. This is the farthest point of the curve from the diagonal line of indiscrimination i.e. the shoulder of the ROC curve.

For our study, the MSIS criteria have been used to be the gold standard, to which the new diagnostic test was compared. Since its publication by Parvizi et al. (2011), MSIS criteria have gained an increasing popularity and they have been used by most of the recent studies assessing new diagnostic tests for PJI (Kwiecien et al., 2017, Shahi et al., 2017, Tischler et al., 2014,

Wouthuyzen-Bakker et al., 2017). As a key to the validity of this study and to avoid observer bias, it has been taken into consideration that the comparison of test results to the gold standard should be done in a blind and independent fashion (Knottnerus et al., 2002).

3.2 Study Approval

The study was approved by Nottingham Health Science Biobank (NHSB) which regulates the donation of surplus tissues for research purposes in the University of Nottingham (Biobank Reference ID ACP0000172; Molecular Diagnosis of Orthopaedic Implant Infections). The application form, as approved by NHSB on the 23rd of January 2016, is presented in the Appendices chapter of this thesis (Appendix 1).

3.3 Participants and Settings

Adult patients presenting to the orthopaedic outpatient clinics for revision arthroplasty procedures were considered for this study. In the first three months of the study, patients with diabetic feet were also considered as a source of clearly infected tissues. Potential participants were counselled by their treating surgeons. If accepting to participate, they were given a patient information sheet issued by the Biobank (Appendix 2) along with a simplified description sheet of this research and its aims (Appendix 3). Participants had to give a written consent (Appendix 4 is an NHSB consent form) to the NHSB for their tissues to be used in research purposes which is lifelong unless withdrawn. The only exclusion criteria that were applied were refusal or lack of capacity to consent.

3.4 Sample Collection, Transport, and Registration.

At the time of surgery, multiple tissue samples were collected in sterile universal containers. Surgeons were advised to collect each sample with a new sterile set of instruments. Each sample was transported in a separate sterile container. Samples were carried in a specimen box containing normal water ice and transferred to NHSB where they were anonymized and barcoded. Retrieved prostheses were collected in large sterile pots (Medfor, Aldershot, UK).

Afterwards, samples were transferred to the Biomaterials-Related Infection microbiology laboratory, registered, and given a serial number according to the laboratory registry protocol (NB…). A sample is defined as a specimen collected from one anatomical site during surgery.

3.5 Conventional Microbiological Methods.

3.5.1 Tissue Processing

After registration, tissue samples were processed under complete aseptic conditions as soon as possible. They were taken out of the container with sterile forceps and placed in a sterile petri dish. A small piece of approximately 5 gm was cut off and the remaining sample was immediately replaced in the container and stored in -20 °C freezer for future use if needed. The 5 gm piece was cut into as many pieces as possible and then approximately 0.15 gm of it was transferred to a 2mL tubes (Sarstedt) along with 1500 µL of PBS to be homogenized.

For homogenizing the samples, Roche magNA Lyser homogenizer was used. Four cycles were run; each lasted for 45 seconds at a speed of 4500

rpm. After each cycle, the lid of the homogenizer was opened making sure that temperature of tubes did not rise. 45 seconds were allowed between cycles.

100 µL of the homogenate was spread on blood agar plates (Oxoid Ltd, Basingstoke, UK). Two plates were prepared; one for aerobic and the other for anaerobic bacterial culture. Both were kept at 37±0.5°C. Aerobic plates were checked daily for 72 hours then discarded if showing no growth. Anaerobic plates were checked after 48 hours, one, and two weeks before being reported as negative.

Any isolated bacteria were identified using the standard microbiological methods i.e. Gram staining, catalase test (hydrogen peroxide- Oxoid), Oxidase test (Oxoid), and analytical profile index micro-organisms identification tests (API, BioMérieux, Basingstoke, UK).

In case of difficult or questionable bacterial identification, isolate was sent to Sheffield Teaching Hospital NHS Foundation Trust to be identified by Matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF, Microflex LT Mass Spectrometer, Bruker Daltronics) using MALDI Biotyper RealTime Classification (Bruker Daltronics) software. A fresh pure culture of the isolate was prepared and sent in a transport swab (Sterilin Ltd, Newport, UK).

The identified isolate was then given an “F” number in the laboratory freezer register and stored in cryobank vials at -20 °C for future use.

One drop of the homogenate was spread on a glass slide, Gram stained, and carefully examined under microscopy for any bacteria. The remaining part of

the homogenate was either immediately used for molecular diagnostic procedures or stored in -20 °C freezer for next day use.

3.5.2 **Prostheses Sonication**

A prosthesis was defined as any retrieved biomaterial from the patient at the time of surgery. This could be one or more component of hip or knee prostheses, polyethylene, or PMMA bone cement.

Retrieved prostheses were collected in sterile wide-mouthed 1-litre polypropylene pots with screw cap (Medfor). Prostheses were processed on the same day of collection.

400 mL of anaerobic basal broth (ABB, Oxoid) were added to the container. The container was then sonicated in precision ultrasonic cleaning sonicator set at 50 Hz (Ultrawave Ltd, Cardiff, UK) for 5 minutes. ErgoOne fast pipette controller (Starlab, Hamburg, Germany) was used to aseptically transfer 20 mL of the sonicate to each of four universal tubes. Universals were then centrifuged for 15 minutes at 3000 rpm. The supernatants of two tubes were discarded and the concentrated sonicate was further used. The other two universals were stored at -20ºC for future use if needed.

100 µL of the concentrated sonicate was cultured on two blood agar plates (Oxoid) for aerobic and anaerobic culture. Any isolated bacteria were identified and stored as described with tissue homogenates. One drop was spread on a glass slide, Gram stained, and examined under microscopy as for tissue homogenates. The remaining part was saved for molecular analysis.

3.6 PMA Treatment

After conventional methods, the stored remaining portions of the samples (homogenate and/or sonicate) were used for the molecular methods. 500 mL of the sample were transferred to each of two sterile Eppendorf tubes. One of those was treated with PMA (Biotium).

A stock of 20 mM PMA was prepared by dissolving 1 mg PMA (as supplied from the manufacturer) in 98 uL dimethyl sulfoxide (DMSO, Sigma-Aldrich). The stock was stored at -20 °C protected from light. A ten-fold dilution was then prepared from the stock at time of use.

For PMA treatment, 5 µL of 2 mM of this photo-reactive dye was added to the sample in a dim-light room for a final concentration of 20 µM/µL. The sample was then incubated in the dark for 5 minutes (covered with aluminium foil), while gently shaking the tubes from time to time to ensure proper mixing. PMA-Lite™ LED Photolysis Device (Biotium) was used for the photo-activation for 10 minutes.

3.7 Molecular Methods.

3.7.1 DNA Extraction

For DNA extraction, GenuElute DNA extraction kit (Sigma-Aldrich) was used starting with 500 µL of the liquid material of interest (homogenate, sonicate, or bacterial suspension) placed in 1.5 mL Eppendorf tubes. According to the manufacturer's instructions, DNA extraction was done in two steps; cell lysis followed by DNA isolation and purification.

To be concentrated, 500 µL of the suspension of interest were centrifuged in an Eppendorf 5415C Centrifuge (Eppendorf, Hamburg, Germany) at 12,000

rpm for 2 minutes. Then, 450 µL of the supernatants were removed. Eppendorf tubes were consistently placed in the centrifuge as the recess of the lid to be facing upwards and outwards. As a result, the pellets were consistently formed in the same site of the tube each time (Figure 3-1). This was important in case of a tiny pellet which could be difficult to be visualized to avoid its disturbance by the tip of the pipette.

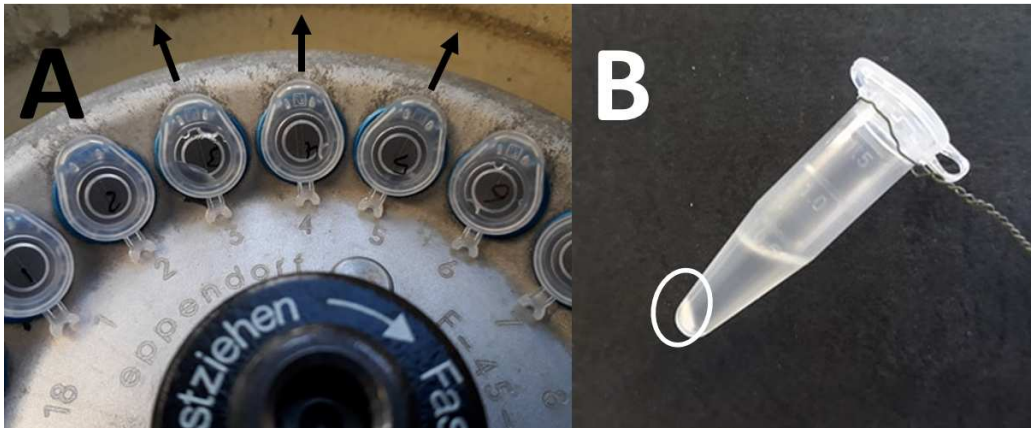


Figure 3-1 A: Eppendorf tubes placed in the same direction in the centrifuge (black arrows). B: pellets were collected in the upper part of the bottom of the tube in the position shown in figure (white circle).

Upon getting concentrated 50 µL aliquots, 200 µL of lysozyme solution were added and incubated at 37ºC for 30 minutes. 20 µL of proteinase K followed by 200 µL of lysis solution C (supplied in the DNA extraction kit) were added. Samples were then vortexed to ensure homogeneity of the mixture which is crucial for efficient lysis and then incubated at 55ºC for 10 minutes. 200µL of ethanol were then added to each sample.

For isolation steps, the samples were transferred to binding columns seated in 2 mL collection tubes after the columns were prepared. To prepare the columns, 500µL of column preparation solution (supplied in the DNA extraction kit) were added to each column and then centrifuged at 12,000

rpm for 1 minute. Samples in the columns were then centrifuged at 9000 rpm for 1 minute for the DNA to be collected at the silica membrane at the base of the columns. Two washing steps were then done; each consisted of adding 500 mL of the relevant washing solution followed by centrifuging. The centrifuging of the second washing step should be 12,000 rpm for 3 minutes to ensure dry column necessary for elution. As the wash solution 2 contained a high ethanol concentration, dryness of columns was crucial prior to elution to avoid co-elution of ethanol which is a strong PCR inhibitor (Rossen et al., 1992). Lastly, elution was done twice in the same tube to increase DNA yield according to the manufacturer's recommendations. 200 µL of elution solution were used each time. The 400 µL end product containing the pure DNA was stored at -20ºC.

3.7.2 **Setting up the PCR Mixture and Protocol**

A final volume of 20 µL was prepared in each tube by mixing 10 µL of SensiFAST™ SYBR® Lo-ROX master mix Kit (Bioline), 0.5 µL of each of the forward and reverse primer, 4 µL nuclease free water and 5 µL template or nuclease free water for non-template control (NTC) preparation. 12 strips, of 8 thin walled PCR tubes (0.2 mL) each, were placed in a PCR plate. 20 µL micropipette set to 10 µL was used for mixing the regents while avoiding introducing bubbles. If bubbles were produced, tube strips were centrifuged prior to running the PCR. It was important to ensure that all the reagents were settling down in the tubes with nothing hanging on the wall for proper mixing. Strips of caps were then used to cover the tubes ensuring that they were not kinked to avoid evaporation of mixture during the PCR. Labelling of the tubes was done away from the lid to avoid interfering with the real-time

detection of fluorescence which is performed by a laser light path that enters through the lid.

Sequences of primers (Eurofins Genomics, Ebersberg, Germany) are shown in Table 3-1. In addition, the human glyceraldehyde-3-phosphate dehydrogenase (GADPH) housekeeping gene was amplified as an internal control of DNA extraction and possible PCR inhibition.

Table 2-5 in Method Development Chapter shows the amplicon size, target gene, and reference for each primer pair.

Table 3-1: Sequences of primers used in the study.

Gene of interest		Primer sequence (5'→3')
Staphylococci: Forward		CAATGCCACAAACTCG
	Reverse	GCTTCAGCGTAGTCTA
Enterococci :	**Forward**	AGAAATTCCAAACGAACTTG
	Reverse	CAGTGCTCTACCTCCATCATT
P.acnes:	**Forward**	GGGTTGTAAACCGCTTTCGCCT
	Reverse	GGCACACCCATCTCTGAGCAC
GADPH*:	**Forward**	TCCCTGAGCTGAACGGGAAG
	Reverse	CGCCTGCTTCACCACCTTCT

* GADPH: Glyceraldehyde-3-phosphate dehydrogenase

SYBR green (with dissociation curve) protocol of MX3005P real time PCR machine (Agilent, California, USA) was used. The following conditions as shown in Figure 1-5 in Introduction Chapter were used for the amplification: 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 30 seconds, 57ºC for 1 minute, and 72ºC for 1 minute. Dissociation (melting) curve followed the 40 amplification cycles immediately.

PCR products were stored at -20ºC for potential agarose gel electrophoresis or any further use in future.

3.7.3 **Standard Curve Preparation**

Standard curves were prepared with the use of ten-fold serial dilution of quantified bacterial DNA. Bacterial suspensions were prepared and quantified as described in 2.1.1 in Method Development Chapter. *S.aureus*, *E.faecalis*, and *P.acnes* were used to prepare standard curves for staphylococci, enterococci, and *P.acnes* PCR assays respectively. Cultures were standardised to have an optical density (490 nm) of between 0.6 - 0.7 which is approximately 10^8 cfu/mL. Plating of serial dilutions of bacterial suspensions was done for confirmation of quantification.

DNA was extracted from the bacterial suspension of 10^8 cfu/mL concentration. Serial dilutions of the extracted DNA were prepared using the appropriate amounts of nuclease free water. Eight dilutions were prepared to cover a range from 10^8 cfu/mL to 10^1 cfu/mL.

PCR was done for the whole prepared range of DNA serial dilution along with a negative control sample. Each sample was run in duplicate and each experiment was done twice to ensure consistency. Standard curves were then generated using the MxPro qPCR software (USA). Slope of the standard curve and PCR efficiency were automatically calculated by the software.

3.7.4 **Setup of PCR Assays and Projects**

In a 96-well PCR plate, forty-six samples (half of the samples were PMA treated and the other half were their non-PMA treated equivalents) were tested in every PCR assay in addition to NTC and a positive control. Each sample and control was run in duplicate. Figure 3-2 is an example of the

outline of the PCR plate. Pre-prepared excel spread sheet was used to import sample numbers as the names of the wells in the assay. A total of 207 samples were divided into nine groups; twenty three samples in each.

PCR project is a group of independent assays in which samples in different experiments can be confidently compared (with the condition of proper setting of threshold was used). The positive control used in each of the nine experiments was a replicate sample that was consistently run in the same well position (H9 and H10 as shown in Figure 3-2). The nine PCR assays of the nine groups of samples with the standard curve experiment were combined in a single project. Three projects were generated; staphylococci, enterococci, and P.acnes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	4266 +	Duplicate of column 1	4266 -	Duplicate of column 3	4280 +	Duplicate of column 5	4280 -	Duplicate of column 7	4291 +	Duplicate of column 9	4291 -	Duplicate of column 11
B	4270 +		4270 -		4281 +		4281 -		4292 +		4292 -	
C	4271 +		4271 -		4283 +		4283 -		4294 +		4294 -	
D	4272 +		4272 -		4284 +		4284 -		4295 +		4295 -	
E	4274 +		4274 -		4285 +		4285 -		4296 +		4296 -	
F	4275 +		4275 -		4286 +		4286 -		4297 +		4297 -	
G	4278 +		4278 -		4287 +		4287 -		4298 +		4298 -	
H	4279 +		4279 -		4290 +		4290 -		PC		NTC	

Figure 3-2 Outline of PCR plate of the second group of samples (from NB4266 to NB4298), every even numbered column is the replicate of its previous odd numbered column ("+": treated with PMA; "-": non-treated with PMA; PC: positive control; NTC: non-template control).

3.7.5 **PCR Data Extraction and Interpretation**

Data generated by the MxPro qPCR software was exported to excel spreadsheet to be validated, analysed, and interpreted. Exported data included the number and name of the well, the melting temperature, and the Ct value.

To be considered valid, the PCR results of every assay had to include an NTC lacking an amplification signal and a positive control sample showing a specific peak in the melting curve. Once the controls proved to be properly working, interpretation of samples were done. The first interpretation step was to discriminate between specific and non-specific products. For each assay, the specific temperature range was calculated from the melting temperature of the positive control ±0.5°C. Positive samples were then quantified by plotting them against the standard curve generated in the relevant PCR project (Case number 15 in the PJI Case Reports Chapter is an example of PCR data analysis). If the specificity of any product was questionable, it was run in agarose gel electrophoresis as described below.

3.7.6 **Agarose Gel Electrophoresis**

2% weight/volume (w/v) agarose gel was prepared with 2 gm agarose (Thermo Fisher) in 100 mL Tris-acetate-EDTA (TAE) buffer in an Erlenmeyer flask. The mixture was then microwaved until complete melting. After cooling, 5µL of 10mg/mL ethidium bromide (Thermo Fisher) was added and the gel was poured in a casting tray. Solidified gel was then transferred to the electrophoresis chamber and covered with TAE buffer.

8 µL of the PCR product of interest was mixed with 2 µL of gel loading dye and then loaded in an indentation in the gel. The same volume of GeneRuler DNA Ladder Mix (Thermo Fisher) was added to one or more indentation. The chamber was then covered and the electrodes were connected. The gel was then run at 90 volts for 42 minutes. Gel was then visualized under an ultraviolet lamp and pictures of the bands were captured and printed.

3.7.7 Effect of PMA on Sensitivity of PCR Assay in Clinical Samples

Ten-fold serial dilution suspensions of fresh culture (18-24 hours old) of *E.faecalis* were prepared from 10^8 cfu/mL to 10^1 cfu/mL. Apparently sterile tissue homogenates were tested with PCR to ensure they are non-infected. 500 mL of each bacterial suspension concentration was added to the same amount of tissue homogenate and then divided; one half was PMA treated and the other left untreated. DNA was then extracted and PCR run. Samples were run in duplicates in each experiment and three independent experiments were carried out in three different days. In addition to a positive control and NTC, pure tissue sample was tested to further ensure lack of contamination. Ct values of PMA treated samples were compared to their untreated equivalents.

3.8 Clinical Data Collection

According to the NHSB's regulations, the principal investigator should not have any identifying data of the patients. NHSB passed the NHS numbers of the participants to a third person, who collected the relevant clinical history and investigations in supplied forms shown in Appendix 5. These data were then received, tabulated, and analysed anonymously. Connection and

correlation between research laboratory results and clinical history were done through the Biobank given numbers.

3.9 Statistical Methods

Graphpad Prism 7 was used to analyse data, produce charts, and generate ROCs. Appropriate statistical tests were used according to the distribution of data. P values <0.05 were considered significant.

Kappa statistic was performed to determine agreement between the MSIS criteria and different diagnostic tests (ESR, CRP, conventional and viability PCR). Landis and Koch (1977) categorized the agreement into poor, slight, fair, moderate, substantial, and almost perfect if the kappa value was <0, 0.0-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80, or 0.81-1.00 respectively.

As our sample size in the PJI and aseptic group are not truly reflecting the prevalence of PJI, the positive and negative predictive values (PPV and NPV respectively) could not be directly calculated. Alternatively, the following formulae were used as recommended by Steinberg et al. (2009):

$$PPV = \frac{sensitivity \times prevalence}{sensitivity \times prevalence + (1 - specificity) \times (1 - prevalence)}$$

$$NPV = \frac{specificity \times (1 - prevalence)}{(1 - sensitivity) \times prevalence + specificity \times (1 - prevalence)}$$

For these formulae, the prevalence of PJI among the knee revision surgeries was assumed to be 15% according to Khan et al. (2016) who used pooled data from joint registries in five countries (UK, Sweden, Australia, New Zealand, and USA).

Chapter 4. Results

This chapter reports the results of conventional and molecular methods applied in this study. It starts with a global overview of the flow of participants and their categorization and demographic description. Results of biomarkers and conventional microbiological methods then follow. For the bacterial cultures, results from the clinical laboratory are also included for comparative purposes. Finally, conventional and viability PCR results are reported and compared to biomarkers and culture results. Results of PJI episodes are individually reported in the next chapter.

4.1 Flow of Participants

Sixty-seven patients were recruited between February 2016 and September 2017. Two patients were excluded as their samples were retained by the NHSB because of typing errors in their consent forms. Five other patients with diabetic feet were recruited and samples were collected from the debridement of their feet for methods validation at the start of the study. Those five patients were not included in the data analysis. Thus, sixty patients who underwent revision arthroplasties were ultimately included in this study. From these patients, 168 tissue samples and 40 retrieved prostheses were collected in sixty-two episodes (samples were collected from two patients on two different occasions from each). Only one prosthesis was excluded as its container was found to be broken on arrival at the laboratory.

Patients were categorized into infected (PJI) and aseptic groups according to the MSIS criteria (Parvizi et al., 2011). Twelve episodes met these criteria while forty-six did not. Among the twelve episodes meeting the MSIS criteria, eleven had sinuses and one episode had two positive cultures with the same organism in absence of a sinus. Among the eleven episodes with sinuses, eight and two episodes were culture-positive and negative respectively from the clinical laboratory while the culture results of one episode were missing. Culture results of the clinical laboratory, rather than the research culture results, were used to satisfy the MSIS criteria. Most of the minor MSIS criteria were not available, so the consensus in the East Midland Specialized Orthopaedic Network (EMSON) was alternatively considered for episodes that did not satisfy the MSIS criteria before being included in the aseptic

group. Three episodes that were considered infected by EMSON despite failing to satisfy the MSIS criteria were included in the PJI group. One episode which was indeterminate as neither satisfying MSIS criteria nor having a consensus in EMSON was included in the PJI group for a more conservative data analysis. This episode was positive on alpha defensin intra-operative testing (Case 29; see PJI Case Reports Chapter). Thus, PJI and aseptic groups included sixteen and forty-six episodes respectively (Figure 4-1 and Table 4-1). Results are reported as per episode rather than per patient.

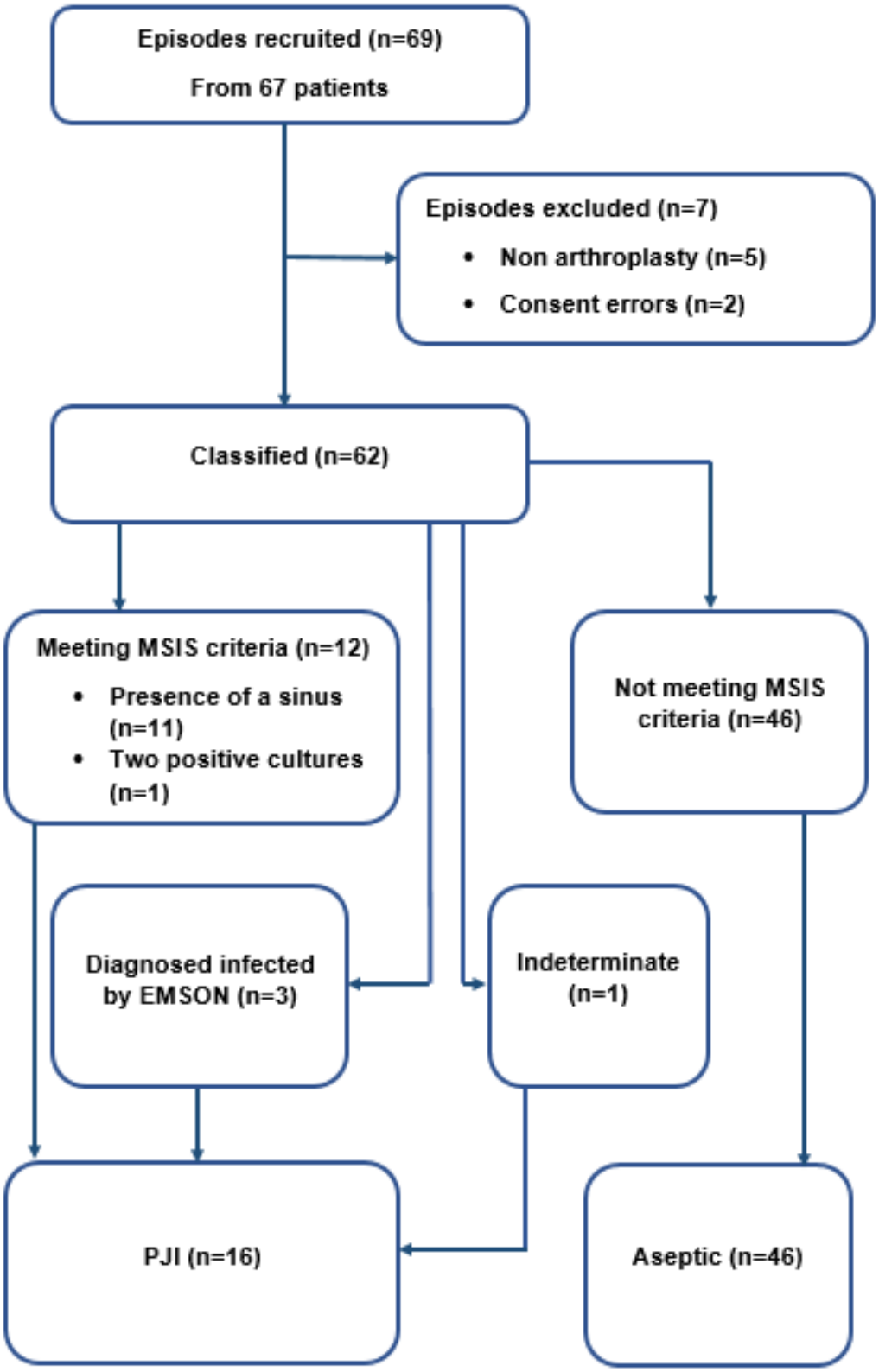


Figure 4-1: Participants flow chart. MSIS: Musculoskeletal Infection Society; PJI: Prosthetic Joint Infection; EMSON: East Midland Specialized Orthopaedic Network.

Table 4-1: Participants classified into PJI and aseptic failure groups.

	PJI	Aseptic failure	Total
Patients	15	45	60
Episodes	16	46	62
Tissue samples	53	115	168
Number of tissue samples per episode: median (range)*	4 (1-7)	2.5 (1-7)	2.7 (1-7)
Prostheses	16	23	39
Percentage of episodes from which prostheses were collected	100%	50%	62.9%
Episodes with only collected prostheses	2	1	3

*Episodes from which only prostheses were collected are excluded.

4.2 Demographics and Characteristics of Participants

The demographic and some other characteristics of the two participant groups are summarized in Table 4-2. Ages were rounded to the nearest year at the time of index surgery. Figure 4-2 shows the type of surgery in the PJI group and Figure 4-3 shows the reason for revision in the aseptic group.

Table 4-2 Demographics of participants.

	PJI	Aseptic failures
Age in years		
Median	68	68
Range	51 - 83	42 - 91
Gender: no. (%)		
Male	7 (44)	19 (41)
Female	9 (56)	27 (59)
Arthroplasty site: no. (%)		
Hip	1 (6)	14 (30.4)
Knee	15 (94)	30 (65.2)
Ankle	0 (0)	2 (4.4)
Time since primary replacement in months*		
Median	28	120
Range	1 – 216	2 – 324
Number of revision: no. (%)		
First	7 (44)	36 (78)
Second	5 (31)	6 (13)
Third or more	4 (25)	4 (9)
Presence of a sinus: no. (%)		
Yes	11 (67)	0 (0)
No	5 (33)	46 (100)

* This data was unavailable in three episodes in the aseptic group.

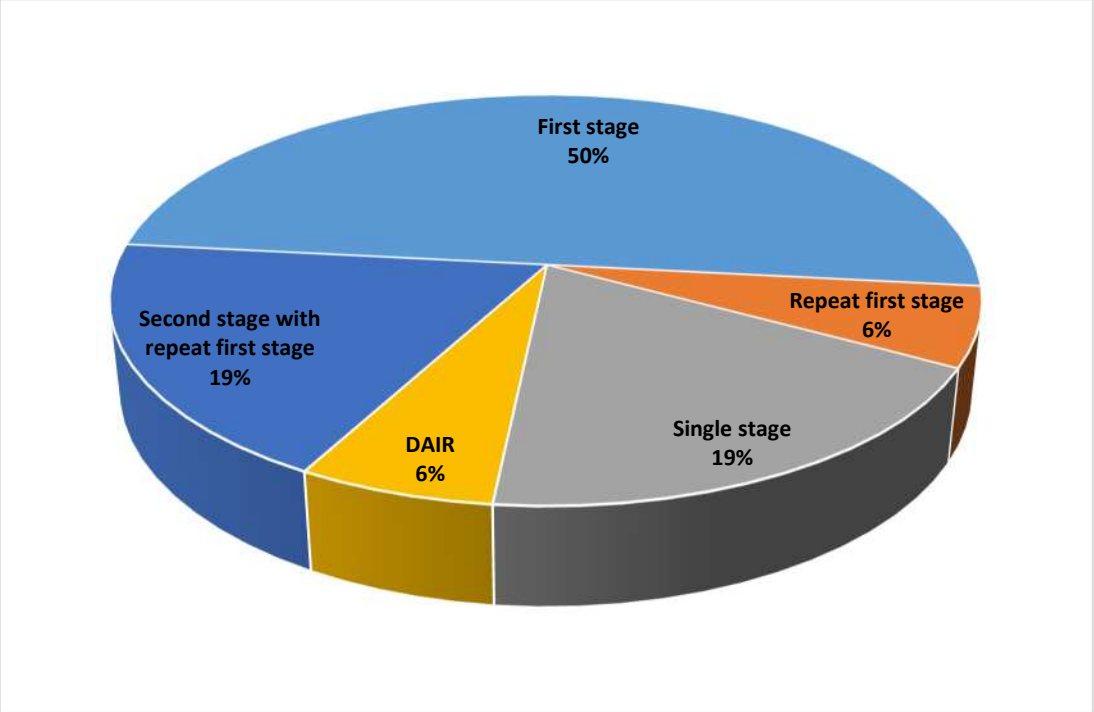


Figure 4-2 Pie chart depicts the type of surgery in PJI group

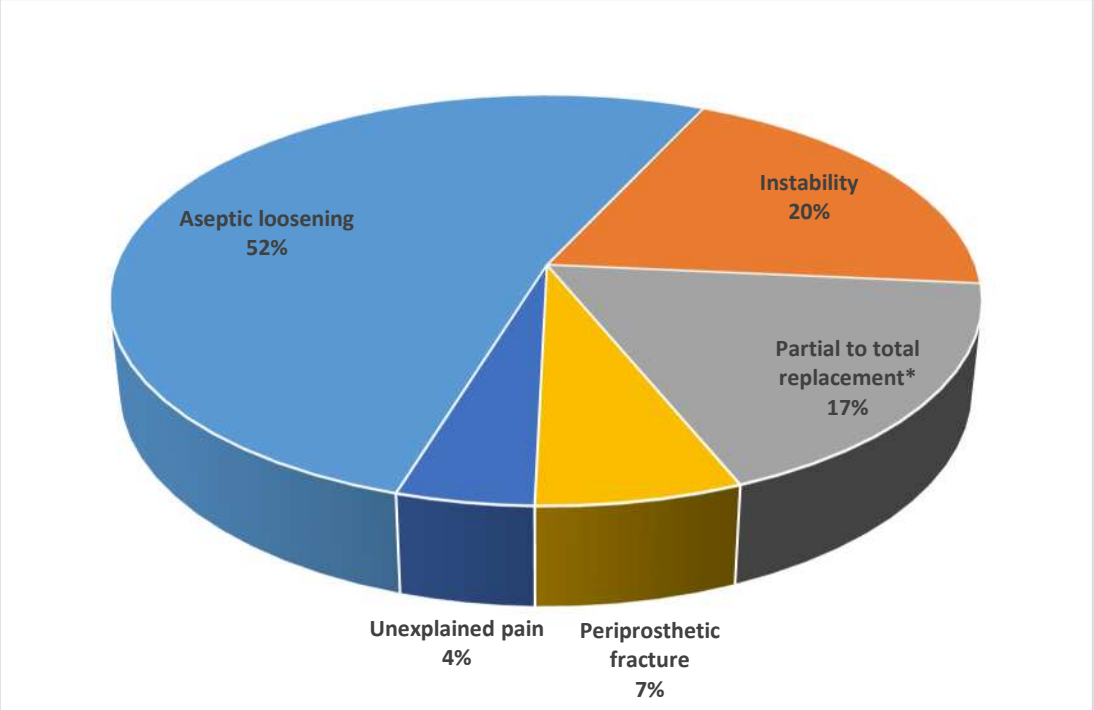


Figure 4-3 Pie chart depicts causes of revision in aseptic group. *Partial to total replacement includes conversions of unicondylar to total knee replacements and resurfacing to total hip replacements.

