Infections in urological practice: bacteraemia and infective endocarditis

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Submitted in accordance with the requirements for the degree of
Doctor of Medicine

The University of Leeds
The Leeds Institute of Molecular Medicine (LIMM)

February 2014

The candidate confirms that the work submitted is his own, except where work that has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Acknowledgements

This research was carried out by a team, which included Mr Ian Eardley, Dr Jonathan Sandoe, Dr Deborah Gascoyne-Binzi and Professor Robert West. My own contributions, fully and explicitly indicated in the thesis, were to design, conceptualise and carry out the project, including participant recruitment and the processing of samples. The other members of the group and their contributions were as follows:

Mr Ian Eardley: supervisory support in the design and completion of the project. He also helped raise funds for the project.

Dr Jonathan Sandoe: supervisory support in the design of the project and help with the culture methodology. He helped in revising the final drafts of the thesis.

Dr Deborah Gascoyne-Binzi: support in the molecular microbiology methodology.

Mr Anthony English: support in sequencing PCR samples.

Professor Robert West: provided statistical support in analysing and interpreting the results.

Dr Selina Bhattarai: helped with the histopathological analysis of the prostate chips.

I would also like to thank the Leeds Charitable Trust, Molzym GmbH & Co. KG, and Abbott Molecular for their financial support. I would like to thank Salem Raman for his help with formatting the final document in keeping with the university regulations. The thesis would not have been completed without the dedicated help of Jonathan Sandoe and Ian Eardley, who devoted countless hours to help me jump the final hurdle. I would also like to thank my parents for pushing me to fulfil my ambitions. Finally, I would like to thank my wife, Dr Samia Mohee for her indomitable support, understanding and advice.

Abstract

Introduction. It is known that infections may occur after urological instrumentation, as some patients develop infective symptoms. The purpose of this study was to investigate bacteraemia in patients undergoing transurethral resection of the prostate (TURP) and catheter manipulation, using contemporary culture methods. Another aim was to explore the potential for molecular methods to detect, identify and quantify bacteraemia. We aim to evaluate the association between urological instrumentation and the development of infective endocarditis (IE).

Methods. Microbiological molecular methods to identify and quantify bacteria in blood were developed. Blood samples were collected at different time points during the procedure from patients undergoing TURP and catheter manipulation to evaluate the presence of bacteria using both the culture and molecular methods. The association between risk factors (patient and procedural) and bacteraemia was analysed statistically. A case-control model was used to assess the association between the development of IE and a number of risk factors, including urological instrumentation.

Results. Bacteraemia occurred in both sets of patients though most patients were asymptomatic. In the TURP group, bacteraemia occurred within the first twenty minutes of the procedure in spite of antibiotics prophylaxis. In the catheter manipulation group, bacteraemia was present even prior to any urological manipulation. The case-control model demonstrated an association between urological instrumentation and the development of IE.

Conclusion. This study has shown that bacteraemia during urological instrumentation is more prevalent than previously thought but is largely asymptomatic. Moreover, antibiotic prophylaxis in TURP patients fails to stop a significant proportion of intra-procedure bacteraemias. Asymptomatic bacteraemia may explain the statistical association between urological instrumentation and IE has been demonstrated.

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Abbreviations

AHA:	American Heart Association
AUA:	American Urological Association
BSAC:	British Society for Antimicrobial Chemotherapy
CBA:	Columbia blood agar
CC:	Catheter change
CFU	Colony-forming unit(s)
CLED:	Cysteine lactose electrolyte deficient
CR:	Catheter removal
CRP:	C-Reactive protein
EAU:	European Association of Urology
ERCP	Endoscopic retrograde cholangiopancreatography
ESC:	European Society of Cardiology
ESR	Erythrocyte sedimentation rate
IE:	Infective endocarditis
IV	Intravenous
IVDU	Intravenous drug user
ICAM:	Intracellular adhesion molecule
NICE:	National Institute of Clinical Excellence
NVE	Native valve endocarditis
OGD	Oesophagogastroduodenoscopy
SPC	Suprapubic catheter
PCR:	Polymerase chain reaction
TOE	Transoesophageal echocardiography
TTE	Transthoracic echocardiography
TURBT	Transurethral resection of bladder tumour

TURP:	Transurethral resection of the prostate
UTI:	Urinary tract infection
VCAM:	Vascular cell adhesion molecule

Chapter 1: Introduction

1.1 Introduction

It is known that patients can develop infections after the instrumentation of the urinary tract. Infective episodes can manifest themselves as simple urinary tract infections (UTIs) to more serious infective episodes like septic shock and infective endocarditis (IE). The rates of infection associated with instrumentation vary and depend on a number of variables including the sterility of urine of the patient and which procedure is being carried out as described in Chapter 2.

However, little is known about the rates of bacteraemia associated with instrumentation of the urinary tract. The literature addresses the issue of bacteraemia in symptomatic patients, with very few studies evaluating bacteraemia in patients irrespective of symptoms in the immediate setting of the procedure. It is known that a bacteraemia is required for the development of bacterial infective endocarditis and the literature is interspersed with case reports and case series of patients having had urological instrumentation and subsequently developing IE. However, a statistical association between urological instrumentation and the development of IE has never been demonstrated.

1.2 Aims and objectives of the study

The purpose of this study is two-fold. Firstly, it assessed whether there is an association between urological instrumentation and the development of IE. The null hypothesis is that urological instrumentation does not cause IE. The reason to study this association was that there has been anecdotal evidence, through case reports and case series, that urological instrumentation may be a precipitating factor for the development of IE.

Secondly, the incidence, nature and duration of bacteraemia during urological procedures were studied. The two procedures that were chosen for study are two of the most common procedures performed in the urological practice. At the Leeds Teaching Hospitals Trust, around two hundred and fifty transurethral resections of the prostate (TURP) operations are performed yearly. Catheter manipulation is done routinely on a daily basis. Different methodologies (the ‘gold standard’ culture method culture method and novel technologies in

molecular microbiology) were employed to assess for the presence of bacteria in blood. The gold standard culture method has been used over many decades but it has its drawbacks, which are described in Chapter 2. This is the reason alternative options to the culture methods were explored.

1.3 Background to infections in urology

The urinary tract is a sterile environment. Instrumentation of the urological tract in the sterile surroundings of an operating theatre should not lead to infective episodes if proper aseptic techniques are used. However, several studies have shown the development of UTIs and systemic symptoms after the instrumentation of the urological tract [1-3]. The existing literature does not provide any significant evidence for the presence of asymptomatic bacteraemia secondary to instrumentation of the urinary tract. However, the presence of bacteraemia has been demonstrated in patients with symptoms. The review of this literature is discussed in the next section. The scope of this study extends primarily to patients undergoing TURP and catheter manipulation, though bacteraemia has been associated with a whole range of urological procedures ranging from cystoscopies to kidney instrumentation and prostatic biopsy [4, 5].

The development of IE requires the prior presence of bacteraemia. IE can be caused by a variety of organisms, the main ones being staphylococci, streptococci and enterococci [6]. Evidence whether instrumentation of body cavities (not including the vascular system) can lead to IE is minimal and the literature concerning this is reviewed in Chapter 2. Antibiotic prophylaxis has been used in surgical practice in order to prevent peri-operative infection since the introduction of systemic antimicrobials [7]. However, there is no clear definition of the type of ‘infection’ that antibiotic prophylaxis prevents. Does antibiotics prophylaxis prevent asymptomatic bacteriuria, urinary tract infections, bacteraemia or sepsis?

In the early part of the last century, investigators started doing work on bacteraemia and urological manipulation. Prior to this, there was anecdotal evidence and isolated case series about episodes of rigors and deaths associated with urological instrumentation. As early as 1810 [8], Moffait described a case in which a patient suffered from rigors and fever following urethral dilatation for a chronic urethral stricture. The patient eventually succumbed to this episode. The cause of death was attributed to sepsis. In 1832, Brodie linked urethral instrumentation (bougie-dilatation) to rigors, describing a patient who experienced rigors with repeated bougie-dilatation. He

went on to hypothesise that the rigors coincided with the passage of urine over the raw urethral mucosa, rather than coinciding with the actual surgery. In 1887, Halle reported bacteraemia in a patient with a urethral stricture and partial urinary retention (presumably high post micturition residual volume). The patient died six days later, and bacteraemia was detected from a blood sample taken several hours after death. Bertelsmann and Mau described a case of death secondary to staphylococcal endocarditis in 1902. The patient reportedly had two urethral dilatations, each associated with rigors prior to his death. The above cases are reported in the paper by Rodin *et al.* [8]. Subsequently, isolated cases of suspected infection related to urological instrumentation have been reported in the literature.

It was only in 1930 that the first structured study was done by Barrington and Wright to investigate the link between urological instrumentation (urethral dilatation) and bacteraemia. Thereafter, more investigators published their case series. Some of these studies are shown in Table 1 (below).

Barrington and Wright found bacteraemia in twelve out of twenty of their patients. The patients had urethral surgery (mainly urethral dilatations) and blood cultures were taken six minutes after surgery. If the patients had subsequent urethral dilatations, repeat cultures were then taken. The study also demonstrated bacteraemia in two out eleven patients after micturition through the dilated 'raw' urethra.

In 1958, Slade commented on the incidence of infection and septicaemia following urological procedures [9]. He quoted figures from the Bristol Royal Infirmary whereby 5000 autopsies, done over twenty years, revealed four cases of infective endocarditis - all linked to previous urological instrumentation. Slade designed protocols with the aim of identifying bacteraemia following micturition through a recently surgically manipulated urethra. Whilst he failed to demonstrate an effect, he did show that there was a high incidence of bacteraemia (ten out of thirty-eight patients) on catheter withdrawal following urological surgery in patients with bacteriuria.

Authors	Date	Procedure		No of patients	Positive Blood Cultures
Barrington &Wright	1930	Operation on urethra or later dilatation		20	12
		Simple dilatation of stricture		3	1
Powers	1936	Dilatation of stricture		16	3
Bjorn (cited by Merritt)	1951	Trans-urethral resection of prostate		106	13
Slade	1958	Removal of catheter		28 infected urine	10
				12 sterile urine	Nil
Mitchell and others	1962	Dilatation of stricture	No prior urethral disinfection	28 infected urine	11
			Prior urethral disinfection	30 infected urine	3
				86 sterile urine	Nil
Tulloch and others	1964	Dilatation of stricture		14 infected urine	5
Rodin and Murray	1966	Dilatation of stricture		15 infected urine	1
				22 sterile urine	Nil
		Prostatic massage		39 (15 with chronic prostatitis)	Nil
		Anterior urethroscopy		22	Nil

Table 1. Early work on bacteraemia (adapted from Rodin and Murray) [8].

The brief description of the historical facts above gives us an inkling that urological instrumentation may lead to bacteraemia and infective endocarditis. However, this is level 3 evidence at best and data that are more robust are required to assess the association between urological instrumentation and bacteraemia and the subsequent development of IE [10].

1.4 Structure of the Thesis

The thesis aims to address the issues related to urological instrumentation, bacteraemia and the development of IE.

Chapter 1 sets the scene of the thesis. It briefly describes the aims and objectives of the study and gives a historical overview of iatrogenic infections in urological practice. Thereafter, a thorough review of the literature regarding the development of IE and its relevance in patients who have undergone urological procedures is described. The chapter examines the development of bacteraemia following urological procedures. This is reviewed in two separate sections: firstly, bacteraemia in patients undergoing TURP and secondly bacteraemia in patients undergoing catheter manipulation.

Chapter 2 depicts the methodology of the epidemiological study used to investigate the association of urological instrumentation and the development of IE. The chapter describes the results obtained in detail and the implications of these results.

Chapter 3 details the methods used to assess bacteraemia. The culture method is described briefly with the major emphasis on the novel molecular microbiological methods used. The different *in vitro* experiments carried out are described.

Chapter 4 highlights two prospective studies, recruiting patients having urological procedures (TURP and catheter manipulation) in order to determine the presence, nature and duration of bacteraemia. The analysis of the results is reported.

Chapter 5 is a detailed discussion and interpretation of the results obtained from the epidemiological study and the prospective studies.

Chapter 6 concludes the thesis by summarising the main findings, addressing the limitations of the work and making suggestions about future possibilities and areas of investigation.

1.5 Infective Endocarditis and Urology

1.5.1 Infective Endocarditis

1.5.1.1 Introduction

Infective endocarditis (IE) is an inflammation of the endocardium caused by pathogens: mainly bacterial or fungal. It is a notoriously hard disease to

diagnose and to treat. In recent years, IE has affected intracardiac implants like valvular prosthesis and pacemaker electrodes in addition to the native endocardium.

William Osler described IE as an entity in 1885 [11] at the Royal College of Physicians. The diagnosis and treatment of the disease have significantly improved since then, with the modern era of advanced diagnostic imaging and antimicrobial therapy. Although a rare disease, IE still carries a significant morbidity and mortality [12-15] despite improvement in antimicrobial therapy and surgical management, as well as improvements in the management of the complications. The mortality associated with IE has not improved over the last thirty years ranging between 20% and 30% with some pathogens causing a higher fatality rate than others [15, 16].

1.5.1.2 Epidemiology

An increase in incidence of IE has been noted since the 1980s, from one to two cases per million to 11 to 26 cases per million [17, 18], which has been attributed partly to an increase in diagnostic and therapeutic procedures done by health-care workers [14]. Most series quote an incidence between 2.3 and 5.9 per 100 000 people, with more elderly than young people being affected [19, 20]. From a urological point of view, urology patients treated tend to be elderly and therefore have and inherent increased risk of developing IE.

This change in epidemiology has been investigated in France by Hoen *et al.* [21] who performed a population study on 26% of the French population, which included patients who first presented with a diagnosis of IE in 1999.

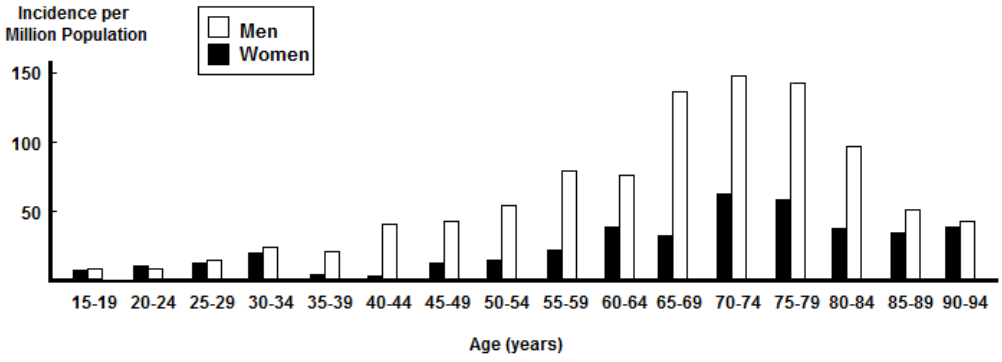


Figure 1. Incidence of Infective Endocarditis in Different Age Groups (from Hoen *et al*. [21])

Figure 1 shows that the majority of the patients were above 64 when they were first diagnosed with IE, which is similar to the age group of patients undergoing

urological instrumentation, the median of which is 61 years [22]. There is also an argument to suggest that this increased incidence can be linked to better diagnostic facilities available to modern medicine. In a series published by the Royal College of Physician Research Unit in 1984, 15% of the IE was attributed to organisms having gained access through the alimentary or genitourinary portals [17]. Men are more likely to suffer from IE with a male to female ratio of 2:1, but the male preponderance is poorly understood [23]. Patients tend to present earlier owing to the easy access of medical facilities. This phenomenon artificially inflates the incidence of IE, especially in the third world countries. It is known that patients who present late tend to have a worse outcome. Therefore, many patients are diagnosed with IE, when previously they would have succumbed to the disease owing to lack of access of medical facilities.

Over the last decade, the profile of the disease has changed, with fewer cases associated with rheumatic fever especially in the developed world and more cases linked to medical and surgical interventions, the elderly population and patients with intracardiac devices or undergoing haemodialysis [16, 23]. From a urological point of view, the fact that the cohort of patients developing IE is becoming older is significant, as patients are more likely to have had instrumentation of their urological tract.

1.5.1.3 Risk factors

Epidemiological studies have shown that patients diagnosed with IE have a higher prevalence of diabetes mellitus and malignancy [24]. These conditions may be linked in the pathogenesis of IE, given that such patients are immunosuppressed. They also tend to be more prone to infections, which may seed the cardiac valves. Furthermore, these patients have a greater need for intravenous access with intravascular devices *in situ* for long periods. Over the next decades, the number of surgical interventions is expected to increase particular in the elderly population, and this may in turn lead to an increase in the incidence of IE.

1.5.1.4 Pathogenesis

The normal endocardium is resistant to colonisation by bacteria circulating in the bloodstream [25]. However, when the endocardium is disrupted, tissue factor activates the coagulation cascade and leads to formation of a thrombus through the deposition of platelets and fibrin [25]. The presence of this sterile thrombus predisposes the endocardium to colonisation by bacteria, leading to infective endocarditis [26].

Endocardial disruption may occur by a variety of mechanisms [27]:

- intrinsic – *e.g.* high pressure turbulent blood flow;
- iatrogenic – *e.g.* intravenous catheters;
- inflammatory – *e.g.* rheumatoid heart disease;
- degenerative – *e.g.* in the geriatric population.

Events that induce bacteraemia have been noted in just 20% of cases of IE [15]. In the other 80% of cases, no causal event was found in the time-line prior to the onset of the disease. This suggests that prior bacteraemia was not detected, as it was not symptomatic. There is growing evidence that certain factors, labelled the 'missing link' in the pathogenesis of IE play a significant role in the development of IE. Host factors like altered immune and inflammatory states and/or associated endothelial dysfunction have been identified as precursors to IE [28]. Inflammatory cytokines and adhesion molecules like the intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) are unregulated in endothelial cells of valves with IE [28].

ICAM-1 is an inflammatory cytokine, which self-perpetuates; it increases a monocyte chemoattractant protein, which in turn increases the levels of ICAM-1 [29]. This inflammatory activation disrupts the endothelial barrier and makes it more prone to adherence of platelets and hence the activation of the coagulation cascade via the tissue factor (extrinsic) arm [28, 29]. This leads to a thrombus formation at the site on the endothelium, which in turn may become colonised by circulating bacteria in blood [26]. Infective endocarditis ensues.

Without an infecting pathogen, the above process leads to non-infective endocarditis. Non-infective endocarditis is a very well documented clinical entity, associated with systemic disease like lupus erythematosus (Libman-Sacks endocarditis), malignancy (marantic endocarditis) and acute rheumatic fever [30]. Therefore, it is clear that bacterial virulence factors are not the only players in leading to endothelial dysfunction, in some cases, inflammatory cytokines may play a role.

This inflammatory model has been tested on two groups of rabbits by Dankert *et al.* [31]. Rabbits in Group A were injected with Interleukin-1α (a pro-inflammatory cytokine) using an intracardiac catheter. Group B was the control group and the intracardiac catheter was sited without injection of any reagent. The intracardiac catheters were then removed in both groups. Bacteria were then injected intravascularly. Their results showed that rabbits in Group A had

bacterial colonies on the endothelium. The rabbits in Group B showed no endocardial bacterial colonisation. This observation can be compared with human patients in a state of chronic inflammation who suffers episode (s) of bacteraemia (*e.g.* critically ill patients in intensive care), who end up developing IE while being in an 'inflammatory' state.

Other factors have been linked to non-infective endocarditis. Haemodynamic stress and turbulence can lead to alterations in endothelial morphology, which makes it more prone to allow the formation of vegetations [32]. This mechanism is associated with the development of endocarditis in patients with cardiac valvular disease. The higher pressures in the left side of the heart in patients with cardiovascular disease may explain the greater prevalence of left-sided endocarditis.

The pathogenesis of endocarditis, in some cases, appears to be linked to systemic inflammatory states and altered endothelial cell morphology. A study by Tonetti [33] demonstrated increased inflammatory markers (PAI-1, E-selectin, CRP, interleukin-6, neutrophils and Von Willebrand factor) in patients with chronic periodontitis. This mechanism has also been linked to the formation of atherosclerosis, with the initial step being endothelial damage.

1.5.1.5 Microbiology of Infective Endocarditis

The common organisms known to cause IE are *Staphylococcus aureus,* "oral streptococci", coagulase-negative staphylococci and enterococci. Less common causes include: *Streptococcus anginosus* group*, Abiotrophia* species, β-haemolytic streptococci (Groups A, B, C, D and G), *Pseudomonas aeruginosa*, HACEK (*Haemophilus aphrophilus, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens* and *Kingella kingae*), *Neisseria gonorrhoeae,* fungi, and *Bartonella* species. Polymicrobial infection is uncommon [15, 34, 35]. In the series of 582 episodes of IE studied by Bayliss in 1984, 27 of the episodes were caused by *Streptococcus bovis* 17 of the episodes were caused by enterococci, 14 by *Streptococcus faecalis* (now described as *Enterococcus faecalis*)*,* 11 by *Escherichia coli* or other Gram-negative bacilli, and 6 by *Streptococcus durans* [17].

Bacteria that end up colonising the endocardium and endocardial thrombi are expected to be resistant to the bactericidal effect of blood, especially platelets [36]. However, a proportion of blood cultures taken from patients suffering from IE are negative for bacterial growth. This may sometimes

adversely affect the clinical management of the patient as antimicrobial therapy is started on an empirical basis, which may not be optimal therapy.

1.5.1.5.1 *Enterococcus* species

Enterococci are part of the normal intestinal microbiota of humans but can also cause serious infections when displaced from their normal locations [37]. The genus *Enterococcus* includes more than 17 species, but only a few cause clinical infections in humans [38]. *Enterococcus* species are facultative anaerobic organisms that can survive and grow in many environments. The most common species associated with humans are *Enterococcus faecalis* and *Enterococcus faecium, a*ccounting for more than 90% of clinical isolates [37]. Other enterococcal species known to cause human infection include *Enterococcus avium, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus durans, Enterococcus raffinosus* and *Enterococcus mundtii* [39]. *Enterococcus faecalis* and other enterococci may also colonise the anterior urethra, the vagina, the cervix and the perineum [17]. It is known that IE can be caused by enterococcus species, mainly *Enterococcus faecalis* [50]. Enterococcal endocarditis is caused by genitourinary and gastrointestinal pathogens in 40-50% of cases [40].

1.5.1.6 Clinical Presentation

The clinical picture may be confusing in many cases but the diagnosis of infective endocarditis should be suspected in the face of unexplained, persistent fever. Fatigue, anorexia, and back pain are all associated with IE, with the classic cardinal symptoms being new onset or worsened heart murmur, night sweats and weight loss. IE manifests in different ways, depending on [23]:

- the underlying cardiac pathology;
- the microbial cause;
- the complications;
- the underlying patient characteristics.

The clinical course of IE can be markedly different in different patients. This often leads to a long latency between the onset of symptoms and a definitive diagnosis of IE, typically one month, which may in turn contribute to the high morbidity associated with the disease [15].

The European Society of Cardiology (ESC) classifies IE as follows [34]:

IE according to localisation of the infection and presence of intracardiac material:

a. left-sided native valve endocarditis;

b. left-sided prosthetic valve endocarditis;

- early endocarditis (< 1 year after valve surgery);
- late endocarditis (> 1 year after valve surgery);

c. right-sided endocarditis;

d. device-related endocarditis.

IE according to mode of acquisition:

a. healthcare-associated endocarditis;

- nosocomial endocarditis;
- non-nosocomial endocarditis;

b. community-acquired endocarditis;

c. endocarditis associated with intravenous drug use.

Active IE – Fever and positive blood cultures, histopathological evidence of IE

Recurrence:

a. relapse;

b. re-infection.

The symptoms of early native valve endocarditis (NVE) are usually subtle and nonspecific. They include low-grade fever (absent in 3-15% of patients), anorexia, weight loss, influenza-like syndromes, polymyalgia-like syndromes, pleuritic pain, and abdominal symptoms (pain and vomiting) [34].

The textbook embolic and immunological signs and symptoms develop much further down the line, especially if no appropriate treatment has been given. Embolic manifestations occur in around 30% of cases [15]. In the contemporary management of IE, treatment is instituted early. This is the reason why it has now become rare to detect signs like Osler's nodes (subcutaneous haemorrhagic nodules linked to immune complex vasculitis or septic embolism),

Janeway lesions (haemorrhage of the palms and soles caused by immune complexes) and Roth's spots.

After the acute episode of IE, long-term complications may persist. These include [41]:

- congestive heart failure and other cardiac pathologies. This is generally linked to the destruction of the chordate tendinae and/or cardiac valves. Eventually the ventricles fail;

- arterial embolisation leading to neurological damage. As discussed above, these are rarely seen nowadays;

- renal insufficiency. The mechanism is linked to the deposition of immune complexes in the glomeruli of the kidneys leading to glomerulonephritis. This can present with frank haematuria.

1.5.1.7 Diagnosis

1.5.1.7.1 Duke criteria

The Duke's criteria were proposed in 1994 by Durack *et al.* from the Duke Endocarditis Service at the Duke School of Medicine in North Carolina [42]. The Duke's criteria consist of a collection of major and minor criteria used to establish a diagnosis of endocarditis. A diagnosis can be reached in any of these three ways: two major criteria, one major and three minor criteria, or five minor criteria. The major criteria include:

- positive blood culture (two separate samples > 12 hours apart) with typical IE microorganism, defined as one of the following:

    - viridans-group streptococci, *S. bovis*, or HACEK group, or
    - community-acquired *Staphylococcus aureus* or enterococci, in the absence of a primary focus;
    - microorganisms consistent with IE from persistently positive blood cultures;

- evidence of endocardial involvement with positive echocardiogram defined as:
    - oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation, or
    - abscess, or
    - new partial dehiscence of prosthetic valve or new valvular regurgitation (worsening or changing of pre-existing murmur is not sufficient).

The minor criteria include:

- predisposing factor: known cardiac lesion, recreational drug injection;
- fever > 38° C;
- evidence of embolism, pulmonary infarcts and haemorrhages (sub-ungal, conjunctival);
- immunological problems like glomerulonephritis, Osler's nodes and Janeway lesions;
- positive blood culture (which does not meet a major criterion);
- positive echocardiogram (which does not meet a major criterion).

Since its introduction in 1994, the Duke's criteria for the diagnosis of IE have been a useful tool to aid the diagnosis of IE. Over the years, the Duke's criteria have been modified to improve sensitivity. Li *et al.* from the same unit in North Carolina published modifications in 2000 [43]. However, the associated increase in sensitivity has come at a price. There has been a corresponding loss of specificity according to a meta-analysis of 3557 patients [15].

1.5.1.7.2 Modified Duke's Criteria

Pathological criteria:

- positive histology or microbiology of pathological material obtained at autopsy or cardiac surgery;
- valve tissue, vegetation, embolic fragments or intracardiac abscess content.

Major Criteria:

- two positive blood cultures showing typical organisms consistent with infective endocarditis, such as viridians streptococci and the HACEK group;
- persistent bacteraemia from two blood cultures taken > 12 hours apart or three or more positive blood cultures where the pathogen is less specific, such as *Staphylococcus aureus* and Coagulase-negative staphylococci;
- positive serology *for Coxiella burnetti*, *Bartonella* species, or *Chlamydia psittaci*;
- positive molecular assays for specific gene targets;
- positive echocardiogram showing oscillating structures, abscess formation, new valvular regurgitation or dehiscence of prosthetic valves.

Minor Criteria:

- predisposing heart disease;
- fever > 38° C;
- immunological phenomena such as glomerulonephritis, Osler's nodes, Roth spots, or positive rheumatoid factor;
- microbiological evidence not fitting major criteria;
- elevated C reactive protein (CRP) or erythrocyte sedimentation rate (ESR);
- vascular phenomena such as major emboli, splenomegaly, clubbing, splinter haemorrhages, petechiae or purpura.

For a definitive diagnosis of infective endocarditis, the following rules must be obeyed:

- pathological criteria positive or
- two major criteria or
- one major criterion and two minor criteria or
- five minor criteria present.

This is the set of rules generally used in clinical practice nowadays in conjunction with other non-specific parameters like:

- a raised white blood cell count (leucocytosis), with a left shift (an increased ratio of immature to mature neutrophils);
- a raised C-reactive protein (CRP);
- a raised erythrocyte sedimentation rate (ESR);
- a raised pro-calcitonin concentration.

1.5.1.8 Echocardiography

Echocardiography is the study of the heart and its related structures with the use of ultrasound waves. It is one of the cornerstones of the modified Duke's criteria for the diagnosis of IE. The procedure can be carried out via two modalities [44]:

- Transthoracic Echocardiography (TTE). The probe is placed on the patient's chest wall. It emits and picks up waves that are then transmitted to a computer. A computer is used to analyse the waves to deliver an image on the screen.
- Transoesophageal Echocardiography (TOE). This is a similar procedure as above, except that the probe is inserted through the mouth into the oesophagus. This allows the probe to be in closer contact to the heart and hence provides more accurate images [45]. Furthermore, owing to the position of the probe, the right side of the heart, not accurately visualised with TTE, is better visualised.

The images obtained through echocardiography provide a pictorial as well as a morphological characterisation of the heart and any associated lesions linked to IE. Erbel *et al*. quote that TOE is generally more sensitive than TTE except for the right side of the heart [46].

Important parameters noted on echocardiography are [47]:

- presence of vegetation (thrombus adherent to the endocardium which is colonised with bacteria);
- size of the vegetation (there is a correlation with complications and patient survival);
- degree of damage to the cardiac tissues (abscess, fistulae, perforations, damage to chordae tendinae, prosthesis dehiscence, and blood flow insufficiency on Doppler scanning);
- ventricular function.

In patients confirmed with a diagnosis of IE, progression of the disease can be monitored with serial echocardiographic imaging.

1.5.1.9 Blood Cultures

Three sets of blood cultures (aerobic and anaerobic) should be taken, prior to the start of any antibiotic therapy. The blood cultures should be taken from adequately disinfected peripheral sites. In patients with IE, there is an assumption that bacteria are constantly being shed into the bloodstream from the vegetations. This leads to continuous bacteraemia, which correlates with the usual associated pyrexia. About 10-30% patients with IE have negative blood cultures, attributed most often to prior antibiotic treatment and/or a fastidious organism as the cause of infection [34]. Ultimately, the diagnosis of IE is based on sound clinical judgment. The Duke's criteria only offer tools to help aid diagnosis.

1.5.1.10 Management

Many patients are started on empirical broad-spectrum antibiotics, pending results from blood cultures. Blood cultures have been the cornerstone of the microbiological diagnosis of IE. Once suitable sensitivities are obtained, patients can be switched to the optimal antibiotic regime. Patients who are unresponsive to medical management (supportive and antibiotic therapy) progress to surgical therapy. This involves the removal of the infective focus and replacement of the infected valve.

1.5.1.11 Prognosis

If left untreated, IE is generally fatal. However, in the modern age of multidisciplinary management of complex conditions, IE is largely a treatable disease. Prognosis depends on the identity of the offending organism, the presence of an intracardiac prosthesis and the timeliness of treatment

(antimicrobial therapy and surgery). IE caused by viridans streptococci has a cure rate of up to 98% whilst fungal infections tend to be harder to treat with a success rate of only 50% [34]. If a prosthetic device is involved in the infection, the cure rate is lower. Patients who end up having surgical management of their infection tend to do less well, as the procedure comes with a mortality of up to 30% [48].

1.5.2 Instrumentation and Infective Endocarditis

It has been hypothesised that IE may follow a transient bacteraemia caused by a surgical procedure involving the dental, alimentary, respiratory, dermatological and genitourinary tract [17, 49]. From a urology perspective, this relates to instrumentation of the urinary tract. Traditionally, antibiotic prophylaxis has been used with a view to either prevent bacteraemia or reduce its magnitude and duration. In urological practice, it is thought that enterococci are the predominant pathogens that lead to bacteraemia [49]. The rationale for using antibiotics prophylaxis in the urological practice is [47]:

- to minimise the infections (UTIs and sepsis) in the acute phase;
- to minimise long-term infections likes IE and infected prosthetics.

However, over the years, it has been proposed that IE can be caused by bacteraemia resulting from normal daily activities [50]. Moreover, not everybody having had an invasive medical procedure develops IE; some people have predisposing factors like underlying valvular pathology and congenital heart malformations [14, 17] with Bayliss reporting a 41% rate of cardiac valve abnormalities in his IE series [17]. The duration and amplitude of the bacteraemia have been proposed as important factors predisposing to IE [14, 51]. Yardena *et al.* showed that 13 out of 212 episodes of IE were preceded by urological procedures [52].

However, it has never been shown whether instrumentation of the urinary tract is statistically associated with the development of IE. The use of antibiotic prophylaxis in procedures causing bacteraemia is controversial, as the scientific literature is inadequate to support or invalidate the practice. There has been no prospective, randomised trial in humans to that effect. Therefore, there is a need to design an epidemiological study to evaluate this link.

1.6 Bacteraemia and TURP

1.6.1 The Prostate and Bladder Outflow Obstruction

The prostate is a pelvic organ, which is traversed by the urethra. The prostate is made up of 70% glandular tissue and 30% fibromuscular tissue. Benign prostatic hyperplasia (BPH) is a condition that affects men as they grow older. The pathophysiology involves detrusor dysfunction and bladder outflow obstruction, and is defined histopathologically by the proliferation of epithelial and stromal cells in the peri-urethral zone of the prostate. Up to 70% of men above 60 years old have a degree of BPH [53].

Symptoms and bother make patients seek treatment for BPH. The management options ranges from medical treatment to surgical management. The surgical management options include open prostatectomy and trans-urethral prostatectomy (resection or vaporisation). Urinary tract infections (UTIs) were an indication for surgical management of BPH in earlier series, though in more contemporary series, surgical management is available for most patients with troubling symptoms, irrespective of the presence of UTIs [54].

1.6.2 Transurethral Resection of the Prostate (TURP)

The transurethral resection of the prostate (TURP) is a procedure introduced in the early part of the last century in the United States of America and has been popularised all over the world. TURP is normally performed under general or spinal anaesthesia and local and national guidelines advise antibiotic prophylaxis (single dose prior to the start of surgery) [55, 56]. If the patient is catheterised prior to the procedure, the catheter is removed. A resectoscope is inserted urethrally until the prostate is visualised. After a cystoscopy of the bladder, prostate resection begins and lasts between thirty to sixty minutes. After resection, the prostatic chips are removed and haemostasis is achieved. The patient is catheterised urethrally and the catheter remains *in situ* until the post-operative haematuria settles (usually 48 hours).

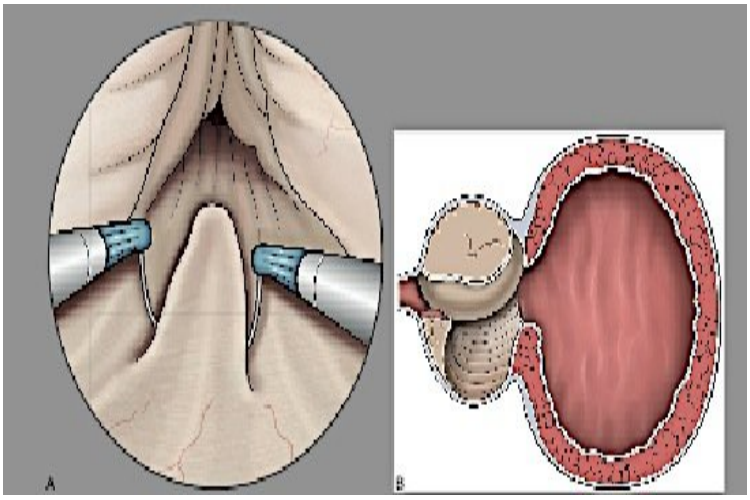


Figure 2. Diagrammatic view of a TURP [57]
The diagram shows the resection loop cutting through the prostate tissue.

TURP is a routine urological procedure, with around two hundred and fifty (250) such procedures performed at the Leeds Teaching Hospitals NHS Trust each year.

1.6.3 Infection and TURP

1.6.3.1 Bacteraemia and TURP: The mechanism

The normal urological tract is a sterile environment, except for the distal urethra, which is often colonised with organisms from the perineal area [58]. Therefore, instrumentation of the urinary tract in the sterile environment of the operating theatre should not lead to bacteraemia. However, bacteraemia has been demonstrated during TURP in several studies and this will be discussed in the next section. In this section, the issue of how bacteria gain access to the bloodstream during a TURP is discussed. The source of the bacteria has been shown to be from [59]:

- urine;
- the urethral meatus;
- the prostate gland;
- instruments and fluids used intraoperatively.

The urinary tract is a normally sterile site and operating theatres are clean environments: nevertheless, there are multiple potential sources of bacteria.

These may have access to the vascular system leading to the potential for bacteraemia during TURP:

1) During a TURP, prostate tissue is resected leading to exposed blood vessels. The natural host barrier is breached allowing easy access for bacteria to gain entry into the bloodstream. During the procedure, fluid is injected continuously through the resectoscope to aid visualisation during the resection of the prostate. This constant flow of fluid retrogradely, from the outside of the patient to inside of the bladder, may provide a route of entry for bacteria residing at the urethral meatus, on the surgical instruments or in irrigation fluid to gain access inside the urinary tract [60].

2) Bacteriuria is one of the main reasons for the development of bacteraemia during urological procedures [2, 61]. Bacteriuria in the peri-procedure period increases the incidence of developing bacteraemia [2] and a UTI (up to 68% in the absence of prophylactic antibiotics use [62]). For this reason, it is generally accepted that non-emergency urological instrumentation should be delayed until bacteriuria is cleared with antibiotic therapy. However, this is not always possible, especially in the presence of a foreign body (*e.g.* a urethral catheter) in the urological tract. Nevertheless, it has also been shown that two-thirds of post-operative bacteriuria are caused by different bacterial strains than pre-operative bacteriuria [58, 63].

3) The prostate. Prescott *et al.* demonstrate that out of 170 patients having prostate surgery, 46% patients had microbial growth from the prostate tissue obtained, with most of these patients (94%) having preoperative sterile urine [58]. The main organism cultured was '*Staphylococcus albus*' (coagulase-negative staphylococci). Although this may represent a contaminant, the methodology used suggests true bacterial growth within the prostate. This defies the commonly accepted truth that the urinary tract is a sterile system. Other studies have also attributed post-operative bacteriuria and UTIs to pathogens residing in the prostate gland [64-66]. Studies have demonstrated bacterial colonisation of the prostate tissue, in spite of no evidence of bacteriuria [65]. Having a urethral catheter *in situ* was associated with prostatic bacterial colonisation [67]. Therefore, if bacteria reside in the prostate with the patient being asymptomatic, breaching the prostate tissue can lead to an escape of those bacteria into the bloodstream [68].

Furthermore, patients undergoing a TURP operation have bladder outflow obstruction. In animal models, there is evidence that obstruction of the urinary

tract, however brief, can lead to bacteriuria and bacteraemia [69]. This may indicate that bacteraemia may be caused by the underlying urological pathology (bladder outflow obstruction) rather than the procedure [70].

1.6.3.2 Bacteraemia and TURP: The Incidence

The reported incidence of patients undergoing TURP with bacteraemia and without antibiotic prophylaxis varies widely (0-31%) decreasing to 1% with antibiotic prophylaxis [2, 68]. There is a wide range of reported bacteraemia incidences as the methodologies and patients groups used were very diverse. However, most published studies consider bacteraemia in clinically affected patients (*e.g.* pyrexial patients postoperatively). Only three studies looked at the incidence of bacteraemia in all patients undergoing TURP, irrespective of clinical symptoms. They are discussed further in Section 1.6.3.3.6. Therefore, it is hard to put a definite value on the incidence of bacteraemia during TURP. The most common organisms cultured in patients with perioperative bacteraemia are listed below, with Gram-negative bacteria being the most common [59, 66, 71, 72]:

- *Escherichia coli*;
- *Enterococcus faecalis* (previously *Streptococcus faecalis*);
- coagulase-negative staphylococci (previously *Staphylococcus albus*);
- *Proteus mirabilis*;
- *Klebsiella* spp.;
- *Pseudomonas aeruginosa*.

1.6.3.3 Infection and TURP

As the literature is sparse concerning studies of asymptomatic bacteraemia in patients undergoing the TURP operation, it is worthwhile reviewing the literature regarding infection associated with TURP. Infections described following TURP include UTIs, peri-prosthetic abscess [73], sepsis, septic shock and infective endocarditis [74]. Tables 2-6 show the most commonly reported infective outcome variables in the literature.

1.6.3.3.1 Bacteriuria

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of Bacteriuria (%)	Main Organisms
Jackaman *et al.* [69]	Retrospective	76% sterile	140	Not known	10	Not known
Mayer *et al.* [70]	Prospective, randomised	75-78% sterile	92	Given	Bactrim: 17.0 Placebo: 20.5	Not known
Collste *et al.* [71]	Prospective	71% sterile	107	Not known	Sterile urine: 32 Infected urine: 100	Not known
Gordon *et al.* [72]	Prospective	100% sterile	122	Nil	28	*Staphylococcus epidermidis*
Botto *et al.* [73]	Prospective, randomised	Not known	176	Cefotaxime Placebo	16.1 57.5	Not known
Grabe *et al.* [74]	Prospective, randomised	Not known	47 49	Cefotaxime Placebo	48 63	Not known

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of Bacteriuria (%)	Main Organisms
Grabe *et al*. [71]	Prospective, randomised	60.9% sterile	179	Cefotaxime Placebo	12.2 26.6	*Escherichia coli* *Enterobacter* sp. *Klebsiella oxytoca* *Proteus mirabilis* *Enterococcus faecalis* *Staphylococcus epidermidis*
Grabe *et al.* [81]	Prospective randomised	Bacteriuria 43% 40%	98 94	Cefotaxime Placebo	18 42	Not known
Charton *et al*. [82]	Prospective, randomised	100% sterile	49 51	Antibiotics Placebo	18.4 51.0	Not known
Fujita *et al.* [83]	Prospective	71.7% sterile	46	Cefotaxime	8.7	Not known
Okamura *et al.* [84]	Prospective	Not known	54	Not known	30	Not known

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of Bacteriuria (%)	Main Organisms
Charton *et al.* [85]	Prospective, randomised	100% sterile	47	Netilmicin	2	Not known
			48	Placebo	34	
Taylor *et al*. [86]	Prospective, randomised	79.9% sterile	158	Temocillin	33	Gram-positive in treatment group
			150	Placebo	87	
Adolfsson *et al*. [87]	Prospective, randomised	0% sterile	343	Trimethoprim+ sulfamethoxazole	21.9	Not known
				Norfloxacin	21.7	
Lepage *et al.* [88]	Prospective, randomised	100% sterile	200	Cefotiam	16	Not known
				Cefazoline		

Table 2. Studies reporting the incidence of bacteriuria following TURP

Bacteriuria, the presence of bacteria in a urine sample, is the most commonly reported infective outcome following TURP. Most studies define bacteriuria as $> 10^4$ colony forming units (cfu) per mL of urine. The rates vary depending on whether antibiotic prophylaxis was used, the sterility of the urine pre-procedure and which definition of bacteriuria has been used. There is a wide range of reported incidence of bacteriuria, as the patients, methods to assess bacteriuria and the reporting of results was heterogeneous. The literature supports the notion that sterile urine preoperatively leads to a lower risk of the patient developing bacteriuria post-operatively. Furthermore, if antibiotics are used as prophylaxis, the incidence of bacteriuria decreases further as shown above. However, it is debateable how important an infective outcome variable bacteriuria is and this will be discussed further in the section 5.3.3.2.

1.6.3.3.2. Urinary Tract Infection (UTIs)

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of UTIs (%)
Gibbons *et al.* [89]	Prospective randomised	All were sterile	100	Antibiotics	Same in both groups
				No antibiotics	
Shah *et al.* [90]	Prospective randomised	Not known	50	Placebo	28
			50	Cephalexin	8
			50	Co-trimoxazole	16
			50	Carfecillin	28
Goldwasser *et al.* [64]	Prospective randomised	Sterile	81	Sulfamethoxazole-trimethoprim (10/7)	3.8
				Sulfamethoxazole-trimethoprim (1/7)	3.8
				Nil	32
Ferrie *et al.* [91]	Prospective randomised	All were sterile	58	Cefuroxime	3.4
				Placebo	6.8
Grabe *et al.* [80]	Prospective randomised	Not known	47	Cefotaxime	2.1
			49	Placebo	10.2

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of UTIs (%)
Finkelstein *et al.* [92]	Prospective randomised	Not known	66	Ceftriaxone	3.0
			63	Placebo	12.7
Grabe *et al.* [81]	Prospective randomised	Bacteriuria			
		43%	98	Cefotaxime	0
		40%	94	Placebo	7.4
Joly-Guillou *et al*. [72]	Prospective randomised	All were sterile	87	Cefotaxime	30
				Placebo	4
Dorflinger *et al.* [93]	Prospective, randomised	Not known	52	Sulbactam+ Ampicillin	8
			51		
				Cefoxitin	4
Prokocimer *et al.* [94]	Prospective, randomised	All were sterile	90	Cefotaxime	30
				Placebo	4
Fujita *et al*. [95]	Retrospective	All were sterile	1251	Heterogeneous	17

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of UTIs (%)
Taylor *et al.* [86]	Prospective, randomised	All were sterile	235	Temocillin	13
				Placebo	24
Stricker *et al.* [96]	Prospective, randomised	All were sterile	100	Ampicillin+ Gentamicin	17
				Placebo	16
Desai *et al.* [97]	Prospective, randomised	Not known	40	Enoxacin	8
			40	Placebo	38
Baert *et al.* [98]	Prospective, randomised	Not known	31	Fosfomycin trometamol	0
			30	Placebo	20

Table 3. Studies reporting the incidence of UTIs following TURP

A urinary tract infection (UTI) is defined as an inflammatory process caused by infectious agents in the urinary tract; it is associated with bacteriuria [93]. A patient should have at least one of the following symptoms or signs, with no other recognised cause to fulfil clinical criteria for a UTI:

- fever> 38° C in a patient aged ≤65 years of age;
- lower urinary tract symptoms (urgency, frequency, dysuria, suprapubic tenderness, loin pain);
- a positive urine culture of ≥ 10^5 cfu/mL with no more than two species present.

This is a contemporary definition and many of the earlier studies investigating UTIs used very broad and heterogeneous definitions of a UTI. Owing to the various definitions of UTI, the literature reports a rate of UTI ranging from 0% to 38% following a TURP. The literature also supports the use of antibiotic prophylaxis to reduce the rate of UTI post TURP as shown in Table 3 above.

1.6.3.3.3. Fever

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of Fever (%)
Gibbons *et al*. [89]	Prospective randomised	100% Sterile	100	Antibiotics	Same in both groups
				No antibiotics	
Nielsen *et al*. [100]	Prospective randomised	100% sterile	110	Cefoxitin	3.9
				Placebo	26.4
Olsen *et al*. [101]	Prospective randomised	0% sterile	22	Cefotaxime	4.5
			20	Methenamine hippurate	45
Qvist *et al*. [102]	Prospective randomised	100% sterile	54	Cefotaxime	13.3
			43	Placebo	18.6
Prescott *et al.* [58]	Prospective randomised	Not known	196	Cephalosporin	0
				Antiseptic	1.7

Table 4. Studies reporting the incidence of fever following TURP

Fever post TURP has been reported in a number of studies, with most studies using a temperature of > 38º C as the cut-off point. Up to 45% of patients receiving no antibiotic prophylaxis during their TURP operation suffer fever and antibiotic prophylaxis decreases the incidence of fever.

