

Understanding Pathogenaemia in Man: A Proof of Concept Study

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Abstract

Introduction: The current understanding of the significance of circulating pathogen DNA in infection is limited. Blood cultures are the current gold standard for pathogen detection. The administration of antibiotics can confound pathogen detection by blood cultures. Polymerase chain reaction assays of circulating pathogen DNA has the potential ability for rapid diagnosis of infection and may be potentially useful in a clinical setting. However, the use of this technology has only recently been used in the study of sepsis.

Animal models for the study of sepsis have added to our understanding. The failure to translate results from animals to humans has been attributed to the disease characteristics of sepsis (complexity and heterogeneity), inappropriate clinical trials (study of ineffective drugs, inadequate clinical trial designs), and animal models that do not fully mimic human sepsis. Therefore, the finding of an easily reproducible *in vivo* human model for pathogenaemia may provide a platform for exploring the host's immune response to circulating pathogen material.

Infection and urosepsis are common complications in diagnostic and therapeutic urological procedures. Urological interventional procedures for the removal of renal stones are commonly done in a controlled operating environment and may potentially be an *in vivo* model for investigating the host immune response to detected pathogen. Antibiotic prophylaxis is routinely given across the world for these urological procedures even though the evidence for their use is weak. If the presence of pathogen is seen to generate an immune response in these *in vivo* human models, it could be argued that there may be a potential benefit to antibiotic prophylaxis.

Aim: To investigate interventional urological stone removal as a model of pathogenaemia in man and, therefore, test the following hypotheses:

1. The detection of circulating pathogen DNA by SeptiFast[®] PCR is an indicator for the infection associated with urological procedures.
2. The presence of circulating pathogen DNA correlates with the host immune and physiological response, supporting the notion that antibiotics prophylaxis is important in urological procedures.

Method: In a prospective group of patients undergoing stone removal, blood samples were taken at five time points, peri-operatively to assess:

- The presence of pathogen by blood culture and pathogen DNA by SeptiFast[®] PCR;
- Circulating IL - 6 and IL - 10 levels to assess activation of host inflammatory response.

Routine peri - operative observations were recorded throughout as measures of physiological responses.

Results: Collected historical data on urological procedures by Salford Royal Foundation Trust urology services show that the SeptiFast[®] assay gave full coverage for pathogens seen in this clinical setting. While positive blood culture was rare, 50% of patients tested positive for pathogen DNAemia and this was associated with increased circulating IL-6 compared to patients with no circulating pathogen DNA. Linkage between pathogen DNA positivity and patient outcome was not established.

Conclusion: These data provide novel evidence that pathogen DNAemia is a common feature of routine urological procedures correlating with an increased systemic

inflammatory response. Endourological stone removal interventions may be a useful model for understanding the role of pathogen DNA in triggering inflammatory responses to infection in man.

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Abbreviations

ARDS	Acute Respiratory Distress Syndrome
BC	Blood culture
BSI	Bacterial bloodstream infection
CLP	Caecal ligation and puncture
CFU	Colony Forming Units
CoNS	Coagulase-negative Staphylococci
COX	Cyclooxygenase
CRP	C Reactive Protein
DNA	Deoxyribonucleic acid
EAU	European Association of Urology
EPR	Electronic Patient Record
ESI	Electro Spray Ionisation
ESIU	EAU Section for Infection in Urology
ESWL	Extracorporeal Shock Wave Lithotripsy
EWS	Early Warning Score
FISH	Fluorescence in-situ hybridization
HMGB-1	High-mobility group box-1
ICU	Intensive Care Unit
IL	Interleukin
IL - 6R	Interleukin 6 receptor
IQR	Interquartile range
ITS	Internal transcribed spacer

kDA	Kilodalton
LPS	Lipopolysaccharide
mls	Millilitres
mg	Milligrams
MODS	Multiple Organ Dysfunction Syndrome
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NAT	Nucleic acid based diagnostic technology
NFkB	Nuclear factor kB
NLR	NOD-like receptor
ng	Nanogram
PAI-1 promoter	Plasminogen activator inhibitor-1 promoter
PAMP	Pathogen-associated molecular pattern
PCWP	Pulmonary capillary wedge pressure
PCNL	Percutaneous nephrolithotomy
PCR	Polymerase Chain reaction
pDC	Plasmacytoid dendritic cells
PG	Peptidoglycan
PNA-FISH	Peptidic nucleic acid fluorescent in-situ hybridisation
RCT	Randomized Controlled Trial
RT PCR	Real time polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCC	Society of Critical Medicine
SIRS	Systemic Inflammatory Response Syndrome
sIL - 6R	Soluble Interleukin 6 receptor
SOAP	Sepsis Occurrence in Acutely ill Patients

SRFT	Salford Royal Foundation Trust
SSC	Surviving Sepsis Campaign
STARD	Standards for Reporting of Diagnostic Accuracy
TNFA	Tumour Necrosis Factor
TLR	Toll like receptor
UNG	Uracil-N-glycosylate
UTI	Urinary Tract Infection

CHAPTER 1

INTRODUCTION

Background

Sepsis is one of the oldest syndromes in medicine. Sepsis has been described in literature since the days of the ancient Greeks. Sepsis is still one of the top 10 causes of death today. The clinical manifestations of sepsis are highly variable, depending on the initial site of infection, the causative organism, the pattern of acute organ dysfunction, the underlying health status of the patient, and the interval before the initiation of treatment (Angus et al, 2013). The 'Surviving Sepsis Campaign' has been instrumental in providing the nomenclature to help physicians understand the pathophysiology of sepsis (Dellinger et al, 2008).

The interaction of infection and immune response plays a decisive role in the pathogenesis of sepsis. Animal and human studies in sepsis have furthered our understanding on the effect of pathogenaemia in sepsis, such as the role of pathogen – associated molecules pattern (PAMP) in the host response in sepsis. The significance of circulating pathogen DNA, a recognised PAMP, in the infection and sepsis process has not been fully defined in these studies. The advancement in nucleic acid technology (NAT) could potentially provide techniques to explore the role of pathogen DNA in the host response in sepsis. The aim of this proof of concept study was to explore the feasible use of a novel *in vivo* human model of pathogenaemia using microbiological, immunochemistry and NAT techniques.

Pathogens, infection and sepsis: A relationship explored

Infection is the invasion and multiplication of pathogens, such as bacteria, viruses, and fungi. If pathogen in blood is present, this represents progression of the infection to pathogenaemia. Patients' responses to pathogenaemia differ and there is no clear answer to why some individuals show only minor symptoms and others progress to a life threatening state of

sepsis (Rao et al, 1991, Mancini et al, 2010). Sepsis is defined as a suspected or proven infection plus a systemic inflammatory response syndrome (e.g. fever, tachycardia, tachypnoea, and leucocytosis) (Bone et al, 1992) Severe sepsis is defined as sepsis with organ dysfunction (hypotension, hypoxemia, oliguria, metabolic acidosis, thrombocytopenia, or obtundation). Septic shock is defined as severe sepsis with hypotension, despite adequate fluid resuscitation.

Septic shock and multiorgan dysfunction are the most common causes of death in patients with sepsis. The mortality rates associated with severe sepsis and septic shock are 25 to 30% and 40 to 70%, respectively (Angus et al 2001, Vincent et al, 2006). There is an estimated annual mortality of between 30 and 50 deaths per population of 100, 000. In Europe alone, an estimated 135,000 patients die each year of sepsis – associated complications, with an overall incidence of sepsis of three cases per 1000 individuals (Lever et al, 2007). In the US, the calculated cost of sepsis is close to 20 billion pounds and is the most common cause of in hospital mortality (Lagu, 2012).

There has been a strong international drive to provide guidelines for clinicians caring for a patient with severe sepsis and septic shock. The on-going motivation for the international drive is that severe sepsis and septic shock are major health care problems, affecting millions of people around the world each year, killing one in four (and often more), and increasing in incidence (Dellinger et al, 2013). In 2004, the surviving sepsis campaign, an international collaboration of professional societies involved in critical care, treatment of infectious diseases and emergency medicine, published the first internationally accepted guidelines to improve outcomes for this group of patients (Dellinger et al, 2008). Although controversially associated with potential conflicts of interest with the drug industry, it served as a nidus for future transparency and guidance in a complex field of medical practice. The following table

(Table 1) highlights the diagnostic criteria for sepsis, severe sepsis and septic shock derived from this group.

Table 1 - Diagnostic criteria for sepsis according to the International Sepsis Definitions Conference (Levy et al, 2003).
<p>Sepsis (documented or suspected infection plus ≥ 1 of the following)[†]</p> <p>General variables</p> <p>Fever (core temperature, $>38.3^{\circ}\text{C}$) Hypothermia (core temperature, $<36^{\circ}\text{C}$) Elevated heart rate (>90 beats per min or >2 SD above the upper limit of the normal range for age) Tachypnoea Altered mental status Substantial oedema or positive fluid balance (>20 ml/kg of body weight over a 24-hr period) Hyperglycaemia (plasma glucose, >120 mg/dl [6.7 mmol/litre]) in the absence of diabetes</p> <p>Inflammatory variables</p> <p>Leucocytosis (white-cell count, $>12,000/\text{mm}^3$) Leukopenia (white-cell count, $<4000/\text{mm}^3$) Normal white-cell count with $>10\%$ immature forms Elevated plasma C-reactive protein (>2 SD above the upper limit of the normal range) Elevated plasma procalcitonin (>2 SD above the upper limit of the normal range)</p> <p>Hemodynamic variables</p> <p>Arterial hypotension (systolic pressure, <90 mm Hg; mean arterial pressure, <70 mm Hg; or decrease in systolic pressure of >40 mm Hg in adults or to >2 SD below the lower limit of the normal range for age) Elevated mixed venous oxygen saturation ($>70\%$) Elevated cardiac index (>3.5 litres/min/square meter of body-surface area)</p> <p>Organ-dysfunction variables</p> <p>Arterial hypoxemia (ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen, <300) Acute oliguria (urine output, <0.5 ml/kg/hr or 45 ml/hr for at least 2 hr) Increase in creatinine level of >0.5 mg/dl (>44 $\mu\text{mol/litre}$) Coagulation abnormalities (international normalized ratio, >1.5; or activated partial-thromboplastin time, >60 sec) Paralytic ileus (absence of bowel sounds) Thrombocytopenia (platelet count, $<100,000/\text{mm}^3$) Hyperbilirubinemia (plasma total bilirubin, >4 mg/dl [68 $\mu\text{mol/litre}$])</p> <p>Tissue-perfusion variables</p> <p>Hyperlactataemia (lactate, >1 mmol/litre) Decreased capillary refill or mottling</p> <p>Severe sepsis (sepsis plus organ dysfunction)</p> <p>Septic shock (sepsis plus either hypotension [refractory to intravenous fluids] or hyperlactataemia)</p>

These definitions give a coherent language to discuss the parameters seen in sepsis which is used globally (Levy et al, 2003). A revised and updated edition of ‘Surviving Sepsis Campaign: International Guidelines for management of severe sepsis and shock: 2012’ was officially released at the Society of Critical Medicine’s (SCC) 42nd Congress (Dellinger et al, 2013), including full disclosures of potential conflicts of interest of the authors. It has been proposed that the host response to pathogenaemia must play a vital defining role in the septic response. To further our understanding on the pathophysiology of sepsis, an *in vivo* model of human pathogenaemia could prove beneficial.

Sepsis: a pathophysiological response to pathogenaemia

Sepsis is the culmination of complex interactions between the infecting microorganism and the host immune, inflammatory, and coagulation responses (James et al, 2006). In the last 10 years, current knowledge of the host’s ability to recognise a pathogen has increased. The expression of common structures known as pathogen – associated molecules pattern (PAMP) are thought to be central to the host response in sepsis (Christaki et al, 2011). These molecules have the potential to trigger a series of events via the immune system giving us the signs and symptoms seen in sepsis and septic shock.

Examples of PAMPs are:

- Lipopolysaccharide (LPS);
- Endotoxins – typically expressed by Gram negative bacteria;
- Peptidoglycans;
- Lipoprotein;
- Flagellin;
- Bacterial DNA;

The use of nucleic acid based diagnostic technology (NAT) such as polymerase chain reaction (PCR) assays has prompted significant interest in the role of bacterial DNA as PAMP. NAT could potentially have clinical significance as a modern day technique for pathogen detection and in the future supplement or even supersede blood cultures in the management of infections and sepsis.

In a recent review (Doi et al, 2009), the course of human sepsis is described as being likely to have an initial pro-inflammatory burst responsible for hypotension and organ dysfunction, followed by a compensatory anti-inflammatory immune response that leads to an immunocompromised state often called immune depression or immune dysfunction.

Figure 1 -Simplified clinical course of sepsis (Doi et al, 2009)

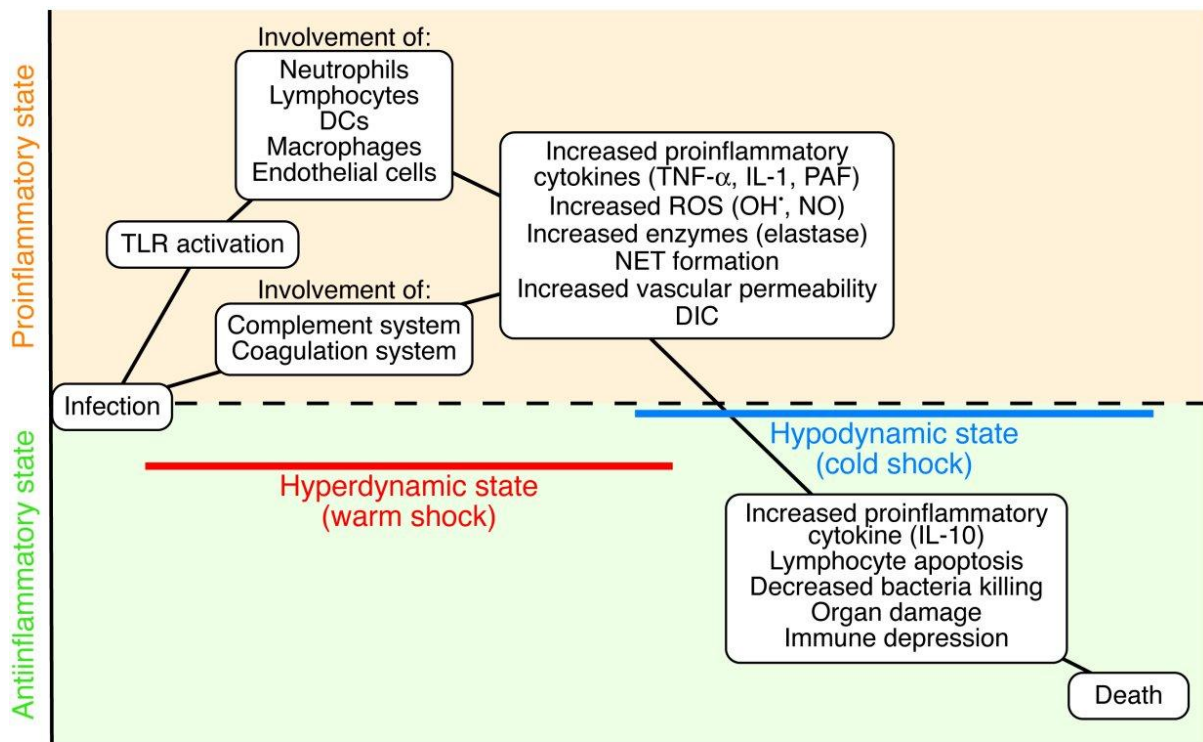


Figure 1. Progression of disease is complex, non-linear and varies from one patient to the next. Shown is an outline of selected landmark events and processes that appear to be common among patients and some animal models.

Although the progression of disease is complex and varies individually, from patient to patient, there are a few common pivotal events seen in all septic patients. A frequent feature in septic shock is a hyper dynamic circulation associated with diminished myocardial function. Blood volume is continually lost into the interstitial space of tissue and intracellular locations, and blood vessels become blocked by dysfunctional coagulation cascades (disseminated intravascular coagulation). Infectious disease, septicaemia in particular, is the most common clinical condition associated with disseminated intravascular coagulation (DIC). Although, virtually all microorganisms can cause DIC, bacterial infection is most frequently related to the development of the syndrome. Clinically onset DIC may occur in 30 to 50 percent of patients with gram negative sepsis (Levi et al, 1999). The mechanism of DIC starts with the systemic activation of coagulation and leads to widespread intravascular deposition of fibrin. Additionally, there is a depletion of platelets and coagulation factors. As a result, thrombosis of small and mid-size vessels may occur, contributing to organ failure and may lead to significant bleeding. Eventually, systolic hypotension and diffuse vasoconstriction lead to a fatal, therapy-refractory ischemia of multiple organs and to organ necrosis (Kruttsagen et al, 2012).

It should be emphasized that the pro-inflammatory and anti-inflammatory 'stages' are not a fixed sequence of events, nor is there an intermediate dissecting point in this diphasic process. Inflammatory responses in sepsis are complex, dynamically evolving, pleiotropic, synergistic and mutually reinforcing (Christaki et al, 2011). In this thesis, most of our work is concerned with analysis of the pro-inflammatory stage/early stage of the host response to a pathogen. In the next section, we will discuss the basis of most of the research into the pathophysiology of sepsis through animal and human models of sepsis.

Previous *in vivo* Models of Sepsis

Animal models of sepsis can be divided into three categories:

- Injection of an exogenous toxin such as lipopolysaccharide;
- Alteration of animal's endogenous protective barrier such as intestinal leaks;
- Infusion or instillation of exogenous bacteria.

Animal studies initially gave credence to the theory that death from sepsis may be due to an overstimulated immune system. A number of animal studies have used large doses of endotoxin or bacteria, leading to situations where circulating levels of cytokines, such as tumour necrosis factor, were exponentially higher in animals than they are in patients with sepsis (Hotchkiss et al, 2003).

LPS infusion/ injection model has been widely used for sepsis research. LPS administration induces systemic inflammation that mimics many of the initial clinical features of sepsis, including increases in pro-inflammatory cytokines such as TNFA and IL-1, but without bacteraemia (Witcherman et al, 1980, Remick et al, 2000, Michie et al, 1988). These results prompt work into possible therapeutic interventions. Treatment of LPS-injected animals with neutralizing antibody against TNFA or IL-1 resulted in improved outcomes for this model (Tracey et al, 1987, McNamara et al, 1993). The most commonly used animal models of sepsis in the last 10 - 20 years have been models which alter the animal's endogenous protective barrier, such as intestinal leak. Caecal ligation and puncture is very straightforward and is the most popular technique used. CLP-induced sepsis models show a cytokine profile similar to that in human sepsis (Remick et al, 2000, Eskandari et al, 1992), and anti-TNFA treatment fails to alleviate sepsis in CLP models as in human sepsis (Eskandari et al, 1992). CLP-induced sepsis increased lymphocyte apoptosis, which mimics immunosuppression at the later phase of human sepsis (Ayala et al, 1996, Dear et al, 2006). In this respect, CLP-induced sepsis is completely different from LPS-induced sepsis and more closely mimics human sepsis. There has been a great deal of disappointment in the inability to extrapolate

these findings to address sepsis in man. A major drawback with animal models is their inability to reflect the complex clinical picture seen in humans. There are clear differences between laboratory animals and patients. Mice and rats are housed in specific pathogen-free areas, may often be inbred strains, have the same age and weight, and most importantly, do not have comorbidities (such as diabetes, hypertension, and pre-existing immunosuppression among others) seen in septic humans. Most humans with sepsis are >50 years old, and most mice used in sepsis are <3 months old (with an average lifespan of 24 months). Furthermore, the experimental models have a precisely known time period. In contrast, we encounter patients of different ethnicities, ages, and weight, and most of the time, we are uncertain when the symptoms first emerged. In addition, there are differences between rodents and humans on the molecular level (Rittirsch et al, 2007). The predominant source of infection in septic patients before the late 1980s was Gram negative bacteraemia. Lipopolysaccharide (LPS), the main component of gram negative bacterial cell wall, was known to stimulate the release of inflammatory mediators from various cell types and induce acute infection symptoms in animals (Riedemann et al, 2003).

