Development of a Rapid Method to Aid in the Diagnosis of Catheter-Associated Infections

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Abstract

One of the most common complications associated with the use of central venous catheters (CVCs) is catheter-associated infection. Traditionally, when an infection is suspected, the CVC is removed and replaced, with the CVC tip being retrospectively analysed. Techniques that involve sampling of the CVC whilst it remains in situ, are hindered by the requirement for conventional laboratory culture of the sample for diagnosis. Some more rapid methods of detecting microorganisms have been developed but none are routinely used CVC management. The aim of this study was to develop a rapid method, which could potentially aid in the diagnosis of catheter-associated infections whilst the CVC remains in situ.

The FAS Endoluminal Brush (FASEB)-retrieved sample has high predictive values for the confirmation or elimination of a catheter-associated infection and it was chosen as the sampling device for use in this study. ATP bioluminescence was chosen as a possible detection technique for use in conjunction with FASEB-retrieved samples.

Much of the development involved, the exposure and degradation of non-microbial ATP and the subsequent lysis and detection of microbial ATP. A TCA-based extraction method was found to be of little clinical utility when evaluated with FASEB samples retrieved from 114 CVC tips. Further work involving a DTAB-based extraction method in combination with α-cyclodextrin, resulted in a sensitivity and specificity of 70.6% and 86.7% respectively, in combination with plate culture, when challenged with 47 FASEB-retrieved samples.

This study indicates that ATP bioluminescence is a useful technique that may have an application for use in conjunction with FASEB-retrieved samples. However, further work is required to improve upon the sensitivity and specificity of the technique, with the main emphasis being on the sample preparation so that contaminating ATP is degraded and all microbial cells are exposed to the microbial lysis agent prior to ATP detection.
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<th>Definition</th>
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<tbody>
<tr>
<td>5'-IMP</td>
<td>Inosine monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ADPase</td>
<td>Adenosine 5'-diphosphatase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AOLC</td>
<td>Acridine orange leucocyte cytospin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine 5’-triphosphatase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td><em>C. albicans</em></td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous ambulatory peritoneal dialysis</td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for Disease Control</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CNS</td>
<td>Coagulase negative staphylococci</td>
</tr>
<tr>
<td>CRBSI</td>
<td>Catheter-related bloodstream infection</td>
</tr>
<tr>
<td>CRI</td>
<td>Catheter-related infection</td>
</tr>
<tr>
<td>CRS</td>
<td>Catheter-related sepsis</td>
</tr>
<tr>
<td>CVC</td>
<td>Central venous catheter</td>
</tr>
<tr>
<td>Diluent C</td>
<td>Supplied reagent for reconstitution of the lyopholised SL reagent</td>
</tr>
<tr>
<td>Diluent E</td>
<td>Supplied reagent for reconstitution of the lyopholised HS reagent</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin, Landau, Verway, Overbeek</td>
</tr>
<tr>
<td>DTAB</td>
<td>Dodecyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DTTP</td>
<td>Differential time to positivity</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EC</td>
<td>Used in enzyme classification, for example apyrase (EC 3.6.1.5)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>FASEB</td>
<td>FAS Endoluminal Brush™</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GWX</td>
<td>Guide wire exchange</td>
</tr>
<tr>
<td>HICPAC</td>
<td>Hospital Infection Control Practices Advisory Committee</td>
</tr>
<tr>
<td>HSLs</td>
<td>N-acyl homoserine lactones</td>
</tr>
<tr>
<td>HS reagent</td>
<td>“High sensitivity” luciferin-luciferase reagent</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
</tbody>
</table>
IV Intravenous
L.1 luminometer Mediators L.1 tube luminometer
MgCl₂ Magnesium chloride
MRSA Methicillin-resistant *Staphylococcus aureus*
PBS Phosphate buffered saline
PBW Phosphate buffered water
Pi Inorganic phosphate
PIA Polysaccharide intercellular adhesin
PICC Peripherally inserted central catheter
PSA Capsular polysaccharide adhesin
*Ps. aeruginosa* *Pseudomonas aeruginosa*
PV⁺ Positive predictive value
PV⁻ Negative predictive value
QBC Quantitative blood culture
RLU Relative light unit
*S. aureus* *Staphylococcus aureus*
*S. epidermidis* *Staphylococcus epidermidis*
SCV Small colony variant
SD Standard deviation
SEM Scanning electron microscopy
SL reagent “Stable light” luciferin-luciferase reagent
Suramin 8-(3-benzoamido-4-methylbenzamido) naphthalene-1,3,5-trisulfonic acid
TCA Trichloroacetic acid
TNF Tumour Necrosis Factor
TNTC Too numerous to count
TPN Total parenteral nutrition
Tris Tris(hydroxymethyl) aminomethane
Triton X-100 t-Octylphenoxypolyethoxyethanol
VAD Vascular access device
VOP Variable orifice probe
AIMS & OBJECTIVES
Context of Study