1.6.3.3.4. Sepsis

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of Sepsis (%)
Schonebeck *et al.* [103]	Prospective, randomized	0% sterile	37	Cephalexin (10/7)	5.4
			42	Methenamine hippurate (10/7)	16.7
Murphy *et al.* [104]	Retrospective	Mixed	1604	Different	1.1 (18)
Grabe *et al.* [80]	Prospective, randomised	Not known	47	Cefotaxime	4.2
			49	Placebo	4.1
		Bacteriuria			
Grabe *et al.* [81]	Prospective, randomised	43%	98	Cefotaxime	0
		40%	94	Placebo	1.1
Shearman *et al.* [62]	Prospective, randomised	81% sterile	110	Ciprofloxacin	1.8
				Placebo	9.1

Table 5. Studies reporting the incidence of sepsis following TURP

Sepsis is defined as the association of a non-specific systemic inflammatory response with evidence, or suspicion, of a microbial cause [105]. This contemporary definition originates from the early 1990s and has remained largely unchanged. However, prior to this, sepsis was defined very loosely. Therefore, comparing contemporary studies to historical studies may not necessarily be addressing the same issue. Sepsis incidence of up to 17% have been reported post TURP, with the presence of a sterile urine sample preoperatively decreasing the chance of developing sepsis post-operatively.

1.6.3.3.5 Bacteraemia in symptomatic patients

Reference	Type of Study	Pre-procedure urine	Number of patients	Antibiotic Prophylaxis	Rate of Bacteraemia (%)
Gordon *et al.* [78]	Prospective	All sterile	122	Nil	3.3
Botto *et al.* [79]	Prospective, randomised	Not known	176	Cefotaxime	0
				Placebo	2.3
Charton *et al.* [85]	Prospective, randomised	All sterile	47	Netilmicin	0
			48	Placebo	2.1

Table 6. Studies reporting bacteraemia rate post TURP

The above studies found a bacteraemia rate between 0 and 3.3% in patients who have symptoms of sepsis after TURP. In these studies, blood cultures were taken in symptomatic patients diagnosed with sepsis after TURP, after they have had antibiotic prophylaxis intraoperatively. The incidence of bacteraemia was very low.

1.6.3.3.6 Bacteraemia and TURP: Asymptomatic patients

The following studies have specifically looked at intra- and peri-operative bacteraemia associated with TURP, irrespective of symptoms. Neilsen *et al*. reported a transient bacteraemia incidence of 46% in patients undergoing TURP, without antibiotic prophylaxis. The recruitment process and microbiological methodologies were not clearly defined [70].

In the 1980s, Robinson *et al.* showed a 36% rate of perioperative bacteraemia in patients undergoing TURP without antibiotic prophylaxis. 5 mL of blood was collected from the antecubital fossa at fifteen minutes after the start of surgery, then on Day 1 and Day 2. The blood cultures were incubated up to 14 days and subcultured at Days 2, 7 and 14 post-operatively [59]. The rate of bacteraemia reflects the surgical practice as well as the microbiological armamentarium available at the time.

Ibrahim *et al.* conducted a randomised controlled trial of patients having TURP [106]. They collected one intraoperative blood sample (5 mL) for culture just

before the end of the operation in a heterogeneous group of one hundred and sixty eight patients with sterile pre-operative urine. Of the one hundred and thirty patients who received no antibiotic prophylaxis, seven (5.4%) developed bacteraemia. None of the thirty-eight patients who received 1 g of IV cephradine developed intraoperative bacteraemia. This study claimed that intraoperative bacteraemia was not linked to postoperative infective complications like septicaemia or epididimo-orchitis. However, the study was designed to collect only one intraoperative blood sample for culture with no negative controls. Though this study showed that antibiotic prophylaxis reduced the incidence of intra-operative bacteraemia, it was not designed to show an association between intraoperative bacteraemia and post-operative infective complications.

The literature is deficient with respect to studies specifically designed to assess bacteraemia related to TURP. Most studies report bacteriuria, UTIs and fever post-TURP; if patients were suspected to have a bacteraemia clinically (*e.g.* fever or rigors), blood cultures were then taken to determine the presence of bacteraemia. Thus, 'symptomatic bacteraemia' has been identified but not studied systematically. By reviewing the literature, it is clear that there is a need for a well-designed study to look at the incidence of bacteraemia associated with a TURP.

1.7 Bacteraemia and Catheter Manipulation

The development of bacteraemia at urethral catheter removal is a well-documented event. Urethral catheters get colonised with multiple microorganisms within 24 hours of insertion and this leads to a 5% increase in risk of bacteriuria per day that the catheter stays *in situ* [38, 107, 109]. Bacteraemia has been noted in 1-4% of catheterised patients with bacteriuria, with certain risk factors like immunosuppression and older age being significant contributory factors [110]. Urethral catheterisation may lead to bacteraemia [110], with 17% of nosocomial bacteraemia being associated with catheterisation [111]. Having a long-term urethral catheter is the leading cause of Gram-negative bacteraemia [112]. The following studies have specifically looked at bacteraemia associated with catheter change and removal.

Ibrahim *et al.* demonstrated bacteraemia in six out of one hundred and sixty eight patients (3.6%) undergoing catheter removal after a TURP [106]. A 5 mL volume of blood was taken for culture just after catheter removal in patients who did not receive any antibiotics prophylaxis for their catheter removal. None of the patients was symptomatic. However, this cohort of patients had a recent

TURP and this may be a confounding factor, assessing bacteraemia in a recently instrumented urological tract, rather than catheter removal *per se*.

Bregenzer *et al.* showed a 4.2% rate of bacteraemia in a geriatric cohort of patients undergoing catheter change [113]. Blood cultures were drawn before and at 5, 15, and 30 minutes after the catheter replacement. Preoperative urine cultures showed growth of one to five different microorganisms. Of four hundred and eighty blood cultured in one hundred and twenty patients, twenty-seven blood cultures (5.6%) were positive. However, the same species grew from blood and urine in only five patients, with no patients having any symptoms suggestive of systemic sepsis. This was a well-designed study.

Polastri *et al.* conducted a prospective study, recruiting thirty-three patients for urinary catheter change [114]. All patients had pre-procedure bacteriuria. Quantitative blood cultures (Isolator) were performed at two time points (during and shortly after urinary catheter removal and insertion). Two out of the forty-six blood cultures were positive and at a very low concentration; one patient had 0.13 cfu/mL *Enterococcus faecalis* (known as *Streptococcus faecalis* at the time) in the blood sample taken 5 minutes after removal of the urinary catheter, and the patient had 0.1 cfu/mL *Proteus mirabilis* 5 minutes after reinsertion of a new urinary catheter. None of the patients were symptomatic. Although this study was small, it described low-level asymptomatic bacteraemia in relation to catheter manipulation for the first time.

Jewes *et al*. conducted a prospective study of one hundred and fifteen patients having one hundred and ninety-seven catheter changes in the community [115]. A pre-procedure urine sample and a 10 mL post-procedure blood sample were taken. Urine was cultured on CLED agar and the blood was incubated in broth for seven days and sub-cultured at Day 2 and Day 7 following inoculation. All patients had polymicrobial bacteriuria. Twenty episodes of bacteraemia, all asymptomatic were noted (10.2%), five of which were polymicrobial.

Again, it must be noted that there are very few studies designed specifically to determine the incidence of bacteraemia associated with catheter manipulation. Most studies are retrospective and report bacteraemia in patients with urinary catheters *in situ*, who present with fever and rigors. In 31 the patients who are asymptomatic, the level of bacteraemia described was very low. One surprising point that emerges from the literature is that bacteraemia and bacteriuria tend to show different isolates. There is the need for a well-designed study to assess the incidence of bacteraemia when catheters are manipulated and its association with bacteriuria.

1.8 Methodology to detect bacteraemia

The vascular system is a sterile closed system and the persistent presence of bacteria in blood is abnormal. 'Bacteraemia' is a term used to describe the presence of bacteria in blood; the number of bacteria per unit volume of blood is irrelevant in the definition of bacteraemia [116]. One viable bacterium is sufficient for bacteraemia to exist.

Blood is a potent medium for proliferation of bacteria, which are pathological to humans, as it is maintained at the optimal temperature and pH. The constituents of blood provide a ready source of energy for bacteria to reproduce [117]. However, blood is an integral part of the immune system that helps the body to fight bacterial proliferation [118]. Figure 3 below shows the basic immune functions of blood responsible in fighting against bacterial proliferation.

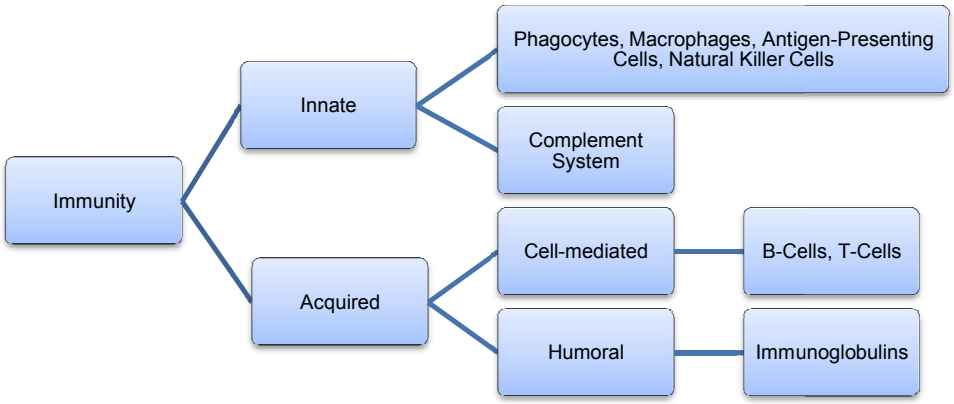


Figure 3. Immune pathways in blood

In spite of this extensive mechanism to fight bacterial proliferation in blood, bacteraemia still occurs. There are several ways to detect bacteraemia in blood, with the traditional 'sampling, incubation and culture' being the gold standard. In order for a diagnostic test to be useful, it should have a high sensitivity, a high specificity and a high positive and negative predictive value [120].

Many other avenues have been explored to detect bacteraemia namely surrogate markers of host response to infection (acute-phase products like C-reactive protein, haematological products like neutrophil count, and other biomarkers including proteomic and genomic markers), PCR-based molecular techniques [120]. This chapter addresses the two methods used to detect bacteraemia, namely the gold standard incubation/culture and the PCR-based molecular technique.

1.8.1 Incubation and Culture

Incubation and culture is regarded as the gold standard technique to detect bacteraemia [121]. A standard protocol for the detection of bacteraemia involves the use of a blood culture system, which should [122]:

- include a culture medium as rich as possible to allow the recovery of very small numbers of a variety of fastidious organisms;
- neutralise antimicrobial substances, either natural blood components or antimicrobial agents;
- minimise contamination;
- allow the detection of bacteria within a reasonable time-frame.

Traditionally, after a specific period, the broth is inspected for its appearance. Microscopy and Gram staining are then performed on a sample of the broth. Broths suspected to be positive are sub-cultured on solid agar. If growth is noted, the growth environment (aerobic and/or anaerobic) and the morphological appearances of the colonies are reported. Gram staining and microscopy are the preliminary tests done on the bacterial colonies before proceeding to more specific biochemical and molecular tests. With the advent of automated systems, the culture bottles are handled slightly differently.

Blood culture systems range from ‘in house’ and commercial manual to fully automated systems. These systems rely on a variety of detection principles and cultural environments to detect bacteria [123]. Most systems utilise both aerobic and anaerobic bottles. The identification of bacteria is affected by a number of clinical factors (method of collection, the number and the timing of samples, the site(s) sampled and previous antimicrobial therapy) and technical factors (the volume of sample, the culture media, neutralisation of antimicrobial agents, incubation time and temperature, agitation of media and headspace atmosphere) [124]. Collection of blood is performed from a suitable venepuncture site, following disinfection of the skin.

The volume of blood collected for incubation plays an important role in the detection of bacteraemia, with an increased volume leading to an increased bacterial yield [125]. The culture media also play an important part in the isolation of bacteria with the ratio of the supplements in the broth, the volume of the broth, the headspace above the fluid broth and the presence of antimicrobial neutralising agents all affecting the outcome. The ratio of blood to broth is also important in determining the viability of the bacteria [124].

The culture media is normally incubated at a temperature of 35° C to 37° C for five to seven days, though a longer incubation time is advocated if fastidious organisms are suspected [124]. The headspace of the culture bottles is an important variable in determining bacterial growth. Aerobic bottles normally have a combination of oxygen and carbon dioxide while anaerobic bottles have carbon dioxide and nitrogen. Agitation of the culture bottles during the incubation period has been shown to increase the yield and time to positivity for aerobic growth [126].

Subculture of culture bottles depends on whether an automated or a manual system is used for the incubation phase. In an automated system, the 'positive' bottles will be flagged up and these bottles are subcultured. Automatic systems flag up positive bottles by the measurement of a change in the components of the broth (for example, carbon dioxide production, increase in fluorescence) [127]. In a manual system, either all bottles are subcultured or bottles with signs of positivity (cloudiness) are subcultured. Subculturing all bottles increases the likelihood of detecting organisms like *Neisseria* spp. and *Haemophilus influenza,* which show little visible signs of growth in the incubated culture bottles [128].

1.8.2 Molecular Assays

Molecular methodology has revolutionised the field of virology over the last two decades. However, in the bacterial field, molecular microbiology is still, rather surprisingly, in its infancy. Broadly speaking, three types of strategies have been described [129]:

- pathogen-specific assays targeting species or genus-specific sequences;
- broad-range assays targeting conserved sequences in the bacterial genome, (panbacterial 16S and 23S rRNA genes);
- multiplex assays allowing the parallel detection of species or genus-specific targets of different pathogens potentially involved in a certain infection type.

Quantitative real-time PCR is a system where amplification and detection of amplified products happens in a single vessel. Two strategies exist for real-time monitoring [130]:

- the use of fluorescent DNA-intercalating dyes, which bind non-specifically to double-stranded DNA generated during amplification;

- the use of DNA probes with specific annealing within the target-amplified region.

The internal probes emit a fluorescent signal during each amplification cycle only in the presence of targeted sequences, with the signal intensity increasing in proportion to the amounts of amplified products generated. Real-time PCR allows for absolute and relative quantification of the target sequence. However, not all molecular methods require the amplification method or PCR [131]. Mass spectrometry (MS) uses the signatures of peptides and carbohydrates or the length, charge and base contents of the nucleic acids present for identification [132]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a contemporary technology for the routine identification of bacteria in clinical microbiology laboratories [133].

1.8.2.1 Bacterial DNA Isolation

To detect and identify the bacteria present in blood, it is imperative to separate the bacteria from the blood products [134]. This is because blood has an inhibitory effect on the different molecular steps [135]. Most methods of DNA isolation need to be carried out as soon as the sample is collected as DNA degradation is noted. Most methods require DNA isolation to take place within twenty-four hours of collection [134]. Once the bacterial DNA is isolated, it needs to be purified to a level that most PCR-inhibitory components are removed. The human genome results in significant signal to noise problems during PCR, which already has inherent limitations like the presence of *Taq* DNA polymerase inhibitors [136]. Another factor that influences PCR is the ratio of bacterial DNA to background DNA, which in the case of blood relates to the human DNA from the blood products [137].

Possible interference in the detection of bacterial DNA can be attributed to human DNA as it has been shown that both have similar base codons and determinants, probably secondary to the evolution [138]. Furthermore, lateral translocation of genes from bacteria and human genome has also been demonstrated, which may increase the false positive rate during bacterial DNA PCR [139].

There are a number of commercially available kits for the isolation of bacterial DNA from blood, for example MolYsis, Molzym GmbH & Co. KG, Bremen and Pureprove, SIRS-Lab GmbH, Jena [134, 140]. Most protocols for the isolation of bacterial DNA have been shown to decrease the amount of human DNA significantly, when isolating bacterial DNA from human samples [141]. However, most of the protocols have limitations, reducing the bacterial yield at the end of

the process. All protocols consist of chaotropic conditions during the DNA degradation stage by DNase [142]. These conditions are very harsh and some bacteria with non-existent, thin or labile cell walls may be lost together with human DNA during this stage. This bacterial isolation process may be biased towards the detection of Gram-positive bacteria, owing to the presence of large amounts of peptidoglycan in their envelopes. The Pureprove technology is based on the selective binding of bacterial DNA to a human protein immobilised on sepharose. Though present more often in bacterial DNA than human DNA, the binding site is not ubiquitous in bacteria. This may lead to the washing away of bacterial DNA together with the human contaminants [141].

1.8.2.2 Bacterial DNA Amplification

Polymerase chain reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double-stranded DNA molecule. PCR entails the use of a pair of primers, each about 20 nucleotides in length, which are complementary to a defined sequence on each of the two strands of the DNA. A DNA polymerase extends these primers, so that a copy is made of the designated sequence, which overlaps on extension [143]. After making one copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to exponential amplification [143].

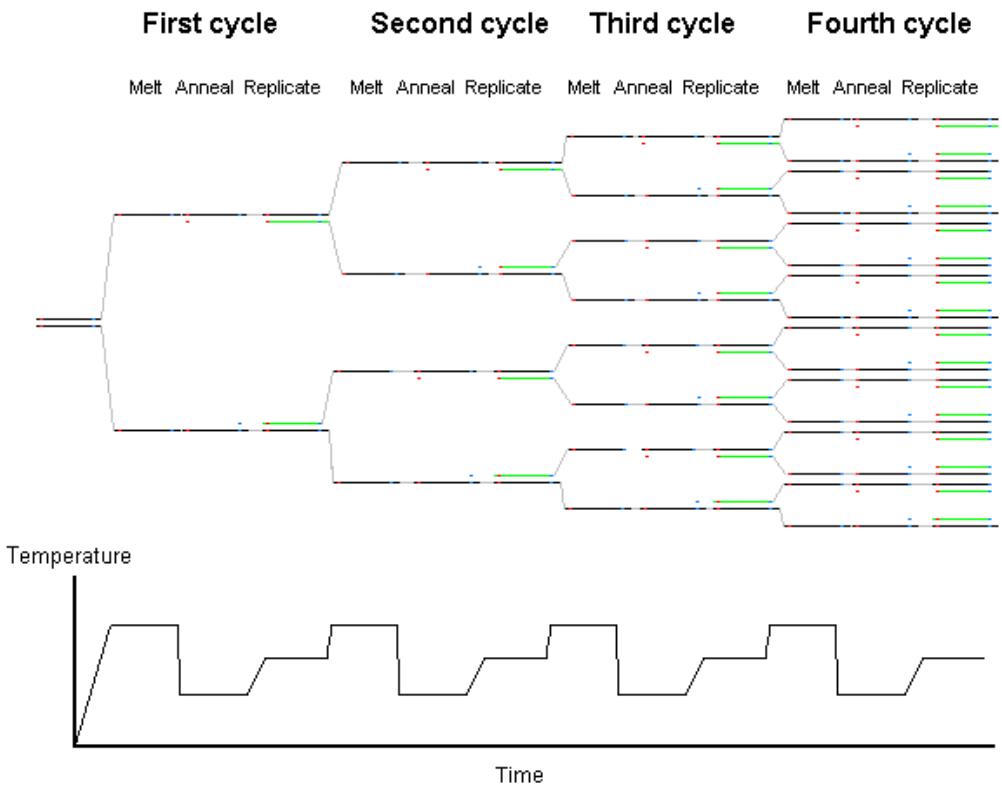


Figure 4. The first four cycles of the polymerase chain reaction

Since it is necessary to raise the temperature to separate the two strands of the double strand DNA in each round of the amplification process, a major step forward was the discovery of a thermo-stable DNA polymerase (*Taq* polymerase), isolated from *Thermus aquaticus,* a bacterium that grows in hot pools. As a result, it is not necessary to add new polymerase in every round of amplification [143]. After several (around 20-40) rounds of amplification, the PCR product is analysed. Chromatography and 16S rRNA sequence determination are the methods that are commonly used [143]. A PCR can be divided into 4 broad phases as shown in the graph below (Figure 5) [144].

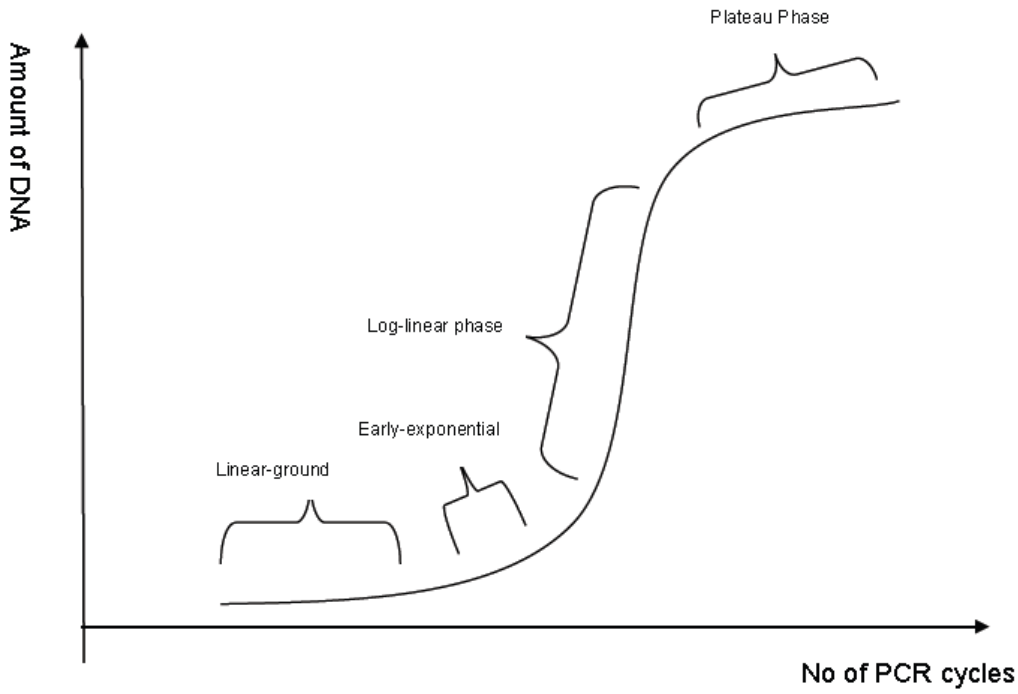


Figure 5. A sigmoid curve showing a typical amplification

The four phases are described below:

Linear phase: During this phase, amplified fluorescence is still below background fluorescence. This normally lasts 10-15 amplification cycles.

Early-exponential phase: The cycle threshold (C_t) value lies in this phase, when amplified fluorescence exceeds background fluorescence. Usually, the fluorescence reaches at least 10 times the standard deviation of the baseline at this point [145].

Log-linear phase: During this phase, the polymerase chain reaction has reached optimal conditions, whereby the amplified products double after each cycle (assuming ideal efficiency of the process).

Plateau phase: During this phase, there are various limiting factors, which restrict the efficiency of the process. For example, the amount of primers to replicate the initial DNA has been used up.

1.8.2.2.1 Real-Time PCR

In real-time PCR, a positive reaction is detected by the accumulation of a fluorescent signal [144]. The cycle threshold (C_t) value is defined as the number of cycles required for the fluorescent signal to cross an arbitrary threshold, which can be defined automatically or manually. This arbitrary value is the value

at which the background fluorescence is exceeded. C_t values are inversely proportional to the amount of target nucleic acids present [144].

Real-time PCR has revolutionised the way in which data are collected during the PCR process. By collecting data at every single step of the process, real-time PCR has combined two previously separate steps (amplification and detection) into one; this constant output from the PCR process allows for the dynamic detection curve plotted above to be achieved [144]. In SYBR Green PCR, a dye that fluoresces maximally when bound to double-stranded DNA is used. However, DNA-binding dyes do not bind in a sequence-specific manner: therefore, there is always the chance of false positive reactions [145]. Real-time PCR has the added benefit of allowing quantification, without significant post-amplification manipulations. However, it is expensive and owing to its extreme sensitivity, proper experiment design is crucial to have the added benefit of high specificity. A melting curve is one of the parameters used in determining specificity [146].

1.8.2.3 Controls

In an experiment, a control is a known variable, which is included to provide a reference set of data that can be compared with the data obtained from the experimental variables [147]. An ideal control is a variable used to account for all the confounding factors except the variable being measured. To ensure that the change noted in the target variable is due to the reason being tested, a controlled experiment is run [148]. A control group is an additional and separate experiment, set up like the others with exactly the same conditions. The only difference is that test variable is changed [148]. Dependable controls are sometimes hard to develop. Without a control, it is impossible to be completely sure that changing the variable causes the observations seen. In molecular microbiology, controls are used in all the various steps from bacterial DNA extraction to sequence determination.

During the bacterial extraction process, extraction and purification controls are used. The controls required are [149]:

- sample-positive control. This control reflects the performance of the extraction process;
- sample-negative control. This control helps to control for cross-contamination during the extraction and purification process.

During the polymerase chain reaction, a number of controls are used to ascertain that bacterial DNA is amplified and detected [150].

- PCR-positive control. This test includes a definite number of target sequence copies to make sure the assay is performing as specified This helps to normalise samples to account for sample-to-sample variation with regards to tissue mass or cell number;
- PCR-negative control. This control contains all the reagents except a target DNA. DNA free water is used instead of the eluate. This control helps to detect any exogenous DNA, which might have been introduced and carried over while pipetting the reagents;
- PCR internal control. Potential inhibition of the PCR process by blood components, which might have been carried over from the extraction and purification process is accounted for by this control.

The meticulous use of the same amount of PCR mastermix should be used for all the experiments in order to standardise the whole process further. Variation in this volume has been shown to deliver different PCR efficiencies though the starting concentration of DNA is the same [151]. As can be seen above, the whole process is fraught with problems at different stages. A tiny variation at the beginning of the process may end up being amplified to significant scales that the results are distorted. Below is a list of the other possible causes of variations that can happen from the initial step of DNA extraction to the final step of amplification and identification.

- PCR inhibitors like the reverse transcriptase enzyme or blood. These may affect the PCR kinetics [136];
- the structure and concentration of the target DNA [152];
- the quality and amount of PCR reagents [152];
- the conditions at which the PCR equipment operates. For example, a slight difference in temperature when measuring fluorescence can lead to a difference in the amount of DNA detected [152];
- human error is by far the most likely cause of variation. Running a standard curve during each reaction may improve this problem though it is very time and labour intensive [153].

The use of the same batch of suspensions and equipment as well as the use of a single investigator help minimise variation in the whole process [154]. Quantifying intra- and inter-assay variation gives a more accurate picture of the actual process.

1.8.2.4 Identification of bacterial DNA

Once the bacterial DNA has been amplified, the amplimers can be identified by a number of methods. In this chapter, two main methods of identification are addressed.

1.8.2.4.1 Sequence determination

Nucleotide sequence determination has been a technology that was first described in 1975 by Sanger in the Croonian lectures at the Royal Society [155]. The technology was subsequently commercialised and made available to the global research community. Applied Biosystems (ABI) commercialised a number of instruments during the 1990s, with a gradually increasing amount of automation [156]. This made the sequence technology less labour and time intensive. Next generation sequencing (NGS) was introduced in the early part of this millennium and has since gained in popularity, mainly through its ability to bring down costs and increase the throughput. This has permitted the determination of entire genome sequences. The second generation NGS can produce up to a billion bases in a single run (Illumina SOLiD). The leading technologies of the second generation NGS are [157]:

- *Illumina*, which uses solid phase amplification and sequences by synthesis and reads around 100 bases;
- *Roche*, which uses emulsion PCR technology and pyro-sequencing and reads around 400-500 bases;
- *ABI* makes use of emulsion PCR and ligation sequencing. It reads around 50 bases.

The third-generation NGS has shown considerable promise and circumvents one of the problems of previous technologies, by not using the PCR step. Previous generations of NGS use PCR, which can introduce base sequence errors owing to the differential amplification of bases. Therefore, by avoiding the PCR phase before identification, third generation NGS allows identification without amplification [158]. The main leading technologies of third generation NGS include [158]:

- *Pacific Biosciences*. This technology involves the incorporation of DNA polymerase with zero-mode wave guides, and then utilises a labelled nucleotide by diffusion. DNA detection happens with the detection of the labelled polymerase;

- *Oxford Nanopore Technologies*. This technology utilises the incorporation of α-haemolysin with exonuclease. A current is used for DNA detection;
- *Visi Gen Biotechnologies* incorporating labelled nucleotide to modified quantum polymerase, which releases energy from a fluorescent labelled base. DNA is identified by light emission;
- *Ion Torrent* uses a semiconductor technology and sequencing detection happens by the release of hydrogen ions during the elongation of the nucleotide chain.

In-depth details of the sequencing determination processes mentioned above is outside the scope of this thesis.

1.8.2.4.2 Mass Spectrometry (MS)

MS is still a novel technology for the detection and identification of bacteria. The basic principle of MS involves the detection of mass to charge ratio of compounds [159]. At present, no system is available for the detection of bacteraemia using MS [160]. However, matrix-assisted laser desorption ionisation (MALDI) time-of-flight (TOF) MS is a relatively recent technology. It utilises the molecular fingerprinting of bacteria, whereby bacteria can be detected by virtue of the different molecular masses of the nucleic acids fragments that constitute them. It has been successfully used in the quick identification of bacteria from colonies [161].

1.8.3 Culture method *versus* Molecular method

Both the culture and the molecular methods have their advantages and disadvantages. One of the common factors to determine positivity in both methods used for the detection of bacteraemia is the volume of blood used. The higher the volume of blood used, the greater the likelihood of detecting bacteria, whether the method used is bacterial growth or bacterial DNA amplification [162]. Another important factor is the time from blood sampling to the time the sample is processed (incubation or bacterial DNA extraction). The sooner processing starts, the higher the positivity rate [163].

One of the advantages of the culture method is it allows the determination of antibiotic susceptibility, which has yet to be reliably reproduced using the molecular methodology [164]. However, the culture method has it disadvantages. It allows detection of only viable bacteria that can be cultured [165]. Therefore, it does not detect the presence of dead bacteria or viable but

non-cultivable pathogens. In the instance when antimicrobial therapy has been started prior to sampling, viable bacteria may not be present in blood, although dead bacteria may be present [166]. Furthermore, fastidious organisms that require very specific conditions to grow may not be detected by the culture method [130]. Once growth is detected in the culture bottle after 24 to 48 hours of incubation, the final identification of the organism by phenotypic characteristics adds a significant time to the processing time. Therefore, the culture method is a process that can be slow to provide definitive results [167].

On the other hand, the molecular method has its own disadvantages. Firstly, the setup is prone to contamination and antimicrobial susceptibility is not possible (thought antimicrobial resistance can be determined by detecting the presence of targeted genes) [168]. However, it detects 'DNAemia' rather than bacteraemia, which allows detection of dead bacteria in the patients undergoing antimicrobial therapy [169]. Furthermore, it is possible to quantify the DNA load and, by inference, quantify the bacterial load [170]. And most significantly, there is no prolonged incubation phase in the molecular processing of the samples, making the molecular method a faster method than the culture method, though it can be more labour-intensive and considerably more expensive when the set-up costs are taken into account [168].

1.9 Conclusions

IE is a condition associated with significant morbidity and mortality. There is some evidence that IE can be caused iatrogenically. However, there is no evidence that urological instrumentation leads of the development of IE. The presence of bacteria in the vascular system is essential in the development of IE. There is evidence that instrumentation of the urinary tract can lead to symptomatic bacteraemia. However, the true incidence of bacteraemia, symptomatic or asymptomatic, associated with urological instrumentation is unknown. Therefore, at present, it is not possible to determine whether urological instrumentation leads to IE though there is low-level evidence suggesting this association.

This study aims to explore different methods to detect bacteraemia. It is abundantly clear from the literature that for a large proportion of septic episodes, an infective pathogen is not identified. Therefore, there is a need to devise a method with high sensitivity and specificity. Results reported in this thesis show that appropriate methods were used to determine the incidence of bacteraemia in patients having TURP and catheter manipulation, two of the

most common urological procedures in modern urology. To determine whether instrumentation of the urinary tract precedes the development of infective endocarditis, a novel approach case-control study was used.

Chapter 2: An association between urological instrumentation and infective endocarditis

2.1 Introduction

Designing a study to investigate IE is a difficult prospect owing to its low incidence [171]. This is why little is known about the associated risk factors that can lead to the development of IE. Allocating a matched control group for a case-control study can be problematic from a study-design point of view. A particular difficulty is selection of patients in the control group with the same predisposing cardiac risk factors as the case group [172]. A study was designed to evaluate the iatrogenic risk factors associated with the development of IE. The null hypothesis that instrumentation of body cavities (the upper and lower gastrointestinal tract and the urinary tract) is not a risk factor for the development of IE was tested. Owing to the difficulty associated with designing such a study, a novel approach to the traditional case-control design was used. The objective was to identify any association between instrumentation and the subsequent development of IE.

2.2 Methods

2.2.1 Justification of Approach

Ethical approval for the study was granted by the Yorkshire and Humber Research Ethics Committee - Bradford (reference 11/YH/0093). Owing to the relative rarity of IE, It was decided that a retrospective approach was the best way to obtain a large cohort of patients. A case-control design was chosen as an appropriate method to analyse risk in an observational epidemiological study [172]. The novel approach to the case-control design used was to select both the ‘case’ and ‘control’ group patients from a population of patients with IE. The outcome measure was IE caused by selected pathogens. A number of possible causative exposures were identified and their rates calculated. In this investigation, the main causative exposures being investigated were instrumentation (urological, upper and lower gastrointestinal).

The IE patients were divided into four distinct patient groups based on the microbiological aetiology of the disease. The four groups were:

- Group A: patients with enterococcal endocarditis
- Group B: patients with coagulase-negative staphylococcal (CoNS) endocarditis
- Group C: patients with *Streptococcus bovis* group endocarditis
- Group D: patients with oral streptococcal endocarditis

Therefore, when Group A (the case group) was being evaluated; Group B, C and D were used as controls (the control group). This approach (assigning case and control groups) was applied to the three other groups.

Case Group	Control Groups
A	B, C and D
B	A, C and D
C	A, B and D
D	B, C and D

Table 7. Table showing the cases and the controls used

This approach to the case-control design, which only included patients who had suffered an episode of IE, bypassed the problem in previous case-control studies of selecting a suitable control group of patients at risk of IE [173- 175]. This is because all patients in this study have developed IE and therefore have predisposing factors to the development of IE. The study design utilises the fact that the normal human microbiota is specific to an anatomical site. Oral streptococci colonise the oral cavity [176, 177] but are rarely cultured from the skin, faeces or urinary tract [178]. The natural habitat of *Streptococcus bovis* group organisms is the bowel, and can be found in 5-16% of stool samples of healthy individuals [179]. However, they are not part of the normal oral microbial flora and rarely cause urinary tract infections [180, 181]. Enterococci are part of the normal microbiota of the lower gastrointestinal tract, with a mean of 10^5-10^7 organisms per gram of human stool [182]. However, enterococci can colonise the mouth, particularly in patients with poor oral health [183] and are a common cause of urinary tract infection [184]. Coagulase-negative staphylococci are members of the normal human skin microbiota and mucous membranes [185]. They are associated with hospital-acquired infections, usually related to medical

devices and community-acquired urinary tract infections *e.g.* caused by *Staphylococcus saprophyticus* [185]. It is highly unlikely that IE caused by bacteria of the *Streptococcus bovis* group could result from a dental procedure because these bacteria reside in the bowel and are not found in the mouth. Similarly, it is highly unlikely that oral streptococcal IE would result from of a lower gastrointestinal procedure. The source of the organism for enterococcal endocarditis is likely to be urological. Coagulase-negative staphylococcal IE is believed to come from the skin arising from vascular access device infections or valve surgery [185, 186].

2.2.2 Population

Adult patients treated for IE at the Leeds Teaching Hospitals NHS Trust (LTHT) between 1st January 2001 and 31st December 2010 from the following groups of organisms (1) enterococci (2) coagulase-negative staphylococci (3) *Streptococcus bovis* group and (4) oral streptococci were included. IE was diagnosed according to the Duke criteria [43]. LTHT comprises two large (> 1000 bed) teaching hospitals, which serve the population of Leeds (approximately 600 000 people) as well as receiving referrals from surrounding hospitals. IE patients included in this analysis came from Leeds and the surrounding locations.

Patients managed by the Leeds Endocarditis Service are routinely asked about potential risk factors for endocarditis in addition to other clinically relevant questions and the data entered onto an audit database. Fully anonymised data extracts from this database were used for statistical analysis. This is a retrospective study utilising fully anonymised, routinely collected clinical data that will not influence the individuals concerned; therefore, patient consent has not been sought.

2.2.3 Study design

An unmatched case-control design was been used to compare the rate of different types of procedure in each group of endocarditis cases. Each group was been compared to the three other groups of patients to investigate any difference in the predisposing factors.

	Study 1	Study 2	Study 3	Study 4
Cases	A (enterococcal IE)	B (CoNS IE)	C (*Streptococcus bovis* IE)	D (oral streptococcal IE)
Controls	B, C & D	A, C & D	A, B & D	A, B &C
Suspected iatrogenic exposure	urological instrumentation	superficial skin instrumentation	lower gastrointestinal instrumentation	oral instrumentation

Table 8. The case-control study design

2.2.4 Variables

Data were collected from the Leeds Teaching Hospitals Endocarditis Service database, patient case notes, the hospital results server and the Patient Pathway Management (PPM) system. In the first instance, the IE database was cross-referenced with patient logbooks, routinely maintained by Dr Jonathan Sandoe (JS, Consultant Microbiologist), who performs the routine endocarditis ward rounds. This process helped resolve any issue with incomplete data. If incomplete data was identified, the PPM system was accessed to search for the missing data (whenever possible). Data on enterococcal bacteriuria were acquired from the hospital results server. The principal investigator (AM) was blinded with respect to the four groups of patients) for this step. The principal investigator was, therefore, not aware data on bacteriuria was being collected for which group of patients. All data was stored on an Excel spreadsheet. The following data were collected:

- age of patient;
- sex of patient;
- the valve affected by IE (Aortic, Mitral, Tricuspid or Pulmonary);
- the Duke criteria for the IE episode;
- the organism responsible for the IE episode;
- the presence of enterococcal bacteriuria with one year of the episode of IE;
- the presence of enterococcal bacteriuria at hospital presentation of the IE episode;
- urological instrumentation within one year of the development of IE;
- any upper GI instrumentation within one year of the development of IE;

- any lower GI instrumentation within one year of the development of IE;
- the presence of an intracardiac prosthetic device (cardiac valvular replacement or intracardiac pacemaker wire) at presentation;
- the use of intravenous drugs;
- any documented valvular heart disease (congenital or acquired);
- the need for haemodialysis preceding the IE episode.

The identity of the causative bacterial pathogen was collected in order to ascribe the patients to the different 'case' groups. The urological procedures included every per-urethra, endoscopic procedure excluding urethral catheterisation and urodynamic studies. Cystoscopy, endoscopic resection of the prostate (TURP), endoscopic resection of bladder tumour (TURBT) and ureterorenoscopy were included. Upper gastrointestinal procedures included oesophagogastroduodenoscopy (OGD) and endoscopic retrograde cholangiopancreatography (ERCP). Lower gastrointestinal procedures included sigmoidoscopy and colonoscopy. Antimicrobial prophylaxis (IV gentamicin 2 mg/kg single dose) is routine practice at the Leeds Teaching Hospitals NHS Trust for all the per-urethra urological procedures included in this study except cystoscopy. Patients undergoing upper and lower GI procedures did not receive routine antibiotic prophylaxis, except for ERCP (ciprofloxacin 750 mg single oral dose).

2.3 Results

2.3.1 Demographics

Table 9 below describes the number of units of observations obtained per variable assessed. All data were available for analysis regarding instrumentation. There were some missing data for the other variables. This issue is further discussed in Section 2.3.2.3.

Variables observed	N=384 (%)
Age of Patient	384 (100)
Enterococcal bacteriuria (within 1 year)	248 (64.6)
Enterococcal bacteriuria (at presentation)	248 (64.6)
Intravenous Drug User	323 (84.1)
Congenital Cardiac Anomaly	324 (84.4)
Acquired Cardiac Disease	325 (84.6)
Intracardiac Prosthesis	325 (84.6)
Haemodyalysis Patient	323 (84.1)
Upper GI Instrumentation	384 (100)
Lower GI Instrumentation	384 (100)
Urological Instrumentation	384 (100)

Table 9. The nature and number of available data for analysis

Variables	**Group A** **N=111** **(28.9%)**	**Group B** **N=86** **(22.4%)**	**Group C** **N=36** **(9.4%)**	**Group D** **N=151** **(39.3%)**	**Total** **N=384 (100%)**
Age>60	79 (28.8)	56 (34.9)	29 (19.4)	59 (60.9)	223 (41.9)
Male sex	80 (72.1)	56 (65.1)	21 (58.3)	122 (81.3)	279 (72.8)
Lower GI Instrumentation	5 (4.5)	3 (3.5)	1 (2.8)	5 (3.3)	14 (3.6)
Upper GI Instrumentation	5 (4.5)	6 (7.0)	2 (5.6)	4 (2.6)	17 (4.4)
Urological Instrumentation	24 (21.6)	4 (4.7)	2 (5.6)	4 (2.6)	34 (8.9)
Enterococcal bacteriuria (within 1 year)	3 (2.7)				
Enterococcal bacteriuria (at presentation)	6 (5.4)				
IVDU	6 (6.7)	2 (2.5)	0 (0)	7 (5.6)	15 (4.7)
Congenital cardiac anomaly	2 (2.2)	5 (6.3)	1 (3.6)	26 (20.6)	34 (10.5)

Variables		Group A N=111 (28.9%)	Group B N=86 (22.4%)	Group C N=36 (9.4%)	Group D N=151 (39.3%)	Total N=384 (100%)
Acquired cardiac anomaly		39 (43.3)	40 (50.6)	14 (48.3)	41 (32.5)	134 (41.4)
Intracardiac prosthetic device		33 (36.7)	42 (53.2)	7 (24.1)	31 (24.6)	113 (34.9)
Haemodyalysis		4 (4.4)	10 (12.7)	1 (3.6)	3 (2.4)	18 (5.6)
Valve	Aortic	61 (55.0)	43 (50.0)	18 (50.0)	75 (49.7)	197 (51.3)
	Mitral	40 (36.0)	25 (29.1)	19 (52.8)	64 (42.4)	148 (38.5)
	Pulmonary	2 (1.8)	0 (0)	0(0)	7 (4.6)	9 (2.3)
	Tricuspid	2 (1.8)	9 (10.5)	0 (0)	7 (4.6)	18 (4.7)
Native valve IE		66 (59.5)	37 (43.0)	27 (75.0)	120 (79.5)	250 (65.1)
Prosthetic valve IE	Early	15 (13.5)	21 (24.4)	5 (13.9)	4 (2.6)	45 (11.7)
	Late	16 (14.4)	7 (8.1)	4 (11.1)	17 (11.3)	44 (11.5)
Pacemaker lead IE		2 (1.8)	11 (12.8)	0 (0)	2 (1.3)	15 (3.9)

Table 10. Demographics of patients included in the case control study of investigating the association of IE and risk factors
Greyed out boxes indicate no data collected.

Three hundred and eighty-four (384) patients were included in this study. Table 10 shows the basic demographics and patient characteristics; 58% of all patients were above 60 years old, with the oral streptococcal IE group having the lowest proportion of patients over 60 years old. The age density histogram in Figure 6 shows the distribution of the patients per age group. The line superimposed on the bar graph shows that there is a sharp rise in patients diagnosed with IE after the age of 50.

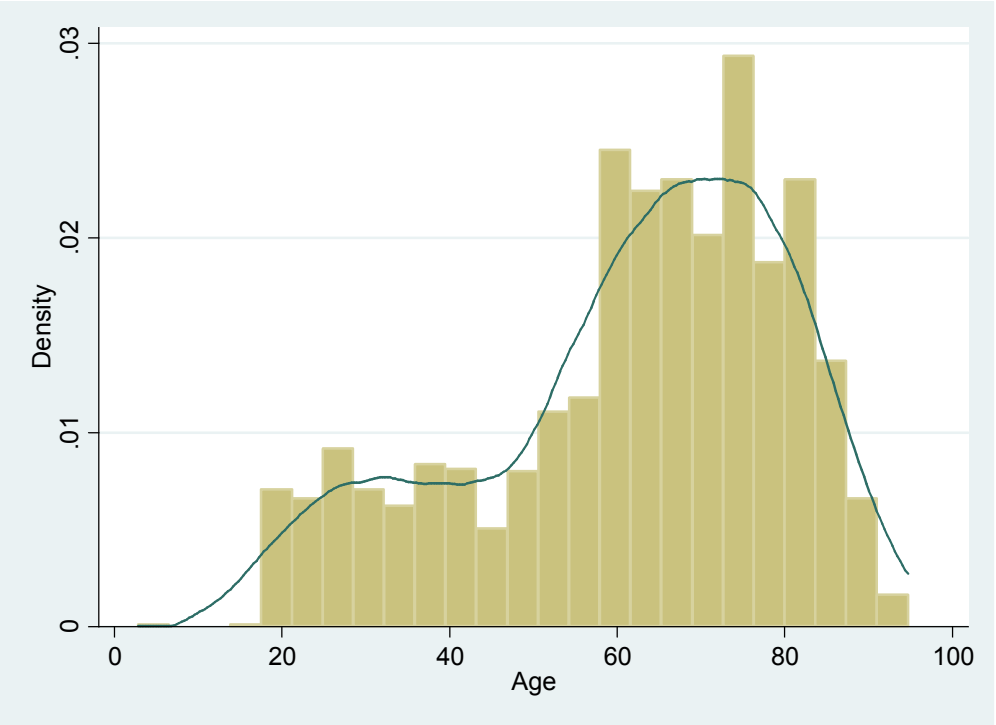


Figure 6. Histogram of age of patients.

Overall, there was a male predominance (72.8% of all patients). The highest proportion of female patients was in Group C. Urological or gastrointestinal procedures were relatively uncommon in the total cohort but over 20% of patients in the enterococcal IE group had undergone a urological procedure in the 12 months prior to presentation. Forty-one percent (41%) of patients had an acquired cardiac abnormality (stenosis or regurgitation) whilst only 10% of the patients were born with a congenital cardiac abnormality. Over a third of the patients had previous cardiac valvular surgery; 53.2% of the patients in Group B had an intracardiac prosthetic device. A minority of patients were intravenous drug users (4.7%) or were dependent on haemodyalysis (5.6%). Most IE episodes affected native cardiac valves (65%), with the aortic valve most commonly affected.