Administration of Gram negative bacterial LPS has been used as a model of severe infection in man and has been shown to reliably induce a febrile systemic inflammatory response with associated hormonal and cytokine changes (Agwunobi et al, 2000). Michie et al (Michie et al, 1988) measured plasma concentrations of circulating tumour necrosis factor alpha (cachectin), interleukin-1 beta, and gamma interferon, together with physiologic and hormonal responses, in 13 healthy men after intravenous administration of *Escherichia coli* endotoxin (4ng per kilogram of body weight) and during a control period of saline administration. The group showed high levels of plasma concentrations of circulating TNFA after the infusion. Results such as these, along with animal studies, prompted the investigation of anti – TNFA and anti-LPS interventions as possible treatments in those with

sepsis but further studies have not shown this. There are a number of plausible explanations for the perceived failure in sepsis trials (Table 2).

Table 2 Possible reasons for failure in sepsis trials (Riedemann et al, 2003)

Development of sepsis model theory	Possible reason for failure
<p style="text-align: center;">Assumptions</p> <ol style="list-style-type: none"> 1. Gram negative bacteria are cause of sepsis 2. Bacteria causing disease shed LPS 3. High levels of serum LPS achieved in septic patients 	<ol style="list-style-type: none"> 1. Incorrect assumptions based on initiating factors of disease 2. Incomplete clinical observation
<p style="text-align: center;">Observation in animals</p> <ol style="list-style-type: none"> 1. High level of TNFA achieved following LPS infusion 	<ol style="list-style-type: none"> 1. Unsuitable animal not translatable to humans 2. High serum level of TNFA not achieved in humans during sepsis
<p style="text-align: center;">Observation of intervention</p> <ol style="list-style-type: none"> 1. Anti - TNFA antibodies increase survival 	<ol style="list-style-type: none"> 1. Unable to block all TNFA 2. Results incorrect
<p style="text-align: center;">Clinical trials in humans in sepsis</p> <ol style="list-style-type: none"> 1. Anti - TNFA antibodies not protective 	<ol style="list-style-type: none"> 1. Anti - TNFA antibodies not protective 2. Study design insufficient 3. Sepsis definition insufficient 4. Drug not working (not tested, etc.) 5. Wrong dose, time point, etc

However, LPS injection in humans is a valid model of endotoxemia and has been used as a model to review stress responses and metabolic response seen in human subjects (Agwunobi et al, 2000). For this thesis, it is proposed that urological interventional procedures involving instrumentation could be a novel *in vivo* model of pathogenaemia in man and provide an easily reproducible model assessing the host immune response to presence of pathogen.

Model of investigation

Introduction

Urology is a surgical speciality which has seen marked changes in the last few decades. Surgical procedures have moved significantly from open to endoscopic and laparoscopic procedures (Bootsma et al, 2008). However, it is well established in the field of urology that there are significant levels of bacteraemia after invasive urological procedures. In one study of 300 patients (Sullivan et al, 1972) the incidence of bacteraemia after urethral dilatation without antibiotic prophylaxis was 24%. It is well known that in urological interventions such as cystoscopy, ureteroscopy and percutaneous nephrolithotomy, the rate of positive blood cultures detected up to 15% (Christanio et al, 2000, Knopf et al, 2003, Doğan et al, 2002 Turan et al, 2006, Rao et al 1991). The incidence of septic shock after endoscopic manipulation for renal stones was about 1% but ‘less serious effects’ were more common (Rao et al 1991). A recent study (Sohn et al, 2013) retrospectively reviewed the medical notes of 531 patients who underwent ureteroscopy and ureteroscopic lithotripsy in their hospital over a nine year period. A total of 20 patients (3.8%) contracted infectious complications after various procedures in the upper urinary tract. The studies above have all been put forward as evidence for the perceived benefit of prophylactic antibiotics before urological procedures. It is thought the use of prophylactic antibiotics will limit post operation infection and urosepsis, which is seen as a common complication of these procedures.

One of the most common urological interventions is the removal of renal stones stuck in the renal tract that cause persistent symptoms. Therefore, more patients have their renal stones removed by ureteroscopy with lasering of the stone or percutaneous nephrolithotomy (PCNL). In PCNL, one third experience some peri-operative complications, the most common being fever secondary to a urinary tract infection (Gutierrez et al, 2013) In two other studies looking at infectious complications in PCNL (Michel et al, 2007, Draga et al, 2009) 21 - 39.8% of patients had a post-operative fever which was transient in most cases. However, 0.3 - 9.3% patients developed sepsis. In both studies, prophylactic antibiotics were used. Post op fevers are not uncommon and have caused people to suggest that this could be an indicator of an early systemic response to infection. Rao et al (Rao et al, 1991) showed that even though post-operative fever was seen in 74% of the patients who had PCNL, only 41% actually had endotoxemia.

There is a significant burden associated with urosepsis and urinary tract infection. Urinary tract infections are the most common cause of hospital associated infections (nosocomial infection). Approximately 80% of nosocomial UTI have been found to be associated with indwelling urinary catheters. Genitourinary interventions appear to be the facilitating factor in 5 – 10% of nosocomial UTI. Patients who have been exposed to some instrumentation are at high risk of urinary tract infections (Turan et al, 2006). In the USA, a study from early 2000s showed that urinary tract infection accounts for 1 million emergency department visits, resulting in 100,000 hospitalizations (Foxman et al, 2003).

Endourology

Endourology is the branch of urologic surgery concerned with closed procedures for visualizing or manipulating the urinary tract. It has lately grown to include all urological minimally invasive surgical procedures. Opposed to open surgery, endourology is performed using small cameras and instruments inserted into the urinary tract. For the purpose of this thesis, ureteroscopy and PCNL are the endourological procedures used and the indication for all of these procedures was for the treatment of renal calculi.

Renal calculi develop from crystals that separate from urine within the urinary tract. The chemical composition of renal stones, typically seen in clinical practice, is highlighted below (Figure 2). There are well-recognised predisposing factors for stone formation which include dehydration, lifestyle, geographical location (dry arid climate), and certain specific risk factors.

Figure 2 - Percentage of kidney stone types

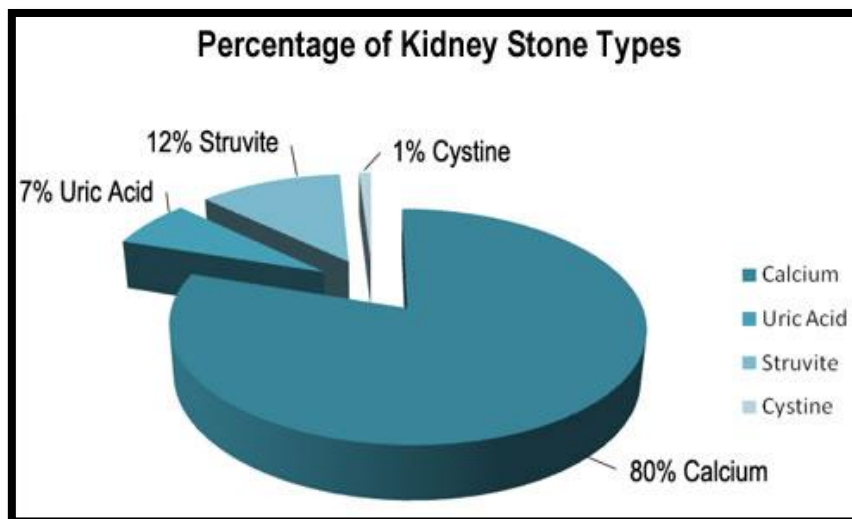


Figure 2. This figure shows the most common composition of kidney stones. The most common type of kidney stones is composed of calcium oxalate in about 75 to 80% of all stones. About 10% of all stones are formed from uric acid.

These factors may include anatomical / structural abnormalities (e.g. ureteropelvic junction obstruction, urinary diversion surgery, horseshoe kidney, calyceal diverticulum), and underlying metabolic conditions (e.g. cystinuria, oxaluria, gout), certain drugs, and urease-producing infective organisms (Tseng et al, 2011).

Endourology procedure - Ureteroscopy

An ureteroscopy is an examination or procedure using an ureteroscope. An ureteroscope, like a cystoscope, is an instrument for examining the inside of the urinary tract. An ureteroscope is longer and thinner than a cystoscope and is used to see beyond the bladder into the ureters.

There are two main types of ureteroscopes (1) flexible like a thin, long straw (2) rigid and firm. Through the ureteroscope, the obstructing stone in the ureter can be visualised and then removed via a small basket at the end of a wire inserted through an extra channel in the ureteroscope. In addition, a separate way to treat urolithiasis through an ureteroscope is to extend a flexible fibre through the scope up to the stone and then, with a laser beam shone through the fibre, break the stone into smaller pieces that can then pass out of the body in the urine. A stent is usually placed to keep ureter patent.

Endourology procedure - Percutaneous nephrolithotomy (PCNL)

Percutaneous nephrolithotomy is a surgical procedure for removing medium-sized or larger renal stones from the patient's urinary tract by means of a nephroscope passed into the kidney through a track created in the patient's back via a small puncture wound (up to about 1cm).

PCNL was first performed in Sweden in 1973 as a less invasive alternative to open surgery on the kidneys. The term "percutaneous" means that the procedure is done through the skin. Nephrolithotomy is a term formed from two Greek words that mean "kidney" and "removing stones by cutting."

With a small 1cm incision in the loin, the percutaneous nephrolithotomy (PCNL) needle is passed into the pelvis of the kidney. The position of the needle is confirmed by fluoroscopy. A guide wire is passed through the needle into the pelvis. The needle is then withdrawn with the guide wire still inside the pelvis. Over the guide wire the dilators are passed and a working sheath is introduced (Fig 3).

Figure 3 - PCNL (taken from www.uroinfo.ca)

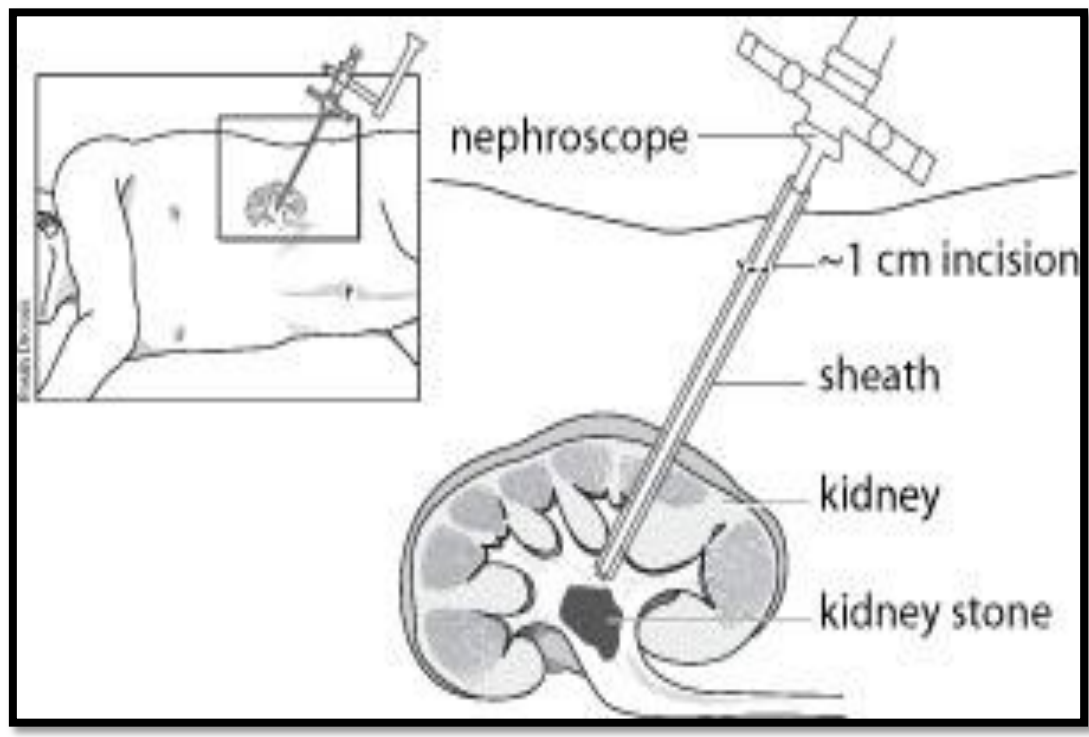


Figure 3. This figure outlines the approach use for stone removal by percutaneous nephrolithotomy (PCNL).

A nephroscope is then passed inside and small stones are taken out. A nephroscope is an instrument with a fiberoptic light source and two additional channels for viewing the inside of the kidney and irrigating (washing out) the area.

The surgeon may use a device with a basket on the end to grasp and remove smaller kidney stones directly. Larger stones are broken up with an ultrasonic or electro hydraulic probe, or via a laser beam. In case the stone is big it may first have to be crushed using ultrasound

probes and then have the stone fragments removed. The procedure can take 1 - 2 hours. (Wynberg et al, 2012). As mentioned earlier, prophylactic antibiotics are commonly used in this procedure and, presently, there is little evidence for their use in the urological procedures mentioned in this proof of concept study. It can be postulated that in our novel *in vivo* mode, if there is a relationship between the presence of detected pathogen and a detected host response to the pathogen, it could be argued that antibiotic prophylaxis may be beneficial in this setting. In the following section, there will be a brief exploration on the current use of antibiotic prophylaxis in urological procedures.

Antibiotic prophylaxis in urology procedures

In urology, the indication for antibiotic prophylactic use is to prevent post-operative infections. A pan-European survey was carried out by the EAU Section for Infection in Urology (ESIU) in a large number of European countries and found that $\geq 10-12\%$ of patients had a healthcare-associated UTI (Bjerklund et al, 2007). The current European Association guidelines on peri-operative antibiotic prophylaxis state that there is no evidence for any benefits of antibiotic prophylaxis in standard non-complicated endoscopic procedures and shockwave lithotripsy (SWL), although it is recommended in complicated procedures and patients with identified risk factors. The EAU guidelines reference papers which are described later in this introduction (Fourcade et al, 1990, Knopf et al, 2003, Rao et al, 1991).

Of all urological surgical procedures, there is strong evidence for a role of antibiotic prophylaxis in transrectal prostate biopsies and transurethral resection of the prostate (Bootsma et al, 2008). However, antibiotic prophylaxis is still widely used with marked differences in the regimens and choice of antibiotics used from one urology department to another. With different approaches, there is the risk of antimicrobial resistance developing.

There are a number of clearly established risk factors for peri-procedural infectious complications. These are highlighted in the table below (Table 3)

Table 3 Generally accepted risk factors for infectious complication (Grabe et al 2012)

General risk factors	Special risk factors associated with an increased bacterial load
Older age	Long pre-operative hospital stay or recent hospitalisation
Deficient nutritional status	History of recurrent urogenital infections
Impaired immune response	Surgery involving bowel segment
Diabetes mellitus	Long term drainage
Smoking	Urinary obstruction
Extreme weight	Urinary stones
Coexisting infection in a remote site	Colonisation with microorganisms

A pan-European study on nosocomial UTI (Bjerklund et al, 2007) has identified the three most important risk factors for infectious complications as:

- An indwelling catheter;
- Previous urogenital infection;
- Long preoperative hospital stay.

The risk of infection varies with the type of intervention undertaken.

There are few randomised control trials looking at antibiotic prophylaxis in ureteroscopy and PNCL procedures. Knopf and his group (Knopf et al, 2003) studied 113 patients undergoing ureteroscopy for stone removal randomised to a single oral dose of levofloxacin versus no antimicrobial. There was a significantly lower incidence of post-operative bacteriuria in those who received the prophylactic antibiotic (1 patient [1.8%] vs. 7 patients [12.5%]) (p=0.026).

Fourcade and his group (Fourcade et al, 1990) compared placebo with antibiotic prophylaxis in both PCNL and ureteroscopy, with separate analysis performed for each intervention group. With such small individual groups, no statistical significant difference was seen between the groups.

Given these findings, the evidence for antibiotic prophylaxis is low to moderate in these endourological procedures. It is proposed that our proof of concept model will identify patients who show a peri-procedural inflammatory response associated with pathogen DNA. This is potentially significant as it could be a first step towards developing targeted antibiotic prophylaxis for urological procedures. This in turn may have both cost-effectiveness benefits and reduce adverse events associated with unnecessary antibiotic use.

Tools for investigation

In this study to achieve our aims, blood cultures and polymerase chain reaction (PCR) assays were used for pathogen and pathogen DNA detection respectively. In order to assess the host immune response amongst the study population, serum interleukin 6 (IL-6) and interleukin 10 (IL - 10) levels were used as markers for the hosts' immune response.

Microbiological investigations – Blood Cultures

The current gold standard of bloodstream microbial detection and identification is blood culture analysis. Blood culture analysis involves the automatic, continuous monitoring of

liquid culture, followed by Gram stain, subculturing and use of phenotypic methods to identify the organism and its susceptibilities. With blood culture analysis, it is very important to differentiate between the presence of true pathogens in blood compared to detection of contaminants. A study of 843 episodes of positive blood cultures in adult inpatients from three hospitals in the US suggested that certain organisms should almost always be thought to represent true bacteraemia or fungaemia when isolated from a blood culture rather than contaminant (Weinstein et al, 1997). These organisms included *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli* and other *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Candida albicans*.

Certain organisms have been found to represent contamination in a significant proportion of cases. These organisms include *coagulase-negative staphylococci*, *Corynebacterium species*, *Bacillus species* other than *Bacillus anthracis*, *Propionibacterium acnes*, *Micrococcus species*, *viridans group streptococci*, *enterococci*, and *Clostridium perfringens* (Weinstein et al, 1997). However, it is crucial to recognize that each of these organisms can also represent true bacteraemias with devastating consequences, particularly if untreated due to misinterpretation as contaminants (Hall et al, 2006).

Limitation of blood cultures

Blood cultures have a central role in the detection of blood borne pathogens in patients with evidence of a systemic inflammatory response (SIRS). SIRS defines a clinical response to a non-specific insult of either infectious or non-infectious (e.g. ischaemia, trauma, inflammation). The detection and identification of pathogens defines such patients as being septic and along with the clinical presentation would prompt appropriate treatment with antimicrobial therapy. However, there are a number of limitations associated with blood cultures.

1. *Timing issues* - A major limitation to blood culture is the time required to complete the process, which ranges from one to five days or more. (Ecker et al, 2010). After a positive signal is given by the automated instrument (usually within 24 to 48 h of incubation), a Gram stain is then performed (together with a preliminary evaluation of the antimicrobial susceptibility) directly from the blood culture bottle.