4.3 Biomarkers (ESR and CRP)

CRP values <5 mg/litre were reported “0”. The median and interquartile range of preoperative CRP values for PJI and aseptic groups were 51 (23 - 82) and 6 (0 - 13) mg/litre respectively (Figure 4-4). The median and interquartile range of postoperative CRP values for PJI were 17 (9 - 39) mg/litre (Figure 4-5). Postoperative values were considered in the time frame between one month and three months after the surgery to allow for normalization after the surgical trauma (Niskanen et al., 1996). Postoperative values could not be calculated for the aseptic group as this data was missing in 56.5% of cases.

The AUC of the ROC curve of preoperative CRP values (Figure 4-6) was 0.882 and the 95% confidence interval (CI) was 0.796 – 0.967 ($p<0.0001$). Table 4-3 shows the sensitivity, specificity, and Youden index for different cutoff values of preoperative CRP.

The median and interquartile range of preoperative ESR values for PJI and aseptic groups were 44.5 (25.25 – 80.5) and 16 (9 - 29) mm/hour respectively (Figure 4-7).

The AUC of the ROC curve of preoperative ESR values (Figure 4-8) was 0.818 and the 95% CI was 0.699 – 0.938 ($p<0.0001$). Table 4-4 shows the sensitivity, specificity, and Youden index for different cutoff values of preoperative ESR.

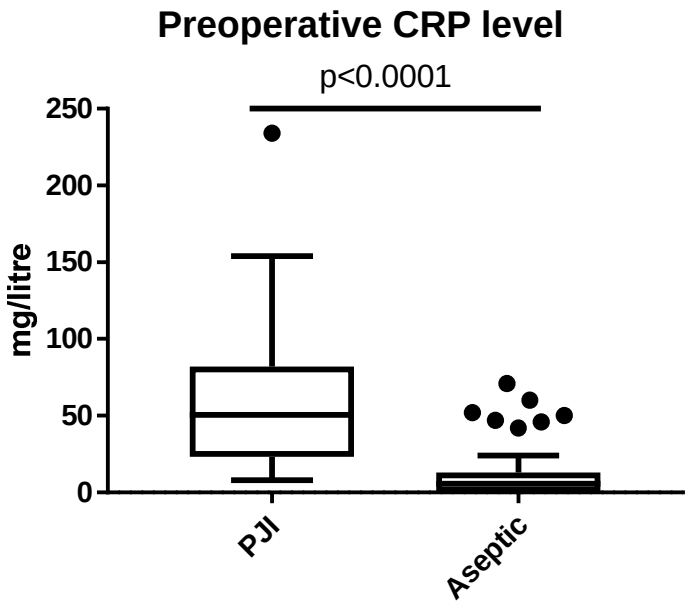


Figure 4-4 Box-whisker plot displaying the preoperative CRP level in the PJI (n = 16) and aseptic group (n = 42); this data was missing in four aseptic episodes (Paired t-test was used).

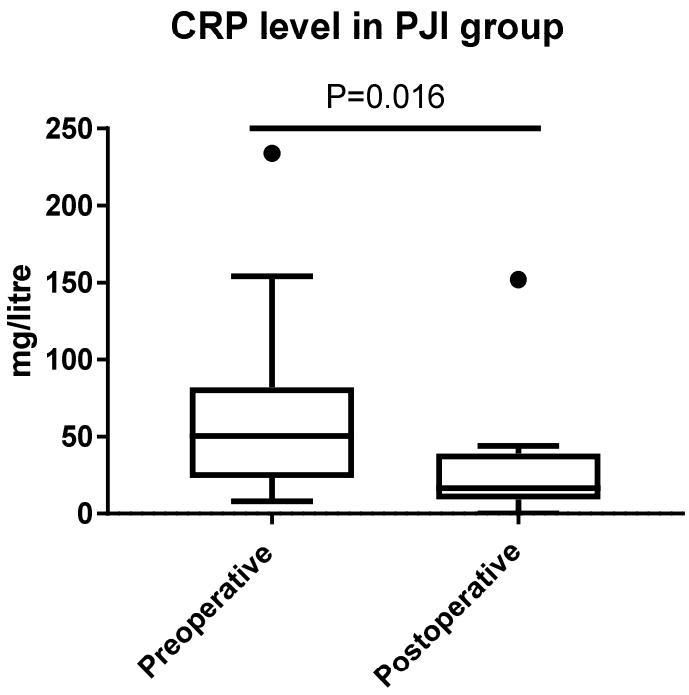


Figure 4-5 Box-whisker plot displaying the preoperative and postoperative CRP level in the PJI group (Paired t-test was used).

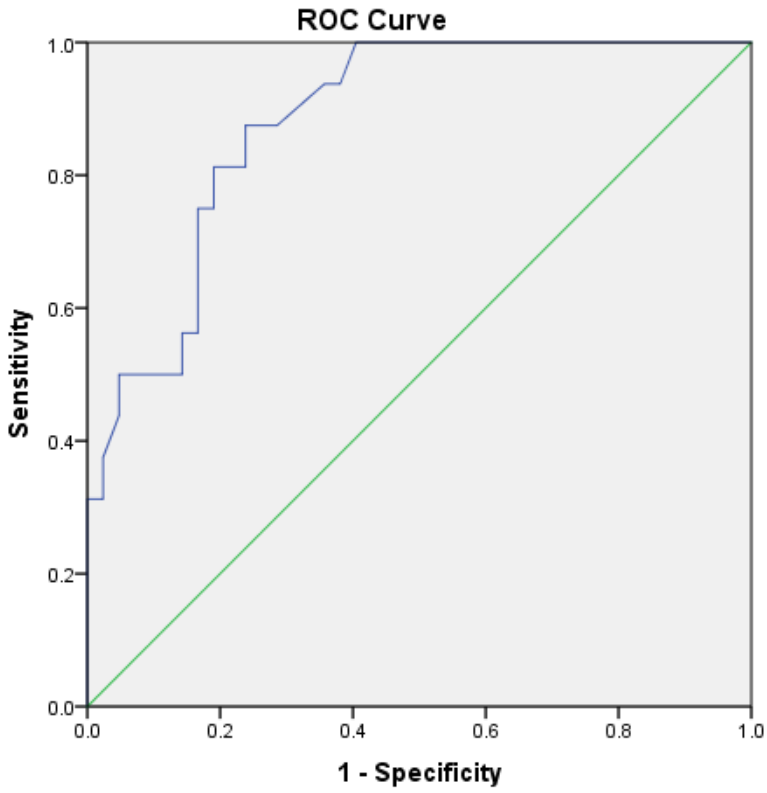


Figure 4-6 Receiver operator characteristic (ROC) curve for preoperative CRP values. AUC=0.882

Table 4-3 Sensitivity, specificity, and Youden index for different cutoff CRP values.

CRP (mg/litre)	Sensitivity	Specificity	Youden index
> 2.5	100	35.71	35.71
> 7.5	100	59.52	59.52
> 10*	87.5	71.43	58.93
> 13	87.5	76.19	63.69
> 27	75	83.33	58.33

* recommended by Bernard et al. (2004) and Miyamae et al. (2013)

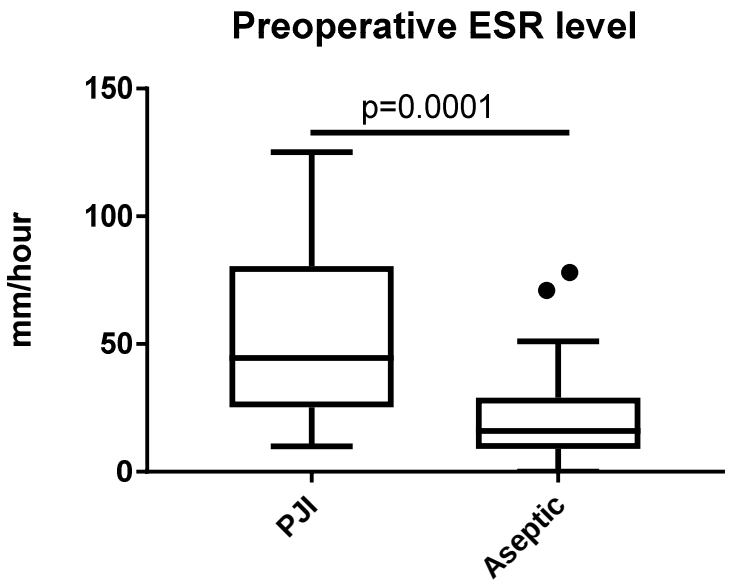


Figure 4-7 Box-whisker plot displaying the preoperative ESR level in the PJI (n = 16) and aseptic group (n = 39); this data was missing in seven aseptic episodes (Paired t-test was used).

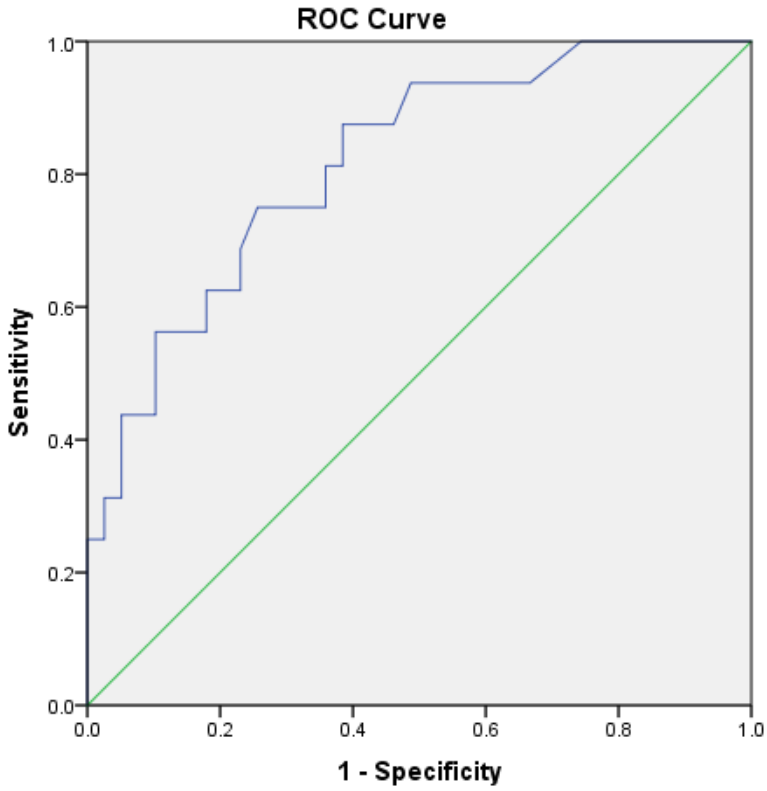


Figure 4-8 Receiver operator characteristic (ROC) curve for preoperative ESR values. AUC=0.818

Table 4-4 Sensitivity, specificity, and Youden index for different cutoff ESR values.

ESR in mm/hr	Sensitivity (%)	Specificity (%)	Youden index
> 15.5	93.75	48.72	42.47
> 28	75	74.36	49.36
> 30*	62.5	76.92	39.42
> 38	56.25	87.18	43.43
> 50	43.75	92.31	36.06

* recommended by Berbari et al. (2010)

4.4 Conventional Culture Results

4.4.1 Clinical Laboratory Results

Organisms were isolated from eight PJI episodes by the clinical laboratory while seven episodes were culture-negative. Result of one episode was unavailable (Case number 19). Figure 4-9 shows the organisms isolated. The clinical laboratory reported the qualitative result of each sample separately but did not quantify the bacterial colonies. In addition, CoNS were not individually speciated. In Cases number 40 and 43, CoNS were isolated from only one sample out of seven and three samples respectively.

CoNS grew from one out of seven samples of an episode in the aseptic group. All other aseptic episodes with available data were culture-negative. Results of nine episodes in this group were missing.

The sensitivity and specificity of the clinical laboratory culture is presented in Table 4-5 according to two different definitions of positive culture. The

definition of two or more positive samples with the same organism was used in the subsequent analysis.

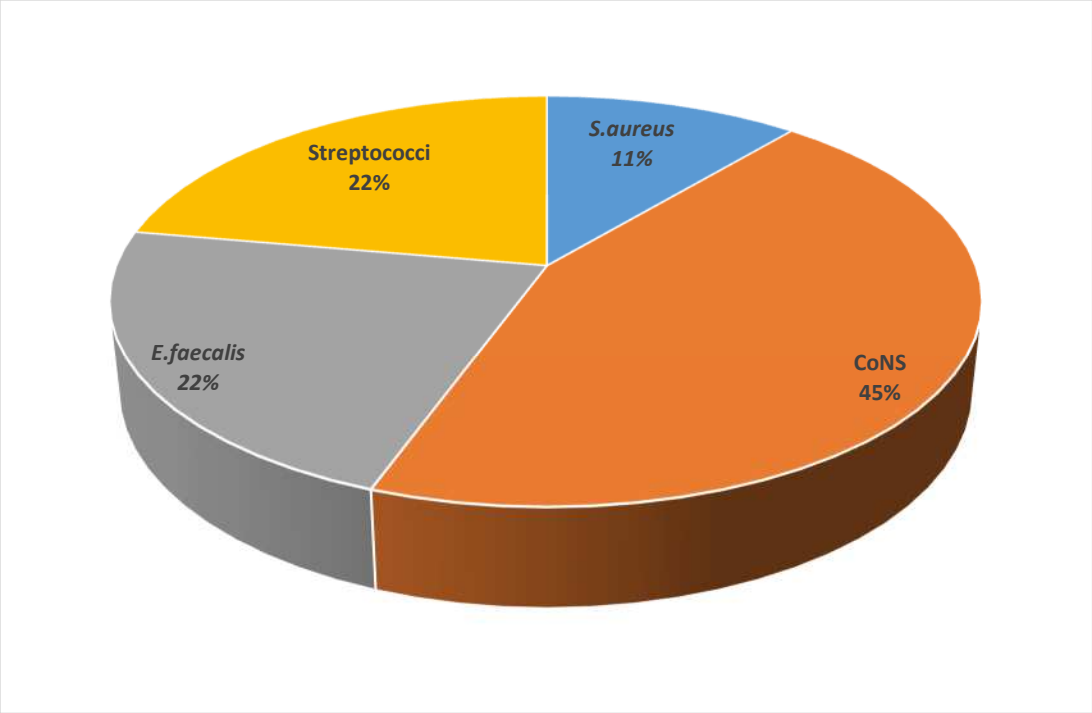


Figure 4-9 Pie chart depicts the bacteria isolated from PJI case tissue samples in the clinical laboratory.

Table 4-5 sensitivity and specificity of the clinical laboratory culture

Definition of positivity	Sensitivity	Specificity
One positive sample*	60%	97.3%
≥ 2 tissue or fluid samples with the same organism**	46.6%	100%

* This is one of the minor MSIS criteria.

** This is one of the major MSIS criteria and it is recommended by Cazanave et al. (2013).

4.4.2 Biomaterials-Related Infection Research Laboratory Results

Figure 4-10 shows the isolated organisms from PJI case tissue samples and/or sonication fluid.

Different definitions for a positive tissue culture have been implied to find out the highest possible sensitivity/specificity pair as shown in Table 4-6. The definition chosen to be used is the isolation of the same organism from two or more tissue samples regardless the number of colonies isolated. This is the definition which has the highest sensitivity (57.1%) along with a 100% specificity. This definition was used in the subsequent analysis. Thus, 42.9% of PJI cases were tissue culture-negative.

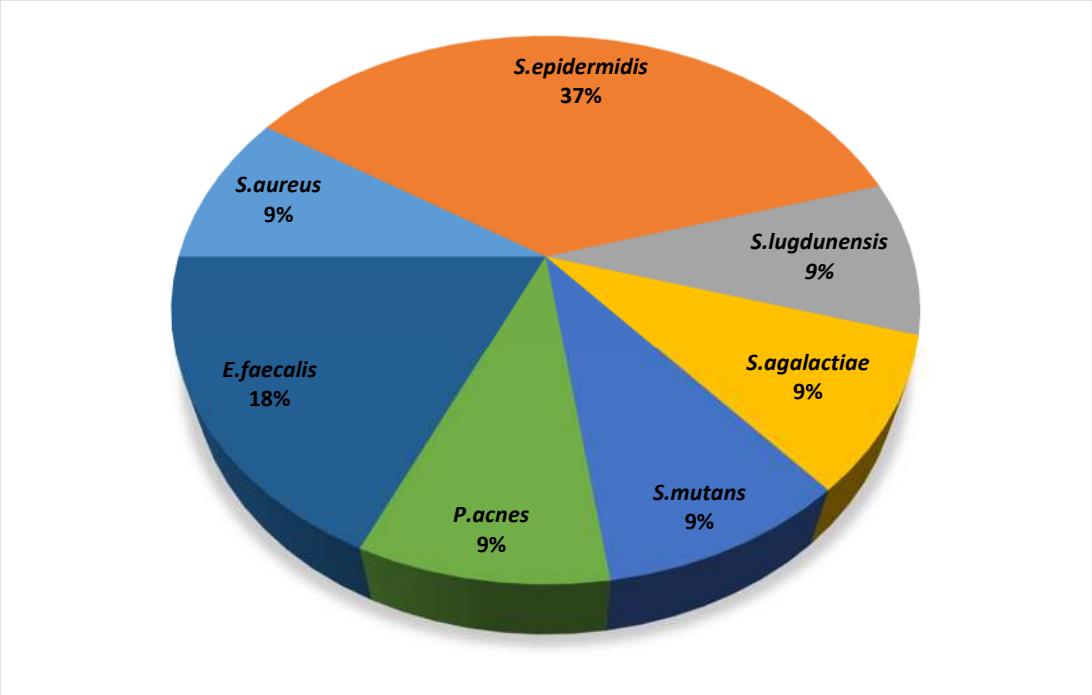


Figure 4-10 Pie chart depicts the organisms isolated from PJI case tissue samples and/or sonication fluid in Biomaterials-Related Infection Laboratory.

Table 4-6 Sensitivity and specificity of the research tissue culture

Definition of positivity	Sensitivity (%)	Specificity (%)
Any growth in one sample	64.3	88.9
Growth ≥ 100 cfu/mL in one sample	50	93.3
Growth ≥ 500 cfu/mL in one sample	42.9	95.6
≥ 2 tissue samples with the same organism	57.1	100
≥ 2 tissue samples with the same organism; each ≥ 100 cfu/mL	35.7	100
≥ 2 tissue samples with the same organism; each ≥ 500 cfu/mL	14.3	100

In addition, different mathematical methods were tested to convert the results of all tissue samples into a single figure per episode. This single figure was then used to generate ROC curves and calculate the AUC for each assumption. Mathematical methods used were the total, median, mean and maximum of colony count of the same organism per millilitre. AUC for all the four assumptions had overlapping 95% confidence intervals as shown in Table 4-7. Different cutoff values for the mean of tissue sample cultures with their corresponding sensitivity, specificity, and Youden index are shown in Table 4-8.

Table 4-7 ROC curve results of different mathematical calculations of research tissue culture.

Method	AUC	95% CI	p value
Total	0.783	0.622 – 0.943	0.002
Median	0.776	0.608 – 0.945	0.002
Mean	0.789	0.627 – 0.950	0.001
Highest value	0.779	0.618 – 0.939	0.002

Table 4-8 Sensitivity, specificity, and Youden index for different cutoff values for the mean of tissue samples culture.

Mean of tissue samples	Sensitivity (%)	Specificity (%)	Youden index
> 10	57.14	93.33	50.47
> 50	42.86	95.56	38.42
> 100	42.86	95.56	38.42
> 250	35.71	100	35.71
> 500	7.143	100	7.143

Using 10^1 cfu/mL as the cutoff value for the sonicate culture positivity as recommended by Trampuz et al. (2007), its sensitivity and specificity were 56.3% and 91.3% respectively.

The number of organisms isolated from sonicates was significantly higher than the mean of organisms isolated from multiple tissue samples. However, upon comparing the quantitative bacterial growth from the sonicates, to the

highest observed growth in multiple tissue samples, the difference was not statistically significant (Figure 4-11).

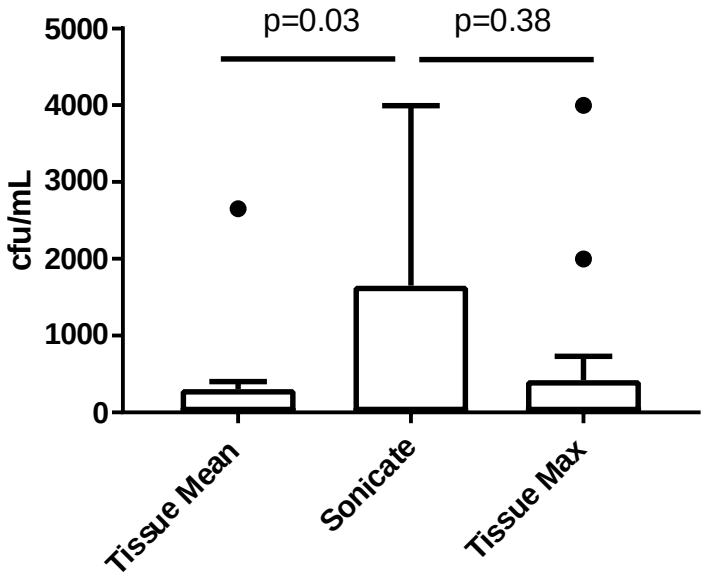


Figure 4-11 Box-whisker plot displaying the number of bacteria isolated from PJI cases tissue samples (mean and maximum) compared to the number isolated from the sonicates of the same episode (Paired t-test was used).

Collectively, a positive culture per episode was defined as either two or more positive tissue samples and/or positive sonicate culture. This definition led to a sensitivity and specificity of 62.5% and 95.7% respectively. Thus, the incidence of culture-negative PJI in our cohort was 37.5%.

4.4.3 **Comparison of Results between Both Laboratories**

Clinical and research laboratory results were concordant qualitatively. However, quantitative comparison could not be done due to lack of quantification in clinical laboratory reports.

In Case 40, CoNS grew from one out of seven tissue samples sent to the clinical laboratory. In the research laboratory, sonicate of the prosthesis grew

100 cfu/mL *S.epidermidis*. No tissue samples were collected from this episode.

In Case 43, while CoNS grew from one out of three tissue samples sent to the clinical laboratory, *S.epidermidis* grew from all the five collected tissue samples and the sonicate in the research laboratory.

4.4.4 Discordant Results between Research Tissues and Sonication Fluid Culture

In two PJI episodes (Cases 19 and 49), sonication fluid was culture-negative and one tissue sample in each episode was positive. *P.acnes* isolated from the tissue sample in Case 19 was unlikely to be the causative organism. Negativity of sonication fluid culture of Case 49 was explained by a presumed initiation and propagation of infection from superficial skin tissues (see Chapter of PJI Case Reports for details).

In two aseptic episodes (Cases 52 and 57), sonication fluid grew *Chryseobacterium takakiae* and *Ralstonia picketti* respectively while all tissue samples were culture-negative.

Index surgery of Case 52 was her third revision after she had her hip resurfaced in 2006 because of psoriatic arthropathy. All the three revisions were due to mechanical problems. She had never been diagnosed with PJI at any of her revisions.

Case 57 was a 59 year old lady who had patellofemoral replacement in March 2017 and developed acute postoperative PJI. Aspiration of the joint, one week after the replacement, grew *S.aureus*. She underwent open washout and synovectomy in March 2017 with the same organism isolated

as before. After the surgery, she was treated with parenteral teicoplanin 800mg OD and oral rifampicin 300mg BD for six weeks followed by oral doxycycline and 100mg BD and oral rifampicin 300mg BD for two weeks. In July 2017, she underwent a revision from which tissue samples were culture-negative (clinical laboratory). She was empirically treated with another cycle of teicoplanin for 6 weeks after this revision.

Organisms isolated in both cases could not be isolated from the water in the sonicator bath. Considering these as true PJI cases, using the same definition as above, would increase the sensitivity and specificity of sonication fluid culture to 61.1% and 100% respectively.

4.5 Molecular Results

The results of the three PCR assays are presented collectively. Case 45 and Case 54 were excluded from this analysis as they were known to be infected by organisms outside the panel of our PCR assays.

4.5.1 Standard Curves and Multi-experiment Projects

The Ct values of the standard positive control sample which was run in every experiment within the same project were compared as a control of inter-experimental consistency. The mean, standard deviation, and 95% CI for positive controls are shown in Table 4-9.

Table 4-9 Representative set of data of positive controls of PCR assays.

Positive control	**Quantity (cfu/mL)**	**Ct value**		
		Mean	Standard deviation	95% CI
S.aureus	10^6	20.28	0.1104	20.2 - 20.37
E.faecalis	$5x10^5$	20.48	0.1689	20.35 - 20.61
P.acnes	10^6	21.13	0.9204	20.36 - 21.9

Figure 4-12 to Figure 4-20 are the amplification plots, dissociation curves, and standard curves generated for staphylococci, enterococci, and *P.acnes*. The detection limit of staphylococci, enterococci, and *P.acnes* PCR assay was 10^2, $5x10^1$, and 10^2 cfu/mL respectively. Melting temperature for staphylococci, enterococci, and *P.acnes* amplicons were approximately 83°C, 81°C, and 89°C respectively. Figure 5-5 in the Chapter of PJI Case Reports is an illustration of how the standard curve generated from an experiment was used to quantify an unknown sample from a different experiment after joining them in one project (and standardizing the threshold of both).

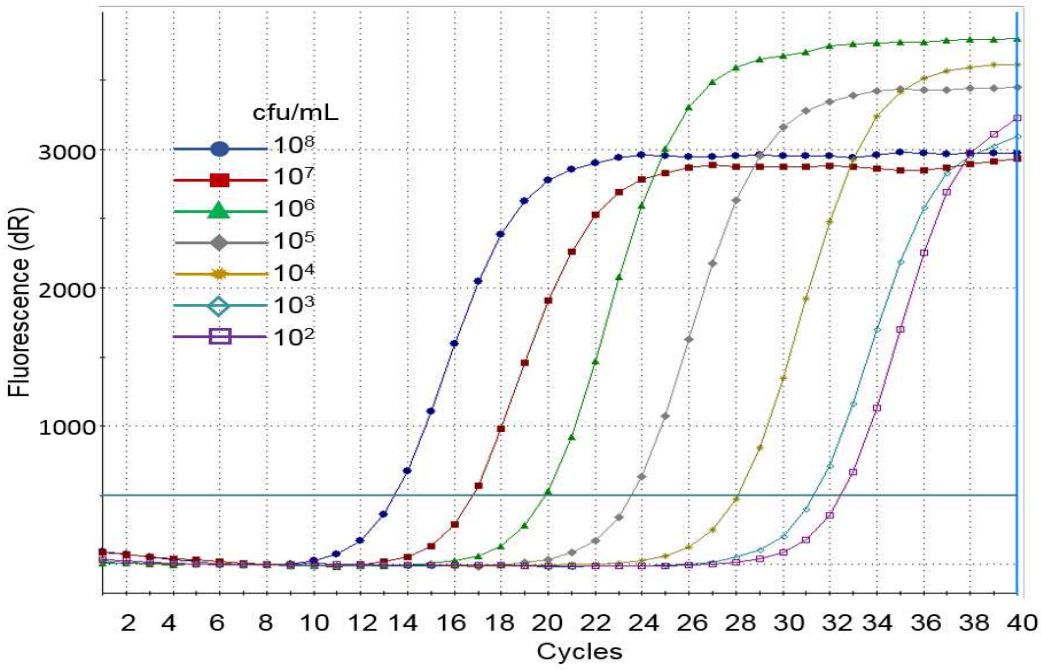


Figure 4-12 Amplification plots of staphylococci.

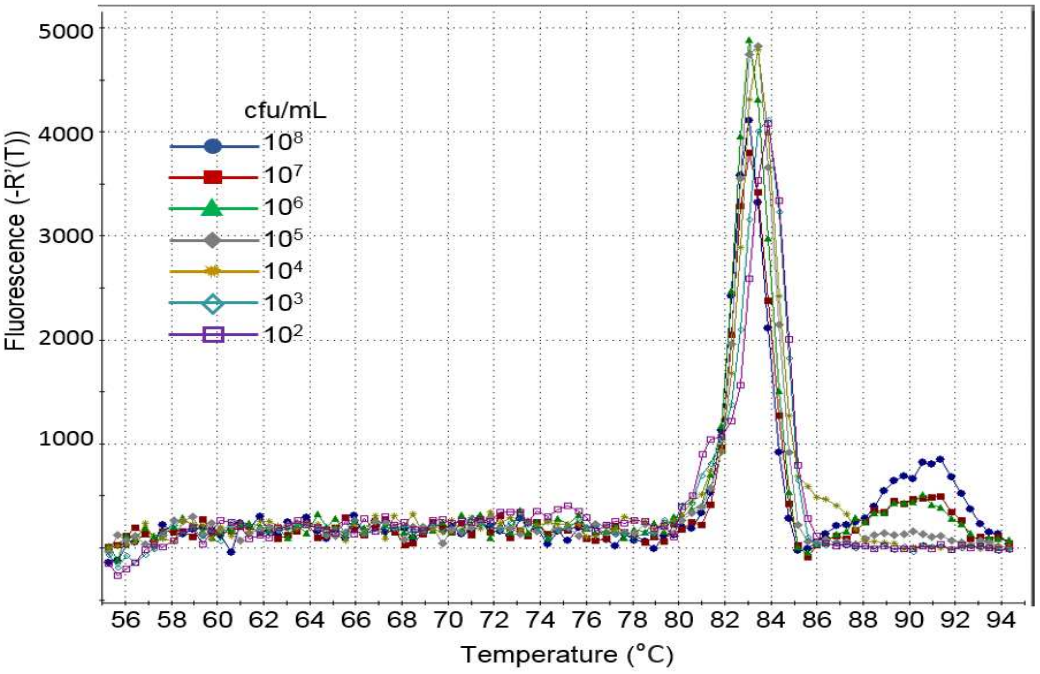


Figure 4-13 Dissociation curves of staphylococci.