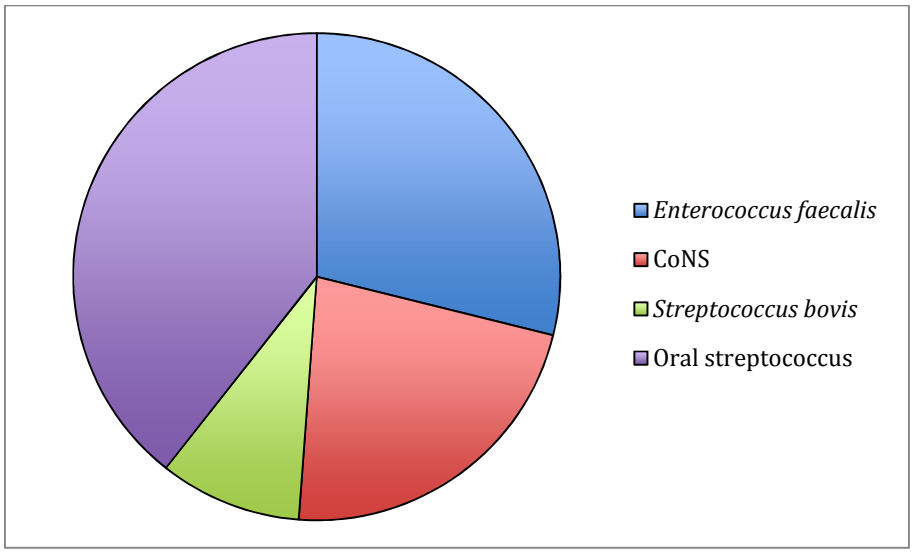


Figure 7. Pie Chart showing the proportion of patients in in the four different groups of patients with IE.

The pie charts above visually demonstrate the proportion of patients per group. The oral streptococcal group was the largest 'case' group with the control group being twice its size. The *Streptococcus bovis* group was the smallest 'case' group with the 'control' group being ten-fold larger.

2.3.2 Statistical analysis

Bradford-Hill criteria were used to investigate the association between the development of IE and the possible risk-factors [187]. The main principles of the Bradford-Hill rule of association stipulate that an association (between instrumentation and the development of IE) can be regarded as causative if:

1. the strength of the association between instrumentation and the development of IE is significant enough (*p* values);
2. the association of instrumentation is repeatedly noted in the group of patients developing IE (consistency);
3. the association of instrumentation is specific to a particular group of patients developing IE. This is accounted for by linking the development of IE by a particular organism to the natural habitat of the said organism in the human body;
4. there is a temporal relationship between the development of IE and instrumentation. It is unlikely that the development of IE would be the cause of instrumentation in the group of patients;

5. an increase in the number of episodes of instrumentation results in an increase in the incidence of IE in the patient group (biological gradient)
6. the plausibility that IE is the result of instrumentation. This is further verified in the second part of the study, which investigates the development of bacteraemia secondary to instrumentation of the urological tract. This bacteraemia in turn may lead to the development of IE.

The data were cleaned up and any discrepancies in the source data were updated by checking the patient pathway manager (PPM) electronic database. It was ascertained that the data from the IE database corroborated with the data available from the patient pathway management system, which tracks the patient journey through the different departments in the hospital. In case of discrepancy from the two data source, the PPM data was used as the actual data. Data missing from the IE database were actively searched for on the PPM database and the IE database updated accordingly.

The data were converted to binary variables (0: absent and 1: present). The software programs used were IBM SPSS Statistics, Version 19.0.0 and R-Studio, Version 0.95.265. Missing data were replaced using the multiple imputations method using SPSS Statistics. Univariate and multivariate analysis were performed using SPSS (Version 19.0.0, IBM Company, Chicago, USA). A logistic regression model was used for the multivariate analysis. Missing data patterns were identified and a multiple imputation method was used to complete the data set.

2.3.2.1 Preliminary Analysis

The variables included in the initial analysis shown in Table 11. Basic statistical analysis was done using a 2 by 2 cross table to investigate a possible association between the development of IE and the different variables.

Variable (Exposure)	Outcome
Urological Instrumentation within 1 year	0 or 1
Upper GI Instrumentation within 1 year	0 or 1
Lower GI instrumentation within 1 year	0 or 1
Age>60	0 or 1
Age	Continuous
Sex (male)	0 or 1
Intravenous drug user (IVDU)	0 or 1
Haemodyalysis	0 or 1
Congenital cardiac anomaly	0 or 1
Acquired cardiac anomaly	0 or 1
Intracardiac prosthesis (valve, pacemaker)	0 or 1
Enterococcal bacteriuria at presentation	0 or 1
Enterococcal bacteriuria within 1 year	0 or 1

Table 11. Variables used to investigate an association with the development of IE

The detailed analyses from the univariate statistical tests performed for patients with enterococcal IE are shown in Tables 12-23. Each variable with a suspected association with the development of enterococcal IE was assessed.

			Enterococcal IE		
			0	1	Total
Sex of Patient	Female	Count	73	31	104
		% within Sex of Patient	70.2%	29.8%	100.0%
	Male	Count	200	80	280
		% within Sex of Patient	71.4%	28.6%	100.0%
Total		Count	273	111	384
		% within Sex of Patient	71.1%	28.9%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Sex of Patient (Female / Male)	0.94	0.58	1.53

Table 12. Risk Estimate (sex and enterococcal IE)

The sex of the patient did not appear to be an important variable in determining whether a patient developed enterococcal IE or one of the three other types of IE (OR: 0.94, CI: 058-1.53).

			Enterococcal IE		
			0	1	Total
Enterococcal Bacteriuria (within 1 year)	0	Count	137	108	245
		% within Enterococcal Bacteriuria (within 1 year)	55.9%	44.1%	100.0 %
	1	Count	0	3	3
		% within Enterococcal Bacteriuria (within 1 year)	.0%	100.0 %	100.0 %
Total		Count	137	111	248
		% within Enterococcal Bacteriuria (within 1 year)	55.2%	44.8%	100.0 %

	Value	95% Confidence Interval	
		Lower	Upper
For cohort Enterococcal IE = 1	0.44	0.38	0.51

Table 13. Risk estimate (enterococcal bacteriuria within a year of presentation and enterococcal IE)

The number of patients with enterococcal bacteriuria identified within one year of presentation of their enterococcal IE episode was too few to perform any meaningful statistical test.

			Enterococcal IE		
			0	1	Total
Enterococcal Bacteriuria (at presentation)	0	Count	135	105	240
		% within Enterococcal Bacteriuria (at presentation)	56.2%	43.8%	100.0%
	1	Count	2	6	8
		% within Enterococcal Bacteriuria (at presentation)	25.0%	75.0%	100.0%
Total		Count	137	111	248
		% within Enterococcal Bacteriuria (at presentation)	55.2%	44.8%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Enterococcal bacteriuria (at presentation) (0 / 1)	3.86	0.76	19.5

Table 14. Risk estimate (enterococcal bacteriuria at presentation and enterococcal IE)

Again, the number of patients presenting with bacteriuria on admission for their IE episode is very small though statistical analysis gives an impression that there is an association with the presence of enterococcal IE (OR: 3.86, CI: 0.76-19.5).

			Enterococcal IE		
			0	1	Total
Urological Instrumentation	0	Count	264	87	351
		% within Urological Instrumentation	75.2%	24.8%	100.0%
	1	Count	10	24	34
		% within Urological Instrumentation	29.4%	70.6%	100.0%
Total		Count	274	111	385
		% within Urological Instrumentation	71.2%	28.8%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Urological Instrumentation (0 / 1)	7.28	3.35	15.8

Table 15. Risk estimate (urological instrumentation and enterococcal IE)

The odds ratio of 7.28 (CI: 3.35-15.8) suggests that patients who have had urological instrumentation are seven times more likely to develop enterococcal IE as opposed to the three other types of IE investigated.

			Enterococcal IE		
			0	1	Total
Lower GI Instrumentation	0	Count	264	106	370
		% within Lower GI Instrumentation	71.4%	28.6%	100.0%
	1	Count	10	5	15
		% within Lower GI Instrumentation	66.7%	33.3%	100.0%
Total		Count	274	111	385
		% within Lower GI Instrumentation	71.2%	28.8%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Lower GI Instrumentation (0 / 1)	1.25	0.41	3.73

Table 16. Risk estimate (lower GI instrumentation and enterococcal IE)

Univariate analysis did not reveal any association between lower gastrointestinal instrumentation and the subsequent development of enterococcal IE.

			Enterococcal IE		
			0	1	Total
Upper GI Instrumentation	0	Count	261	106	367
		% within Upper GI Instrumentation	71.1%	28.9%	100.0%
	1	Count	13	5	18
		% within Upper GI Instrumentation	72.2%	27.8%	100.0%
Total		Count	274	111	385
		% within Upper GI Instrumentation	71.2%	28.8%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Upper GI Instrumentation (0 / 1)	0.95	0.33	2.72

Table 17. Risk estimate (Upper GI instrumentation and enterococcal IE)

Again, univariate analysis shows no statistical association between upper gastrointestinal instrumentation and the development of enterococcal IE.

It was decided to investigate whether older patients were more likely to develop enterococcal IE as opposed to the three other kinds of IE. A threshold of 60

years was used to divide the patient cohort into two approximately equal groups (the median age for the whole cohort: 60.2 years)

			Enterococcal IE		Total
			0	1	
Age>60	0	Count	129	32	161
		% within Age>60	80.1%	19.9%	100.0%
	1	Count	145	79	224
		% within Age>60	64.7%	35.3%	100.0%
Total		Count	274	111	385
		% within Age>60	71.2%	28.8%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Age>60 (0 / 1)	2.20	1.37	3.53

Table 18. Risk estimate (age>60 and enterococcal IE)

An association between the development of enterococcal IE and the patient's age being greater than 60 was noted with an OR: 2.20, CI 1.37-3.53.

			Enterococcal IE		Total
			0	1	
Intravenous Drug User	0	Count	224	84	308
		% within Intravenous Drug User	72.7%	27.3%	100.0%
	1	Count	9	6	15
		% within Intravenous Drug User	60.0%	40.0%	100.0%
Total		Count	233	90	323
		% within Intravenous Drug User	72.1%	27.9%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Intravenous Drug User (0 / 1)	1.78	0.61	5.15

Table 19. Risk estimate (intravenous drug user and enterococcal IE)

Univariate analysis did not reveal any statistical association between being an intravenous drug user and the subsequent development of enterococcal IE.

			Enterococcal IE		
			0	1	Total
Congenital Cardiac Anomaly	0	Count	202	88	290
		% within Congenital Cardiac Anomaly	69.7%	30.3%	100.0%
	1	Count	32	2	34
		% within Congenital Cardiac Anomaly	94.1%	5.9%	100.0%
Total		Count	234	90	324
		% within Congenital Cardiac Anomaly	72.2%	27.8%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Congenital Cardiac Anomaly (0 / 1)	0.14	0.03	0.61

Table 20. Risk estimate (congenital cardiac anomaly and enterococcal IE)

Univariate analysis showed that enterococcal IE was less likely to develop in patients a congenital cardiac anomaly.

			Enterococcal IE		
			0	1	Total
Acquired Cardiac Disease	0	Count	139	51	190
		% within Acquired Cardiac Disease	73.2%	26.8%	100.0%
	1	Count	96	39	135
		% within Acquired Cardiac Disease	71.1%	28.9%	100.0%
Total		Count	235	90	325
		% within Acquired Cardiac Disease	72.3%	27.7%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Acquired Cardiac Disease (0 / 1)	1.11	0.68	1.81

Table 21. Risk estimate (acquired cardiac anomaly and enterococcal IE)

Acquired cardiac anomaly did not have any bearing on the subsequent development of enterococcal IE.

			Enterococcal IE		
			0	1	Total
Intracardiac Prosthesis	0	Count	154	57	211
		% within Intracardiac Prosthesis	73.0%	27.0%	100.0%
	1	Count	81	33	114
		% within Intracardiac Prosthesis	71.1%	28.9%	100.0%
Total		Count	235	90	325
		% within Intracardiac Prosthesis	72.3%	27.7%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Intracardiac Prosthesis (0 / 1)	1.10	0.66	1.83

Table 22. Risk estimate (intracardiac prosthesis and enterococcal IE)

Univariate analysis did not demonstrate any association between the presence of an intracardiac device and the development of enterococcal IE.

			Enterococcal IE		
			0	1	Total
Haemodyalysis Patient	0	Count	219	86	305
		% within Haemodyalysis Patient	71.8%	28.2%	100.0%
	1	Count	14	4	18
		% within Haemodyalysis Patient	77.8%	22.2%	100.0%
Total		Count	233	90	323
		% within Haemodyalysis Patient	72.1%	27.9%	100.0%

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Haemodialysis Patient (0 / 1)	0.73	0.23	2.27

Table 23. Risk estimate (haemodyalysis and enterococcal IE)

There was no statistical association between the development of enterococcal IE and the patient having haemodyalysis.

In conclusion, univariate analysis showed that enterococcal IE was associated with urological instrumentation and increasing age, whilst no statistical association was found between the other suspected risk factors. Then, the

same univariate analysis was carried out for the three other groups and the results are shown in Table 24.

	Enterococcal IE group		**CoNS IE group**		***Streptococcus bovis* IE group**		**Oral streptococcal IE group**	
Variable	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval
Sex	0.94	0.58-1.54	0.62	0.37-1.03	0.48	0.24-0.97	**2.10**	**1.28-3.43**
Age >60	**2.20**	**1.37-3.53**	1.46	0.88-2.40	**3.27**	**1.40-7.67**	0.27	0.17-0.49
Enterococcal Bacteriuria (at presentation)	3.86	0.76-19.5						
Enterococcal Bacteriuria (within 1 year)	0.44	0.38-0.51						
IVDU	1.78	0.61-5.15	0.46	0.10-2.10	0.91	0.88-0.94	1.39	0.49-3.93
Congenital cardiac anomaly	0.14	0.03-0.61	0.50	0.19-1.35	0.30	0.04-2.24	**6.18**	**2.70-14.1**
Acquired cardiac anomaly	1.11	0.68-1.81	1.63	0.98-2.72	1.35	0.63-2.90	0.54	0.34-0.86
Intracardiac prosthesis	1.10	0.66-1.83	**2.74**	**1.63-4.62**	0.56	0.23-1.36	0.46	0.28-0.80
Haemodyalysis	0.73	0.23-2.27	**4.28**	**1.63-11.3**	0.61	0.08-4.73	0.30	0.09-1.06
Upper GI Procedures	0.95	0.33-2.72	1.79	0.65-4.93	1.22	0.27-5.55	0.43	0.14-1.33

	Enterococcal IE group		CoNS IE group		*Streptococcus bovis* IE group		Oral streptococcal IE group	
Variable	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval
Lower GI Procedures	1.25	0.41-3.73	0.86	0.24-3.14	0.68	0.09-5.36	0.77	0.26-2.29
Urological Procedures	**7.28**	**3.35-15.8**	0.44	0.15-1.28	0.58	0.13-2.54	0.19	0.06-0.54

Table 24. Univariate analysis evaluating the risk factors in the development of IE. Data on enterococcal bacteriuria was collected for the enterococcal IE group only. The 'greyed out' boxes indicate that no data was available.

Patients with enterococcal IE were significantly more likely to be over 60 years of age (OR: 2.20) and to have undergone urological procedures (OR: 7.28) compared with patients in the other three IE groups. There was an association between the presence of enterococcal bacteriuria, at the time of the hospital admission when the IE episode was diagnosed, and enterococcal IE. The detailed analyses are described in more details above. The presence of an intracardiac prosthesis and being haemodyalysis dependent were associated (OR 2.74 and 4.28 respectively) with the development of CoNS IE compared to IE caused by the other pathogen groups. Increasing age was also associated with *Streptococcus bovis* group IE, whereas being male and having a congenital cardiac anomaly made patients more likely to develop oral streptococcal IE rather than the three other types of IE. Upper and lower gastrointestinal procedures were not associated with any of the four different types of IE.

To strengthen the statistical analysis, unnecessary details were dropped (removed from the analysis). The following data were dropped:

- the timing of urological, upper and lower GI instrumentation was documented as 'within 1 year', 'within 6 month' and 'within 3 month' of the subsequent development of IE. All the instrumentation was documented as 0 or 1, irrespective of the timing of the said exposure;
- age was initially considered as a binary data (Age > 60, Age ≤ 60) for the χ^2 crosstabs 2 by 2 analysis. In subsequent analysis (logistic regression), age was used as a continuous variable;
- upper and lower gastrointestinal instrumentation was no included in further analyses as univariate analyses revealed no statistical association with the subsequent development of IE.

2.3.2.2 Logistic regression model

A binomial logistic regression statistical model was used to evaluate the association between the development of the four disease possibilities and the variables deemed significant from the univariate analyses. The variables not deemed significant (based on the 2 by 2 χ^2 test) were not included to avoid confounding the effects of the included variables on the final outcome. Multivariate analysis using a logistic regression model was performed using SPSS (Version 19.0.0, IBM Company, Chicago, USA).

2.3.2.3 Missing data

Missing data were completed using a multiple imputations method, resulting in seven data sets (the original data set, five imputed data sets and one pooled data set). Imputations were carried out using the whole data set originally available. The standard imputation protocol inbuilt in SPSS was used to complete the data set. Table 25 below shows the patterns of missing data. Sensitivity analysis was undertaken to investigate if a bias might have arisen from some of the data being incomplete [188]. No evidence for bias was found. Sensitivity analysis yielded the same conclusions as the analysis on the incomplete data; the fitted values and their confidence intervals were not

substantially changed as shown by the results based on the original data and the pooled data after multiple imputations.

N	Missing Patterns										
	Upper GI instrumentation	Lower GI instrumentation	Urological instrumentation	Sex	Age	Intracardiac prosthetic device	Acquired cardiac anomaly	Congenital cardiac anomaly	Haemodyalysis	IVDU	Complete if ...
314											314
70						X	X	X	X	X	384

Table 25. Patterns of missing data

Three hundred and fourteen out of the 384 entries had a complete dataset, with 70 entries having missing data. All the entries had a complete data set for the following variables:

- upper GI instrumentation;
- lower GI instrumentation;
- urological instrumentation;
- sex;
- age.

This was made possible as three different data sources were crosschecked to gather the data:

- the endocarditis spreadsheet;
- the patient pathway management (PPM) system;
- the hospital results database.

As the hospital notes of patients were not accessed, it was not possible to gather all the data with respect to cardiac history, haemodyalysis and intravenous drug usage.

2.3.2.4 Multivariate Analysis

Multivariate analysis was performed on the initial data set, the five imputed data sets and the final pooled data set. The tables below show the results from the initial data set and the pooled data set for each of the four groups of patients.

Variable		Model Coefficient	Standard Error	p-value	Odds Ratio	95% CI for Odds Ratio	
						Lower	Upper
Original data	**Age**	**0.03**	**0.01**	**0.005**	**1.03**	**1.01**	**1.05**
	Sex	0.32	0.30	0.300	1.37	0.76	2.49
	IVDU	**1.90**	**0.69**	**0.006**	**6.69**	**1.72**	**26.1**
	Congenital Cardiac Anomaly	-0.78	0.82	0.343	0.46	0.09	2.30
	Acquired Cardiac Anomaly	-0.17	0.37	0.642	0.84	0.41	1.73
	Intracardiac prosthetic device	0.25	0.38	0.505	1.28	0.62	2.68
	Haemodialysis	-0.43	0.64	0.499	0.65	0.19	2.27
	Urological Procedures	**2.11**	**0.43**	**0.000**	**8.21**	**3.54**	**19.05**
	Constant	-3.26	0.76	0.000	0.04		
Pooled Imputed data	**Age**	**0.03**	**0.01**	**0.007**	**1.03**	**1.01**	**1.05**
	Sex	0.35	0.31	0.251	1.42	0.78	2.58
	IVDU	**1.74**	**0.68**	**0.011**	**5.72**	**1.50**	**21.77**
	Congenital Cardiac Anomaly	-0.86	0.82	0.297	0.43	0.09	2.12
	Acquired Cardiac Anomaly	-0.25	0.37	0.499	0.78	0.38	1.60
	Intracardiac prosthetic device	0.31	0.37	0.407	1.36	0.66	2.84
	Haemodialysis	-0.45	0.64	0.480	0.64	0.18	2.23
	Urological Procedures	**2.15**	**0.43**	**0.000**	**8.56**	**3.69**	**19.85**
	Constant	-3.18	0.75	0.000	0.04	0.01	0.18

Table 26. Multivariate analysis on the data for patients with enterococcal IE group. Bold rows indicate variables that achieved statistical significance.

Table 26 shows the results of the multivariate analysis on the data relating to patients diagnosed with enterococcal IE. The analysis indicates that urological

procedures, within the one year preceding the diagnosis, were statistically associated with the development of enterococcal IE (OR: 8.21, CI: 3.54-19.05, $p < 0.05$). Enterococcal IE was also associated with increasing age (OR: 1.03, CI: 1.01–1.05, $p < 0.05$) and the patient being an intravenous drug user (OR: 6.69, CI: 1.72–26.1, $p < 0.05$).

Variable		Model coefficient	Standard Error	p-value	Odds Ratio	95% CI for Odds Ratio	
						Lower	Upper
Original data	Age	-0.01	0.01	0.231	0.99	0.97	1.01
	Sex	0.16	0.30	0.606	1.17	0.65	2.10
	IVDU	-0.90	0.86	0.297	0.41	0.08	2.21
	Congenital Cardiac Anomaly	-1.22	0.66	0.064	0.30	0.08	1.07
	Acquired Cardiac Anomaly	-.257	0.38	0.504	0.77	0.36	1.64
	Intracardiac Prosthetic Device	**1.46**	**0.39**	**0.000**	**4.28**	**2.00**	**9.16**
	Haemodialysis	**1.96**	**0.54**	**0.000**	**7.06**	**2.48**	**20.1**
	Urological Procedures	-0.98	0.59	0.096	0.38	0.12	1.19
	Constant	-0.86	0.65	0.188	0.423		
Pooled Imputed Data	Age	-0.01	0.01	0.241	0.99	0.97	1.01
	Sex	0.17	0.30	0.564	1.19	0.66	2.15
	IVDU	-0.91	0.86	0.292	0.40	0.08	2.18
	Congenital Cardiac Anomaly	-1.24	0.65	0.059	0.29	0.08	1.05
	Acquired Cardiac Anomaly	-0.28	0.38	0.468	0.76	0.36	1.60
	Intracardiac Prosthetic Device	**1.47**	**0.39**	**0.000**	**4.34**	**2.04**	**9.23**
	Haemodialysis	**1.96**	**0.54**	**0.000**	**7.06**	**2.48**	**20.1**
	Urological Procedures	-0.98	0.59	0.094	0.38	0.12	1.15
	Constant	-0.88	0.65	0.178	0.42	0.12	1.49

Table 27. Multivariate analysis on the data for patients with CoNS IE group. Bold rows indicate variables that achieved statistical significance.

The analysis shows that CoNS IE was associated with the presence of an intracardiac prosthetic device (OR: 4.28, CI: 2.00–9.16, $p < 0.05$) and haemodialysis (OR: 7.06, CI: 2.48–20.1, $p < 0.05$).

Variables		Model coefficient	Standard Error	p-value	Odds Ratio	95% CI for Odds Ratio	
						Lower	Upper
Original data	**Age**	**0.05**	**0.017**	**0.008**	**1.05**	**1.01**	**1.08**
	Sex	0.85	0.438	0.052	2.34	0.99	5.53
	IVDU	-17.2	10245	0.999	0.00	0.00	Infinity
	Congenital Cardiac Anomaly	0.18	1.16	0.876	1.20	0.12	11.6
	Acquired Cardiac Anomaly	0.45	0.51	0.380	1.56	0.58	4.22
	Intracardiac prosthetic Device	**-1.42**	**0.58**	**0.015**	**0.24**	**0.08**	**0.76**
	Haemodyalysis	-0.87	1.09	0.423	0.42	0.05	3.52
	Urological Procedures	-0.73	0.80	0.362	0.48	0.10	2.31
	Constant	-5.47	1.290	0.000	0.00		
Pooled Imputed Data	**Age**	**0.05**	**0.02**	**0.003**	**1.05**	**1.02**	**1.09**
	Sex	0.83	0.44	0.059	2.29	0.97	5.42
	IVDU	-6.25	6465	0.999	0.00	0.00	Infinity
	Congenital Cardiac Anomaly	0.30	1.16	0.797	1.35	0.14	13.2
	Acquired Cardiac Anomaly	0.57	0.50	0.250	1.77	0.67	4.68
	Intracardiac prosthetic Device	**-1.52**	**0.58**	**0.008**	**0.22**	**0.07**	**0.68**
	Haemodyalysis	-0.87	1.09	0.425	0.42	0.05	3.54
	Urological Procedures	-0.74	0.80	0.352	0.48	.010	2.27
	Constant	-5.85	1.31	0.000	0.00	0.00	0.04

Table 28. Multivariate analysis on the data for patients with *Streptococcus bovis* IE group. Bold rows indicate variables that achieved statistical significance.

The results from the multivariable analysis show that *Streptococcus bovis* group IE was associated with increasing age (OR: 1.05, CI: 1.01–1.10, $p < 0.05$). An intracardiac prosthetic device appeared to be protective to the development of *Streptococcus bovis* IE with respect to the three other types of IE (OR: 0.24, CI: 0.08–0.76, $p < 0.05$).

Variables		Model Coefficient	Standard Error	p-value	Odds Ratio	95% CI for Odds Ratio	
						Lower	Upper
Original data	**Age**	**-0.03**	**0.01**	**0.001**	**0.97**	**0.96**	**0.99**
	Sex	**-0.80**	**0.30**	**0.009**	**0.45**	**0.25**	**0.82**
	IVDU	-1.05	0.63	0.098	0.35	0.10	1.21
	Congenital cardiac anomaly	0.82	0.54	0.128	2.26	0.79	6.47
	Acquired Cardiac Anomaly	0.11	0.34	0.739	1.12	0.57	2.19
	Intracardiac Prosthetic Device	**-0.93**	**0.35**	**0.009**	**0.40**	**0.20**	**0.79**
	Haemodyalysis	**-1.41**	**0.68**	**0.038**	**0.24**	**0.06**	**0.92**
	Urological Procedures	**-1.70**	**0.57**	**0.003**	**0.18**	**0.06**	**0.56**
	Constant	1.96	0.60	0.001	7.12		
Pooled Imputed Data	**Age**	**-0.03**	**0.01**	**0.001**	**0.97**	**0.95**	**0.99**
	Sex	**-0.85**	**0.31**	**0.006**	**0.43**	**0.23**	**0.78**
	IVDU	-1.14	0.63	0.071	0.32	0.09	1.10
	Congenital cardiac anomaly	0.79	0.53	0.137	2.21	0.78	6.29
	Acquired Cardiac Anomaly	0.11	0.34	0.740	1.12	0.58	2.17
	Intracardiac Prosthetic Device	**-0.91**	**0.35**	**0.009**	**0.40**	**0.20**	**0.80**
	Haemodyalysis	**-1.41**	**0.68**	**0.038**	**0.24**	**0.06**	**0.93**
	Urological Procedures	**-1.73**	**0.57**	**0.002**	**0.18**	**0.06**	**0.54**
	Constant	2.07	0.60	0.001	7.92	2.42	25.9

Table 29. Multivariate analysis on the data for patients with oral streptococcal IE group. Bold rows indicate variables that achieved statistical significance.

Table 29 shows that age, intracardiac prosthetic device, male sex, haemodialysis and urological procedures seem to have a protective effect against developing oral streptococcal IE. These results can be interpreted in the light that the 'case' group (oral streptococcal IE) was being compared of the three other 'control groups' (A, B and C). Therefore, one can deduce that older and male patients having urological procedures and dialysis are more likely to be in the control group than in the oral streptococcal group.

Infective Endocarditis	Urological Instrumentation	Age	Sex (Male)	Intracardiac prosthetic device	IV Drug User	Haemodyalysis
Enterococcal	↑↑	↑			↑↑	
Staphylococcal				↑↑		↑↑
Streptococcus bovis		↑		↓		
Oral Streptococcal	↓↓	↓	↓	↓		↓

Table 30. Diagrammatic representation showing the association between the development of the four different types of IE and the variables investigated

Table 30 is a diagrammatic representation of the different associations between the development of IE and the variables investigated. The key results are:

- this study shows that there is a strong association between urological instrumentation and the development of IE caused by enterococci (Group A). There is also an association between increasing age and IV drug usage and the development of IE caused by enterococci;
- staphylococcal IE is associated with prosthetic devices (haemodialysis lines and intracardiac prosthetic devices);
- *Streptococcus bovis* group IE is associated with increasing age;
- there does not seem to be a positive association between the development of oral streptococcal IE and the variables collected in this study.

2.4 Discussion

Endocarditis prophylaxis guidelines from the National Institute of Health and Clinical Excellence (NICE) no longer recommend antibiotic prophylaxis for routine urological and gastrointestinal procedures on the basis of a lack of evidence of a link between these procedures and IE [189]. Other national or international guideline development groups have taken a similar stand

[23, 190-198]. Infective endocarditis appears to be increasing in incidence in England [199], but the reasons for this can only be speculated upon [200]. The authors speculate that an ageing population, with more risk factors for the development of IE may in part explain the increase in incidence of IE. The increase in incidence appears to be independent of a huge reduction in prescriptions for antimicrobial prophylaxis by dental practitioners [199] but NICE recommendations remain controversial and many clinicians continue to prescribe IE prophylaxis for a variety of conditions. NICE included just three case–control studies that analysed the risk of developing IE after various procedures; two of these included gastrointestinal procedures and one procedure studied dental procedures. There has been no case–control study investigating urological investigations and subsequent development of IE.

This is, therefore, the first case–control study that has focussed on preceding urological and gastrointestinal investigations and development of IE. It is also the first case–control study to include only patients who have suffered from IE, thereby removing the problems of selecting an appropriate control group of 'at risk' patients. The validity of this approach is supported by the fact that the analysis confirms several findings that are already known such as the association between increasing age or IVDU and the development of enterococcal IE [201-203]; the association between CoNS IE and dependence upon haemodialysis (which can be explained by the ease of access of skin microbiota to the vascular system via the vascular catheters often used for haemodialysis [204-206]); and the well-known association between presence of an intracardiac prosthetic device and CoNS IE [23]. Increasing age and the development of *Streptococcus bovis* group IE has also been reported [207], which may be explained by the fact that *Streptococcus bovis* IE has been linked with bowel malignancy, with the incidence of the latter increasing with age [208, 209]. It was speculated that patients with bowel symptoms and gastrointestinal neoplasia might be more likely to have gastrointestinal procedures and *Streptococcus bovis* group IE, in a similar manner to urological procedures and enterococcal IE, but this was not demonstrated in the analysis.

This study also demonstrates that patients diagnosed with enterococcal IE were more likely to have enterococcal bacteriuria at the time of presentation compared with the other patients diagnosed with IE, though statistical

significance was not achieved (OR: 3.86, CI: 0.76–19.5). This may be due to the relatively small number of patients with enterococcal IE who actually had documented enterococcal bacteriuria. This association between enterococcal bacteraemia and bacteriuria may be a similar phenomenon to *Staphylococcus aureus* bacteraemia and bacteriuria whereby bacteria in the blood can appear in the urine as a result of glomerular filtration [210]. The former hypothesis reinforces the theory that patients with enterococcal IE who undergo urological procedures have underlying urological pathology, which predisposes them to bacteriuria and subsequent bacteraemia. Another possible explanation for the association between the development of IE and prior urological procedures revolves around the inflammatory response mediated by the body during a procedure [33, 211]. This may explain why bacteraemia linked to iatrogenic procedures, which are associated with a state of inflammation, might have a higher likelihood of leading to infective endocarditis than bacteraemia secondary to mundane daily activities. However, there is no conclusive evidence of this hypothesis.

2.4.1 Interpretations

This study supports the NICE, American Heart Association (AHA) and European Society of Cardiology (ESC) recommendations not to give antimicrobial prophylaxis for gastrointestinal procedures. It does, however, demonstrate a statistical association between urological procedures and the development of enterococcal IE, which raises questions about the role of urological investigations in the development of enterococcal IE and the need for more information about the infective risks of such procedures. Similar to arguments relating to dentistry and IE, two possible explanations are proposed: 1) urological procedures can cause an enterococcal bacteraemia, which (rarely) results in IE; or 2) patients with urological pathology are predisposed to enterococcal urinary tract infection (or colonisation) and spontaneous enterococcal bacteraemias, which may result in IE. The lack of consistent findings of previous enterococcal bacteriuria in enterococcal IE patients, suggests asymptomatic infection or colonisation may be involved.

2.4.2 Limitations

This is a retrospective study from a single hospital trust, but it should be noted that patients came from two large teaching hospitals as well from the

surrounding area. Although the cohort of patients may have been biased towards more complex cases, this will have applied to all cases of IE in the analysis. A retrospective, case–control design will always be subject to potential bias but a prospective randomised study would be impractical owing to the relative rarity of enterococcal IE. The case–control study design that has been used has overcome concerns about the validity of the control group, in terms of comparable risk of developing IE. By including only patients with IE, patients in both the 'case' and 'control' groups had risk factors for developing IE. This design has limitations: only cases caused by selected groups of pathogens were included. These groups were chosen because their natural habitats are well recognised and anatomically confined; *Staphylococcus aureus* was not included because of the wide variety of body sites that may be colonised or infected with the organism. The validity of this methodology is supported by the fact that the analysis confirms a number of previously reported associations.

The majority patients undergoing urological procedures included in this study will have received antibiotics prophylaxis as described in the Methods section though it was not possible to record the compliance with antimicrobial prophylaxis regimens reliably. It is noteworthy that these regimens do not have activity against enterococci. There were some missing data, but inclusion of a multiple imputations method and re-analysis of the data did not alter the findings. A sensitivity analysis based on completed datasets was undertaken; this showed no evidence of bias and consequently this sensitivity has provided greater confidence in the findings.

2.4.3 Generalisability

Further studies are required to evaluate the presence of bacteraemia during urological procedures, and cardiac valvular bacterial seeding pre- and post-procedure, in patients with underlying urological pathology. An understanding of the incidence of bacteraemia during daily activities like urination would help guide further research so that more focussed recommendations about antibiotic prophylaxis could then be made.

2.5 Conclusions

This is the first case-control study to examine urological procedures as a risk factor in the development of IE. An association between urological procedures and enterococcal IE has been found. This study has also not demonstrated any association between gastrointestinal procedures and the development of IE.

The association between enterococcal IE and urological procedures raises questions about the pathogenesis of enterococcal IE. Can enterococcal IE result from bacteraemia caused by the procedure or are patients who undergo urological procedures more likely to have an underlying urological pathology that causes repeated bacteraemias in the period preceding the procedures? Both mechanisms may lead to the bacterial seeding of cardiac valves, but would warrant different approaches to prophylaxis.

Chapter 3: Methods to detect bacteraemia

3.1 Introduction

This chapter describes the different methods used to detect, identify and quantify bacteria and bacteraemia. The process of determining the optimal methods for assessing bacteraemia in the context of this study is described. The main technologies used were:

- incubation and culture of blood;
- extraction of bacterial DNA from blood;
    - amplification of bacterial DNA by PCR;
    - identification of bacterial DNA by sequencing and mass-spectrometry technology (PLEX-ID).

The evolution of the methodologies used to refine the final methodology used for the clinical trial (Chapter 4) is described in this chapter.

3.2 Methods

3.2.1 *In Vitro* Studies

The aim of the *in vitro* studies was to evaluate a number of techniques to identify the most appropriate for use in the detection of bacteraemia during the prospective clinical study (Chapter 4). As low-level bacteraemias were expected from the study patients, an assay that is able to detect low levels of bacteria in blood consistently was required. Different methods to quantify the number of bacteria in blood were explored, to evaluate whether it was possible to measure the bacterial load in the patients recruited in the study.

3.2.2 Development of an assay to detect low level bacteraemias

Different techniques can be used to detect bacteria in blood. The schematic diagram below shows the basic pathways. In the hospital setting, the 'culture method' arm is traditionally used to detect and identify bacteraemia. However, this method becomes unreliable if the patient has been given antibiotic therapy prior to collection of the blood for culture [212]. Furthermore, some bacteria are unreliably detected using this method, especially if the incubation time is short [213]. The 'molecular method' offers the potential benefit of additional

sensitivity as well as the possibility to detect fastidious bacteria, even in the setting whereby the patients have received prior antibiotics therapy [212].

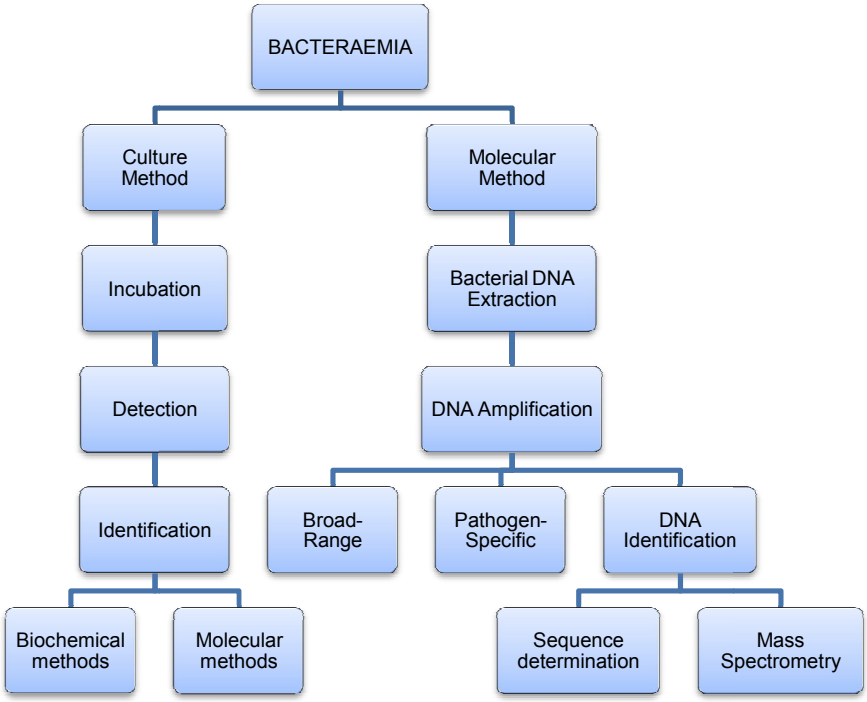


Figure 8. Methods to detect bacteraemia

A broad-range PCR method to detect and identify low levels of bacteria in blood has been designed and validated. The use of broad-range PCR to identify bacteria from positive culture bottles is widely reported [214]. Furthermore, broad-range PCR has also been shown to be an effective method in determining the nature of the pathogen in joint infection and culture negative IE [130]. However, the use of broad-range PCR on clinical blood samples to detect bacteraemia is fraught with complications relating to contamination of the DNA sample [214]. At each of the steps involved in the molecular method (broadly DNA extraction, DNA amplification, sequence determination), contamination may occur leading to false positive results. Furthermore, at each step, the loss of bacterial DNA material can occur, leading to false negative results.

3.2.2.1 Incubation and Culture

In the first instance, the author needed to become accustomed to the basic principles of incubation and plating bacterial colonies. Basic techniques like Gram staining and microscopy to identify the biochemical properties of various commonly encountered human bacterial pathogens were practised. Use of a number of commercially available biochemical identification kits was explored.

Thereafter, the Miles & Misra bacterial quantification method to determine the number of viable bacteria in broth was used [215]. Blood was seeded with bacteria to attempt to quantify the number of viable bacteria present using the Miles & Misra method of bacterial quantification.

3.2.2.2 Samples

Three of the most common organisms involved in infection in urological practice are *Escherichia coli, Enterococcus faecalis and Staphylococcus epidermidis* [55]. Therefore, it was decided that *in vitro* experiments would mainly involve these three organisms.

In the first instance, inocula of the organisms in Table 31 were acquired from the organism bank at the Leeds Teaching Hospitals NHS Trust. All the above organisms were plated on Columbia blood agar (CBA) and cysteine lactose electrolyte deficient (CLED) plates. All the organisms were incubated at 37° C in air. The plates were read after 24 hours. Growth was noted on all the plates. Each organism was inoculated in a tube of glycerol broth and stored in a freezer at around -70° C to -80° C. This set of tubes was used as the organism bank for subsequent experiments.

Gram stained preparations of all the organisms were examined according to the standard operating protocol from the Health Protection Agency, now Public Health England. The results are tabulated below.

ORGANISM	Microscopy and Gram Stain
Enterococcus faecalis	GP coccus
Escherichia coli	GN rod
Extended spectrum β-lactamase *Escherichia coli*	GN rod
Haemophilus influenzae	GN rod
Klebsiella edwardsii	GN rod
Pseudomonas aeruginosa	GN rod
Staphylococcus epidermidis	GP coccus
Staphylococcus aureus	GP coccus
Meticillin-resistant *Staphylococcus aureus*	GP coccus
Streptococcus penumoniae	GP coccus

Table 31. Organisms acquired from the organism bank (GP: Gram-positive, GN: Gram-negative)

The next step was for the author to become familiar with the analytical profile index (API) protocols by BioMerieux SA, Marcy-l'Etoile, France. These are bacterial identification systems, developed in the 1970s, that allow for rapid identification of bacteria to species level. The kits include strips that contain up to 20 miniature cupules in which biochemical reactions are tested. Dilute bacterial suspensions are inoculated in each slot cupule of the API strip, which is then incubated appropriately, Following suitable incubation and the addition of certain reagents when appropriate, the results are read and noted on a scoring sheet. This uses an octal scoring system to provide a unique number between 0 and 7 for each set of three tests. APIweb™ is a software product containing all of the API databases that allows for an automated interpretation of API strip results [216].

The strips that were used were:

API® Gram-negative Identification:

- API 20E – 18-24 hour identification of Enterobacteriaceae and other non-fastidious gram negative bacteria;
- API Rapid 20E – 4-hour identification of Enterobacteriaceae;

- API 20NE – 24 to 48-hour identification of Gram-negative non-Enterobacteriacae.

API® Gram-positive Identification:

- API Staph – Overnight identification of clinical staphylococci and micrococci;
- RAPIDEC® Staph – 2-hour identification of the commonly occurring staphylococci;
- API 20 Strep – 4 or 24-hour identification of streptococci and enterococci API®.

Anaerobe Identification:

- API 20A® – 24-hour identification of anaerobes;
- Rapid ID 32A – 4-hour identification of anaerobes.

This process allowed insight in the different biochemical reactions available for the identification of bacteria to species level. However, owing to the funding restrictions, a decision was made not to continue using these methods.

3.3 Experiment 1 – Quantification of bacteria

An experiment to be able to quantify the number of bacterial colonies from a broth suspension was designed.

3.3.1 Method

The first step was to measure 900 µL sterile water in ten sterile tubes. The tubes were labelled A-J. Tube A was inoculated with *Enterococcus faecalis*. A 100 µL volume of the contents from Tube A was pipetted into Tube B; 100 µL of the contents of Tube B was pipetted into Tube C and so on, until multiple ten ten-fold serial dilutions of the initial inoculum were obtained. Volumes of 200 µL from each tube were plated on CLED plates, which were then incubated at 37° C in air for 24 hours.

3.3.2 Results

All the bacterial growth on the CBA plates for any of the Tubes A-J was confluent and viable counts could thus not be made. The above experiment was repeated but, instead, only 20 µL sample volumes were used, incubated on CLED agar for 24 hours in air at 37° C. The results are shown in Table 32

below. Tube E was used to calculate the number of bacterial cfu present in the initial broth suspension.

The mean count in Tube E was

(33 + 41 + 39 + 32 + 42 + 38) / 6 = 37.5 cfu/mL

Therefore, the bacterial count in the initial suspension was

(37.5 / 20 x 1000) x 10^4 = 1.9 x 10^7 cfu/mL

Tube	Count on Plate 1	Count on Plate 2	Count on Plate 3	Count on Plate 4	Count on Plate 5	Count on Plate 6
A (original)	Confluent	Confluent	Confluent	Confluent	Confluent	Confluent
B (x 10^{-1})	Confluent	Confluent	Confluent	Confluent	Confluent	Confluent
C (x 10^{-2})	Confluent	Confluent	Confluent	Confluent	Confluent	Confluent
D (x 10^{-3})	Confluent	Confluent	Confluent	Confluent	Confluent	Confluent
E (x 10^{-4})	33	41	39	32	42	38
F (x 10^{-5})	<20	<20	<20	<20	<20	<20
G (x 10^{-6})	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
H (x 10^{-7})	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

Table 32. Quantification of *Enterococcus faecalis*

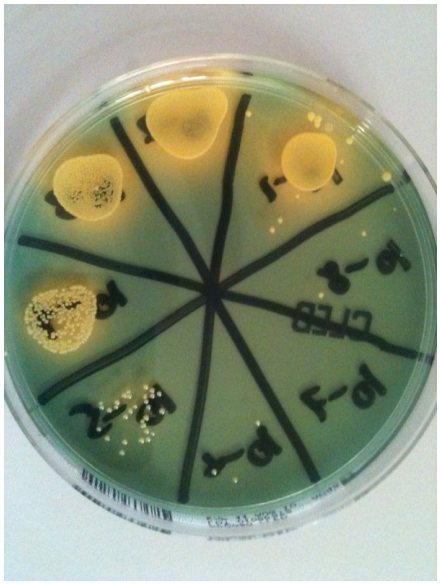


Figure 9. CLED plate showing the results of a Miles and Misra dilution series to determine viable bacterial counts

3.4 Experiment 2 – Quantification of bacteria from incubated broth

The next experiment was to quantify the number of bacteria in an incubated broth.

3.4.1 Method

Two brain heart infusion (BHI) broth tubes (10 ml), labelled Tube X and Y, were inoculated with *Enterococcus faecalis*. Both tubes were incubated at 37° C in air. After 6 hours of incubation, Tube X was taken out of the incubator and serially diluted tenfold with sterile water to obtain eight successive suspensions (A-H). The eight suspensions were plated on CLED plates in 20 µL volumes to allow quantification. The plates were incubated at 37° C in air and read at 24 hours. Tube Y was incubated for 18 hours and the above process repeated.

3.4.2 Results

Tubes X, A, B, C and D yielded confluent bacterial growth making it impossible to count the number of bacterial colonies. Tubes F, G and H grew less than 20 cfu. Therefore, Tube E was used for quantification purposes.

The mean count in Tube E was

[(33 + 33 + 27 + 34 + 33 + 36) / 6] / 20 x 100 = 1.8 x 10^3 cfu/mL

Therefore, the mean count in the initial suspension was

= 1.8 x 10^3 x 10^5 = 1.8 x 10^8 cfu/mL

The mean count in Tube Y was

9.8 x 10^8 cfu/mL

Experiment 2 was repeated three times with the three common organisms encountered in urology (*Enterococcus faecalis, Escherichia coli* and *Staphylococcus epidermidis*). The bacterial growth curve below shows that the stationary phase is reached after 5 to 6 hours of incubation.