The pathogen is then identified by biochemical tests. Rapid phenotypic tests may allow the identification of a large percentage of pathogens commonly recovered from blood cultures (usually within 18 to 24 h); however, more time is often needed for the final identification and for antimicrobial susceptibility evaluation of a given isolate, especially when slow-growing pathogens such as yeasts or anaerobes are present (Mancini et al, 2010).

2. *Sensitivity and false positive* - Sensitivity of blood cultures for slow-growing and fastidious organisms can be poor. Blood cultures miss fastidious organisms that are difficult or impossible to culture such as *Legionella pneumophila*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae* (Socan et al, 1999).

The culture diagnosis of invasive fungal infections has low sensitivity and the results are not usually available for many days in an important number of cases. From a clinical view point, these infections are seen amongst neutropenic patients frequently and mortality from untreated infection is high (Peters et al, 2004). Reportedly, more than 50% of blood cultures are negative where true bacterial or fungal sepsis is believed to exist (Ecker et al, 2010). In addition to this, as many as half of the cultures that are positive, represent contaminants organisms inoculated from the skin into culture bottles at the time of sample collection. Such results are false-positive blood cultures that can lead to unnecessary investigations and treatments.

3. *Low impact on clinical management* - It has been shown that there are a number of medical disorders where blood cultures have little influence on clinical management such as non – severe community acquired pneumonia and cellulitis (Peters et al 2004). It has been shown that the most therapeutic interventions occur immediately after collection of blood samples for culture and that the number of intervention decreased rapidly with time (Munson et al, 2003).

Blood cultures remain central to care of septic patients; however, molecular techniques aimed at complementing and negating the limitations of blood cultures are likely to be pivotal in the future, especially for ‘time-critical’ decision making and diagnosis. Polymerase chain reaction (PCR) assays of circulating pathogen DNA is seen as a potential technology which can be utilised for the rapid detection of infection.

Molecular investigation – Nucleic acid based diagnostic technology

Molecular methods based on nucleic acid based diagnostic technology (NAT) have been developed for the diagnosis of infection and pathogen identification. Polymerase chain reaction (PCR) is an example of a nucleic acid based diagnostic technology (NAT). PCR is a biochemical technology in molecular biology, which amplifies a single (or a few copies) of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Several pathogen-specific, broad range, and multiplex PCR-based amplification strategies have been used for positive blood cultures (Mancini et al, 2010). An example of a more recent process used for the direct molecular detection of pathogens is shown below (Fig 4)

Figure 4 - Identification and genotyping of microbes by PCR/ ESI/ mass (Ecker et al, 2010)

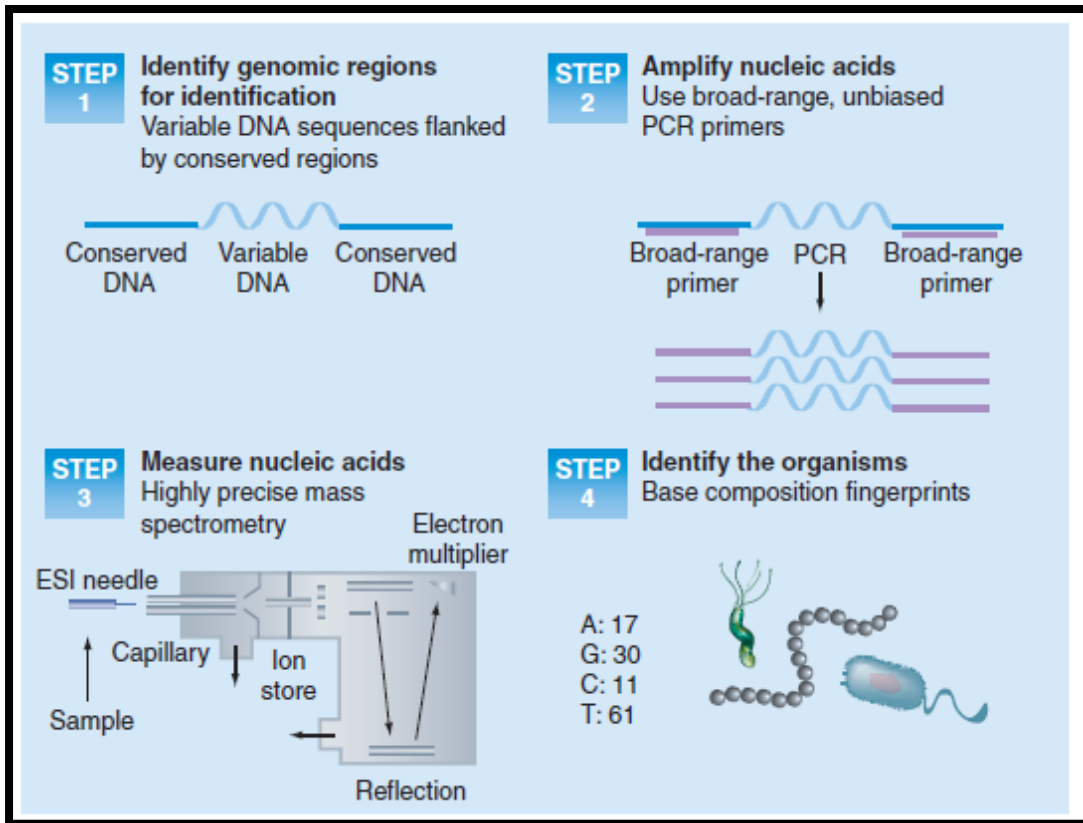


Figure 4 – This schematic shows the stepwise processes involved in the identification of microorganisms by PCR. ESI – electro spray ionisation

NAT applied to sepsis and detection of blood borne micro organisms can be divided into two main categories which will be discussed next.

NAT assays for the detection and identification of pathogens from blood culture bottles

In the first main category, molecular detection and speciation after an initial growth in blood culture medium occurs most easily with either hybridisation based or amplification based techniques (Peters et al, 2004). After the initial growth in blood culture medium, the hybridisation based technique provides identification of most pathogens within two hours. An example is fluorescence in situ hybridization (FISH) with oligonucleotide probes targeting

bacterial or fungal genes (typically rRNA genes). However, these methods can be technically difficult and need advanced detection systems and also require skilled laboratory staff.

Pathogen-specific assays is limited due to the high variety of pathogens, potentially responsible for blood borne infections. The main disadvantage of broad-range approaches are that after the PCR amplification of a target sequence, further identification procedures are necessary. The broad assay approach could be more useful for persistently negative blood cultures in the presence of a strong clinical suspicion of bacteraemia and fungaemia, as in the case of infective endocarditis (Mancini et al, 2010). Typically, blood cultures samples are taken multiple times for improved detection of organisms which are located in regions with little blood supply such as heart valves.

The risk of false-positive results due to environmental bacterial or fungal DNA contamination on sampling bloods needs to be considered when these panbacterial or panfungal approaches are used (Ng, 2006). The multiplex PCR approach targets different genes of those pathogens most frequently isolated from blood stream infections.

After the amplification process, there is sequencing analysis of the microorganism. The above methods of molecular identification of micro organisms on positive blood cultures are not routinely practiced in microbiology labs as they do not show greater or parallel clinical benefit or cost effectiveness compared with conventional methods (Peters et al, 2004).

Direct molecular detection of pathogens in blood with PCR

In 1993, two separate groups published the first use of pathogen specific PCR assay to detect bacteria in blood. In the first study to be published (Song et al, 1993), a *Salmonella typhi* PCR assays was described. The number of clinical samples was small. However, the peripheral mononuclear cells from 11 of 12 patients with typhoid fever confirmed by blood

culture were positive for DNA fragment of the flagellin gene of *Salmonella typhi*, whereas 10 blood specimens of patients with other febrile diseases were negative. This shows that the sensitivity of the PCR assay was 92% compared with blood cultures. In addition, *Salmonella typhi* DNA were detected from blood specimens of four patients with suspected typhoid fever on the basis of clinical features but with negative cultures. Interestingly, on the basis of the results of the PCR, these patients were treated with ciprofloxacin for 14 days with an excellent outcome.

There are four different molecular diagnostic approaches for detection of bacterial and fungal DNA in whole blood samples that are currently approved for clinical use by European regulatory authorities (Pletz et al, 2011):-

- A multiplex real – time PCR that simultaneously detects a pre defined panel of the most important sepsis pathogens by species- or genus-specific fluorescent probes (SeptiFast[®]; Roche).
- A eubacterial and panfungal real – time PCR that is able to detect nearly all known bacterial and fungal pathogens by a 16S and 18S ribosomal RNA (rRNA) gene-based universal PCR followed by sequencing of the amplification product for species identification (SepsiTest[™]; Molzym). The term eubacteria is commonly used to describe 'true bacteria' which includes all bacteria except archaeobacteria.
- A multiplex PCR that detects a predefined panel of the most important sepsis pathogens by electrophoretic separation of target-specific amplicons. An amplicon is a piece of DNA or RNA that is the source and/or product of natural or artificial amplification or replication events. (VYOO[®]; SIRS Lab)

- A eubacterial and panfungal PCR that is able to detect nearly all known bacterial and fungal pathogens by genome – specific targets followed by mass spectrometry for species identification (Plex-ID; Abbott).

The majority of clinical studies which compared PCR based diagnostics with conventional blood culture showed that more pathogens were detected by PCR techniques (Pletz et al, 2011).

SeptiFast[®] assay

The Roche product SeptiFast[®] has been available longer than other molecular-based tests and is the assay used in our proof of concept model. SeptiFast[®] uses real-time PCR in a nonquantitative mode to identify ten bacteria at the species level, several more at the genus level, as well as five *Candida species* and *Aspergillus fumigatus*. This assay reportedly identifies the 25 organisms that account for more than 90% of the culturable pathogens associated with sepsis (Ecker et al, 2010). No unculturable organisms are identified nor are most of the highly fastidious organisms that are difficult to culture. Whilst the majority of studies have reported that SeptiFast[®] has a higher pathogen detection rate compared with blood culture analysis (Dierkes et al, 2009, Lehmann et al, 2010, Louie et al, 2008, Mancini et al, 2008, Westh et al, 2009), other studies have disputed this (Bloos et al, 2010).

Furthermore, it has been reported that SeptiFast[®] is more sensitive in detecting fungal pathogens, such as *Candida species* and *Aspergillus fumigatus* when compared with conventional blood culture (Dierkes et al, 2009, Westh et al, 2009).

A number of studies have described the occasional incidence of negative PCR results associated with a simultaneous positive blood culture, so-called ‘false’ negative results (Lehmann et al 2010, Louie et al 2008, Yanagihara et al, 2010). This can occur because the aetiologic organism is not found in the SeptiFast[®] Masterlist (which is an obvious limitation

of the assay), or because of a technical fault with the platform not detecting the presence of the organism. Low concentration of the organisms in the blood sample can result in a 'false' negative result due to the limit of detection of SeptiFast[®] (Yanagihara et al, 2010). Excess total DNA in the sample can lead to saturation to the enzyme which interferes with the amplification and signaling to produce a negative result (Louie et al, 2008).

As SeptiFast[®] has an analytical sensitivity of 3 to 30 CFU/ml (Lehmann et al, 2008, Pletz et al, 2011), the diagnostic capabilities of the assay is limited to some extent, compared with the theoretical sensitivity of one CFU per culture bottle in conventional blood culture after inoculating approximately 10ml whole blood (Lehmann et al, 2010). This is potentially problematic as quantitative blood culture studies have shown that the majority of clinically significant bacteraemia in adults are characterised by circulating low numbers of bacteria (Lehmann et al, 2010). There have been a number of studies which have looked at the clinical application on the SeptiFast[®] assay in medical practice. Avolio et al (Avolio et al, 2010) compared traditional blood cultures with SeptiFast[®] in patients with suspected blood stream infections (BSI) arriving at the emergency department of a regional Italian hospital. In this study population, not all pathogens detected by blood cultures were also found by the PCR technique used even when those pathogens are on the PCR panel profile. Of 144 blood samples examined, 13 cases (24.5%) blood culture identified organisms which were not detected by real time PCR and similar findings have been seen in other studies.

In Avolio et al's study (Avolio et al, 2010), the SeptiFast[®] PCR assay gave positive results where the blood culture was negative in 10 cases. In these 10 cases there was a microbiology confirmation of infection by pathogen isolation from other sites with diagnoses ranging from pneumonia (two cases), meningitis (two cases), aortic prosthesis infection (one case), necrotising fasciitis (one case), or urosepsis (three cases). Therefore, in this population the

SeptiFast[®] results positively impacted the therapeutic choices and clinical outcome of the patients (Avolio et al, 2010).

Effect of antimicrobial administration pre testing on both microbiological and molecular approaches

The most recent international guidelines for sepsis (Dellinger et al, 2013), recommend obtaining blood cultures before antimicrobial therapy is initiated if such cultures do not significantly delay (>45 minutes) the start of antimicrobial(s) administration. However, a substantial number of blood cultures are taken from pretreated patients or patients developing sepsis despite antibiotic prophylaxis, i.e. pre-operative or after solid organ transplants. A perceived advantage of a DNA based detection system, compared to blood cultures, is that the pathogen does not have to be viable at the time of sampling. A Danish study (Westh et al, 2009), compared SeptiFast[®] with blood cultures in a multicentre trial of patients with suspected bacterial and fungal sepsis. 558 samples from 359 patients were evaluated. The rate of positivity was 17% from blood cultures and 26% for SeptiFast[®]. The administration of antibiotics did not affect the ability of DNA detection by the SeptiFast[®] assay and the SeptiFast[®] assay had a better pick up rate for pathogen detection. This study highlights the fact that blood culture positivity and pathogen DNA positivity are not equivalent measures of infection.

There are situations where PCR detection occurs in those with negative cultures. It is commonly known that our current gold standard for detecting pathogens in blood is blood cultures. Blood cultures fail to identify more than 50% of the cases of sepsis, believed to be caused by bacteria or fungi based on clinical and other criteria (Ecker et al, 2010). In numerous studies, as mentioned previously, SeptiFast[®] consistently identified more positive specimens than blood cultures. These potential ‘false positives’ were frequently deemed clinically significant based on chart reviews of clinical data, other analytical evidence of

infection or disease severity and were often subsequently confirmed after isolation of the pathogen from relevant clinical samples from other sources (Ecker et al, 2010). SeptiFast[®] - positive / culture-negative results could conceivably come from non-viable organisms in the blood (resulting from ongoing antibiotic treatment), cell-free DNA released from infected or colonized remote infection sites, or antibiotic interference with culture. With this variation in the potential role of circulating pathogen DNA, at present molecular PCR techniques would have a role as an adjunct to blood cultures.

For the purpose of this proof of concept study, it was vital to choose a method of detecting pathogen DNA in this model which would be robust enough to detect the organisms typical seen in this study population. It is widely established that the SeptiFast[®] assay covers the majority of bacteria and fungi seen in intensive care patients. It is also important that this coverage is broad enough for the urology patient population in this study. At Salford Royal Foundation Trust, where the study was undertaken, the urology service maintains a clinical database comprising clinical infection and microbiological data on interventional renal stone treatment dating from 2004 to 2011. The data establishes that SeptiFast[®] has suitable coverage of microbial detection to make this a viable tool for investigating infection in this study population.

Table 4 summarises the comparative advantages and disadvantages between blood culture and molecular methods in identifying blood stream infection (CFU - colony forming unit).

Table 4 - Needs and current status of methods to identify bloodstream infections

(Adapted from Ecker et al, 2010)

NEED	CURRENT STATUS		FUTURE OPPORTUNITIES
	<u>Culture</u>	<u>Molecular methods</u>	<u>Molecular methods</u>
Identify all bacterial and fungal infection	Identifies only culturable organisms	Varies with method from 25 frequently cultured organisms to panbacterial; limited or no fungal	Panbacterial and panfungal identification
High sensitivity: bloodstream infections can be caused by less than 10 CFU/ml in adults	Blood cultures are negative in >50% of clinically sepsis cases	Mixed results. Blood culture is more sensitive in some cases, but molecular methods identify organisms missed by culture; reported sensitivities as low as 3 CFU/ml	Mixed results. Blood culture is more sensitive in some cases, but molecular methods identify organisms missed by culture; reported sensitivities as low as 3 CFU/ml
Rapid identification; mortality increases hourly in the absence of appropriate antimicrobial therapy	Requires 1–5 days	Requires 1 day	< 1 hr
Antimicrobial resistance determination	Requires 1 additional day after obtaining culture isolate	A few major resistance determinants measured	Significant opportunity with future understanding of molecular mechanisms
Quantitative assessment of pathogen load; load correlates with disease severity	Quantitative culture methods too difficult for routine practice	Not quantitative	Quantitative results
Low labour requirements,	Labour intensive	Labour intensive	Fully automated

Cytokine driven immune responses

The body has developed intricate pathways to protect and defend against pathogenic organisms. Cytokines are one of the main protagonists in these processes. Cytokines are small cell-signalling protein molecules that are secreted by numerous cells and used extensively in intercellular communication. Cytokines can be proteins, peptides, or glycoproteins; the term "cytokine" encompasses a large and diverse family of regulators produced throughout the body by cells of diverse embryological origin (Kruttschnitt et al, 2012).

To understand the role of important cytokines such as IL - 6 and IL - 10, it is important to understand the early stages of the host immune response to common pathogens such as bacteria and fungi. The expression of common structures, known as PAMP, is thought to be central to the host response in sepsis (see earlier). The release and increase in serum concentration pro – inflammatory cytokines (cytokine storm) in sepsis has been shown to be triggered by release of bacterial endotoxin (Hack, 1989, Krettschnitt et al, 2012).

In gram positive sepsis, a similar role is seen with lipoteichoic acid and peptidoglycans (Schmidt et al, 2011). Both LPS and lipoteichoic acids bind to members of a family of PAMP receptors. These receptors are known as the toll like receptors (TLR). The combination of the receptor and its ligand alerts the innate immune response system to presence of invading pathogen. The innate immune system comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner and is the first line of the host's defence .

TLRs trigger intracellular pathways involving the signaling molecules MyD88 or TRIF and leading to activation of the transcription factors c – Jun N – terminal kinase and NF- κ B, thereby initiating the transcription of pro – inflammatory cytokine genes and production of pro – inflammatory cytokines such as interleukin - 6 (IL-6) (Stearns – Kurosawa, 2011).

Interleukin 6

IL-6 is a cytokine that acts as both a pro – inflammatory and anti – inflammatory cytokine.

IL-6 is a cytokine linked to sepsis and is one of the major NF – kB target genes. IL-6 is a member of 4 helical cytokine families, which signals via an 80 kDA cytokine receptor (IL – 6R). Once IL – 6 binds to IL – 6R, the resultant complex associates with the signalling receptor subunit gp130. IL – 6 signals by two mechanisms (1) via the ubiquitous trans membrane gp130: ‘classic’ signalling using membrane –bound IL – 6R (gp80) and (2) via trans-signalling using soluble IL – 6R (sIL – 6R).