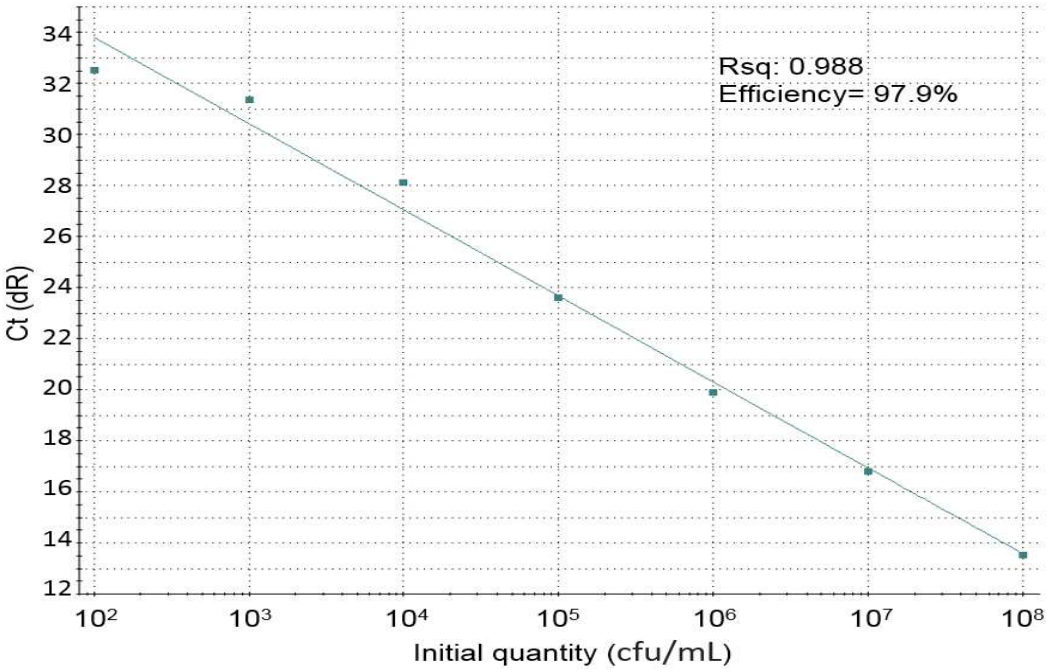


Figure 4-14 Standard curve of Staphylococci.

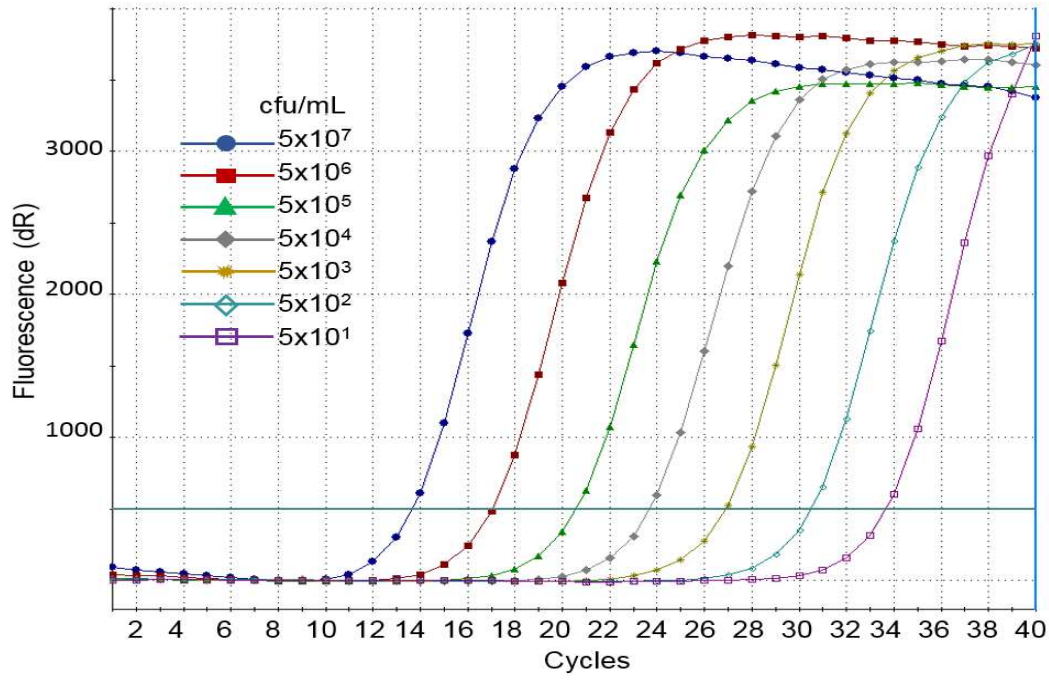


Figure 4-15 Amplification plots of enterococci.

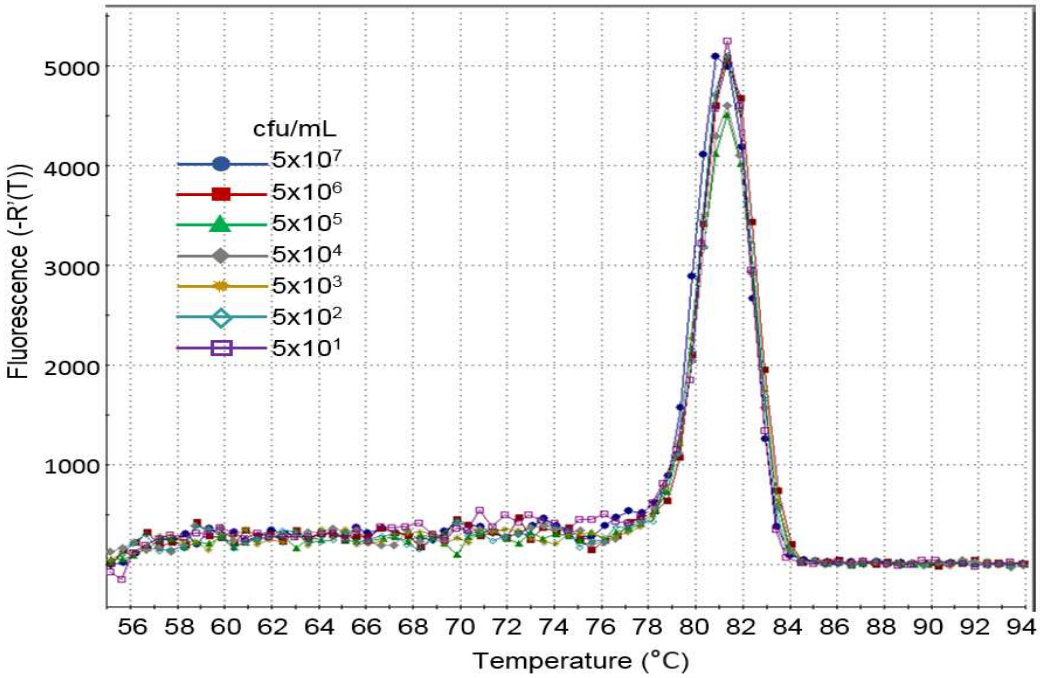


Figure 4-16 Dissociation curves of enterococci.

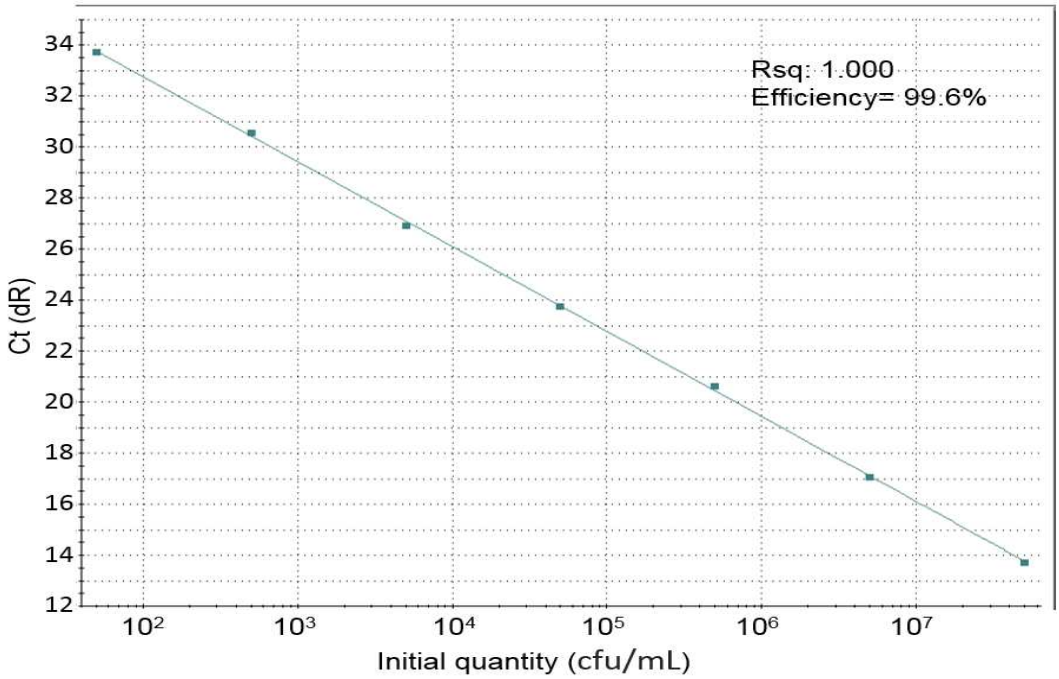


Figure 4-17 Standard curve of enterococci.

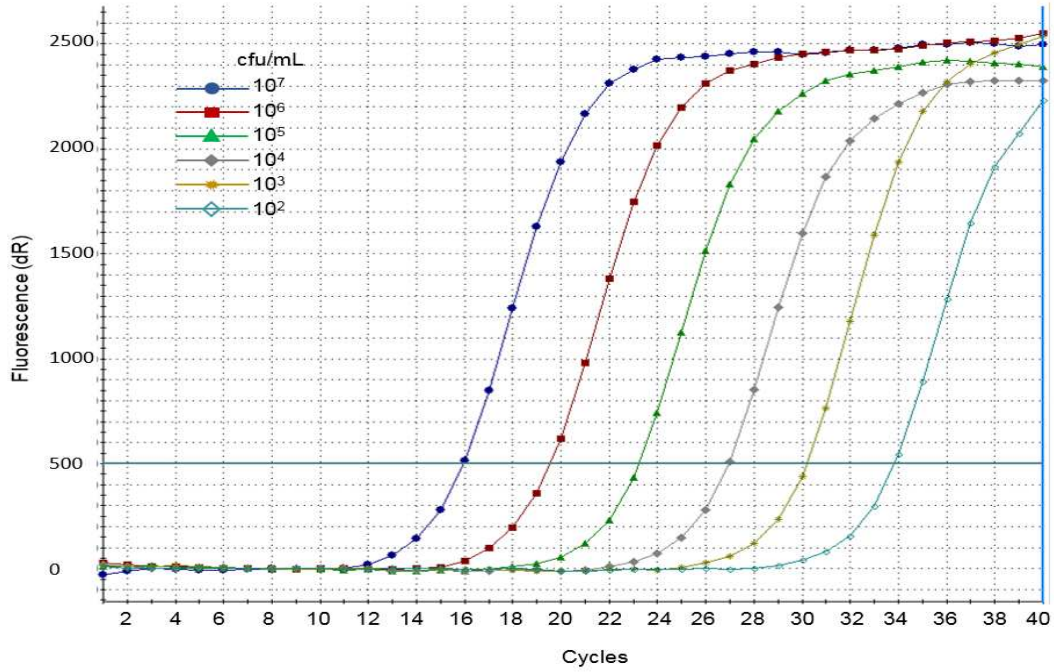


Figure 4-18 Amplification plots of *P.acnes*.

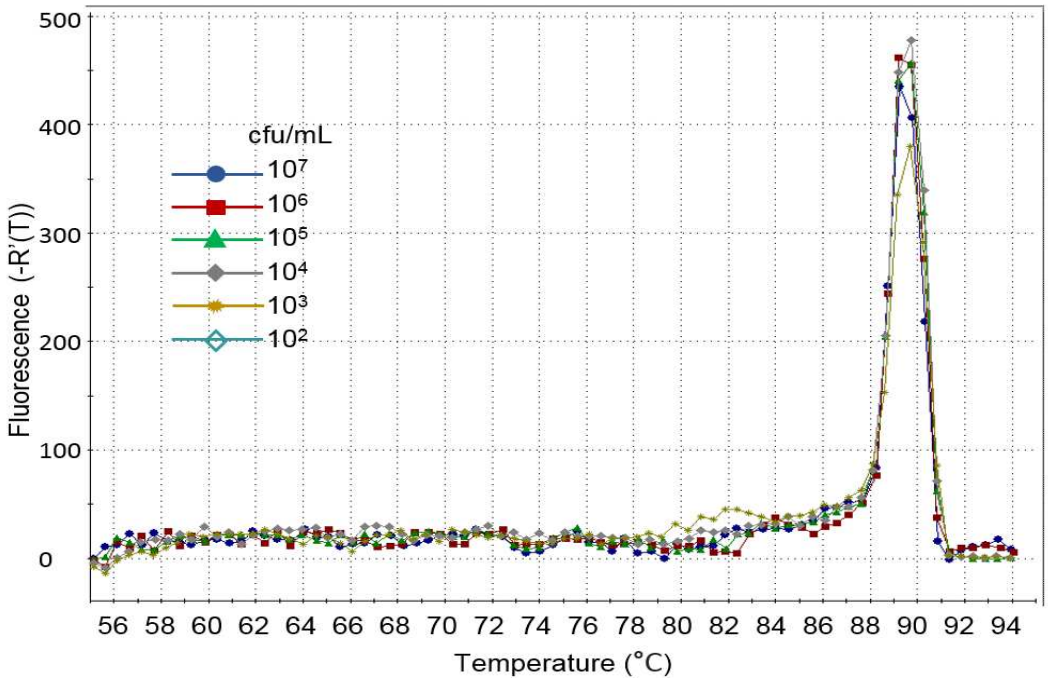


Figure 4-19 Dissociation curves of *P.acnes*.

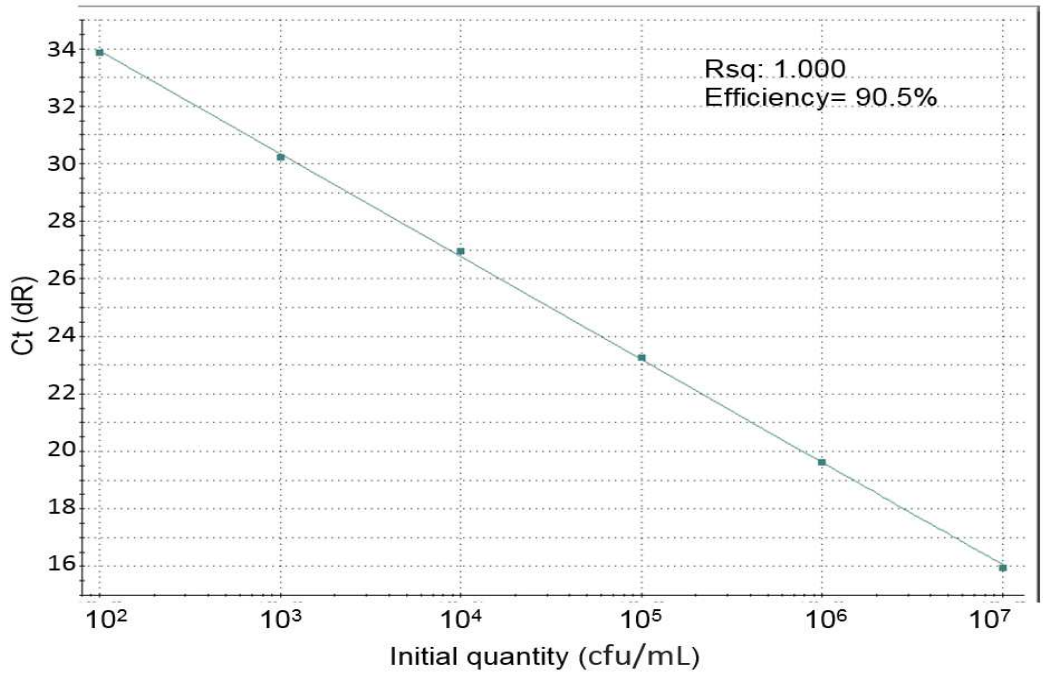


Figure 4-20 Standard curve of *P.acnes*.

4.5.2 **Conventional real-time PCR**

Positive PCR sample was defined as any significant amplification (using the dissociation curve and/or the gel electrophoresis) above the detection limit (threshold). Definition of positive PCR episode was adopted from the definition used in culture. A positive PCR episode is an episode with two or more positive tissue samples and/or positive sonicate in the same assay. Among the 14 episodes of PJI included in the analysis, conventional PCR of periprosthetic tissues was positive in only two episodes while conventional PCR of sonicates was positive in nine episodes. Conventional PCR results were calculated for periprosthetic tissue homogenates and implants sonicates separately for comparative purposes as shown in Table 4-10 and Table 4-11 respectively. Table 4-12 shows the combined results of conventional PCR using periprosthetic tissue and sonication fluid. These

aforementioned three tables are two-by-two (2x2) tables which were used to calculate the sensitivity and specificity of conventional PCR using tissue samples, sonicates, or both combined as shown in Table 4-13. The 95% confidence intervals were also calculated. It is noteworthy that the combined sensitivity using both the tissues and sonicates is higher than either of them separately. However, sonicates were significantly more sensitive than tissue homogenates.

The next step was trying to find if there is any correlation pattern between the conventional PCR results and biomarkers (ESR or CRP) or culture results. The significance of establishing this correlation with the biomarkers is to be used as surrogate markers to predict the conventional PCR results. On the other hand, the significance of establishing this correlation with the culture results is to evaluate the performance of PCR in culture-positive and culture-negative PJI. Because of the relatively small sample size of this cohort, we could not establish obvious correlations. Alternatively, “correlation” tables were generated as below to show the relationships between PCR results and biomarkers and culture results.

Four correlation tables were generated; Table 4-14 and Table 4-15 show the number of positive conventional PCR results in PJI group in comparison to biomarkers and culture results respectively while Table 4-16 and Table 4-17 show the number of negative conventional PCR results in the aseptic group in comparison to biomarkers and culture results respectively. Each of these tables is composed of four columns and four rows (4x4) excluding the “total” column and row. Conventional PCR results are shown in columns; the first and second show the results of tissue homogenates and implants sonicates

respectively. The third column shows the results of either of them satisfying the required condition (positive result in Table 4-14 and Table 4-15 and negative results in Table 4-16 and Table 4-17). The fourth column is the combined result of both tissue homogenates and implants sonicates. Biomarkers or culture results were presented in rows. The first and second rows show the results of ESR and CRP or the culture results of tissues or sonicates respectively. The third row is either of them and the fourth row is both of them combined.

From Table 4-14 and Table 4-16, it is noted that a potential correlation exist between the conventional PCR results of sonicates and the CRP. Similarly, there is a correlation between the conventional PCR and culture results of sonicates as noticed in Table 4-15 and Table 4-17.

Table 4-10 Results of conventional PCR using periprosthetic tissues.

	Positive	Negative	Total
PJI	2	11	13*
Aseptic	0	45	45**

* No tissue samples were collected from Case number 40 in this group.

** No tissue samples were collected from Case number 58 in this group.

Table 4-11 Results of conventional PCR using sonication fluid.

	Positive	Negative	Total
PJI	9	5	14
Aseptic	10	13	23

Table 4-12 Results of conventional PCR using periprosthetic tissues and sonication fluid.

	Positive	Negative	Total
PJI	10	4	14
Aseptic	13	33	46

Table 4-13 Sensitivity and specificity of conventional PCR.

Sample	% Sensitivity (95% CI)	% Specificity (95% CI)
Periprosthetic tissue	15.38 (1.92 to 45.45)	100 (92.13 to 100)
Sonication fluid	64.29 (35.14 to 87.24)	56.52 (34.49 to 76.81)
Either of both	71.43 (41.90 to 91.61)	71.74 (56.54 to 84.01)

Table 4-14 Number of conventional PCR positive results in PJI group compared to serum markers positive results.

		Conventional PCR positive				
		Tissue (n=13)	Sonicate (n=14)	Either (n=14)	Both (n=13)	Total
Serum markers positive	ESR (n=14)	1	6	7	0	8
	CRP (n=14)	1	8	9	1	12
	Either (n=14)	2	8	9	1	12
	Both (n=14)	1	6	7	0	8
	Total	2	9	10	1	

Table 4-15 Number of conventional PCR positive results in PJI group compared to culture-positive results.

		Conventional PCR positive				
		Tissue (n=13)	Sonicate (n=14)	Either (n=14)	Both (n=13)	Total
Culture-positive	Tissue (n=13)	1	6	6	1	9
	Sonicate (n=14)	1	6	6	1	12
	Either (n=14)	1	7	7	1	12
	Both (n=13)	1	5	5	1	9
	Total	2	9	10	1	

Table 4-16 Number of conventional PCR negative results in aseptic group compared to serum markers negative results.

		Conventional PCR negative				
		Tissue (n=45)	Sonicate (n=23)	Either (n=46)	Both (n=22)	Total
Serum markers negative	ESR (n=39)	29	6	30	5	30
	CRP (n=42)	30	8	30	8	30
	Either (n=42)	33	9	34	8	37
	Both (n=39)	26	5	26	5	26
	Total	45	13	46	12	

Table 4-17 Number of conventional PCR negative results in aseptic group compared to culture-negative results.

		Conventional PCR negative				
		Tissue (n=45)	Sonicate (n=23)	Either (n=46)	Both (n=22)	Total
Culture-negative	Tissue (n=45)	45	12	45	12	45
	Sonicate (n=23)	20	13	21	12	21
	Either (n=46)	45	13	46	12	46
	Both (n=45)	20	12	20	12	20
	Total	45	13	46	12	

4.5.3 **PMA-PCR (viability PCR)**

With the same definition of positive PCR sample and episode applied as above, results of viability PCR using periprosthetic tissue, sonication fluid, or both are shown in Table 4-18, Table 4-19, and Table 4-20 respectively. Among the 14 episodes of PJI included in the analysis, viability PCR of periprosthetic tissues was positive in three episodes while viability PCR of sonicates was positive in ten episodes.

The previous three tables were used to calculate the sensitivity and specificity of viability PCR using tissue samples, sonicates, or both combined as shown in Table 4-21.

In addition, four correlation tables were generated as previously described in 4.5.2. Table 4-22 and Table 4-23 show the number of positive viability PCR results in PJI group in comparison to serum markers and culture results respectively.

Table 4-24 and Table 4-25 show the number of negative viability PCR results in the aseptic group in comparison to serum markers and culture results respectively.

It is noteworthy that the highest correlations were between viability PCR results of sonicates and CRP and between viability PCR and culture results of sonicates.

Table 4-18 Results of viability PCR using periprosthetic tissues.

	Positive	Negative	Total
PJI	3	10	13*
Aseptic	0	45	45**

* No tissue samples were collected from Case number 40 in this group.

** No tissue samples were collected from Case number 58 in this group.

Table 4-19 Results of viability PCR using sonication fluid.

	Positive	Negative	Total
PJI	10	4	14
Aseptic	4	19	23

Table 4-20 Results of conventional PCR using periprosthetic tissues and sonication fluid.

	Positive	Negative	Total
PJI	11	3	14
Aseptic	5	41	46

Table 4-21 Sensitivity and specificity of viability PCR.

Sample	% Sensitivity (95% CI)	% Specificity (95% CI)
Periprosthetic tissue	23.08 (5.04 to 53.81)	100 (92.13 to 100)
Sonication fluid	71.43 (41.90 to 91.61)	82.61 (61.22 to 95.05)
Either of both	78.57 (49.20 to 95.34)	89.13 (76.43 to 96.38)

Table 4-22 Number of viability PCR positive results in PJI group compared to serum markers results.

		Viability PCR positive				
		Tissue (n=13)	Sonicate (n=14)	Either (n=14)	Both (n=13)	Total
Serum markers positive	ESR (n=14)	2	6	7	1	8
	CRP (n=14)	2	8	9	2	12
	Either (n=14)	3	8	9	2	12
	Both (n=14)	2	6	7	1	8
	Total	3	10	11	2	

Table 4-23 Number of viability PCR positive results in PJI group compared to culture-positive results.

		Viability PCR positive				
		Tissue (n=13)	Sonicate (n=14)	Either (n=14)	Both (n=13)	Total
Culture-positive	Tissue (n=13)	2	6	6	2	9
	Sonicate (n=14)	2	6	6	2	12
	Either (n=14)	2	7	7	2	12
	Both (n=13)	2	5	5	2	9
	Total	3	10	11	2	

Table 4-24 Number of viability PCR negative results in aseptic group compared to serum markers negative results.

		Viability PCR negative				
		Tissue (n=45)	Sonicate (n=23)	Either (n=46)	Both (n=22)	Total
Serum markers negative	ESR (n=39)	29	12	30	11	30
	CRP (n=42)	30	13	30	13	30
	Either (n=42)	33	15	34	14	37
	Both (n=39)	26	10	26	10	26
	Total	45	19	46	19	

Table 4-25 Number of viability PCR negative results in aseptic group compared to culture-negative results.

		Viability PCR negative				
		Tissue (n=45)	Sonicate (n=23)	Either (n=46)	Both (n=22)	Total
Culture-negative	Tissue (n=45)	45	18	45	18	45
	Sonicate (n=23)	20	17	21	16	21
	Either (n=46)	45	19	46	18	46
	Both (n=45)	20	20	20	16	20
	Total	45	19	46	19	

4.5.4 Concordance of Molecular Methods with Culture in PJI group

Only one episode (Case 56) had negative PCR results despite positive culture results. Three episodes (Cases 24, 49, and 59) were culture-negative but both conventional and viability PCR positive. One episode (Case 60) was culture-negative, conventional PCR negative, but had positive viability PCR result. These results are discussed in detail in the Chapter of PJI Case Reports.

Apart from one episode (Case 19), molecular and culture methods were matching to the genus level as genus-specific primers were used for staphylococci and enterococci. For Case 19, *P.acnes* (mostly contaminants) grew in culture while PCR was positive for enterococci.

4.5.5 Concordance of Molecular Methods with Culture in Aseptic Failure group

Sonicates were culture-positive in two episodes as discussed in 4.4.4 above. PCR was negative in these episodes as the isolated organisms were outside the assay panel.

On the other hand, conventional PCR was positive in 13 out of 46 aseptic episode giving a specificity of 71.1%. The use of PMA, in the viability PCR, converted 8 of these results into negative. The five episodes with both positive conventional and viability PCR were Cases 1, 21, 23, 26, and 30. Case 30 had a remote CoNS-caused PJI episode. He had his primary TKR in 2006 which was revised in a single stage one year later because of infection. This infection was then clinically and laboratory proven to be eradicated. In August 2016, he was re-admitted with cellulitis around his

knee and he was treated with a 4-week course of antibiotics. The index surgery (from which samples were collected) was performed in January 2017. After the surgery, because of persistent wound discharge for four weeks, the patient was empirically treated with vancomycin and meropenem. There was no history of infection in the other four episodes. Cases 1 and 21 were knee revisions due to aseptic loosening, Case 23 was a hip revision due to periprosthetic fracture, and Case 26 was an ankle revision for a 10-year old replacement.

4.5.6 Time Needed from Attainment of Tissue Sample or Prosthesis until Getting the Viability PCR Results.

The turnaround time for the whole process was approximately 5 hours and 20 minutes. Table 4-26 shows the breakdown of this time into the different steps. In our case, samples were collected at Nottingham University City Hospital and processed in Biomaterials-Related Infection Laboratory based in the Queen's Medical Centre which is 30 minutes away.

Table 4-26 Turnaround time for viability PCR.

Step	Time
Transport	30 min.
Homogenization/Sonication	10 min.
PMA treatment	20 min.
DNA extraction	90 min.
PCR	2 hr and 50 min.
Total	5 hr and 20 min.

4.5.7 **Effect of PMA on Sensitivity of PCR Assay**

Unexpectedly, 10 (out of 207) samples were found to be positive in viability PCR and negative in conventional PCR. These samples were re-tested to exclude incorrect labelling or other human errors. For further confirmation of the significance of the viability PCR products, they were run on gel. For illustration, dissociation curve and gel electrophoresis of the PMA-treated and untreated equivalents of the sample NB 4330 (sonicate of Case 30) are shown in Figure 4-21 and Figure 4-22 respectively.

Differences in Ct values between PMA-treated and untreated simulated infected human tissue samples for the concentrations between 10^8 to 10^2 cfu/mL were less than one cycle. However, the 10^1 cfu/mL concentration was detected only in PMA treated samples. The end reaction product was consistently higher for PMA-treated samples for the whole range of concentrations ($p=0.01$). Figure 5-3 in the Chapter of PJI Case Reports shows an example of this difference in the end reaction product.

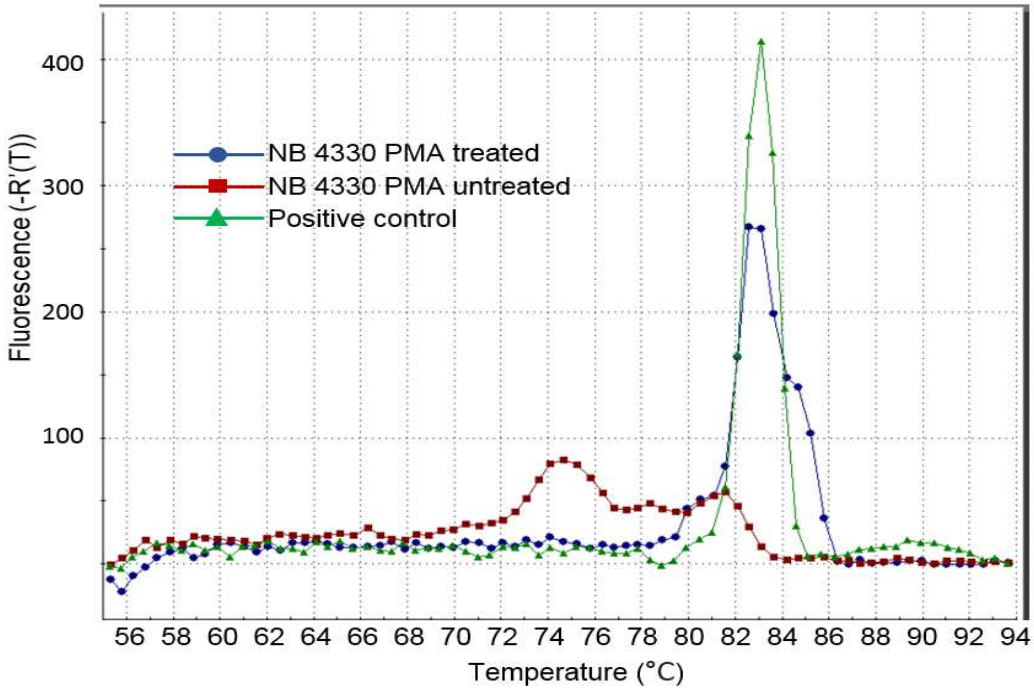


Figure 4-21 Dissociation curve of PMA-treated and untreated sample.