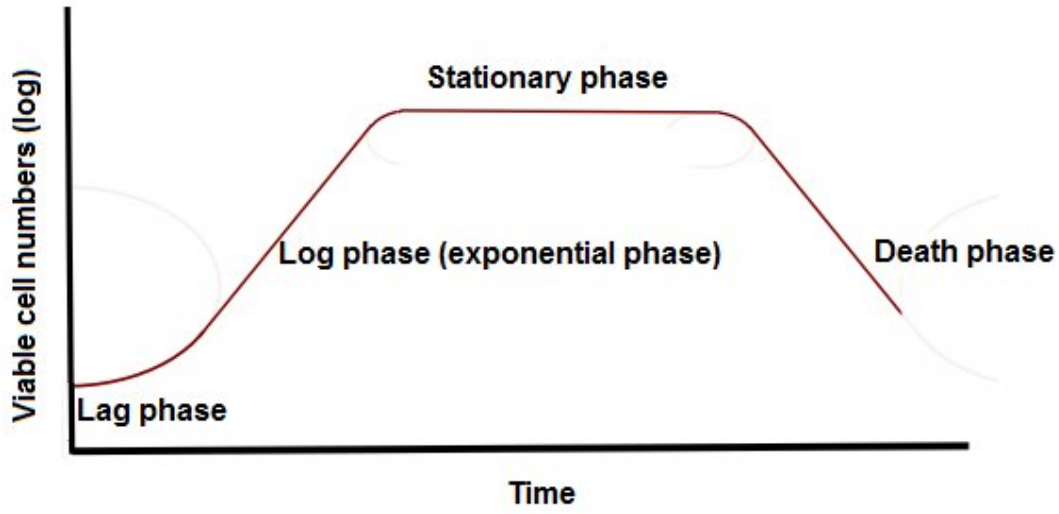


Figure 10. A typical bacterial growth curve

3.5 Experiment 3 – Quantification of bacteria from blood

The next step was to quantify the number of bacteria in blood. Whole research blood was ordered from the NHS Blood and Transplant Service for the following experiments.

3.5.1 Method

Enterococcus faecalis was inoculated in BHI broths (bottles containing 10 ml each), which were incubated at 37° C in air for 6 hours. The initial suspension was serially diluted tenfold with sterile molecular grade water to obtain eight different suspensions (Tubes A-G).

The following tubes of blood spiked with bacteria were obtained:

4 mL of Tube B + 4 mL of Blood = Tube X

4 mL of Tube D + 4 mL of Blood = Tube Y

4 mL of molecular grade water + 4 mL of Blood = Tube Z

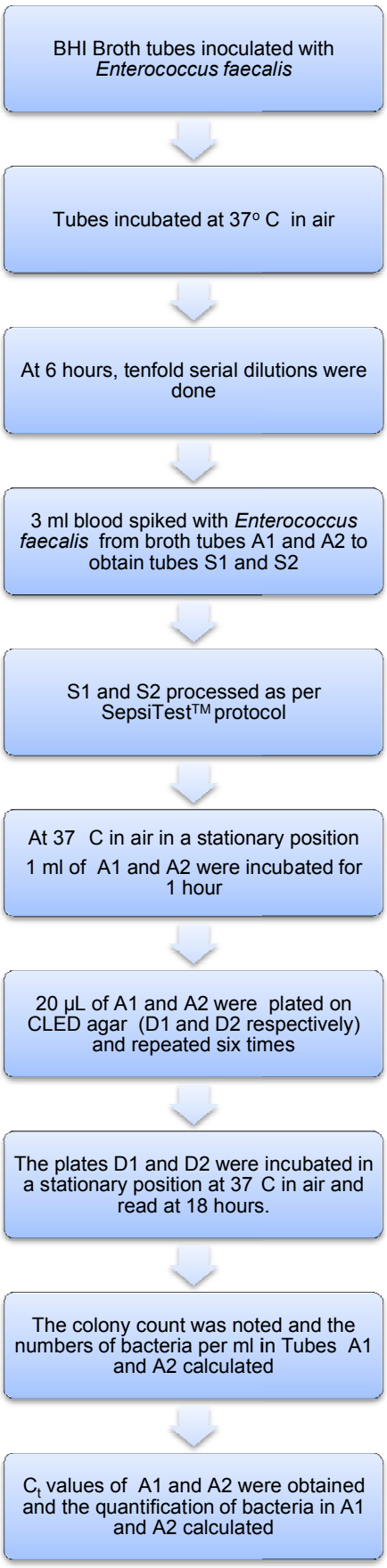
Six inocula of 20 µL of the suspensions from Tube X, Y and Z were plated on CLED and incubated for 24 hours in air at 37° C. Tube E and F were then serially diluted tenfold using blood to obtain eight diluted blood suspensions from each initial tube. Six inocula of 20 µL each of the suspensions from Tube X, Y and Z were plated on CLED and incubated for 24 hours in air at 37° C. The suspensions (in six 20 µL volumes) were plated for quantification purposes.

3.5.2 Results

The results are shows in the Table 33 below.

Plates	Quantitation	Plates	Quantitation
Tube X	Confluent	Tube Y	Confluent
Tube X x 10^{-1}	Confluent	Tube Y x 10^{-1}	Confluent
Tube X x 10^{-2}	Confluent	Tube Y x 10^{-2}	Confluent
Tube X x 10^{-3}	Confluent	Tube Y x 10^{-3}	Confluent
Tube X x 10^{-4}	Confluent	Tube Y x 10^{-4}	(22+34+27+52+22+32)/6 =31.5 cfu
Tube X x 10^{-5}	Confluent	Tube Y x 10^{-5}	Too few
Tube X x 10^{-6}	(19+29+28+26+26+32)/6 =26.2 cfu	Tube Y x 10^{-6}	No growth
Tube X x 10^{-7}	Too few	Tube Y x 10^{-7}	No growth
Tube E x 10^{-8}	No growth	Tube Y x 10^{-8}	No growth
		Tube Z	No growth

Table 33. Suspensions plated in 6 x 20 µL volumes for quantification



Therefore, the numbers of *Enterococcus faecalis* in the spiked blood samples were:

Sample X = (26.2/20) x 1000 x 10^6 = 1.3 x 10^9 cfu/mL

Sample Y = (31.5/20) x 1000 x 10^4 = 2.9 x 10^7 cfu/mL

Experiment 3 showed that quantification was possible from blood spiked with *Enterococcus faecalis*.

3.6 Experiment 4 – Molecular microbiological method to detect bacteraemia

Experiment 4 was designed to evaluate the use of molecular methods to detect bacteraemia. SepsiTest™ by Molzym was used to detect bacterial DNA. SepsiTest™ is a kit developed by Molzym GmbH & Co. KG, Bremen, Germany, with a protocol to identify bacteria from blood. SepsiTest™ is based on the real-time PCR detection and sequence identification of organisms. This molecular method provides the first results of positivity after only four hours. In positive cases, sequence analysis of the amplimers results in the readout of the species. SepsiTest™ requires 1 mL of blood and can detect three hundred and forty-five (345) human pathogens.

Figure 11. Algorithm for Experiment 4

3.6.1 Method – Part 1

The following consumables were required for the experiment:

- 12 mL (4 mL x 3) of fresh human blood
- green-top blood bottles (containing lithium heparin) from Becton Dickinson (BD), UK
- BHI Broth tubes x 2
- Dilution bijoux bottles
- Distilled water
- CLED and CBA agar plates
- Molzym SepsiTest™ kit

Two BHI broth tubes were each inoculated with one 1 mm colony of *Enterococcus faecalis* grown on a CLED agar plate. The two tubes were labelled A1 and A2. A third BHI broth was labelled A3. This tube was not inoculated with any organism.

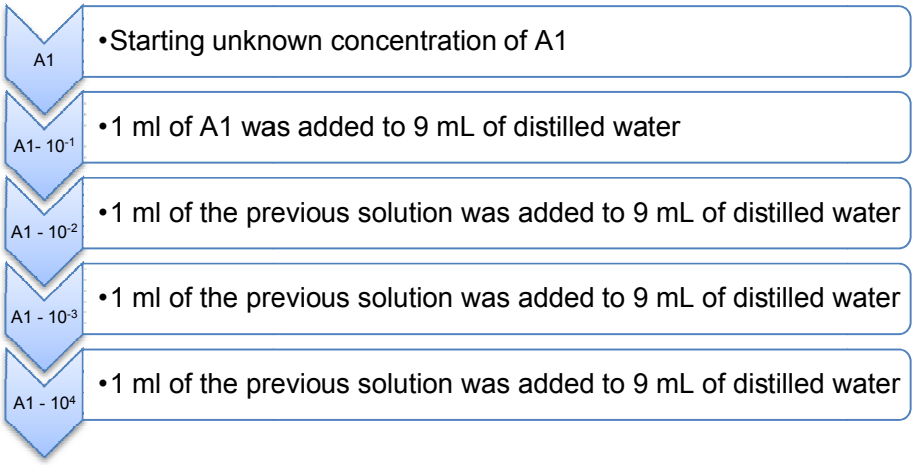


Figure 12. Serial dilutions of A1

A1, A2 and A3 were thoroughly shaken and were then incubated in a stationary position at 37° C for four hours. At 4 hours, A1, A2 and A3 were taken out of the incubator, shaken, placed on the laboratory bench and observed.

3.6.2 Results – Part 1

Tubes	*Appearance*
A1	Cloudy
A2	Cloudy
A3	Clear

Table 34. Appearance of BHI broths tubes after 4 hours of incubation At 37° C

Cloudiness in the broths indicated bacterial proliferation. Tube A1 was used for further experiments. Tubes A2 (as Tube A1 was deemed adequate for the further experiments) and A3 were discarded.

3.6.3 Method – Part 2

The contents of Tube A1 were serially diluted tenfold using sterile 20 mL bijoux tubes as per the algorithm in Figure 12. Three green-top tubes were labelled B, C and D and 4 mL of blood was added to each tube. A 4 mL volume of the second (A1-10^{-2}) and fourth dilution (A1-10^{-4}) were pipetted in Tubes B and C respectively. A 4 mL volume of distilled water was pipetted to Tube D.

Three CLED agar plates and three CBA agar plates were labelled. The six plates were divided into twelve segments each. In each segment of plates B-CBA and B-CLED, 20 µL of the suspension from Tube B was pipetted. This procedure was repeated for suspensions in Tubes C and D until all six plates were inoculated with their corresponding suspensions. The plates were incubated for 18 hours at 37° C in air.

3.6.4 Results – Part 2

All the segments on plates B-CLED, B-CBA, C-CLED and C-CBA were confluent. There was no growth on the bacteria-free control plates.

3.6.5 Methods – Part 3

As all the bacterial growth was confluent and precluded quantification, Tube B was diluted serially as follows.

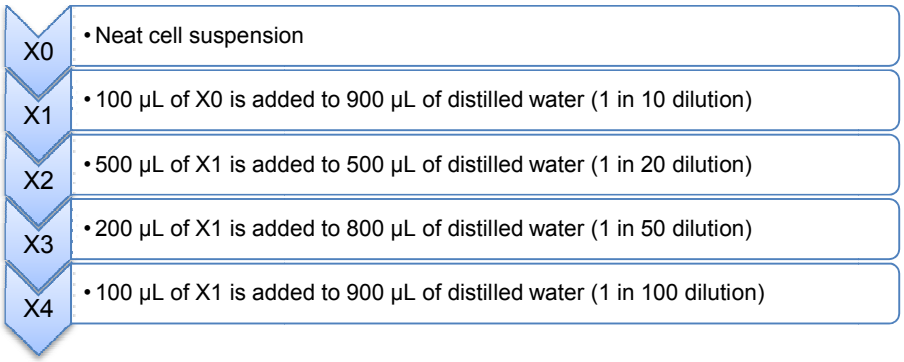


Figure 13. Serial dilutions of Tube B

Four CLED and four CBA agar plates were divided into six segments each as described above. Samples from tubes X1, X2, X3 and X4 were plated on the both CBA and CLED as follows.

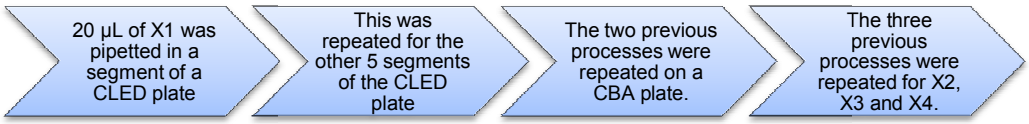


Figure 14. Algorithm for plating the diluted suspensions of Tube B

The eight plates obtained were labelled. Tube C was handled slightly differently and was diluted serially as shown in Figure 15 below.

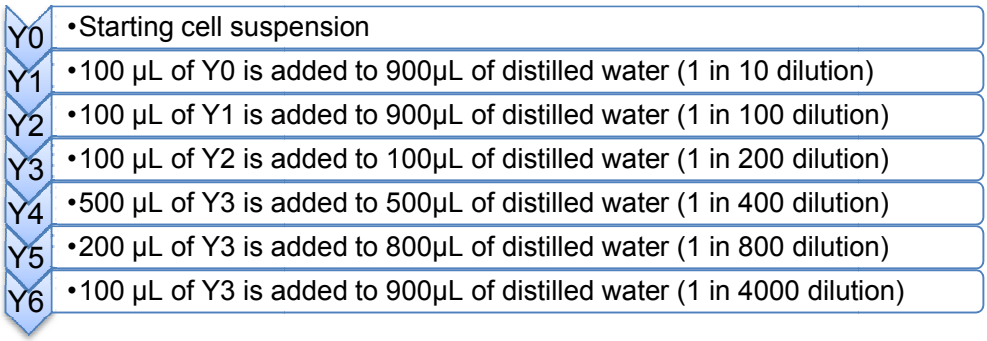


Figure 15. Serial dilutions of Tube C

Four CLED and four CBA agar plates were divided into six segments each as described above. Samples from Y2, Y3, Y4 and Y5 were plated on the both CBA and CLED agar as follows. The eight plates thus obtained were labelled. The sixteen plates were incubated for 18 hours at 37° C. Plates X1-4 and Y2- 5 were read by counting the number of cfu per 20 µL inoculants on the CLED and CBA plates.

3.6.6 Results – Part 3

The results are shown in Table 35 below.

	CLED Plate						**CBA Plate**					
	X1-CLED						X1-CBA					
Count per segment	54	59	47	55	N	N	48	58	56	44	N	N
	X2-CLED						X2-CBA					
Count per segment	29	29	30	39	28	26	21	32	20	18	27	31
	X3-CLED						X3-CBA					
Count per segment	F	F	F	F	F	F	F	F	F	F	F	F
	X4-CLED						X4-CBA					
Count per segment	F	F	F	F	F	F	F	F	F	F	F	F

Table 35. Quantification on plates X1-4
F = too few to count; N = not inoculated; T = too many to count

The results for plates Y are shown below.

	CLED Plate						CBA Plate					
	Y2-CLED						Y2-CBA					
Count per segment	T	T	T	T	N	N	T	T	T	T	N	N
	Y3-CLED						Y3-CBA					
Count per segment	26	31	28	26	37	24	39	35	28	32	33	37
	Y4-CLED						Y4-CBA					
Count per segment	9	13	10	13	8	9	9	15	9	13	14	11
	Y5-CLED						Y5-CBA					
Count per segment	F	F	F	F	F	F	F	F	F	F	F	F

Table 36. Quantification on plates Y2-5
F = too few to count; N = not inoculated; T = too many to count

The mean bacterial counts and the concentrations of the initial suspensions are shown in Table 37 below.

Plate	CLED		CBA	
	Average Count/CFU	Number of bacteria in the initial suspension /ml	Average Count/CFU	Number of bacteria in the initial suspension/ml
X1 (1/10)	53.75	2.69×10^8	51.5	2.57×10^8
X2 (1/20)	30.2	3.02×10^8	24.8	2.48×10^8
Y3 (1/200)	28.7	2.87×10^8	34	3.40×10^8
Y4 (1/400)	10.3	2.57×10^8	11.8	2.95×10^8

Table 37. Mean counts and bacterial concentrations.

Therefore, the mean number of bacteria per mL in suspension B (X0) = $\{(2.69+2.57+3.02+2.48)/4\} \times 10^8 = 2.69 \times 10^8$ cfu/mL

and, the mean number of bacteria per mL in suspension C (Y0) = $\{(2.87+3.40+2.57+2.95)/4\} \times 10^8 = 2.94 \times 10^8$ cfu/mL

3.6.7 Methods – Part 4

Once the growth and quantification methods were performed using the traditional culture methods described above, samples B, C and D were also used to identify and quantify the bacteraemia present using SepsiTest™. The three samples (B, C and D) were processed in duplicates. The SepsiTest™ methodology was used and the samples in Table 37 above were amplified using the MxPro-Mx3000P thermocycler from Stratagene. Owing to time constraints, the process was run over three separate days as follows.

- Day 1: Step 1-7 for bacterial DNA extraction. The samples were then stored at -15° C;
- Day 2: Step 8-18 for the completion of the DNA extraction. The samples were stored at -15° C;
- Day 3: DNA amplification using the thermal profile advised by the SepsiTest™ manual.

3.6.8 Results – Part 4

The results of the molecular experiments good amplification of the bacterial DNA was noted with C_t values as low as nineteen cycles noted. Sharp and narrow melting curves were noted at temperatures between 88-92° C.

Sample	Contents	C_t Values
NC	Negative Control (Bacteria Mastermix + DNA free water)	22.98
IC-B1	Internal Control (IC Mastermix + B1 DNA)	26.51
IC-B2	Internal Control (IC Mastermix + B2 DNA)	26.52
IC-C1	Internal Control (IC Mastermix + C1 DNA)	26.01
IC-C2	Internal Control (IC Mastermix + C2 DNA)	27.91
IC-D1	Internal Control (IC Mastermix + D1 DNA)	28.63
IC-D2	Internal Control (IC Mastermix + D2 DNA)	25.75
IC-Water	Internal Control (IC Mastermix + DNA-free water)	27.17
B1	Bacteria Mastermix + B1 DNA	26.27
B2	Bacteria Mastermix + B2 DNA	25.31
C1	Bacteria Mastermix + C1 DNA	25.90
C2	Bacteria Mastermix + C2 DNA	19.72
D1	Bacteria Mastermix + D1 DNA	23.34
D2	Bacteria Mastermix + D2 DNA	23.55
P1	Bacteria Mastermix + P1 DNA	22.84
P2	Bacteria Mastermix + P2 DNA	29.28

Table 38. Samples processed by the SepsiTest™ method and their C_t values

Table 38 above shows the C_t values of the samples processed using the SepsiTest™ method. Experiment 4 showed that it was possible to quantify the number of bacteria using the culture method. The molecular method was able to detect the bacteria in blood and further work was required to evaluate whether quantification using the molecular method was possible.

3.7 Experiment 5 - SepsiTest™

Experiment 5 was designed to evaluate whether it was possible to quantify low-level bacteraemia using the SepsiTest™ kit. The organism used was *Enterococcus faecalis* from a previous plate growth.

3.7.1 Method

Three tubes of BHI broths, containing 10 ml of broth each, were labelled A, B and C. Tube A was inoculated with two 1 mm colony of the *Enterococcus faecalis*. Tube B was inoculated with twenty 1 mm colonies of the *Enterococcus faecalis*. Tube C was not inoculated with any organism. Tubes A, B and C were incubated for 6 hours at 37° C in air. At 6 hours, Tube A was mildly cloudy, Tube B was very cloudy and Tube C was clear. Suspension B was used for further tests. Tube B was re-labelled as Y and serially diluted as shown in Figure 18 below.

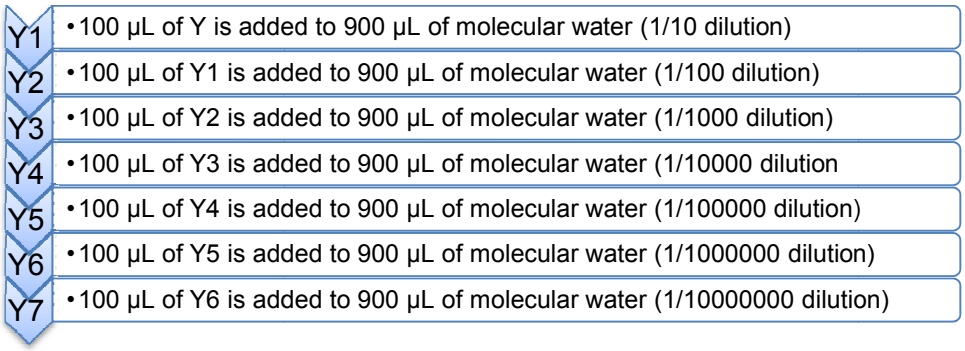


Figure 16. Dilutions of the BHI broth from Tube B

A 200 µL volume of suspension Y was pipetted into 1.8 mL of bacteria-free blood to produce suspension PY and subsequent tenfold dilutions were performed as shown in Figure 19 below.

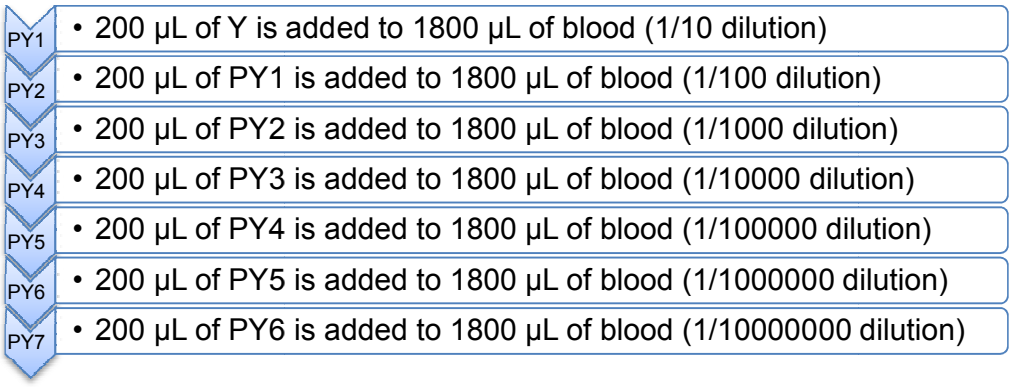


Figure 17. Serial dilutions of spiked blood

The following suspensions were used for molecular processing:

- PY, PY1, PY2, PY3, PY4, PY5, PY6, PY7 and C, with C being 2 mL of blood without any inoculant.

For the Miles and Misra method of quantification, the following suspensions were used:

- Y1, Y2, Y3, Y4, Y5, Y6 and Y7.

Bacterial DNA from the nine samples (C, PY, PY1, PY2, PY3, PY4, PY5, PY6 and PY7) was extracted in duplicates using the SepsiTest™ protocol. The extracted bacterial DNA was, in turn, amplified in triplicate using the SepsiTest™ protocol. This process resulted in forty-eight separate samples and C_t values were obtained for the forty-eight samples. A 20 µL volume of each of Y1-7 were plated on CBA Agar plates in sextuplicate and incubated for 18 hours at 37° C in air to allow quantification.

3.7.2 Results

The concentrations of the eight starting suspensions were calculated and tabulated, using the Miles and Misra quantification method.

Suspension	Number of cfu/ml
Y	8.25×10^8
Y1	8.25×10^7
Y2	8.25×10^6
Y3	8.25×10^5
Y4	8.25×10^4
Y5	8.25×10^3
Y6	8.25×10^2
Y7	8.25×10^1

Table 39. Quantification using the culture method

The SepsiTest™ method yielded the following C_t values (Table 40).

Initial Suspension	Duplicates after extraction	Triplicate after PCR	C_t values
C	Cα	Cα-A	25.63
		Cα-B	26.51
		Cα-C	29.00
	Cβ	Cβ-A	26.31
		Cβ-B	28.69
		Cβ-C	27.79
Y	Yα	Yα-A	12.87
		Yα-B	14.23
		Yα-C	13.85
	Yβ	Yβ-A	12.65
		Yβ-B	13.26
		Yβ-C	13.60
Y1	Y1α	Y1α-A	14.72
		Y1α-B	15.36
		Y1α-C	15.81
	Y1β	Y1β-A	14.87
		Y1β-B	15.54
		Y1β-C	15.55
Y2	Y2α	Y2α-A	20.47
		Y2α-B	20.04
		Y2α-C	19.72
	Y2β	Y2β-A	22.27
		Y2β-B	23.53
		Y2β-C	26.83

Initial Suspension	Duplicates after extraction	Triplicate after PCR	C_t values
Y3	Y3α	Y3α-A	23.13
		Y3α-B	23.31
		Y3α-C	24.11
	Y3β	Y3β-A	21.42
		Y3β-B	22.05
		Y3β-C	22.78
Y4	Y4α	Y4α-A	26.09
		Y4α-B	25.93
		Y4α-C	26.58
	Y4β	Y4β-A	26.77
		Y4β-B	26.44
		Y4β-C	Failed well
Y5	Y5α	Y5α-A	29.01
		Y5α-B	28.46
		Y5α-C	27.40
	Y5β	Y5β-A	27.62
		Y5β-B	28.27
		Y5β-C	27.81
Y6	Y6α	Y6α-A	28.37
		Y6α-B	29.96
		Y6α-C	27.32
	Y6β	Y6β-A	28.37
		Y6β-B	34.04
		Y6β-C	27.37

Initial Suspension	Duplicates after extraction	Triplicate after PCR	C_t values
Y7	Y7α	Y7α-A	28.04
		Y7α-B	35.88
		Y7α-C	27.37
	Y7β	Y7β-A	27.84
		Y7β-B	27.38
		Y7β-C	35.70

Table 40. C_t values using the SepsiTest™ method of bacterial DNA amplification

The results obtained from the Miles and Misra and the SepsiTest™ methods were used to construct a standard curve, which was used for bacterial quantification. The standard curve was plotted with the x-axis being Log_{10} (number of bacteria) and the y-axis being the C_t values obtained. For the purpose of this experiment, C_t values above 27 were considered too high to be able to identify and quantify *Enterococcus faecalis* reliably, as the mean C_t value for the control sample, C, was 27.3. Therefore, the standard curve was plotted for the five samples with mean C_t values of 27 or less (Figure 20).

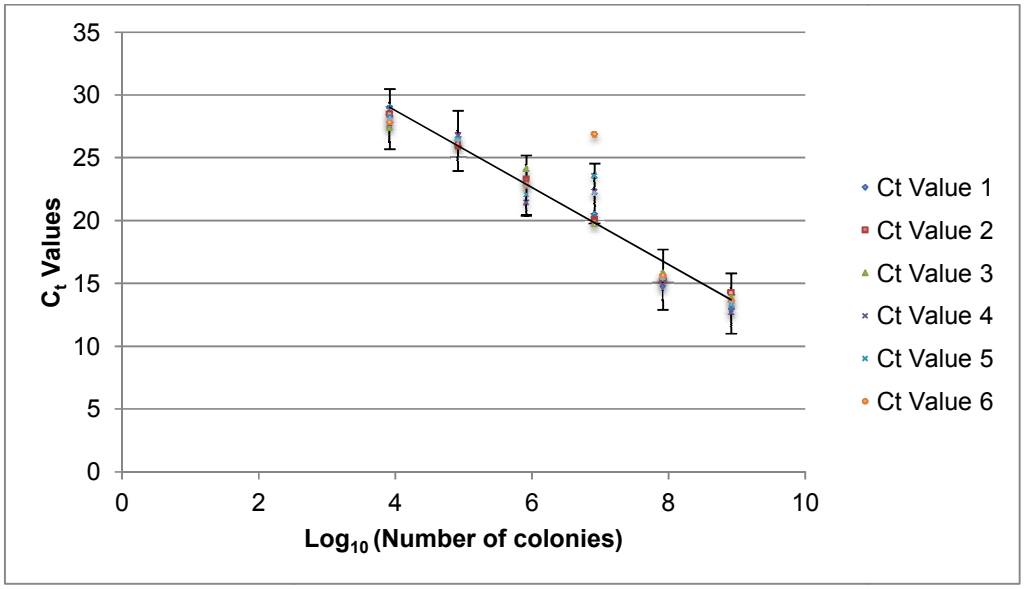


Figure 18. Standard curve for *Enterococcus faecalis* using Sepsitest™

The molecular method used was not sensitive enough to detect low levels of bacteria in the blood samples. Levels greater than 10^4 cfu/mL could be reliably detected using this broad-range PCR method, in line with reported studies in the literature [8]. However, a more sensitive method was required, that was able to detect bacteraemia of < 10^4 cfu/mL

3.8 Experiment 6 – MolYsis Complete 1 mL protocol

Experiment 5 was repeated using a different molecular microbiological kit. The kit used for bacterial DNA extraction from blood was the MolYsis Complete 1 mL protocol. DNA amplification was done using an in-house amplification protocol using the in-house short 16S primers.

3.8.1 Method

The methods used in Experiment 5 were repeated using a different molecular kit (MolYsis Complete 1 mL).

3.8.2 Results

The standard curve obtained is shown below (Figure 19).

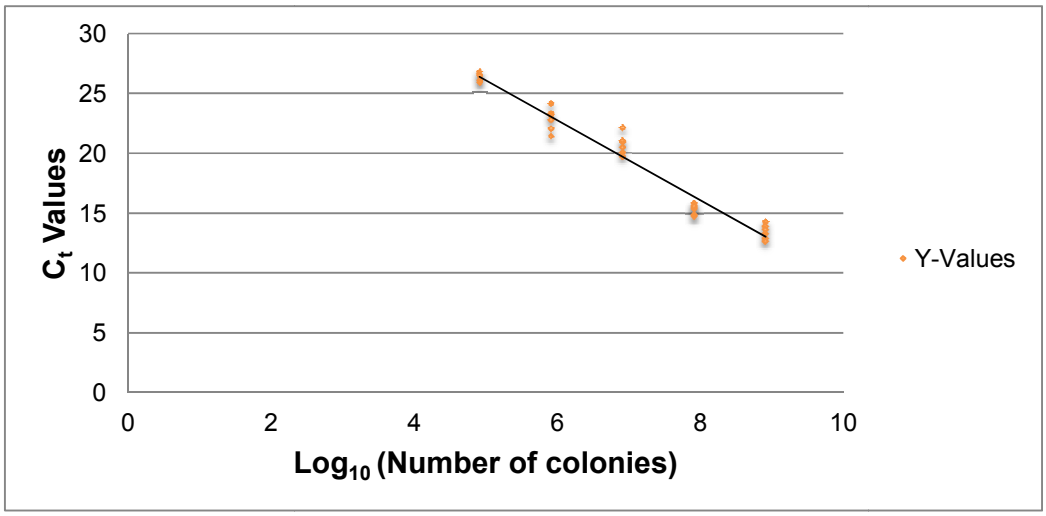


Figure 19. Standard Curve for *Enterococcus faecalis* using the 1 mL MolYsis Complete5 protocol

The standard curve allowed quantification of *Enterococcus faecalis* down to 10^4 cfu/mL but no improvement was possible.

3.9 Experiment 7 – MolYsis Complete5

This experiment was designed to be able to improve the detection and quantification of bacteria in blood using molecular methods, particular when the

number of bacteria present in blood was low. One of the problems encountered in previous experiments was the inability to detect low-level bacteraemia. Therefore, a decision was made to use larger volumes of blood for the molecular processing. The kit identified was MolYsis Complete5 by Molzym GmbH & Co. KG, Bremen, Germany.

The SepsiTest™ bacterial DNA extraction protocol processed 1 mL of blood and yielded 100 µL of eluate used for amplification. The same concentration gradient was possible using 1 mL of blood and the MolYsis Complete kit. Both these protocols allowed quantification to 10^4 cfu/mL but no better. MolYsis Complete5 uses 5 mL of blood instead of 1 mL of blood and yields 100 µL of eluate as in the previous methods. Therefore, in theory, five times more bacterial DNA was harnessed by the MolYsis Complete5 method, improving sensitivity and the ability to detect low levels bacteraemia.

3.9.1 Method

The organisms used were *Enterococcus faecalis* (A), *Escherichia coli* (B) and *Staphylococcus epidermidis* (C). The colonies were obtained from previous plate growth. Seven tubes of BHI broth (each containing 10 ml of broth) were labelled A1, A2, B1, B2, C1, C2 and D. Tube A1 was inoculated with two 1 mm colony of *Enterococcus faecalis*. Tube A2 was inoculated with twenty 1 mm colony of *Enterococcus faecalis*. Tube B1 was inoculated with two 1 mm colony of *Escherichia coli*. Tube B2 was inoculated with twenty 1 mm colony of *Escherichia coli*. Tube C1 was inoculated with two 1 mm colony of *Staphylococcus epidermidis*. Tube C2 was inoculated with twenty 1 mm colony of the *Staphylococcus epidermidis*. Tube D was not inoculated with any organism. The seven tubes were incubated for 6 hours at 37° C in air.

3.9.2 Results

At 6 hours, Tube A1 was mildly cloudy, Tube A2 was very cloudy, Tube B1 was mildly cloudy, Tube B2 was very cloudy, Tube C1 was very cloudy, Tube C2 was cloudy and Tube D was clear. Therefore, suspensions A2, B2, C2 and D were used for further tests.

3.9.3 Method

Tube A2 was relabelled as X, B2 as Y, and C2 as Z. Suspension X was serially diluted as per the algorithm below (Figure 22) and this process was repeated with suspensions Y and Z.

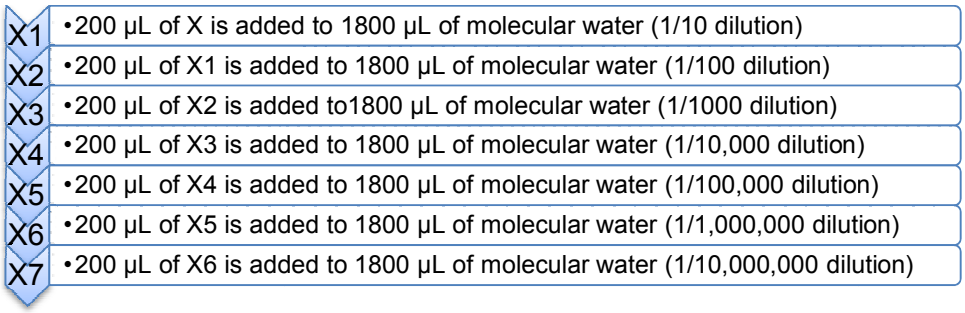


Figure 20. Dilution of sample X

A 1 mL volume of X was pipetted into 9 mL bacteria-free blood to produce suspension PX. A 5 mL volume of suspension PX was pipetted into a 15 mL Falcon™ tube; the remaining 5 mL of suspension PX was pipetted in another 15 mL Falcon™ tube. The tubes were labelled PXα and PXβ. A 1 mL volume of X1 was pipetted into 9 mL bacteria-free blood to produce PX1. The 10 mL of PX1 was divided into two 15 mL Falcon™ tubes, each containing 5 mL of suspension and labelled PX1α and PX1β respectively. This process was repeated with X1-X7, Y-Y7 and Z-Z7. A 10 mL volume of fresh 'unseeded' blood was also divided into tubes labelled Cα and Cβ. Therefore, the following samples were obtained for DNA extraction (Table 41). Bacterial DNA was extracted from the following samples using the Molzym MolYsis Complete5 protocol. The eluates obtained were stored at -70° C.

Samples with bacteraemia							
PXα	PX1α	PX2α	PX3α	PX4α	PX5α	PX6α	PX7α
PXβ	PX1β	PX2β	PX3β	PX4β	PX5β	PX6β	PX7β
PYα	PY1α	PY2α	PY3α	PY4α	PY5α	PY6α	PY7α
PYβ	PY1β	PY2β	PY3β	PY4β	PY5β	PY6β	PY7β
PZα	PZ1α	PZ2α	PZ3α	PZ4α	PZ5α	PZ6α	PZ7α
PZβ	PZ1β	PZ2β	PZ3β	PZ4β	PZ5β	PZ6β	PZ7β
Cα	Cβ						

Table 41. Spiked blood samples for bacterial DNA extraction

X was serially diluted tenfold as per the algorithm below. This process was repeated for samples Y and Z.

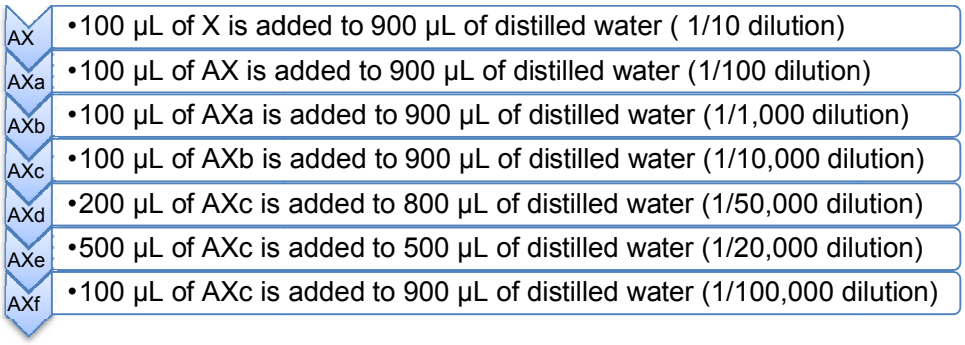


Figure 21. Dilutions of the initial sample

The following samples (Table 42) were obtained. A 20 µL volume of each of the samples was plated on CBA Agar plates in triplicate and incubated for 18 hours at 37° C. The plates were used for bacterial quantification using the Miles and Misra method. Sample D was a control sample.

SAMPLES							
X	AX	AXa	AXb	AXc	AXd	AXe	AXf
Y	AY	AYa	AYb	AYc	AYd	AYe	AYf
Z	AZ	AZa	AZb	AZc	AZd	AZe	AZf
D							

Table 42. Samples for Miles and Misra quantification

Viable counts were obtained in Samples AXf, AYc and AZb. All the other samples had either too many or too few to quantify. Bacterial quantification using the Miles and Misra method was as shown in Table 43 below.

Samples	**CFU Counts on plate**	**Mean count**	**Concentration of initial suspension (cfu/ml)**
AXf	152-150-142	148.0	X: 7.4×10^8
AYc	41-41-40	40.7	Y: 2.0×10^7
AZb	190-186-195	190.3	Z: 9.5×10^6

Table 43. Bacterial count using the Miles and Misra method

Experiment 7 above was repeated to obtain further bacterial eluates. The samples were labelled F (*Enterococcus faecalis*), G (*Escherichia coli*) and H (*Staphylococcus epidermidis*). The concentrations of the different suspensions are as follow:

Concentration of *Enterococcus faecalis*: 1.32×10^8 cfu/mL

Concentration of *Escherichia coli*: 1.59×10^7 cfu/mL

Concentration of *Staphylococcus epidermidis*: 1.78×10^7 cfu/mL

The samples in Table 44 below were available for bacterial DNA extraction and amplification.

Enterococcus faecalis	**Concentration (Miles& Misra cfu/ml)**	***Escherichia coli***	**Concentration (Miles & Misra cfu/ml)**	***Staphylococcus epidermidis***	**Concentration (Miles & Misra cfu/ml)**
PXα	7.4×10^7	PYα	2.0×10^6	PZα	9.5×10^5
PXβ		PYβ		PZβ	
PFα	1.3×10^7	PGα	1.6×10^6	PHα	1.8×10^6
PFβ		PGβ		PHβ	
PX1α	7.4×10^6	PY1α	2.0×10^5	PZ1α	9.5×10^4
PX1β		PY1β		PZ1β	
PF1α	1.3×10^6	PG1α	1.6×10^5	PH1α	1.8×10^5
PF1β		PG1β		PH1β	
PX2α	7.4×10^5	PY2α	2.0×10^4	PZ2α	9.5×10^3
PX12β		PY12β		PZ12β	
PF2α	1.3×10^5	PG2α	1.6×10^4	PH2α	1.8×10^4
PF2β		PG2β		PH2β	
PX3α	7.4×10^4	PY3α	2.0×10^3	PZ3α	9.5×10^2
PX3β		PY3β		PZ3β	
PF3α	1.3×10^4	PG3α	1.6×10^3	PH3α	1.8×10^3
PF3β		PG3β		PH3β	
PX4α	7.4×10^3	PY4α	2.0×10^2	PZ4α	9.5×10^1
PX4β		PY4β		PZ4β	
PF4α	1.3×10^3	PG4α	1.6×10^2	PH4α	1.8×10^2
PF4β		PG4β		PH4β	
PX5α	7.4×10^2	PY5α	2.0×10^1	PZ5α	10
PX5β		PY5β		PZ5β	
PF5α	13×10^2	PG5α	16	PH5α	18
PF5β		PG5β		PH5β	
PX6α	74	PY6α	2	PZ6α	1
PX6β		PY6β		PZ6β	

Enterococcus faecalis	Concentration (Miles& Misra cfu/ml)	*Escherichia coli*	Concentration (Miles & Misra cfu/ml)	*Staphylococcus epidermidis*	Concentration (Miles & Misra cfu/ml)
PF6α	13	PG6α	1	PH6α	2
PF6β		PG6β		PH6β	
PX7α	7	PY7α	Nil	PZ7α	Nil
PX7β		PY7β		PZ7β	
PF7α	1	PG7α		PH7α	
PF7β		PG7β		PH7β	

Table 44. Samples available for amplification with the concentration of bacteria

All the samples in Table 44 above were stored at -70° C for further molecular processing.

3.10 Experiment 8 – Polymerase Chain Reaction

All the samples from Table 44 above were amplified in triplicate using the PCR protocol used in the Department (Appendix 4).

3.10.1 Results

The results from the *Enterococcus faecalis* samples are tabulated below.

Sample	Concentration (CFU/ml)	Ct values			Ct values for unspiked Blood	Ct values for NTC
X-A	$7.4x10^7$	14.81	15.42	15.32	29.63	27.91
X-B		11.98	12.17	12.16	29.36	
X1-A	$7.4x10^6$	15.09	15.31	15.15	25.98	25.18
X1-B		13.76	13.46	13.88		
X2-A	$7.4x10^5$	18.06	20.35	21.64	29.35	33.66
X2-B		19.84	18.73	19.92		
X3-A	$7.4x10^4$	24.16	23.83	23.77	30.72	30.57
X3-B		20.12	22.06	22.17		29.03
X4-A	$7.4x10^3$	28.54	26.63	25.85	27.99	27.57
X4-B		24.60	26.22	25.62	28.40	
X5-A	$7.4x10^2$	25.72	26.23	26.43	29.56	25.20
		26.63	26.66	26.17	30.09	
X5-B		27.89	27.84	26.54	29.68	
		27.65	27.61	27.58	26.81	
X6-A	$7.4x10^1$	30.77	27.82	29.98	29.98	29.98
X6-B		29.25	30.76	31.28		
X7-A	7	26.50	28.64	27.23	28.59	29.65
X7-B		28.15	29.91	28.78		

Table 45. C_t values for the *Enterococcus faecalis* samples from the first extraction experiment (shading represent C_t values within three cycles of the C_t value of the negative control)

Samples having C_t values within three cycles of the negative control (unspiked blood) were not used for further analysis in plotting the standard curve. This strict criterion allowed the subsequent use of the standard curve to be more

accurate. Figure 24 below shows the standard curve using C_t values not within three cycles of the negative controls.

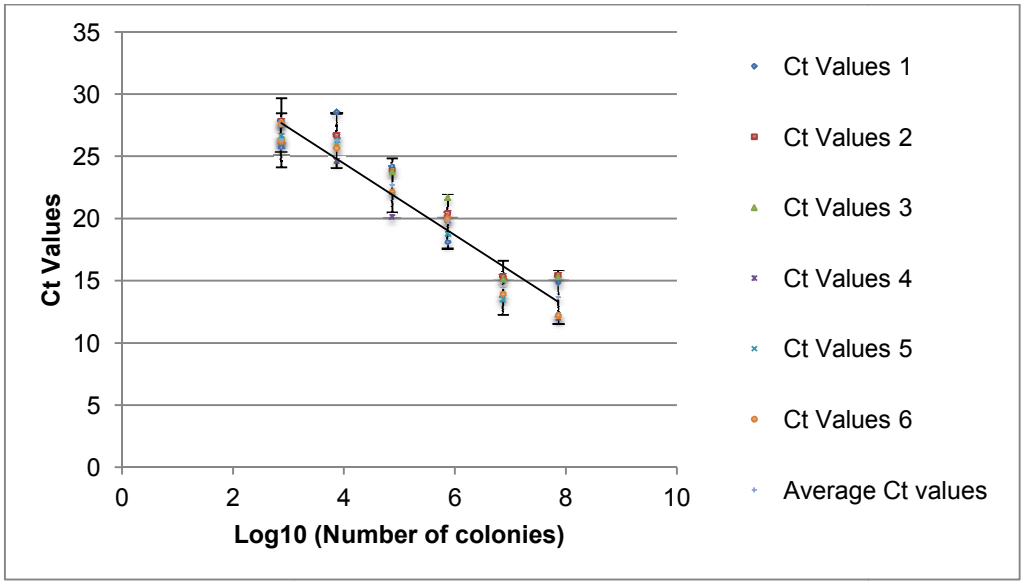


Figure 22. Standard Curve for X samples

The above analysis was repeated for the F samples (*Enterococcus faecalis*). The results and the resulting standard curve are shown below.