Research has shown that pro – inflammatory activities of IL – 6 are mainly driven by IL – 6 trans – signalling via the sIL – 6R, whereas anti – inflammatory or regenerative functions rely on classic IL – 6 signalling via the membrane bound receptor (Scheller et al, 2011). In a study (Waage et al, 1989), serum samples from patients with meningococcal disease were examined for the presence of IL - 6, TNFA and LPS. Median serum concentration of IL - 6 was 1,000 times higher in patients with septic shock (189 ng/ml) than in patients with bacteraemia or meningitis alone. This suggests that IL - 6 has an important role in the sepsis process. It was concluded that a complex pattern of cytokine exists in patients with fatal sepsis in those with meningococcal infection, and that the release of IL - 6 as well as interleukin 1 (IL - 1) is associated with a fatal outcome.

For a number of years, IL - 6 has been established as a prognostic marker for mortality in sepsis. In a study (Hack et al, 1989), a group measured levels of IL - 6 in plasma samples from 37 patients with sepsis or septic shock obtained at the time of admission to the intensive care unit and related these levels to hemodynamic and biochemical parameters as well as to clinical outcome. In 32 of the 37 patients, increased levels of IL - 6 were found, occasionally up to 7,500 times the normal level (Fig 5).

Figure 5 - IL – 6 and sepsis - associated mortality (Hack et al, 1989)

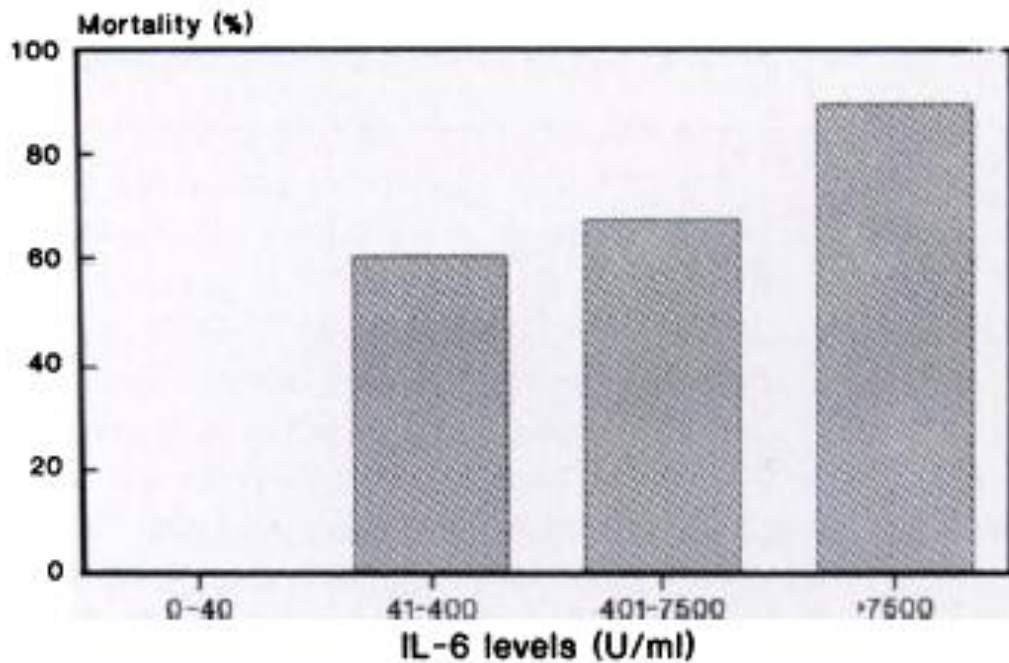


Figure 5 – Mortality in 37 patients with sepsis as a function of IL- 6 levels on admission. Patients were divided into four groups according to the IL- 6 levels as indicated

Importantly, IL - 6 on admission appeared to be of prognostic significance: levels were higher in septic patients who subsequently died than in those who survived ($P = .0003$), particularly when only patients with septic shock were considered (P less than $.0001$). All nine septic patients with levels of less than 40 U/mL on admission survived, whereas 89% of the nine patients with levels exceeding 7,500 U/mL died.

Research in the oncology field has raised a possible role for IL – 6 in the hemodynamic response typically seen in the sepsis process. IL – 6 trans signalling was found to increase endothelial permeability by phosphorylation of VE – cadherin (Kruttscham et al, 2012). This process could lead to vascular leakage and may play a vital role in the life threatening refractory drop in blood pressure seen in shocked patients.

Interleukin 10

Interleukin 10 is an anti-inflammatory cytokine. Interleukin 10 levels are undetectable in healthy individuals. The IL – 10 protein is a homodimer; each of its subunits is 178 amino acids long, as shown in the figure below (Figure 6).

Figure 6 – Interleukin 10 and receptor (Kotenko et al, 1997)

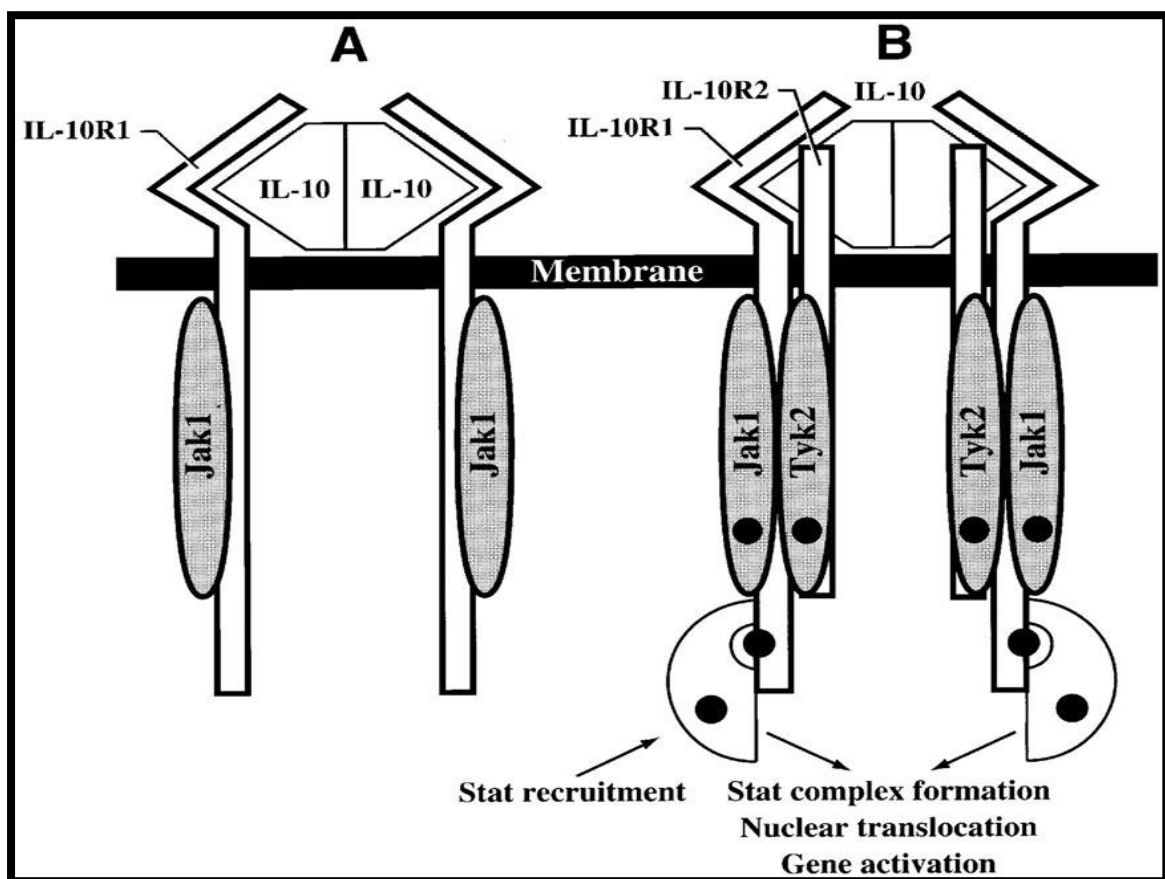


Figure 6 – This figure shows diagrammatically the structure of IL – 10 and IL – 10 receptor and the subsequent intercellular signalling from their interaction

IL – 10 is a pleiotropic cytokine with important immunoregulatory functions whose actions influence activities of many cell types in the immune system (Couper et al, 2008). IL – 10 is capable of repressing synthesis of pro-inflammatory cytokines such as TNFA, IL – 2 and interferon gamma made by macrophages and regulatory T – cells.

IL-10 has the ability to suppress the antigen presentation capacity of antigen presenting cells as well. Conversely, IL – 10 stimulates a number of inflammatory cells as well. IL – 10 has been seen to be elevated in the state of sepsis with an association between its concentration, severity of sepsis and death (Friedman et al, 1997, Giannodous et al, 2000).

Summary

Sepsis is the leading cause of death in critically ill patients. Sepsis has been described in medical literature for over 2000 years but is still a leading cause of both economic burden and patient morbidity and mortality. All episodes of sepsis are associated with infection and the presence of pathogen in body tissue. The complex interaction between pathogen and the host response has been extensively investigated for decades. However, the role of circulating pathogen DNA in the early triggering of the host response to infection is not understood fully.

Blood culture is the current gold standard method for pathogen detection in blood but cannot provide time – critical results that impact on the initial management of a patient (Dark et al, 2009). In addition, this method of pathogen detection is particularly susceptible to false negative results following antimicrobial use. Molecular methods like PCR have the potential ability to rapidly detect (or rule out) the presence of illness causing organisms. These molecular methods could facilitate effective immediate management of the infection and influence the subsequent clinical outcome. These molecular methods are not influenced by antibiotic use. However, this expensive technology has a number of limitations.

In recent years, urology as a surgical speciality has moved away from open surgery, towards endourological procedures for the treatment of renal stones. As with any procedure, these interventions are not without complications. One of the most commonly seen complications is infection and urosepsis. To combat this complication, it is common practice to give prophylactic antibiotics. There is a long established association between bacteraemia and

invasive instrumentation during urological procedures. However, there is no clear evidence on the use of prophylactic antibiotics in all urological procedures, particularly not on those commonly used in stone removal.

Therefore, in this thesis, it is proposed that interventional urological stone removal can be a model to establish if the detection of pathogen by PCR is an indicator for infection in man. In this clinical setting, we can explore the patient's physiological response (peri-operative observations) and immune response (serum IL - 6 and IL-10 levels) in those who have pathogen detected by blood culture and more importantly, PCR assay. The PCR assay used in this study was the SeptiFast[®] assay. SeptiFast[®] is a multiplex RT - PCR assay that has a broad detection coverage of the 25 most commonly detected pathogens in critically ill patients seen in intensive care settings.

Aim of study

To appraise the presence and significance of blood borne microorganisms that may appear in patients during interventional urological procedure involving urinary tract instrumentation with:-

1. A multiplex real – time PCR that simultaneously detects a pre-defined panel of the most important sepsis pathogens by species- or genus-specific fluorescent probes (**SeptiFast[®]; Roche**);
2. Blood cultures.

To appraise the host immune responses and clinical relevance of urological interventional procedures as a model of human pathogenaemia by:-

1. Measuring the serum levels of IL - 6 and IL – 10 at five time points;
2. Measurements of patient clinical observation both pre, during and after procedure.

To help understand the significance of the presence of antibiotics in the laboratory detection of microorganisms using the complementary techniques of culture and microorganism DNA or RNA analysis.

The hypotheses being tested were, therefore, that:

1. The detection of circulating pathogen DNA by SeptiFast[®] PCR is an indicator for infection associated with urological procedures.
2. The presence of circulating pathogen DNA correlates with host immune and physiological response supporting the notion that antibiotic prophylaxis is important in urological procedures.

CHAPTER 2:
METHODS

Participant recruitment

All eligible participants were identified through either urology outpatient clinics, elective or emergency admission under the Salford Royal Urology team. Potential participants were provided with a written information sheet giving details of the research question and protocol and were given at least 24 hours to decide on whether to participate in the study. The potential eligible participants had been formally listed for either ureteroscopy or percutaneous nephrolithotomy. Informed written consent were obtained from eligible participant (REC reference 10/H1016/135)

The inclusion criteria were that all participants were older than 16, were due to be listed for the aforementioned urological procedures for ureteric stones and showed capacity to consent to participation in the study. Interpreter facilities were made available for potential participants who were unable to understand English.

Study design

The following diagram shows the study design for this proof of concept study and highlights when microbiological and immunochemical sampling occurred. In addition, the figure shows when data was collected on the physiological status of the patient during the pre procedure, peri procedure and post procedure phases (Figure 7).

Figure 7 Study Design

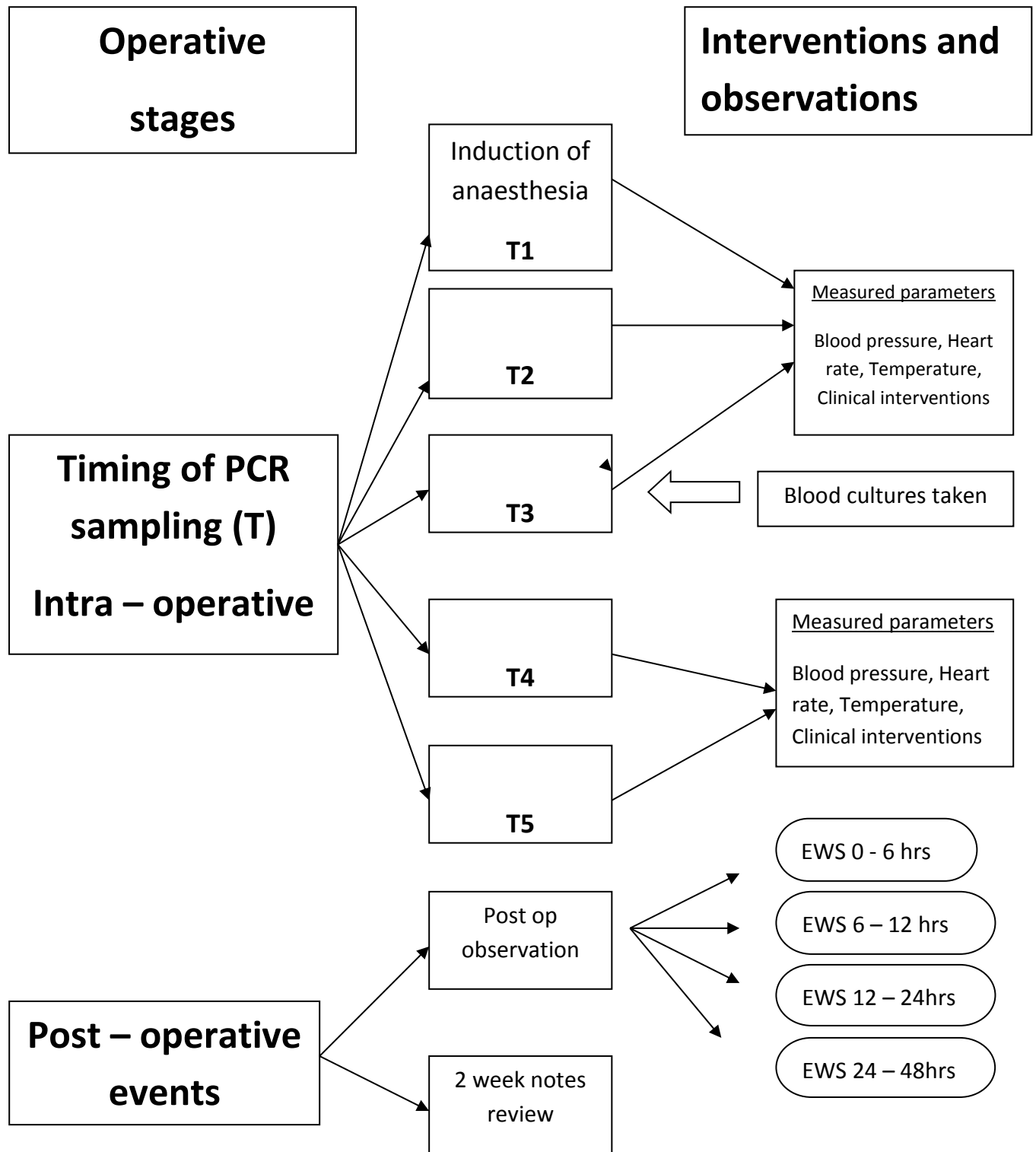


Figure 7 – The figure highlights the pivotal time points within the study

Blood sampling

From each participant, 5 mls of blood was taken at five distinct referenced time points for analysis of pathogen DNA by SeptiFast[®] PCR assay. These five specific time points represented times during the procedure which were proposed as the times when there would be an increased likelihood of pathogen DNA material being shed into the participant's blood stream. An additional 20mls of blood was taken at the point of maximal urological instrumentation use (time point T3). This time point was postulated as the time where maximal pathogen DNA shedding would likely occur and this 20mls was used for blood culture analysis. The timing schedule for blood sampling for all participants in the study is shown below (Table 5).

Table 5 – Timing schedule for blood sampling for RT-PCR analysis

Patient	Timing (t)				
	T1	T2	T3	T4	T5
A	Induction	At visualisation of renal stone	During laser	On stenting	Post op in recovery
B	Induction	Puncture	lithotripsy	Suturing patient for closure at end of operation	Post op in recovery
C	Induction	At visualisation of renal stone	During laser	On stenting	Post op in recovery
D	Induction	At visualisation of renal stone	During laser	On stenting	Post op in recovery
E	Induction	At visualisation of renal stone	During laser	On stenting	Post op in recovery
F	Induction	At visualisation of renal stone	During laser	On stenting	Post op in recovery
G	Induction	At visualisation of renal stone	During laser	On stenting	Post op in recovery
H	Induction	At visualisation of renal stone	During laser	On stenting	Post op in recovery

I	Induction	At visualisation of renal stone	Just after laser and basketing	Post stent insertion	Post op in recovery
J	Induction	As first removing papillae	Mid – removing papillae	Not done*	Not done*
K	Induction	Puncture	Lithotripsy	Suturing patient for closure at end of operation	Post op in recovery
L	Induction	At visualisation of renal stone	During laser	At stenting	Post op in recovery

All patients had blood sampled at the point of anaesthesia as time point T1. In the cohort, 2 patients had PCNL and the other 10 patients had ureteroscopy procedures, so the T2 - T4 time points were different in the PCNL patients from the ureteroscopy patients. In planning the study, there was a clear discussion with our urology team who proposed that the inflammatory response generated by PCNL was likely to be similar to the inflammatory response seen in the ureteroscopy procedures (personal communications with Mr Chris Betts, consultant urologist).

The blood samples were transported immediately to the on-site Biomedical Research Facility laboratories for further processing. One aliquot of blood (~2.5ml) was collected in EDTA tubes and stored at -80°C for subsequent analysis of pathogen DNA by SeptiFast® multiplex PCR. A second aliquot (~2.5ml) was collected in lithium-heparin tubes and plasma prepared by centrifuging the blood at 1500g for 10 minutes at 40°C. The plasma was removed from the cell pellet using a plastic pastette and stored in ~1ml aliquots at -80°C for analysis of immune-inflammatory markers, IL-6 and IL-10 by ELISA.

Data collection

For all study participants, a series of basic demographic information was obtained from our electronic patient record systems including age, sex and urological diagnosis. At each blood

sampling time point, the following parameters were recorded: blood pressure, temperature, pulse rate and clinical observations such as use of inotropic drugs and intravenous fluid administration. Post operative, the early warning score (EWS) for each participant over a 48 hour period post procedure was recorded.