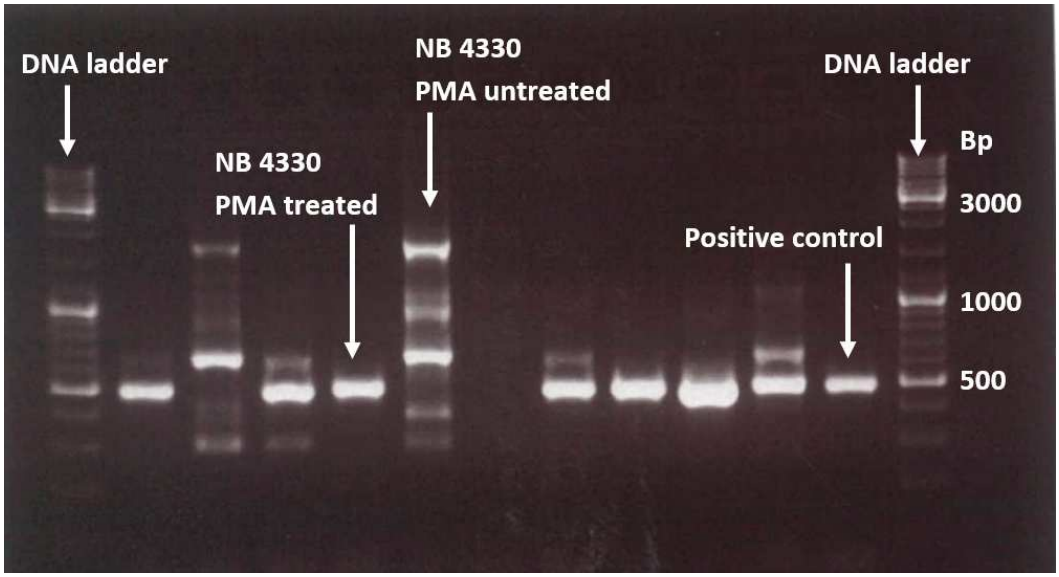


Figure 4-22 Gel electrophoresis of PMA-treated and untreated sample.

4.6 Consistency between MSIS Criteria and Different Diagnostic Tests

Cohen's kappa (κ) was run to determine the degree of agreement between the MSIS criteria and different diagnostic tests and the results are shown in Table 4-27.

Table 4-27 Agreement between the MSIS criteria and different diagnostic tests.

	κ	**Agreement***	**p value**
ESR	0.374	Fair	= 0.005
CRP	0.494	Moderate	< 0.001
Conventional PCR	0.300	Fair	= 0.015
Viability PCR	0.579	Moderate	< 0.001

* According to Landis and Koch (1977)

4.7 Consolidation of Results

Table 4-28 summarizes the sensitivity and specificity of different diagnostic tests and depicts the positive and negative likelihood ratios and predictive values for each. It is noteworthy that the generation of ROC curves for culture and PCR (conventional and viability) necessitates the conversion of a two-step diagnostic test into one step. These two steps are determining the cutoff of a positive sample and then the cutoff of a positive episode. Given the relatively small sample size of episodes in this study, this conversion

could not be accomplished. Hence, the AUC of each diagnostic test is not presented in Table 4-28.

Table 4-28 Statistical results of different diagnostic tests (LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; PPV: Positive predictive value; NPV: Negative predictive value).

	ESR	CRP	Culture*	Conventional PCR	Viability PCR
Sensitivity (%)	62.5	87.5	62.5	71.43	78.57
Specificity (%)	76.92	71.43	95.65	71.74	89.13
LR+ (%)	2.71	3.06	14.38	2.53	7.23
LR- (%)	0.49	0.17	0.39	0.40	0.24
PPV (%) **	32.34	35.08	71.72	30.85	56.05
NPP (%) **	92.08	97	93.53	93.43	95.93

* These are the research laboratory results.

** Calculated as recommended by Steinberg et al. (2009).

Chapter 5. PJI Case Reports

In this chapter, fifteen case reports are presented to illustrate the dilemma of diagnosis of PJI and the potential benefit of using the conventional and viability PCR to expedite and improve it. Some patients had multiple surgeries. So, the operation from which samples were collected for this study was referred to as the “index surgery”. Case 47 had two index surgeries as samples were collected from the same patient on two different occasions.

Precise dates of surgical interventions were excluded to avoid patients’ identification. Numbers were allocated to cases in a chronological fashion according to the time of sample collection.

Clinical microbiology laboratory results are given within the clinical history while results from this study at the Biomaterials-Related Infection laboratory are separately reported in the “laboratory results” subheading in each case. ESR and CRP are collectively referred to as biomarkers. Viability and conventional PCR are used to describe qPCR with and without prior PMA treatment of the samples respectively.

5.1 Case Number 15

5.1.1 Clinical History

An 80 year old man underwent left sided primary TKR in April 2014. He was allergic to penicillin and under medical treatment for asthma, hypertension, coronary heart disease, and depression. His knee replacement started to become painful nearly one month before he attended the emergency department (ED) in March 2016. Aspiration of the knee at that time grew *S.aureus* sensitive to erythromycin and flucloxacillin along with elevated biomarkers. The prosthesis was well fixed and he underwent a DAIR procedure with exchange of the polyethylene tibial insert in March 2016. Tissue samples from the DAIR procedure grew the same organism with the same antibiotic susceptibility. After the surgery, he was treated with 2gm IV ceftriaxone OD for six weeks then 500 mg oral ciprofloxacin for another 6 weeks. In addition, he received 300 mg oral rifampicin BD for 12 weeks. The patient slightly improved after this procedure but he continued to complain of intermittent pain and the biomarkers did not normalize. A sinus developed 4 months later. In August 2016, he underwent a first stage revision (index surgery) in which, after debridement and sinus excision, a standard PFC Sigma TKR prosthesis (DePuy Synthes, Warsaw, Indiana) was used as an articulating spacer. The knee was fixed in place using Palacos R+G (Hereaus Medical, Wehrheim, Germany) with 2g vancomycin added. *S.aureus* (with the same antibiotic susceptibility as before) was isolated in few numbers, yet from all tissue samples obtained intraoperatively. Following the surgery, the patient was initially treated with parenteral 2g ceftriaxone OD and 300mg rifampicin BD for three weeks and these were stopped because

he developed a rash. IV 400mg teicoplaninn OD was then prescribed for two weeks. This was followed by six weeks of oral clindamycin 450 mg QDS. Since the first stage revision, the patient has been satisfied and is functioning well with his temporary knee. He is not keen to have a second stage revision. His biomarkers went back to normal. Figure 5-1 summarizes this patient's surgical history and Figure 5-2 and illustrates the CRP level in the perioperative period of the index surgery.

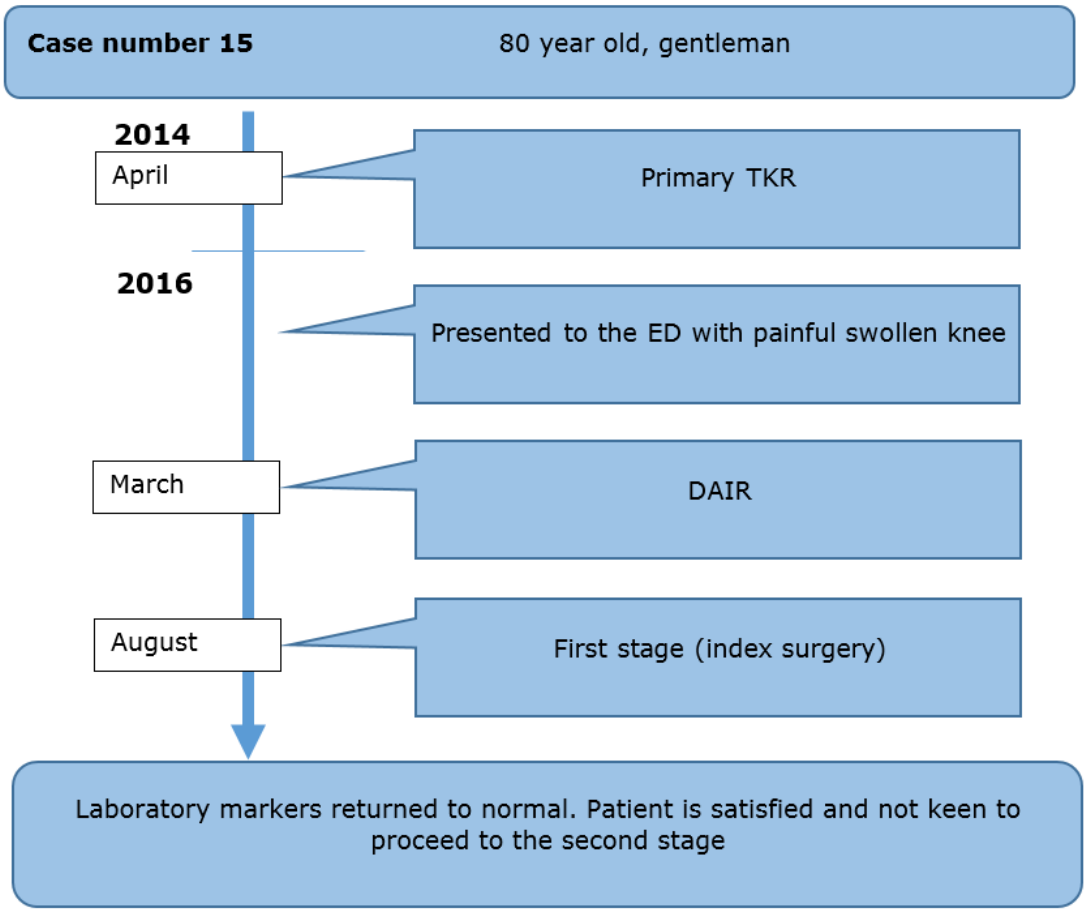


Figure 5-1 Timeline of patient 15.

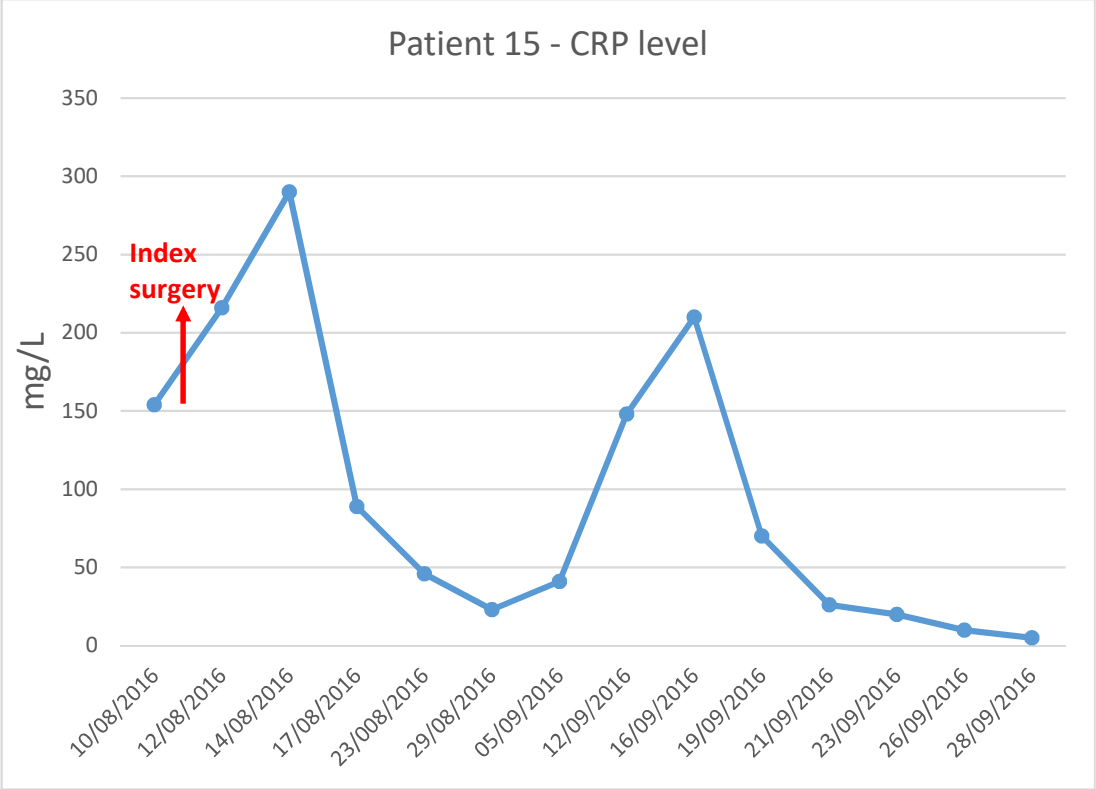


Figure 5-2 CRP level of patient 15 starting just prior to the index surgery until returning back to normal.

5.1.2 **Laboratory Results**

Both tissue samples and sonicate grew *S.aureus*. Bacterial growth from the sonicate was four-fold higher than the tissue homogenate, $3x10^2$ and $12x10^2$ cfu/mL respectively. All PCR results of the tissues were negative. For the sonicate, staphylococcal conventional and viability PCR detected $2x10^5$ and $5x10^4$ cfu/mL respectively. Enterococcal viability PCR of the sonicate detected $4x10^2$ cfu/mL while the conventional PCR was negative. Figure 5-3, Figure 5-4, and Figure 5-5 illustrate the PCR results of the sonicate.

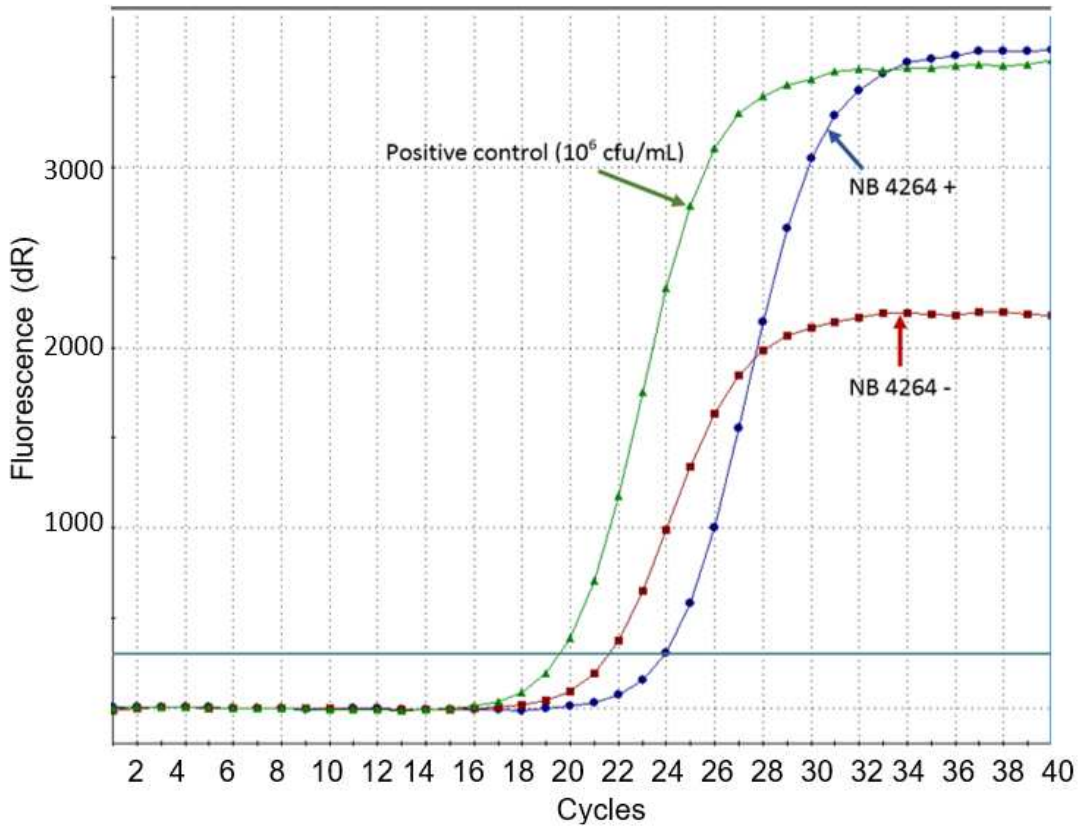


Figure 5-3 Amplification plots of PMA treated (blue) and untreated (red) sonication fluid of case 15. Note the difference in Ct values and fluorescence of end reaction products between the two samples. The green curve is a positive control sample.

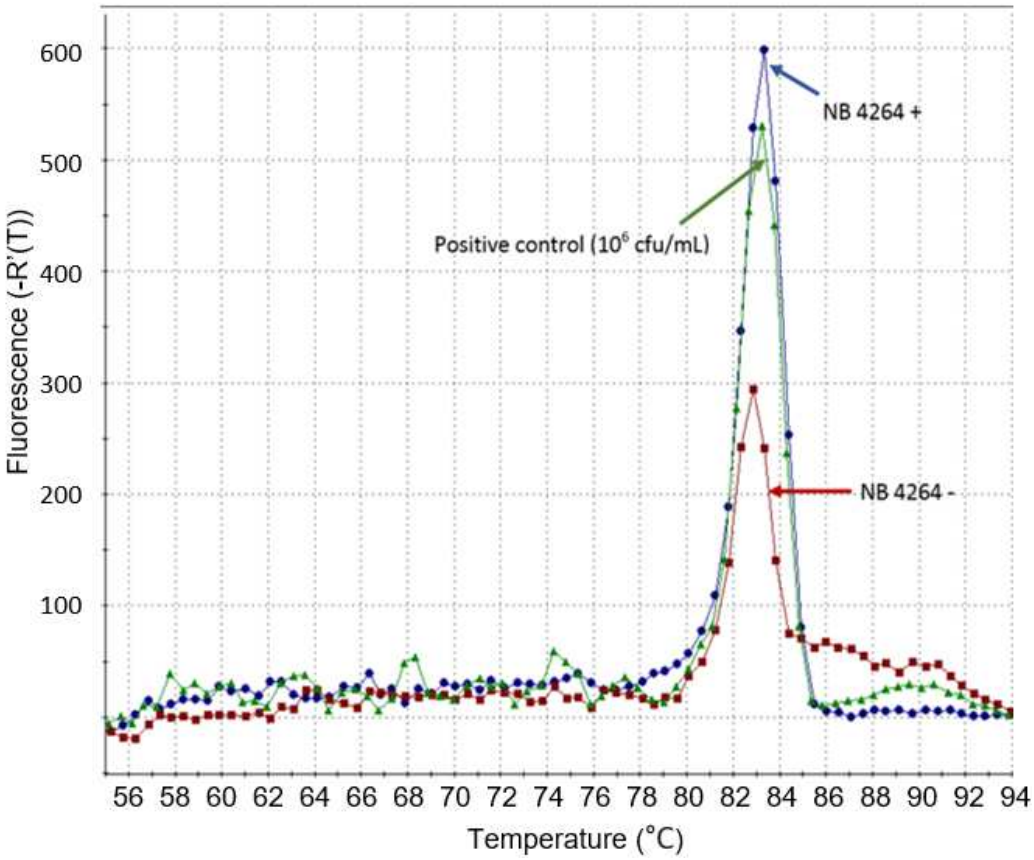


Figure 5-4 Dissociation curves of PMA treated (blue) and untreated (red) sonication fluid of patient 15. Both have the same melting temperature as the positive control i.e. significant products.

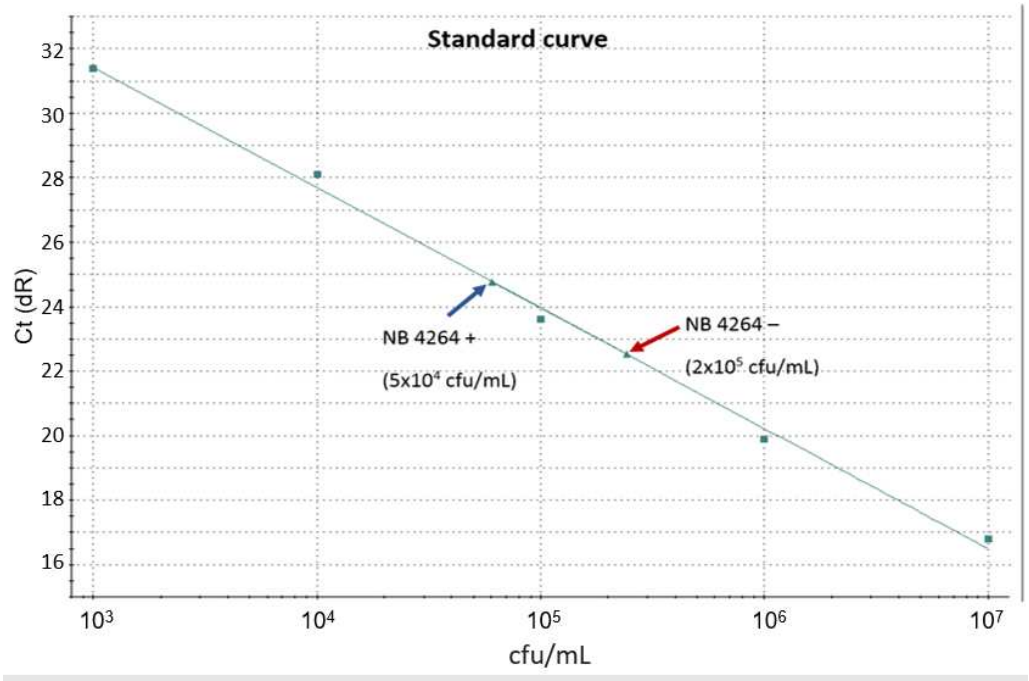


Figure 5-5 Standard curve used for quantification of PMA treated (blue) and untreated (red) sonication fluid of patient 15.

5.1.3 **Discussion**

This is a case of haematogenous PJI which developed nearly two years after the primary replacement. DAIR is a recognised modality of treatment in such cases (Vilchez et al., 2011b, Geurts et al., 2013). However, the infection was not eradicated by this procedure as the symptoms persisted and a sinus developed.

CRP is a non-specific inflammatory marker that can be raised in different conditions including systemic inflammatory disorders (e.g. rheumatoid arthritis), burns, trauma, kidney and liver failure, and some malignancies (Du Clos and Mold, 2004). The second peak in the CRP curve shown in Figure 5-2 could be explained by an episode of urinary tract infection that the patient experienced at that time. Postoperative CRP level interpretation should take

into account the duration needed to be normalized after the trauma of the surgery which is usually between 2 and 4 weeks (Niskanen et al., 1996).

As depicted in Figure 5-3, the amplification from the sonicate is different between PMA treated and untreated samples. The cycle threshold (Ct) value of untreated sample is lower than its treated equivalent i.e. higher DNA quantity, which is expected, as it is reflecting both dead and live bacteria. However, the end reaction product of the treated sample is higher than the untreated sample. In other words, the amplification curve of the untreated sample came to the plateau stage earlier. This can be caused by either earlier consumptions of reagents or accumulation of inhibitors. The first possibility is less likely as the quantification difference between treated and untreated samples is not wide (nearly 2 cycles). Reduction in inhibitor accumulation could be an advantageous incidental finding of PMA treatment as discussed later.

5.2 Case Number 19

5.2.1 Clinical History

A 61 year old man who receives synthetic testosterone injections for primary hypogonadism had a primary TKR in February 2015. This was a complex, long surgery which included tibial osteotomy for correction of a post-traumatic knee deformity. Unfortunately, the wound did not heal properly and the edges became necrotic with haematoma collection underneath. In March 2015, the haematoma was evacuated, the necrotic edges were debrided, gentamicin beads were placed under the wound, and a negative-pressure wound therapy known as VAC (vacuum-assisted closure) was used. Three days later, a washout of the knee was carried out with change of the

gentamicin beads and VAC. Tissue samples at this time grew *S.epidermidis* sensitive to vancomycin. However, the serosanguinous discharge persisted and a DAIR procedure was done after 4 days. During this intervention, the tibial insert was changed and the fixing wires of the tibial tuberosity were removed while the fixed knee components were kept in place.

The wound condition improved afterwards but frequent flares of swelling and redness of the knee persisted. Wound swabs at the time isolated *Serratia marcescens,* which is a known coloniser of moist sites. One more trial of getting his condition to settle down was tried in June 2015 with a second look debridement procedure. However, the condition kept deteriorating until a continuously draining sinus developed.

In an EMSON meeting, a two-stage revision strategy was agreed on. A long complex first stage was done in July 2015. A vascularized skin flap was inevitable for the wound closure after the aggressive debridement. Tissue samples obtained at this surgery were culture-negative. After the surgery, his clinical situation improved and biomarkers were approaching their normal values. An aspiration in September 2015 was culture-negative.

Having a deformed tibial and femoral bone put him in need for a specific custom-made prosthesis for the second stage which would take much longer time to be re-implanted with increasing risk of recurrent infection. At that stage, for a safer outcome, the patient was counselled for a knee fusion. However, he refused the fusion at any cost and preferred to have above knee amputation instead if the custom-made prosthesis did not fit.

The second stage (index surgery) was done in September 2016. However, the tissues looked grossly unhealthy so the surgeon performed another debridement before the re-implantation. Furthermore, the popliteal artery was injured and had to be repaired intraoperatively which lengthened the procedure further.

The patient did well afterwards until he fell down and his wound was opened in October 2016. X-ray taken at that time ensured that his prosthesis was in place and he received wound care until healing. Figure 5-6 summarizes the surgical history of this patient.

It is noteworthy that this patient has had a PJI of his contralateral TKR too which has been treated with two-stage revision as well.

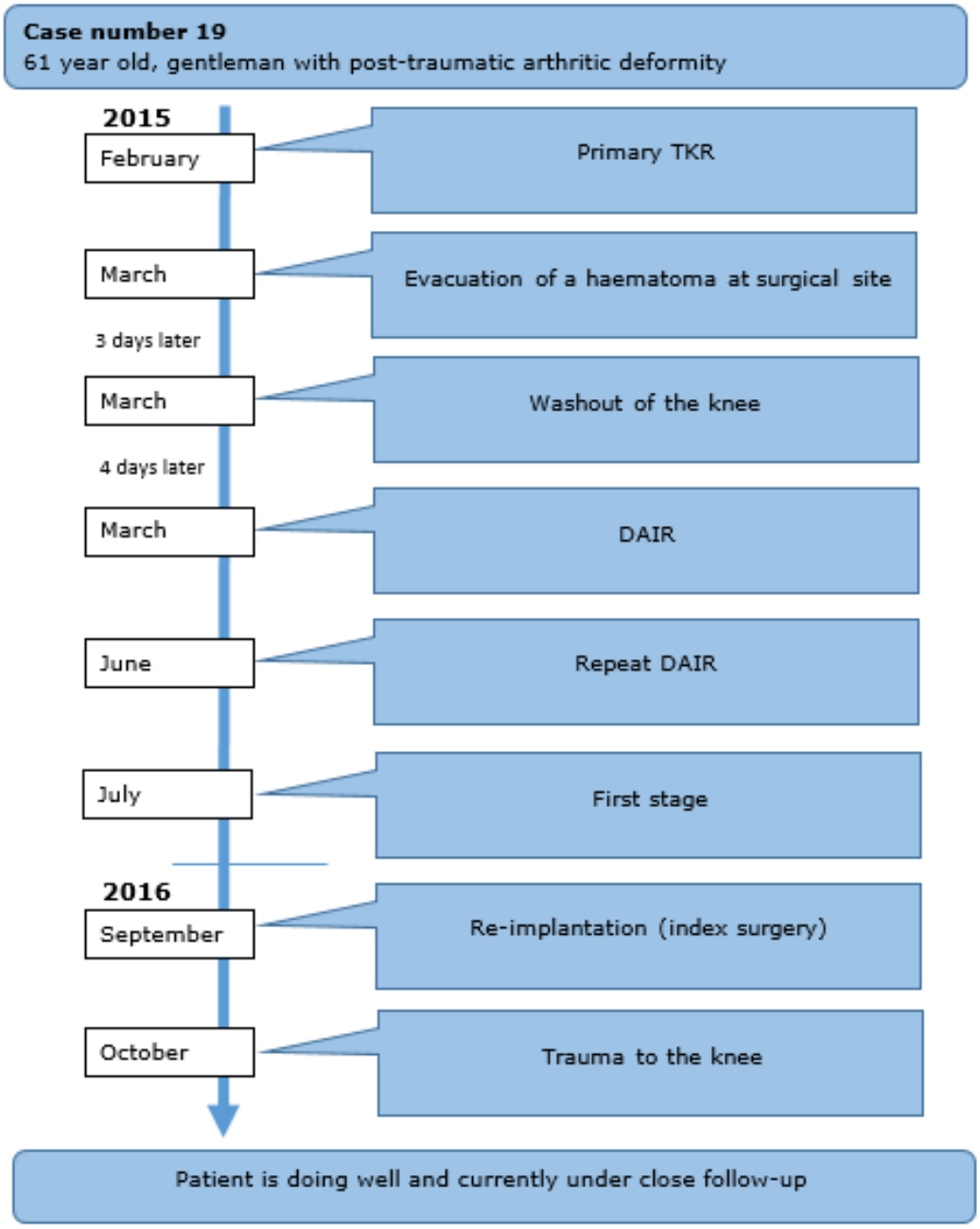


Figure 5-6 Timeline of patient 19.

5.2.2 **Laboratory Results**

One tissue sample grew 20 cfu/mL *P.acnes,* while sonicate of the retrieved spacer was culture-negative. Enterococcal conventional and viability PCR of the sonicate were positive (100 cfu/mL).

5.2.3 **Discussion**

This was a case of acute PJI with many interventions within a short time frame. The lack of strong evidence of the causative organism and the uncertainty of the ideal procedure and antibiotic therapy along with the difficulty in surgical procedure since the primary replacement because of a deformed knee have all contributed to this unfavourable situation.

Oily skin and development of acne are common side effects of testosterone replacement therapy (Surampudi et al., 2014, Bassil et al., 2009). However, the low number of isolated *P.acnes* makes it unlikely to be the causative organism.

Bereza et al. (2016) emphasized the importance of considering a positive sonication culture and/or molecular testing as real risk factors for infection recurrence after the second stage. However, this should be cautiously interpreted in light of the context of clinical situation to avoid the inevitable possibility of false negative and false positive results. Positive PCR results could be ascribed to dead bacterial DNA (Cazanave et al., 2013). The potential beneficial outcome of this study is that it is restricting the positive results of PCR to the live bacteria eliminating such confusion.

5.3 Case Number 20

5.3.1 **Clinical History**

This 51 year old woman with a long history of knee problems, was admitted with pain around her right knee replacement and a draining sinus. Her previous surgical history is shown in Figure 5-7. She attended the ED with an increase in pain, and a draining sinus at the inferior portion of the wound.

This had started as an indurated area seven days ago. Her CRP was 30 mg/L and ESR was 42 mm/hr.

She underwent a first stage revision of the TKR. At the revision surgery, while the femoral and patellar components were well fixed, the tibial component was grossly loose. Following debridement, an articulating spacer was created using a standard PFC Sigma TKR prosthesis (DePuy Synthes, Warsaw, Indiana). The tibial component had a small cemented stem to provide additional diaphyseal support, and the knee was cemented in place using Palacos R+G (Hereaus Medical, Wehrheim, Germany) with 2g vancomycin added. The sinus was excised and wound primarily closed.

Tissue samples obtained at time of surgery grew CoNS. Following the surgery, she was initially treated with ceftriaxone 2g OD and rifampicin 300mg BD, but ceftriaxone was changed to teicoplanin 800mg OD after 5 weeks because of a rash. After a 6-week course of IV antibiotics, a further 6 weeks of oral flucloxacillin 1g QDS and rifampicin 300mg BD were prescribed. She has done well following her revision and has found that the articulating spacer provides her with adequate function. She has therefore not yet proceeded to the second stage. She has been followed up for more than a year at the time of writing. Her CRP and ESR have returned to normal and the wound has completely healed.