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
F-A	$1.32X10^7$	13.21	13.90	13.77	29.47	29.96
F-B		10.47	10.17	10.32		
F1-A	$1.32X10^6$	13.31	13.13	13.19		
F1-B		13.35	13.54	13.06	30.00	32.65
F2-A	$1.32X10^5$	18.92	18.40	18.43	27.83	28.75
F2-B		18.01	18.27	17.81		
F3-A	$1.32X10^4$	20.85	21.58	21.28	29.64	26.85
F3-B		22.69	22.89	22.84		39.36
F4-A	$1.32X10^3$	24.34	25.28	25.64	28.22	29.38
F4-B		25.57	24.69	25.94		

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
F5-A	$1.32X10^2$	27.81	29.02	26.95	30.55	31.89
F5-B		28.41	27.41	28.52		
F6-A	$1.32X10^1$	28.83	27.68	29.89	28.50	30.17
F6-B		28.74	29.16	29.38		
F7-A	1	29.11	30.77	31.62	32.11	35.07
F7-B		31.00	24.51	30.41		

Table 46. C_t values for the *Enterococcus faecalis* samples from the second extraction experiment (shading represent C_t values within three cycles of the C_t value of the negative control)

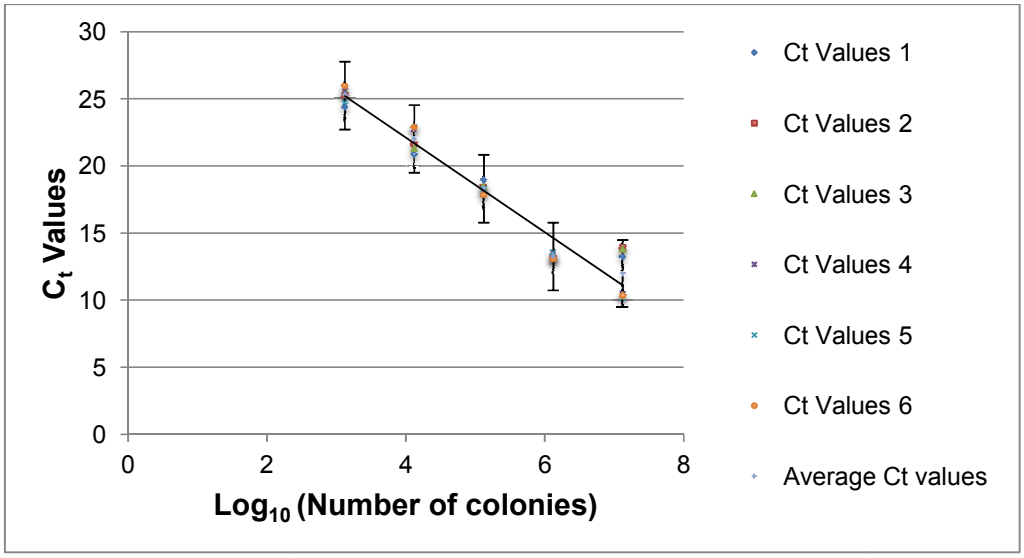


Figure 23. Standard curve for *Enterococcus faecalis* using values from F samples

Using values from both the Z and the F samples for *Enterococcus faecalis*, the standard curve below was obtained (Figure 26):

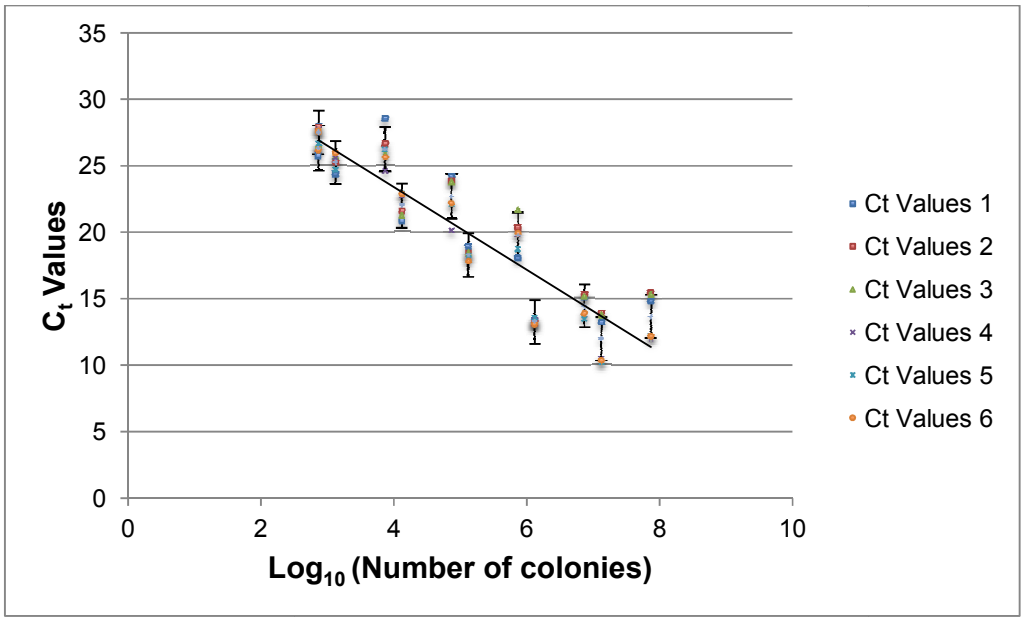


Figure 24. Standard curve for *Enterococcus faecalis* using values from X and F samples

The standard curves above shows that the molecular method that was used allowed reliable detection and quantification of *Enterococcus faecalis* bacteraemia of an order of 10^3 cfu/mL. Samples containing 10^2 cfu/mL bacteria were reliably detected but not reliably quantified as the C_t values corresponding to 10^2 cfu/mL samples were within three cycles of the negative control. The above analysis was repeated for the *Escherichia coli* and the *Staphylococcus epidermidis* samples obtained from Experiment 7. The results are shown below.

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
Y-A	2.0×10^6	22.69	22.50	22.94	27.41	29.65
Y-B		23.39	23.36	23.53		
Y1-A	2.0×10^5	25.08	25.58	25.61		
Y1-B		26.90	27.95	28.09	29.34	37.23

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
Y2-A	2.0×10^4	30.29	29.89	30.37	33.84	31.84
Y2-B		29.58	31.19	30.54		31.26
Y3-A	2.0×10^3	27.17	29.74	30.48	32.62	28.90
Y3-B		29.95	30.00	30.49		30.78
Y4-A	2.0×10^2	29.85	28.69	28.46		
Y4-B		29.63	32.51	31.29		33.33
Y5-A	2.0×10^1	28.20	31.16	30.29	32.81	35.43
Y5-B		27.57	31.86	33.82		
Y6-A	2	30.52	31.03	31.25	26.08	27.00
Y6-B		30.83	29.89	32.20		
Y7-A	Nil	31.92	31.78	30.12	29.23	29.32
Y7-B		29.09	28.82	30.97		

Table 47. C_t values of Y samples (shading represent C_t values within three cycles of the C_t value of the negative control)

The C_t values of the Y samples above were used to construct a standard curve to allow quantification based on C_t values. C_t values of samples within three cycles of the negative controls were disregarded in the construction of the standard curve.

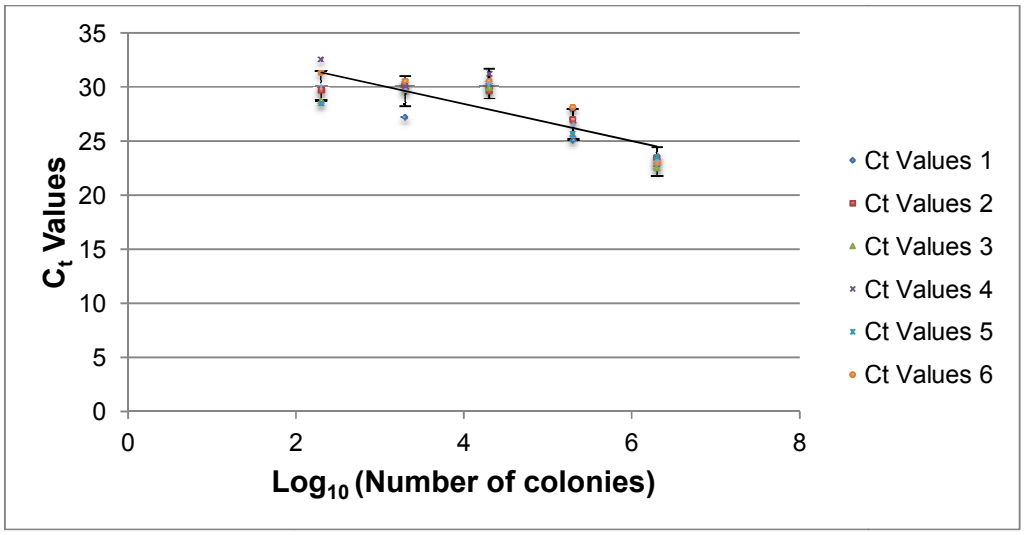


Figure 25. Standard Curve for Y samples

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
G-A	$1.59x10^6$	10.71	10.61	10.57	31.17	31.79
G-B		13.09	13.17	13.03		30.19
G1-A	$1.59x10^5$	16.09	15.88	15.43	30.04	32.75
G1-B		15.31	15.44	15.57		32.45
G2-A	$1.59x10^4$	19.82	19.70	19.54		31.01
G2-B		19.46	18.95	19.16	27.09	29.50
G3-A	$1.59x10^3$	25.05	25.29	25.42	30.92	31.09
G3-B		26.25	26.79	26.55		
G4-A	$1.59x10^2$	26.91	26.91	26.26	30.78	30.78
G4-B		27.27	27.24	27.06		

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
G5-A	$1.59x10^1$	28.40	27.75	28.38	28.97	30.48
G5-B		28.95	28.62	28.99		
G6-A	2	31.69	31.43	32.41	32.05	31.62
G6-B		29.25	31.57	30.83		
G7-A	Nil	32.77	31.99	29.83	29.22	33.79
G7-B		32.32	29.77	30.53		

Table 48. C_t values of G samples (shading represent C_t values within three cycles of the C_t value of the negative control)

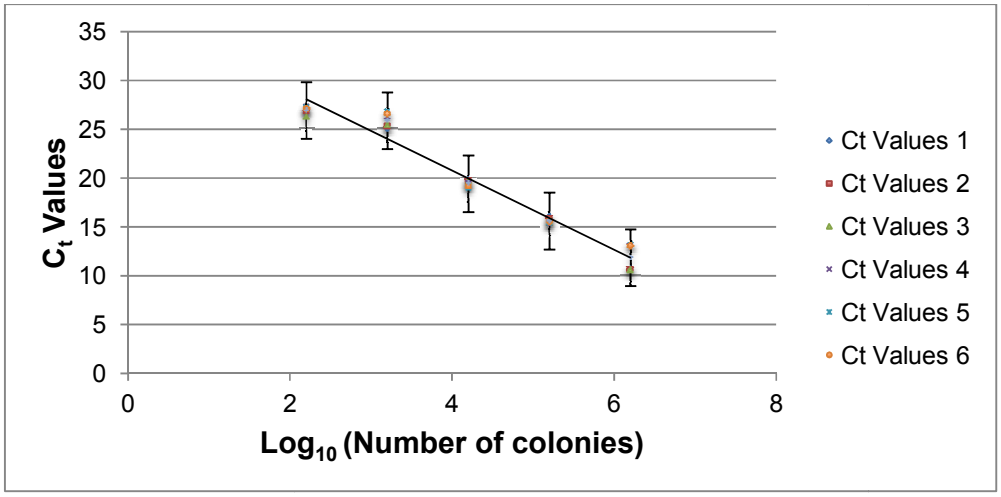


Figure 26. Standard curve for G samples

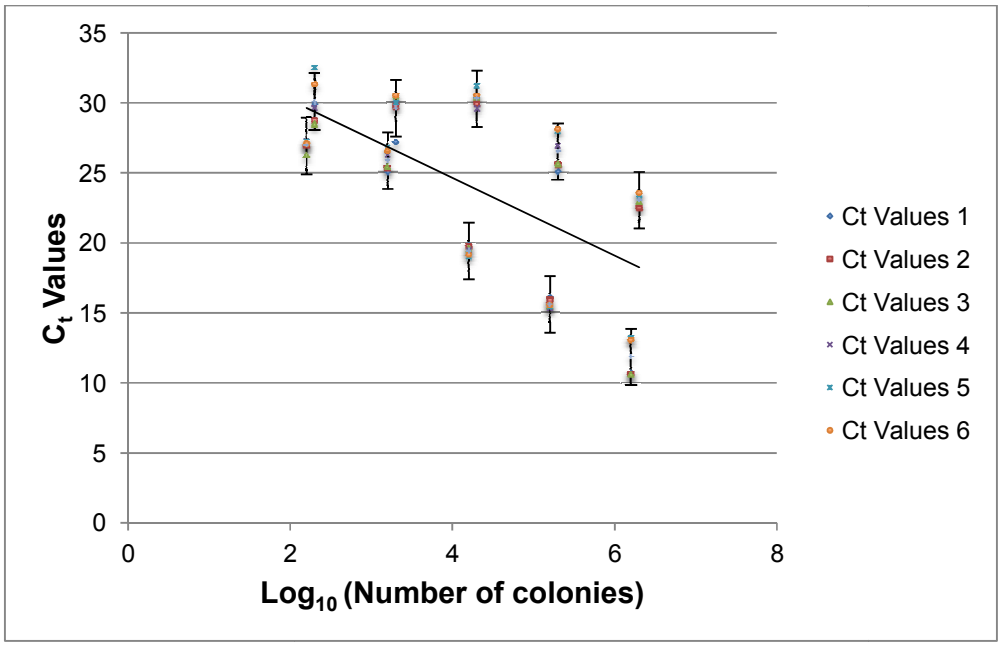


Figure 27. Standard curve for *Escherichia coli* using values from Y and G samples

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
Z-A	9.5×10^5	22.69	22.50	22.94	27.41	29.65
Z-B		23.39	23.36	23.53		
Z1-A	9.5×10^4	25.08	25.58	25.61		
Z1-B		26.90	27.95	28.09	29.34	37.23
Z2-A	9.5×10^3	30.29	29.89	30.37	33.84	31.84
Z2-B		29.58	31.19	30.54		31.26

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
Z3-A	9.5×10^2	27.17	29.74	30.48	32.62	28.90
Z3-B		29.95	30.00	30.49		30.78
Z4-A	9.5×10^1	29.85	28.69	28.46		
Z4-B		29.63	32.51	31.29		33.33
Z5-A	10	28.20	31.16	30.29	32.81	35.43
Z5-B		27.57	31.86	33.82		
Z6-A		30.52	31.03	31.25	26.08	27.00
Z6-B		30.83	29.89	32.20		
Z7-A		31.92	31.78	30.12	29.23	29.32
Z7-B		29.09	28.82	30.97		

Table 49. C_t values of Z samples (shading represent C_t values within three cycles of the C_t value of the negative control)

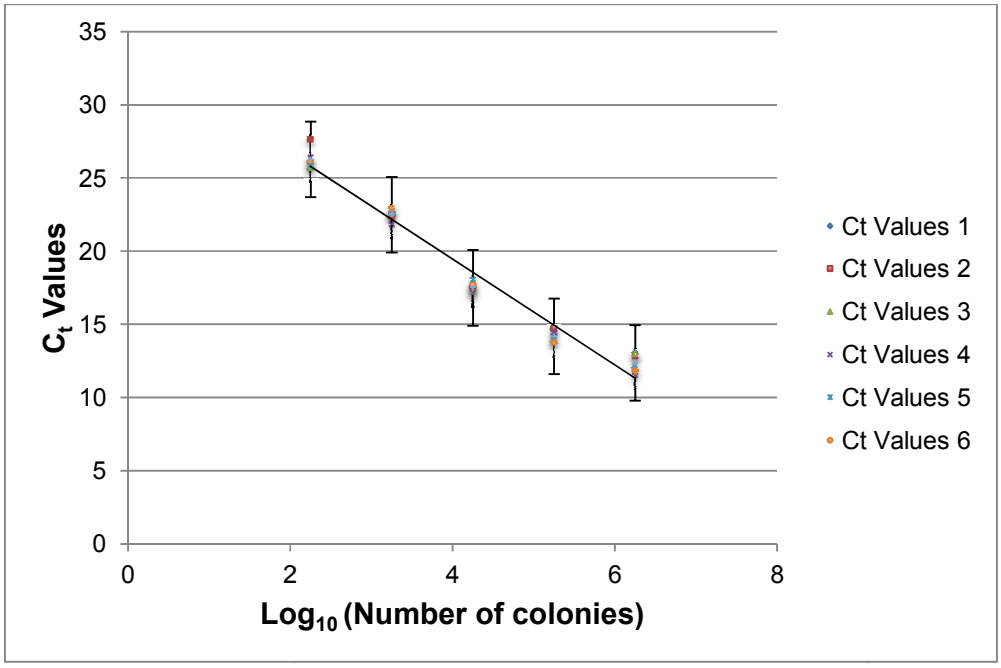


Figure 28. Standard curve for *Staphylococcus epidermidis* using values from Z samples

Sample	Concentration (CFU/ml)	Ct values			Ct values for Blood	Ct values for NTC
H-A	1.8×10^6	13.03	12.81	12.97	28.92	28.33
H-B		11.48	12.15	11.82	30.20	
H1-A	1.78×10^5	14.31	14.70	13.99		
H1-B		14.47	13.93	13.73		
H2-A	1.78×10^4	17.47	17.51	17.19		
H2-B		17.14	18.04	17.67		
H3-A	1.78×10^3	22.66	22.38	22.66		
H3-B		21.82	22.56	22.90		
H4-A	1.78×10^2	26.06	27.60	25.71		
H4-B		26.38	25.79	26.13		
H5-A	1.78×10^1	29.97	28.52	30.01		
H5-B		29.11	29.33	26.01		
H6-A	2	25.82	25.07	23.69		
H6-B		30.00	29.51	23.57		
H7-A	Nil	24.90	30.22	22.78		
H7-B		25.51	28.74	27.92		

Table 50. C_t values of H samples (shading represent C_t values within three cycles of the C_t value of the negative control)

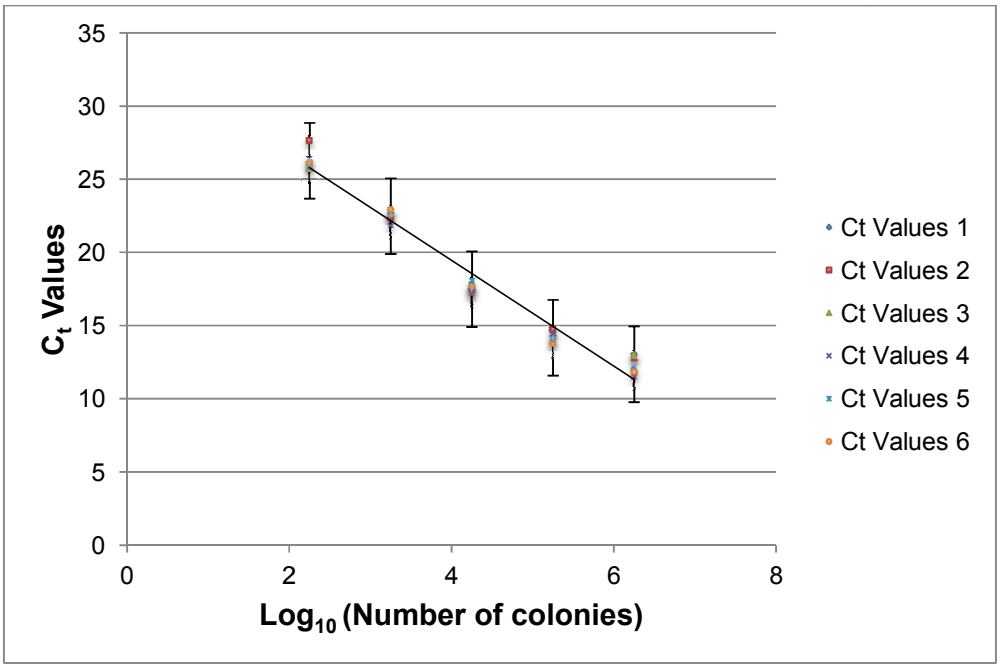


Figure 29. Standard curve for *Staphylococcus epidermidis* using values from H samples

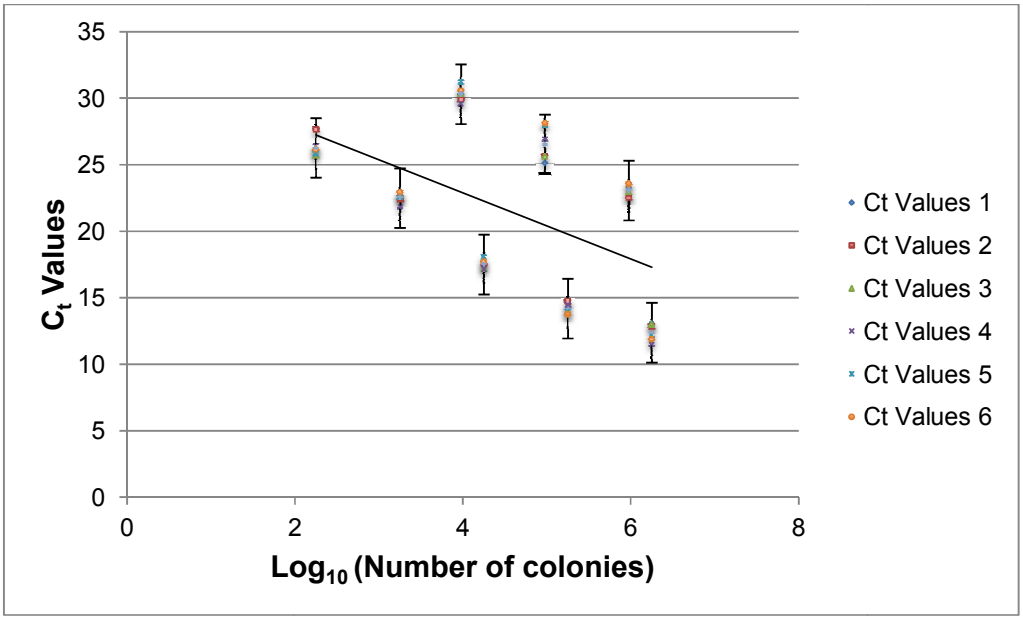


Figure 30. Standard curve for *Staphylococcus epidermidis* using values from Z and H samples

Experiment 8 has demonstrated that it is possible to detect and quantify the three most prevalent 'urological bacteria' from blood reliably and consistently, using molecular microbiological methods. Furthermore, there was at least three cycles of amplification between the detection of a 10^3 cfu/mL sample and the

negative controls. Therefore, it is possible to use this assay (used under the above conditions) for the detection of 10^3 cfu/mL or more bacteraemia, in patients with suspected urological infections.

3.11 Experiment 9 – The Plex-ID

The final experiment described was to evaluate the use of the Plex-ID in the detection and quantification of bacterial DNA obtained via extraction from blood. The samples used were the *Enterococcus faecalis* (X and F), *Escherichia coli* (Y and G) *Staphylococcus epidermidis* (Z and H) samples obtained in Experiment 7. Ibis Biosciences, now part of Abbott Molecular, developed the IbisT5000, based on a technology known as the triangulation identification for the genetic evaluations of risks (TIGER). This research programme was mandated by the biodefense agencies in the USA [9]. The commercialised version of the IbisT5000 is the Plex-ID, marketed by Abbott Molecular. It is a nearly fully automated system, which uses PCR ESI-MS technology to detect amplified nucleic acids from bacteria. A computerised triangulation of the nucleic acid detected allows the detection of the organisms present, down to species level, in the Plex-ID databases. A broad-range PCR is used on the DNA extracts which targets ribosomal and genes expressing housekeeping proteins. The PCR uses multiple primers and allows hybridisation during the first cycles. The Plex-ID allows a degree of quantification based on the peak heights of the mass spectra [10] and provides information on typing, virulence and resistance. However, in-depth discussion of this technology is not within the scope of this thesis.

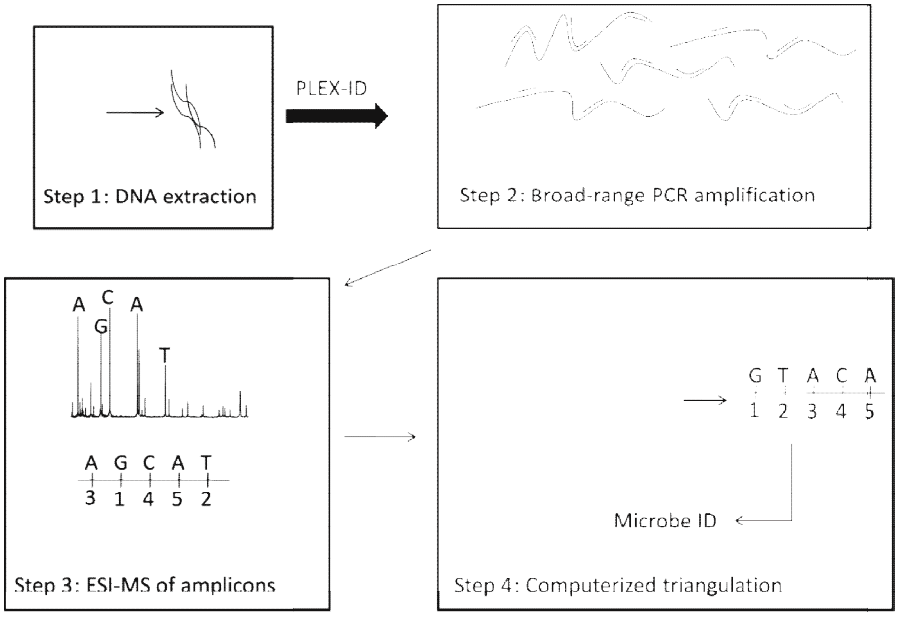


Figure 31. Diagrammatic representation of the Plex-ID technology

3.11.1 Results

Tables 51-56 below shows the results obtained processing samples, X, Y, Z, F, G and H as per the standard protocol for the Plex-ID. The results were compared with the quantification obtained from the Miles and Misra method as well as the C_t values obtained from the broad-range PCR experiments (Experiments 7 and 8). All the samples were identified correctly by the Plex-ID system. Bacteraemia down to the level of < 10 cfu/mL were detected and quantified.

Enterococcus faecalis (Extraction 1)					
Sample	**Miles & Misra Concentration (CFU/ml)**	**Ct values**			**Plex-ID value**
X-A	$7.4x10^{7}$	14.81	15.42	15.32	194
X-B		11.98	12.17	12.16	-
X1-A	$7.4x10^{6}$	15.09	15.31	15.15	200
X1-B		13.76	13.46	13.88	-
X2-A	$7.4x10^{5}$	18.06	20.35	21.64	311
X2-B		19.84	18.73	19.92	
X3-A	$7.4x10^{4}$	24.16	23.83	23.77	210
X3-B		20.12	22.06	22.17	181
X4-A	$7.4x10^{3}$	28.54	26.63	25.85	217
X4-B		24.60	26.22	25.62	195
X5-A	$7.4x10^{2}$	25.72	26.23	26.43	172
		26.63	26.66	26.17	
X5-B		27.89	27.84	26.54	119
		27.65	27.61	27.58	
X6-A	$7.4x10^{1}$	30.77	27.82	29.98	5
X6-B		29.25	30.76	31.28	17
X7-A	7.4	26.50	28.64	27.23	4
X7-B		28.15	29.91	28.78	-

Table 51. Comparison between conventional methods and molecular method (shading represent C_t values within three cycles of the C_t value of the negative control)

Enterococcus faecalis (Extraction 2)					
Sample	**Miles & Misra Concentration (CFU/ml)**	**Ct values**			**Plex-ID value**
F-A	$1.3x10^7$	13.21	13.90	13.77	-
F-B		10.47	10.17	10.32	-
F1-A	$1.3x10^6$	13.31	13.13	13.19	-
F1-B		13.35	13.54	13.06	-
F2-A	$1.3x10^5$	18.92	18.40	18.43	-
F2-B		18.01	18.27	17.81	-
F3-A	$1.3x10^4$	20.85	21.58	21.28	229
F3-B		22.69	22.89	22.84	230
F4-A	$1.3x10^3$	24.34	25.28	25.64	197
F4-B		25.57	24.69	25.94	376
F5-A	$1.3x10^2$	27.81	29.02	26.95	149
F5-B		28.41	27.41	28.52	68
F6-A	13	28.83	27.68	29.89	22
F6-B		28.74	29.16	29.38	10
F7-A	1	29.11	30.77	31.62	19
F7-B		31.00	24.51	30.41	4

Table 52. Comparison between conventional methods and molecular methods (shading represents C_t values within three cycles of the C_t value of the negative control)

Escherichia coli (Extraction 1)					
Sample	**Miles & Misra Concentration (CFU/ml)**	**Ct values**			**Plex-ID value**
Y-A	$2x10^6$	22.69	22.50	22.94	195
Y-B		23.39	23.36	23.53	-
Y1-A	$2x10^5$	25.08	25.58	25.61	187
Y1-B		26.90	27.95	28.09	-
Y2-A	$2x10^4$	30.29	29.89	30.37	191
Y2-B		29.58	31.19	30.54	171
Y3-A	$2x10^3$	27.17	29.74	30.48	187
Y3-B		29.95	30.00	30.49	180
Y4-A	$2x10^2$	29.85	28.69	28.46	186
Y4-B		29.63	32.51	31.29	-
Y5-A	20	28.20	31.16	30.29	-
Y5-B		27.57	31.86	33.82	-
Y6-A	2	30.52	31.03	31.25	10
Y6-B		30.83	29.89	32.20	-
Y7-A	-	31.92	31.78	30.12	13
Y7-B		29.09	28.82	30.97	-

Table 53. Comparison between conventional methods and molecular methods (shading represents C_t values within three cycles of the C_t value of the negative control).

Escherichia coli (Extraction 2)					
Sample	**Miles & Misra Concentration (CFU/ml)**	**Ct values**			**Plex-ID value**
G-A	$1.6x10^6$	10.71	10.61	10.57	-
G-B		13.09	13.17	13.03	-
G1-A	$1.6x10^5$	16.09	15.88	15.43	-
G1-B		15.31	15.44	15.57	-
G2-A	$1.6x10^4$	19.82	19.70	19.54	-
G2-B		19.46	18.95	19.16	-
G3-A	$1.6x10^3$	25.05	25.29	25.42	160
G3-B		26.25	26.79	26.55	-
G4-A	$1.6x10^2$	26.91	26.91	26.26	116
G4-B		27.27	27.24	27.06	-
G5-A	16	28.40	27.75	28.38	42
G5-B		28.95	28.62	28.99	-
G6-A	2	31.69	31.43	32.41	7
G6-B		29.25	31.57	30.83	-
G7-A	-	32.77	31.99	29.83	3
G7-B		32.32	29.77	30.53	-

Table 54. Comparison between conventional methods and molecular methods (shading represents C_t values within three cycles of the C_t value of the negative control).

Staphylococcus epidermidis (Extraction 1)					
Sample	**Miles & Misra Concentration (CFU/ml)**	**Ct values**			**Plex-ID value**
Z-A	$9.5x10^5$	22.69	22.50	22.94	-
Z-B		23.39	23.36	23.53	-
Z1-A	$9.5x10^4$	25.08	25.58	25.61	-
Z1-B		26.90	27.95	28.09	-
Z2-A	$9.5x10^3$	30.29	29.89	30.37	13
Z2-B		29.58	31.19	30.54	-
Z3-A	$9.5x10^2$	27.17	29.74	30.48	4
Z3-B		29.95	30.00	30.49	
Z4-A	95	29.85	28.69	28.46	-
Z4-B		29.63	32.51	31.29	-
Z5-A	10	28.20	31.16	30.29	-
Z5-B		27.57	31.86	33.82	-
Z6-A	1	30.52	31.03	31.25	-
Z6-B		30.83	29.89	32.20	-
Z7-A	1	31.92	31.78	30.12	-
Z7-B		29.09	28.82	30.97	-

Table 55. Comparison between conventional methods and molecular methods (shading represents C_t values within three cycles of the C_t value of the negative control)

Staphylococcus Epidermidis (Extraction 2)					
Sample	**Miles & Misra Concentration (CFU/ml)**	**Ct values**			**Plex-ID value**
H-A	$1.8x10^6$	13.03	12.81	12.97	-
H-B		11.48	12.15	11.82	-
H1-A	$1.8x10^5$	14.31	14.70	13.99	-
H1-B		14.47	13.93	13.73	-
H2-A	$1.8x10^4$	17.47	17.51	17.19	
H2-B		17.14	18.04	17.67	-
H3-A	$1.8x10^3$	22.66	22.38	22.66	193
H3-B		21.82	22.56	22.90	

Sample	Miles & Misra Concentration (CFU/ml)	Ct values			Plex-ID value
H4-A	$1.8x10^2$	26.06	27.60	25.71	203
H4-B		26.38	25.79	26.13	-
H5-A	18	29.97	28.52	30.01	99
H5-B		29.11	29.33	26.01	-
H6-A	2	25.82	25.07	23.69	30
H6-B		30.00	29.51	23.57	-
H7-A	-	24.90	30.22	22.78	21
H7-B		25.51	28.74	27.92	-

Table 56. Comparison between conventional methods and molecular methods (shading represents C_t values within three cycles of the C_t value of the negative control)

The results from Experiment 9 above demonstrate an alternative, novel molecular microbiological technology that can detect and quantify low-level bacteraemia reliably and consistently. The Plex-ID technology, like other molecular technologies, is more labour-intensive than the conventional culture and Miles and Misra method.

3.12 Conclusions

The experiments carried out and reported in this chapter have shown that it is possible to detect, quantify and identify bacteraemia by different methods. The traditional incubation and culture method allowed the detection of bacteria using conventional methods. The molecular techniques detected bacterial DNA. The broad-range PCR method that was optimised allowed the detection and quantification of bacteria to a level of 10^3 cfu/mL. The Plex-ID method allowed detection and quantification to a level of < 10 cfu/mL, which is a major improvement on the broad-range PCR methods described in the initial experiments. Identification was integrated in the Plex-ID method whereas further sequence analysis was required for the broad-range PCR methods. Experimenting and validating sequencing methodology for the detection of bacterial DNA was not within the remit of this thesis.

Chapter 4: Bacteraemia during TURP and Catheter Manipulation

4.1 Introduction

The literature suggests that bacteraemia does occur during urological procedures [14, 219-221]. However, most studies report bacteraemia in patients who become symptomatic after urological instrumentation. An absolute figure for the presence of bacteraemia during urological procedures is not reported in the literature. Therefore, the aim of the work presented in this chapter was to determine the incidence, amplitude, timing and causative organisms of bacteraemia occurring during urological procedures, using the methods described in Chapter 3. The significance of asymptomatic bacteraemia is not known but is discussed further in Chapter 5.

4.2 Ethics

Ethics approval was obtained from the Leeds West Research Ethics Committee (REC) (REC number: 10/H1307/5). Research and Development approval was obtained from the Leeds Teaching Hospitals NHS Trust Research and Development (R&D) department (R&D number: UR09/9173). The trial was also registered on the International Standard Randomised Controlled Trial Number (ISRCTN) Register (ISRCTN89902973).

4.3 The pilot study: is it feasible to recruit urology patients in a study to detect bacteraemia?

4.3.1 Introduction

To determine the feasibility of a prospective study to recruit patients having urological procedures, a pilot study was designed and run. Patients having transurethral resection of the prostate (TURP), extracorporeal shockwave lithotripsy (ESWL) and catheter manipulation were recruited and blood samples were obtained from the recruited patients. The three procedures above were

identified as they were commonly carried out in the urological department at the Leeds Teaching Hospitals Trust.

In the first instance, in the catheter manipulation group, it was decided to include patients having only catheter change rather than catheter removal. This was done in order to avoid recruiting patients who had been catheterised for a short period, where bacteraemia is rarely reported [222].

4.3.2 Methods

4.3.2.1 Identification of the participants and processing of samples

A urology research nurse identified patients. The inclusion criteria were adult patients attending the Leeds Teaching Hospital NHS Trust (LTHT) for TURP, catheter manipulation and ESWL. The research nurse approached and invited the patient to take part in the study. If a patient showed interest in taking part in the study, they were put in touch with the principal investigator (the author). Patient consent was obtained and allocated a unique identification number. Blood samples were obtained according to the schedules shown in Table 57. Samples were set up for culture according to the method described in Chapter 3. Blood culture isolates were processed by Gram stain and were identified according to the methods in Section 4.3.3.2. For the purpose of the pilot study, the blood samples obtained were processed using only the culture method, as the molecular methodologies had not been developed at the time this study was carried out. Demographic and clinical data were collected using a *pro forma* (Appendix 7).

4.3.2.2 Timing of blood samples

For the purpose of the pilot study, blood was acquired at the following time points for the TURP group of patients:

	A	B	C	D	E	F	G
Time Point	Pre-procedure	Start of procedure	5 minutes into procedure	15-20 minutes into procedure	End of procedure	10 minutes following procedure	20 minutes following procedure

Table 57. Schedule for timing of blood sampling in the pilot study of bacteraemia during TURP

For the catheter-change group, time-points C and D were excluded owing to the duration of the procedure.

4.3.3 Results

4.3.3.1 Demographics of the patients recruited

Table 58 shows the basic demographics of the patients recruited in the pilot study.

Procedure		**TURP N=11**	**Catheter change N=5**	**ESWL N=5**
Male sex (%)		11 (100)	3 (60)	4 (80)
Mean age ± SD (years)		71.3 ± 10.2	60.0 ± 15.5	41.0 ± 14.8
Mean weight ± SD (kg)		81 ± 15.5	83.6 ± 11.3	69.9 ± 14.1
Urine dipstick Negative (%)		11 (100)	4 (80)	5 (100)
Within 2 weeks of the procedure	Antibiotics Therapy (%)	0 (0)	1 (20)	1 (20)
	Instrumentation (%)	0 (0)	0 (0)	0 (0)
	Symptomatic UTIs (%)	1 (9.1)	0 (0)	0 (0)
Immunosuppressant (%)		0 (0)	1 (20)	0 (0)
Diabetes Mellitus (%)		0 (0)	1 (20)	0 (0)

Procedure	TURP N=11	Catheter change N=5	ESWL N=5
Smoking (%)	2 (18.1)	1 (20)	2 (40)
Co-existing Infection (%)	3 (27.3)	2 (20)	0 (0)
Recent hospital stay (%)	5 (45.4)	0 (0)	0 (0)
Mean length of stay ± SD (days)	15.6 ± 12.0	n/a	n/a
Recurrent UTIs (%)	0 (0)	1 (20)	1 (20)
Urinary stones (%)	1 (9.1)	1 (20)	5 (100)
Catheter-in-situ (%)	7 (63.6)	5 (100)	0 (0)
Mean length of catheterisation ± SD (days)	84 ± 118	92±24	84 ± 118
Cardiac History (%)	1 (9.1)	0 (0)	0 (0)
Endocarditis (%)	0 (0)	0 (0)	0 (0)
Prosthetic device – cardiac and non-cardiac (%)	1 (9.1)	0 (0)	0 (0)
Organ transplant (%)	0 (0)	0 (0)	0 (0)

Table 58. Demographics of patients recruited in the pilot study of patients undergoing TURP, catheter change and ESWL

Figure 32 below shows the patients recruited as part of the pilot study.

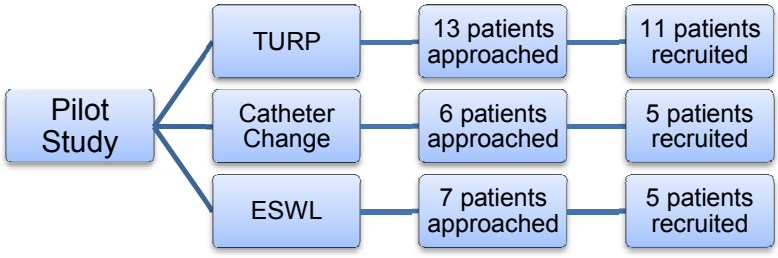


Figure 32. The number of patients recruited during the pilot study

4.3.3.2 Results of the sampling pilot study in patients having TURP

The samples acquired for the TURP group are shown in Table 59 below.

RECRUIT	A	B	C	D	E	F	G
TURP001	√	X	X	X	X	X	X
TURP002	√	√	√	√	√	√	√
TURP003	√	√	√	√	√	X	X
TURP004	X	X	X	X	X	X	X
TURP005	√	√	√	√	√	√	X
TURP006	X	X	X	X	X	X	X
TURP007	√	√	√	√	√	√	√
TURP008	√	√	√	√	√	√	√
TURP009	X	X	X	X	X	X	X
TURP010	√	√	√	X	X	X	X
TURP011	X	X	X	X	X	X	X

Table 59. Blood samples obtained from TURP patients

A complete set of blood samples was not obtained from all the participants. The reasons for the failure to complete the recruiting protocol are given in Table 60 (over the page).

Recruit	Reason for failure
TURP001	The cannula fell out and was not re-sited
TURP003	The cannula failed to bleed for time-points F and G
TURP004	Patient was cancelled owing to lack of space on the theatre list
TURP 005	The cannula failed to bleed at time-point G
TURP006	Failure owing to logistics in operating theatre: unable to get samples
TURP009	The cannula failed to bleed
TURP010	The cannula fell out after time-point C
TURP 011	The cannula failed to bleed

Table 60. The reasons for the failure of the recruitment process during the TURP pilot study

Thirty-seven out of a maximum seventy-seven samples (48.1%) were obtained. Cannula failure was the most common reason for the inability to obtain the blood samples as shown in Table 60 above.

4.3.3.3 Results of sampling pilot study in patients having catheter change

The samples obtained for the catheter-change group are shown in Table 61 (over the page). Patients who had a urethral catheter change were annotated with the letter U (*e.g.* CATH003-U) and the patients having a suprapubic catheter change were annotated with S (*e.g.* CATH001-S).

RECRUIT	A	B	C	D	E	F	G
CATH001-S	√	√	√	√	√	√	√
CATH002-S	√	√	X	X	√	√	√
CATH003-S	√	√	X	X	√	√	X
CATH004-U	X	X	X	X	X	X	X
CATH005-U	√	√	√	X	√	√	√

Table 61. Samples from catheter change patients

The reasons for the failure to complete the recruiting protocol are given in Table 62.

Recruit	Reason for failure
Cath003-S	The cannula failed to bleed at time-point G
Cath004-U	Failure to gain venous access with participant

Table 62. The reasons for the failure of the recruitment process during the catheter change pilot study

The same issue arose for the catheter change participants (cannula failure) to explain the inability to acquire the complete set of blood samples.

4.3.3.4 Results of the sampling pilot study in patients having ESWL

The samples obtained from the ESWL patients are shown in the Table 63.

RECRUIT	A	B	C	D	E	F	G
ESWL001	√	√	X	X	X	X	X
ESWL002	√	√	√	√	√	√	X
ESWL003	√	X	X	X	X	X	X
ESWL004	√	√	√	√	√	√	√
ESWL005	√	√	√	√	√	√	√

Table 63. Samples from ESWL patients

The reasons for the failure to complete the recruiting protocol are given below.

Recruit	Reason for failure
ESWL001	The cannula fell out after time-point B
ESWL002	The cannula failed to bleed after time-point F
ESWL003	The cannula failed to bleed after time-point A

Table 64. The reasons for the failure of the recruitment process during the ESWL pilot study

4.3.3.5 Evidence of bacteraemia in the pilot study for TURP, catheter change and ESWL patients

Five positive samples were obtained in the pilot study and further investigations were carried out to allow identification of the bacteria found in the sample. The results are tabulated below (Table 65).

	Gram-Staining	Oxidase Test	Catalase Test	Identification by PCR
Control Samples				
S. aureus	Positive Coccus	X	√	
P. aeruginosa	Negative Rod	√	√	
Test Samples				
TURP003 C	GN Rod	√	√ (slow)	*P. aeruginosa*
TURP005 B	GN Rod	X	√	*Enterobacter* sp.
TURP007 C	GN Rod	√	√	*P. aeruginosa*
TURP007 D	GN Rod	√	√	*P. aeruginosa*
TURP007 G	GN Rod	√	√	*P. aeruginosa*

Table 65. Bacteraemia detected in the pilot study of patients having TURP

4.3.4 Conclusions of the pilot study

All the bacteraemias detected were in the patients undergoing TURP. Table 65 shows the organisms detected in the five patients with bacteraemia.

Pseudomonas aeruginosa was the most common organism detected in the pilot study. The pilot study showed a good recruitment rate. However, a few issues were noted which were improved in the prospective study. This is discussed in Sections 4.3.4.1-6.

4.3.4.1 Patient contact

It was deemed too time consuming for the urology research nurse to seek patients and approach them actively. Therefore, it was decided that the treating clinician would make the first approach with the patient and briefly explain the study being carried out. If the patient expressed an interest in taking part in the study, he/she was put in touch with the principal investigator (the author). If the patient wished to take part in the study after the first contact with the principal investigator, full consent was obtained.

4.3.4.2 Problems with the venous cannulation

One of the main issues encountered in the pilot study was cannula failure. Therefore, a large proportion of the recruited participants failed to provide a complete set of blood samples for this reason. It was decided that improvement of cannulation skills should be attempted by the author attending a course organised by the 'in-house' phlebotomy service and planned to use the biggest possible cannula that the patient's vein will allow and where possible get experts (*e.g.* anaesthetists in the operating theatre for TURP patients) to insert the cannula.

4.3.4.3 Keeping cannula patent with IV fluid

A slow intravenous infusion of normal saline was used in the pilot study to keep the cannula patent. This extra step did not improve the patency of the cannulae. In fact, this extra step made the whole process cumbersome, with the additional need for a drip stand. Therefore, it was decided to drop this step and not utilise IV fluid for the prospective study.

4.3.4.4 Timing of blood samples

It was decided to obtain only one rather than three post-procedure samples. It made the prospect of entering the study easier for patients, especially those scheduled for catheter manipulation. They did not have to wait long after their procedure before they could go back home. Furthermore, to cut costs, it was felt

that having three post-procedure samples would not provide any additional information, as only one post-procedure sample in the pilot study was positive for bacteraemia. The shorter *in situ* time for the cannula improved its patency for the duration of the recruitment period.

4.3.4.5 Urine samples

Obtaining a pre-procedure urine sample was felt to be important to be able to interpret the bacteraemia result. Therefore, it was decided to acquire a urine sample to check for bacteriuria for the prospective study.

4.3.4.6 Procedure

Running three prospective studies for three different procedures was too time-consuming, both in terms of recruiting patients and processing the samples. It proved to be too costly as well. A decision was made only to recruit patients having TURP and catheter manipulation. In the pilot study, only patients having catheter change were recruited. This group was broadened to patients having catheter manipulation (catheter change and removal) to improve the recruitment rate.

4.4 Prospective studies looking at bacteraemia during TURP and catheter manipulation

4.4.1 Introduction

A study protocol was designed to answer the questions:

- 'Does bacteraemia occur during urological procedures? If so, when does it occur, how many bacteria are involved, for how long does it last and which bacteria are implicated?'

As discussed previously, the two categories of patients studied were patients having TURP and catheter manipulation. Two prospective cohort studies were designed to evaluate bacteraemia in patients undergoing TURP and catheter manipulation.

4.4.2 Methods

4.4.2.1 The recruitment of patients

Patients recruited in the study were counselled about the study prior to their contact with the research team. Potential TURP recruits were approached in the outpatient department by the clinician listing the patient for the procedure. The urology nurse specialist approached potential recruits undergoing catheter manipulation before they had any contact with the research team. If the potential recruits expressed an interest in taking part in the study, they were put in touch with the principal investigator.

4.4.2.1.1 Recruitment for the TURP group

Patients having TURP were identified via number of different sources:

- the admissions list circulated weekly by the urology admissions office. This document was used to identify patients having a scheduled TURP on an elective urology list. The date of the procedure, the operating theatre and the admitting ward were identified;

- patients having TURP after an acute admission for urinary retention were identified via word-of-mouth from the ward staff;

- patients having a day-case TURP were identified through the secretaries of the urological surgeon choosing to list the patient to have the operation.

4.4.2.1.2 Recruitment for the catheter manipulation group

Patients having catheter manipulation were identified through the urology nurse specialist who performed all the scheduled catheter manipulations (for patients in the community, who require their catheter to be manipulated by the urology department in Leeds) at the Leeds Teaching Hospitals NHS Trust.

4.4.2.1.3 Inclusion/Exclusion Criteria

The inclusion criteria for the patients recruited into the study were:

- adults (> 18 years old);
- both male and female[1];
- patients undergoing the following procedures (transurethral resection of the prostate and urinary catheter change or removal) at the Leeds Teaching Hospital NHS Trust.

The absolute exclusion criteria for the study were:

- patient aged less than 18 years;
- patients who were not competent to consent for enrolment in the study;
- signs and symptoms of an ongoing infection (of any source) at the admission to the hospital.

There were some relative exclusion criteria and each patient was assessed individually. The relative exclusion criteria were:

- the use of systemic antibiotics within the two weeks of presentation to the hospital;
- recent (within two weeks) instrumentation of the urinary tract (not including urethral or suprapubic catheterisation);
- patients with poor veins, leading to difficult venous cannulation.

Based on these inclusion and exclusion criteria, there was an expectation to recruit fifty participants in each of the two groups of patients.

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[1] Only male patients have a TURP

4.4.2.2 Consent

Once the patient expressed a wish to take part in the study, their full consented to participate in the study was obtained using the consent form included in Appendix 6. At the same sitting as consent was obtained, patients were given a patient information sheet, describing the study and their involvement in the study (Appendix 5). Both the consent form and the patient information sheet were vetted and approved by the ethics committee and the R&D department at the Leeds Teaching Hospitals NHS Trust. Once consented, the patient was assigned a research identification number. The TURP patients were assigned T-identification numbers (*e.g.* T001) and the catheter manipulation patients were assigned C-identification numbers (*e.g.* C001).