When a CVC is suspected to be involved in a bloodstream infection, it is often removed from the patient and retrospectively analysed. Studies have shown that up to 80% of CVCs that are removed from catheterised patients on suspicion of being implicated in an episode of bloodstream infection are removed unnecessarily (Kite et al. 1997, Padberg et al. 1981, Pettigrew et al. 1985, Ryan et al. 1974). Catheter removal is generally based on the clinician’s judgement as to whether the clinical signs of fever are related to the CVC. Various groups have attempted to develop methods that involve the sampling of a CVC whilst it remains in situ within the patient, thus obviating CVC removal. One such group (Markus and Buday 1989) developed a device known as the FASEB that could be used to sample the internal lumen of a CVC whilst it remains within the patient. FAS Medical Ltd, UK, have carried out further developed work on this device, achieving FDA and CE mark approvals, in addition to proving its efficacy in independent clinical studies (Dobbins and Kite 1999, Kite et al. 1997). However, FASEB use has not become routine in contemporary catheter management, as one of the major objections to its use is the fact that the CVC will remain within the patient until the FASEB-retrieved sample is cultured. Sample culture often requires 24 hours and therefore the CVC would need to be left in situ overnight. In reality, this rarely happens, as the Consultant in charge of the patient will initiate treatment on the basis of clinical judgement. Therefore the culture result from the FASEB-retrieved sample is often retrospective.

Current techniques used to rapidly detect the presence of microorganisms associated with CVCs (Blot et al. 1999, Kite et al. 1999) are not routinely used in clinical microbiology laboratories. Consequently, there is a requirement for an adequate rapid technique, which should not require CVC removal from the patient (Farr 1999). In order to meet this requirement, the directors of FAS Medical Ltd formed a strategy to develop a rapid laboratory-based method that could be used in conjunction with a FASEB-retrieved sample. Through an agreement between the RCPEH at the University of Surrey and FAS Medical Ltd, the present study was conducted with research and development work carried out in an attempt to achieve the goal of developing a rapid test for catheter-associated infections. This study was funded by FAS Medical Ltd, with clinical samples supplied by St George’s NHS Trust, London.
Aims & Objectives

Aims of this study

The overall aim of this study was to develop a laboratory-based technique to rapidly detect microorganisms that have colonised a CVC without having to remove that CVC. Such a technique could be used to diagnose or exclude a catheter-associated infection. The technique should not only reduce the number of CVCs that are removed unnecessarily, but it would also provide information that could lead to earlier intervention and correct diagnosis, should the CVC not be the source of the problem. In the present study the aim could be simplified into the following two questions:

• Have any microorganisms colonised the CVC?

• If there are, then how many organisms are present?

It is therefore critical that the sampling method and the detection technology that are chosen will be appropriate to answer these questions.

A successful rapid technique that would be used routinely in the laboratory should have a simple enough methodology so that it could to be routinely carried out both by trained staff and “on call” staff who provide emergency cover outside the normal 9 to 5pm working day. Other features that are important include:

• An assay should not be labour intensive,

• It should be cost-effective in terms of initial capital outlay on equipment, reagents and consumables,

• The turn-around time between sampling and obtaining a result should be within the working day.

• The entire system should at least equal, if not improve, on the current technology with respect to sensitivity and specificity and it is important that the system has a low rate of false negative results.
Objectives

The major objectives that were identified at the beginning of this project were to:

1. Conduct a review of relevant literature in the areas of catheters, catheter-associated infections, catheter colonisation, diagnosis of catheter infections and rapid methods used to detect microorganisms.

2. Obtain information on procedures that do not involve CVC removal in order to obtain a sample. Review these techniques and choose an appropriate method for use in this study.

3. Obtain and review technical information on "rapid" technologies that are currently available to detect microorganisms. Conduct a search for information on clinical as well as environmental and food-related industries.