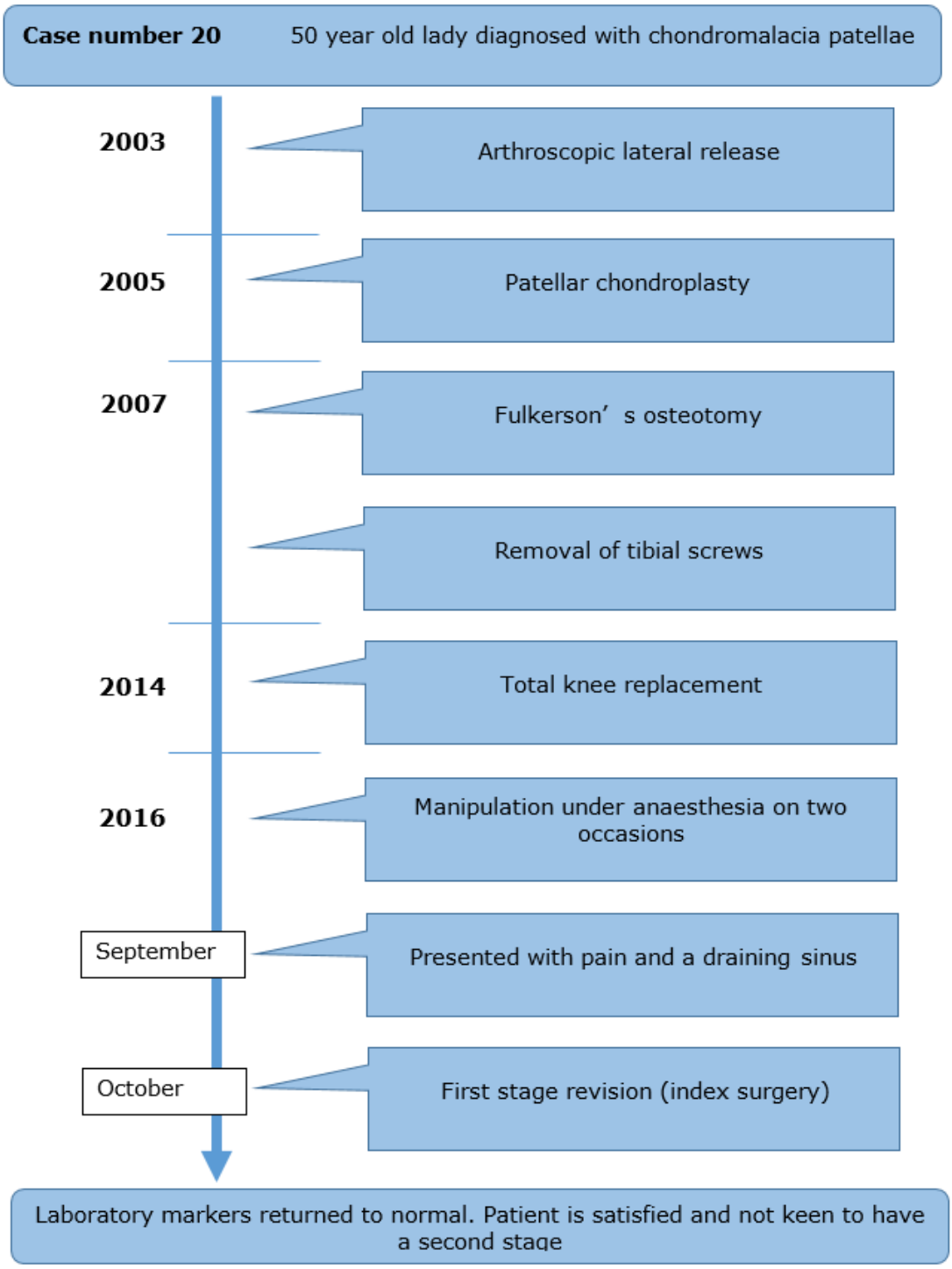


Figure 5-7 Timeline of patient 20.

5.3.2 **Laboratory Results**

Tissue samples grew 30-40 cfu/mL of *S. lugdunensis*, and sonicate grew 350 cfu/mL of full size parent colonies scattered on a background of a large number of SCVs which were not clearly evident until 72 hours of incubation

(Figure 5-8). Matrix-assisted laser desorption ionization–time of flight mass spectroscopy (MALDI-TOF MS) showed that both parent and SCV were indistinguishable and were *S. lugdunensis*.

PCR and conventional results of the sonicate matched. Conventional and viability PCR quantification was $9x10^3$ and $2x10^3$ cfu/mL respectively. PCR failed to detect the organism from the tissue homogenate.

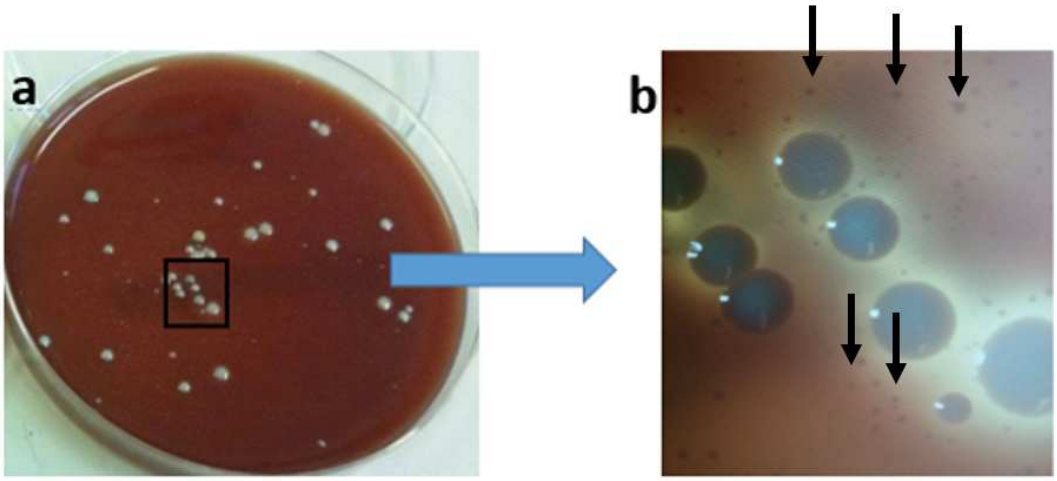


Figure 5-8 Culture of the sonicate after 72 hours of incubation, showing large colonies surrounded by high numbers of very small colonies (SCVs). "b" is 10-time magnification of the square bordered in "a" with arrows pointing out some SCVs.

5.3.3 **Discussion**

S.lugdunensis can produce clumping factor (bound coagulase) and hence can be misidentified as *S. aureus* if the slide coagulase test is used rather than the tube test (Sampathkumar et al., 2000). This misidentification has been reported in the literature and can be compounded by yellow pigment and DNAse production by some strains (Szabados et al., 2011). The appearance of SCVs can also give an impression of a mixed culture, with possible interpretation of contamination. The phenotypic changes in *S.*

lugdunensis SCVs can also give rise to misidentification as *Staphylococcus hominis* or other CoNS in certain commercial identification systems (Seifert et al., 2005, Ben-Ami et al., 2003). In such situations MALDI-TOF MS is able to provide more confident identification (Harris et al., 2010, Szabados et al., 2011, Dupont et al., 2010). *S. lugdunensis* resembles *S. aureus* in pathogenicity (Shah et al., 2010). In addition, *S. lugdunensis* isolates are usually β-lactamase deficient and susceptible to penicillins (Tan et al., 2008). Hence, recognition of SCVs and species identification of *S. lugdunensis* is crucial for proper treatment. Although being clonally similar, their biochemical characterisation can vary as well as their antibiotic susceptibilities. Therefore, antibiotic susceptibility testing should be done for each phenotype separately (Tande et al., 2014). SCV of *S. aureus hemB* mutants have been shown to exhibit more adhesiveness to surfaces than the parent strain (Vaudaux et al., 2002).

S. lugdunensis appears to have a preference to infect knees more than hips. This observation was previously made by the authors of the largest two series of *S. lugdunensis* PJI (Shah et al., 2010, Lourtet-Hascoet et al., 2016). An explanation could be the more thorough pre-operative preparation of the perineo-inguinal region in hip than in knee surgeries. Presentation of PJI due to *S. lugdunensis* can vary widely between acute symptoms such as fever and local site inflammation, to unexplained dull aching pain at the site of surgery. Delay between time of surgery and presentation can be as short as three weeks (Lourtet-Hascoet et al., 2016), or as long as 10 years (Merino et al., 2010).

In a report of 38 PJI patients infected with staphylococcal SCVs, 3 patients were infected by *S.lugdunensis* SCV (Tande et al., 2014). More recently, a case of normal variant *S.lugdunensis* PJI who was initially treated by DAIR, developed a persistent infection one year later and SCV were isolated (Argemi et al., 2017). This highlights the importance of not underestimating the pathogenicity of *S.lugdunensis*. Unlike our case, emergence of SCVs was mostly due to prior antibiotic use after the DAIR procedure (ofloxacin and rifampicin for three months). In our case, SCVs had similar susceptibilities to antibiotics as normal variants which could be explained by not being induced by antibiotics.

5.4 Case Number 24

5.4.1 Clinical History

A 83 year old woman with several comorbidities had a primary left and right TKR in 2004 and 2005 respectively. She was doing very well until her left TKR started to bother her in early 2016. In April 2016, she was presented to the ED with a painful swollen knee. Her CRP was over 200 mg/L and WCC (white cell count) was over $15x10^9$/L. She underwent a DAIR procedure and beta haemolytic streptococci were isolated from the joint aspirate. She was treated with 2gm IV ceftriaxone OD for six weeks.

Unfortunately, this lady did not improve after the DAIR procedure and her symptoms persisted. Joint aspiration was performed in July 2016 and it was culture-negative.

Because of the persistence of symptoms and the high biomarkers, the patient underwent a single stage revision (index surgery) in October 2016.

Tissue samples were culture-negative. Figure 5-9 summarizes the surgical history of this patient.

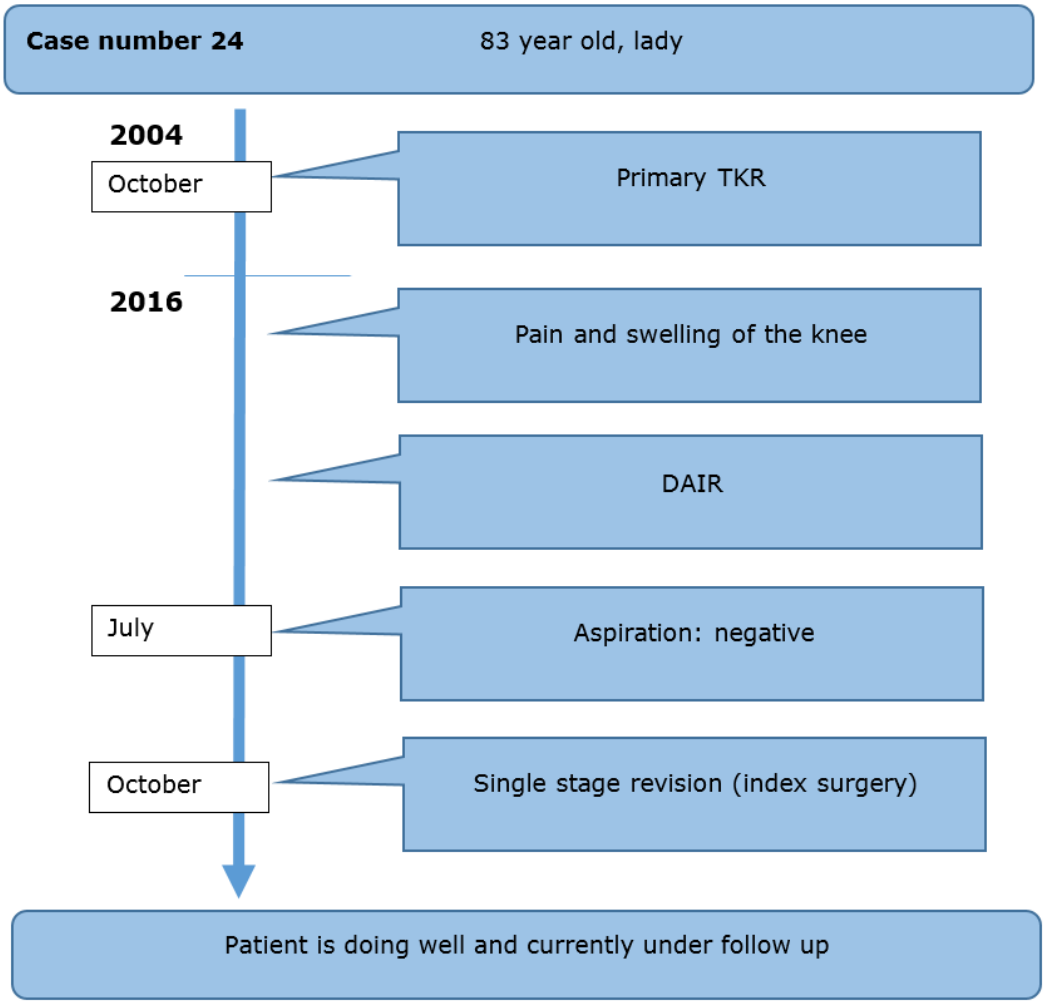


Figure 5-9 Timeline of patient 24.

5.4.2 **Laboratory Results**

Tissue samples were negative by both culture and molecular methods. Sonicate was culture-negative but staphylococci qPCR resulted in $3x10^2$ cfu/mL with negative PMA-qPCR. In addition, sonicate had a positive enterococci qPCR and PMA-qPCR low similar results (around 100 cfu/mL).

5.4.3 **Discussion**

This was a case of culture-negative PJI. In such cases, prior antibiotic use could be the reason for the non-cultivability of bacteria (Berbari et al., 2007). However, in this case, there were 4 antibiotic free weeks at least before the culture-negative aspiration and the patient did not receive antibiotics since then until after the first stage revision which was culture-negative as well.

Although there is no definite proof, it is likely that this is a case of persistent rather than recurrent PJI after the DAIR procedure. This opinion is justified by lack of clinical improvement after DAIR and the continuity of symptoms. Therefore, persistence of the beta haemolytic streptococci could be assumed.

The positive enterococcal PCR results could be a cross-amplification of the streptococci by the enterococci primers because of the genetic similarity between them (Yother et al., 2002). *E.faecalis* was once known as *Streptococcus faecalis* (Ike et al., 1983). Enterococci genus-specific primers used in this study were shown to have a low level of cross amplification with non-enterococcal species (Ryu et al., 2013). Sequencing of the amplicon of interest in this case could prove or refute this possibility of cross amplifications.

Given the small size of the amplicon of interest (92bp), its Sanger sequencing would be unreliable. Size of the amplicon available for sequencing after exclusion of 15-40 nucleotides from each of the forward and reverse direction would be too short to be informative (Ebili et al., 2017).

Next generation sequencing offers a reliable, but expensive, alternative in such cases.

5.5 Case Number 29: Indeterminate Case

5.5.1 Clinical History

A 56 year old woman with multiple co-morbidities had a primary right-sided TKR in December 2015. Her co-morbidities included hypertension, obesity (BMI>30), depression, penicillin allergy, previous history of transient ischaemic attack (TIA) and perforated peptic duodenal ulcer.

In May 2016, she presented with discomfort and swelling of her knee. Her CRP and ESR were 18 mg/L and 38 mm/hour respectively and there was no sinus. Aspiration of her joint was culture-negative. However, it was positive on the qualitative alpha-defensin testing, Synovasure (Zimmer Biomet, Warsaw, USA). X-ray showed lysis under the tibial component.

A two-stage revision was agreed on in an EMSON meeting, the first stage (index surgery) of which was done in January 2017. A standard PFC Sigma TKR prosthesis (DePuy Synthes) was used. Multiple tissue samples were collected and all of them were culture-negative. Furthermore, these tissue samples were sent by the clinical laboratory for broad-range PCR targeting the 16S rDNA bacterial gene and this was negative too.

After surgery, the patient was treated with 800 mg teicoplanin OD for six weeks. The patient has been improving and she is satisfied with her temporary knee implant. She is not keen on the second stage revision. Figure 5-10 summarizes the surgical history of this patient.

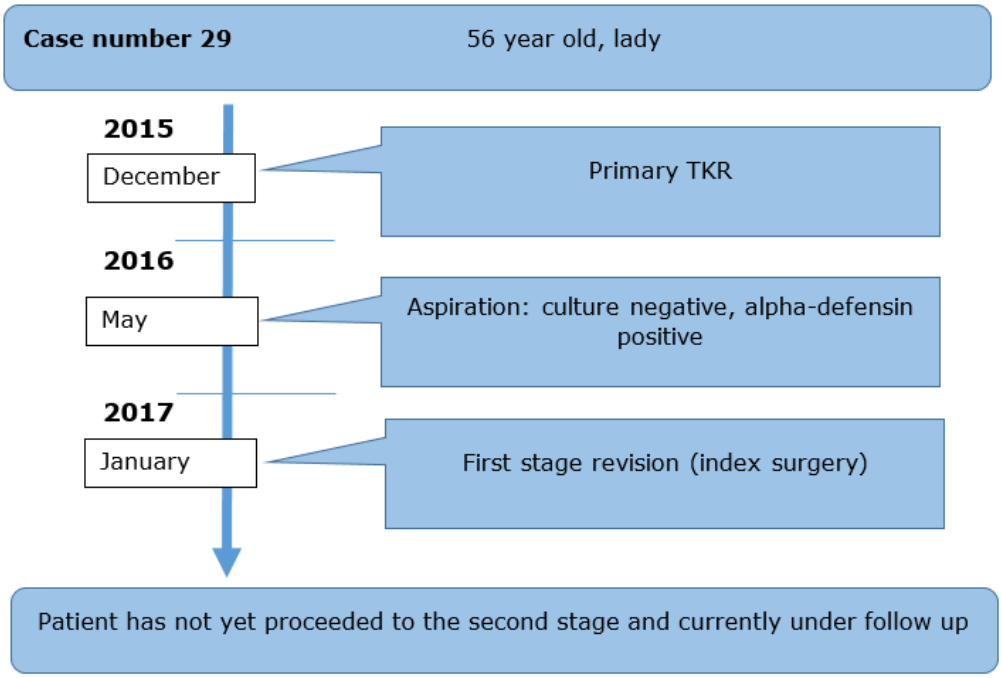


Figure 5-10 Timeline of patient 29.

5.5.2 **Laboratory Results**

All tissue samples and sonicate tested negative on both culture and PCR. No organisms could be identified on microscopic examination of Gram stained films of any of the samples.

5.5.3 **Discussion**

This case has neither met the MSIS criteria nor received a consensus on diagnosis in the EMSON. However, given the repeatedly reported high specificity of alpha defensin (see Table 1-2), this lady was included in the PJI group for a more conservative data analysis.

Therefore, this is a Culture-Negative PJI with no antecedent antibiotic use. Mycoplasmas and Ureaplasmas are among the smallest known free living organisms and can cause PJI (Thoendel et al., 2017, Rouard et al., 2017, Farrell et al., 2014). However, suspecting a non-cultivable bacteria in this

situation cannot be justified because of the negative results of 16S rDNA broad-range PCR.

5.6 Case Number 40

5.6.1 Clinical History

A 68 year old hypertensive and chronically anaemic woman had a primary TKR on November 2010 which was complicated by acute infection, wound dehiscence, and patellar tendon rupture. Patellar tendon reconstruction with Achilles tendon graft was tried along with single stage revision for the PJI. While the tendon reconstruction failed, infection came to a quiescent state. Later, the patient fell down and had a periprosthetic fracture of the distal femur which was internally fixed with plate and screws. Deterioration continued and a sinus developed. CRP, ESR, and white blood cell (WBC) were 60 mg/L, 47 mm/hour, and $10.6x10^9$/L respectively.

A two-stage revision was decided at that time. The first stage (index surgery) was done in February 2017 in which all the hardware and the infected Achilles tendon allograft were removed with an aggressive debridement. Then, a fusion nail was used and surrounded by Palacos R+G cement with 2 gm vancomycin added. A vascularized skin flap was necessary for wound closure. Scanty growth of CoNS was observed in one out of seven tissue samples sent to the clinical laboratory which was considered to be an insignificant contaminant by the microbiologists (antibiotic susceptibility testing was not done).

After the surgery, the patient was treated with IV 600mg teicoplanin OD for six weeks. Biomarkers came down to 5 mg/L, 23 mm/hour, and $9.3x10^9$/L for

the CRP, ESR, and WBC respectively. The second stage was done in September 2017 which included extensor mechanism reconstruction. Tissue samples as well as joint aspirate were culture-negative. The patient improved and the biomarkers were down to normal. Figure 5-11 summarizes the surgical history of this patient.

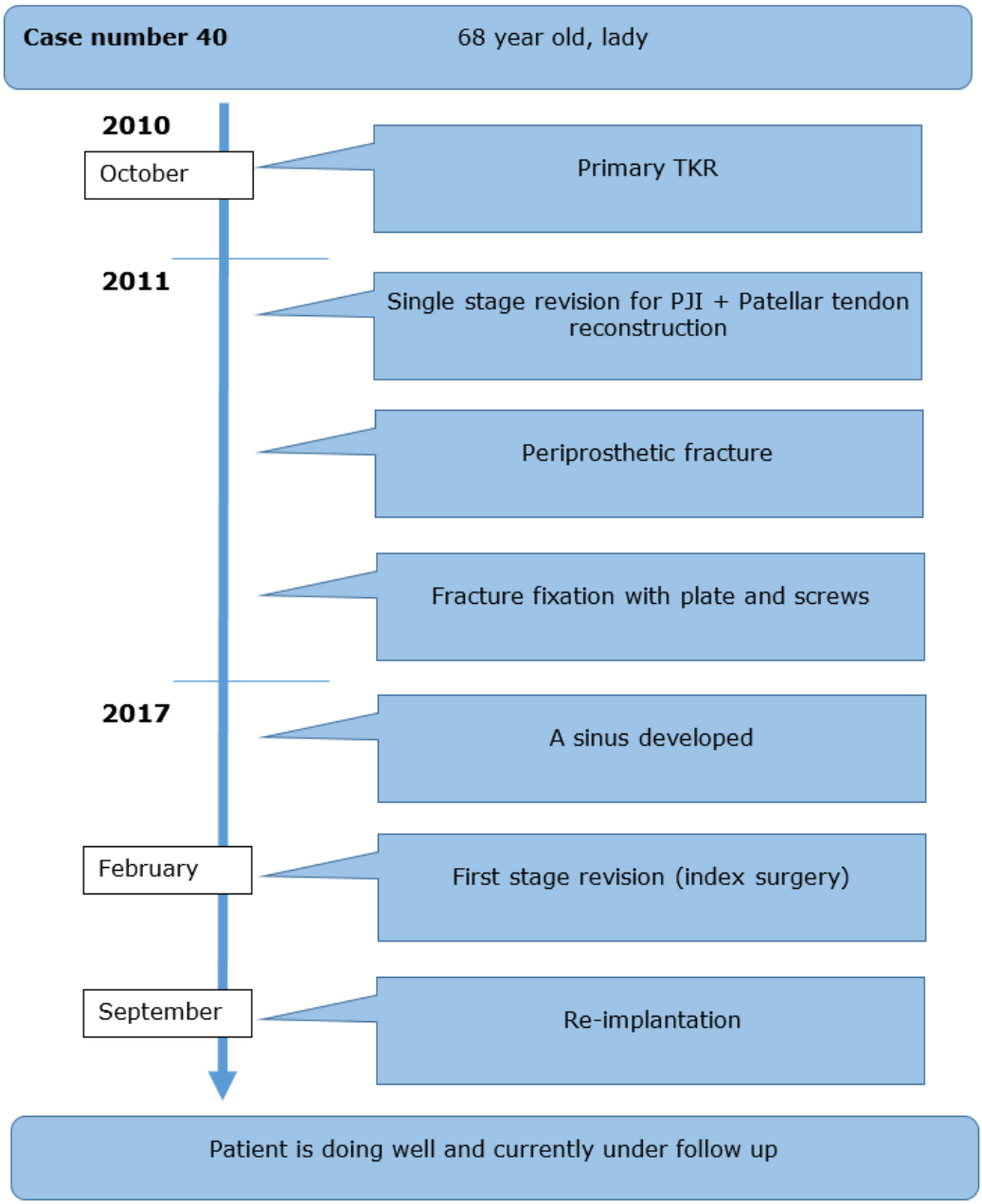


Figure 5-11 Timeline of patient 40.

5.6.2 **Laboratory Results**

Only the prosthesis was collected from this patient at the time of first stage revision, sonication of which grew 100 cfu/mL *S.epidermidis*. Conventional and viability PCR of this sonicate detected $8x10^2$ and $3x10^2$ cfu/mL staphylococci respectively.

5.6.3 **Discussion**

Scanty growth of a common contaminant in one out of seven tissue samples could be interpreted as a non-infected case or that the isolated bacterium is not the infective organism. A low virulent organism, such as *S.epidermidis*, is known to cause a long history of vague symptoms of pain and discomfort as in this case. In addition, isolation of the same organism from the sonication fluid enhances the possibility of being the causative organism. A well-studied cause of negative culture is prior antimicrobial use which affects the validity of tissue samples more than sonicates (Trampuz et al., 2007). In such cases, PCR is a promising tool that is not influenced by prior antibiotic use.

5.7 Case Number 42

5.7.1 **Clinical History**

A 53 year old man had a primary TKR in July 2016. He developed an acute postoperative superficial wound infection swabbing of which revealed *S,aureus* sensitive to flucloxacillin. So, he was treated with flucloxacillin and augmentin (amoxicillin and clavulanate). Unfortunately, the infection did not subside, and a sinus developed. CRP and ESR were 25 mg/L and 23 mm/hour. The patient had a DAIR procedure in September 2016 and was empirically treated with flucloxacillin and gentamicin until the culture grew

E.faecalis. His treatment was then changed to co-amoxiclav for 2 weeks IV then 4 weeks oral. His CRP and ESR went down to 6 mg/L and 18 mm/hour respectively. In December 2016, the patient received a trauma to his knee leading to swelling. X-ray at that time showed radiolucency under the tibial component. Aspiration of the swelling grew *E.faecalis*. CRP and ESR were 54 mg/L and 44 mm/hour after the trauma and continued to be elevated until February 2017 when they were 83 mg/L and 30 mm/hour respectively. Lack of improvement prompted the surgeon to perform a first stage (index surgery) of a two-stage revision in March 2017. Tissue samples grew *E.faecalis* again and histopathology examination showed significant neutrophil infiltration which is consistent with chronic infection. The patient was treated with 2mg IV amoxicillin TDS for six weeks. Figure 5-12 summarizes the surgical history of this patient.

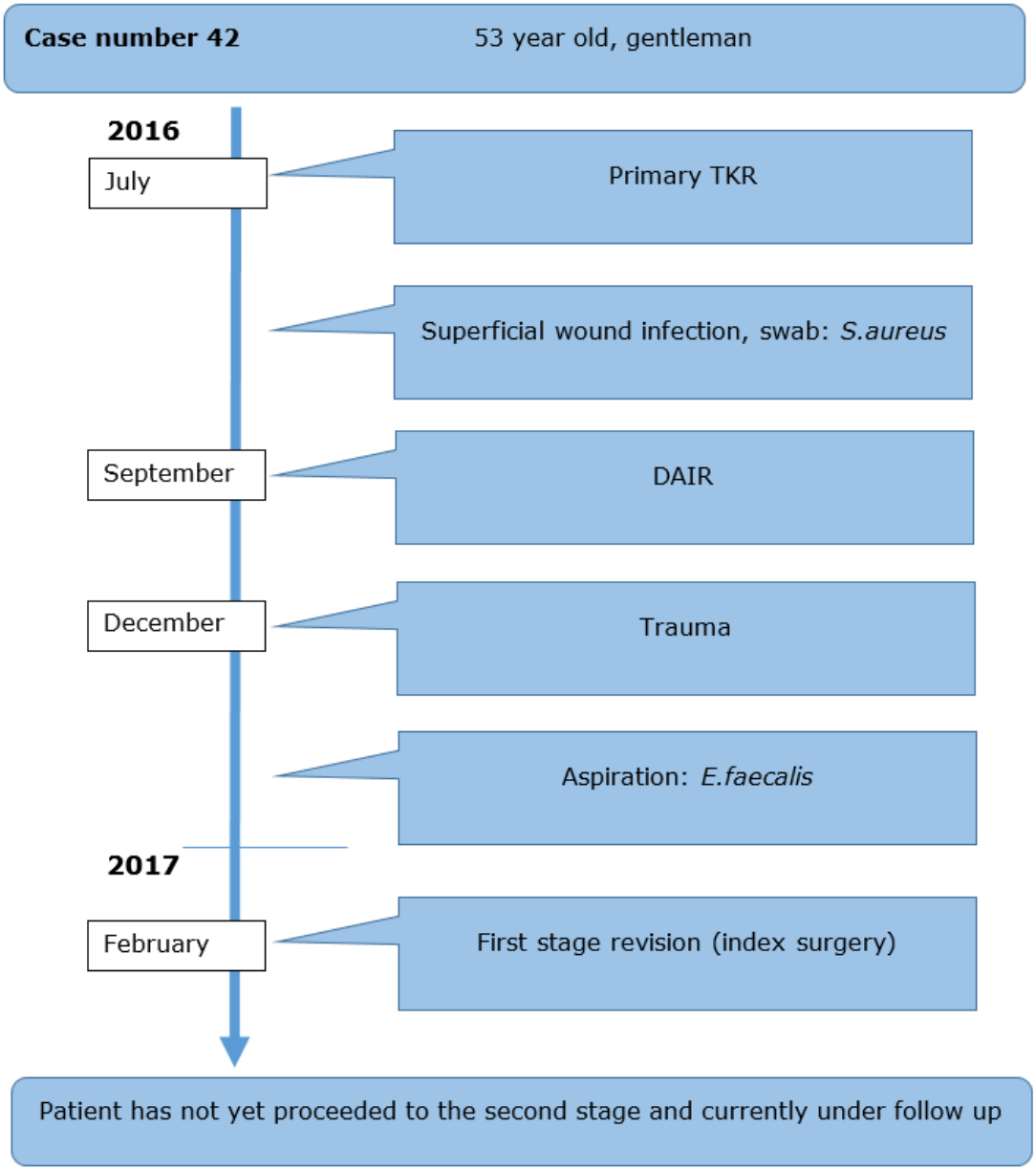


Figure 5-12 Timeline of patient 42.

5.7.2 **Laboratory Results**

E.faecalis was isolated from all tissue samples and sonicate of the retrieved prosthesis showing around 3-4x10^3 cfu/mL except for one tissue sample from which only 600 cfu/mL were isolated. Enterococcal conventional PCR was positive for sonicate and all tissue samples except the tissue sample with the low bacterial count while viability PCR was positive for all samples.

5.7.3 **Discussion**

In this case, the isolation of *S.aureus* at the early start of PJI might have been a distractor from the true infective organism (*E.faecalis*) which had not been isolated at that time. Current literature recommends against the use of wound swabs due to high percentage of false positive results.

The tissue samples with positive viability PCR and negative qPCR is one of the samples that raised the probability of enhancement of the PCR sensitivity by PMA treatment as discussed later in the discussion chapter.

5.8 Case Number 43

5.8.1 **Clinical History**

A 71 year old man had a primary TKR in February 2014. He had never been happy with his replaced knee and kept complaining of continuing pain. A fluctuant collection was noted in his knee and he underwent incision and drainage of wound abscess in June 2016. Tissue samples revealed scanty growth of CoNS. The patient had a two-week course of flucloxacillin. One month later, in July, aspirate of the knee grew two colony types of CoNS from broth enrichment only. A single stage revision (index surgery) was done in March 2017 and scanty CoNS was isolated from one out of three tissue samples collected at that surgery. The patient was treated with 2gm IV ceftriaxone OD and 300mg oral rifampicin BD for six weeks followed by 100mg doxycycline BD and 300mg oral rifampicin BD for another six weeks. CRP and ESR before and after the index surgery were 8 mg/L and 24 mm/hour and <5 mg/L and 6 mm/hour respectively. Figure 5-13 summarizes the surgical history of the patient.