4.4.2.3 Medical History to collect data on potential risk factors for developing bacteraemia

On the day of their procedure, a detailed medical history was obtained using the specially designed data collection form (Appendix 7). The form was divided into three main sections (exclusion criteria, general risk factors for bacteraemia, specific risk factors for infective endocarditis).

4.4.2.4 Processing of urine samples

4.4.2.4.1 Obtaining the urine sample

Once the medical history form was completed, a urine sample was collected from the patient in a standard, sterile urine pot. The pot was labelled using the patient identification number. The date of the collection of the sample was also recorded on the pot. The pot was stored in a bio-sample transportation bag (as per the Trust policy). If the patient was not catheterised, the patient was asked to void spontaneously and a mid-stream urine sample (MSU) collected. The patient was given clear verbal instructions of how to collect the urine sample. If the patient was catheterised, a urine sample was obtained from the catheter bag. The urine sample obtained was processed in the laboratory within 24 hours of collection.

4.4.2.4.2 Handling of the urine sample in the laboratory

The urine sample was processed as follows. The urine pot was shaken and a 10 µL inoculating loop was dipped in the urine. The loop was spread on a CLED agar plate (Thermo Scientific, Basingstoke, UK). The CLED agar plate as incubated in air at 37° C for 24 hours and then read. If growth was noted, the following identification protocol was performed:

- the colony morphology on the CLED agar plate was noted;
- the isolate was Gram stained and microscopy was performed (as per the protocol in Chapter 4);
- simple preliminary identification tests (oxidase, catalase) were performed depending on the results obtained in the previous steps;
- the number of colony forming units (cfu) was counted to provide a quantitative value to the bacteriuria. Since the number of cfu per 10 µL was known, the concentration of bacteria in the original urine was calculated. For practical purposes, a cut-off value of 10^4 cfu/mL was used to define bacteriuria.
- the organism(s) grown was stored in glycerol broth at -70° C for further processing.

Once a reasonable number (thirty) of stored bacterial samples were obtained, all the stored organisms were inoculated on CBA plates (Thermo Scientific, Basingstoke, UK) and incubated at 37° C in air for 24 hours. The fresh cultures were processed for bacterial DNA amplification as follows:

- a 10 µL inoculating loop was used to touch a single colony on the CBA plate and then transferred into a tube containing sterile water. The mixture was homogenised by stirring the loop in the tube. This mixture was used for bacterial DNA amplification as per the standard protocol. Once the bacterial DNA was amplified and C_t values obtained, the bacterial DNA present in the sample was identified by the sequence

determination method using the BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.4.2.5 Processing of the blood samples

The next step was to obtain blood samples. This procedure was done using standard aseptic technique in compliance with local guidelines.

4.4.2.5.1 Cannulation of the patient

The method used in the pilot study was modified to insert a cannula, which was left *in situ* for the duration of the study, rather than using a syringe and a needle. This was done using a 16G, 18G or 20G cannula, inserted in the antecubital fossa of the patient. The size of the cannula depended on the size of the veins available. This step was done by the principal investigator or an experienced anaesthetist, if available. Once the cannula was in place and fixed, a three-way tap was connected. Patients having TURP under general or spinal anaesthesia had their arms out on an arm board during the procedure, to provide easy access to of the cannula in the antecubital fossa. Patients in the catheter manipulation group did not require their arms out on an arm board as the patients were not anaesthetised and were compliant to instructions.

4.4.2.5.2 Obtaining the blood samples

Firstly, the nozzle of the three-way tap was cleaned with an 'alco-wipe' (alcoholic 2% chlorhexidine skin wipes by Clinell®). Then, 2 mL of blood was aspirated using a 5 mL syringe after opening the three-way tap. This blood sample was discarded and 20 mL of blood was aspirated using a 20 mL syringe. The cannula was flushed with 2 mL of normal saline and the three-way tap closed.

4.4.2.5.3 Timing of the blood samples

This above process was repeated at each time point that a blood sample was required, shown in Table 66. For the TURP group, patients who had a urethral catheter *in situ* prior to the procedure had an extra time point at which an extra blood sample was obtained. This was done in order to assess whether the

catheter removal just prior to TURP influenced the development of bacteraemia in this group of patients. For the catheter manipulation group, patients having catheter removal only missed time point C as shown in Table 66.

Procedure	A (pre-procedure)	B (Catheter out)	C (5 min into procedure)	D (10 min into procedure)	E (20 min into procedure)	F (Post procedure)
TURP (no catheter)	✓	✗	✓	✓	✓	✓
TURP (urethral catheter)	✓	✓	✓	✓	✓	✓
Catheter Manipulation	✓	✓	✓	✓		

Table 66. Time points for blood sample collection in the two prospective studies

The 20 mL of blood obtained at each time point were transferred in three separate tubes as per the algorithm shown in Figure 33.

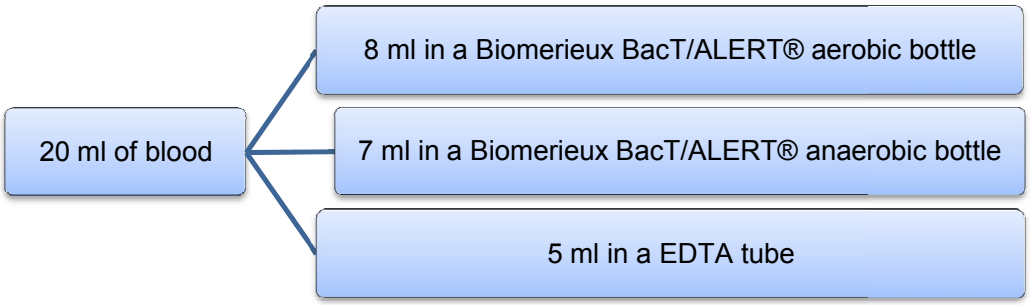

Figure 33. Distribution of the 20 mL of blood obtained at each time point

Once all the blood samples were acquired, the cannula was removed from the antecubital fossa of the patient and haemostasis was achieved with pressure. The blood samples obtained were processed in the laboratory within 24 hours of collection.

4.4.2.5.4 Processing the blood samples by the culture method

The two inoculated blood culture bottles obtained at each time point were placed in the BACTEC™ 9050 Blood Culture instrument. The BACTEC™ 9050 Blood Culture System is an automated system for incubation and agitation of culture bottles. However, the automation setting was disabled and only the incubation and agitation properties of the instrument were used. After ten days, all samples were plated as described in the Table 67. Ten days (as opposed the standard four to five days) was chosen to improve sensitivity. Samples were obtained from the blood culture bottles using the venting unit provided by the manufacturer of the blood culture bottles. The blood culture bottles were then discarded.

Standard Medium	**Incubation**			**Plates Read**
	Temperature	Atmosphere	Time	
Blood (CBA)		5-10% CO_2		
FAA	35-37° C	Anaerobic	48 hours	48-72 hours
CLED		Air		

Table 67. Media for subculture (adapted from 'Investigation of Blood Cultures by The Health Protection Agency – now Public Health England)

At 48-72 hours, all the plates were read. Any bacterial growth was noted and characterised as follows:

- the colony morphology on the plate was noted
- the organism was Gram stained and microscopy was performed (as per the protocol in Chapter 4);
- simple tests (oxidase, catalase) were performed depending on the results obtained in the previous steps.

Thereafter, a loop was used to sample any bacterial growth obtained and this was stored at -70° C in tubes containing glycerol broth. Once a reasonable

number (ten to twelve samples) of stored bacterial samples were obtained, they were processed as for the bacterial samples obtained from urine (described in Section 4.4.7.1). This process allowed the identification of all bacteria grown from blood samples that were incubated and cultured.

4.4.2.5.5 Extracting bacterial DNA from blood

Bacterial DNA was extracted from the 5 mL of blood the EDTA tube. This process was carried out within 24 hours of the collection of the sample, using the standard protocol for MolYsis5 Complete by Molzym GmbH & Co, Bremen, Germany. Each 5 mL blood sample yielded 100 µL of eluate. The eluate was stored at -70° C and bacterial DNA was amplified and the sources identified in batches of twenty-four. For further processing, the eluate was thawed and diluted with molecular grade water to a volume of 220 µL.

4.4.2.5.6 Processing the extracted bacterial DNA

4.4.2.5.6.1 Processing the extracted bacterial DNA by the Plex-ID System

The diluted eluate was plated according to the Plex-ID automated protocol (see Appendix 5). Samples were processed in batches of twenty-four, as this was the maximum number of samples that could be plated by the automated Plex-ID fluid handler system. A 220 µL volume of the eluate was placed in the designated well on the 24-well plate. The fluid handler was loaded with four BAC-SF plates, which had previously been thawed according to the protocol.

Each sample was plated into the sixteen corresponding wells on BAC-SF plates in 10 µL volumes, using a total of 160 µL. Allowing for pipetting error, 40 µL of the eluate was left, which was used for the further processing (Section 4.4.7.2.2.2). A positive control (a sample with confirmed bacterial DNA) and negative control (molecular grade water) was used for each plating run. The loaded BAC-SF plates were processed according to the standard protocol, going through the following steps:

- sealing of BAC-SF plates;
- PCR amplification in the thermocycler;
- detection, identification and quantification of the products in the Plex-ID analyzer.

The results obtained from this method are shown in section 4.4.3.3.2. The Plex software allocated any organism that was detected two parameters. For example, 0.90 and 14; the first parameter (0.90) refers to the probability of the organism detected by the Plex-ID system being a correct identification. A probability of > 0.85 is considered a positive result. The second parameter (14) is a semi-quantitative measure of the amount of bacterial DNA present in the sample. For the purposes of this study, it was decided that a level of < 10 should be considered a negative result to avoid false positives, especially relating to skin contaminants.

4.4.2.5.6.2 Processing the extracted bacterial DNA by the 16S PCR method

The 40 µL of the eluate left from the Plex-ID processing was used for the 16S broad-range PCR process. PCR was performed using the in-house 16S primer and the eluate. The protocol and primers used for the PCR is described in Appendix 4. The C_t values and fluorescence level for each sample were ascertained.

The set of rules below was used to process the results from the 16S PCR (Figure 34). This algorithm was devised with the help from Dr Deborah Gascoyne-Binzi, a senior clinical scientist in the molecular laboratory. By following these criteria, the aim was to:

- maximise true positive results from the samples processed;
- minimise the number of samples processed in an attempt to avoid unnecessary costs.

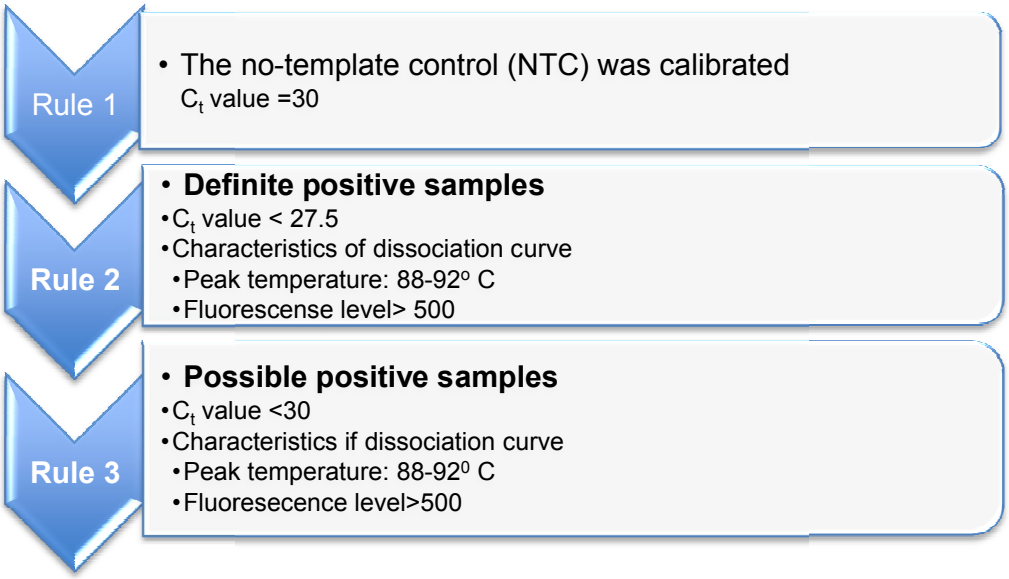

Figure 34. Criteria for defining sample positivity after real-time 16S PCR

These rules yielded a large number of false positive results after the initial batch of sequencing. Therefore, all the definite and possible results (C_t values, melting curves and fluorescence level) were reviewed by Dr Deborah Gascoyne-Binzi, an experienced clinical scientist, to determine which samples were likely to produce a positive result on sequence analysis. This step was done in an attempt to keep the costs down and to avoid the unnecessary, labour-intensive processing of the samples. Once the samples were sequenced, identification was done using Basic Local Alignment Search Tool (BLAST). BLAST is an online website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) that provides an algorithm for the identification of biological sequences, including bacterial nucleotide sequences [223].

4.4.2.6 Follow-up of patients after their procedure

Three months after their procedure, participants were contacted by telephone and a semi-structured telephone interview was carried out, using a questionnaire (Appendix 8). The data collected from the telephone interview included whether, within the preceding three months, the patient:

- felt unwell;
- had lower urinary tract symptoms;
- developed a post-procedure UTI;
- had antibiotic therapy for an infective cause;
- was re-admitted to hospital.

The patient management server and the results server at the hospital were also accessed to obtain further information regarding:

- the timing of discharge from hospital;
- urine culture post-procedure sent by either the general practitioner or hospital staff;
- blood culture sent by either the general practitioner or hospital staff.

4.4.2.6.1 Histological analysis of prostatic tissue from the TURP group

After presenting the above data at learned conferences, one issue that was raised was the presence of inflammation and calcification in the prostate tissue and the association with the development of bacteraemia. Inflammation and calcification can be surrogate markers for infections [224]. Therefore, the prostatic tissues collected at the TURP operation were re-examined by Dr Selina Bhattarai, an experienced consultant uro-histopathologist, for signs of malignancy, inflammation and calcification.

4.4.2.7 Statistical comparison of the three different methods used to detect bacteraemia

To compare how the three tests to detect bacteraemia performed, McNemar's test was used. It was used to compare the frequencies in dichotomous data sets. SPSS Version 20.0.0, IBM Corporation was used to perform the tests. The culture method was considered the gold standard against which the other tests were compared.

4.4.2.8 Multi-level modelling to evaluate the association between the development of bacteraemia and the timings of the blood samples statistically

The model was designed to determine whether there was an association between the development of bacteraemia and the time point at which the blood samples were collected. It was hypothesised that both the procedure (TURP or catheter manipulation) and the timing of collection of the blood sample had a bearing on the development of bacteraemia. To test this hypothesis, a multi-level model was designed, with the help of Professor Robert West, professor in biostatistics, using the open source statistical programme R, Version 2.15 (http://www.r-project.org). The model was designed as shown diagrammatically below.

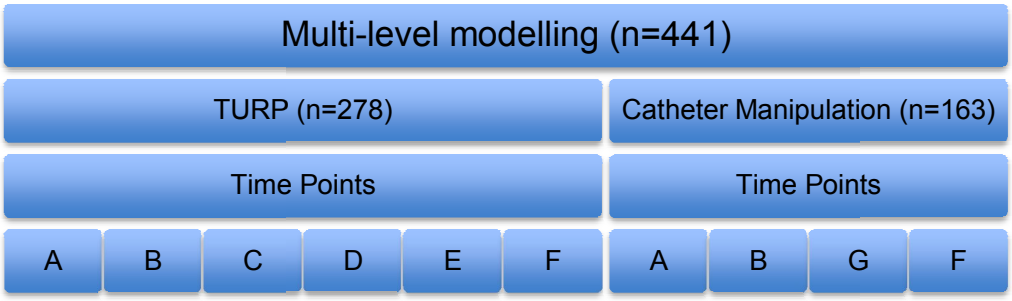

Figure 35. Multi-level model design to show evaluate the association between the development of bacteraemia and the timings of collection of the blood samples

The model assumes that the presence or absence of bacteraemia is influenced by the nature of the procedure and the timings of when the blood samples were taken. The model developed by the software was a generalised linear mixed model fit by the Laplace approximation (a statistical method to smooth out large differences in by normalising the data).

The formula of the modelled could be summarised as:

$$\text{Bacteraemia} \approx \text{Procedure} + \text{Time} + (1/\text{sample})$$

4.4.2.9 Statistical analysis to evaluate the association between bacteriuria and bacteraemia

To assess the association between the presence of bacteriuria and bacteraemia, Pearson χ^2 test was performed using SPSS, Version 20.0.0, IBM Company.

4.4.2.10 Data collection and analysis following the procedure

During the recruitment process, data were collected regarding possible 'patient-related' risk factors, using the history form (Appendix 7). The variables are shown in Table 68 below.

Risk factors			
Mean age (years)	Diabetes	Recurrent UTIs	Infective endocarditis
Mean weight (kg)	Smoking	Urinary calculus	Prosthetic device
Recent antibiotics	Co-existing infection	Urinary catheter	Organ transplant
Immunosuppression	Long hospital stay	Cardiac history	

Table 68. Patient-related risk factors to developing bacteraemia

The following data regarding the journey of the patient following the procedure was also collected (Table 69, over the page), via the telephone interview and the hospital results and patient management server.

Parameters from hospital server	
Patients discharged within two days	
Patients with positive MSU within three months of procedure	
Patients with a positive blood culture within three months of the procedure	
Malignant prostate histology	
Parameters from telephone interview	
Felt unwell	
Lower urinary tract symptoms	
Urine sample to GP	
Antibiotics from GP	
Readmission	For Infection
	For other causes

Table 69. Data regarding the post-procedure journey of the patient

A multivariate logistic regression model (SPSS Version 20.0.0, IBM Company) was used to assess the association between 'patient-related' risk factors and the development of intra-procedure bacteraemia. To improve the accuracy of the logistic regression model, not all of the collected parameters were included in the analysis. The variables deemed to be significant on univariate analysis were included.

4.4.3 Results

4.4.3.1 Demographics of patients recruited in the studies

The demographics obtained from the medical history form are described in this section. The two algorithms below summarise the number of participants at the various stages of the two prospective studies.

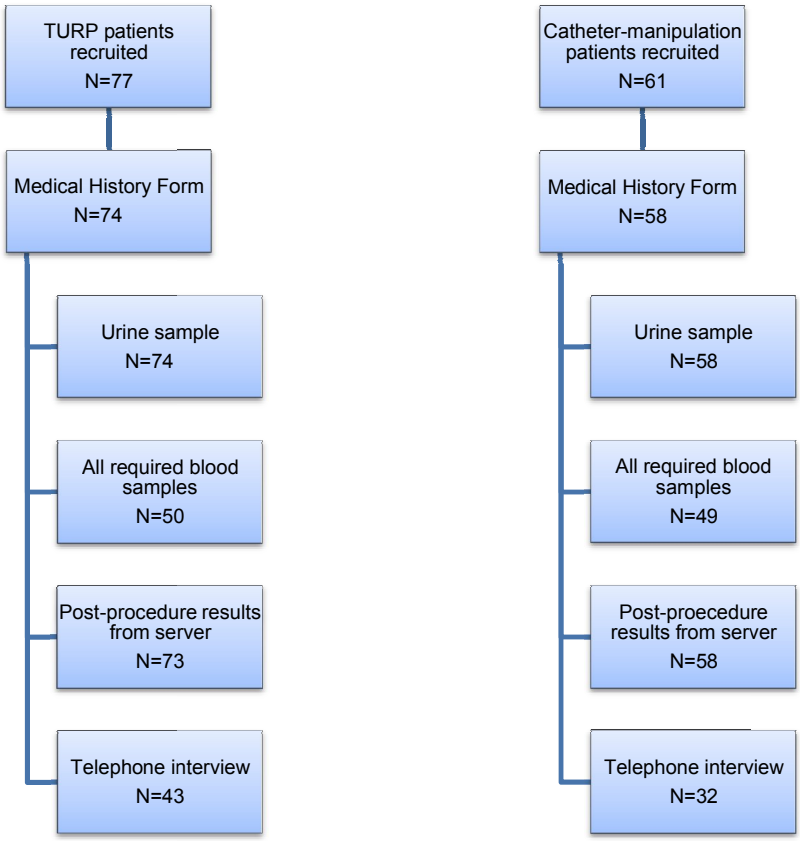

Figure 36. Algorithm showing the patients recruited in the two studies

Figure 36 shows that fifty patients in the TURP group had all the required blood samples to assess for bacteraemia. In the catheter manipulation group, forty-nine patients provided all the required blood samples. The post-procedure telephone interview had a low 'pick-up' rate. The reasons why patients were not contactable by telephone included the wrong telephone number being held in records, no answer despite multiple attempts (at least three separate occasions), change of address and death.

4.4.3.1.1 Demographics of the TURP Group

Parameter		N=73
Mean age (years)		72.7
Mean weight (kg)		86.6
Within two weeks, the number of patients who had:	Antibiotics	18
	Urological instrumentation	3
	a UTI	10
Immunosuppression		2
Diabetes		6
Smoking		8
Co-existing infection		3
Long hospital stay		20
Recurrent UTIs		9
Urinary calculus		4
Urinary catheter		38
Cardiac history		29
Infective endocarditis		0
Prosthetic device		21
Organ transplant		0

Table 70. Basic demographics of patients recruited into a study of bacteraemia caused by TURP

A minority of patients had a UTI, urological instrumentation and antibiotic therapy within two weeks of the TURP. Twenty patients (27.3%) had a long (greater than three days) inpatient hospital stay within three months of the procedure, for various medical conditions including urological pathologies. Only nine patients (12.3%) had a history of documented recurrent UTIs. The majority of patients (52.0%) had a urinary catheter *in situ* at the time of their surgery. The range of duration of catheterisation varied from a few days to many

months. In this cohort of patients, it should be noted that almost 40% (29) of the patients who went on to have a TURP operation had an underlying cardiac pathology. This figure is in line with the big epidemiological studies investigating the prevalence of cardiac disease as reported by the National Academy of an Aging Society (http://www.agingsociety.org/agingsociety). It must also be noted that 28.8% of the cohort of patients had a prosthetic device *in situ*.

4.4.3.1.2 Demographics of the Catheter manipulation Group

Parameter			N=57
Male Sex			52
Mean age (years)			72.3
Mean weight (kg)			84.4
Type of catheter manipulation	Urethral	Removal	39
		Change	10
	Suprapubic	Removal	1
		Change	7
Within 2 weeks, the number of patients who had	Antibiotics		18
	Urological instrumentation		3
	a UTI		5
Immunosuppression			2
Diabetes			10
Smoking			8
Co-existing infection			3
Long hospital stay			5
Recurrent UTIs			8
Urinary calculus			5
Cardiac history			19
Infective endocarditis			0
Prosthetic device			14
Organ transplant			0

Table 71. Basic demographics of patients recruited in the catheter manipulation group

In the catheter manipulation group, most patients had a urethral catheter removal. All the catheters had been *in situ* for at least three weeks. In some cases, the patient has had catheters for many years. Most patients had their current catheters in situ for a period of three months. Ten out of the fifty-seven patients (17.5%) had diabetes in this group. A third of the patients had an underlying cardiac pathology and a quarter had prosthetic devices *in situ*.

None of the patients recruited in the two groups have had previous episodes of infective endocarditis or organ transplant. Most of the patients recruited were aged above 60 years and were male.

4.4.3.1.3 The number of recruited participants

Table 72 shows the patients recruited in the study. Only ninety-nine out of the one hundred and thirty eight patients recruited had a full data set (a completed medical history form, a urine sample, and all the required blood samples). The main reason for the inability to obtain a complete data set was the failure of the cannula, which resulted in the failure to obtain blood samples.

Procedure	Patients recruited	Full data set	Incomplete set of blood samples	No blood samples
TURP Group	**77**	50	3	24
Catheter manipulation Group	**61**	49	4	8
Total	**138**	99	7	32

Table 72. The number of patients recruited for the two prospective studies

4.4.3.2 Bacteriuria from urine samples

4.4.3.2.1 Bacteriuria in the TURP Group

All the isolates from the positive urine samples from the TURP patients were identified and are shown in Table 73. Most of the urine samples demonstrated mixed growth of bacteria. The predominant organism cultured was identified. In two samples, it was not possible to differentiate between the different organisms present.

Identification of urine culture samples from TURP patients		
Sample	**Origin**	**Predominant organism (s)**
T005	MSU	*Escherichia coli* + *Shigella* sp.
T013	Urethral catheter	Failed well
T015	Urethral catheter	*Staphylococcus epidermidis*
T017	Urethral catheter	*Enterococcus faecalis*
T018	Urethral catheter	*Enterococcus faecalis*
T020	MSU	*Escherichia coli*
T021	Urethral catheter	*Corynebacterium amycolatum*
T023	Urethral catheter	*Staphylococcus hominis*
T024	Urethral catheter	*Enterococcus faecalis + Pseudomonas aeruginosa*
T027	Urethral catheter	*Enterococcus faecalis*
T028	MSU	*Streptococcus agalactiae*
T030	Urethral catheter	*Escherichia coli*
T031	MSU	*Enterococcus faecalis*
T033	MSU	*Klebsiella oxytoca*
T034	Urethral catheter	*Enterococcus faecalis*
T043	Urethral catheter	*Corynebacterium amycolatum*
T044	MSU	*Morganella morganii*
T045	Urethral catheter	*Pseudomonas aeruginosa*
T046	Urethral catheter	*Proteus mirabilis*
T049	Urethral catheter	*Enterococcus faecalis*
T052	MSU	*Staphylococcus aureus*
T053	Urethral catheter	*Pseudomonas aeruginosa*
T054	MSU	*Escherichia coli*
T055	Urethral catheter	*Pseudomonas aeruginosa*
T059	Urethral catheter	Mixed

Sample	Origin	Predominant organism (s)
T061	Urethral catheter	*Enterococcus faecalis*
T065	Urethral catheter	*Pseudomonas aeruginosa*
T067	Urethral catheter	*Klebsiella penumoniae*
T069	MSU	*Klebsiella oxytoca*
T071	Urethral catheter	Failed well
T072	MSU	*Enterococcus faecalis*
T075	MSU	*Enterococcus faecalis*
T076	Urethral catheter	*Klebsiella penumoniae*
T077	Suprapubic catheter	Mixed

Table 73. The identity of bacteriuria in TURP patients (Mixed: more than one sequence that could not be separated)

Thirty-four (45.9%) patients undergoing TURP had preoperative bacteriuria, with *Enterococcus faecalis* being the most common organism encountered in this group accounting for 29.4% of patients with bacteriuria. Eleven of the thirty-four patients (32.3%) with bacteriuria did not have a urinary catheter in situ, though four out of those eleven patients (36.4%) performed clean intermittent self-catheterisation on a regular basis. Two samples yielded mixed sequences that could not be separated without the use of commercially available tools. However, this study did not have the funding to use these tools.

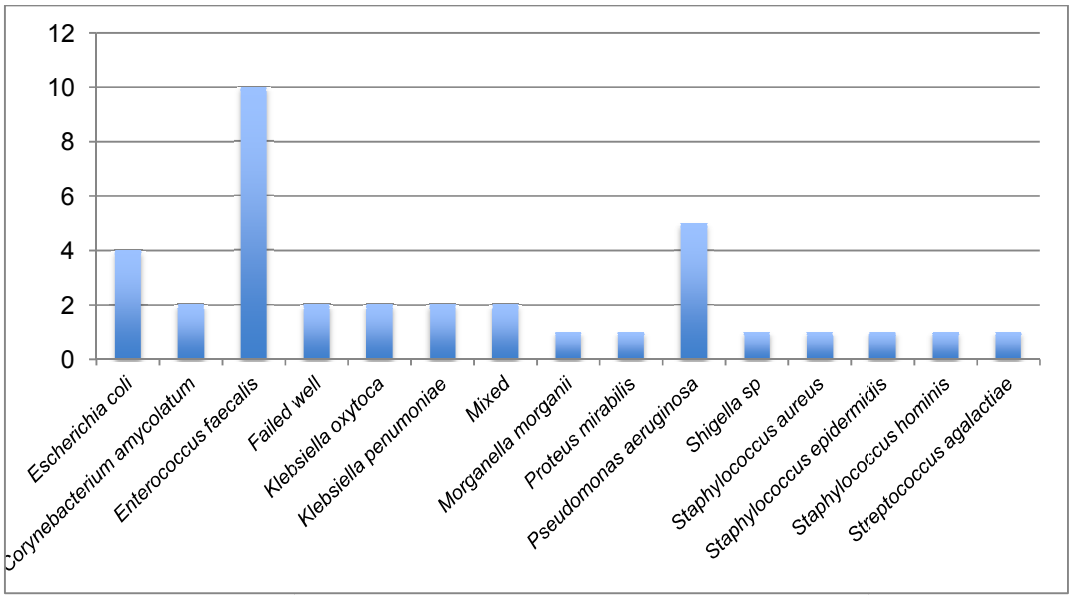

Figure 37. Histogram showing distribution of organisms causing bacteriuria in patients undergoing TURP

4.4.3.2.2 Bacteriuria in the Catheter Manipulation Group

Bacteriuria of > 10^4 cfu/mL were detected in all the patients. Therefore, the identity of the bacteriuria was determined only in patients who were noted to have bacteraemia during the procedure. Table 77 (over the page) shows the results of the bacteriuria in bacteraemic patients.

Identification of urine culture samples in catheter manipulation patients		
Sample	**Origin**	**Predominant organism (s)**
C004	Suprapubic catheter	*Staphylococcus epidermidis + Pseudomonas putida*
C019	Urethral catheter	*Proteus mirabilis*
C020	Urethral catheter	Mixed
C022	Urethral catheter	*Enterococcus faecalis*
C024	Urethral catheter	*Klebsiella oxytoca*
C025	Urethral catheter	*Propionibacterium acnes*
C034	Urethral catheter	*Escherichia coli*
C036	Urethral catheter	*Klebsiella oxytoca*

Table 74. Identity of bacteriuria in the catheter manipulation group

Seven out of the eight patients with both bacteriuria and bacteraemia had a urethral catheter *in situ*. The other patient had a suprapubic catheter. The organisms found in the urine were quite diverse as shown in the table. One sample yielded mixed sequences that could not be separated without the use of commercially available tools. However, this study did not have the funding to use these tools.

4.4.3.3 The detection of bacteraemia in the two prospective studies

Bacteraemia was detected by three different methods: the culture method, the broad-range 16S method and the Plex ID methods as described in Section 4.4.2.5. The results of the different methods are described in Section 4.4.3.1-3.

4.4.3.3.1 The detection of bacteraemia by the culture method

4.4.3.3.1.1 The detection of bacteraemia by the culture method in the TURP group

Table 75 shows the results of bacteraemia in patients undergoing TURP, detected from blood samples processed by incubation and culture. The bacteria grown in the culture broth was identified using molecular methods (described in

Section 4.4.7.1). To validate the identity of the bacteria further, simple biochemical tests were also performed. The results are shown in Table 75.

TURP Group (Recruited 77, Full data set 50)			
Samples	**Presence of catheter**	**Identity of Bacteraemia**	
		Basic Identification	**16S PCR on culture**
T012A	No	GP cocci, catalase positive, coagulase negative	*Staphylococcus caprae*
T021D	Yes	GP rods, catalase negative	*Actinomyces turicensis*
T021E		GP rods, catalase negative	*Actinomyces turicensis*
T021F		GP rods, catalase negative	*Actinomyces turicensis*
T024D	Yes	GN rods, oxidase positive	*Pseudomonas aeruginosa*
T024E	Yes	GN rods, oxidase positive	*Pseudomonas aeruginosa*
T027C	Yes	GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
T028D	No (CISC)	GP cocci, beta haemolytic	*Staphylococcus epidermidis*
T028E		GP cocci, beta haemolytic	*Staphylococcus epidermidis*
T028F		GP cocci, beta haemolytic	*Staphylococcus epidermidis*
T033A	No (CISC)	GP rods, catalase negative	*Actinomyces neuii*
T033C		GP rods, catalase negative	*Actinomyces neuii*
T033D		GP rods, catalase negative	*Actinomyces neuii*
T033E		GP rods, catalase negative	*Actinomyces neuii*
T033F		GP rods, catalase negative	*Actinomyces neuii*
T039C	No	GP coccobacilli, catalase negative, oxidase negative	*Actinobaculum massiliense*
T039D		GP coccobacilli, catalase negative, oxidase negative	*Actinobaculum massiliense*
T040F	No	GP coccobacilli, catalase positive, oxidase negative	*Corynebacterium glucuronolyticum*
T045C	Yes	GN cocci, anaerobic growth	*Veillonella dispar*

Samples	Presence of catheter	Identity of Bacteraemia	
		Basic Identification	16S PCR on culture
T046B	Yes	GN rods, oxidase negative	*Proteus vulgaris*
T046C		GN rods, oxidase negative	*Proteus vulgaris*
T046D		GN rods, oxidase negative	*Proteus vulgaris*
T046E		GN rods, oxidase negative	*Proteus vulgaris*
T046F		GN rods, oxidase negative	*Proteus vulgaris*
T049A	Yes	GP cocci, catalase negative	*Enterococcus faecalis*
T049B		GP cocci, catalase negative	*Enterococcus faecalis*
T049C		GP cocci, catalase negative	*Enterococcus faecalis*
T049F		GP cocci, catalase negative	*Enterococcus faecalis*
T050E	Yes	GP cocci, catalase negative	*Streptococcus anginosus*
T053D	Yes	GN rods, oxidase positive	*Pseudomonas aeruginosa*
T053E		GN rods, oxidase positive	*Pseudomonas aeruginosa*
T053F		GN rods, oxidase positive	*Pseudomonas aeruginosa*
T055C	Yes	GN rods, oxidase positive	*Pseudomonas aeruginosa*
T055D		GN rods, oxidase positive	*Pseudomonas aeruginosa*
T055E		GN rods, oxidase positive	*Pseudomonas aeruginosa*
T055F		GN rods, oxidase positive	*Pseudomonas aeruginosa*
T061	Yes	GP cocci, catalase negative	*Enterococcus faecalis*
T064	Yes	GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
T065D	Yes	GP cocci, beta haemolytic	*Streptococcus agalactiae*
T065E		GP cocci, beta haemolytic	*Streptococcus agalactiae*

Table 75. Identity of positive blood cultures from TURP patients

Fifty participants provided a total of two hundred and seventy six (276) blood samples. Fourteen out of the seventeen (82.4%) patients who developed bacteraemia during their procedure had a urinary catheter *in situ* prior to the

procedure or were performing CISC prior to the procedure. Of the forty-one positive blood cultures, two were regarded as contaminants (T012A and T040F) as the organisms detected (*Staphylococcus caprae* and *Corynebacterium glucuronolyticum* respectively) are known skin contaminants and were recovered only from single samples [225]. Of the thirty-nine true bacteraemia cases (those that did not include skin contaminants), the most common organisms detected in the TURP group were *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Fourteen out of the fifty patients (28.0%) undergoing TURP had bacteraemia peri-operatively, detected using the culture method.

4.4.3.3.1.2 The detection of bacteraemia by the culture method in the catheter manipulation group

Catheter Group (Recruited 61, Full data set 49)			
Sample	**Type of catheter**	**Identity of Bacteraemia**	
		Basic Identification	**16S PCR on culture**
C002A	Urethral	GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
C004A	Suprapubic	GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
C004B		GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
C004C		GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
C004D		GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
C005D	Urethral	GP coccobacilli, catalase positive, oxidase negative	*Corynebacterium amycolatum*
C007D	Suprapubic	GP rods, anaerobic growth	*Propionibacterium acnes*
C019A	Urethral	GP cocci, not bile soluble	*Streptococcus pseudopneumoniae*
C019B		GP cocci, not bile soluble	*Streptococcus pseudopneumoniae*

Sample	Type of catheter	Identity of Bacteraemia	
		Basic Identification	16S PCR on culture
C020B	Urethral	GP coccus, catalase negative	*Streptococcus mitis*
C020B		GP coccus, catalase negative	*Streptococcus mitis*
C022A	Urethral	GP coccus, coagulase negative	*Staphylococcus warneri/pasteuri*
C022D		GP coccus, coagulase negative	*Staphylococcus warneri/pasteuri*
C024B	Urethral	GN rods	*Klebsiella oxytoca*
C024D		GN rods	*Klebsiella oxytoca*
C025B	Urethral	GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*
C034A	Urethral	GN cocci, aerobic	*Neisseria flavescens*
C036D	Urethral	GN cocci, aerobic	*Neisseria flavescens*
C047A	Suprapubic	GP cocci, oxidase positive	*Micrococcus* species
C050D	Urethral	GP cocci, catalase positive, coagulase negative	*Staphylococcus epidermidis*

Table 76. Identity of bacteraemia in patients in the catheter manipulation group

Forty-nine participants in the catheter manipulation group provided a total of one hundred and sixty-three (163) blood samples. Of the forty-nine participants recruited, thirty-four patients had urethral catheter removal, seven had urethral catheter change, seven had suprapubic catheter change and one had suprapubic catheter removal. Seven out of thirty-four patients (20.6%) in the urethral catheter removal group and one out of the seven patients (14.3%) in the suprapubic catheter change group had bacteraemia. No bacteraemia was noted in the urethral catheter change and the suprapubic catheter removal groups. The main organisms causing bacteraemia were *Staphylococcus epidermidis* and *Klebsiella oxytoca*.

4.4.3.3.2 The detection of bacteraemia by the Plex-ID System

4.4.3.3.2.1 The detection of bacteraemia by the Plex-ID System in the TURP group

The Plex-ID detected thirty-four positive samples in the TURP group. However, based on the quantification cut-off of < 10 and the knowledge of skin contaminants, only four samples were considered true positives (T028D, T028E, T028F and T049B). The organisms detected were *Streptococcus agalactiae* and *Streptococcus penumoniae.* The thirty other positive samples were considered to be false positives as they were either skin contaminants and/or were detected at levels < 10. Table 77 shows all the results not reported as 'negative' by the Plex-ID.

Blood Sample	Identification of bacteraemia using the Plex-ID
T005A	*Staphylococcus hominis* (0.99; 24)
T015D	*Bacillus subtilis* (0.97; 45)
T015F	*Bacillus subtilis* (0.93; 46)
T017C	Well failure
T020A	*Staphylococcus aureus* (0.90; 5)
T021E	Well failure
T022C	Well failure
T022D	Well failure
T023A	*Staphylococcus aureus* (0.99; 4)
T023C	*Staphylococcus aureus* (0.99; 3)
T023E	Plate failure
T023F	Plate failure
T024A	Plate failure
T024B	Plate failure
T024C	Plate failure
T024D	Plate failure
T024E	Plate failure
T024F	Plate failure
T026A	Plate failure
T026B	Plate failure
T026C	Plate failure
T026D	Plate failure
T026E	Plate failure
T026F	Plate failure
T027A	Plate failure

Blood Sample	Identification of bacteraemia using the Plex-ID
T027B	Plate failure
T028D	*Streptococcus agalactiae* (1.00; 69)
T028E	*Streptococcus agalactiae* (0.99; 43)
T028F	*Streptococcus agalactiae* (1.00; 22)
T043F	*Pseudomonas putida/stutzeri* (0.93; 27)
T044D	*Pseudomonas putida/stutzeri/entomophilia* (0.94; 22)
T045A	*Pseudomonas putida/entomophilia/stutzeri* (0.94; 38)
T045C	*Pseudomonas putida/entomophilia/stutzeri* (0.93; 29)
T046A	*Pseudomonas putida/entomophilia/stutzeri* (0.94; 32)
T046E	*Streptococcus oralis/sanguinis* (0.93; 5)
T049A	*Pseudomonas putida/stutzeri* (0.93; 37)
T049B	*Streptococcus penumoniae* (0.93; 11)
T049D	*Staphylococcus saprophyticus* (0.99; 11)
T050B	*Pseudomonas putida/entomophilia/stutzeri* (0.94; 24)
T053C	*Pseudomonas putida/stutzeri* (0.94; 53)
T055A	*Propionibacterium acnes* (0.98; 22)
T055B	*Corynebacterium xerosis* (0.93; 10)
T055D	*Micrococcus luteus* (0.96; 25)
T059A	Propionibacterium acnes (0.98; 30)
T059E	*Acinetobacter baumannii* (0.94; 3)
T062B	*Staphylococcus hominis* (0.99; 12)
T062C	*Bacillus cereus group* (0.96; 7)
T062F	*Pseudomonas stutzeri* (0.98; 56)
T064C	*Staphylococcus hominis* (1.00; 40)
T067B	*Staphylococcus aureus* (0.92; 3)
T067E	*Cellulomonas humilata* (0.94; 44)
T071C	*Staphylococcus aureus* (0.90; 3)
T076E	*Propionibacterium acnes* (0.93; 21)
T076F	*Propionibacterium acnes* (0.97; 21)

Table 77. Identities of bacteria from blood samples taken from patients undergoing TURP as detected by the Plex-ID

4.4.3.3.2.2 The detection of bacteraemia by the Plex-ID System in the catheter manipulation group

In the catheter manipulation group, twenty-four samples were positive. However, all the samples were considered false positives as the organisms

detected were skin contaminants and/or were too few. Therefore, the Plex- ID did not pick up any bacteraemia in the catheter manipulation group. Table 78 shows all the results not reported as 'negative' by the Plex-ID.

Blood Sample	Identification of bacteraemia using the Plex-ID
C002A	*Lactobacillus acidophilus* (0.94; 17) + *Enterococcus durans/haire* (0.97; 17)
C002C	*Weissella confusa* (0.97; 13)
C005B	*Staphylococcus aureus* (0.93; 7)
C006B	*Macrococcus caseolyticus* (0.92; 8)
C009D	*Pseudomonas putida/stutzeri* (0.96; 69)
C013A	*Staphylococcus aureus* (0.95; 7)
C015B	*Staphylococcus aureus* (0.91; 4)
C016B	*Staphylococcus haemolyticus* (1.00; 11)
C019A	*Staphylococcus hominis* (0.98; 15)
C026A	*Methylobacterium mesophilicum* (0.93; 8)
C026D	*Staphylococcus aureus* (0.91; 4)
C029B	*Staphylococcus hominis* (0.99; 16)
C032B	*Staphylococcus epidermidis* (0.92; 12)
C035B	*Staphylococcus aureus/haemolyticus* (0.95; 10)
C037D	*Staphylococcus aureus* (0.95; 16)
C038A	*Staphylococcus aureus* (0.95; 6)
C041A	*Streptococcus vestibularis* (0.91; 6)
C041D	*Propionibacterium acnes* (0.95; 21)
C047C	*Streptococcus penumoniae* (0.92; 4)
C053A	*Propionibacterium acnes* (0.97; 24)
C055A	*Staphylococcus aureus* (0.94;8)
C057A	*Staphylococcus aureus* (0.99; 13)
C057D	*Acidovorax temperans* (0.91; 7)
C058B	*Staphylococcus aureus* (0.92; 7)

Table 78. Identities of bacteria from blood samples taken from patients undergoing catheter manipulation as detected by the Plex-ID

4.4.3.3.3 The detection of bacteraemia by the 16S PCR method

By following the criteria described in Section 4.4.2.5.6.2, forty-five samples in the TURP group were 'possible' positive samples and fifty-three samples were 'definite' positive samples. In the catheter manipulation group, forty-one samples were 'possible' positive samples and thirteen were 'definite' positive

samples. The detailed results are shown in Appendix 9. The following samples were identified for sequence determination and analysis (as described in Section 4.4.2.5.6.2) after review by the experienced molecular microbiology scientist, Dr Deborah Gascoyne-Binzi.

Positive samples (Expert review)	C_t Value
T017E	26.74
T017F	26.90
T018E	23.28
T020A	26.88
T020C	24.33
T020F	24.24
T021A	24.18
T021D	24.50
T021E	24.13
T021F	24.22
T022C	24.19
T022D	26.68
T027F	25.63
T031F	25.75
T036D	25.37
T038E	27.49
T043A	24.02
T044A	23.87

Table 79. Samples determined that eventually had their sequences determined

Out of the eighteen samples examined, three of the samples yielded sequences that could be read and compared with sequences from BLAST (http://blast.ncbi.nlm.nih.gov).

Sample	C_t Value	Identity from BLAST
T027F	25.63	*Streptococcus agalactiae*
T031F	25.75	Enterobacteriacae
T036D	25.37	*Acinetobacter* spp.

Table 80. Identification of the positive sample using BLAST

The three samples identified were not positive when using the two other methods (culture and Plex-ID). The broad-range 16S PCR method demonstrated that three samples had evidence of bacteraemia.

4.4.3.4 Results from the follow-up of patients following the procedure

A summary of the results from the post-procedure telephone interview, the results server and the review of the prostate histology are shown below.

4.4.3.4.1 Follow-up data from the TURP group

Parameters from hospital server		***n=73***
Patients discharged within two days		52
Patients with positive MSU within three months of procedure		41
Patients with a positive blood culture within 3 month of the procedure		7
Malignant prostate histology		20
Parameters from telephone interview		**n=43**
Felt unwell		15
Lower urinary tract symptoms		16
Urine sample to GP		15
Antibiotics from GP		15
Readmission	For Infection	7
	For other causes	6

Table 81. Data from the telephone interview from patients in the TURP group

Fifty-two patients (71.2%) having a TURP operation were discharged within two days of the procedure, with haematuria being the main reason for delays in discharge from the hospital. Forty-one patients (56.1%) had a documented bacteriuria within three months of the procedure. Twenty out of the seventy-three patients (27.4%) recruited had prostate cancer, based on histopathological results of the prostate chips obtained from the TURP operation. Forty-three of the recruited patients (58.9%) were contactable by telephone for an interview. Sixteen of the patients contacted (37.2%) said they felt unwell following the procedure, mainly from lower urinary tract symptoms. Their general practitioner reviewed them, and a urine sample was sent for culture. Fifteen patients (34.9%) were prescribed antibiotics for their symptoms. Thirteen of the patients contacted (27.1%) were readmitted to the hospital. Seven of the patients who were readmitted to hospital, presented with infective symptoms. Data from four patients were missing and, therefore, it was possible to obtain post-operative parameters for seventy-three of the seventy-seven patients recruited

4.4.3.4.1.1 Prostate histology from the TURP group

Variables	***N=73 (%)***
Malignant histology	21 (28.8)
Presence of acute inflammation	3 (4.1)
Presence of chronic inflammation	23 (35.6)
Presence of calcification	3 (4.1)
Number of patients with bacteraemia	15 (20.5)

Table 82. The presence of inflammation and calcification in TURP patients based on histopathological analysis following the procedure

Twenty-six patients had evidence of prostatic inflammation, twenty-three patients had chronic inflammation, one had acute inflammation and two patients had both acute and chronic. Only three patients had evidence of prostatic calcification.

Univariate analysis (Pearson χ^2 test) showed that there was an association between the development of intraoperative bacteraemia and prostatic inflammation (χ^2 (1) = 4.97, p = 0.026). The frequency of calcification was too low to detect any statistical association with intraoperative bacteraemia.