The EWS tool is used in Salford Royal Foundation Trust to assess the degree of illness of patients. It is based on data derived from four physiological readings (systolic blood pressure, heart rate, respiratory rate, body temperature) and one observation (level of consciousness, AVPU). The resulting observations are compared to a normal range to generate a single composite score with the higher the score the higher the level of medical care or risk of death. This data was obtained by review of the electronic patient records (EPR) used in Salford Royal Foundation trust. A subsequent review of the EPR system was done to see if any of the study participants were readmitted within two weeks from the date of their urological procedure. In addition, urine culture and stone debris results sent on the day of the participant's procedures were obtained and analysed. If no urine culture results from the day of the procedure were obtained a retrospectively review for any recent mid-stream urine results was performed.

Laboratory analyses

Analysis of pathogen DNA by SeptiFast[®] PCR assay

SeptiFast[®] is a CE - marked assay for detection of a panel of bacterial and fungal pathogens in blood. The organisms covered by SeptiFast[®] represents > 95% of pathogens commonly found in healthcare-associated blood stream infections (Table 6). The assay involves steps for extraction of pathogen DNA and pathogen DNA analysis by real - time and was performed according to the manufacturer's detailed protocol.

Table 6 - SeptiFast® Pathogen Detection Panel

Gram negative	Gram positive	Fungi
<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Candida albicans</i>
<i>Klebsiella (pneumoniae/oxytoca)</i>	CoNS	<i>Candida tropicalis</i>
<i>Serratia marcescens</i>	<i>Staphylococci</i> *	<i>Candida parapsilosis</i>
<i>Enterobacter (cloacae/aerogenes)</i>	<i>Streptococcus pneumoniae</i>	<i>Candida krusei</i>
<i>Proteus mirabilis</i>	<i>Streptococcus spp</i> **	<i>Candida glabrata</i>
<i>Pseudomonas aeruginosa</i>	<i>Enterococcus faecium</i>	<i>Aspergillus fumigatus</i>
<i>Acinetobacter baumannii</i>	<i>Enterococcus faecalis</i>	
<i>Stenotrophomonas maltophila</i>		
* <i>S.epidermis, S. haemolyticus</i> ** <i>S.pyogenes. S.agalactae, S.mitis</i>		

Pathogen DNA extraction from blood

In order to initiate DNA extraction, whole blood samples were mixed on a bottle roller for 30 minutes. 1.5ml of each whole blood specimen was placed in SeptiFast® Lys Kit MGRAD tubes and subjected to mechanical lysis with ceramic beads in a MagNALyzer® instrument (Roche Diagnostics GmbH, Mannheim, Germany) for 70 sec at 7000 rpm and left to stand in the MagNALyzer for 10 min.

SeptiFast® preparation M Grade kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to extract the DNA using the protocol provided by the manufacturers. 1ml of the lysed specimen was vortexed with 150ul of proteinase K and an internal control (10ul; IC) and 1500ul of chaotropic lysis buffer (50% guanidinium, Tris-HCL buffer, thiocyanate and 20% Triton X-100) were then added.

To promote the release of the DNA, this mixture was incubated for 15 min at 56°C with gentle mixing at 500rpm. Following mixing with the binding buffer, the extract was

transferred onto spin columns designed to adsorb genomic DNA to the glass fibre matrix. Unbound substances were removed by washing the spin columns sequentially with 1.8ml inhibition removal buffer (containing 50% guanidinium HCL, 40% ethanol and Tris-HCl buffer) and 1.6ml wash buffer (containing 0.2% sodium chloride, Tris-HCl buffer and 80% ethanol). Finally, the adsorbed genomic DNA was eluted into 1.5ml DNA-free reagent tubes by incubation with 300µl of preheated (70°C) elution buffer (containing Tris-HCl) for 5 min followed by centrifugation for 2 min at 4200xg. This extract contained pathogen DNA which was then assayed by SeptiFast®.

SeptiFast® real-time PCR of pathogen DNA

Real-time PCR was carried out in 100µl glass capillary tubes using the LightCycler®2.0 instrument. Three capillary tubes were used per specimen for detection of Gram positive bacteria, Gram negative bacteria and Fungi with appropriate controls. 50µl of ready-to-use master mix (Roche Diagnostics) was added to 50µl of the target DNA.

The process of PCR amplification begins with a Pre-UNG incubation cycle at 40°C for 5 min. This cycle is carried out for the activation of Uracil-N-glycosylase that recognizes and catalyses the destruction of DNA strands containing deoxyuridine, but not DNA deoxythymidine. Since, PCR products contain uracil while genomic DNA contains thymidine; this procedure reduces the risk of carry-over contamination in the assay.

PCR was initiated by a denaturation cycle at 95°C for 10 min followed by two amplification programs (1-2) and a single melting curve profile (3).

1. 15 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 50 cycles and extension at 72°C for 40 sec.
2. 30 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 50 sec and extension at 72°C for 40 sec.

3. Denaturation at 95°C for 60 sec, annealing at 40°C for 60 sec and melting at 80°C at a temperature change rate of 0.1°C/sec.

SeptiFast[®] - Identification of pathogen species and controls.

The measured fluorescence (emitted during the annealing phase when the fluorescent probe hybridizes to the PCR amplicons) in four different detection channels was detected.

The assay is provided with specialised software (SeptiFast[®] Identification Software, SIS) which facilitates automated identification of species and controls by analysing the melting temperatures of the amplicons produced and a report for the pathogen status for each sample is obtained. The analysis protocol adopted by SeptiFast[®] is based on the study by Lehmann et al who elegantly showed that melting centre analysis permits rapid simultaneous detection of 25 different microorganisms using wide signal-to-signal melting peak differences (Lehmann et al, 2008). The specific melting temperatures for the PCR products and probes derived from the different organisms are shown in Figure 8 overleaf (Lehmann et al, 2008).

Figure 8 - Distribution of melting temperature and respective detection channels for all microorganisms and internal control in the SeptiFast® assay (Lehmann et al, 2008)

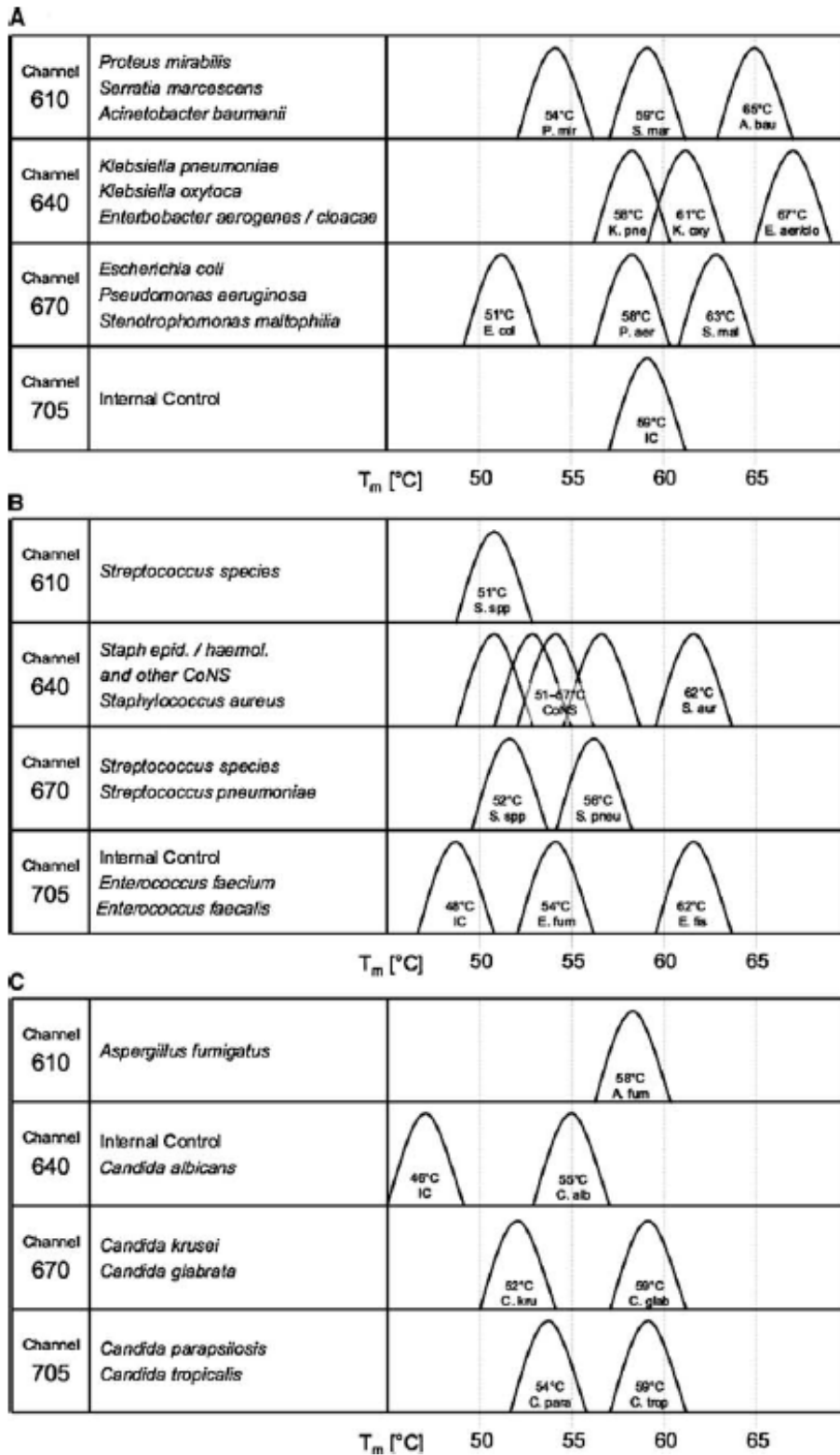


Figure 8 – Distribution of melting temperature (T_m) and respective detection channels for all microorganism and internal controls. A= Gram negative bacteria; B= Gram positive bacteria; C=Fungi

Cytokine analysis

IL-6 ELISA

IL-6 was measured using a Pelikine compact human sandwich enzyme-linked immunosorbent assay (ELISA) kit (Mast Diagnostics M1916). All solutions were made up according to the manufacturer's instructions.

A ninety six well plate was coated with the monoclonal antibody anti-human IL-6 coat antibody diluted 1:100 in coating buffer and 100µl was added to each well. The covered plate was incubated at room temperature (18-25) overnight. The plate was washed five times with PBS using an automatic plate washer. 200µl of blocking buffer was added to each well and the plate was incubated at room temperature for one hour. A standard curve was prepared as per the specification sheet, and the plate was washed five times with wash buffer (PBS with 0.005% TWEEN 20).

The substrate blank wells were left empty and 100µl of standards and diluted samples were added to the appropriate wells. The plate was re-covered and incubated at room temperature for one hour, with continuous shaking at 700rpm. Biotinylated antibody was diluted 1:100 in working-strength high performance ELISA dilution buffer (HPE). The plate was washed five times in wash buffer, the substrate blank wells were left empty and 100µl diluted biotinylated antibody was added to the remaining wells. The plate was re-covered and again incubated at room temperature for one hour, whilst being shaken at 700rpm. The streptavidin-horseradish peroxidase (HRP) conjugate was diluted 1:10 000 in working strength HPE dilution buffer. The plate was washed a further five times in wash buffer, and 100µl streptavidin-HRP conjugate was added to all wells except the substrate blank wells.

The covered plate was incubated at room temperature for 30 minutes, whilst being shaken at 700rpm. A suitable volume of TMB (Invitrogen 00-2023) was brought to room temperature ensuring that it was not exposed to light. A stop solution (1.8M sulphuric acid) was also prepared. The plate was re-washed five times and 100µl substrate was added to all wells. The plate was incubated in the dark at room temperature for 30 minutes, whilst being shaken. 100µl of stop solution was added to all wells and the plate was read at 450nm using a Victor multi-label counter (Wallac, Milton Keynes, UK). The cytokine concentrations were determined using a four parameter curve fit algorithm (Delta Soft analysis software, BioMetallics Inc, Princeton, NJ).

Figure 9 - The principle of sandwich ELISA (adapted from www.lenico.com)

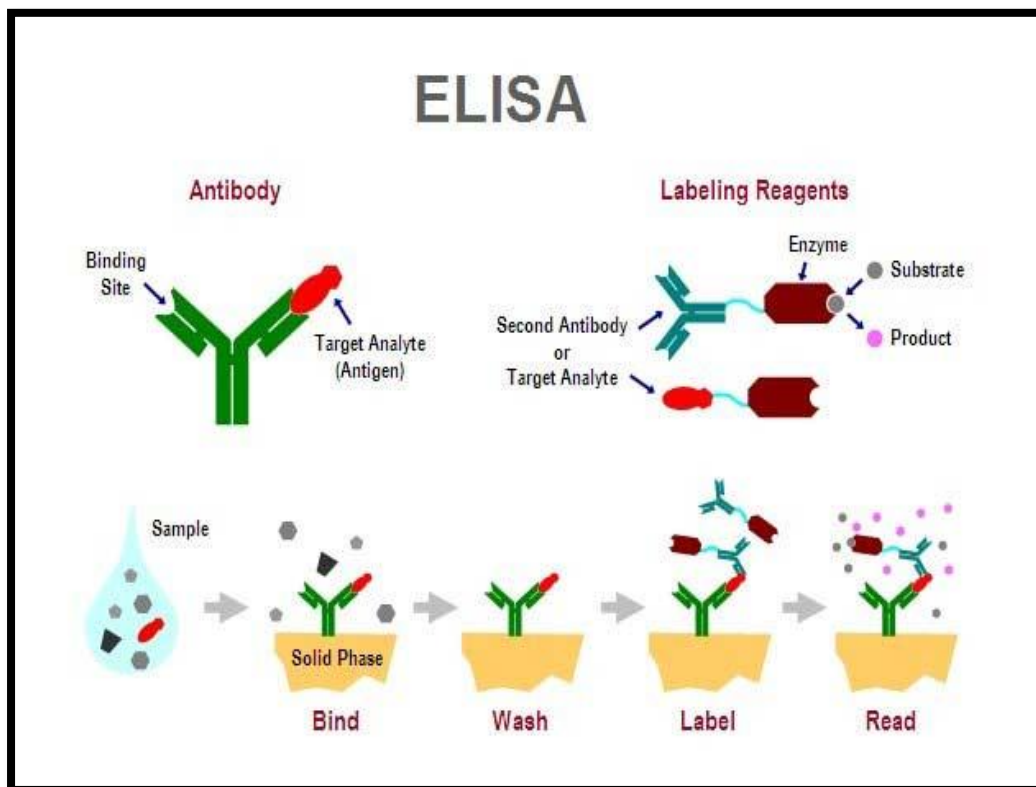


Figure 9 –An enzyme-linked immunosorbent assay (ELISA) is typically performed to detect the presence and / or amount of a target protein

IL-10 ELISA

IL-10 was measured using a Pelikine compact human sandwich ELISA kit (Mast Diagnostics M1910), following the same protocol as used for IL-6 (Fig 9). The sandwich ELISA process initially starts with a plate being coated with capture antibody. Blocking buffer is added to block remaining protein-binding sites on plate. A sample is added to plate and any antigen present is bound by the capture antibody. A washing stage follows to remove any non-binding antigen. The next step usually involves a labelling reagent which can be a labelled detection antibody which when added to the plate binds to any antigen present and triggers an enzymatic reaction to produce a detectable product for analysis.

Statistical analysis

In looking for an association between the detection of circulating pathogen DNA and interleukin levels, I used a simple unpaired t test at each time point to compare those with positive and negative pathogen DNA at each time point.

CHAPTER 3:

RESULTS

SeptiFast® assay coverage and historic data on positive cultures

Before commencing the study, it was necessary to validate the use of the SeptiFast® assay in this study population by ensuring that it detects those organisms typically associated with bacteriuria after stone extraction at Salford Royal Foundation Trust (SRFT). The urological services at SRFT have collected a database containing clinical infection and microbiological data for all patients who underwent renal stone removal surgery from 2004 – 2011. The SeptiFast® assay was designed to detect and identify 25 bacterial and fungal species that make up to greater than 90% of the pathogens causing bloodstream infections in critical care. By comparing these 25 microorganisms against those found in the SRFT urology database, it was possible to ascertain whether SeptiFast® was the right research tool to use to test the hypotheses.

Table 7 - List of positive organism growth from mid-stream urine sample from all patients who underwent renal stone removal surgery at Salford Royal Foundation Trust from 2004 – 2011.

Gram negative bacteria	Gram positive bacteria	Fungi
<i>Stenotrophomonas.maltophilia</i>	<i>Staphylococcal.sp</i>	
<i>Pseudomonas.sp</i>	<i>Enterococcus.sp</i>	
<i>Escherischia.coli</i>		
<i>Proteus.sp</i>		

The above table (Table 7) shows the list of positive organism growth from mid-stream urine samples from all patients who underwent renal stone removal surgery in the trust from 2004 –

2011. Comparing with the SeptiFast[®] Pathogen Detection Panel (Table 5), it can be seen that the SeptiFast[®] assay's coverage of organisms, has 100% overlap with the organisms detected via mid-stream urine results from the historical data compiled by urology services of SRFT.

Study Population

Twelve patients were consented for participation in the study. These 12 patients all underwent a urological procedure between 27 September 2011 and 14 November 2011. Ten patients (83%) underwent ureteroscopy with laser treatment to renal stones followed by ureteric stent insertion and two of 12 patients underwent PCNL. The median age of the study population was 54.5 years (interquartile range 24 - 61 yrs.).

The study population comprised of five male patients and seven female patients. For the purpose of this proof of concept study, we assume that similar inflammatory reactions occur with both described urological intervention as both procedures are using instrumentation in removal of renal stones. The basic demographics data for the 12 patients included in the study are shown in the following table (Table 8)

Table 8 - Patient demographic and operation detail

Patient identification	Age	Gender	Operation
A	77	M	Left ureteroscopy and laser to ureteric stone, basket extraction and insertion of stent
B	20	F	Left PCNL
C	59	M	Left ureteroscopy and laser to ureteric stone extraction, insertion of stent
D	62	M	Right ureteroscopy and laser to stone, insertion of stent
E	61	F	Left ureteroscopy and laser to ureteric stone, insertion of stent
F	50	M	Left ureteroscopy and laser to ureteric stone, basket extraction of fragments, insertion of stent
G	61	F	Right ureteroscopy, removal of stent, laser to stone, insertion of stent
H	24	F	Left ureteroscopy and laser to ureteric stone, basket extraction of fragments, insertion of stent
I	75	F	Right ureteroscopy and laser to stone, basket extraction of fragments and washout, insertion of stent
J	25	F	Left ureteroscopy and laser to stone
K	20	F	Left PCNL
L	32	M	Left ureteroscopy, removal of stent, basket extraction of fragments and laser to stone, insertion of stent

Pathogen species identified by SeptiFast[®] assay and blood culture

Using the SeptiFast[®] assay, the detection of a PCR signal was either classified as positive (signal present) or negative (signal absent). A positive blood culture result occurred in one out of the 12 patients (9%). The organism detected was the yeast, *Candida glabrata*.

A total of six patients (50% of cohort) returned a positive signal for the presence of circulating pathogen DNA. It can be argued from the data that the absence of PCR signal for pathogen DNA is likely to be an indicator of the absence of infection. In the case of patient J, there was a positive signal for circulating pathogen DNA and positive blood culture (Table 8). Post operatively, Patient J was transferred to a critical care setting for severe urosepsis.