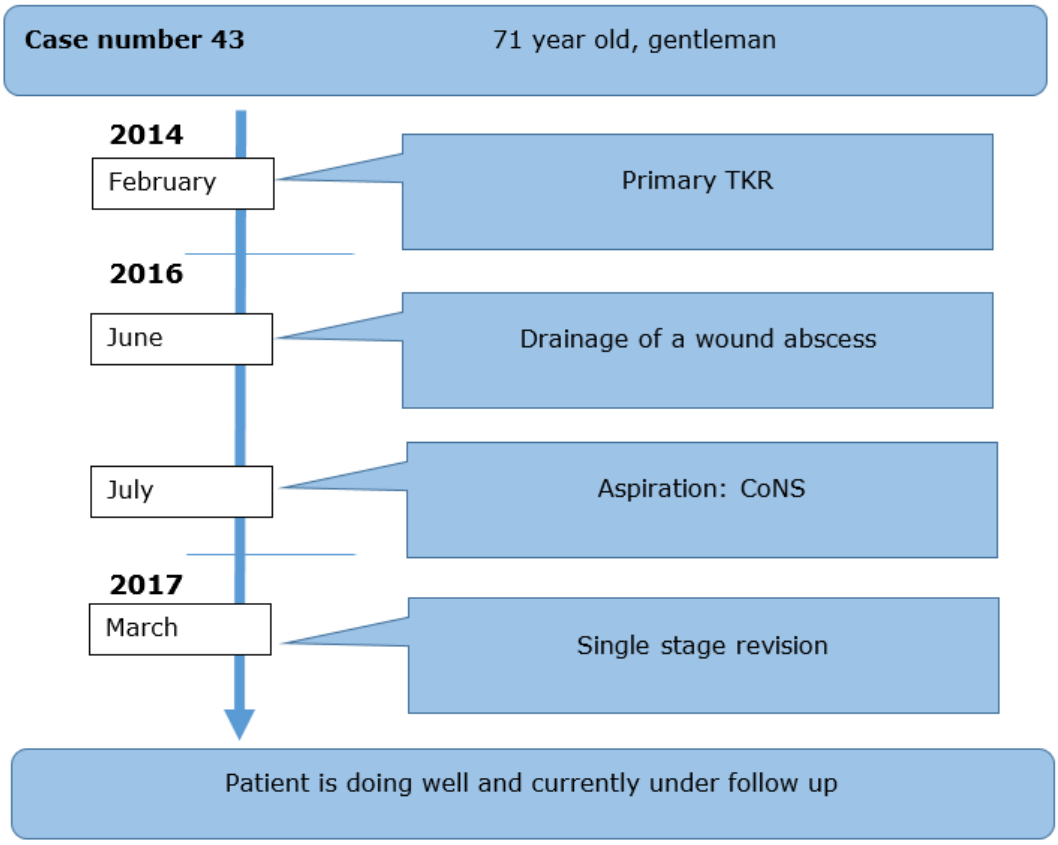


Figure 5-13 Timeline of patient 43

5.8.2 **Laboratory Results**

Low numbers of *S.epidermidis* colonies (10-40 cfu/mL) were observed on plates of tissue samples. All of these tissue samples tested negative on both types of PCR used in this study. Quantification of *S.epidermidis* in the sonicate was 650 cfu/mL and $2x10^3$ cfu/mL by culture and qPCR respectively. PMA-qPCR of the sonicate was negative.

5.8.3 **Discussion**

This is a case of late PJI in which there was no pain-free period after the primary replacement. The low number of isolated *S.epidermidis* colonies could explain these negative PCR results. These false negative results could be caused by either poor DNA extraction or PCR inhibition.

The positive qPCR result of the sonicate in the time of negative results of tissues support the conclusion made by Rak et al. (2016) that sonicate is a more sensitive sample for PCR in PJI diagnosis.

5.9 Case Number 45

5.9.1 Clinical History

An 83 year old woman had a primary THA 18 years ago. Because of early instability, she underwent a revision within the first year of her THA. She had been satisfied with her hip for 16 years until it became painful during 2017. In February 2017, a pus-draining sinus developed. A single stage revision (index surgery) was carried out in March, 2017. Her pre-operative CRP and ESR levels were 44 mg/ L and 60 mm/hour respectively. Out of the five tissue samples collected at the time of surgery, two samples had scanty growth of *Streptococcus mutans* sensitive to penicillin, vancomycin, and rifampicin. She was treated with 2gm IV ceftriaxone OD for six weeks followed by another six weeks of oral amoxicillin 500mg TDS. She has been improving since surgery and her wound healed properly. Figure 5-14 summarizes the surgical history of the patient.

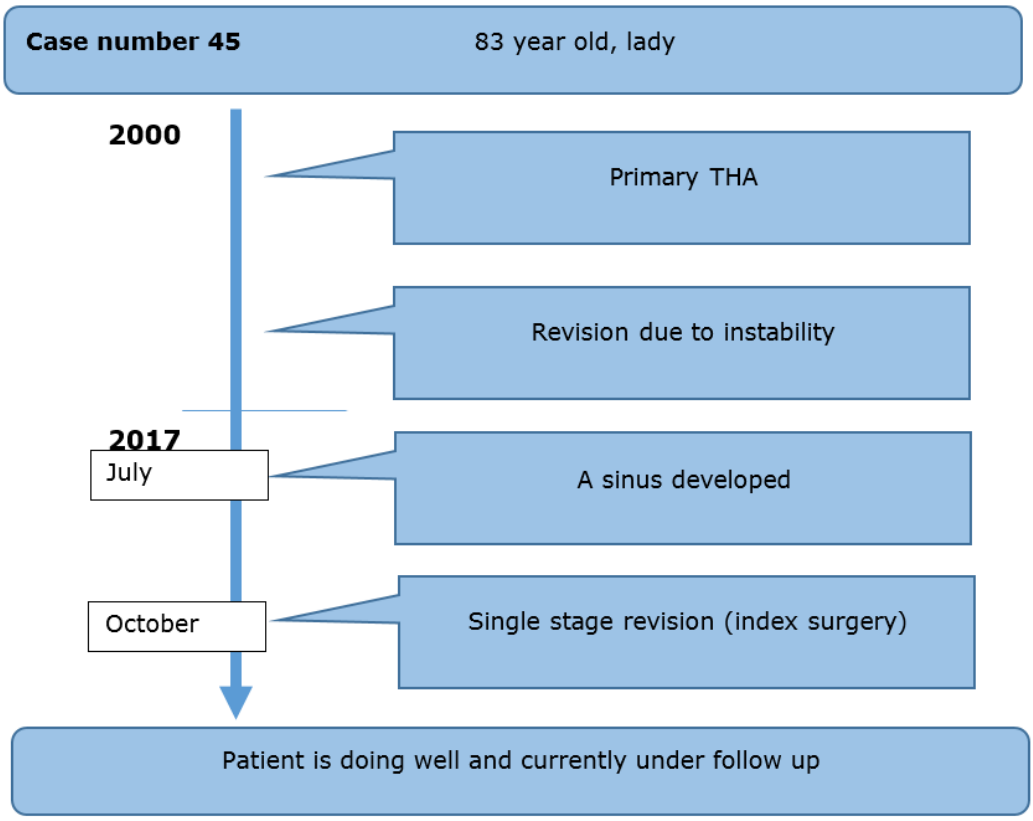


Figure 5-14 Timeline of patient 45

5.9.2 **Laboratory Results**

Three out of four tissue samples and the sonicate grew Gram positive rods/elongated cocci (Figure 5-15) which were catalase and coagulase negative. API coryne (for coryneform bacteria) was inconclusive. This organism was identified as *Streptococcus mutans* by MALDI-TOF MS and later by API strep.

PCR was not helpful in this case as its panel did not include primers for streptococci.

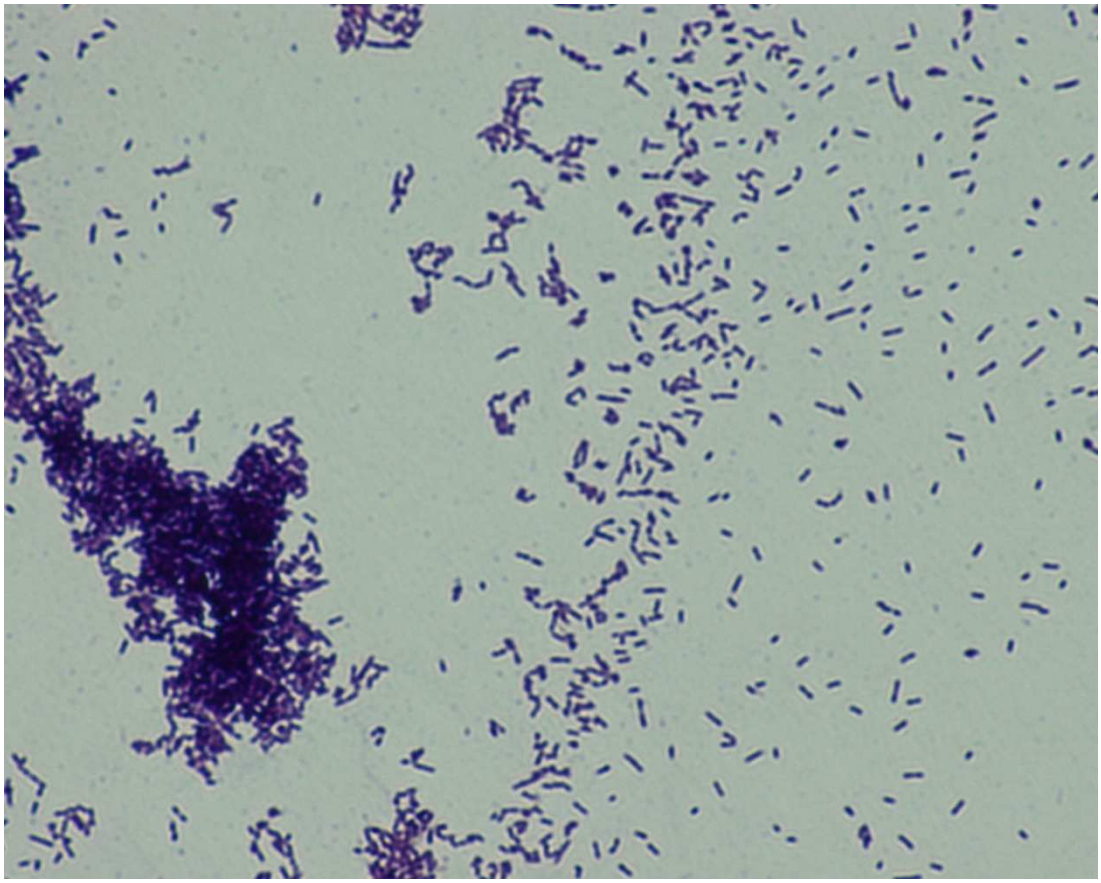


Figure 5-15 Gram positive elongated cocci/rods.

5.9.3 **Discussion**

This is a case of haematogenous PJI. After 16 years free of pain since the primary replacement, the patient started to complain of painful swollen knee. Her teeth are a likely source of infection as *S.mutans* commonly inhabits the oral cavity and cause caries (Kumarasamy et al., 2014, Loesche, 1986). Microbes causing dental caries, including *S.mutans*, produce acids upon exposure to carbohydrates in the diet (Loesche, 1986). In an acidic medium, *S.mutans* assumes a bacillus shape with a 3 μm length or more (Clarke, 1924).

5.10 Case Number 47

5.10.1 Clinical History

A 65 year old man, who was diabetic and on warfarin for a heart valve replacement, underwent a primary knee replacement in February 2017. Two weeks later, he presented with a painful erythematous area surrounding the surgical wound with no discharge. His CRP was 83 mg/L and ESR 42 mm/hr. Aspiration of the knee was done at that stage and grew CoNS. Therefore, he underwent an urgent DAIR procedure (index surgery 1) and multiple tissue samples grew Enterococcus species. Following the surgery, he was treated with daptomycin 700mg OD and amoxycillin 2 g TDS for 6 weeks guided by the antibiotic susceptibility testing.

Unfortunately, the infection did not settle, high biomarkers persisted, and he developed a sinus draining pus at a later stage. He was scheduled for a formal first stage revision (index surgery 2) with an aggressive debridement and explantation of the whole prosthesis. At this stage, all tissue samples were culture-negative. Therefore, the patient was treated with the same antibiotic regimen used after the DAIR. Figure 5-16 summarizes the surgical history of the patient.

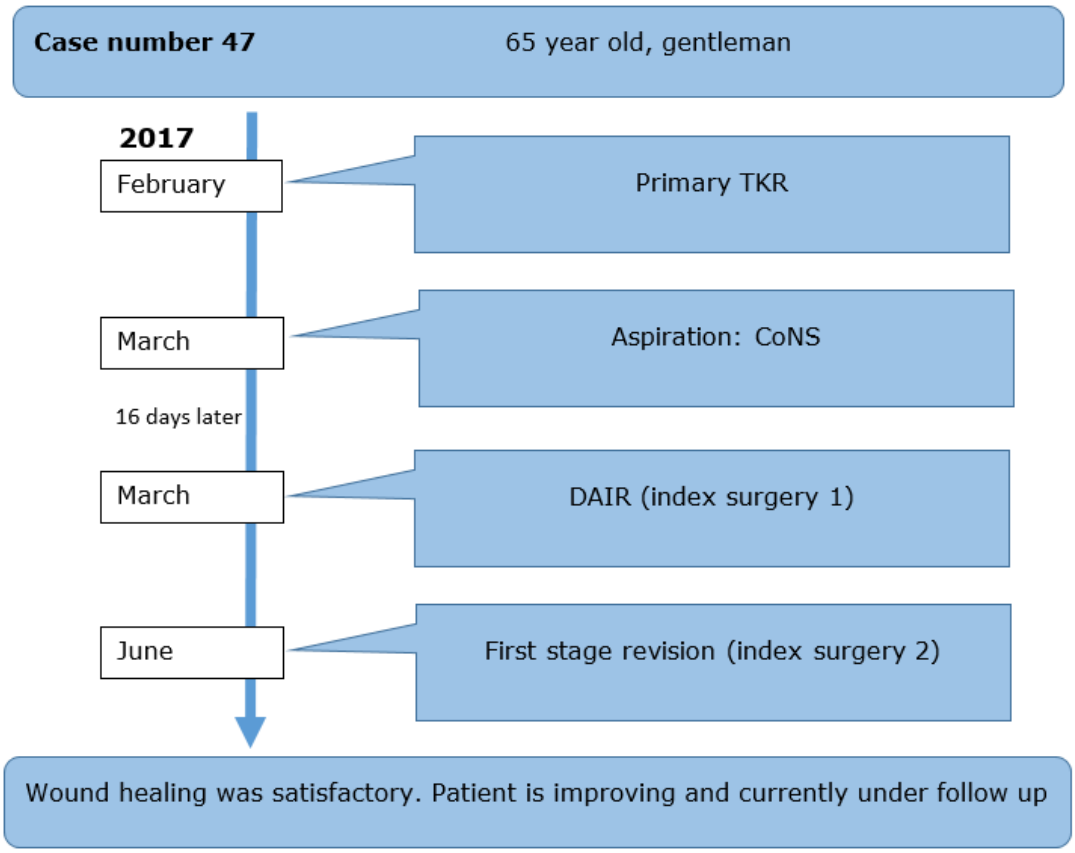


Figure 5-16 Timeline of patient 47.

5.10.2 Laboratory Results

All tissue samples collected at time of index surgery 1 grew *E.faecalis* (60 – 600 cful/mL). In addition, sonicate grew $4x10^3$ cfu/mL *E.faecalis* which were 5 to 10 fold the number grown from tissue samples. However, all tissue samples and sonication fluid from the index surgery 2 were culture-negative.

Furthermore, conventional and viability PCR quantification from the sonicate of the index surgery 1 were $2x10^4$ and $9x10^3$ cfu/mL enterococci respectively. Three out of four tissue samples were positive on viability but not conventional PCR. The fourth tissue sample was negative on both conventional and viability PCR. PCR failed to detect the organism from either tissue samples or sonication fluid at the index surgery 2.

5.10.3 Discussion

This case highlights the dilemma of culture-negative PJI (CN-PJI). In the acute stage of infection, *E.faecalis* was isolated from tissue samples collected during the DAIR procedure. However, with more progression of the infection and deterioration of the patient's condition to the stage of sinus development, no organism was identified by culture. Prior antibiotic use has been recognized as a risk factor for CN-PJI (Malekzadeh et al., 2010, Trampuz et al., 2007). In a study by Berbari et al. (2007), 53% of CN-PJI episodes were treated with antibiotics within three month of the index surgery. Likewise, prior antibiotics use in our case is an acceptable explanation for negative culture results at index surgery 2. Possibilities like unusual or fastidious organisms or ineffective tissue sample processing are unlikely given the positive culture results earlier at index surgery 1.

Other less likely possibilities could also be considered. First, the enterococcal infection might have been treated by the surgical debridement and antibiotic prescription and another de novo infection with non-culturable organism developed. Furthermore, death of bacteria could have occurred at any stage between harvesting and culturing the samples because of local release of antibiotics from the old cement at time of surgery (Powles et al., 1998).

Negative PCR results could be explained by the fact that mutations of the 23rRNA gene, which is targeted by the enterococci primers used in this study, have been associated with antimicrobial resistance of *E.faecalis* (Marshall et al., 2002).

5.11 Case Number 49

5.11.1 Clinical History

A 71 year old woman had a primary TKA in 2009 with which she was satisfied until October 2015 when it started to be painful. An abscess in the wound site was noticed before development of a sinus discharging a scanty amount of pus in December 2015. Because of the general medical condition of the patient, antibiotic suppression was tried but unfortunately it was unsuccessful. Culture of the joint aspirate (not a swab from the sinus tract) isolated CoNS after broth enrichment only. This CoNS which was not individually speciated was sensitive to vancomycin. At that stage, the CRP was 87 mg/L and a formal two-stage revision was decided. The first stage was done in April 2017 (index surgery). Culture of the tissue samples from the first stage was negative. Unfortunately, the patient did not improve clinically and her biomarkers did not drop either. So, a washout with harvesting other tissue samples was carried out in May 2017. Culture of periprosthetic tissue samples continued to be negative. The joint was aspirated one month after this washout and the aspirate culture grew *E.coli* and *Klebsiella* after broth enrichment only. The patient was then treated with 2gm ceftriaxone OD. As the patient did not improve and her CRP was 100 mg/L, a repeat first stage with a vascularized skin flap was done in June 2017 and no growth was noted from the tissue samples for the third time. The patient was then retreated with 2gm ceftriaxone OD. After this repeat first stage, although the wound healing was satisfactory and the biomarkers were decreasing, patient has not yet proceeded to the second stage. Figure 5-17 summarizes the surgical history.

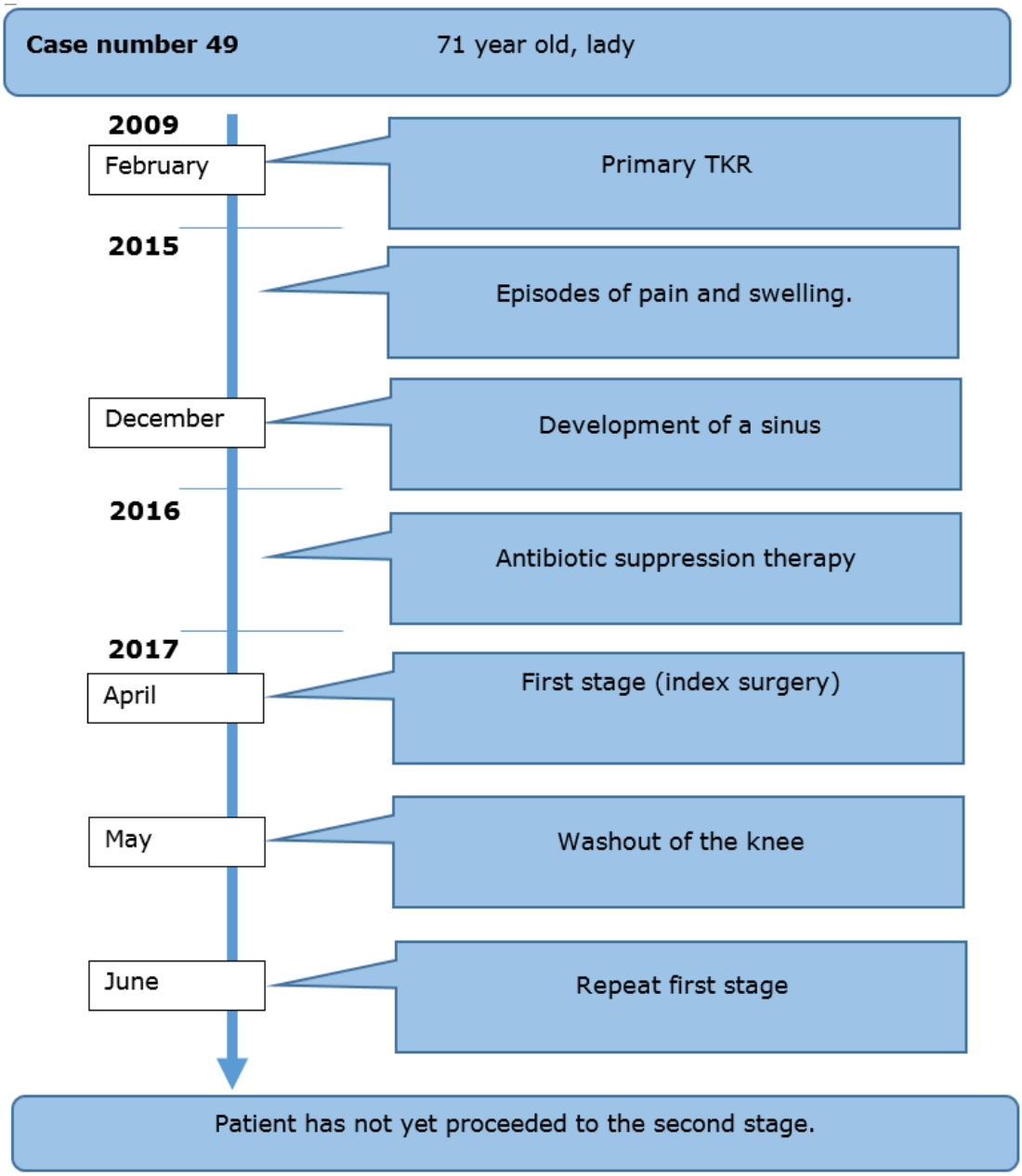


Figure 5-17 Timeline of patient 49.

5.11.2 Laboratory Results

One out of seven tissue samples grew $2x10^3$ cfu/mL *S.epidermidis.* Molecular quantification of staphylococci from this culture-positive sample was $2x10^4$ and $7x10^2$ cfu/mL by conventional and viability PCR respectively. Another sample showed 10^4 and $3x10^3$ cfu/mL on staphylococcal quantification by conventional and viability PCR respectively. A third sample

was positive with viability PCR only ($4x10^2$ cfu/mL). Sonicate was culture and PCR negative.

5.11.3 Discussion

In this case, a superficial abscess over the joint is likely to be the source of infection given the clinical scenario and the isolation of *S.epidermidis* which are commonly present as part of human skin flora.

The isolation of *S.epidermidis* from only one out of seven tissue samples might raise the possibility of contamination. However, the probable concordance with previous isolation from joint aspirate, relatively large number of the isolate, and positive PCR results of multiple tissue sample would favour its clinical significance. Individual organisms within CoNS group should be individually speciated to enable comparison of isolates and to help studying the pathogenicity of each.

Multiple tissue samples are required to enhance the sensitivity of the culture in PJI diagnosis (Atkins et al., 1998, Trampuz et al., 2007). The variability of results of different tissue sample could be explained by the postulated process of infection initiation and propagation. In this case, infection is likely to have spread from a superficial skin lesion to deeper tissues and joint space subsequently. Clear precise labelling of tissue samples along with a hand drawn diagram would help to accept or reject this explanation.

5.12 Case Number 54

5.12.1 Clinical History

A 78 year old woman underwent the first stage revision (index surgery) in May 2017 for her infected TKR which was implanted 16 years ago. She had

been troubled with pain for the last two years with development of multiple sinuses. Her pre-operative CRP and ESR values were 64 mg/L and 73 mm/hour respectively. One month postoperatively, they dropped to 11 mg/L and 32 mm/hour respectively. This diabetic and hypertensive patient had a BMI of 39 and also suffered from congestive heart failure and atrial fibrillation. From six out of the nine tissue samples sent to the microbiology laboratory, beta-haemolytic streptococci were isolated only after broth enrichment and were sensitive to penicillin, amoxicillin, and vancomycin. Figure 5-18 summarizes the surgical history.

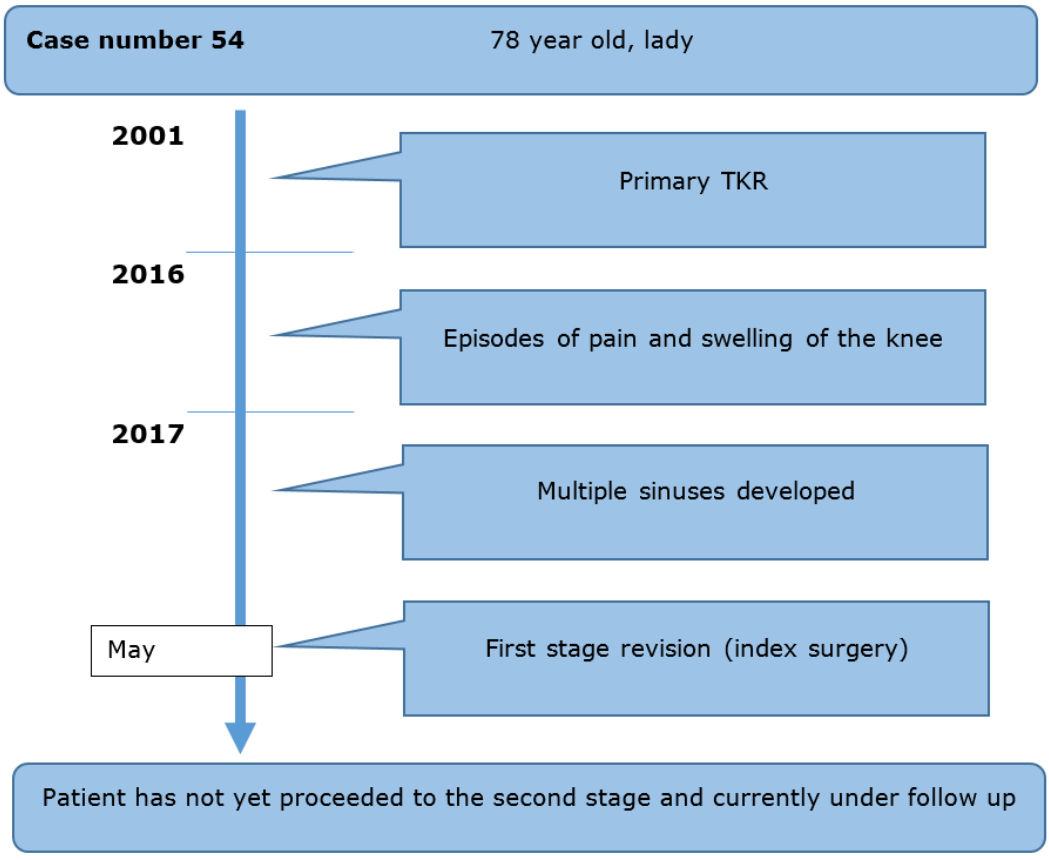


Figure 5-18 Timeline of patient 54.

5.12.2 Laboratory Results

Only the prosthesis was collected from this patient. Culture of sonicate grew $15x10^2$ cfu/mL *Streptococcus agalactiae*. PCR results were negative.

5.12.3 Discussion

This is a case of haematogenous PJI in a patient with multiple co-morbidities. *Streptococcus agalactiae* is a known causative organism of PJI (Duggan et al., 2001). Antibiotic treatment should be guided by susceptibility testing as some penicillin-resistant *S.agalactiae* have been reported (Cunningham et al., 1992, Gaudreau et al., 2010).

Negative PCR results were expected as the causative organism was outside the tested panel.

5.13 Case Number 56

5.13.1 Clinical History

A 67 year old man, using inhaled corticosteroids for asthma, had a primary TKA for an osteoarthritic knee in February 2017. He presented, four weeks later, with multiple sinuses. He underwent a first stage revision in April 2017 followed by the second stage (index surgery) which included a vascularized skin flap in July 2017. In between stages, the patient developed peripherally inserted central catheter (PICC) associated thrombosis and he was treated with low molecular weight heparin. Culture of tissue samples retrieved CoNS (without speciation of individual organism) sensitive to teicoplanin, vancomycin, and daptomycin. The patient's antibiotic treatment regimen with IV teicoplanin 800 mg OD continued for six weeks followed by oral linezolid

600 mg BD and rifampicin 300 mg BD. Figure 5-19 summarizes the surgical history of the patient.

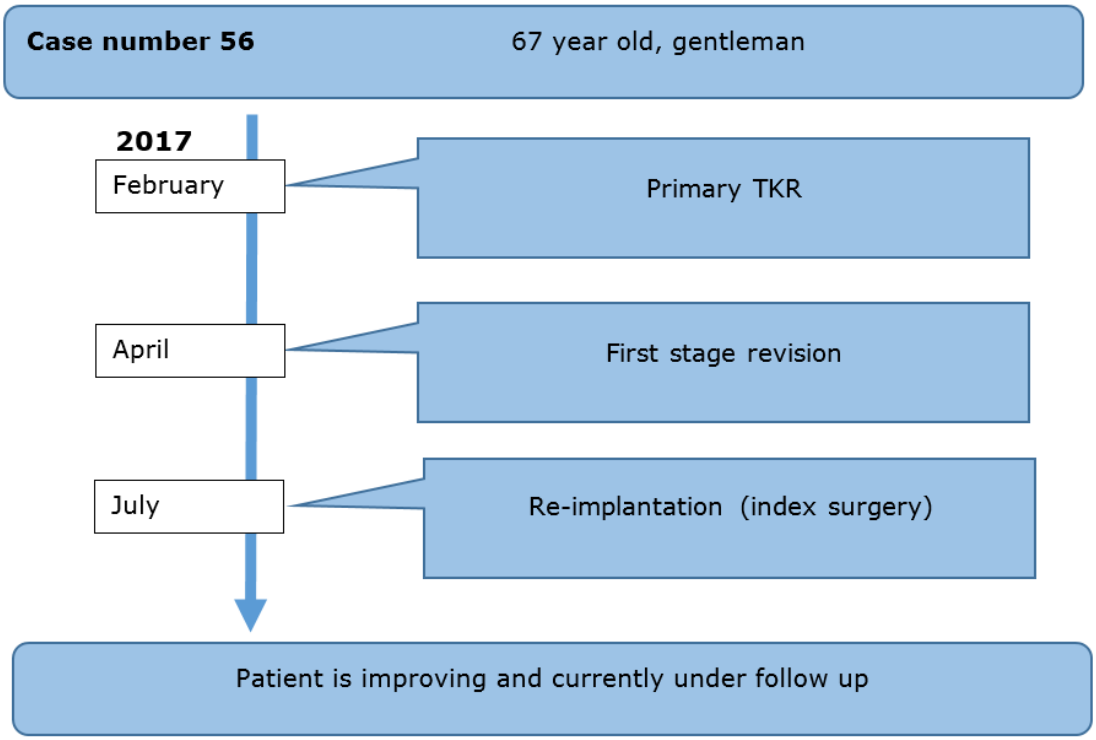


Figure 5-19 Timeline of patient 56.

5.13.2 Laboratory Results

S.epidermidis was isolated from the sonication fluid of retrieved spacer (720 cfu/mL) and tissue samples (50-230 cfu/mL). All PCR results were negative. GADPH gene was amplified from all samples.

5.13.3 Discussion

Long term systemic, but not inhaled, corticosteroids use has been considered a risk factor for PJI (Eka and Chen, 2015).