4.4.3.4.2 Follow-up data from the catheter manipulation group

Parameter from hospital server		n=57
Patients with positive MSU within three months of procedure		10
Patients with a positive blood culture within three months of the procedure		1
Parameters from telephone interview		**n=32**
Felt unwell		5
Lower urinary tract symptoms		6
Urine sample to GP		8
Parameters from telephone interview		**n=32**
Antibiotics from GP		6
Readmission	For Infection	0
	For other causes (e.g. pain)	3

Table 83. Follow-up data from the catheter manipulation group

Examination of the results server revealed that only a minority of patients who had catheter manipulation had urine and blood samples sent within three months of their procedure. Thirty-two participants (56.1%) responded to the telephone interview with less than 20% of the respondents reporting any urological complaint. Three of the respondents were readmitted within three months of their procedure but none of these admissions were owing to infective complications.

4.4.3.5 Methods for the detection of bacteraemia detection: how do they compare?

The bacteraemia results obtained using the three different methods (culture, broad-range 16S PCR and Plex-ID) are compared in Table 84 below. Four hundred and forty-one blood samples were obtained in all. All the results were

available for the culture and 16S PCR methods. Twenty results were missing from the Plex-ID methodology owing to plate failure during the amplification process. The results of the three different methods are shown in Table 84.

Blood Sample	Culture+ 16S PCR	DNA Extraction + 16S PCR	DNA Extraction + Plex-ID
T005A	Negative	Negative	*Staphylococcus hominis* (0.99; 24)
T012A	*Staphylococcus caprae*	Negative	Negative
T015D	N/A	Negative	*Bacillus subtilis* (0.97; 45)
T015F	N/A	Negative	*Bacillus subtilis* (0.93; 46)
T020A	Negative	Negative	*Staphylococcus aureus* (0.90; 5)
T021D	*Actinomyces turicensis*	Negative	Negative
T021E	*Actinomyces turicensis*	Negative	Well failure
T021F	*Actinomyces turicensis*	Negative	Negative
T023A	Negative	Negative	*Staphylococcus aureus* (0.99; 4)
T023C	Negative	Negative	*Staphylococcus aureus* (0.99; 3)
T024D	*Pseudomonas aeruginosa*	Negative	Plate failure
T024E	*Pseudomonas aeruginosa*	Negative	Plate failure
T027C	*Staphylococcus epidermidis*	Negative	Negative
T027F	Negative	*Streptococcus agalactiae* (C_t: 25.63)	Negative
T028D	*Staphylococcus epidermidis*	Negative	*Streptococcus agalactiae* (1.00; 69)
T028E	*Staphylococcus epidermidis*	Negative	*Streptococcus agalactiae* (0.99; 43)
T028F	*Staphylococcus epidermidis*	Negative	*Streptococcus agalactiae* (1.00; 22)
T031F	Negative	*Enterobacteriacae* (C_t:25.75)	Negative
T033A	*Actinomyces neuii*	Negative	Negative
T033C	*Actinomyces neuii*	Negative	Negative
T033D	*Actinomyces neuii*	Negative	Negative
T033E	*Actinomyces neuii*	Negative	Negative
T033F	*Actinomyces neuii*	Negative	Negative
T036D	Negative	*Actinobacter* (C_t: 25.37)	Negative
T039C	*Actinobaculum massiliense*	Negative	Negative
T039D	*Actinobaculum massiliense*	Negative	Negative
T040F	*Corynebacterium glucuronolyticum*	Negative	Negative
T043F	Negative	Negative	*Pseudomonas putida/stutzeri /entomophilia* (0.93; 27)
T044D	Negative	Negative	*Pseudomonas putida/stutzeri/entemophilia* (0.94; 22)
T045A	Negative	Negative	*Pseudomonas putida/sturtzeri/entomophilia*(0.94; 38)

Blood Sample	Culture+ 16S PCR	DNA Extraction + 16S PCR	DNA Extraction + Plex-ID
T045C	*Veillonella dispar*	Negative	ASA (0.93; 29)
T046A	Negative	Negative	*Pseudomonas putida/stutzeri/entomophilia* (0.94; 32)
T046B	*Proteus vulgaris*	Negative	Negative
T046C	*Proteus vulgaris*	Negative	Negative
T046D	*Proteus vulgaris*	Negative	Negative
T046E	*Proteus vulgaris*	Negative	*Streptococcus oralis/sanguinis* (0.93; 5)
T046F	*Proteus vulgaris*	Negative	Negative
T049A	*Enterococcus faecalis*	Negative	*Pseudomonas putida/stutzeri*/entemophilia (0.93; 37)
T049B	*Enterococcus faecalis*	Negative	*Streptococcus penumoniae* (0.93; 11)
T049C	*Enterococcus faecalis*	Negative	Negative
T049D	Negative	Negative	*Staphylococcus saprophyticus* (0.99; 11)
T049F	*Enterococcus faecalis*	Negative	Negative
T050B	Negative	Negative	*Pseudomonas putida/stutzeri*/entemophilia (0.94; 24)
T050D	Negative	Negative	*Propionibacterium acnes* (0.97; 9)
T050E	*Streptococcus anginosus*	Negative	Negative
T053C	Negative	Negative	*Pseudomonas putida/stutzeri/entemophilia* (0.94; 53)
T053D	*Pseudomonas aeruginosa*	Negative	Negative
T053E	*Pseudomonas aeruginosa*	Negative	Negative
T053F	*Pseudomonas aeruginosa*	Negative	Negative
T055A	Negative	Negative	*Propionibacterium acnes* (0.98; 22)
T055B	Negative	Negative	*Corynebacterium xerosis* (0.93; 10)
T055C	*Pseudomonas aeruginosa*	Negative	Negative
T055D	*Pseudomonas aeruginosa*	Negative	*Micrococcus luteus* (0.96; 25)
T055E	*Pseudomonas aeruginosa*	Negative	Negative
T055F	*Pseudomonas aeruginosa*	Negative	Negative
T059A	Negative	Negative	*Propionibacterium acnes* (0.98; 30)
T059E	Negative	Negative	*Acinetobacter baumannii* (0.94; 3)
T061C	*Enterococcus faecalis*	Negative	Negative
T062B	Negative	Negative	*Staphylococcus hominis* (0.99; 12)
T062C	Negative	Negative	*Bacillus cereus* group (0.96; 7)
T062F	Negative	Negative	Pseudomonas stutzeri (0.98; 56)
T064A	*Staphylococcus epidermidis*	Negative	Negative
T064C	Negative	Negative	*Staphylococcus hominis* (1.00; 40)
T065D	*Streptococcus agalactiae*	Negative	Negative
T065E	*Streptococcus agalactiae*	Negative	Negative
T067B	Negative	Negative	*Staphylococcus aureus* (0.92; 3)
T067E	Negative	Negative	*Cellulomonas humilata* (0.94; 44)
T071C	Negative	Negative	*Staphylococcus aureus* (0.90; 3)

Blood Sample	Culture+ 16S PCR	DNA Extraction + 16S PCR	*DNA Extraction + Plex-ID*
T076E	Negative	Negative	*Propionibacterium acnes* (0.93; 21)
T076F	Negative	Negative	*Propionibacterium acnes* (0.97; 21)
C002A	*Staphylococcus epidermidis*	Negative	*Lactobacillus acidophilus* (0.94; 17) *Enterococcus durans/haire* (0.97; 17)
C002C	Negative	Negative	*Weissella confusa* (0.97; 13)
C004A	*Staphylococcus epidermidis*	Negative	Negative
C004B	*Staphylococcus epidermidis*	Negative	Negative
C004C	*Staphylococcus epidermidis*	Negative	Negative
C004D	*Staphylococcus epidermidis*	Negative	Negative
C005B	Negative	Negative	*Staphylococcus aureus* (0.93; 7)
C005D	*Corynebacterium amycolatum*	Negative	Negative
C006B	Negative	Negative	*Macrococcus caseolyticus* (0.92; 8)
C007D	*Propionibacterium acnes*	Negative	Negative
C009D	Negative	Negative	*Pseudomonas putida/stutzeri/entemophilia* (0.96; 69)
C013A	Negative	Negative	*Staphylococcus aureus* (0.95; 7)
C015B	Negative	Negative	*Staphylococcus aureus* (0.91; 4)
C016B	Negative	Negative	*Staphylococcus haemolyticus* (1.00; 11)
C019A	*Streptococcus pseudopneumoniae*	Negative	*Staphylococcus hominis* (0.98; 15)
C019B	*Streptococcus pseudopneumoniae*	Negative	Negative
C020B	*Streptococcus mitis*	Negative	Negative
C020D	*Streptococcus mitis*	Negative	Negative
C022A	*Staphylococcus warneri/pasteuri*	Negative	Negative
C022D	*Staphylococcus warneri/pasteuri*	Negative	Negative
C024B	*Klebsiella oxytoca*	Negative	Negative
C024D	*Klebsiella oxytoca*	Negative	Negative
C025B	*Staphylococcus epidermidis*	Negative	Negative
C026A	Negative	Negative	*Methylobacterium mesophilicum* (0.93; 8)
C026D	Negative	Negative	*Staphylococcus aureus* (0.91; 4)
C029B	Negative	Negative	*Staphylococcus hominis* (0.99; 16)
C032B	Negative	Negative	*Staphylococcus epidermidis* (0.92; 12)
C034A	*Neisseria flavescens*	Negative	Negative
C035B	Negative	Negative	*Staphylococcus aureus/haemolyticus* (0.95; 10)
C036D	*Neisseria flavescens*	Negative	Negative
C037D	Negative	Negative	*Staphylococcus aureus* (0.95; 16)
C038A	Negative	Negative	*Staphylococcus aureus* (0.95; 6)
C041A	Negative	Negative	*Streptococcus vestibularis* (0.91; 6)

Blood Sample	Culture+ 16S PCR	DNA Extraction + 16S PCR	DNA Extraction + Plex-ID
C041D	Negative	Negative	*Propionibacterium acnes* (0.95; 21)
C047A	*Micrococcus* species	Negative	Negative
C047C	Negative	Negative	*Streptococcus penumoniae* (0.92; 4)
C050D	*Staphylococcus epidermidis*	Negative	Negative
C053A	Negative	Negative	*Propionibacterium acnes* (0.97; 24)
C055A	Negative	Negative	*Staphylococcus aureus* (0.94;8)
C057A	Negative	Negative	*Staphylococcus aureus* (0.99; 13)
C057D	Negative	Negative	*Acidovorax temperans* (0.91; 7)
C058B	Negative	Negative	*Staphylococcus aureus* (0.92; 7)

Table 84. Bacteraemia in all the blood samples obtained using the three different methods to detect bacteraemia (culture, 16S PCR and Plex- ID)

The three methods performed very differently. The 'gold standard' culture method produced the most positive results. This may be owing to the fact that the incubation period was extended to achieve maximal sensitivity. However, sensitivity tests were not done during the in-vitro studies to establish how sensitive the 'culture method' was. The Plex-ID method detected a significant number of false positives, likely to be contaminants [226]. These bacterial contaminants were largely skin commensals, which may have been introduced during the large number of steps needed to extract bacterial DNA from blood and the subsequent identification of the nucleotides present. This processing problem was not picked up by the other molecular method (16S PCR) as only a small, selected number of the samples had their sequences determined. None of the 16S PCR tests produced positive results from the catheter manipulation samples. The Plex-ID method also detected a number of skin contaminants. The culture method also picked up a number of contaminants.

4.4.3.5.1 Statistical comparison of the different methods used to detect bacteraemia

McNemar's Test to compare the different methods (Culture *vs* 16S PCR, Culture *vs* Pled-ID and 16S PCR *vs* Plex-ID) showed no concordance between the three methods of detecting bacteraemia. The gold standard method in this study was the culture method. Therefore, bacteraemia results from this method were used for further analysis.

4.4.3.6 Statistical evaluation of the association between the development of bacteraemia and the timings of the blood samples

Four hundred and thirty-three observations from one hundred and five patients were included in the model. Eight samples were not included for the analysis as the bacteraemia results were not available from the culture method. The results are shown in Table 85 below. All the variables were compared with Time point A. Time point A is the pre-procedure time point and was used as the negative control as it is assumed that no bacteraemia was present at that point. This is based on the premise that under normal conditions, blood is a sterile environment [227]. For the purposes of this statistical analysis, the time point when the catheter was re-inserted was renamed Time Point G (instead of C), as shown in Table 85.

Time Points	Time when the blood sample was taken
A	Before the procedure
B	When catheter comes out
C	5 minutes into procedure
D	10 minutes into procedure
E	20 minutes into procedure
F	End of procedure
G	When catheter is re-inserted

Table 85. Definitions of the time points at which blood samples were taken

From Table 85 above, it can be shown that time point G occurred only in patients having catheter change. Time points C, D and E only occurred in patients having a TURP.

Variable	Estimate	Standard Error	*z*-value	*p*-value
Procedure (TURP)	-0.56	1.08	-0.52	0.61
Time points B	-0.27	0.70	-0.40	0.69
Time points C	1.20	0.77	1.56	0.12
Time point D	**1.48**	**0.76**	**1.94**	**0.05**
Time point E	**1.67**	**0.79**	**2.12**	**0.03**
Time Point F	1.01	0.61	1.65	0.09
Time Point G	-1.48	1.86	-0.79	0.43

Table 86. Results from multi-level model evaluating the association between the development of bacteraemia and the timings of the blood samples. Results in bold are statistically significant.

Table 86 shows the results from the linear model. The model shows that time points D and E are important in determining the presence of bacteraemia compared with time point A. The procedure (TURP or catheter manipulation) and the other time points (B, C, F and G) were not statistically significant in determining the presence of bacteraemia. These results are interpreted as follows:

- blood samples taken 10 minutes (Time point C) and 20 minutes (Time point D) into a TURP operation are more likely to have bacteraemia compared to a blood sample taken before the procedure;
- the removal of a catheter (Time point B) did not increase the likelihood of developing bacteraemia in either a patient having catheter manipulation or a patient having a TURP;
- catheter manipulation did not have any statistical association with the development of bacteraemia.

The odds of developing bacteraemia during TURP are shown in Figure 38. At time point D, it was 4.39 times more likely to detect bacteraemia compared to time point A. At time point E, it was 5.31 times more likely to detect bacteraemia compared with time point A.

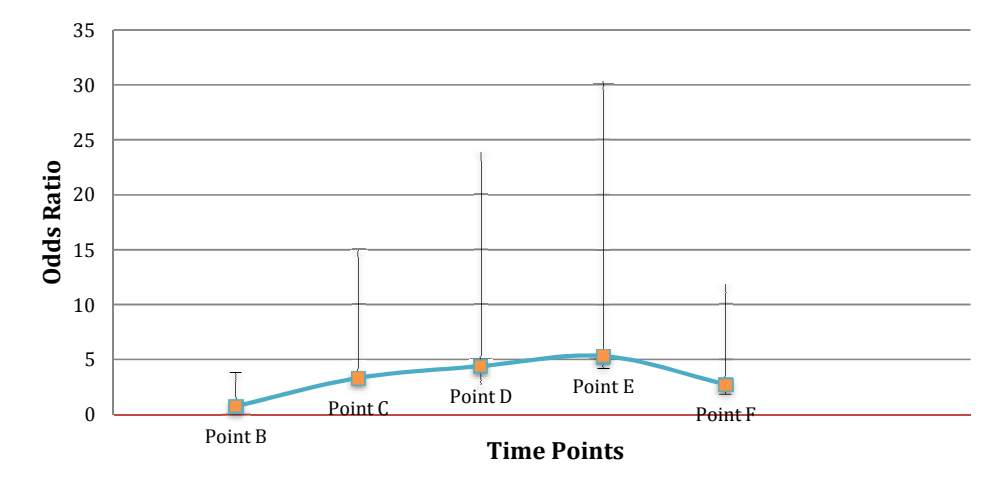

Figure 38. Odds ratio of developing bacteraemia at different time points during a TURP compared with time point A

Figure 38 above demonstrates that the likelihood of developing bacteraemia increases as the TURP operation proceeds, with bacteraemia most likely to be detected twenty minutes into the procedure. This study has demonstrated that having a TURP leads to a transient bacteraemia, as the likelihood of detecting bacteraemia at the end of the procedure (Time point F) is the same as the likelihood of detecting bacteraemia before the procedure (Time point A).

4.4.3.7 Relationship between bacteriuria and bacteraemia in the TURP group

The results of χ^2 test performed to evaluate the association between pre-procedure bacteriuria and intra-procedure bacteraemia is shown in Table 87 (over the page).

		Bacteraemia		
		No	Yes	Total
Bacteriuria	No	35	6	41
	Yes	22	11	33
Total		57	17	74
		Value	df	Asymp. Sig (2-sided)
Pearson χ^2		3.74	2	0.15
Likelihood ratio		3.94	2	0.14

Table 87. χ^2 tests to compare bacteriuria and bacteraemia in patients with TURP

No association was found between the presence of bacteriuria and the subsequent development of bacteraemia during a TURP. This goes against traditional teaching as described in Chapter 1. In the catheter group, statistical analysis was not carried out to evaluate the association between bacteriuria and the subsequent development of bacteraemia as every patient in this group had bacteriuria.

4.4.3.8 Risk factors, bacteraemia and post-procedure infections

This work established that asymptomatic bacteraemia happens both during TURP and catheter manipulation. The rate of asymptomatic bacteraemia was higher than the previously reported rate of symptomatic bacteraemia. TURP was a cause of asymptomatic bacteraemia confirmed by the timing of bacteraemia during the procedure. The next step was to establish whether there was any relationship between 'patient-related' risk factors and the development of intra-procedure bacteraemia, using the logistic regression model described in Section 4.4.2.10.

4.4.3.8.1 The risk factors in the TURP group and the association with bacteraemia and post-procedure infection

Three risk factors were chosen out of the all the risk factors collected, based on the estimated likelihood of impact on bacteraemia, in an attempt to make the logistic regression model as accurate as possible. Antibiotic use within two weeks of the procedure can affect the development of bacteraemia [230]. Histological changes in the prostate gland (benign or malignant) can be a surrogate marker of initial bacterial colonisation of the prostate [229]. The presence of a urinary catheter is known to predispose patients to bacteriuria [112], and the prolonged presence of the catheter place patients at risk of developing bacteraemia [115].

Risk factors		Frequency
Antibiotics use within two weeks of the procedure	No	55
	Yes	18
Prostatic histology	No	51
	Yes	22
Presence of a catheter	No	35
	Yes	38

Table 88. Frequency table of the risk factors used in the logistic regression model.

	Coefficient	Standard Error	*p*-value	OR	95% C.I. for OR	
					Lower	Upper
Antibiotics Use	-1.47	0.69	0.032	4.34	1.14	16.62
Catheter	1.59	0.75	0.034	4.92	1.13	21.51
Histology	1.59	0.68	0.019	4.90	1.30	18.46

Table 89. Multivariate analysis using the logistic regression model to evaluate the association between the development of bacteraemia and the associated risk factors

A patient with histological evidence of malignancy was five times more likely to develop bacteraemia than a patient with no evidence of malignancy in the prostatic tissue (OR: 4.90, CI: 1.30-18.46, $p = 0.019$). This result suggests that the presence of a prostate adenocarcinoma increases the likelihood of developing bacteraemia during a TURP procedure. A patient who had antibiotics within two weeks of a TURP was four times less likely to develop bacteraemia than a patient who did not receive antibiotics (OR: 4.34, CI 1.14-16.62 p = 0.032). This may be explained by the fact that patients who were administered antibiotics were more likely to have any bacteria present in the system eradicated during the use of the antibiotics. Therefore, there is less chance of detecting bacteria in the blood. Having a urinary catheter in situ was associated with the development of intra-TURP bacteraemia (OR: 4.92, CI 1.13-21.51, p = 0.034). This association has been described in the literature previously [112,115].

It was not possible to fit prostatic inflammation in the model owing to the multi-level association between antibiotic use, prostatic malignancy, age and bacteraemia. On univariate analysis, an association between prostatic inflammation and intraoperative bacteraemia was found (χ^2 (1) = 4.97, $p = 0.026$). Further interpretation of this result is difficult in the sample population as univariate analysis showed that a malignant prostatic histology was associated with prostatic inflammation (χ^2 (1) = 7.46, $p = 0.006$).

4.4.3.8.2 The risk factors in the catheter manipulation group and the association with bacteraemia and post-procedure infection

For the catheter manipulation group, the following risk factors were selected to evaluate an association with bacteraemia, as shown in Table 90.

		Frequency
Suprapubic catheter	No	37
	Yes	20
Antibiotics use	No	39
	Yes	18
Long hospital stay	No	52
	Yes	5
Duration of catheterisation (> 3 months)	No	48
	Yes	9

Table 90. Frequency table of the risk factors in the logistic regression model

	Coefficient	Standard error	*p*-value	Exp(B)	95% C.I. for EXP(B)	
					Lower	Upper
Suprapubic catheter	-0.63	0.88	0.47	0.53	0.10	2.97
Antibiotics use	0.96	0.86	0.26	2.60	0.49	13.97
Age	0.02	0.03	0.51	1.02	0.96	1.09
Recent Hospital Stay	0.52	1.28	0.68	1.69	0.14	20.65
Duration of catheter (>3 month)	0.02	0.73	0.98	1.02	0.25	4.27
Constant	-3.54	3.02	0.24	0.03		

Table 91. Results from the logistic regression model

The logistic regression model did not show any association between the development of bacteraemia and the risks factors. It could be that there was no association or the numbers were not sufficient to show any association.

4.4.4 Discussion

The studies reported above have demonstrated that bacteraemia is prevalent during both TURP and catheter manipulation, when the results from the culture method are analysed. All the episodes of bacteraemia detected in this study were asymptomatic. In the TURP group, two patients had symptomatic bacteraemia following the procedure and were treated by the clinical team. The issues related to bacteraemia, risk factors and symptoms are discussed further in Chapter 5.

4.4.4.1 Issues with the interpretation of the prostate histology

It is possible that during the fixing process of the prostatic tissue, tissue that was hard and appeared calcified was not embedded. This was a protective measure utilised by the histopathologist to avoid damaging the microtome knife. Therefore, the incidence of calcification of the prostate may be higher than reported in this study, though it is not possible to have an exact figure.

4.4.4.2 Relationship between bacteriuria and bacteraemia

Many patients had bacteriuria and bacteraemia in this study. Were patients with bacteriuria more likely to develop bacteraemia? The literature suggests that the presence of pre-operative bacteriuria in patients having transurethral procedures makes them more likely to develop a symptomatic inflammatory response (pyrexia with or without a positive blood culture) following the procedure [2, 70, 228-9]. According to traditional teaching, patients with preoperative bacteriuria require antibiotic treatment or prophylaxis to eradicate their bacteriuria before proceeding to transurethral surgery. However, this study failed to demonstrate an association between pre-procedure bacteriuria and intra-procedure bacteraemia. This lack of association is discussed further in Chapter 5.

4.5 Conclusions

The prospective studies evaluating the incidence of bacteraemia in patients who had TURP and catheter manipulation showed some surprising results. The traditional ‘culture’ method to detect bacteraemia outperformed the contemporary ‘molecular’ methods. Many patients having urological procedures

had bacteriuria. Bacteraemia was more prevalent during the two urological procedures than previously thought. The bacteraemia present was largely asymptomatic. Contrary to perception, the presence of bacteriuria did not influence the development of bacteraemia in this study. In the TURP group of patients, the timing of the blood samples was related to the development of bacteraemia. The presence of prostate adenocarcinoma was associated with the development of bacteraemia during a TURP. The salient point of the two clinical studies was the presence of asymptomatic bacteraemia in urological patients having procedures. The relevance of this bacteraemia is discussed further in the Chapter 5.

Chapter 5: Discussion

5.1 Introduction

This thesis addresses a number of important issues related to infections in urological practice. The largest case-control study in the published literature investigating urological procedures and infective endocarditis has been carried out. The study found a statistically significant association between urological procedures and the development of infective endocarditis. Infective endocarditis normally requires the presence of bacteraemia, though the latter is not essential in the diagnosis of IE when using the modified Duke's criteria [231]. To evaluate the association between infection and urological procedures further, two studies were designed to determine the incidence of bacteraemia during TURP and catheter manipulation. The reported incidence of sepsis following urological procedures is less than 1% [71, 104]. However, the studies in this thesis have shown that bacteraemia is more common than previously thought, but with the bacteraemia being largely asymptomatic and detectable during the procedure.

5.2 Urological procedures and infective endocarditis

A review of the literature demonstrated a number of case reports and case series linking urological instrumentation to the development of infective endocarditis. At the time of writing, the author was made aware of a case of IE developing in the immediate post-operative period for a patient undergoing a TURP. The published literature shows that the incidence of sepsis, pyrexia, UTIs and even bacteriuria can be reduced with antibiotic prophylaxis, but there is no evidence to suggest that antibiotic prophylaxis prevents or decreases the rate of infective endocarditis in patients having urological procedures, largely because this has not been studied. Traditionally, urological surgeons have been very wary of performing procedures on patients at high risk of developing infective endocarditis without the use of antibiotic prophylaxis [232]. However, this cautious approach has not been supported by a high level of evidence. For this reason, the advice published in the guidelines pertaining to urological practice (EAU, AUA and NICE guidelines) and infective endocarditis (AHA, BSAC guidelines) has recently changed [191, 200, 233-5]. None of the major

guidelines at the time of writing advocated the use of antibiotic prophylaxis for the prevention of IE post urological procedures.

It has been demonstrated that there is a statistically significant association between the development of IE and prior urological procedures in patients who developed IE subsequent to prior urological procedures. The study demonstrated that a patient who had developed enterococcal IE was eight times more likely to have had one or more urological procedures within the year preceding the diagnosis of the disease, than a patient who had developed IE caused by CoNS, *Streptococcal bovis* group or oral streptococci. Enterococci are commonly found in the human gastrointestinal tract and associated with urinary tract infections [182]. In fact, enterococci are among the top three most common organisms causing urinary tract infections, together with *Escherichia coli* and *Staphylococcus epidermidis* [182]. Because of the association between enterococci and both urinary tract infection and endocarditis and because of anecdotal experience of cases of enterococcal IE developing after urological procedures (Sandoe, *pers comm*), it was decided to study the link between urological instrumentation and enterococcal IE. It was hypothesised that a urological procedure might cause an enterococcal bacteraemia and that this may lead to the subsequent development of IE.

In the case-control study, an odds ratio of 8.21 (CI: 3.54–19.05, $p < 0.05$) for patients with preceding urological procedures to develop enterococcal IE was suggested. It is possible that the design of the study may have also contributed to the results, with an odds ratio of 8.21 when no previous study has demonstrated any statistical association. It is important to point out that the control group consisted of patients who had all developed IE caused by organisms other than enterococci rather than patients who had not developed IE. Therefore, it must be stressed that the odds ratio of 8.21 does not mean that a patient is eight times more likely to develop enterococcal IE if he or she underwent a urological procedure within one year. This would have been true for a conventional case-control study. It has been demonstrated that if a patient developed IE caused by an enterococcus, they were more likely to have had a urological procedure than were patients with IE caused by one of the comparator groups of organisms.

It is not known how long the incubation period is prior to the development of symptoms in IE, but it is known that patients with enterococcal IE may have

symptoms for many months before the diagnosis is made [236]. There is therefore a potentially long period prior to presentation. In order to encompass this uncertainty and maximise the chances of identifying the initial event in the development of IE, It was assumed that any bacteraemia that may have occurred within the year preceding the development of IE might be that initial event. This is the reason data were collected about urological procedures that had occurred within one year of the diagnosis of the IE episode, rather than over a shorter period. During that one year, the patient may have had one or more procedures affecting the urinary tract and it is impossible to determine which one of the instrumentation episodes, if any, caused the bacteraemia that eventually led to the development of the IE episode. This argument can be extended further by inferring that the reason the patient was having the instrumentation of his urinary tract in the first place was because of urological symptoms. One can speculate that a patient having urological procedures may have an underlying urological pathology, which would be the reason why the patient is having a urological procedure. This underlying urological pathology may be the cause of the bacteraemia, rather than the procedure *per se*. One may even contemplate that repeated episodes of bacteraemia from urological pathogens occur in a patient with a urological pathology, similar to a patient with gingival pathology developing repeated episodes of bacteraemia from oral organisms [237-8].

It is not known whether repeated bacteraemia episodes occur in patients with urological pathology of non-infective origin. There is evidence that bacteraemia does occur during urological procedures (see Chapter 1). Is it possible that an episode of bacteraemia during urological instrumentation is sufficient to lead to the development of IE in a patient who is not immunocompromised? One could argue that the host defences would be capable of dealing with such an insult and would not be overwhelmed, especially if the bacteraemia is temporary and of a small magnitude. It is also possible that repeated episodes of bacteraemia, before the host defences had a chance to overcome the episode of asymptomatic bacteraemia, cause repeated insults to the endocardium eventually to lead to the bacterial seeding of the heart valves and the development of IE. Elucidating these issues has proved difficult for researchers

and clinicians to date as such studies would have to be longitudinal and require massive resources. A related issue that is discussed in the next section is the potential association between prostatic adenocarcinoma and the development of bacteraemia. This reinforces the hypothesis that urological pathology, possibly prostatic adenocarcinoma, makes patients more prone to bacteraemia and the subsequent bacterial seeding of the endocardium.

Epidemiological studies have shown that the incidence of IE is increasing [239]. One of the possible reasons is that the population is growing older and hence frailer. Older patients tend to have a larger number of medical problems, including urological and cardiac pathologies [240]. Therefore, it is not unreasonable to speculate that the aging population is undergoing more and more procedures to investigate and treat their pathologies. At the same time, this ageing population would have a greater number of underlying risks factors for the development of IE. In the face of this 'at-risk' population undergoing a larger number of procedures, it is possible that this is contributing to the increasing incidence of IE. There may be alternative explanations: It is known that the ageing population have a larger number of pathologies and these pathologies may predispose them to repeated episodes of bacteraemia. For example, with respect to urological pathologies, the incidence of bladder outflow obstruction secondary to benign prostatic hyperplasia is known to increase with age [53]. Therefore, more patients are requiring procedures to treat this pathology as the population ages. It is also possible that this obstructive picture may cause repeated bacteraemia episodes in these patients, leading to bacterial seeding of the endocardium.

The studies that are reported in this thesis have also shown that enterococcal bacteriuria was present in a proportion of patients who developed enterococcal IE. In the TURP group of patient, ten patients (13.0 %) patients had enterococci in urine at presentation and just two patients (2.6%) had documented evidence of an enterococcal UTI in the twelve months prior to presentation. It is not known which condition might have occurred first: the development of bacteriuria that subsequently led to the bacteraemia and thereafter the development of IE or the development of IE, which then led to the development of bacteriuria owing to the bacterial load in blood. The studies presented herein were not

designed to determine whether asymptomatic enterococcal bacteriuria was more prevalent prior to their diagnosis, in patients who developed enterococcal IE. It was possible to study patients from whom cultures had been sent but, owing to the small number of patients with documented enterococcal bacteriuria, this seemed unlikely to be a key event in the pathogenesis. By definition, symptomatic bacteriuria, if it occurred in these patients, would not have been noticed and would therefore not be detected clinically. There is evidence that patients with a urological pathology suffer from a higher incidence of UTIs [241]. It is possible, therefore, that there is a higher incidence of bacteriuria in patients with urological pathology, thereby subjecting the patient to repeated subclinical episodes of bacteraemia. These episodes of asymptomatic bacteraemia may eventually lead to the development of IE in the group of patients with urological pathology that go on to be investigated by urological procedures.

In conclusion, this work has led to two main theories to explain the initial events which may lead to the development of IE in patients having urological procedures: 1) bacterial seeding of the endocardium occurs secondary to bacteraemia caused by urological procedures and 2) bacterial seeding of the endocardium occurs secondary to repeated episodes of (a) symptomatic bacteraemia in patients with underlying urological pathology. The next section addressed the first of these theories (urological procedures cause bacteraemia) suggested to explain the statistical association found between urological procedure and the development of enterococcal IE.

5.3 Methods to detect bacteraemia in urological practice

The traditional method to detect bacteraemia (the culture method) in patients undergoing urological procedures has been described in the literature. Newer, more contemporary technologies to detect bacteraemia have mushroomed over the last ten to fifteen years [130, 134, 242], which have shown promise. To date, these new technologies have not gained widespread adoption in diagnostic bacteriology owing to on-going technical difficulties. To date, there are no reports of the use of the new molecular technologies, such as broad-range 16S PCR examination, to detect bacteraemia in urological practice.

The study presented in this thesis is the first of its kind to use the newer molecular technologies to detect bacteraemia in the urological setting, although further work is required to optimise these techniques before they will be available for routine use.

A decision was made to evaluate the use of DNA detection methods to detect bacteraemia in urological practice, primarily to determine if it had greater sensitivity for detection of bacteraemia, particularly as many of the study patients would have received antimicrobials. Firstly, the molecular technology chosen is a relatively novel technology, which has never been used in urological practice before. Secondly, this molecular technology had the potential to detect and quantify bacteraemia, even when the bacterial load in the blood sample was low, as well as the detection of non-cultivable bacteria (such as those affected by antimicrobials). Thirdly, molecular methods offer the promise of relatively quicker result reporting than the traditional culture method. In the context of this study, the rapidity of the process was not crucial. However, in clinical practice, the sooner bacteraemia can be detected, the quicker the patient can be started on the optimal treatment. This will, however, involve further challenges since there is currently no easy way of predicting antimicrobial susceptibilities of pathogens identified using DNA detection technology. A decision was made to explore the merits of the molecular technologies in providing a quick answer to patients having low-level bacteraemia. Fourthly, the molecular methods employed to detect bacteraemia utilised a smaller volume of blood than the traditional culture method and therefore, allowed both the traditional culture method and a molecular method to be performed in parallel. And finally, the molecular methods offered the possibility of detecting bacterial DNA irrespective of whether the bacteria were alive or dead. Therefore, it detected the presence of bacteria by using bacterial DNA as a surrogate marker for the presence of bacteria, in spite of the use of antimicrobial prophylaxis, which may have killed the bacteria. This was particularly relevant in the TURP group of patients, where all patients at the Leeds Teaching Hospitals NHS Trust received gentamicin as antibiotic prophylaxis. Developmental work in the laboratory confirmed that the molecular technology was able to detect low-level bacteraemia (the Plex-ID was able to

detect and identify bacterial suspensions with concentration of 10 cfu/ml), albeit with an uncertain reliability, and provided results quicker than the culture method. Experiments using different volumes of blood using the molecular technology were undertaken to see if use of larger volumes of blood would improve the sensitivity. The blood culture method used 10 mL blood, whereas methods of detection of microbial DNA usually use much smaller volumes (1-5 mL); a fairer comparison of the molecular and culture methods was considered desirable. However, the molecular method did not perform as well as the simpler culture methodology during the *in vivo* trial samples. The reasons for this are discussed below.

5.3.1 Bacterial DNA extraction

In routine clinical practice in the UK, it is uncommon to use diagnostic tests that rely on the extraction of bacterial DNA from blood. The molecular technology has, to date, been largely devoted to the extraction of bacterial DNA from 'cultured' samples, with the diagnostic pathway of meningitis and mycobacterial infections being exceptions [242]. For example, bacterial DNA has been extracted from blood or human samples that had been incubated (in order to increase the bacterial yield) [214]. The work in this part of the thesis focussed on the extraction of bacterial DNA from blood that had not been previously cultured. Therefore, the expectation was that the amount of bacterial DNA would be significantly less than in previously incubated blood. Developmental work began using 1 mL of 'spiked' blood. Although it was easier to handle this small volume of blood in the molecular laboratory, to make a fair comparison with the blood culture method and maximise sensitivity by using larger volumes of blood was considered desirable. Utilising a larger volume of blood for bacterial DNA extraction allowed the molecular method to become more comparable to the gold standard culture method, which utilised larger volumes of blood. The DNA extraction process starts with a relatively large sample volume and it ends with a relatively small sample. This concentration process consisted of multiple steps and each of the steps has the potential for loss of bacterial DNA. Therefore, it was possible for no bacterial DNA to be left in the extract after the extraction process because the entire sampled DNA had been lost during the process. By using a larger volume of blood, it was hypothesised

that we would be working with a larger amount of target bacterial DNA. Although some bacterial DNA was presumably lost during the extraction process, the extract (which was of exactly the same volume as previously obtained when using the smaller starting volume of blood for processing) contained enough bacterial DNA for further processing (amplification and detection). In the final protocol, 5 mL of blood for the molecular processing was examined compared with 7-8 mL used for the culture bottles.

One of the major issues when extracting bacterial DNA from a larger volume of blood is the increasing presence of "background" DNA, especially the human DNA present in blood cells. During the extraction process, the main aim was to get rid of the maximum amount of background (human) DNA whilst preserving the maximum amount of bacterial DNA. Studies have shown that Molzym's MolYsis offered a consistent technology to this effect [141, 243]. However, because larger volumes of blood (5 mL) were being used, the amount of human DNA was significantly larger than reported in the previous studies (which had used small volume samples from the oral cavity). At the time of the study, using large volumes of blood (> 1 mL) for bacterial DNA extraction was uncommon, even in the research setting. The *in vitro* studies (described in Chapter 3), showed that it was possible to extract and detect tiny amounts of bacterial DNA reliably (as little as < 100 copies/mL) from large volumes (5 mL) of blood, without significant noise to signal issues. This means that the process was able to detect bacterial DNA without much interference from the background DNA. Therefore, a standard operating procedure (SOP) was developed to extract bacterial DNA reliably from 5 mL of blood, which was later used during the clinical trial on patients undergoing TURP operations and catheter manipulation, although it still does not currently out-perform standard culture and more work is required before it can be used routinely.

5.3.2 Bacterial DNA amplification and identification

During the developmental work, it was found that the process of broad range amplification of sequences encoding bacterial 16S rRNA from blood was complicated by the cross-reactivity of the primer with background human DNA. This led to ambiguous chromatograms on sequence analysis. This made

sequence interpretation hard and unreliable. Furthermore, in SYBR green real-time PCR assays, human DNA can also affect crossing threshold (C_t) values and consequently affect the sensitivity of the assay [244]. In order to avoid the issues relating to cross-reactivity, a number of primers that bind to and amplify sequences encoding bacterial rRNA were considered, including long and short 16S primers. Bacteria-specific, genus specific, Gram-positive/negative primers were also evaluated as described in Chapter 3. It was concluded that the in-house 16S primer would provide optimal results. Firstly, restricting the target to one bacterium or one genus of bacteria was not considered desirable, as it was not possible to predict the identity of the pathogen causing bacteraemia that would be encountered during urological procedures. In fact, this standpoint was validated as bacteraemia caused by *Actinobaculum massiliense*, a bacterium not routinely detected from clinical urological samples was identified using a 16S primer pair (forward primer: AGAGTTTGATCCTGGCTCAG, reverse primer CTACGCATTTCACCGCTACAC). UTIs caused by *Actinobaculum massiliense* have been reported but this study is the first to identify bacteraemia caused by this organism in the urological setting [245]. The bacterial species was detected in the culture arm of the study in a patient undergoing TURP. Secondly, by using the in-house 16S primer pair (forward primer AACTGGAGGAAGGTGGGGAT reverse primer AGGAGGTGATCCAACCGCA) [246], which is a short primer pair (producing an amplimer of less than 300 base pairs), it was hoped to detect as many bacteria as possible, while minimising cross-reactivity and the pick-up of “background” human DNA.

Primer length and the target region were important considerations when deciding which primer would be the most suitable for this study. The in-house 16S primer pair provided the most consistent results during the *in vitro* experiments for the amplification of sequences encoding bacterial 16S rRNA, without sacrificing the sensitivity of the assay (Chapter 3). Optimising the PCR conditions allowed the assay to perform to maximum sensitivity without the problem of background human DNA. All assays were amplified over forty cycles, and the negative blood control was assigned a fixed C_t value of 30. These strict criteria to assess all the samples ensured that only a minimum

number of samples had to be cleaned up for sequence determination reactions, therefore increasing the specificity of the test. This strict regimen was followed in an attempt to minimise the cost of processing a large number of samples, with only a few positive results. In fact, during the developmental process, the molecular methods detected bacterial level as low as 10 cfu/mL. However, this was not replicated in the blood samples from the clinical trial. It was speculated that the reason for this disparity is that the level of bacteraemia in the blood samples from the two studies was very low in both groups of patients. The 5 mL of blood used for the molecular methodology was insufficient to account for the bacterial DNA loss that invariably occurred during the extraction process. The *in vitro* experiments were carried within 1-2 hours after the seeding of blood. The samples from the studies were processed within 24 hours of collection, with most samples processed within six hours. This delay in processing between the *in vitro* and *in vivo* samples may explain the inability of the same methodology to detect low levels of bacteraemia owing to the phenomenon bacterial DNA degradation and background DNA increase, which have been described previously [247]. Furthermore, it is likely that the bacteraemia in the clinical samples was not constant but intermittent and at very low-levels. It was likely the blood sampling managed to capture only one or two of the organisms, if any. However, it is possible that the patient still had bacteraemia, which was not captured during the sampling process owing to the small number of bacteria involved.

Whilst the culture method, which was optimised for maximum sensitivity (an extended incubation period), was able to amplify this small number of bacteria during the incubation process, the molecular method instead failed to do so as it progressively lost the low number of bacterial DNA during the extraction process. This may explain the discrepancy between the two methods. The culture method was not designed to quantify the bacteria present during episodes of bacteraemia; it was designed with sensitivity as the primary concern. As the bacteraemia was not detected by the molecular methods, it was impossible to quantify the bacteraemia encountered during the study for TURP and catheter manipulation patients. This is a failure of the molecular methodology as it is not entirely efficient in the detection of bacteria. The results

from the *in vitro* experiments were not reproduced *in vivo* during the trial. It is also possible that background DNA, mainly human DNA masked the presence of bacterial DNA. When the results from the 16S PCR amplifications were processed, eighteen samples were believed to be positive based on C_t values and the shape and temperature of the melting curve. However, bacterial sequences were identified in only three of these eighteen samples. The fifteen other samples were considered negative as no bacterial DNA could be identified. The reason for this could be two-fold: either no bacterial DNA was present at all, or the bacterial DNA present was masked by the presence of human DNA and could not be detected.

5.3.3 Bacteraemia and urology

Multiple methods were used to evaluate the presence of bacteraemia in patients having urological procedures. The two studies conducted revealed surprising results. The most important result was the fact that bacteraemia occurred in 30% of patient having a TURP, in spite of antimicrobial prophylaxis. This level of asymptomatic bacteraemia has never been reported before. The significance of this bacteraemia can only be speculated upon at this stage.

5.3.3.1 TURP: Risk factors, bacteraemia and symptoms

There are a number of studies that have investigated the incidence of infection during TURP. There is evidence that bacteriuria, UTIs, sepsis and symptomatic bacteraemia can occur during the procedures, with varying incidence and significance, which is discussed at length in Chapter 1. Guidelines therefore recommend the use of antimicrobial prophylaxis to prevent these infective episodes [233, 235]. However, the evidence to support this practice is poor; there are some randomised studies to show that antimicrobial prophylaxis prevents the development of infective episodes, pyrexia and bacteriuria (Chapter 1). However, there is no evidence that antibiotics prophylaxis can prevent the development of infective endocarditis following urological procedures. In fact, there is no conclusive published evidence to link the development of infective endocarditis to previous urological procedures. Therefore, all the major guidelines are in agreement in not recommending

antimicrobial prophylaxis in patients having urological procedures for the prevention of infective endocarditis.

Procedure	Anecdotally associated with endocarditis	% Bacteraemia	Requires IE prophylaxis	Comment
Cystoscopy	NK	0-26	Yes	Standard perioperative prophylaxis may need modification Risk of bacteraemia increases with presence of bacteriuria. If possible, treat bacteriuria before the procedure
Urethral catheterization	Yes	0-17	No [a]	
Urethral dilation	Yes	18-33	Yes	Standard perioperative prophylaxis may need modification
Transurethral prostatic resection	Yes	70-76	Yes	Standard perioperative prophylaxis may need modification
Transurethral prostatic biopsy	Yes	12-46	Yes	Standard perioperative prophylaxis may need modification
Vasectomy	Yes	NK	No	Cases developing after vasectomy have been reported without known cardiac defects
Lithotripsy of renal stones	Yes	8	No [a]	
Circumcision	Yes	NK	No	
Cosmetic piercing involving urethral mucosa	No	NK	No	

[a] Risk of bacteraemia increases with presence of bacteriuria. Treatment is recommended pre-procedure
NK, Not Known

Table 92. Bacteraemia and infective endocarditis in urology (adapted from Gould *et al*. [194])

Table 92 shows the recommendations for antibiotic prophylaxis based on the anecdotally reported risks of infective endocarditis from various urology procedures. The incidence of bacteraemia in TURP patients from this table is 70-76%, even higher than transrectal biopsy of the prostate. This high figure might seem counter-intuitive as the urinary tract is perceived to be a sterile while transrectal biopsy of the prostate is a ‘dirty’ procedure. It may be a reflection of the pathogenesis of the urological pathology that leads to the

TURP, whereby patients have underlying bacteriuria, causing bacteraemia. In fact, the available evidence shows that pre-procedure bacteriuria is an independent factor for the development of post-procedure infective episodes. However, the study presented in this thesis did not show an association between bacteriuria and bacteraemia. It is possible that asymptomatic bacteraemia occurs irrespective of the presence or absence of bacteriuria. It is speculated that the source of the bacteria leading to the bacteraemia is not the urine but the prostate gland instead. This concept is discussed further in the next section.

In this study, a third of patients developed bacteraemia during the procedure. Only two patients showed symptoms of sepsis in the immediate post-operative period, but no intra-operative bacteraemia was detected in either patient. These two patients required an extended course of antimicrobial therapy following TURP. However, the thirteen patients with intraoperative bacteraemia were asymptomatic perioperatively. It was not possible to determine the amplitude of bacteraemia (see next paragraph), but it was possible to determine the duration of the bacteraemia. Bacteraemia occurred after the insertion of the instrument used for the procedure. The bacteraemia did not last for the duration of the procedure. Patients were more likely to develop bacteraemia between ten to twenty minutes after the start of the procedure. Thereafter, the risk of bacteraemia fell to pre-procedure levels. Presumably, the bacteraemia was ‘mopped up’ by the host defences once the presence of bacteria in the bloodstream has been detected; this would explain why most of the patients did not have symptoms of sepsis perioperatively. It has been demonstrated that it is possible to have asymptomatic episodes of bacteraemia during urological procedures. It is possible that spontaneous bacteraemia may occur in patients in the community, who have an underlying urological pathology and develop intermittent, transient and asymptomatic bacteraemia, but confirming this would require a separate study. Interestingly, the removal of a ‘colonised’ urethral catheter and the potential trauma it may have caused to the urothelium was not associated with the development of bacteraemia. This reinforces the hypothesis that the source of the bacteria was other than bacteriuria and colonised foreign material.