4. Identify at least two technologies and evaluate which would be the most appropriate technology for detecting organisms within a clinical sample, based on ease of use, cost and potential performance.

5. Choose one of the technologies and develop a method based upon this technology which could be used to detect the presence of microorganisms in a laboratory simulated environment.

6. Optimise the developed method so that it could be used routinely within a clinical pathology laboratory. In doing this, identify a hospital where it may be possible to obtain true clinical samples (CVC tips) and fulfil appropriate hospital approvals to secure the samples.

7. Evaluate the developed method using samples obtained from catheterised patients.
Plan of thesis

This thesis describes the development and evaluation of a technique for rapidly detecting microbial colonisation associated with CVCs. The thesis is made up of seven chapters, the contents of which are summarised below.

Chapter 1 is a review of literature, which focused on CVCs, the types that are available and their use in modern medical practice. The pathogenesis of catheter-associated infections was reviewed as well as the techniques that have been developed to accurately diagnose catheter-associated infections.

Chapter 2 reviewed the techniques that are currently available to obtain a sample from a CVC without having to remove the CVC from the patient. The most appropriate technique, based on published studies, was chosen for use in this study. Three technologies used to rapidly detect microorganisms in both clinical and environment samples were compared and one chosen for further evaluation in this study.

Chapter 3 details all the method and materials used in the course of conducting this study.

The Results section is made up of three chapters (Chapters 4, 5 and 6).

• Chapter 4 details research conducted using an off-the-shelf luminometer system and a stand-alone tube luminometer with recommended reagents. The off-the-shelf system was found to be inappropriate as few parameters could be varied and therefore optimisation of the technique was not possible. The tube luminometer was selected for further development.

• Chapter 5 details research carried out using a tube luminometer and laboratory-developed reagents. A method was developed and evaluated; however areas of improvement were identified so as to increase the performance of the technique.

• Chapter 6 describes the further development and optimisation of a technique evaluated in Chapter 5. This technique was evaluated and the results analysed to determine the potential usefulness of the assay.

Chapter 7 contains a discussion of the findings of this study both in the context of the study and in the context of the literature reviewed in Chapter 1. The major findings are presented as well as recommendations for future work.
CHAPTER ONE

Literature Review
1.1 Overview

This review of literature will focus on central venous catheters, the types that are available, their use in medical practice and complications associated with their use. Much of the review will centre on the post-insertion complication of catheter-associated infection, where the pathogenesis of this infection will be discussed, as well as measures and strategies to prevent such infections. Finally, the techniques that have been developed for use in diagnosis of catheter-associated infections will be reviewed.

1.2 Catheters and Catheterisation

A catheter is a tube, which allows fluid to be delivered to, or taken from, the body. Meyers (1945) first described catheterisation of human blood vessels, where a technique for extending intravenous (IV) therapy by introducing a 9-12 inch plastic catheter through a steel needle was described. Since then the use of catheters or vascular access devices (VADs) has grown quite dramatically, with over 150 million used per year in the USA alone (Raad 1998). By 1996, it was observed that IV catheters were used in more than 50% of hospitalised patients in the USA (Adal and Farr 1996).

One particular type of VAD, the central venous catheter (CVC), has become increasingly important in modern medical practice. Some 200,000 CVCs (Waghorn 1994) and 5 million CVCs (Maki 1991) are inserted in the UK and USA respectively each year. Although CVCs have revolutionised patient care in the second half of the twentieth century, a number of problems, such as sepsis and thrombosis, are associated with their use (Decker and Edwards 1988) and there continue to be major complications of CVC application (Fletcher and Bodenham 1999a).

There are many other different types of VADs, including Pulmonary Artery catheters, which are used in an acute setting when an accurate assessment of the pulmonary and cardiac status is necessary (Dawkins 1987, Mermel and Maki 1994) and specialist haemodialysis catheters used by patients with renal failure (Canaud 1999). However it is central venous VADs or CVCs, used throughout medical practice, with which this study is most concerned.
Since 1945, CVCs have gone through a series of design changes, modifications and improvements (Broviac et al. 1973, Hickman et al. 1979, Trooskin et al. 1985). They can now be used for the administration of different therapies and are tailored for specific functions and patient types (Orr and Ryder 1993). They have had an enormous impact on many branches of medicine, such as intensive care, oncology, gastroenterology, haematology, nephrology, trauma and burns and modern medical practice is dependent upon their use (Dobbins et al. 1999).