Five out of the six patients with a positive signal from SeptiFast[®] assay had a detectable signal at a single time point. One patient had a positive signal at anaesthetic induction (Patient H, T1), another at the time of maximal instrumentation (Patient J, T3) whilst two separate patients had positive signals in post recovery (Patients F & L, T5). Patient I was the only patient to elicit positive DNA signals at two separate time points in the procedure (T3 and T4 respectively). These results are all highlighted in the next the table overleaf (Table 9).

Table 9 - Detection of pathogen DNA signal RT - PCR on blood samples at different time points

Patient	Frequency of sampling for PCR analysis detected (n)	Blood Culture positive	PCR signal T1	PCR signal T2	PCR signal T3	PCR signal T4	PCR signal T5
A	5	No	Negative	Negative	Negative	Negative	Negative
B	5	No	Negative	Negative	Negative	Negative	Negative
C	5	No	Negative	Negative	Negative	Negative	Negative
D	5	No	Negative	Negative	Negative	Negative	Negative
E	5	No	Negative	Negative	Negative	Negative	Negative
F	5	No	Negative	Negative	Negative	Negative	Positive
G	5	No	Negative	Negative	Negative	Negative	Negative
H	5	No	Positive	Negative	Negative	Negative	Negative
I	5	No	Negative	Negative	Positive	Positive	Negative
J	3	Yes	Negative	Negative	Positive	Not applicable	Not applicable
K	5	No	Negative	Positive	Negative	Negative	Negative
L	5	No	Negative	Negative	Negative	Negative	Positive

PCR positive signal patients vs. PCR negative signal patients

The background characteristics between those where the SeptiFast[®] PCR assay detected circulating DNA compared to those with a negative signal from the PCR assay was compared. The PCR negative group were our control group in the study (Table 10).

Table 10 - Characteristic of PCR positive patients vs. PCR negative patients

Characteristic	PCR signal detection	
	PCR positive	PCR negative
Female : Male	4 : 2	3 : 3
Median age, range (yrs.)	28.5 years (20 - 75)	61 years, (20 - 77)
Percutaneous nephrolithotomy (PCNL)	1 /6	1/6
Vasopressor use in procedure	1/6	2/6
Gentamicin administered at T1	6/6 ^a	6/6
Tazocin administered at T1	1/6 ^b	1/6 ^b
Augmentin administered at T1	2/6	0/6
Mean LOS for readmission directly related to infectious complication of procedures	7 days	4 days
Key:		
a - Patient J received 300mg compared to 120mg given to the other 11 patients.		
b - given to PCNL patients		

There are more females than males in the PCR positive signal group compared to the PCR negative group. The mean age in the positive signal group was 38 years compared to 57 years in the negative signal group. The proportion of PCNL to ureteroscopy/ laser treatment/ ureteric stent insertion procedures was identical in both groups.

In both groups all patients received gentamicin. All patients received 120 mg gentamicin, apart from patient J who received 300 mg gentamicin and was clinically unwell throughout the procedure. In the two patients who had PCNL, each were also given two doses of Piperacillin/ tazobactam 4.5 grams as prophylaxis. Piperacillin/ tazobactam is a combination broad spectrum antibiotic containing the extended-spectrum penicillin antibiotic piperacillin and the β -lactamase inhibitor tazobactam. In addition, two of those in the PCR positive group were also given a dose of Augmentin 1.2g in the T1 stage. These results highlight that the antibiotic strategies used in these procedures are not fixed and universal.

Relationship between pathogen isolate and clinical outcome

In the group with a positive signal detected on PCR, three patients were admitted with infective episodes within 30 days of their initial procedures (table 11),

Table 11 - Correlation between PCR positivity and clinical outcome

Patient	Organism detected by blood culture	Organism detected by SeptiFast® analysis	Post - operative event
F	Nil detected	<i>Streptococcal sp</i> <i>S.pneumoniae</i>	Had subsequent EWSL. 2 weeks later stent removal then Readmitted next day with clinical episode of urosepsis - within 30 days of initial procedure
H	Not performed	<i>K.pneumoniae / oxytoca</i>	No clinical episodes of urosepsis
I	Nil detected	<i>E. Faecalis</i>	No clinical episodes of urosepsis
J	<i>Candida glabrata</i>	<i>Candida glabrata</i>	Clinical episode of urosepsis – stent removal, post op admission
K	Nil detected	<i>E.coli</i>	Subsequent admission with pyelonephritis – required readmission
L	Nil detected	<i>S.aureus</i>	No clinical episodes of urosepsis

In the PCR positive group, Patient F was readmitted with urosepsis. After the patient's initial interventional procedure, the patient underwent a non-invasive urological procedure for stone removal, Extracorporeal Shock Wave Lithotripsy (EWSL) and subsequently had the stent removed. The day after removal of stent the patient was admitted with urosepsis.

Urine samples taken at the time of the EWSL and on admission with urosepsis showed only pyuria. If the urine culture had grown *streptococcal sp* and/or *S.pneumoniae*, it would have provided evidence that the interventional procedure may have directly contributed to the episode of urosepsis.

Within the PCR positive group, patient K was admitted with a clinical diagnosis of pyelonephritis directly related to the urological intervention. This patient was readmitted 12 days post procedure. The patient had a positive PCR signal for *E.coli* but on readmission had no growth in blood culture. Urine cultures from readmission episodes were positive for skin flora and pyuria.

The third patient, patient J, had a clinical diagnosis of urosepsis. The patient developed septic shock post procedure and required admission to intensive care unit. In this patient, *Candida glabrata* was detected on both SeptiFast[®] assay and blood cultures. Regarding the length of stay seen, the total extra in-patient bed days attributed to infection in the PCR positive group was 14 days with the mean stay in the two patients with an infection episode being seven days.

In the PCR negative group (table 12 overleaf), one patient was re-admitted for urosepsis 12 days post procedure. Urine was sent from two separate sites, a urostomy sample and a sample from the patient's urinary catheter. *E.coli* was grown from a sample sent from urostomy whilst the urinary catheter sample showed pyuria only. In the PCR negative group, the length of stay in the only patient with a clinical episode of infection was four days. These results suggest that PCR positive patients have a poorer clinical outcome regarding increased frequency of infections and longer length of stay.

Table 12 - Correlation between PCR negativity and clinical outcome

Patient	Organism detected by blood culture	Organism detected by SeptiFast® analysis	Post - operative event
A	Nil detected	Nil detected	No clinical episode of urosepsis
B	Nil detected	Nil detected	Clinical episode of urosepsis - antibiotics and subsequent repeat PCNL.
C	Nil detected	Nil detected	No clinical episode of urosepsis
D	Nil detected	Nil detected	No clinical episode of urosepsis
E	Nil detected	Nil detected	No clinical episode of urosepsis
G	Nil detected	Nil detected	No clinical episode of urosepsis

Analysis of circulating IL-6 and IL-10 levels

Immunochemical analysis was performed to establish if there was any detectable relationship between the presence of pathogen DNA and the level of plasma IL-6 and IL-10 in patients undergoing these interventional urological procedures. Plasma IL - 6 and plasma IL - 10 levels were measured in the T1 - T5 samples and direct comparisons were made between the PCR positive group and PCR negative group (Fig 10).

Figure 10 - Mean IL – 6 and IL-10 in PCR positive and PCR negative patients

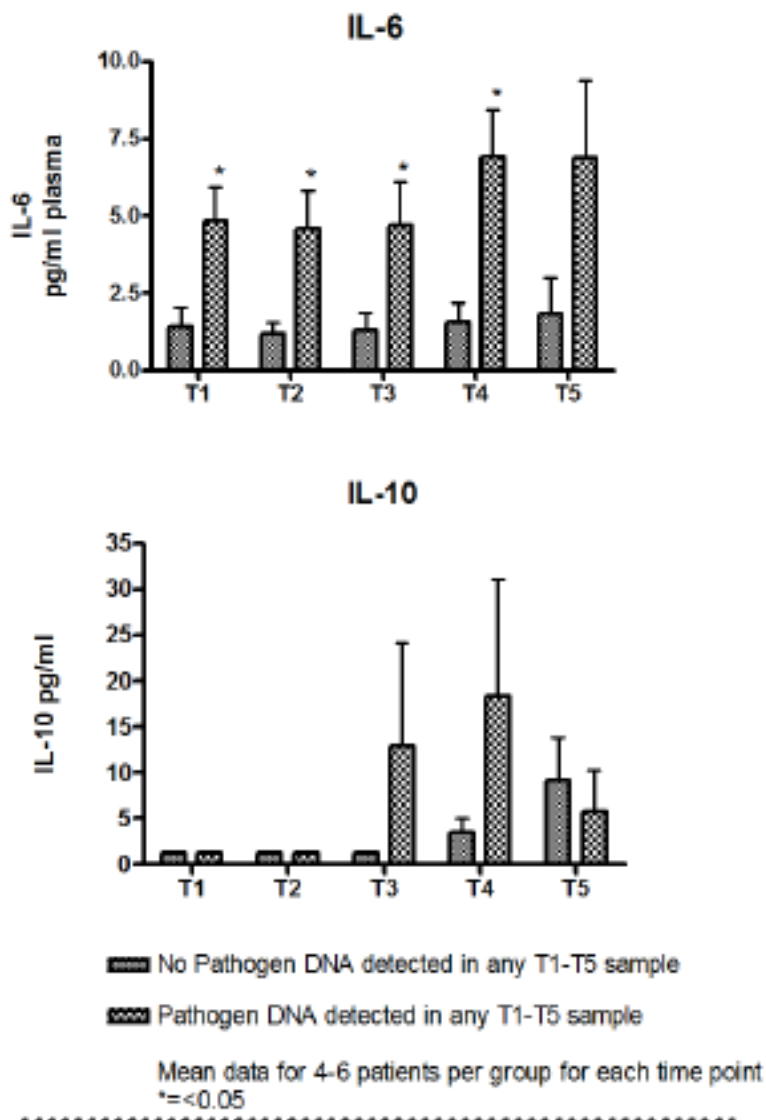


Figure 10 - This figure clearly shows that at each time point the detected level of IL – 6 is higher in those with a positive signal compared to those where no signal was detected. This pattern was not seen with IL – 10.

The mean level of IL - 6 in PCR - positive group was higher by a factor of five at all time points compared to the level found in PCR - negative group. This effect was statistically significant ($p < 0.05$) at all time points except for T5 (Figure 12) A different pattern was observed with IL-10 (Figure 12). There was no statistic difference ($p > 0.05$) between the

mean plasma levels of IL - 10 between the PCR - positive and PCR - negative group at any time points, although there was some qualitative evidence of higher levels at T3 and T4 in the PCR - positive groups.

From these results, there seems to be an association between the detection of circulating pathogen DNA and elevated pro inflammatory cytokine levels. The results demonstrate a likely link between the presence of circulating pathogen DNA and the host immune response in this study population.

Pathogen detection and physiological response

All patients had bedside observations and this helped to assess if there was an association between the peri procedure observations such as temperature, pulse and blood pressure (markers of physiological response) and PCR positivity. Blood pressure was expressed as the mean arterial blood pressure (MAP). The MAP is a measure of the perfusion pressure to vital organs of the body.

MAP is derived from the formula:

Mean arterial pressure = diastolic blood pressure + 0.33 (systolic blood pressure - diastolic blood pressure) in mmHg

The measured pulse rate is a measure of the heart's response during the procedures and is expressed in beats per minute. There was no clear pattern in the physiological responses seen in the PCR positive or PCR negative group. There seems to be no clear difference in the witnessed variation in the MAP across the time points when comparing those who were PCR positive with those who were PCR negative (Fig 11 and 12). When the mean MAP across each study group is compared at all time points, there was no clear difference seen (Fig 13).

Figure 11 - Variations of mean arterial blood pressures in PCR negative group

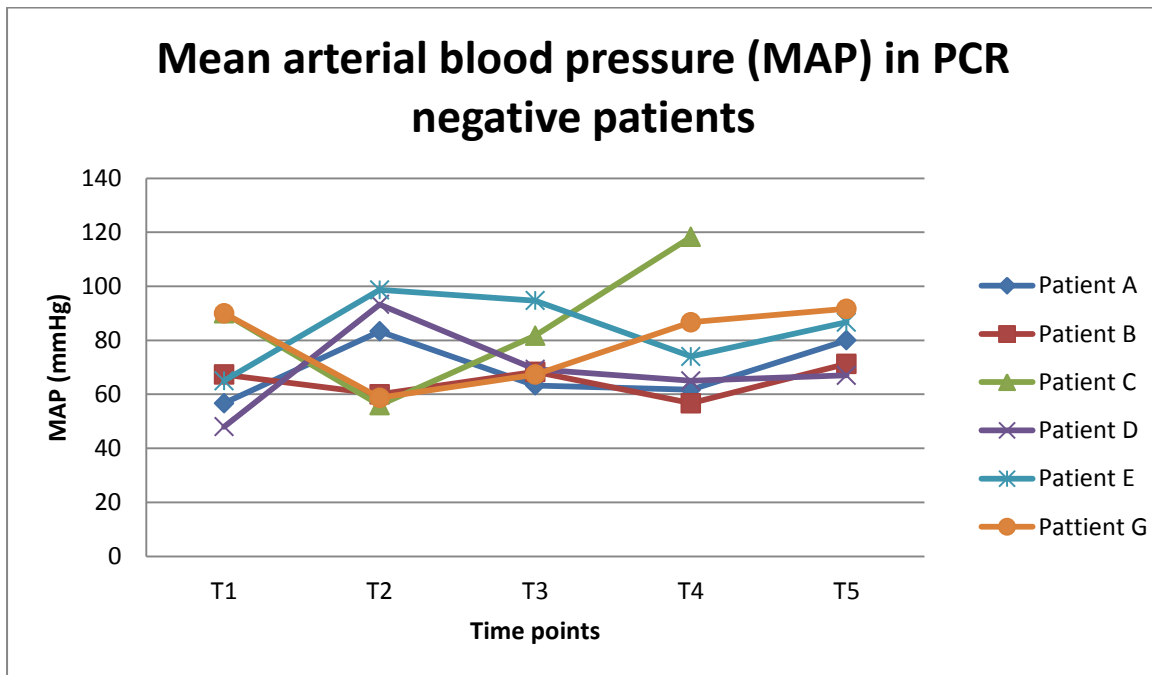


Figure 11 – In the 6 patients who were PCR signal negative, there is no clear variation in MAP with the time points.

Figure 12 - Variation of mean arterial blood pressure in PCR positive group

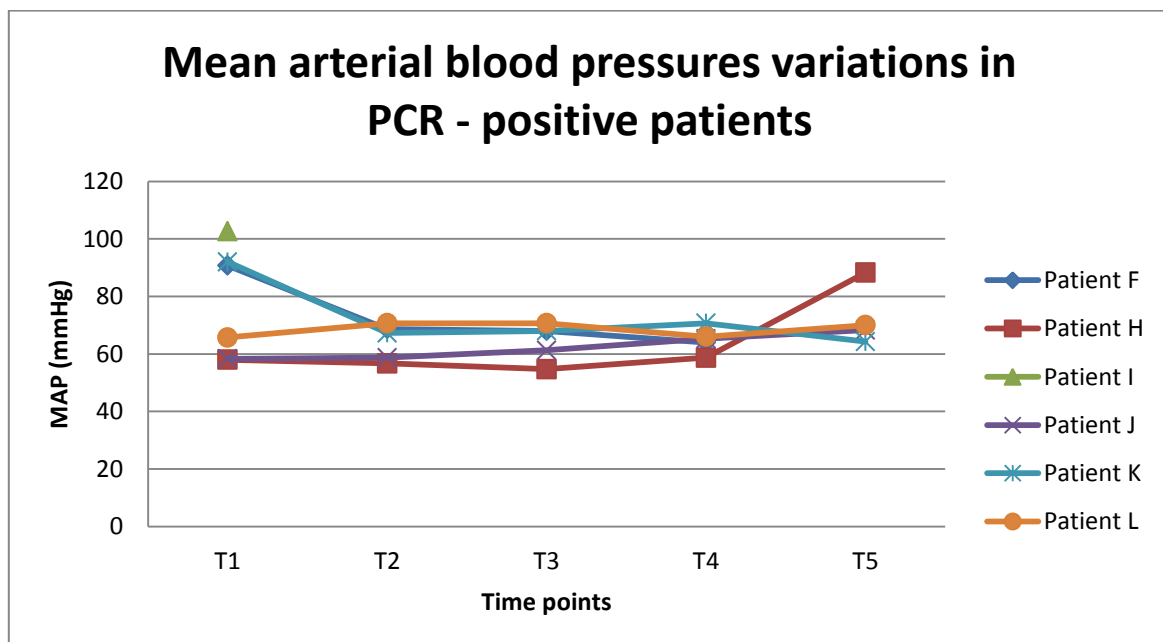


Figure 12 – The PCR signal positive patients have differing MAPs across the different time points with no clear pattern of change across the timepoints for each patient.

Figure 13 - Comparison of the variation of mean MAP between PCR positive patient group and PCR negative patient group

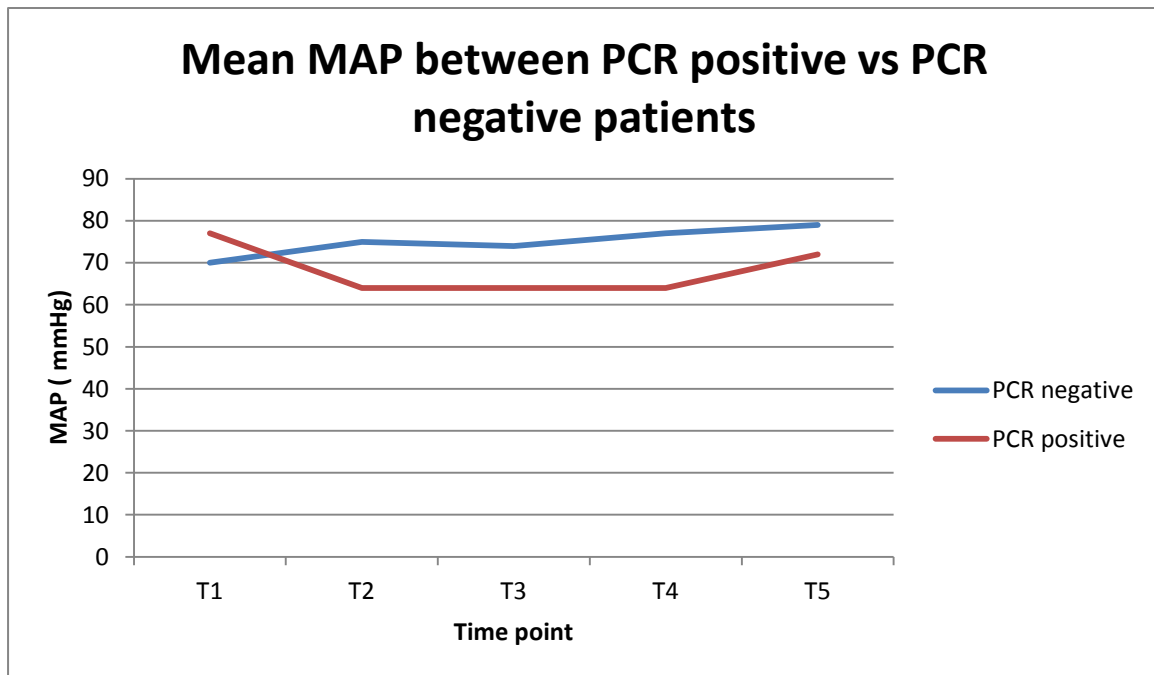


Figure 13 – The mean MAP at each time point showed no difference between patients with a positive signal and those patients with no signal detected.