The methodology that was used for this study did not, in the end, allow an accurate quantification of the bacteraemia. The 'culture' methodology was a qualitative method but it was hoped that the molecular methodology would give an idea of the amplitude of the bacteraemia by using surrogate parameters like C_t values from the 16S PCR and 'quantity' value from the Plex-ID. Unfortunately, the molecular methods failed to detect the bacteraemias that were identified by the culture method and quantification was therefore not possible. The reasons for this are discussed in Section 5.2

5.3.3.1.1 Prostate adenocarcinoma and bacteraemia

A noteworthy finding from the studies we carried out was a statistically significant association between the presence of prostate adenocarcinoma and the development of bacteraemia. Why do patients who have carcinoma in the prostate at the time of TURP more likely to develop bacteraemia during the procedure? It was felt that bacteria may be present in the prostate gland of these patients and these bacteria were introduced into the bloodstream during the procedure. If this was the case, bacteria may have caused colonisation in the abnormal gland (intermittently or constantly for an undetermined period). If longstanding infection had been present in the prostate gland of these patients, there is a possibility that this might contribute to prostatic tumourgenesis, leading to prostatic adenocarcinoma. As mentioned in Chapter 1, previous literature has shown that almost half of the prostate tissue obtained at TURP and cultured demonstrated evidence of bacteria [58, 62]. The study in this thesis has suggested a link between prostatic adenocarcinoma and intra-operative bacteraemia that warrants further investigation. There is a study that alludes to a bacterial aetiology of prostatic adenocarcinoma [248]. However, an association between prostatic inflammation and the presence of prostatic adenocarcinoma was demonstrated on univariate analysis. Inflammation, prostatic adenocarcinoma and bacteraemia could not be incorporated in a multivariate analysis owing to the possible bi-directional relationships between the variables. It may be that the inflammation plays a role in the development of the adenocarcinoma. It is also possible that the inflammation is the result of adenocarcinoma in the prostate. However, without microbiological samples of the prostate only speculation about the association

between prostatic adenocarcinoma, inflammation and bacteraemia is possible. These issues should be explored in further studies to shed more light on the relationship between bacteraemia, prostatic inflammation and prostate adenocarcinoma, because an infective aetiology would potentially enable earlier diagnosis and preventative strategies.

5.3.3.2 Catheter manipulation: risk factors, bacteraemia and symptoms

The catheter manipulation group involved patients, both male and female, having a urinary catheter changed or removed. This group of patients was heterogeneous. A urinary catheter (urethral or suprapubic) offers easy access for bacteria into the bladder. A urethral catheter is a recognised risk factor for UTIs. It is thought that a urethral catheter carries a greater risk of infection than a suprapubic catheter as it lies in the perineal area, which has a larger concentration of bacterial commensals compared with the anterior abdominal wall skin [249]. However, a recent review of the literature showed that the incidence of urinary tract infections was similar in both urethrally and suprapubically catheterised patients [250]. Male and female patients have different lengths of urethra. The shorter urethra in females offers relatively little barrier to bacterial migration from the outside body (mainly from the vagina and rectum) to the bladder. Therefore, a urethral catheter in a woman is a 'better' portal of access for bacteria into the bladder than a urethral catheter in a man. There is also the argument that the prostate surrounds the male urethra at its junction with the bladder and may act as a defence mechanism against infection (physically acting as a barrier to the bladder but also via its zinc-enriched secretions). However, it is hypothesised that the prostate is not a sterile organ. Bacteria may be present on or in the prostate gland commensally, either as colonies or as biofilms, without acute injury to the human host. This notion has been reinforced by studies that have shown that a significant proportion of TURP chips grew bacteria when cultured [58, 67]. Therefore, male catheterisation may carry a higher infective burden that previously suspected. However, the microbiological methods used in this study did not allow this issue to be addressed sufficiently. Further studies looking specifically at the microbiology of the prostate are required.

Bacteraemia can happen during catheter manipulation, however minor the procedure is as demonstrated in this study. No statistical association between catheter manipulation and the development of bacteraemia was identified. In fact, it is possible to speculate that the mere presence of a urethral catheter may predispose a patient to bacteraemia, as demonstrated in Patient C002, who had a bacteraemia from *Staphylococcus epidermidis* prior to any catheter manipulation and this bacteraemia persisted after the catheter change. Though this bacteraemia may have been a contaminant, it is unlikely that the same bacteria would grow from four separate blood samples if it were the case. However, this finding needs to be demonstrated in further studies to validate and strengthen this argument. It may not be unrealistic to envisage that many patients with indwelling urinary catheters have intermittent, asymptomatic bacteraemia. This thesis may have captured a small snapshot in time of the real picture with respect to bacteraemia and the presence of a urinary catheter. There is good evidence that the presence of a urinary catheter predisposes a patient to bacteriuria [112, 251-2] and it may be that this constant bacteriuria leads to repeated, transient episodes of bacteraemia. This postulated mechanism is akin to a patient with a urological pathology. The underlying urological pathology may predispose the patient to unrecorded episodes of bacteriuria, which in turn predisposes the patient to episodes of asymptomatic bacteraemia. The telephone interview following the procedure did not reveal any significant septic episodes, confirming that patients tend to be asymptomatic, even if they have episodes of bacteraemia.

5.4 Asymptomatic bacteraemia: the unifying theory for IE and urology?

The bacteriuria associated with urinary catheterisation, particularly urethral catheterisation, is of unknown significance. It is commonly accepted that this asymptomatic bacteriuria does not have any detrimental effect on the patient and should not be treated. The study of catheter manipulation undertaken for this thesis found one patient with a *Staphylococcus epidermidis* bacteraemia, which was detected before any catheter manipulation and persisted following catheter change. The urine sample taken from this patient confirmed *Staphylococcus epidermidis* bacteriuria. This patient was completely

asymptomatic. Therefore, it is possible that this patient has been having multiple, repeated episodes of asymptomatic bacteraemia during the duration of urinary catheterisation. As this patient has had a catheter for over a year, it is possible that the occurrence of this bacteraemia has been repeatedly happening over a prolonged period. A similar phenomenon has been described in the dental literature, evaluating patients with periodontal disease and tooth brushing [237-8, 253]. A meta-analysis showed that the presence of bacteraemia was associated with tooth brushing in patients with plaque (OR: 2.6, CI: 1.4-4.7) [253]. The significance of such bacteraemias is not known.

A high incidence of bacteriuria post TURP is well documented and the duration of this bacteriuria can last for months (Section 1.2.3.3.1). Therefore, it is plausible that these patients may be having repeated episodes of asymptomatic bacteraemia. This bacteraemia may have long-term sequelae. For example, having repeated bacteraemias may eventually lead to cardiac valvular seeding and the onset of IE. It has also been shown that there is a statistical association between urological procedures and IE [254]. Patients with underlying urological pathology may also have recurrent asymptomatic bacteraemias in addition to those associated with urological procedures and devices. Therefore, it is possible that patients in the community, with underlying urological pathologies, are having repeated asymptomatic bacteraemias of unknown duration. Repeated episodes of bacteraemia may have far-reaching consequences that can only be speculated on. This may explain the association between urological instrumentation and the development of enterococcal IE. It is also possible that other infective episodes that stem from repeated bacteraemias may develop in this group of patients. For example, patients with prostheses (metal joint replacements, vascular grafts, *etc*.) may have delayed seeding of these prostheses from the repeated asymptomatic bacteraemias.

This argument goes against the popular view that the vascular system is a sterile environment and the presence of bacteria is, by definition, pathological. Any bacteria gaining access to the vascular system is dealt with by the immune system and other host defences, rendering blood a sterile environment. It is not known how quickly bacteraemia is dealt with once bacteria gain access to the

vascular system. We assume that a host response is mounted for every bacterium that gains access to the vascular system, so that every foreign body is 'mopped up' to maintain the sterility of the vascular system. However, it may be possible that if an immune response is not raised, the patient does not show the stigmata of a bloodstream infection, for example, pyrexia. This may involve a scenario whereby asymptomatic bacteraemia prevail. This may be a 'learned' phenomenon whereby the immune system detects that this low-level bacteraemia does not respond as if this poses an imminent, major threat to the body and thus allows its presence without invoking all the energy-dependent processes to mount a full response. Although the body is safe from any immediate danger, this 'under-the-radar' bacteraemia may pose a threat in the long term. For example, the heart valves may be seeded, especially in 'at risk' patients, leading to the development of infective endocarditis.

There is a growing consensus that bacteraemia can result from mundane activities of daily living [194]. For example, tooth brushing and chewing can cause bacteraemias. There is a school of thought that believes that cumulative bacteraemias is more significant in the pathogenesis of infective endocarditis than one single episode of bacteraemia that occurs during a procedure. This reinforces the hypothesis that patients who are undergoing urological procedures may have underlying urological pathologies that may predispose them to repeated bacteraemias over time.

5.5 Conclusions

This present study confirms the presence of asymptomatic bacteraemia in patients having urological procedures, at a rate higher than previously thought. A link between urological procedures and IE has been identified. It has been postulated that the presence of asymptomatic bacteraemia in patients with urological pathologies offers a possible explanation for the link between IE and urological patients. This concept of asymptomatic bacteraemia, though referenced in the literature, has not been described outside the dental literature. It is thus postulated that asymptomatic bacteraemia exists in patients with urological pathologies.

Chapter 6: Conclusion

6.1 Introduction

An aim of the work reported in this thesis was to evaluate whether there was an association between having a urological procedure and the subsequent development of infective endocarditis. A novel design for a case-control study was utilised to perform the study. The second part of the project focussed on bacteraemia during urological procedures. It is known that some urological procedures cause bacteraemia, which may, in turn, explain the connection between urological procedures and infective endocarditis. A second aim was to evaluate different methods to detect and possibly to quantify bacteraemia. Thereafter, the optimised methods used for the detection of bacteraemia were used to evaluate the presence of bacteraemia in patients undergoing TURP and catheter manipulation.

6.2 Main findings

The main findings of this thesis are briefly described below. The two main questions were:

- are urological procedures associated with the development of infective endocarditis?
- do urological procedures (TURP and catheter manipulation) lead to bacteraemia? If so, what is the cause, the amplitude and duration of the bacteraemia?

6.2.1 Hypothesis: there is no association between urological procedures and the subsequent development of infective endocarditis

The case-control study is the first study of its kind to show a statistical association between the development of enterococcal infective endocarditis and prior urological procedures. The fundamental assumption of the study is that members of the normal human microbiota occupy specific ecological niches in

the human body. The literature supports the assumption that enterococci are the most likely organisms to be introduced into the urinary tract during urological procedures. The high odds ratio and low *p* value reinforced the strong association between the urological procedures and enterococcal IE. Therefore, the first hypothesis was not supported. The association between prior urological instrumentation and the development of enterococcal IE does not necessarily mean that there is a causal association between the two episodes. However, it is not unreasonable to assume that there might be a link between the two instances and we speculate about the possible reasons for this association in Chapter 2.

6.2.2 Hypothesis: molecular methods are better than the traditional culture method to detect and quantify bacteraemia in urological patients

The initial experiments carried out in the laboratory showed that the molecular methods were able to detect low levels of bacteraemia. The 16S PCR method reliably detected and quantified bacteraemia to a level of 10^3 cfu/mL. The Plex-ID performed even better detecting and giving a rough estimate of the amplitude of bacteraemia; bacteraemia of < 10 cfu/mL were detected. Based on these results, it was decided to use the molecular as well as the culture methods to identify bacteraemia during the clinical studies. However, the molecular methods performed poorly during the clinical studies. This may be because the levels of bacteraemia were so low that the methods were unable to detect it. The culture method was optimised for maximum sensitivity explaining the reason why it performed better than the molecular methods. Therefore, the hypothesis that molecular methods to detect bacteraemia outperform traditional culture methods was not supported.

6.2.3 Hypothesis: the incidence of bacteraemia is high during urological instrumentation and it lasts for the duration of the procedure

Two of the most common procedures performed in a urology department were investigated: transurethral resection of the prostate (TURP) and urological catheter manipulation. Fifty patients in the TURP group and forty-nine patients in the catheter manipulation group were recruited to the study.

6.2.3.1 Transurethral resection of the Prostate (TURP)

Fifty patients were recruited in this group and an incidence of bacteraemia of 28% was noted in spite of the use of standard antibiotic prophylaxis. This bacteraemia was largely asymptomatic. The bacteria detected were common urological pathogens. The bacteraemia started after the start of the procedure and was transient and cleared before the end of the procedure. The methodological methods we used did not allow determination of the scale of the bacteraemia. This was probably because the levels of bacteraemia were too low to allow the molecular methods to quantify the bacteria present during bacteraemia. A high incidence of transient, asymptomatic bacteraemia in patients undergoing TURP was demonstrated, in spite of the use of antibiotic prophylaxis; this has not been previously reported in the literature.

6.2.3.2 Catheter Manipulation

Forty-nine patients in this group were recruited and the incidence of bacteraemia was 16.3%. This shows that a simple procedure like catheter manipulation, which lasts less than a few minutes, can still be associated with a transient, asymptomatic bacteraemia. The organisms cultured (the molecular methods did not detect any bacteraemia) were bacteria likely to cause urinary infections. It is speculated that it is possible for patients to have long-term asymptomatic, bacteraemia irrespective of catheter manipulation. The mere presence of a urinary catheter may be the cause of the repeated, asymptomatic bacteraemia.

6.3 Contributions and novelty of the study

This study has highlighted the fact that bacteraemia may be more prevalent in patients undergoing urological procedures than previously thought. The most important finding is that almost a third of patients undergoing TURP develop bacteraemia in spite of the current recommendations of antibiotic prophylaxis. This study highlights the fact that current antibiotic prophylaxis is inadequate in preventing episodes of asymptomatic bacteraemias during TURP, though it did prevent symptomatic infective episodes. The possibility of recurrent, transient, asymptomatic bacteraemia as a concept in patients with urological pathology

has also been discussed. This may explain the second finding, which has never been reported previously, that there is a statistical association between urological procedures and the development of enterococcal infective endocarditis. Although the literature is interspersed with case reports and case series about the association of urological procedures with the development of infective endocarditis, a statistical association has never been shown to exist. This study, by virtue of its novel design as a case-control study, has finally provided some evidence of a possible link between urological instrumentation and infective endocarditis.

6.4 The future

There are still many unanswered questions regarding infection and urology. Is there really an association between urological procedures and the development of IE? Does one cause the other? Other researchers should investigate the association between infective endocarditis and urological procedures. The findings of our study should be confirmed or disproved by studying different patient populations, in different settings. A national database of patients with endocarditis has recently been setup with a number of centres in the United Kingdom collaborating and is currently being populated. This database will hopefully provide more evidence on the interaction between iatrogenic procedures and the subsequent development of infective endocarditis.

It is also important to investigate the incidence of bacteraemia in different urological procedures, including upper urinary tract instrumentation. It may be possible that previous series have underestimated the incidence of bacteraemia, particularly asymptomatic bacteraemia. It is also possible that current antibiotic prophylaxis regimes are not adequate to the prevention of symptomatic bacteraemia (though they prevent sepsis) and head-to-head trials between newer antibiotic regimes are required. Bigger series from different centres will shed more light on the incidence of asymptomatic bacteraemia during urological procedures. Furthermore, newer methodologies to detect bacteraemia should be used to provide contemporary figures about the incidence of bacteraemia. Most studies relating to urology and bacteraemia have been done decades ago, using technology available at the time.

The other important issue that needs resolving is the presence of asymptomatic bacteraemia in patients with urological pathologies, irrespective of instrumentation. Does asymptomatic bacteraemia occur more regularly than traditional teaching has led us to believe? It may be possible that the prostate gland is not a bacteria-free organ. Although there are a few studies looking at culture of prostate chips and tissue from prostate biopsies, newer methodologies like PCR and electron microscopy may help shed light on whether bacteria reside in or on the prostate, and whether they reside as colonies or biofilms that are hard to culture. A non-urological question that needs to be answered is the relevance of asymptomatic bacteraemia. What are the implications of recurrent, asymptomatic bacteraemia?

At present, it is not possible to answer all these questions adequately and it is incumbent on future researchers to resolve these issues.

6.5 Conclusions

This study has shown that patients who undergo urological procedures are more likely to develop enterococcal infective endocarditis than any other kind of infective endocarditis. The reasons for this can only be speculated upon and revolve around the actual procedure causing the bacteraemia or the underlying urological disease process causing the bacteraemia. This study has also demonstrated that bacteraemia is more prevalent during TURP operations and urinary catheter manipulation than previously thought. The bacteraemia is largely asymptomatic but may have long-standing implications like the development of infective processes such as infective endocarditis.

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Appendix 1: Patient Information Sheet

REC Number 10/H137/5, R&D number UR09/9173, ISRCTN89902973

Infection after urological procedures: is there a risk to the heart?

PART 1

The information in Part 1 should allow you to decide whether the study is of interest to you. If you are interested, please read Part 2 and discuss with your study doctor.

Invitation to take part in this research study

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

- Part 1 tells you the purpose of this study and what will happen to you if you take part
- Part 2 gives you more detailed information about the conduct of the study.

If there is anything that is not clear to you or you would like more information, ask your study doctor and his team. Take time to decide whether or not you wish to take part.

1. **What is the purpose of the study?**

The purpose of the study is to determine whether having urological procedures -Transurethral Resection of the Prostate (TURP) and Urinary Catheter Change - allows bacteria to enter the blood stream. If so, to what extent, and whether this has any bearing on developing infective endocarditis (infection of the inner lining of the heart) later on in life.

Infection after surgery occurs in 1-3% of cases. Recognising this early and taking the necessary precautions minimise the infective risk caused by having

surgery. The information collected from this study will give us a clearer picture about bacteria in the blood stream during the three above urological procedures. It will allow us to formulate a more scientific approach to managing infection during the operative period and hence minimise further infective complications further down the line.

2. **Why have I been invited to take part?**

You are being asked to take part in this study as you are undergoing one of the following three procedures: Transurethral Resection of the Prostate (TURP) and Urinary Catheter Change at the Leeds Teaching Hospitals NHS Trust.

We aim to recruit between 50 patients for each of the above procedures.

3. **Do I have to take part?**

No. It is up to you to decide whether to take part or not. If you decide to take part, you will be given this information sheet to keep and asked to sign a consent form. You are still free to withdraw from the study at any time without giving any reason. If you decide not to take part in the study or withdraw at any time, this will not affect the standard of care you receive.

4. **What will happen to me if I take part?**

After you have signed the consent form, your study doctor will give you contact details of medical staff to contact if you have any questions about the study or any problems you want to ask them about.

If you agree to take part in the study, you will be involved in the study for a total of 1 year. You will be in contact with the Research Team on 2 separate occasions.

<u>Occasion 1</u>:

This is the day you come in the hospital for the procedure planned for you by your Urological Surgeon.

Before any study-related procedures are carried out, you will be asked to read and sign the consent form. If you wish to participate, you will be asked a set of questions to determine your suitability.

If you are a suitable candidate, a urine sample will be collected from you before the start of your planned procedure. Then, a cannula will be inserted in your arm, which will be connected to a drip. At specific time points before, during and after your planned procedure, blood will be withdrawn from the cannula and transferred to blood bottles for the purposes of the study. The total volume of blood we will need for the study is 160 ml (6oz). This corresponds to around 30 'spoonful' of blood. The cannula will be removed once we have gathered all the blood for the study.

Occasion 2:

Within one year after Occasion 1, a member of the Research Team will give you a telephone call and mail you (a self-addressed envelope) to ask you a few questions about any episodes of infection (especially of the heart) you suffered in the last year requiring hospital admission. The telephone call is expected to last 5 minutes.

5. **Expenses and Payments**

There will be no remuneration for participating in this study. Providing us with your blood and urine for Occasion 1 is on a completely voluntary basis for the purpose of the study. The follow-up telephone call is completely voluntary as well.

6. **What do I have to do?**

The study does not preclude you from doing your normal daily activities at any point in time.

7. **What are the possible disadvantages and risks of taking part?**

We do not anticipate any major disadvantage or risk of taking part in this study. For some patients, there may be slight bruising in the arm at the site where the cannula went in.

8. **What are the possible benefits of taking part in this study?**

There will be no benefits for the participants of the study. However, the information obtained from the study will help patients undergoing the following procedures (Transurethral Resection of the Prostate (TURP) and Urinary Catheter Change) in the future.

9. **What happens when the research study stops?**

If you want to know the results of the study, you will be able to contact the Research Team to have a copy of any results published in the medical literature.

10. **What if there is a problem?**

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be taken very seriously. The detailed information on this is given in Part 2.

11. **Will my taking part in the study be kept confidential?**

Yes. We will follow ethical and legal practice and all the information about you will be handled in confidence. The details are included in Part 2.

THIS COMPLETES PART 1

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

PART 2

The additional information in Part 2 should be read and understood before you decide whether to take part in the study and give informed consent.

12. **What will happen if I don't want to carry on with the study?**

If you decide that you don't want to carry on with the study, then you can withdraw at any time, without any prejudice to your treatment.

13. **What if there is a problem?**

If you are worried about your health during the study, please contact your study doctor. Should you need to consult another doctor during the study, please inform him/her that you are taking part in the research study. You may even show this information leaflet for more clarification.

If you have a concern about any aspect of this study, you should speak to your Study Doctor who will do his best to answer your questions. If you remain unhappy and wish to complain formally, you can do this by following

the complaints procedure in place at the Leeds Teaching Hospitals NHS Trust.

The Leeds Teaching Hospitals NHS Trust provides compensation and indemnity for this study under the NHS Indemnity Arrangement for clinical negligence claims in the NHS, issued under cover of HSG96/48. Your right in law to claim compensation for injury where you can prove negligence is not affected. Copies of these guidelines are available on request from your Study Doctor.

14. **Will my taking part in this study be kept confidential?**

All information concerning your participation in this study will be kept confidential. If you consent to take part in the research, any of your medical records may be inspected by the Research Team for the purpose of analysing the results. They may also be looked at by the people from regulatory authorities to check that the study is being carried out correctly. Your name, however, will not be disclosed. In the event that the results of this study are published, confidentiality will be maintained. During the study, your study number only will identify you. In signing the consent form, you are authorising your Study Doctor to disclose details of your relevant medical history in strict confidence. In signing this form, you are agreeing to co-operate with your doctor and for your General Practitioner (GP) to be informed that you are taking part in this study.

15. **What will happen to any samples I give?**

The blood and urine samples you give will be identified by the study number only. Your name will not be given outside of the Leeds Teaching Hospitals NHS Trust. Once the analysis of the blood and urine samples is complete, they will be destroyed. No long-term storage of samples is planned for this study. No human genetic testing will be performed.

16. **What will happen to the results of the research study?**

Results of the study will be written up in a report and may be published in the medical literature and/or presented at medical conferences. You will not be identified by name in any study results. When available, a summary of the results of the study may be obtained from a member of the Research Team.

17. **Who is organising and funding the research?**

This study has been conceived by Dr Amar Mohee, the Chief Investigator and his supervisors, Mr Ian Eardley, Consultant Urological Surgeon and Dr Jonathan Sandoe, Consultant Microbiologist. This study is being done in conjunction with the University of Leeds. The study is being funded by the Pyrah Department of Urology at the St James University Hospital NHS Trust in Leeds. The study is registered with the University of Leeds and the chief investigator will obtain a higher degree (MD) at the submission of all the data to the university.

You will not receive payment for taking part in this study.

18. **Who has reviewed the study?**

Before the study started, it was reviewed and approved by an independent Ethics Committee: the Leeds (West) Research Ethics Committee (REC). The study has also been reviewed by the Research and Development Department at the Leeds Teaching Hospitals NHS Trust.

19. **Further information and contact details**

If you have any questions about the study or your health at any time during the study, please contact one of the named people below. You should also contact one of these people in an emergency. They will answer your questions or give you advice.

Doctor: Dr Amar Mohee	Phone Number: 0113 206 6994
Nurse: Ms Lorraine Lamb	Phone Number: 0113 206 4883

Appendix 2: Consent Form

REC Number 10/H137/5, R&D number UR09/9173, ISRCTN89902973

Patient Identification Number: .....

CONSENT FORM

Full Title: A One-Year, Prospective, Observational, Cohort, Single-Centre Study on the Incidence, Intensity, Duration and Identity of Bacteraemia in Patients undergoing Transurethral Resection of the Prostate (TURP) and Urinary Catheter Change.

Short Title: Bacteraemia post-urological instrumentation: endocarditis risk?

Name of Researcher: .....

Please Initial Boxes

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.	
2. I understand that my participation is voluntary and that I am free to withdraw any time, without giving any reason, without medical care or legal right being affected.	
3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from the Research team, from the regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4. I have been given adequate time to consider my decision and have been given a copy of the participant information leaflet and a copy of this form.	

5. I agree to take part in the above study.	
6. I give permission for my GP to be informed of my participation in this study.	
7. I give permission for the collection and use of my samples. I understand that the Research Team will take all reasonable steps to protect my right to privacy.	

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Name of Patient	Date	Signature

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Name of Person taking consent	Date	Signature

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Name of Researcher	Date	Signature

Appendix 3: PCR for the identification of positive cultures

Specimens

Cultures for identification may be referred from blood cultures and routine

Only use pure cultures.

Prepare culture for direct use as template **immediately prior to use**: make a slightly turbid suspension of the culture in PCR water. Use neat and 1/10 dilutions directly in place of DNA extract.

16S PCR analysis

The preparation of PCR reactions and use of the Stratagene MX3000P thermocycler are described in MBNTP64 Real-time PCR detection of bacteria using SYBR green and the Stratagene MX3000P.

Whilst preparing the reagents, turn on the MXP3000P thermocycler to warm up.

In the PCR clean room, prepare the reagents required for PCR amplification of a short segment of the 16S rRNA gene as described in MBNTP64 using primers and conditions described below:

Forward primer: (MycF) AGAGTTTGATCCTGGCTCAG

Reverse primer: (Uni690R) CTACGCATTTCACCGCTACAC

Primer mixes are prepared by mixing equal volumes of forward and reverse primers and diluting 1/10 (*i.e.* 10 µl of each primer and 180 µl PCR water). These are then stored at -20° C.

Use the following table to calculate the volumes of reagents required:

Each sample is to be run using 3 µl of extracted template extractions and following dilution at 1/5 or culture at neat and 1/10. The final reaction volume is 30 µl.

(Number of reactions = 2 x number of samples (n) + Negative control + Positive controls + No template control (NTC) *i.e.* 2 x n + 3).

Table of reagents required in reagent mix for 27 µl volumes (3 µl template)

Number of reactions	1	2	3	4	5	6	7	8	9	10	11	12
Mastermix	15	30	45	60	75	90	105	120	150	165	180	195
Primer mix	0.8	1.5	2.3	3.0	3.7	4.5	5.3	6.0	7.5	8.3	9	9.8
H_2O	11	22	34	44	55	66	77	88	110	121	132	142
UNG	0.3	0.6	0.9	1.2	1.5	1.8	2.1	2.4	3	3.3	3.6	3.9

Allowance has been made for pipetting errors, *etc*.

In the designated setting up area within the extraction room, add the template to the prepared reagents, cap the tubes and briefly centrifuge so that the template and reagents are in the bottom of the tubes. Place the tubes into the wells in the Stratagene MX3000P and set up the machine using the thermal profile below, stored as “Tony/MR Final”:

37° C 10 min; 95° C 10 min; (95° C 30 sec, 62° C 1 min, 72° C 1.5 min) x 40; 72° C 10 min (fluorescence collected at end of extension)

95° C 1 min, ramp down to 55° C then ramp up to 95° C (collecting readings at ALL points)

Data analysis and interpretation

The PCR products should be analysed according to the protocol described in MBNTP64 Real-time PCR detection of bacteria using SYBR green 1 and the Stratagene MX3000P.

Check that the positive amplification plots have a lower C_t than the negative and NTC controls. The C_t of the negative controls must be >30 cycles. Where there is not a clear distinction between the C_t and the negative controls (minimum 2 cycles), then the result should be interpreted as equivocal and the PCR repeated.

For cultures, the amplification curves should have a low C_t of **< 27 cycles** and the melting curve of the PCR products should have a **sharp** peak – with a high T_m (> 82° C). If there is no sharp peak visible, the preparative PCR should be repeated.

For clinical specimens, the amplification curves must have a C_t of **< 30 cycles**. Where the C_t > 30 cycles, the amplification may be repeated once more. If C_t > 30 cycles then the results is NEGATIVE and a comment (code: 16S) added to the report:

To avoid the detection of contaminating bacteria, the sensitivity of 16S-PCR is lower than PCR for specific bacterial targets. A negative PCR result cannot entirely exclude bacterial infection.

All positive PCR products should be stored at -20° C until they are cleaned and sent for nucleotide sequence determination and analysis.

Appendix 4: PCR Protocol for bacterial DNA extracted from blood

PCR for bacterial 16S rDNA

Primers:

"Short 16S" (Greisen et al. (1994): *J Clin Microbiol* 32: 335-351)

RW01 (F) AACTGGAGGAAGGTGGGGAT

DG74 ® AGGAGGTGATCCAACCGCA

Product length ~390

Kathryn Harris (Harris & Hartley (2003): *J Med Microbiol* 52: 685-691)

16S Fa (F) GCTCAGATTGAACGCTGG

16SFb (F) GCTCAGGAYGAACGCTGG

16SR TACTGCTGCCTCCCGTA

Product length ~320bp

"Enteric 16S" (Greisen et al. (1994): *J Clin Microbiol* 32: 335-351)

RW01

RDR140 (reversed) CATGAATCACAAAGTGGTAAGCGCC

Product length ~312bp

Ensure all tubes, pipettors and racks are clean before use (use DNAse Away and UV light).

	Per 25 µl Rx
2.5 x Mastermix	10
50 pmol/µl combined primers*	0.65
Staining solution	2.5
Water	8.6
MolTaq	0.8
Template/DNA	2.5

For each reaction (25 µl):

Primer mixes are prepared by mixing 10 µl of each primer, make up to 200 µl with PCR water. Store at -20° C.

Make up a pool of reagents ensuring included positive, negative and no template controls, and allow at least 1 reaction for pipetting errors.

N.B. water and template volumes can be varied according to the proportion of template that is required in the final reaction.

Tubes come in strips of eight - used pipette boxes can be used as a rack to transport them.

Prepare a plan for the positions of each tube in the PCR machine (A1-A12; G1-G12).

Dispense the reagent pool (*e.g.* 22.5 µl allows room for 2.5 µl template) into Stratagene tubes. Minimise labelling of the tubes if possible – the caps can be labelled at the ends.

Take tubes to the place where the DNA template can be added (extraction lab).

In the PCR machine room

1. Switch on the Stratagene MX3000P at the back (rocker switch, left hand side above power cable) and then the computer.
2. Double click MX3000P icon to open up software.
3. Select experiment type –SYBR green with dissociation curve.
4. If the top light bulb icon is red (and red LAMP OFF sign at bottom of screen is indicated), click the icon – it should turn yellow. The lamp takes about 20 min to warm up.

In the extraction room

1. Prepare a list of all templates before starting, to ensure that the correct sample is loaded into the correct tube.
2. Add appropriate volume of template to each tube.
3. Orientate the caps so that the "A" on the end of the strip lies with the first tube of the strip. Carefully cap each strip of tubes, ensuring that the caps lie flat. If more than one strip is used, alter the label of the tab of the subsequent strips to B, C….*etc*. Tube 1 will be the one nearest to the labelled tab.
4. Briefly spin tubes in the special centrifuge to ensure that all contents are at the bottom of the tubes.

In the PCR machine room

1. Place the tubes into the PCR machine – pull the black "handle" forward and lift up to reveal the locations. Arrange the strips from left to right with tube "A1" in the top left hand corner.
2. On the "**Plate setup**" screen, select the wells on the grid that correspond to the samples and positive controls and assign these as "*unknown"*; select dye as *FAM*. Assign the NTC wells as "*NTC*"; dye *FAM*.
3. (This can be completed once the programme is running)

   To label the wells individually double click on the well and type into the dialogue box. Alternatively to label multiple wells, you need to click the

"Full screen Plate" box, select wells and click "name and comments" box to open the dialogue box.

4. Select the "**Thermal profile**" button. Import the required programme using the import button and double clicking on the file. Alternatively, the machine can be programmed by editing the default programme.

   Cycling conditions (Harris & Hartley):

   3 min @ 94º C

   40 cycles: 30 sec 94º C; 1 min 60º C; 1 min 72º C

   - Stratagene melt curve.

5. Select "**Start run**" button bottom left hand side of screen to start the programme. Tick the "**Turn off lamp at end**" box. If the lamp has not warmed up, you will get a dialogue box requesting information about when to start run. Cancel the run and then tick the "Turn off lamp…" box and then select the "Start run" button to start run. When the dialogue box reappears, select the option "start run when lamp has warmed up".
6. If the run has begun the "*raw data plots*" will allow real time monitoring of the reactions.

Expected running time for 40 cycles with melting curve analysis 2 hr 30 min

Data analysis

1. Select plateau for amplification analysis by the clicking the "amplification" button. This will be found in **segment 2**. Click OK
2. Select ramp for dissociation analysis by the clicking the "dissociation" button. This will be found in **segment 4**. Click OK
3. Select wells for data analysis on the grid, click "data analysis" button.

If there are several different assays, it is helpful to select and print off each group individually.

4. Working from the top of the list on the right hand side down, click on each of the options:

Amplification: Print off and label any positives (hover mouse over the curve). Check that the threshold line lies above the background readings, if not it can be moved by dragging. A summary of the Ct values are displayed in the box on the right hand side, if there are no Ct values where there are good curves, the threshold should be adjust. If the threshold is set too high, then late Ct values will be counted as negative.

Dissociation curve: The colours of the curves will correspond to those from the amplification plots. Print off and label any positives. Check whether the characteristic “double peak” is visible for any of the specimens (not just those with positive amplification).

Text report. Tick the box for **Tm product 1** to be reported along with the defaults. Print out report.

You can print off a **composite report** by ticking the information required on the final report, selecting the number of areas per page and printing off.

Data Interpretation

1. Check that the positive amplification plots have lower Ct than the negative and no template controls. Where there is not a clear distinction between the Ct and the negative control, then the result should not be interpreted as positive. It is good practice to ensure there are >3 C_t s between amplification plot and the NTC/neg control.
2. Check all positive samples have a sharp peak at the appropriate Tm for the melting curve. Primer dimer and non-specific DNA is usually present with a lower TM. Samples where there is a mixed PCR product are unlikely to have a sharp peak.

Both the dissociation curve and the Tm of the text report should be considered when interpreting the result.

Appendix 5: Protocol for using PLEX System with BAC SF kit

PCR Setup Procedure (manual)

Remove SF plate from freezer and allow to thaw before use. Ensure that contents are in the bottom of the wells. Remove the protective foil. Turn on the plate sealer to warm up (see 1.1 below).

For each sample pipette 10 µl of eluate into the wells of the designated column – note "column 1 and column 7" = sample 1; "column 2 and column 8" = sample 2; *etc*. *(8 wells per column)* piercing the foil cover.

If you are only using the first 6 columns, ensure that you use PLEX-ID reagent in the unused wells.

Resealing PCR plates

Seal the PLEX-ID Assay Plate(s) with Foil Seals after PCR setup is complete.

NOTE: Gloves should be worn during the heat sealing process to avoid contaminating the seal.

- Depress the heat button on the Heat Sealer to initiate the warming cycle. While the Heat Sealer is warming, the light will blink on and off. When the Heat Sealer has reached the set point of 180° C, the light will remain on.
- Place the PLEX-ID Assay Plate on the Heat Sealer.
- Immediately prior to use, remove a foil seal from the bag, and apply to the plate, making sure the seal is centered for minimal overlap on either side and the proper side of the seal is Up as indicated on the seal bag.
- Lower the handle to engage the heat block and hold until the timer has counted down to zero and **three audible beeps** have been heard.

- Raise the handle to disengage the heat block, and allow plate to cool slightly. Carefully remove the plate from the Heat Sealer. *Check sealed properly or turn and return plate briefly to sealer.*
- If more than one plate is to be sealed, allow Heat Sealer to warm back up to the set point before sealing subsequent plates.

Plates can be frozen if they are not to be used immediately.

PLEX-ID TC Operation

- Slide open PLEX-ID TC lid, if closed, and place PLEX-ID BAC Spectrum SF Assay Plate in the block. Slide lid completely closed.
- Repeat for each Assay Plate to be loaded.
- Using arrow buttons on control panel, select **PLEX-ID** directory, then "**Diagnostic Programs"** then "**BAC_SF_TCv01**" for each Thermal Cycler used.
- Press **Start** button.
- Select cycler that has been loaded with the PLEX-ID BAC Spectrum SF Assay Plate. (*If only one thermocycler is on, this will be omitted)*
- Press **OK** button.

NOTE: Thermal cycled plates can be stored up to 24 hours in the thermal cycler before being placed on the PLEX-ID Analyzer. If plates are not placed on the analyzer within 24 hours, they can be stored for up to 48 hours at 2-8° C.

Transport plates in designated PCR box

Spin plates for 1 min at 800 rpm in plate centrifuge in Sequencing Lab.

Preparation of the PLEX-ID Analyzer

- Select completed plates from the corresponding Thermal Cycler and press Mark Completed.
- Follow the procedure described in the Plex-ID operations manual for instructions on how to run the Plex-ID analyser.
- Provision the Plex-ID analyser with clean-up micro particles, cleanup 1,2,3 and 4 if necessary.

NOTE: A PLEX-ID Analyzer Check Kit plate should be run weekly or immediately following each Clean-up Reagent change on the PLEX-ID Analyzer. While the PLEX-ID BAC Spectrum BC Assay Plates are being processed in the PLEX-ID TC, load and run a PLEX-ID Analyzer Check plate (if required). Results from the PLEX-ID Analyzer Check plate should be obtained before loading the PLEX-ID BAC Spectrum BC Assay Plates

Calibration plates (in clean room freezer or PCR machine room freezer) can be placed directly into the loading area of the Plex.

Barcode should be on the side with A1 and faces the back of the loader.

Sample Clean-up and Analysis on the PLEX-ID Analyzer

If the PLEX-ID Analyzer Check plate is run, the results should be obtained before proceeding to run the PLEX-ID BAC Spectrum BC Assay Plates. If the PLEX-ID Analyzer Check plate report indicates that the results are not satisfactory, do **NOT** proceed to run PLEX-ID BAC Spectrum BC Assay Plates. Contact your Abbott Representative for troubleshooting.

- Load the amplified PLEX-ID BAC Spectrum BC Assay Plates in the input stacker of the PLEX-ID Analyzer for sample clean-up and analysis.
- Select plate order from the available plates in the menu – note you need to have told the equipment that the PCR has been completed.
- Select “Run” on the user console.

Logging process

Computer: logon using username: Plex-ID

p/w: Plex-ID

Log in to the PLEX-ID Front-End Software on the PLEX-ID computer workstation and select the **"Pre-Extraction"** step on the menu.

NOTE*: For step-by step instructions on the PLEX-ID Front-End Software, see the PLEX-ID Front-End Processing Guide.*

- Select PLEX-ID BAC Spectrum (no EC) protocol and create a batch.
- Put in sample ID then press "**+**", then number for the specimen tube (this should be noted by the computer) and press "**+**"

  Press Done when batch completed.
- Return to the workstation and select the "**Extraction**" step from the software menu.
- Select the created batch and "**auto-assign**" the process tubes to the process tube block for the PLEX-ID FH. Press Next.
- Elution Plate number (see note by computer) and scan the PLEX-ID BAC Spectrum SF Assay Amplification Reagent Kit Lot Number when prompted. Press "Next". Follow instruction on the screen for the extraction procedure.

1. Return to the workstation and select **PCR Setup**.
2. Identify batch and add in the Scan Elution plate number (above).
3. Scan Barcode on the PLEX-ID BAC Spectrum SF Assay Plate(s).
4. Number BAC Spectrum BC Assay Plate(s) as indicated on the screen. Press Done.

1. Return to the workstation and select **Amplification**. Select the Thermal Cycler to be used.
2. Select the PLEX-ID BAC Spectrum BC Assay Plate(s). Press Done.
3. Also Select Complete Amplification at this point!

Appendix 6: Protocol for collecting blood from patients (Local Guidelines)

Using a needle and syringe. *N.B.* Using vacuum-assisted blood collection system is the preferred technique and the needle and syringe method should only be used as a last resort.

1. Prepare all the equipment you will require.
2. Remove the caps from the blood culture bottles and wipe the bottle tops with a sterile wipe containing 2% chlorhexidine in 70% isopropyl alcohol. Allow to air dry.
3. Identify the correct patient e.g. name band and verbally where possible and explain the procedure and obtain verbal consent where appropriate.
4. Wash hands with soap and water, then dry hands and put on disposable apron.
5. Selecting a venepuncture site. *N.B.* Use the femoral vein only if venepuncture is not possible at other sites. In this case, clean the skin as below (7) and repeat.
6. Apply a tourniquet if required.
7. Clean the venepuncture site using a 2% chlorhexidine in 70% isopropyl alcohol wipe. [1,2] Apply the disinfectant by pressing the swab in the centre of chosen venepuncture site. Then apply the disinfectant with a spiral outward motion from the centre of the venepuncture covering 1-2 finger breadth to each side. Allow to air dry (the drying process kills the bacteria).
8. Put on sterile examination gloves while skin disinfectants dry.
9. Attach a butterfly needle or straight needle to a Mel (or larger) syringe.
10. Perform venepuncture and withdraw 16-Mel of blood for blood culture sampling and more if other tests are required.
11. Remove tourniquet

12. Place a swab or cotton wool over the venepuncture site and apply gentle pressure while withdrawing the needle. Press firmly over the venepuncture site until bleeding has stopped (the patient can be asked to do this if appropriate).
13. Transfer 8-10 ml of blood into each bottle. NB. If the peripheral blood cultures are being taken as part of a set to identify intravascular catheter colonisation, the volume of blood needs to be the same as that collected from the central venous catheter (CVC). Fill anaerobic bottle first then aerobic. DO NOT change needle between sample collection and inoculation.
14. Discard needle and syringe into a sharps bin.
15. Write patient details and clinical information on blood culture bottles according to Trust policy.
16. Wash hands with soap and water, then dry hands.
17. Arrange transport of the sample to the laboratory.
18. Ensure that sampling details and any subsequent positive results communicated by the microbiology department are accurately documented in the patients' notes and advice is acted on.

Appendix 7: Medical history form

Patient Identification Number: .....

Name: **Age:**

Sex: **Weight:**

Telephone:

Planned procedure:

Date of planned procedure:

Within 2 weeks of the above date:

1. **Have you had any antibiotics therapy? :** **Yes or No**

   Is Yes, what was the indication? _____
2. **Any instrumentation of the urological tract? :** **Yes or No**
3. **Any symptomatic UTIs?** **Yes or No**

-

General questions

1. **Any immunosuppressants?** **Yes** **or** **No**

   _____
2. **Diabetes Mellitus?** **Yes** **or** **No**

   _____
3. **Smoking?** **Yes** **or** **No**

   _____
4. **Co-existing infection?** **Yes** **or** **No**

   _____
5. **Recent long hospital stay?** **Yes** **or** **No**

   _____

6. **Recurrent UTIs?** **Yes or No**

_____

7. **Urinary Stones?** **Yes or No**

_____

8. **Catheter *in situ*?** **Yes or No**

If catheter present, for how long? _____

Specific Questions

1. **Cardiac History?** **Yes or No**

_____

2. **Previous Endocarditis?** **Yes or No**

_____

3. **Prosthesis (*e.g.* joint, pacemaker, penile)?** **Yes or No**

_____

4. **Organ Transplant?** **Yes or No**

_____

Appendix 8: Interview Questionnaire

Good morning Mr/Mrs

I am Dr Amar Mohee from the urology research team at St James Hospital.

You took part in a study when you came for your catheter change/removal/prostate operation. We took some blood and a urine sample from you. Do you remember?

We are just calling to see how you are doing and we want to ask you a few questions. Is it ok?

1. Firstly, have you felt unwell or flu-ey after your procedure?

(if yes, elaborate)

2. Have you had any water infection after your procedure?

(if yes, elaborate)

3. Did you have to go to your GP
    a. For antibiotics? (yes/no)
    b. To provide a urine sample (yes/no)
4. Did you have to be re-admitted after your procedure?

(if yes, when and why?)

5. Did you come into hospital for treatment of any infection?

- (if yes, when and what type of infection?)

That is all.

Thank you once again for taking part in our study.

I wish you a good day.

Good Bye

Appendix 9: 16S PCR results

Catheter Manipulation Samples			
Possible positive samples	**C_t Value**	**Definite positive samples**	**C_t Value**
C003A	28.81	C002C	27.28
C004C	27.64	C005A	20.62
C006C	28.50	C005B	22.45
C007B	28.73	C005C	22.60
C009A	29.76	C005D	21.13
C023A	29.53	C006A	26.07
C023B	29.32	C009D	26.69
C024D	29.58	C025D	25.46
C026D	28.46	C032D	27.36
C030B	28.92	C037D	26.10
C030D	29.43	C042D	22.41
C033D	29.43	C048B	27.30
C034B	29.45	C055A	24.99
C035B	29.95		
C035D	28.28		
C038A	28.70		
C038B	29.38		
C038D	28.11		
C039A	28.69		
C039B	29.98		
C039D	28.41		
C041A	28.74		
C042A	29.19		

Possible positive samples	C_t Value	Definite positive samples	C_t Value
C042B	27.67		
C045A	28.07		
C045D	29.28		
C046A	29.35		
C047A	29.34		
C047D	28.73		
C048A	28.11		
C048C	27.60		
C050A	28.26		
C050D	29.15		
C052B	28.68		
C054D	27.52		
C056B	29.59		
C057B	29.58		
C057C	29.53		
C060A	29.61		
C060B	29.59		
C060D	29.16		

TURP samples			
Possible positive samples	**C_t Value**	**Definite positive samples**	**C_t Value**
T005A	28.99	T005D	27.22
T005E	27.62	T017C	25.10
T009E	29.74	T017E	26.74
T012E	29.79	T017F	26.90
T018F	27.94	T018E	23.28
T021C	27.59	T020A	26.88
T024C	29.32	T020C	24.33
T027C	28.85	T020F	24.24
T028E	29.44	T021A	24.18
T028F	29.96	T021D	24.50
T030A	28.88	T021E	24.13
T031C	29.17	T021F	24.22
T033D	28.98	T022C	24.19
T033E	29.65	T022D	26.68
T034F	28.65	T026F	25.37
T039C	28.18	T027D	26.40
T039D	27.96	T027F	25.63
T039E	29.04	T031F	25.75
T039F	29.63	T035C	26.37
T042F	28.22	T036D	25.37
T044D	28.59	T038E	27.49
T044E	27.88	T040A	24.11

Possible positive samples	Ct Value	Definite positive samples	Ct Value
T045A	27.87	T040C	26.57
T045C	29.11	T040D	25.41
T045F	28.37	T040E	25.28
T046A	28.09	T041D	25.81
T046B	28.28	T041F	25.91
T046C	28.01	T042C	25.60
T046F	28.62	T043A	24.02
T049B	29.27	T043C	26.68
T049C	29.25	T043D	26.70
T052D	27.57	T043E	25.44
T053C	27.82	T044A	23.87
T053D	29.38	T049D	26.32
T054A	29.48	T049E	27.49
T054D	29.78	T050C	24.33
T059E	28.79	T050D	25.12
T060C	29.77	T050F	25.41
T060D	29.75	T052F	27.49
T063D	29.28	T054C	26.82
T064A	29.21	T055B	26.97
T064B	28.50	T055C	23.87
T064E	28.67	T055D	26.98
T064F	29.84	T062B	24.94
		TO62F	27.17
		T063A	26.24

Possible positive samples	Ct Value	Definite positive samples	Ct Value
		T064C	26.99
		T065B	26.44
		T065F	24.69
		T071C	20.01
		T075A	19.57
		T077F	27.41