This was a case of acute PJI which was treated with a two-stage protocol because of the aggressiveness of infection as evidenced by development of

multiple sinuses. *S.epidermidis* is the most common CoNS causing PJI (Harris et al., 2010).

In this scenario, with significant positive culture from tissue samples and sonicate, PCR results should be interpreted cautiously. These negative PCR results are very likely to be false negative. PCR inhibition could still be considered despite the amplification of the housekeeping gene (see 6.3.3.4). A postulated reason for these false negative PCR results is the prior prolonged use of heparin. Heparin is a known PCR inhibitor (Yokota et al., 1999, Garcia et al., 2002) that can accumulate in tissues (Gurbuz et al., 2013). This case is an example of situations that justify the use of PCR as an adjunct to the conventional microbiological methods to expedite and complement the diagnosis of PJI but not to replace them, a recommendation previously made by Vandercam et al. (2008).

5.14 Case Number 59

5.14.1 Clinical History

A 68 year old woman underwent bilateral TKR on two separate occasions in 2014. She presented with an infected right TKR one year after the surgery. *S.aureus* was isolated from the joint aspirate in September 2015. She was treated with a two-stage revision protocol of which the first stage was done in October 2015. At that stage, *S.aureus* was re-isolated from intra-operative tissue samples. Prior to the second stage, aspiration of the knee was done and it grew *S.aureus* again. So, in April 2016, the second stage was performed and it involved an aggressive re-debridement before the re-implantation. Tissue samples obtained at the time of the second stage were culture-negative. Unfortunately, the infection was not eradicated as a third

aspiration in August 2017 revealed *S.aureus*. She had to undergo a repeat first stage revision (index surgery) in September 2017. No growth was detected from the tissue samples obtained at this time, so the patient was treated with empirical antibiotics for six weeks. The second stage was done in December 2017 with postoperative antibiotics for three days. Figure 5-20 summarizes the surgical history of the patient.

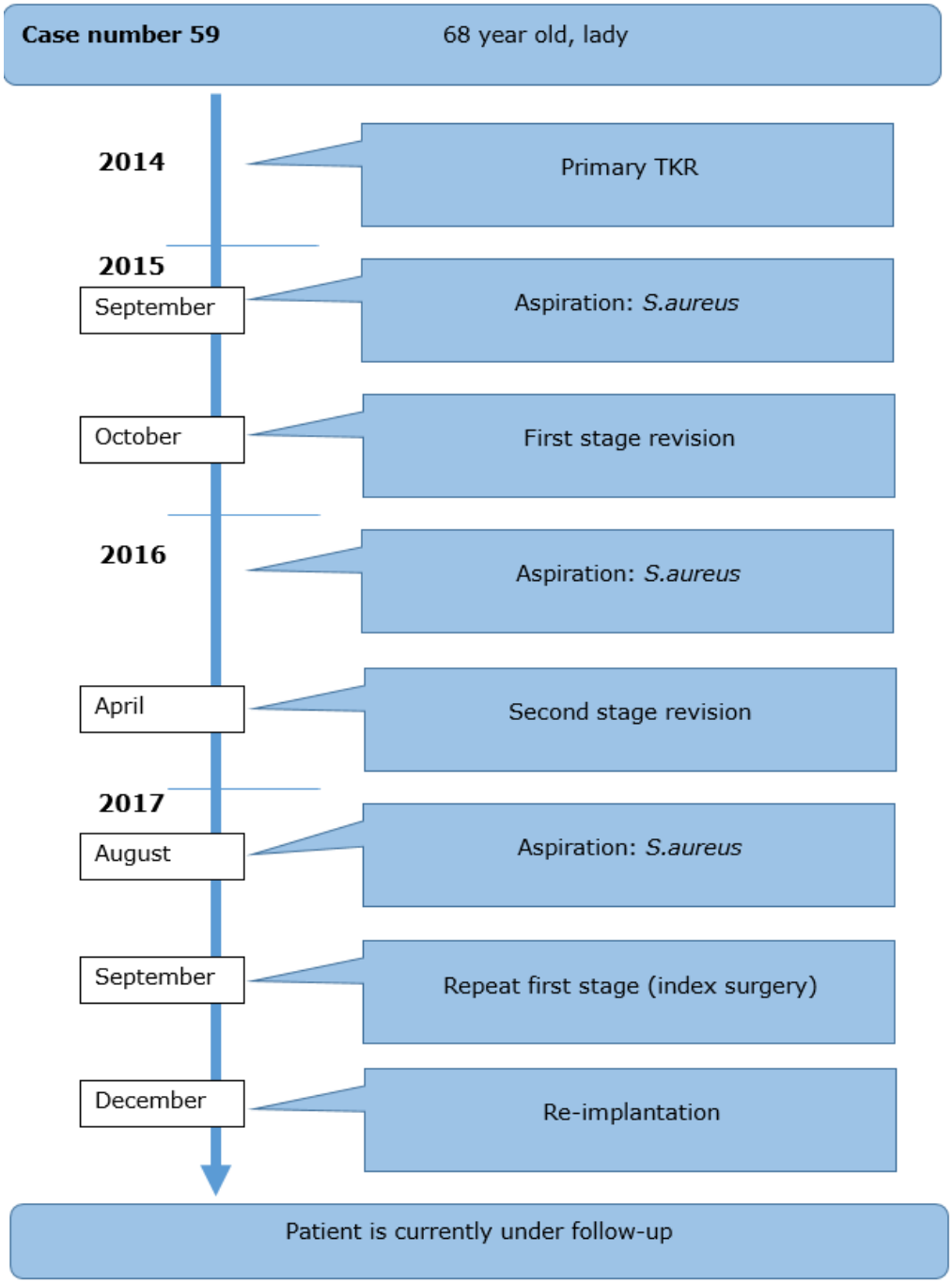


Figure 5-20 Timeline of patient 59.

5.14.2 Laboratory Results

Cultures of all tissue samples and sonicate were negative. Staphylococci conventional and viability PCR of the sonication fluid of retrieved prosthesis detected $7x10^4$ and $3x10^3$ cfu/mL respectively.

5.14.3 Discussion

This is an example of cases in which qPCR could detect the infecting organism, *S.aureus* in this case, when the culture methods failed to do so. In similar situations, in which there is a long history of multiple surgical intervention with long periods of antibiotic treatment, diagnosis of PJI should not solely rely on culture methods (Berbari et al., 2007). In these cases, a culture independent method, like PCR, could be of preference as it is not affected by prior antibiotic treatment.

The qPCR positive result in this case could be arguably ascribed to DNA from dead bacteria while the viability qPCR positive result should reflect only live bacteria. These results are in agreement with previous studies concluding that sonicates are more sensitive not only in conventional culture methods as repeatedly reported (Portillo et al., 2013, Trampuz et al., 2007) but also in molecular diagnosis (Rak et al., 2016).

5.15 Case Number 60

5.15.1 Clinical History

A 78 year old woman had a left-sided primary TKR in 2003 which “has never been right” in the patient’s own words. In 2007, she was diagnosed with PJI of her TKR and underwent single stage revision for that reason. Her knee continued to be painful with fluctuating swelling. In September 2016, she developed a draining sinus that reached a size of 3x3 cm. The first stage of a two-stage revision was carried out in February 2017 in which a fusion intramedullary nail surrounded by Palacos R+G (Hereaus Medical) cement with added 2 gm vancomycin was used. In addition, a vascularized muscle flap was necessary for tension-free wound closure. Culture of intraoperative

tissue samples revealed polymicrobial infection including *E.coli* (sensitive to amoxicillin, cefuroxime, and gentamicin), *E.faecalis* (sensitive to amoxicillin and vancomycin), and *Streptococcus viridans* (sensitive to erythromycin and penicillin). Following the surgery, she was treated with amoxicillin TDS, parenteral 2gm for six weeks followed by oral 1 gm for another six weeks. Her wound healed well and biomarkers were declining. Re-implantation of a silver-impregnated prosthesis with repair of the extensor mechanism of the knee (index surgery) was done in September 2017. Postoperatively, she received empirical antibiotics for three days until the culture of the tissue samples were reported as negative. Figure 5-21 summarizes the surgical history of the patient.

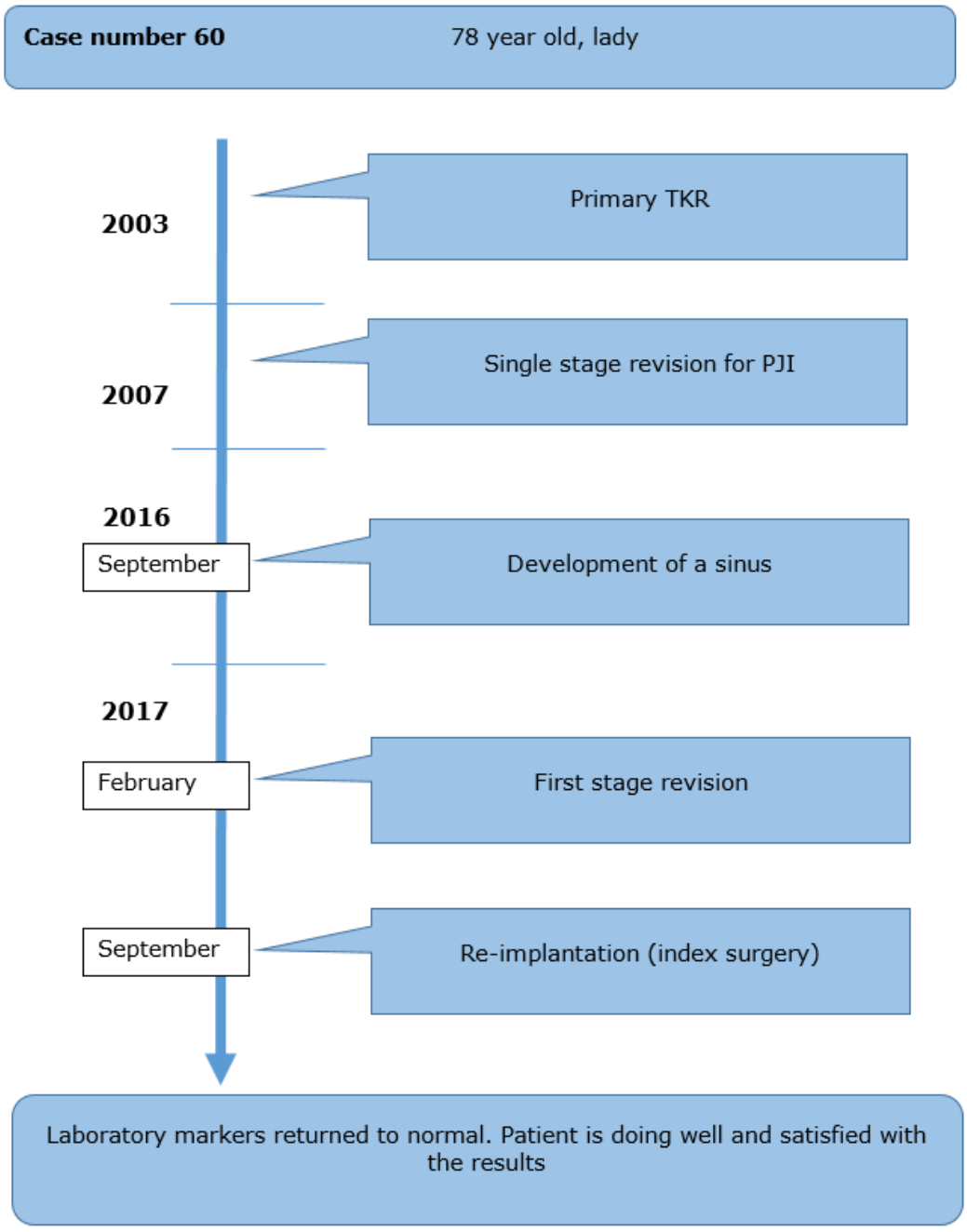


Figure 5-21 Timeline of patient 60.

5.15.2 Laboratory Results

Cultures of all tissue samples and sonicate were negative. QPCR was also negative. PMA-qPCR of the sonicate detected $6x10^2$ cfu/mL Enterococci.

5.15.3 Discussion

This is a case of late polymicrobial PJI which is a well-known risk factor for failure of the first stage revision in a two-stage protocol (Bozhkova et al., 2016). The positive result of the viability PCR for Enterococci is a red flag which should prompt close follow-up and lower the threshold of suspicion for infection persistence. As other organisms isolated at the time of first stage are not covered by the panel of PCR assay in this study, it cannot be argued whether these organisms had been eradicated or not. However, this case is an example of cases in which the PMA could enhance the sensitivity of PCR testing of the sonicates as discussed later in the Discussion Chapter.

5.16 Conclusion of the Chapter

Diagnosis of PJI can be challenging. This chapter illustrates that every patient is unique in presentation and diagnosis, and that treatment needs to be personalised accordingly. Interpretation of the conventional and molecular microbiological tests should be done in light of the clinical presentation. Therefore, a multidisciplinary team approach is essential for PJI management.

Sonicates have been shown to have higher sensitivity than tissues in both culture and molecular diagnostic modalities. Cases 19, 20, 43, and 59 are examples of cases with positive sonicate and negative tissue PCR results. It is highly recommended to routinely include prosthesis sonication in the diagnosis of PJI.

PCR outperformed the culture methods in some culture-negative episodes as in Cases 24, 59, and 60. The short turnaround time is an advantage of

PCR that enables earlier targeted treatment. However, neither the culture nor the PCR could identify the causative organisms in some clinically proven PJI cases as in the second episode of Case 47. Furthermore, false negative results of PCR could not be avoided in one culture-positive episode (Case 56).

Viability PCR could identify organisms in samples with low bacterial count as in Case 43 and first episode of Case 47. The increase in PCR sensitivity by PMA is an accidental finding that needs further investigation in order to be proved or refuted.

In conclusion, PCR is a powerful tool for diagnosis of PJI. Yet, it cannot replace culture. PMA has the potential to improve the performance of PCR in PJI diagnosis and should be investigated on a larger scale. Table 5-1 summarizes the conclusion of each PJI episode presented in this Chapter and whether the viability PCR would have improved the diagnosis or not.

Table 5-1 Summary of PJI episodes.

Case	Conclusion	PMA-qPCR improved diagnosis
15	Sonicate is more sensitive than tissue samples in PJI diagnosis using PCR.	Yes
19	Sonication of retrieved spacer at the second stage is crucial to confirm eradication of infection.	Yes
20	SCVs can be difficult to detect and identify by culture in comparison to the parent strain.	Yes
24	PCR can identify the causative organism in some episodes of culture negatives PJI.	Yes
29	Alpha-defensin is highly sensitive in PJI diagnosis	No
40	PCR, being not affected by prior antibiotics use, is more sensitive in CN-PJI.	Yes
42	PMA can enhance the sensitivity of PCR in PJI diagnosis.	Yes
43	Sonicate is more sensitive than tissue samples in PJI diagnosis using PCR.	Yes
45	This episode was excluded from data analysis as the causative organism was outside the PCR panel.	No
47 (1)	PMA can enhance the sensitivity of PCR in PJI diagnosis.	Yes

47 (2)	PCR is a sensitive diagnostic tool of PJI. Yet, false negative results can still be encountered.	No
49	This is the only episode with negative PCR result of sonicate and positive result of tissue homogenates.	Yes
54	This episode was excluded from data analysis as the causative organism was outside the PCR panel.	No
56	PCR is a sensitive diagnostic tool of PJI. Yet, false negative results can still be encountered.	No
59	Sonicate is more sensitive than tissue samples in PJI diagnosis using PCR.	Yes
60	PMA can enhance the sensitivity of PCR in PJI diagnosis.	Yes

Chapter 6. Discussion

The results of this study are discussed in this chapter in light of the aims and objectives stated in the Introduction Chapter and in the context of the literature. Potential clinical applications of this study are also suggested. Study limitations are described along with recommended techniques to overcome these limitations in the future studies.

6.1 Statement of Main Findings

In this study, the ESR and CRP levels varied significantly between the PJI and aseptic groups (See 4.3). In addition, the calculated cutoff levels were very close to the values published in literature.

The incidence of culture-negative PJI in our cohort was 37.5% (See 4.2.2). Our culture methods were 95.7% specific. Sonication fluid outperformed periprosthetic tissue samples significantly in both conventional culture and molecular techniques.

The conventional PCR had a sensitivity and specificity of just above 70% which increased upon pre-treatment of the samples with PMA to 79% and 89% respectively (See Table 4-28). Thus, PMA was able to significantly enhance the accuracy of PCR in this small-scale study. The agreement between the MSIS criteria and the viability PCR was higher than their agreement with the conventional PCR.

The unexpected finding of increased PCR sensitivity with PMA treatment was interesting. This increase was shown to be genuine in laboratory-generated simulated infected tissue samples with *E.faecalis*. PCR tested positive in PMA-treated samples of tissue homogenate spiked with 10^1 cfu/mL concentration, while untreated samples were negative. Furthermore, the end reaction product was consistently higher for PMA-treated samples for the whole range of concentrations (p=0.01). See 4.5.7 for details.

6.2 Number of Samples per Episode

In this study, average samples per episode was higher in the infected group as surgeons were willing to harvest more samples when they were highly

suspicious of infection in order not to miss a relevant sample for the diagnosis. In aseptic cases, where surgeons were expecting some mechanical causes for failure, they preferred to send the explanted prosthesis to the London Implant Retrieval Centre (LIRC) for mechanical investigation and that is why fewer prostheses were collected from this category.

6.3 Discussion of the Results in the Context of the Literature

6.3.1 **Biomarkers (ESR and CRP)**

ESR and CRP are ubiquitously used to diagnose and follow-up PJI with different cutoff values. In our study, we tested different cutoff values for each of them. For the ESR, the highest AUC was achieved with 28 mm/hour, very close to the MSIS recommendation for chronic PJI diagnosis and recommendations by Berbari et al. (2010) which is 30 mm/hour.

Similarly, for the CRP, the highest AUC was achieved with 13 mg/L. This is also in close proximity to the recommended cutoff by the MSIS, Bernard et al. (2004) and Miyamae et al. (2013) which is 10 mg/L. In addition, the preoperative and postoperative CRP values in the PJI group varied significantly ($p=0.016$). However, its role to predict the prognosis and confirm the infection eradication is unclear and debatable in the literature (Kusuma et al., 2011, Schinsky et al., 2008, Larsson et al., 1992).

Currently, biomarkers play a pivotal role in the timing of reimplantation in the two-stage protocol for PJI treatment due to lack of a better alternative. Along with the clinical improvement, their sequential decline is used as a proxy for

treatment success. However, their use is hindered by their inherent low specificity and occasional lack of normalization even with eradicated infections (Ghanem et al., 2009b, Shukla et al., 2010). This highlights the need for a more specific alternative to judge the success of treatment and determine the ideal time for reimplantation (Parvizi et al., 2013).

6.3.2 **Discussion of Culture Results**

6.3.2.1 Low Sensitivity is a Major Limitation of the Culture

Sensitivity of culture-dependent diagnosis of PJI is known to be limited. This observation was also noted in our study. The incidence of culture-negative PJI in our cohort was 37.5% (See page 118) highlighting the need for a more sensitive diagnostic tool. In fact, this limited sensitivity has long been the trigger to investigate other diagnostic measures such as PCR. The prevalence of culture-negative PJI in the literature ranges from 7% (Berbari et al., 2007) to more than 40% (Bejon et al., 2010, Kim et al., 2015). Our results came at the higher end of that range. We had this relatively high percentage despite our endeavour to optimise tissue processing. In addition, it is a routine practice in our institution to have an antibiotic-free period of at least two weeks before revision surgery. However, Malekzadeh et al. (2010) found that the use of antibiotics within three months prior to diagnosis is a risk factor for CN-PJI. Nevertheless, most of the cases managed in our institute are referred cases from other centres after failure of previous trials of treatment and exposure to different antimicrobials and this could explain, at least in part, the high incidence of CN-PJI in this cohort. In addition, the sensitivity of culture methods in our research was higher than the clinical

laboratory due to optimisation of tissue processing and performing sonication of implants.

6.3.2.2 Sonicates versus Tissue Homogenates

Sonication fluid has been repeatedly reported to have a higher sensitivity than periprosthetic tissues in PJI diagnosis. Our results support these observations. The threshold of a positive sonication fluid culture recommended by Trampuz et al. (2007) is 10^1 cfu/mL. Upon applying this cutoff in our cohort, we had a sensitivity and specificity of 56.3% and 91.3% respectively.

Culture of the sonication fluid of the cement spacers has been tried to predict the prognosis of treatment of PJI. This approach can guide the postoperative antimicrobial prescription when culture-positive. However, it is time-consuming and cannot aid the decision of proper timing of reimplantation. Alternatively, PCR of the spacers' sonicates might provide a more convenient and speedy tool to help the surgeon to decide either to repeat the first stage or to proceed to the second stage. In our cohort, we collected only four cement spacers and/or primary knee implants used as spacers (from Cases 19, 56, 59, and 60). Thus, we were unable to compare the performance of spacer sonication to others' results. In our institution, loosely cemented implants of primary knee replacement have been used an alternative to cement spacers. The use of this technique led to adequate functional outcomes and reduced the number of patients proceeding to the second stage revision (Marson et al., 2017). Hence, the low number of spacers collected in this study.

6.3.3 **Discussion of Molecular Results**

Heterogeneity of studies of molecular methods in PJI diagnosis made their evaluation and comparison very difficult. Studies used different PJI definitions as the gold standard. They were also different in the type of the sample used (tissues, sonication fluid, joint aspirate, or swab), the prior processing of samples, the number of samples processed, the number of subjects recruited, the type of PCR used, the definition of a positive PCR result, and the protocol design for bacterial DNA extraction, purification, and amplification.

6.3.3.1 Different PJI definitions

To start with, the lack of a comprehensive definition of PJI led different authors to consider different definitions. In our study, we used the MSIS criteria (Parvizi et al., 2013), being the most comprehensive available definition. It is noteworthy that the experts who postulated these criteria acknowledged the possibility of presence of infection even if the proposed criteria have not been fully met. One drawback of these criteria is that they do not consider the implant sonication despite its recognised usefulness. It is crucial to admit the possibility of the gold standard being wrong in some instances and the newly developed diagnostic tool could be outperforming this gold standard, otherwise, the argument about the low specificity of the new test would be unjustified. In which case, the experts of the field including microbiologists and orthopaedic surgeons should use their clinical judgement to modify the gold standard when deemed necessary (Knottnerus et al., 2002).

6.3.3.2 Type of PCR

Broad-range PCR (BR-PCR) has been used in the diagnosis of PJI more frequently than specific PCR. However, we chose to use the specific PCR in our study to avoid many inherent limitations of the BR-PCR. Among these limitations are, being more prone to contamination due to presence of traces of bacterial DNA in the PCR reagents or their accidental introduction into clinical samples during preparation. Unsurprisingly, adding external fluid as in sonication would increase the risk of contamination (Hartley and Harris, 2014). Techniques used to remove the bacterial DNA remnants in PCR reagents (e.g. UV irradiation) were associated with dramatic decrease in the PCR sensitivity (Harris and Hartley, 2003). Secondly, BR-PCR per se is a truncated procedure. To be meaningful and interpretable, it has to be followed by a sequencing step to ensure the clinical significance of the amplified bacterial DNA as done in many previous studies (Marin et al., 2012, Fihman et al., 2007, Gomez et al., 2012, De Man et al., 2009). Sequencing would make the diagnosis more time-consuming and costly. Last but not least, is the difficulty in diagnosis of mixed infection in BR-PCR due to generation of uninterpretable data upon sequencing. In a prospective multicentre study by Bemer et al. (2014), approximately one-quarter of polymicrobial PJI cases (as identified by culture) tested negative on BR-PCR and two-thirds of them yielded one organism only. Furthermore, if this obstacle was mitigated by more sophisticated sequence analyses, the problem of identifying large numbers of commensals and unrecognized pathogens would be encountered (Hartley and Harris, 2014).

For the previous reasons, a specific PCR was used in this study. Using this type of PCR provides more control of contamination. Additionally, to avoid the need of using multiple species-specific PCR, we used genus-specific primers to identify the commonest PJI causative organisms (staphylococci and enterococci).

6.3.3.3 What Defines a Positive PCR Result?

Data in the literature about the definition of a positive PCR result is very scarce. Only two studies could be identified to interpret their PCR results according to the number of positive samples per patient. Marin et al. (2012) obtained the best combination of their results when only one positive sample was considered significant. Identification of the same species from at least two samples reduced the sensitivity of their assay significantly. On the other hand, Rak et al. (2015) recommended the identification of the same species from at least two samples to be the cutoff for a positive PCR sample. However, both of these studies used BR-PCR. Each of them used a different definition of PJI and processed samples differently. In addition, neither of them used sonication fluid. All these variations made the application of either recommendation to our study unjustified. Nevertheless, to mimic the definition used in culture methods, we defined a positive PCR result as two or more positive tissue samples and/or positive sonicate in the same assay. This approach has been previously used by Bemer et al. (2014). Obviously, this is an area of weakness which needs to be addressed in future studies.

6.3.3.4 PCR of Periprosthetic Tissues

Few studies using periprosthetic tissues for PCR were identified. Our results showed that periprosthetic tissues have poor sensitivity (15.4% for

conventional PCR and 23% for viability PCR) as previously found by Ryu et al. (2014), Suda et al. (2017), and Huang et al. (2018). They reported sensitivities of 16%, 30.8%, and 34% respectively. Unlike our study, Ryu et al. (2014) used their own criteria rather than the MSIS definition of PJI which they admitted as a limitation of their study that probably resulted in misclassification. In addition, they used a wider PCR panel than ours with no improvement in the sensitivity of tissue PCR. Furthermore, the use of a much wider commercially available multiplex PCR panel known as Unyvero i60 implant and tissue infection (ITI) multiplex PCR system (Curetis, Holzgerlingen, Germany) by Suda et al. (2017) did not improve this poor sensitivity. Similarly, Huang et al. (2018) reported low sensitivity of tissue PCR and recommended against its use despite the use of BR-PCR. In a prospective study by Rak et al. (2013), both broad range and species-specific PCR of tissues were inferior to culture. These studies jointly suggest that this low sensitivity is inherently related to the tissues rather than the type of PCR used. In our results, as mentioned in the previous Chapter of PJI Case Reports, both specific and BR-PCR of tissue samples from Case number 29 were negative.

6.3.3.5 Causes of the low sensitivity of periprosthetic tissue PCR

Different factors could contribute to the poor sensitivity of tissue PCR. These factors could be classified into two main categories; those that can adversely affect the use of tissues in the diagnosis of PJI regardless the nature of the diagnostic test and other factors influencing the PCR in particular. In the first group, regarding the distribution of bacteria; as the pathogenesis of PJI is known to be linked to development of biofilms on the surface of implants,

bacteria might exist in tissues only in low numbers. In addition, the unequal distribution of bacteria in tissues makes this technique liable to sampling errors. However, the higher sensitivity of the tissue culture than the tissue PCR, as in our study and other studies, make these explanations unsatisfactory to justify the low sensitivity of tissue PCR. In the second group, the relatively small volume of samples used in PCR makes it more liable to sampling errors. Relatively difficult extraction of bacterial DNA because of the high viscosity of homogenate is another reason. Using larger volumes or higher concentrations may enhance the tissue PCR sensitivity. On the other hand, tissue samples as such would be overwhelmed with human genome and loaded with PCR inhibitors. Potential abundance of PCR inhibitors in tissues also contributes significantly in this poor sensitivity. There is a need for techniques for purification of bacterial DNA and removal of human DNA for better results.

In this study, we used an “all or none” method to test for the PCR inhibition. In this method, any amplification of the GADPH gene was accepted as an indicator of lack of inhibition. We chose this external control (i.e. done in a separate aliquot) to avoid the potential interference of the target gene amplification by the co-amplification of the internal control (Hofmann, 2003). However, the nature of PCR inhibition as being a relative rather than an absolute process (King et al., 2009) would make this technique less reliable. Spiking of samples with known amounts of external DNA and subsequent measurement of Ct shifting relative to a control reaction would be a more reliable but more technically demanding method which is mostly used in absence of an endogenetic control (Schrader et al., 2012).

On the contrary, Moojen et al. (2007) reported higher sensitivity of tissue PCR than culture. They used a combined BR-PCR and reverse blot line hybridization to identify different bacterial species. One possible explanation for this high sensitivity is the reference used to diagnose infection which relied solely on clinical diagnosis. The value of PCR in such clinically evident PJI cases is questionable. Furthermore, the use of different tissue samples for culture and PCR in their study, makes the comparison unreliable. Likewise, Miyamae et al. (2013) reported a 90% sensitivity of tissue BR-PCR. Interestingly, this was combined with 45% specificity which could be ascribed to the tissue processing technique. They sonicated tissues in bags, a method which is more liable to contamination than tubes (Trampuz et al., 2006). In addition, our comparison of different tissue processing methods showed that sonication was significantly inferior to homogenization in bacterial recovery from tissues (refer to Method Development Chapter).

6.3.3.6 PCR of Sonication Fluid

With better understanding of the pathogenesis of PJI and biofilm development, retrieved prostheses have been appreciated as very valuable material for diagnosis of PJI. Different methods were tested for biofilm removal from the surfaces of the implants. Sonication has proved to be reliable, effective, and reproducible (Trampuz et al., 2007, Portillo et al., 2013). Sonication fluids of implants have been used in both conventional and molecular microbiological methods. Sonicates were used more frequently than periprosthetic tissues as PCR material for PJI diagnosis and have been reported to be more sensitive (Bjerkan et al., 2012, Ryu et al., 2014, Rak et al., 2016). Our results are in agreement with these studies as the sensitivity

of sonicate PCR was 64.3% and 71.4% for the conventional and viability PCR respectively in comparison to 15.4% and 23% for the periprosthetic tissues.

Sensitivity of sonication fluid PCR in the literature varied widely from a 34.2% (Villa et al., 2017) to 95% (Portillo et al., 2012) with most of the studies on the higher side relative to periprosthetic tissues.

In our study, implants were sonicated in properly locked containers, rather than bags, to reduce the risk of contamination (Trampuz et al., 2006). In addition, careful cleaning of the water bath was done regularly. Furthermore, in case of isolation of water-borne bacteria, culture of samples from the water bath was done to investigate on the probability of contamination.

Villa et al. (2017) treated the retrieved implants with dithiothreitol (DTT) and reported low sensitivity of the PCR of DDT elute which they ascribed to failure to detect *S.aureus*, one of the most common PJI causative organisms. Suda et al. (2013) used surgical knives to scratch the surface of the implants and performed PCR on the scratch material with good results. Given the availability, safety, and relatively affordable price of sonicators, there is no current need for an alternative unless proved to have a higher performance.

6.3.3.7 Positive Results in Presumed Aseptic Cases

The interest of employing the PCR in diagnosis PJI arises from the high incidence of culture false negative results. This is particularly important when considering a patient in the interim period of a two-stage revision. A false negative result, e.g. of joint aspirate, would give spurious evidence of successful eradication of infection leading to inappropriately proceeding to

reimplantation with potentially disastrous sequelae. As well as false negative results, false positive results can also have a very deleterious impact on the patient management. Unnecessarily, such a patient would be exposed to a lengthy and costly treatment. Hence, the importance of achieving the highest possible accuracy of a diagnostic tool in PJI.

Obtaining positive microbiological results (either in culture or PCR) in apparently aseptic cases is a real challenge. It has been described in multiple previous studies not only in prosthetic joints but also in a wider range of implants such as pacemakers (Mason et al., 2011, Rohacek et al., 2010) and breast implants (Pajkos et al., 2003, Rieger et al., 2009).