1.2.1 Development and functions of CVCs

CVCs are used to administer medications such as antibiotics, antimitotics (drugs used to treat cancer) or inotropes (drugs to stimulate the heart). In addition CVCs are used for the administration of total parenteral nutrition (TPN), or for specialised functions such as haemodialysis (Adal and Farr 1996, Elliott et al. 1994a, Maki 1991, Olson et al. 1992).

As can be seen in Figure 1.1, CVCs consist of a tube, referred to as a lumen, ending in a capped hub, which is situated at the proximal end. The catheter extends from the hub to the distal end – known as the catheter “tip”. A single catheter may have up to four lumens, each of which may be used separately, and independently, to infuse solutions, take venous pressures or withdraw blood samples. CVCs are manufactured for all patient groups, from paediatric to adult. They generally differ in the number of lumens that are present (from single lumen CVCs to those with multiple lumens), lumen diameter and length, and according to their intended use (Goldmann and Pier 1993, Orr and Ryder 1993). The hub has a Luer lock, which aids attachment of the device to an external supply of nutrition or therapy. Many CVCs are radiopaque, so that they can be x-rayed and their internal position determined post-insertion (Campisi et al. 2000, Elliott 1993). CVCs vary in the number of lumens found within the catheter,

CVCs are inserted into the major veins, as described in Table 1.1, with the tip located in the superior vena cava above its junction with the right atrium and the distal portion placed parallel to the vessel wall. Access to the large veins is important as better dilution of the fluid is achieved and repeated venepuncture is avoided, thus preventing peripheral vein sclerosis (Brovaic et al. 1973). The insertion of a CVC should always be performed under aseptic conditions by trained personnel (Collins 1991, Elliott et al. 1994a).
Figure 1.1  Triple lumen CVC (Arrow 7Fr, 20cm). This particular CVC is 20cm long (manifold to tip) and has three lumens extending into three hubs; brown is distal (16G), white is proximal (18G) and blue is medial (18G). The manifold is located at the junction of the leader tubes from the hubs to the 7Fr tube. The black line on the yellow tube marks 10cm from the tip (blue) to the manifold.
Table 1.1 Location of CVC insertion sites in the human body (Martin 1998).

<table>
<thead>
<tr>
<th>Vein</th>
<th>Description and location</th>
</tr>
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<tbody>
<tr>
<td>Right and Left Internal Jugular Veins</td>
<td>Large veins running vertically down the side of the neck draining blood from the brain and face</td>
</tr>
<tr>
<td>Right and Left Subclavian Veins</td>
<td>Large veins located in the chest and draining blood from the head, neck and upper limbs</td>
</tr>
<tr>
<td>Right and Left Femoral Veins</td>
<td>Long veins with one located in each thigh, where blood is removed from the legs</td>
</tr>
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</table>
The most frequently used insertion sites include the right and left internal jugular veins, the right and left subclavian veins shown in Figure 1.2 and the right and left femoral veins (Reed et al. 1995). On occasion the supra-clavicular and ante-cubital fossa can also be used (Elliott et al. 1994a).

\textit{a) A single lumen CVC}

Single lumen CVCs were the first variant of catheters to be developed (Broviac et al. 1973, Meyers 1945) and they are frequently used in an acute setting or for the provision of long-term access to a central vein for nutritional support, antibiotics or cancer chemotherapy (Adal and Farr 1996). Should this type of catheter be in place for a prolonged period of time, then it may be tunnelled under the skin prior to entry into the vein (Broviac et al. 1973, Hickman et al. 1979).

\textit{b) A multiple lumen CVC}

Multiple-lumen CVCs vary in the lumen number from at least two to four (generally named double, triple and quad-lumen catheters). In cases where a patient may require the administration of two or more incompatible infusions, it is preferable to employ a multi-lumen CVC. This avoids the use of multiple access sites for a number of single lumen CVCs. Multiple-lumen catheters have widespread use and are important in providing all the fluids that a patient requires, both in acute care settings and in long-term delivery of antibiotics and chemotherapy (Orr and Ryder 1993). The Arrow triple lumen CVC shown in Figure 1.1 is a frequently used multiple-lumen CVC within acute care settings.