The cardiovascular system response can be assessed by both MAP and heart rates. In the PCR negative group, there was a generalised high starting pulse rate at T1 amongst the PCR negative patients with a lower pulse rate recorded at T5 in these patients but not to a point of bradycardia (pulse rate below 60 beats per minute).

Figure 14 - Variations in pulse rate in PCR negative group

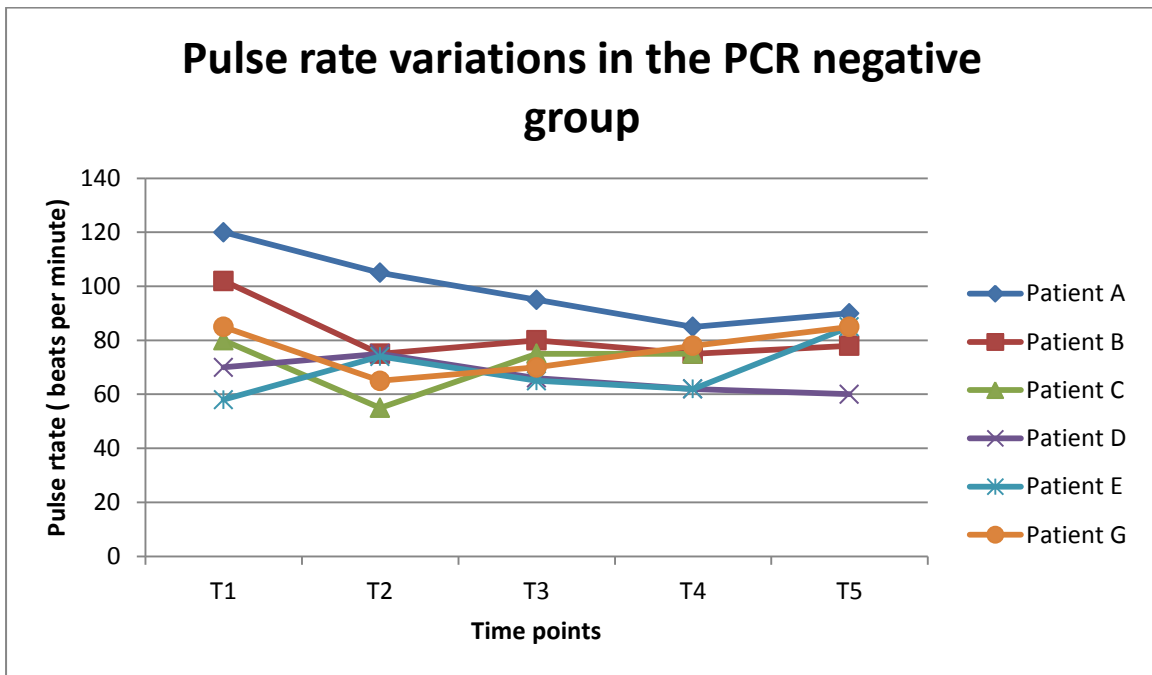


Figure14 – There was no consistent pulse variations in those who do not have DNA detected by PCR across the 5 time points.

In the PCR positive group, 3/6 patients exhibited a rise in their pulse rate with two patients exhibiting tachycardia (pulse rate higher than 100 beats per minute). However, there was no uniform or observed pattern to the change in pulse rate for either the PCR positive or PCR negative patients (Fig 16). This is highlighted when, from these results, there is no marked difference between the blood pressure and pulse responses seen between the PCR positive and the PCR negative group through the procedures.

Figure 15 - Variations in pulse rate in PCR positive groups

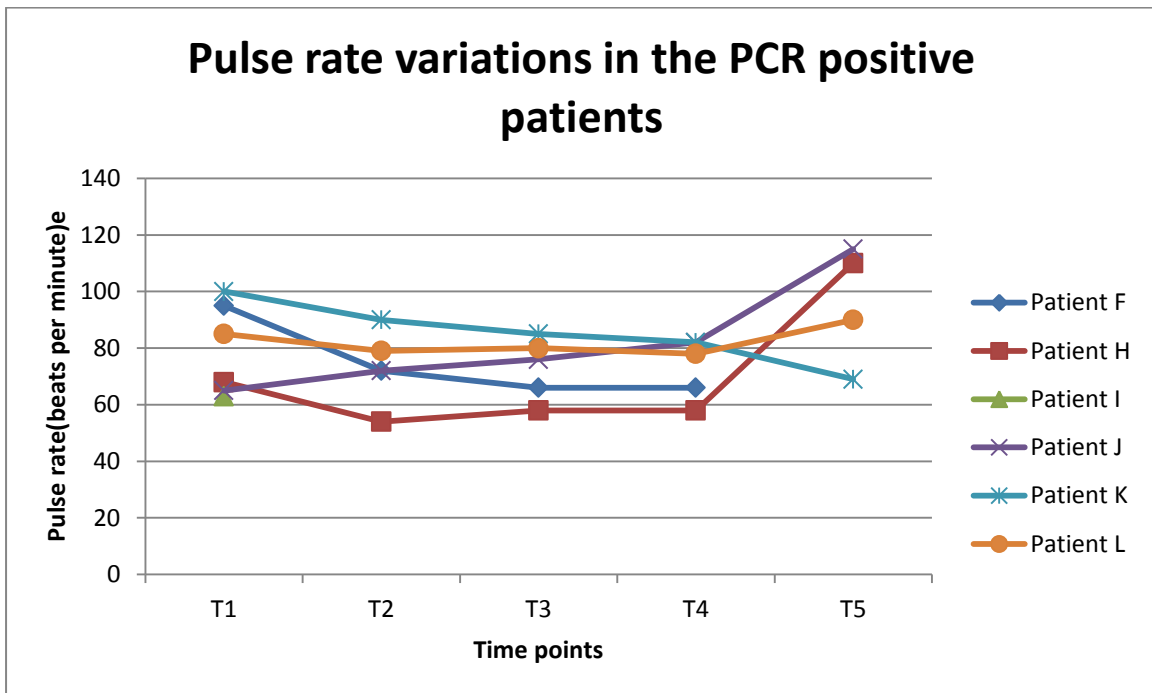


Figure 15 – In the 6 patients who had a PCR positive signal, there was no observed pattern across the patients in the response of their pulse rates across the different time points.

Figure 16 - A comparison of the variation between mean pulse rate between PCR negative patients and PCR positive patients

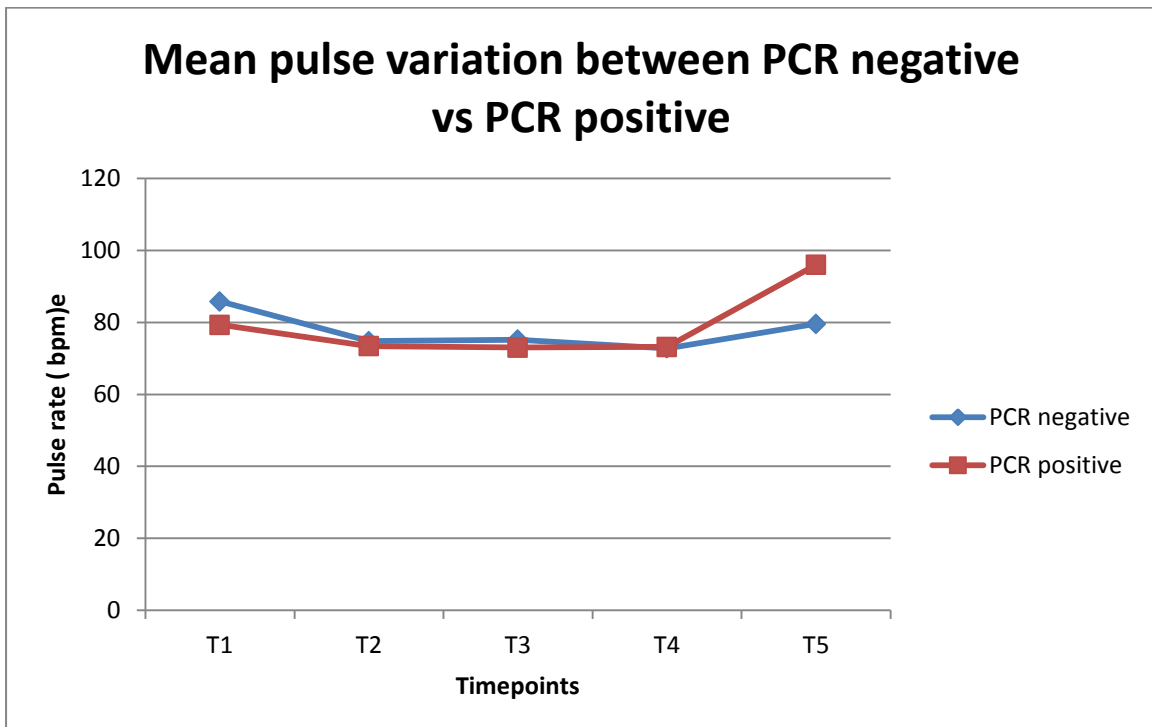


Figure 16 – This figure shows that there is no difference between the mean pulse rates at different time points between the two groups

Case specific findings - PCR positive patients

Case J had a positive blood culture and positive DNA signal at time point T3 and subsequently had a significant rise in temperature and pulse rate in recovery room post procedure. The patient became more unwell and deteriorated leading to sample at T4 and T5 not being taken. This patient progressed rapidly from a SIRS response to septic shock, and required admission to intensive care for organ support. Case J needed aggressive fluid resuscitation throughout the procedure and had a total length of stay in hospital of eight days. Case F had *Streptococcal sp* and *S pneumoniae* detected with the SeptiFast[®] assay. This patient experienced a steady drop in blood pressure across the five time points associated with a similar drop in pulse rate as well but no recorded bradycardia (pulse rate less than 60 beats per minute). Case H had a positive signal for *K.pneumoniae /oxytoca* by the SeptiFast[®] assay. Case F was administered metaraminol at T1 stage. It is a well-known phenomenon for blood pressure to be low due to the anaesthetics. Metaraminol is an alpha 1 adrenergic receptor agonist and is used by anaesthetists for this clinical indication.

Case specific findings - PCR negative patients

Patient A was hemodynamically unstable during the procedure. Patient A had a tachycardia (120 beats per minute) and a reduced MAP (56.7). At each of the first four time points, the patient received vasopressor therapy - ephedrine (T1 and T3) and metaraminol (T2 and T4). This patient, however, had no post operative complications and did not require hospital admission. The patient was both PCR and blood culture negative. This hemodynamic instability was not associated with an inflammatory response to a pathogen.

Patient B had a clinical episode of urosepsis and was readmitted for IV antibiotics treatment 12 days after the procedure. The patient had six units of blood loss through the procedure and required four litres of intravenous fluids. No vasopressor medication was administered during the procedure and the patient's total length of stay on readmission was four days.

Stone composition and pathogen detection

There is evidence in the literature that stones of certain compositions, such as struvite stones are associated with pathogens such as Klebsiella and Proteus. (Tseng et al, 2011)

These urease producing bacteria are well-recognised predisposing factors for stone formation (Tseng et al 2011). In our study there was a clear association between the detection of circulating pathogen DNA and the presence of calcium oxalate stones (Table 13). There was no clear evidence of any growth from the stone particles sent for analysis (Table 13). With the study population being so small, care needs to be taken from drawing conclusions and larger study would further prove if there is an association between stone composition and pathogen detection.

Table 13 - Stone composition and organism detection

Patient	Stone composition (percentage)	Record of culture from stone	Organism detected by blood culture	Organism detected by SeptiFast[®] analysis
A	Uric acid (99%)	No	Nil detected	Nil detected
B	Calcium phosphate (47%) Magnesium ammonium phosphate (53%)	No	Nil detected	Nil detected
D	Calcium oxalate (98%)	No	Nil detected	Nil detected
E	Calcium oxalate monohydrate (96%)	No	Nil detected	Nil detected
F	Calcium oxalate (98%)	No	Nil detected	Streptococcal <i>sp</i> <i>S.pneumoniae</i>
I	Calcium oxalate (97%)	No	Nil detected	<i>E. Faecalis</i>
L	Calcium oxalate (21%) Urate (79%)	No	Nil detected	<i>S.aureus</i>

CHAPTER 4
DISCUSSION

Background of the study

This is the first proof of concept study to propose that urological procedures involving instrumentation could be a reproducible model to study pathogenesis in man. If there was a better understanding of the role pathogens play in triggering the host immune response, we would be able to further our knowledge on the pathogenesis of infection and sepsis. Sepsis is clinically defined when a patient shows at least two of the criteria for systemic inflammatory response (SIRS) with evidence of systemic infection (Mancini et al, 2010).

The systemic inflammatory response (SIRS) is activated in patients in response to either a non-infectious (e.g. tissue injury, trauma) or an infectious aetiology; however, it is not uncommon for both forms of aetiology to co-exist. The difficulty from a diagnostic point of view is determining when the inflammatory response is caused by infection (i.e. sepsis).

Blood cultures are the current gold standard for microbial detection in the blood stream.

Blood culture analysis is often unreliable, particularly where patients have received antimicrobial therapy. Recent advances in molecular diagnostics allow the measurement of DNA from pathogens in clinical samples (i.e. blood). These approaches are less likely to be affected by antibiotic use and may have great benefit in the diagnostic process.

Currently, the relationship between circulating pathogen DNA, the presence of infection and the impact on the systemic inflammatory response is not fully understood. There have been a number of both animal and human models of sepsis described in the literature. In this thesis, I propose that an easily reproducible model for exploring the relationship between circulating pathogen DNA and the subsequent inflammatory response could be urological procedures involving instrumentation.

It was initially thought that, endourological surgery were considered clean contaminated procedures and did not require antibiotic coverage. A clean-contaminated surgical site is seen

when the operative procedure enters into a colonized viscus or cavity of the body, but under elective and controlled circumstances. However, evidence in the literature dating back decades shows that urological instrumentation is associated with increased incidence of urinary tract infection and bacteraemia (Alsaywid et al, 2013).

Infections and urosepsis is one of the most common complications seen in urological procedures. The rate of infection complication in all urological procedures in the literature is as high as 12 - 14 % in the literature (Bjerklund et al 2007). Following ureteroscopy, the reported incidences of UTI range between 3.9% and 25%. (Rao et al, 1991, Puppo et al, 1991, Hendrickx et al, 1999).

A number of studies have previously highlighted the significant inflammatory response in patients undergoing endourological manipulations for urinary stones. A frequently referenced study on this was published by Rao et al in 1991 (Rao et al, 1991). In an effort to predict septicaemia following endourological manipulation for stones in the upper urinary tract, 117 patients were studied and classified according to the procedure performed (Rao et al, 1991). In this study, fever was used as a proxy for infection and 74% of the PCNL patients in their study developed post-operative fever; however, only 41% had endotoxemia. The only patient in the Rao's study to develop septic shock had a PCNL.

Antibiotic prophylaxis is commonly used perioperative in urological surgery and procedures to alleviate the occurrence of infection. Antibiotic prophylaxis aims to prevent healthcare-associated infections that result from diagnostic and therapeutic procedures (Grabe et al, 2012). I have highlighted the prevalence and incidence of infection and sepsis in those undergoing endourological procedures however regarding evidence of countering infection with antibiotic prophylaxis, there is no clear-cut evidence that exists on the use of antibiotic prophylaxis in this population (Knopf et al, 2003, Fourcade et al, 1990).

In this study, ureteroscopy and PCNL are the two urological interventions which may be useful *in vivo* models for investigating pathogenesis in man. If this model shows that there is a relationship between the presence of a detectable level of circulating pathogen DNA and a witnessed immune and physiological response, this would suggest a possible beneficial need for the use of prophylactic antibiotics. In this study, there was evidence of a relationship between the levels of cytokines particularly IL - 6 and the presence of circulating pathogen DNA, however in this study the only patient to develop severe sepsis (to prompt an admission to ICU) did not receive antimicrobial to cover the causative organism i.e. fungi. As a result of our study I therefore propose that a randomised controlled trial on antimicrobial prophylaxis in this population is warranted.

Current international guidelines state that it is important to distinguish urological interventions into low-risk procedures (e.g. simple diagnostic and distal stone treatment) and higher-risk procedures (e.g. treatment of proximal impacted stones and intrarenal interventions) when considering prophylactic antibiotics (Grabe et al, 2012). In higher-risk procedures, there would be more of an indication to use antibiotic prophylaxis.

In this study, all patients received some form of antibiotic prophylaxis; however, the antibiotic regimen was variable 25% of the study population (3/12 patients) went on to develop an infective episode despite the use of prophylactic antibiotics. However, it is hard to provide strong arguments against prophylactic antibiotics in a study of this size with difference in the choice of antibiotics, doses and time of administration of antibiotics.

In a recent worldwide study, (Gutierrez et al, 2013) 5803 consecutive PCNL patients were enrolled from 96 centres over 12 months to participate in the PCNL Global Study. The presence of a post-operative fever of $\geq 38.5^{\circ}\text{C}$ was used as a proxy for infection in this study

and the main finding was that a10% of PCNL-treated patients developed fever in the post-operative period despite receiving antibiotic prophylaxis.

In this proof of concept study, my aim was:-

1. To appraise the presence and significance of blood borne microorganisms that may appear in patients during interventional urological surgery involving urinary tract instrumentation by (blood culture and by multiplex real time polymerase chain reaction assay (SeptiFast[®] Roche).
2. To appraise the host immune response and physiological response in the study population to the presence of circulating pathogen DNA and if this provides evidence for the use of antibiotics prophylactically in these patients.

On-going research using PCR technology in an *in vivo* human model of pathogenaemia could help determine if PCR technology has a role in the detection of circulating pathogen DNA in infective and septic patients. PCR technology could supersede the current gold standard of pathogen detection in blood in clinical settings and provide rapid detection of pathogen regardless of whether antibiotics have been administered.

Key findings of the study

The key findings from the study are that the SeptiFast[®] assay had broad pathogen coverage for this study population. In the group of patients with circulating pathogen DNA detected by SeptiFast[®], there was a clear immune response elicited (elevated cytokine levels) but no witnessed physiological response in this group, when compared with patients with no detectable circulating pathogen DNA.

SeptiFast[®] - Role in circulating pathogen DNA detection in infection ?

The SeptiFast[®] assay was chosen as the assay to test the study hypotheses. The SeptiFast[®] assay has been available longer than any other molecular-based tests. SeptiFast[®] uses real-time PCR in a non-quantitative mode to identify ten bacteria at the species level, several more at the genus level, as well as five *Candida* species and *Aspergillus fumigatus*. This assay reportedly identifies the 25 organisms that account for more than 90% of the culturable pathogens associated with sepsis (Ecker et al, 2010).

Prior to commencing this study, a review of the collected historical data on urological procedures at Salford Royal Foundation Trust urology services show that the SeptiFast[®] assay has complete coverage of all the pathogens present in urine cultures commonly seen in the urology patients treated at Salford Royal. This confirms the suitability of this potential diagnostic tool in this study population. Notably, the SeptiFast[®] assay did not detect any circulating pathogen DNA from a species not normally seen in this clinical setting.