In this study, we tried to address two major causes of false positive results; contaminants and dead bacterial DNA detection by PCR. For the former, we used specific rather than BR-PCR as described earlier. The core of this study was geared towards restricting the PCR detectability to the DNA of viable bacteria. In this study, thirteen episodes tested positive with conventional PCR. Eight of them became negative with viability PCR leading to a higher specificity. Although this is a small-scale study and these findings cannot be generalized until applied to a larger cohort of patients, it provides a promising tool to avoid the inherently low specificity of PCR while not affecting its sensitivity. On the contrary, the sensitivity has also been unexpectedly increased as discussed later in this Chapter.

6.4 A continuum of Bacterial Existence; Wild, SCVs, VBNC, and Dead Bacteria

The use of the terms “alive” and “dead” has proved very confusing and lacking a standard definition. This could be ascribed to the lack of specific

criteria that are common to all those regarded as “living”. Hence, trials to define live objects usually use a combination of attributes, some of which might apply to non-living objects and none of them would be shared by all living objects (Pirie, 1937). Nevertheless, the question about life and death of bacterial cells has long been interesting and of major clinical importance because of the implication of its answer on the patients’ treatment. A pragmatic definition of living bacteria that is usually adopted by microbiologists is the capability of multiplication (Bogosian and Bourneuf, 2001). This is usually evidenced by producing colonies on culture plates or causing broth turbidity (Sheridan et al., 1998). A major drawback of this approach is the inability to accommodate viable but non-culturable (VBNC) bacteria (Roszak and Colwell, 1987, Ramamurthy et al., 2014).

Furthermore, death of bacteria is a process rather than an event. In this “scale of life”, the wild phenotype could be placed on the top, followed by SCVs, then by VBNC bacteria, and the dead bacteria positioned at the bottom. Currently, there is no approved relationship between the SCVs and the VBNC bacteria. However, both are known to be survival strategies adopted by bacteria when exposed to starvation or other environmental stresses (Li et al., 2014b, Kim et al., 2016).

Viability PCR, as described in this study, has the ability to detect the slowly-growing SCVs and the non-growing VBNC bacteria as it is culture-independent. Furthermore, this technique will not amplify the DNA of dead harmless bacteria, thus, avoiding both the false negative results of culture techniques and false positive results of conventional PCR.

6.5 PMA for Live/Dead Discrimination and its Effect on the PCR Performance in the Diagnosis of PJI

Persistence of dead bacterial DNA up to several months after clinical eradication of infection has been previously reported in PJI (Bemer et al., 2014), septic arthritis (van der Heijden et al., 1999, Canvin et al., 1997), and infective endocarditis (Branger et al., 2003). Amplification of these DNA remnants by the PCR can lead to false positive results in cured PJI cases and thus compromise its specificity.

A major goal of this work was to determine the effect of prior PMA treatment of clinical samples on the performance of PCR in the diagnosis of PJI. Our results showed an increased specificity of PCR with PMA treatment. Viability PCR was better at matching with the MSIS definition of PJI than conventional PCR.

6.5.1 Comparison between PMA and RNA-based PCR for Live/Dead Discrimination

To the best of our knowledge, this is the first clinical study investigating this viability PCR in the diagnosis of PJI. However, some other studies tried to overcome the problem of false positive PCR results caused by dead bacterial DNA (Lee et al., 2013, Fang et al., 2018, Ince et al., 2004). All these other studies tested the detection of different types of ribonucleic acid (RNA) as a marker of viability. Birmingham et al. (2008) performed real-time quantitative reverse transcription PCR on twelve simulated infected joint fluid samples to quantify the bacterial messenger RNA (mRNA). Their results were promising as they found a decrease in the bacterial mRNA concentration after treatment with antibiotics in a timely fashion. However, another study coming

out of the same laboratory admitted the limitations of using mRNA in the first study as a viability marker because of its compromised sensitivity due to low concentration and high rate of degradation, and lack of universal sequence (Bergin et al., 2010). Alternatively, in this later study, they targeted the ribosomal RNA (rRNA) which constitutes more than 95% of the total RNA. Furthermore, Kobayashi et al. (2009) described the use of mRNA as “tedious” as several DNase treatments were necessary to eliminate the DNA contamination.

It is not clear which of mRNA or rRNA, if either, is more suitable for use as a bacterial viability marker. Different studies have found that the rRNA can still be detected from dead bacteria after treatment with antibiotics (Sheridan et al., 1998, van der Heijden et al., 1999). Similarly, some authors reported the persistence of mRNA in dead cells for an extended duration (Birch et al., 2001, Sung et al., 2005). These observations, if proved true, would significantly undermine their use for that purpose. Nevertheless, mRNA might be a less favourable target as it constitutes less than 1% of the total RNA and its rate of degradation after bacterial death is higher than rRNA, and both would compromise the sensitivity of its detection and usability as a viability marker (Bergin et al., 2010). In addition, there are some common drawbacks for using either as a viability marker. Firstly, RNA extraction is more complicated and time-consuming than DNA extraction which would make it less favourable in a situation of intraoperative assessment of infection persistence. Secondly, the level of these products is dependent on the metabolic activity of bacteria. Variation in their levels would primarily reflect the change in the metabolic activity rather than the live/dead state. In

SCV and VBNC state, the metabolic activity is expected to be suppressed while the bacteria are still alive. Unlike rRNA and mRNA, tRNA has not been tested in the diagnosis of PJI and data about its persistence after cell death is very scarce. However, a study by Davis et al. (1986) found that it survived even longer than rRNA. Hence, it is very unlikely to perform well as a viability indicator.

Given the aforementioned limitations, PMA appears to outperform all RNA types in investigating bacterial viability. In this study, the use of PMA converted the positive PCR results of eight aseptic episodes into negative, thus, increasing the specificity of PCR from 71.7% to 89.1%. Interestingly, five out of these episodes had a suspicion of infection at some point of their clinical history (i.e. prolonged wound discharge or unexplained pain). This kind of presentation without proof of infection is usually referred to as “subclinical” infection. In which case, it is quite challenging and crucial to decide how such a patient should be treated. Limiting the positive PCR results to viable bacteria can help to avoid unnecessary prolonged and costly treatment. On the other hand, persistence of the positive signal in PCR after PMA treatment, as in five aseptic episodes in our cohort, could identify these episodes as “at risk”. In which case, health carers should be alert for the possibility of probable or impending infection and prompted to closer and more frequent follow-up.

6.5.2 **Enhanced Sensitivity of PCR with the Use of PMA**

Unexpectedly, the sensitivity of the viability PCR was found to be higher than the conventional PCR (78.57% vs 71.43%). Ten samples tested positive with viability PCR and negative with conventional PCR. At first sight, this might be

explained by technical error (e.g. incorrect labelling). However, the same results were obtained on repeating the experiments. So, we hypothesized that PMA can increase the sensitivity of PCR and lower its detection limit. PMA does not bind to prokaryotic DNA selectively. Rather, it can interact with DNA from any source such as damaged eukaryotic cells (Taylor et al., 2014). As such, PMA could interact with the abundant human DNA in clinical samples preventing or limiting the generation and accumulation of PCR inhibitors. This could explain the observed increased sensitivity. To prove or refute this hypothesis, an experiment was designed to study the effect of PMA on PCR in simulated clinical samples in a trial to explain these unexpected results. Interestingly, the 10^1 cfu/mL concentration was detected only by viability PCR in our simulated infected clinical samples. These results were explained by a possibility of the PMA inhibition of generation and/or action of PCR inhibitors. One more finding supporting this hypothesis is the end reaction product in the previous experiment which was consistently higher for PMA-treated samples for the whole range of bacterial concentrations ($p=0.01$). Lower end reaction product (i.e. the reaction came to a plateau phase earlier) could be caused by either earlier consumption of reagents or accumulation of inhibitors. Given the equal initial DNA templates in our experiment, variation in PCR inhibitor generation and accumulation would be a more logical explanation.

6.5.3 Recommendations for Interpretation of Viability PCR Results

To get the maximum potential benefit of this modified PCR technique and provide a full picture of the PJI status, we recommend the use of both

conventional and viability PCR. Our suggestion for the interpretation of results has four possibilities; first and second, both are negative or positive, which are straightforward cases for aseptic and infected conditions respectively. Third, positive conventional and negative viability PCR should be interpreted as either cured or remote episode of infection. Lastly, positive conventional and negative viability PCR should be cautiously scrutinized. In this last case, if the bacterium quantified by viability PCR is of low number, it could be ascribed to true existence of bacteria in the clinical sample and interpreted in the light of clinical scenario. However, if this quantity is high, this should raise the suspicion of faulty technique or contamination. The cutoff between this high and low level needs further studies to be determined. Finally, it should be emphasized that PJI diagnosis cannot be dependent on a single test alone and it should rather be discussed in a multidisciplinary strategy in the context of the clinical presentation.

6.6 Potential Clinical Applications of the Study

The clinical significance of this study is quite interesting. This technique could be used for the follow-up of PJI cases during antibiotic therapy and witnessing the decline in viable bacterial load or the development of resistance and the need to change the antimicrobials. Thus, it could be used to monitor a patient after the first stage and help to determine the optimum duration of a course of antibiotics before the new joint is inserted in a second stage revision.

A clinical trial using this technique could also be designed. In this trial, different groups of patients who are treated with antimicrobials for different durations could be recruited. Samples would be collected from each group at

different time intervals to decide the optimum length of antibiotic course in between the first and second stage revision. This interim duration is currently 6 weeks at least (Bernard et al., 2010), but this is arbitrarily derived and there are tentative moves to shorten the course (Stockley et al., 2008). The viability PCR would become a valuable tool in this venture, potentially providing evidential support for shorter courses, with less patient exposure to antibiotics, fewer adverse effects, and reduction of antibiotic resistance. The patient would recover mobility earlier and overall outcomes would improve.

Another potential clinical impact of this study is assessing the safety of prosthetic joint replacement in prior septic arthritis patients. Different diagnostic tests have been tried to detect the persistence of active infection such as biomarkers (Li et al., 2007), joint aspiration, and multiple intra-operative samples for culture (Parvizi et al., 2013). However, none of the aforementioned tools has proved satisfactory. Viability PCR should provide an accurate measure for the success of infection eradication prior to the replacement.

Additionally, this research will satisfy an unmet need for a more accurate method for earlier and faster detection of infection which can remove the uncertainties surrounding some of the difficult-to-diagnose cases such as culture-negative PJI. It will also shed the light on some of the causes of infection recurrence. As such, it can lead to modification of the international guidelines for definition and diagnosis of PJI.

We are aiming to use this study, along with other similar ongoing studies in the Biomaterial Related Infection Laboratory in University of Nottingham, to

persuade the NHS to start applying implant sonication in clinical laboratories and to be included in National Institute for Health and Care Excellence (NICE) guidelines. We can provide robust evidence that the cost would be greatly outweighed by the success of infection treatment benefits.

6.7 Study Caveats

In our institute, most of the parameters used to satisfy the minor MSIS criteria are not routinely performed which might have led to missing some PJI cases. Neither microscopic examination and leucocyte count of the synovial fluid nor the histopathologic tissue examination is ordered except in very limited situations. In a trial to overcome this obstacle, agreement in EMSON was accepted as an alternative. It is recommended that different studies use the same definition to have a common platform to allow for results comparison.

Additionally, this is a single centre study with a relatively small number of participants making it liable to bias. Small sample size resulted in wide confidence intervals of the calculated sensitivities and specificities (du Prel et al., 2009). For that particular reason, we are planning to launch a larger multicentre study. This pilot study will be used to calculate the sample size needed to generate more statistically significant results.

In addition, no synovial fluid samples were collected as surgeons were keen to send all the aspirates to the clinical laboratory. Thus, we were unable to investigate the performance of our viability PCR in these samples.

Another limitation is the narrow PCR panel that might have negatively affect its sensitivity. However, the primary focus of this research was to evaluate

the effect of PMA on the performance of PCR in the diagnosis of PJI rather than testing the PCR per se. However, broadening this panel in a future study should be easily achievable.

Another technical limitation of this study is the qualitative “all or none” testing of PCR inhibition as it does not provide enough data about the percentage of PCR inhibition. A more informative test should be in place to enable more precise judgement of DNA extraction and PCR performance. Although technically demanding, spiking clinical samples with known amounts of exogenous DNA is worth a trial of optimisation.

6.8 Study Strengths

To the best of our knowledge, this is the first study to apply the use of PMA in clinical samples to enhance the specificity of PCR in PJI diagnosis. Nevertheless, it has been applied in environmental and food samples. Clinical samples are notorious for their complexity. Method optimisation for clinical samples is challenging, costly, and time-consuming being dependent on a trial and error approach. In spite of the several limitations mentioned above, our results were promising and encouraging to launch a larger scale multicentre study.

The comparison of different tissue processing methods done at the early stage of this study is another point of strength. It gives a clear idea on the impact of processing on bacterial viability and provides a solid platform to start any further investigation on the tissue products. Homogenization has proved to have the highest retention of bacterial viability. However, a swifter

method, when available, would enhance the release of bacteria and the sensitivity of every subsequent step in the workflow.

6.9 Summary

The diagnosis of PJI is challenging. There is no standalone test that can solve this dilemma. PCR is a useful complement to conventional microbiological methods that allows a faster diagnosis. Yet, it cannot replace culture methods.

PMA has the potential to enhance the performance of PCR in PJI diagnosis by increasing not only its specificity but also its sensitivity. The merit of the use of PMA is its ability to discriminate between live and dead bacteria. Thus, eliminating the risk of false positive results of the PCR However, a larger multicentre study is needed before recommending its clinical use.

Sonication fluid has higher sensitivity than periprosthetic tissue in both culture and PCR. However, efforts should be exerted to increase the sensitivity of tissue as the implant might not be available in every case to be sonicated.

6.10 Future Work

This current study helps broaden our understanding of the different phases of PJI pathogenesis; initiation, propagation, and persistence. It contributes significantly to the current literature regarding the optimisation of PCR for a rapid and accurate diagnosis of PJI. Our results are promising and show a potential to move this diagnostic tool one step ahead.

The next stage in this project is the launch of a multicentre study to enhance the statistical power of our findings and to conclude a clear definition of

positive PCR result per sample and per episode. In this intended larger project, a number of modifications should be considered to avoid some of the limitations encountered in this study. Firstly, a wider panel including primers for most of the known PJI pathogens should replace the current limited assay. For that purpose, the Unyvero i60 implant and tissue infection (ITI) multiplex PCR system (Curetis) could be a good starting point that could be modified as needed. Secondly, a quantitative assessment of PCR inhibition should be introduced. Different strategies for mitigating or abolishing the PCR inhibition could be developed and tested. In addition, the very valuable and easily accessible samples of joint aspirates would be collected. Furthermore, the recruited patients, in particular those with apparent false negative and false positive results, will be followed up for a period of 3-5 years to correlate their outcome with our results. Last but not least, we would consider obtaining some samples from patients while receiving antibiotic therapy at different points down the line of treatment, albeit being subject to ethical and institutional approval. This strategy might help to determine the optimum duration of antimicrobials.

For this multicentre study, we are intending to approach some large centres in the UK with significant flow of PJI cases such as Oxford Bone Infection Unit, Sheffield Hallam University Hospitals, and University College London Hospitals. A quality control assay would be set up to continuously assess, monitor, and validate the results obtained from different laboratories.

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Appendices

Appendix 1: Application form for the NHSB



APPLICATION FORM FOR ACCESS TO NHSB SAMPLES

Biobank Reference ID ACP0000172 Molecular Diagnosis of Orthopaedic Implant Infections

Date application approved 23rd January 2016

Principal Investigator (PI)

Name and Title Roger Bayston, Professor of Surgical Infection

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Post Code NG7 2UH

Email roger.bayston@nottingham.ac.uk

Phone/Fax 0115 82 31115

Contact Person (if different from above)

Name and Title

Address

Post Code

Email

Phone/Fax
R&D approval Number
Grant Code
Invoice details
Name
Address
E-mail

Research Project details

1. Title of the project: Molecular Diagnosis of Orthopaedic Implant Infections

2. Introduction: Approximately 180,000 joint replacements are performed in UK each year. The two most serious complications are infection and loosening of the implants. A preferred form of treatment for infection is to remove the implant, give antibiotics and then replace with a new implant once the infection has resolved. However, it is difficult to be sure that there are no living bacteria left, which might cause a second infection. We now know that infection may play a role in “aseptic” loosening too. The bacteria that cause the infections are again difficult to find on conventional investigation.

A method for detecting bacteria based on bacterial genetic material (DNA) replication (PCR) can be used. However, this is so sensitive that it can detect even DNA from contaminants or dead bacteria, leading to incorrect diagnosis (DNA from dead bacteria can be left over at the end of successful treatment).

This study will evaluate and develop a new PCR technique that overcomes this problem. The technique distinguishes between contaminants and infection, and between live and dead bacteria. A modification prevents DNA from dead bacteria from taking part in the PCR.

We have evaluated the technique in the laboratory and shown that it works. We now wish to apply the technique to clinical samples from infected hip, knee and shoulder joint replacements and from spine instrumentation, to determine its usefulness in patients at risk.

3. Aims: The study will compare a modified method for detection of bacterial DNA with a conventional method involving culture techniques:

- *Can the modified molecular technique detect bacteria where culture methods fail to do so?*
- *Does the molecular method over - diagnose infection compared to the culture method?*
- *Does it distinguish between live bacteria surviving prior antibiotic treatment, and dead ones killed by the treatment but whose DNA*

is still present?

4. Specimens requested

Approximately 60 samples are expected in the year available, and consultants in orthopaedics and spinal surgery have agreed to contribute material.

- **Non-infected Group**

A series of control patients undergoing primary arthroplasty, where bone and surplus tissue are removed as part of the procedure, will provide uninfected tissue.

- **Infected Group**

Surplus tissue obtained from adult patients with suspected arthroplasty infection or aseptic loosening, spinal instrumentation infection, or undergoing two-stage revision surgery for infection.

Surplus Tissue samples

Surplus tissue samples (any membranes formed between the implant and bone, pus etc) taken from patients with suspected arthroplasty infection or aseptic (non-infected) loosening, spinal instrumentation infection, or undergoing two-stage revision surgery, will be taken aseptically and transported to the Nottingham Health Science Biobank (NHSB). Clinical staff will contribute relevant clinical data that will be subsequently anonymized and assigned the same unique study number as the tissue.

Appendix 2: NHSB Patient's Information Sheet

Nottingham University Hospitals NHS
NHS Trust

nottingham health science biobank

Donation of surplus tissues for research purpose

The Nottingham University Hospitals NHS Trust has established a Biorepository, which stores human tissue samples for medical research. We would like to invite you to consider donating surplus tissues, which would otherwise be disposed of, and also providing a blood, urine or other required samples to the Nottingham Health Science Biobank. Before you make a decision please read the following information carefully and discuss it with your family, friends or other people that you think appropriate, if you wish.

If you have any questions at a later date please contact Nottingham Health Science Biobank on 0115 9691169 Ext. 56507 or 56517

This document can be provided in different languages and formats. For more information please contact:
The Nottingham Health Science Biobank
City Hospital campus
Tel: 0115 9691169 Ext. 56507 or 56517

Nottingham University Hospitals NHS
NHS Trust

What is Nottingham Health Science Biobank?
The Nottingham Health Science Biobank (NHSB) is based within the laboratories at the Nottingham University Hospitals and is a store for tissue and body fluids which have been consented for research. It is run by the Nottingham University Hospital NHS Trust and has broad ethical approval to make tissue available for certain types of research projects. The aim is to act as a resource of high quality samples for use in medical research. It is anticipated that tissues will be made available for research into a wide number of diseases, thereby helping to improve patient care and treatments in the future.

What will happen if I agree?

- When a patient undergoes surgery or a clinical procedure which involves the removal of tissue or body fluids, samples are usually sent to the pathology laboratory in the hospital to help with your diagnosis. In many cases there will be a surplus of the sample that is not required for diagnosis and this would normally be disposed of. These samples would be transferred to the NHSB, where they will be stored in a facility that is licensed by the Human Tissue Authority, in a way that maintains the quality and integrity of the tissues. The tissues removed during the course of your treatment will be used only for ethically approved research projects in the future, including genetic studies and medical research which may use animals.

- Part of the research may involve extracts of donated tissue being grown in a laboratory and used to develop new tests or treatments. These tissues may be used to form experimental cell lines which are a very effective means of testing new therapies. Your tissue will not be used for research that involves reproductive cloning.

- If you have had previous surgery, the pathology laboratory may also have kept some of your samples as part of your records. Some of these tissues can still be very useful for researchers. With your permission, we would like to be able to access these samples on behalf of researchers. The pathology department will ensure that there are always sufficient samples remaining for the primary storage purpose, which is for your records.

- In addition to using surplus samples, we request permission to take a small amount of extra blood as part of the routine samples taken for your care. We would also like permission to ask you to donate other body fluids such as urine which may not be taken as part of your care. These samples will be used for the purposes of medical research.

- When your sample arrives at the NHSB it will be given a unique code linked to your NHS or hospital number. A link to your identity will be retained within the hospital for audit purposes and to prove that the necessary consent is in place.

- Information about your condition, other disease(s) and your past and current treatment and follow up information are scientifically useful to researchers. The member of staff who is taking the consent will ask questions about your medical history during the interview and will ask permission to view your hospital records. Sometimes it may also be helpful to contact your GP for further information related to your condition and you will be asked to agree to this. There will be no further direct contact with you from the NHSB or any researchers using your tissues.

- Your samples and the associated clinical information will be used mainly by local researchers but may also be made available to researchers outside of Nottingham, in the UK or overseas. The researchers may work in universities, hospitals or private/commercial companies that undertake medical research.

Do I have to say yes?
No it is entirely up to you whether you agree to allow surplus and other samples to be used in this way.

Is there any research that my samples will not be used for?
With your consent the NHSB Access Committee, comprising NHSB senior staff, professional researchers and service providers as well as lay persons, will look at applications for access to your tissue according to HTA regulations and the NHSB Tissue Access Policy. As long as the project fits within the ethical approval already granted to the biorepository then your tissue will be made available. However, the ethical approval of the Biobank specifically forbids us to make any tissue available for reproductive cloning, research into termination of pregnancy or non-clinical research such as testing cosmetic products. Your tissue will also not be used for transplantation purposes. When we provide samples to researchers they are obliged to only use the samples for the research they said they would do. They will be bound by a strict agreement to ensure this.

Will information about me be given to people outside of the trust?
All information about you will remain confidential and will be stored in accordance with the UK Data Protection Act 1998. Under the Data Protection Act you are entitled to see what is recorded about you. No information will ever be released to an insurance company. Your tissue is given a unique identifying number and this allows us to collect data relating to your medical records, such as diagnosis, treatment and demographic details which we store within a secure database. We need this information to keep accurate records on the tissue collected, and to help make the most out of the research done on your tissue donation.

Researchers will NOT be provided with any personal information such as your name, address or phone number. The information they will be given for their work will relate to your disease and treatment and will NOT be directly linked to your identity. Access to personal identifiable data will be restricted to approved sources within the trust only. Approved sources are limited to Nottingham University Hospitals NHS Trust staff or those with honorary contracts authorised to work at Nottingham University Hospitals.

Breast Cancer Now Tissue Bank
The NHSB is a founding member of the Breast Cancer Now Tissue Bank (BCNTB). Any tissue you donate may be used by the BCNTB. Should our collaborative arrangement end with the Breast Cancer Now, some of your tissue and data may be transferred to one of the hospitals, charities or universities associated with the BCNTB. If your tissue and information is transferred it will be held in the same way as we currently hold it and can only be used for further medical research.

Do I benefit in any way?
Humanity as a whole stands to benefit in the long term from your donation but it is unlikely that there will be any direct benefit to you. You will not receive any personal financial reward for donating your tissue. Your donation could help forward medical knowledge and expertise, especially in the area of drug development. However, whilst this may ultimately provide a profit for an organisation, it takes many years using many tissue samples and financial investment to get to this stage, so individual donors cannot be rewarded.

Does the NHSB benefit financially?
The NHSB is part of the Nottingham University Hospital Trust, and is therefore non-profit making. We are able to recover the costs from researchers for work carried out on their behalf. The benefit to the Trust is that tissues are made available for research purposes, which helps to improve medical knowledge and ultimately aims to improve patient care.

What are the risks to me?
There is NO increased risk to your health. You will only be allowing us to use tissue samples which would be collected anyway as part of your normal care and there are no more risks of giving blood for research than there are for giving any routine blood sample.

Will I be told the results of individual research tests on my samples?
No. Individual results of research on your samples will not be given to you or your doctors because this research is not part of your routine medical treatment. The overall results of the research projects may be published as research papers in scientific and medical journals.

What happens if I change my mind?
You can change your mind at any time by contacting the NHSB on Tel: 0115 9691169 ext 56507 or 56517. You do not have to give a reason why. If you tell us you have changed your mind, this will not affect your care or treatment in any way now or in the future and all samples in storage will be disposed of according to hospital policy. Similarly, the information we store about you will be deleted so that it cannot be used again. We will also contact any research groups using your tissue and ensure that they destroy any unused samples and associated data. If you change your mind after a long period of time, the samples may already have been used. We cannot recall samples or information from researchers if this is the case.

What else do I need to do now?
If you have received enough information and have decided that you would like to agree, then you will be asked to sign a consent form. If you do not agree, then you need do nothing. No tissue will be stored or used unless you have specifically signed an agreement.

Contact information
If you require any further information or have any questions, please contact the NHSB, Nottingham University Hospitals NHS Trust, David Evans Medical Research Centre, City Campus, Hucknall Road, Nottingham, NG5 1PB. Tel: 0115 9691169 ext 56507 or 56517.

Feedback
We appreciate and encourage feedback. If you need advice or are concerned about any aspect of care or treatment please speak to a member of staff or contact the Patient Advice and Liaison Service (PALS):
Freephone (City Hospital campus): 0800 052 1195
Freephone (QMC campus): 0800 183 0204

From a mobile or abroad: 0115 924 9924 ext 65412 or 62301
Minicom: 0800 183 0204

E-mail: pals@nuh.nhs.uk
Letter: NUH NHS Trust, c/o PALS, Freepost NEA 14614,
Nottingham NG7 1BR
www.nuh.nhs.uk

Appendix 3: Research Description Sheet for Patient's Information

Nottingham University Hospitals NHS
NHS Trust

Donation of surplus tissue for the research of:

"Molecular detection of living bacteria in arthroplasty"

The department of academic orthopaedics in the Queen's Medical centre, University of Nottingham is inviting you to consider donating surplus tissue, **which would otherwise be discarded**, in order to be used for that research aiming at improving the diagnosis of infected orthopaedic cases.

What is this research about?

The most widely accepted method of treatment of a patient with infected orthopaedic implant is the "two-stage" strategy. Firstly, the infected material should be explanted along with surrounding "unclean" tissues. Secondly, after ensuring that the infection has completely subsided, another brand new sterile material will be implanted again (in cases of arthroplasty or if your broken bone not yet united).Otherwise, infection can recur.

The aim of this research is to help surgeons to be quite confident that the infection has been totally controlled before proceeding to the second stage.

What will happen if I agree?

When a patient, who has infected orthopaedic implant, undergoes surgery for the purpose of infection eradication, the infected tissue are taken out and discarded, a process which is called debridement.

If you agree to participate in our research, these tissues will be transferred to our lab to carry out the experimental “molecular” diagnosis.

Do I have to say yes?

No, it is entirely up to you whether you agree for us to collect your surplus tissue to be used for that purpose.

Will information about me be given to people outside the NHS trust?

No, information will never be released outside the trust. It is part of your confidentiality.

Researchers will **not** be provided with any personal information such as your name, address or phone number. The information they will be given for their work will relate to your disease and treatment and will **not** be directly linked to your identity.

Do I benefit in any way?

Humanity as a whole stands to benefit in the long term from your donation but it is unlikely that there will be any direct benefit to you. You will not receive any personal financial reward for donating your tissue.

What are the risks to me?

There is **NO** increased risk to your health.

Will I be told the results of individual research tests on my samples?

NO. The individual results of research on your samples will not be given to you or your doctors as this research is not part of your routine medical treatment.

Nottingham University Hospitals NHS
NHS Trust

What if I change my mind?

All what you need to do is to let your doctors know that you do not want to participate in that research anymore. You do NOT have to give a reason for that.

What else do I need to do now?

If you agree, you will be asked to sign a consent form.

If you do not agree, then you need to do nothing.

Contact information:

You have to contact your surgeon.

Appendix 4: NHSB Consent Form

nottingham health science biobank

Nottingham University Hospitals NHS
NHS Trust

Consent Form

Patient Identification Number for this project:.....

Contact telephone number: 0115 969 1169 ext. 56507 or 56517

Please affix patient label

Patient Name

D.O.B

NHS/ K Number

To confirm agreement with each of the statements below, please initial in each box

1. I confirm that I have read, understood and have had time to consider the information sheet and I have had the opportunity to ask questions. ☐

2. I understand that my donation is voluntary and will not affect my medical treatment or legal rights in any way and that I may withdraw my consent at any time. ☐

3. I consent to the storage and use of any tissues that are removed during the course of my treatment and are not required for diagnostics for approved research projects including medical research which may use animals. ☐

4. I consent to extra samples of blood and body fluids being taken and used as explained to me. ☐

5. I understand that my tissue may be used to create experimental cell lines (tissue that can be specifically grown in a laboratory from your cells) ☐

6. I understand that relevant sections of my medical notes may be looked at now or in the future by personnel from Nottingham Health Science Biobank and researchers contracted through the Nottingham University Hospitals NHS Trust. Parts of my medical information may be passed to researchers external to the Trust on the understanding that the data will only be released in a non-identifiable format. ☐

7. I understand, and agree to, data relating to my donated samples being stored electronically. ☐

8. I understand and agree that researchers in Nottingham, outside of Nottingham, in the UK or overseas; including those in Universities, NHS, and charities or commercial organisations, may have access to samples / data however no personally identifiable information will be released to researchers. ☐

9. I understand that donated samples are not sold, but costs are recovered on a non-profit-making basis and that I will not gain financially in any way from this donation. ☐

Name of patient:.....**Date:**.....**Signature:**.....

Person taking consent:.....**Date:**.....**Signature:**.....

Key: Copies - White: Biobank, Pink: Patient, Blue: Patient's case notes, Green: Biobank

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Appendix 5: Anonymous Patient Data Form

These data are to be used only for research purposes.
To be completed by the lead surgeon and anaesthetist.
Researcher will NOT have an access to any patient personal data.

This specimen is collected on / / (2017-2018)

1. Basic Data

Patient Gender ..... Age …….

Specimen No.....

NHSB No.....

2. Clinical Data

According to MSIS, Is it infection:	YES		According to Surgeon, Is it infection:	YES	
	NO			NO	
Diabetes Mellitus	YES		If YES	TYPE I	
	NO			TYPE II	
Renal Failure	YES		Cancer	YES	
	NO			NO	
Long term Steroid intake	YES		Other immunosuppressive conditions	YES (Please specify)	
	NO			NO	

3. Antibiotics:

Is the patient currently on antibiotics	YES		
	NO		
When was the last time to be on antibiotics:	DAY	MONTH	YEAR
What is that			
For how long	DAY	MONTH	YEAR

4. Surgery:

Type of surgery	**Hip**		
	Knee		
	Other (please specify)		
Is this surgery	**Primary**		
	Revision		
Is this surgery	**First stage**		
	Second stage		
	Repeat first stage		
Date of first surgery (if applicable)	DAY	MONTH	YEAR
Reason for first surgery	**Osteoarthritis**		
	Rheumatoid Arthritis		
	Posttraumatic		
	Other		

5. Lab:

	Preoperative	**Postoperative**
HB		
ESR		
CRP		
WBC		