\textbf{1.2.2 CVCs currently used}

In addition to the number of lumens that are present, CVCs also differ in function due to lumen size, proposed insertion site and their "life expectancy". There are a wide variety of CVCs used in modern medicine and it is important that the most appropriate CVC is used in a particular patient (Elliott et al. 1994a). The choice of CVC will depend upon the chosen method of CVC introduction, the catheter material, its composition and design, as well as the intended function of the catheter (Elliott et al. 1994a).
Figure 1.2  Two common insertion sites for a double lumen permanent haemodialysis catheter (Quinton PermCath). On the left the CVC is tunnelled into the internal jugular vein, whilst on the right the CVC is inserted into the subclavian vein. In both cases the CVC tip is placed at the junction of the superior vena cava and the right atrium. (Source: promotional poster from Sherwood Davis and Geck, USA).
a) **Long-term (tunnelled) CVCs**

During prolonged therapy, the CVC is often implanted or tunnelled under the skin. This involves the catheter being inserted into the vein at a site distal to the point at which it exits the skin. These catheters, which are generally made of silicon, are specially adapted and have the distal tip positioned in the central vein, near the right atrium (Broviac *et al.* 1973, Tenenbaum and Brennan-Scelsi 1994). The proximal end of the catheter generally consists of a manifold which gives rise to multiple hubs.

It was Broviac and colleagues who first reported the use of a narrow (1mm diameter) silicone rubber catheter, which was inserted into a central-vein and exited the skin at a distant site, after passing through a subcutaneous tunnel (Broviac *et al.* 1973). This catheter was developed specifically to administer TPN. It has a small Dacron® cuff, which encircles the catheter at the point where it becomes fixed, by the fibrotic tissue, to the subcutaneous portion. It holds the catheter in place (Johnson and Oppenheim 1992) and acts as a physical barrier to prevent microorganisms from migrating down the outside of the catheter (Decker and Edwards 1988). Silicone rubber is used in this CVC as it is a less thrombogenic alternative to polyvinyl chloride (Bozzetti *et al.* 1983, Broviac *et al.* 1973). Hickman *et al.* (1979) introduced a modified Broviac catheter which is basically a wider bore catheter - a larger internal diameter of 1.6mm instead of 1mm - and is used to administer blood products, drugs and IV infusion; also blood can be withdrawn through it (Johnson and Oppenheim 1992). The two most popular commercially available tunnelled CVCs are the Groshong® CVC (Campisi *et al.* 2000), shown in Figure 1.3 and the Hickman® CVC (Hickman *et al.* 1979), shown in Figure 1.4. These can be used for long-term administration of antibiotics, TPN, cancer chemotherapy or other fluids and are generally placed for periods of more than 10 days (Crnich and Maki 2002b).

b) **Short-term catheterisation**

"Short-term" CVCs are used mostly in acute care settings, such as the intensive care unit (ICU) of a hospital (Cyna *et al.* 1998) and are generally placed for periods of 10 days or less (Crnich and Maki 2002a). They can have up to four lumens and generally do not have the Dacron® cuff that is associated with the long-term tunnelled CVCs. They are
Figure 1.3 Groshong double lumen catheter (BARD, 10Fr, 40cm). This particular CVC is 40cm long (manifold to tip) and has two lumens extending into two hubs; red is distal and green is proximal. The black tip contains a valve, which is the unique feature of the Groshong catheter. Note that the Dacron cuff is located midway between the manifold and the tip.
Figure 1.4 Double lumen Hickman catheter (BARD, 9Fr, 60cm). This particular CVC is 60cm long (manifold to tip) and has two lumens extending into two hubs. Both lumens are the same length. Note that the Dacron cuff is located approximately 15cm between the manifold and the tip.
usually made of polyurethane or Teflon (Keegan-Wells and Stewart 1992) and are placed within a patient for administration of antibiotic therapy inotropic drugs, and to provide nutritional support whilst in the ICU. They are generally placed for up to seven days (Elliott et al. 1994a) although they can stay in situ longer if they are properly cared for and maintained (Cyna et al. 1998). The disadvantage of using these CVCs is an increased risk of infection because they are neither tunnelled under the skin nor have a Dacron® cuff (Keegan-Wells and Stewart 1992). However, they are much more easily removed than the cuffed catheters. As well as the Arrow catheter shown in Figure 1.1, the Quinton Mahurker® catheter shown in Figure 1.5 is another example. This particular catheter is used when patients require dialysis after acute renal failure.

c) Haemodialysis Catheters

CVCs used for haemodialysis may be used to either;

- provide temporary vascular access for haemodialysis, until permanent access via a native fistula or graft is available, or

- provide permanent access for haemodialysis (Canaud 1999).