In our study, 1 patient (9%) had a positive growth from blood culture and the organism detected was *Candida glabrata*. This figure is well within the range for rate of positive cultures associated with urological surgery in the literature (Knopf et al, 2003, Dogan et al, 2002). This patient was admitted to intensive care for organ support due to urosepsis. In a number of papers looking specifically at those with urosepsis after endourological intervention for stones, blood cultures tend not to be positive and sepsis rates are 0.25 - 1.5 % (Demirtas et al, 2012, Dogan et al, 2002). Interestingly, with studies looking specifically at PCNL, between 0.3 and 9.3% of patients can develop potentially life-threatening sepsis (Michel et al, 2007, Draga et al, 2009). In this proof of concept study, we have assumed that PCNL and ureteroscopy produce similar results and further work would need to be done to establish if this assumption is valid.

In our thesis, there is a low level of blood culture positivity compared to PCR positivity from the SeptiFast[®] assay and there are a number of possible explanations for this observation. A proposed reason for this finding could relate to the administration of antibiotics pre procedure which can affect the detection of pathogen by blood cultures. In our study, out of the four patients in the study who had infective and / or septic episodes post procedure, 50% were PCR positive blood culture negative, 25% were PCR positive blood culture positive. Blood cultures are reported to be negative in more than 50% of the cases where true bacterial or fungal sepsis is believed to exist (Ecker et al, 2010). A theoretical explanation between the difference in circulating pathogen DNA detection (by SeptiFast[®]) and intact pathogen detection (by blood cultures) could relate directly to the urological procedure. In these endourological procedures, the use of instrumentation could lead to the introduction of fragments of pathogen DNA particles into the blood stream rather than intact pathogen.

SeptiFast[®] - A place in clinical practice?

There are no described studies in the literature on the use of the SeptiFast[®] assay in the setting of urological surgery. There have been a number of studies looking at the use of SeptiFast[®] assay in other clinical settings. In a recent Danish study (Westh et al, 2009) SeptiFast[®] was compared directly against blood cultures in patients with suspected bacterial and fungal sepsis. The aim of the study was to observe if non viable pathogen detection by SeptiFast[®] would aid in the clinical decision making process. The group postulated that one advantage of PCR assays over blood cultures is that DNA based detection systems can detect pathogens causing sepsis at the time of sampling if they are viable or non viable.

558 samples from 359 patients were evaluated. The rate of positivity was 17% from blood cultures (BC) and 26% from SeptiFast[®] assay. 96 microorganisms were isolated with BC, and 186 microorganisms were identified with SeptiFast[®]; 231 microorganisms were found by combining the two tests. Of the 96 isolates identified with blood culture, 22 isolates were

considered to be contaminants. Of the remaining 74 non-contaminant BC isolates available for comparison with SeptiFast[®], 50 were identified as a species identical to the species identified with SeptiFast[®] in the paired sample. Of the remaining 24 BC isolates for which the species, identified in the BC, could not be detected in the paired SeptiFast[®] sample. 18 BC isolates were identified as a species included in the SeptiFast[®] masterlist, and six blood culture isolate were not organism detected by SeptiFast[®]. With SeptiFast[®], 186 microorganisms were identified, 12 of which were considered to be contaminants. Of the 174 clinically relevant microorganisms identified with SeptiFast[®], 50 (29%) were detected by BC. More than half of the remaining microorganisms identified with SeptiFast[®] (but not isolated after BC) were also found in routine cultures of other relevant samples taken from the patients.

Dierkes et al (Dierkes et al, 2009) performed a retrospective analysis of PCR results on the clinical management of patients with presumed sepsis. Of the 101 blood samples from 77 patients with presumed sepsis, 39 samples had pathogens identified from either the use of blood cultures or the SeptiFast[®] assay. From the samples, 63 (62%) yielded concordant negative results, 14 (13 %) concordant positive and 9 (9%) were blood culture positive only. In 14 (13%) samples pathogen was detected only by PCR assay, resulting in adjustment to therapy in five patients. In three samples a treatment adjustment would have been made earlier resulting in a total of 8 adjustment in all 101 samples (8%). In this single centre study with small number, the group have suggested that rapid molecular diagnostic tests may lead to a higher rate of early adequate antibiotic therapy in approximately 8% of patients with suspected sepsis. This could have had a potential therapeutic impact on clinical outcomes.

The SeptiFast[®] assay ability for pathogen detection has also been used in onco-haematological patients (Mancini et al, 2008). These patients are susceptible to neutropenia. The term 'neutropenia' describes low levels of neutrophils in the blood. Neutrophils are

important in fighting bacterial infections. Among the 103 blood samples, 35 (34%) samples were positive by at least one of the two methods. Twenty-one (20.4%) samples were positive by blood culture and 34 (33%) with SeptiFast[®]. The analysis of concordance evidenced a low correlation between the two approaches. When comparing the six hour turnaround time for the SeptiFast[®] assay with blood cultures, even in the two observed cases of fast-growing *Escherichia coli*, a mean of 10 h of incubation was needed, with definitive identification after an additional 36 h (Mancini et al, 2008). As evident from these three separate studies, there is potential in the utilisation of the SeptiFast[®] assay in the management of infection. In this thesis, one of our main hypotheses was to ascertain if the detection of circulating DNA by the SeptiFast[®] assay was an indicator of infection.

In this study, two out of the three patients who went on to have an infective or septic episode only had an organism detected by the SeptiFast[®] assay alone and not by blood cultures. Only one patient who was PCR signal and blood culture negative, went on to have a clinical episode of urosepsis. Half of the patients in the study with a positive signal for an organism detected by the SeptiFast[®] assay did not go on to develop an infective or septic episode. In this small study, there was a low level of blood culture positivity and I was unable to do a direct assessment of concordance between PCR assay and blood cultures as diagnostic tools. In the study, the one patient who had a fulminant fungal septicaemia gave a positive signal via the SeptiFast[®] assay and had a positive BC.

In this study, the presence of detectable DNA seem to stimulate an inflammatory response. The clinical relevance of this is that potentially we have a technology which will greatly speed up the identification of pathogenic organisms and facilitate the prompt and targeted treatment in patients with sepsis and infection.

Using blood culture as a reference ‘gold standard’ to compare molecular methods has been shown to be problematic in the literature. It is well recognised that blood culture fails to identify more than 50% of the cases of sepsis believed to be caused by bacteria or fungi based on clinical and other criteria (Ecker et al, 2010). In most studies, SeptiFast[®] consistently identified more positive specimens than blood culture methods (Pletz et al, 2011) and this was seen in this study. An obvious limitation of the SeptiFast[®] as a diagnostic tool is that the assay does not provide information about antimicrobial susceptibilities of the organisms detected.

In this study, half of the cases with positive PCR signal can be classed as ‘false’ positive results. The definition of PCR ‘false’ positives is those cases in which the presence of pathogen DNA is associated with no growth in simultaneous blood cultures. In this study, our controls were those who were negative for DNA signal. The lack of normal control group makes it difficult to assess the SeptiFast[®] assay with respect to false positive outcomes. There has however been a recent study (Warhurst et al, 2015) which has set out to determine the accuracy of SeptiFast[®] real-time PCR for the detection of health care associated BSI against standard microbiological culture.

Of 1006 new patient episodes of systemic inflammation in 853 patients, 922 (92%) met the inclusion criteria and provided sufficient information for analysis. Adult patients had been exposed to a median of 8 days (interquartile range 4–16 days) of hospital care, had high levels of organ support activities and recent antibiotic exposure. SeptiFast[®] real-time PCR, when compared with culture-proven bloodstream infection at species/genus level, had better specificity (85.8%, 95% CI 83.3% to 88.1%) than sensitivity (50%, 95% CI 39.1% to 60.8%). When compared with pooled diagnostic metrics derived from their systematic review, their clinical study revealed lower test accuracy of SeptiFast[®] real-time PCR, mainly as a result of low diagnostic sensitivity. There was a low prevalence of BC-proven pathogens in these

patients (9.2%, 95% CI 7.4% to 11.2%) such that the post-test probabilities of both a positive (26.3%, 95% CI 19.8% to 33.7%) and a negative SeptiFast[®] test (5.6%, 95% CI 4.1% to 7.4%) indicate the potential limitations of this technology in the diagnosis of bloodstream infection. Using this analysis approach, the sensitivity of the SeptiFast[®] test was low but also appeared significantly better than BC. Blood samples identified as positive by either culture or SeptiFast[®] real-time PCR were associated with a high probability (> 95%) of infection, indicating higher diagnostic rule-in utility than was apparent using conventional analyses of diagnostic accuracy. In my study, all of the urology patients were subject to sterile injury, had evidence of systemic inflammation and had prophylactic antibiotics administered so the data from the aforementioned study (Warhurst et al, 2015) data could be applied to our population. The presence of false positive patients poses more questions than answers regarding the detecting of circulating DNA by the SeptiFast[®] assay as a potential indicator of infection. Avolio et al (Avolio et al, 2010) compared traditional blood cultures with SeptiFast[®] in cases suspected to have blood borne infection. There were 10 patients who had an organism detected by SeptiFast[®] but not by blood culture that subsequently had microbiology confirmation from other sites. It would be important, therefore, to differentiate between DNA associated with intact pathogens from free pathogen DNA detected by PCR, if pathogen DNAemia is to be a marker of infection. PCR analysis may be a sensitive method of detecting pathogen DNA released in to circulation at levels which would:

1. Not be detected by sampling volumes obtained for blood cultures;
2. From sites with restricted or poor blood flow.

Importantly, PCR assays are not affected by antimicrobials given (Ecker et al, 2010, Mancini et al, 2010).

A separate issue raised by false positive cases is that, presently, we cannot ascertain through the assays if the circulating pathogen DNA detected is associated with viable bacteria rather than non-viable bacteria destroyed by host immune system +/- antimicrobial therapy. So we cannot, in this study, definitely say that the detected circulating pathogen in the case which was PCR signal positive and blood culture negative was acting as a pathogen.

In the study amongst all the patients who were PCR signal positive, a positive signal for pathogen DNA was detected at all time points. However, it was often noted that it was only present transiently in each of the six PCR positive cases. There are a number of likely possibilities for these findings. Firstly, there is the possibility that pathogen DNA is only found fleetingly in circulation. A second viable explanation is that circulating pathogen DNA levels may be low or close to limits of detection by the SeptiFast[®] assay so that in some of the cases DNA may not have been detected even though present. Thirdly, sampling may be an issue and a larger study population may help to explain this.

SeptiFast[®] consistently identified more positive specimens than blood culture methods; however, the seminal paper on SeptiFast[®] (Lehmann et al, 2008) showed that culture consistently identified some organisms that were not identified by SeptiFast[®]. There are a number of possible explanations such as the larger volume of blood analysed by culture and the lower limit of SeptiFast[®] detection of approximately 3–30 CFU/ml. Ideally, we would want to use as sensitive a PCR assay as possible and, presently, the most sensitive is the SeptiFast[®] assay.

Circulating pathogen DNA and the immune response

In this study, there was a strong correlation between the detection of circulating pathogen DNA and increased level of IL - 6. These results suggest that in these urology patients there is a positive association between the presence of circulating pathogen DNA and the witnessed

immune response. However, there was no direct association between circulating DNA and the physiological response in these patients.

It has been shown that bacterial - derived short DNA fragments are able to stimulate immune cells to promote the release of IL-6 from human mono nuclear cells (Schindler et al, 2004). In this study, we have shown an association between the presence of circulating pathogen DNA and increased IL- 6 detected levels during urological procedures in this study population. In our study, cytokine levels were reviewed over the period of the procedure and in post operation (Mokart et al, 2002). In this study, serial blood samples were collected from 30 consecutive patients for determination of serum cytokine levels. Healthy volunteers were used as the control group. Eleven patients developed no complications (group 1), 14 developed sepsis or severe sepsis (group 2), and five developed septic shock (group 3).

After operation IL-6 levels in group 1 were increased in comparison with day 0 but normalized with time, suggesting that surgical trauma induced the immediate and temporary postoperative increase in IL-6 concentration. However, on day 1, patients in groups 2 and 3 had IL-6 levels that were respectively, two and six times higher than those in group 1 patients. In addition, they remained high during the course of the study. These high levels of IL-6 were independently associated with postoperative septic events and correlated with post-operative morbidity (length of ICU stay and duration of mechanical ventilation). As septic events always occurred after day 2 the group it suggested that, IL-6 is probably an early indicator of post-operative infection following the trauma of major oncological surgery (Mokart et al, 2002). It would be interesting to use our study population to confirm these findings.

The findings in this thesis suggest that the detectable presence of pathogen DNA could stimulate an inflammatory response. In the literature, there have been advances in our

knowledge on how the cells of our immune system detect microbial pathogens. The ability of the host's immune system to sense nucleic acid is one such mechanism which has been extensively studied. For detecting microbial DNA, toll - like receptor 6 (TLR6) in endosomes and numerous cytoplasmic DNA binding proteins have been discovered (Holm et al, 2013).

Toll-like receptors (TLRs) are a class of pattern recognition molecules that play a unique function in the innate immune system. This system is the first line of defence against microorganisms that initiate cellular signal in response to pathogen-associated molecular patterns (PAMPs) and induce expression of genes involved in the inflammatory process, therefore, it plays a crucial role initiating and directing the adaptive immune system (Jahantigh et al, 2013).

Ten functional TLR members (TLR1–TLR13) have been identified in humans. TLR9, an endosomal localized receptor on B cells, plasmacytoid dendritic cells (pDCs), and monocytes/ macrophages, recognizes unmethylated nucleic acid motifs, especially Cytosine-phosphate-Guanine motifs, in bacterial DNA and it is one of the most important receptors in the initiation of protective immunity against intracellular pathogens by activation signalling cascade of intracellular receptor signalling (Jahantigh et al, 2013). From our study, we detected similar DNA levels in the patients with a positive signal from the SeptiFast® study; however, we do not currently know whether this level of pathogen DNA could activate the TLR9.

The urology patient: Host response, sepsis and antibiotic prophylaxis

This study is not the first to look at the host inflammatory response and sepsis in urology patients. Rao et al's (Rao et al 1991) study is one of the most cited studies in urology that looked directly at systemic inflammatory responses and sepsis in patients undergoing

urological manipulation. In the individuals in this study who had PCNL, despite use of a prophylactic antibiotic, postoperative bacteraemia and fever are reported as 37% and 74%.

Antibiotic prophylaxis aims to prevent healthcare associated infections that result from diagnostic and therapeutic procedures. Antibiotic prophylaxis is only one of several measures to prevent infections and can never compensate for poor hygiene and operative technique.

The principle of antibiotic prophylaxis is to provide protection to the patient with creating an environment to promote antibiotic resistance (Grabe et al, 2012). Unfortunately, the benefit of antibiotic prophylaxis for most modern urological procedures has not yet been established by well-designed intervention studies (Grabe et al, 2012). Antibiotic prophylaxis in urology has been controversial for many years. In the literature regarding PCNL case studies, there is conflicting evidence for the role of prophylactic antibiotics evidence in PCNL procedures.

Charton et al (Charton et al, 1986) reviewed 126 cases of percutaneous extraction of renal stones by PCNL that no major septic complication was observed without prophylaxis. Only 10% of the patients were exposed to fever and 35% had bacteriuria. Mariappan et al (Mariappan et al, 2006) presented results showing that treating patients who have dilated pelvicalyceal systems and / or stones of ≥ 20 mm before PCNL with ciprofloxacin 250 mg twice daily for 1 week significantly reduces the risk of upper UTI and urosepsis. Eighteen of 46 (39%) patients in the control group developed SIRS, whereas only 7 of 52 (13.4%) patients in the treatment group developed SIRS. Mariappan's group reported that one-week administration of ciprofloxacin prophylaxis decreased positivity of pelvicalyceal culture by three times, stone culture positivity by two times, and risk of developing SIRS by three times.

In this study, circulating pathogen DNA is definitely seen to stimulate a significant inflammatory response in this urology population. Within the study, a quarter of our patients

went on to develop an infective episode despite the use of prophylactic antibiotics. So, in this proof of concept study, even though we have been able to appraise the host immune response to the presence of circulating pathogen DNA the study cannot put forward evidence for the use of antibiotics prophylactically in his population. This study has not provided concrete evidence to support the use of prophylactic antibiotics in this group but has stimulated enough questions to suggest the need to design a RCT in this population. The model may be a useful system to explore this but we would need to look in a larger study population with set antibiotic protocol regarding antibiotic use, dose administered and time of antibiotic administration during the procedure.

It is widely appreciated that the presence of urinary stones is a risk factor for infectious complication in urological procedures and is associated with increased bacterial load (Grabe et al, 2012). There has also been literature which has looked at the association of the composition of the stones and infection (Tseng et al, 2011). In our study, there was no association between stone composition and the circulating pathogen DNAemia.

Strength and weakness of the study

The major strength of this proof of concept study is that even with this small number of patients we have been able to address the hypotheses above and have provided results which suggest that this study should be scaled up in regards to study number to find if these results are reproducible in larger cohorts. Compared to animal study, this *in vivo* study gives us results in the clinical setting which is a strength of the study.

There were limitations in this study. In obtaining the ethics for this study, our study size was targeted to a small number of 12. The assay cost are high and not in routine use and it was decided when obtaining our ethics approval that a first in man proof of concept study of this

size was deemed acceptable. However, with such a small number, it can be difficult to ascertain the true significance from the data obtained in our study.

In our group of 12, there were two patients who had PCNL, not out of design, but due to the nature of the cases encountered. Prior to the study commencing discussions were had with the urology department (personal communication with Mr Chris Betts, consultant urologist) and it was felt that the similar surgical insult between the two urological procedures would likely produce a comparable inflammatory response. If the study was scaled up, it would be ideal to compare these procedures head to head to prove this postulation.

In regards to other weaknesses to the study, it would have been ideal to compare urinary samples for growth to see if there was any concordance with Septifast[®] assay. The fact that a complete database of results was not present on all patients slightly weakens the findings presented.

Future work

This proof of study has set up the potential for further work.

It is important to take this work forward by using the same study design but to recruit enough patients so the study is well powered to:

1. Show that the findings in this study are reproducible;
2. Have sufficient numbers to show if the observed findings are truly statistically significant.

Hopefully, in future studies we could test if there is concordance between the detection of circulating pathogen DNA and other microbiological samples (e.g. mid-stream urine samples peri - operatively or pathogens in stone debris). In future testing, it would be good to explore the use of prophylactic antibiotics in this model by having patients split into an arm having a prophylactic antibiotics pre procedure and an arm which does not receive prophylactic

antibiotics. It would show if there is any effect of the antibiotics on pathogen detection by blood culture and SeptiFast[®] and if the immune response effects seen are different. This would add further evidence on the role of prophylactic antibiotics in this population.

Conclusion

This was a proof of concept study looking to see if urology patients having instrumental stone removal procedures could potentially be an *in vivo* model for exploring pathogenaemia in humans. The data from this study provides novel evidence that presence of pathogen DNAemia correlates with an increased systemic inflammatory response. This may be a useful model system for understanding the role of pathogen DNA in triggering inflammatory responses to infection in man. Additionally, further studies using this model may address the issue of the role of antibiotic prophylaxis in endourological procedures. However, larger studies are needed for this work to progress further.

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