Therefore these types of CVC can be inserted for a short period of time, like the Quinton Mahurker® catheter shown in Figure 1.5, or they can be placed indefinitely. Generally such CVCs are double lumen catheters with two large-bore lumens (the arterial and venous lumens). These lumens are connected to the blood tubing of the dialysis machine in order to complete a circuit for the removal and return of the patient’s blood during the dialysis procedure. Examples of haemodialysis catheters are shown in Figure 1.6 and 1.7, the Quinton Permcath® and MedComp Ashsplit® catheters respectively. Another type is the Tesio® catheter where two single lumen CVCs are placed within the patient in parallel (Figure 1.8).

d) Peripherally Inserted Central Catheters (PICC)

PICCs are inserted into the ante-cubital fossa (at the elbow) and advanced until the end of the catheter is located in the central vein (Ryder 1993). They can be single or double lumen and are used to deliver either continuous or intermittent therapy. PICCs can be
Figure 1.5  Quinton Mahurkar® catheter (Quinton, 12Fr, 24cm). This particular short-term CVC is 24cm long (manifold to tip) and has two lumens extending into two hubs; blue is distal which is used for the venous side and brown is proximal which is the arterial side.
Figure 1.6 The PermCath® (Quinton, 16Fr, 28cm). This particular long-term CVC is 28cm long (manifold to tip) and has two lumens extending into two hubs; blue is distal which is used for the venous side and brown is proximal which is the arterial side. There is a Dacron cuff located between the manifold and the tip.
The Ashsplit® catheter (MedComp, 14Fr, 28cm) is a long-term CVC which is 28cm long (manifold to tip) and has two lumens extending into two hubs; blue is distal which is used for the venous side and red is proximal which is the arterial side. There is a Dacron cuff located between the manifold and the tip.
Figure 1.8 The Tesio® catheters (MedComp, 10Fr, 50cm) are long-term, single lumen CVCs, which are supplied up to 55cm long and are designed to be trimmed at time of insertion for an appropriate fit. Again the lumen of each extends into a hub; with the blue CVC used for the venous side and red for the arterial side. Again the Dacron cuff is located between the manifold and the tip.
used in an acute setting, although, they are commonly used for long-term venous access providing nutritional support, antibiotic therapy or cancer chemotherapy (Ryder 1993). PICCs gained popularity in the late 1980's due to the increase of home intravenous therapies (Orr and Ryder 1993) and the fact that nurses in addition to surgeons are now inserting PICCs (Ryder 1993). The advantages of PICCs include the elimination of insertion-related complications of the neck and chest (Tenenbaum and Brennan-Scelsi 1994), a decrease in the cost of therapy (reduced hospital stay, with more patients in home care environments) and a reduced potential for catheter-related infection (Fletcher and Bodenham 1999a, Orr and Ryder 1993). However, PICCs are not useful for high volume exchange and their use can induce stenosis of the smaller veins.

e) Implantable Ports

A port is a device that is a totally implanted under the skin (subcutaneous) and consists of a small reservoir with a rubber seal to which is attached an outlet catheter (Keegan-Wells and Stewart 1992, Levin et al. 1998). Ports are ideal for patients who do not require continuous medication but only require intermittent therapy. The Dialock™ port (shown in Figure 1.9) is used for haemodialysis access and it consists of a titanium reservoir to which two silicon catheters are attached (Canaud et al. 1999, Levin et al. 1998, Megerman et al. 1998). Administration to, or flushing of, the port is relatively simple, as it requires a subcutaneous injection using a special needle to pierce the rubber seal (Canaud et al. 1999, Keegan-Wells and Stewart 1992). Whilst ports offer some advantages to the patient over tunnelled CVCs, such as giving the patient the freedom to bath or swim (Tenenbaum and Brennan-Scelsi 1994), they do require significant surgical intervention if they become infected or blocked (Whitman and Boatman 1995).

1.2.3 Materials used in CVC manufacture

Intravascular catheters need to be made from biocompatible materials which can withstand conditions inside the patient’s body without causing further complications: additional desirable properties include thromboresistance, flexibility, a smooth surface and a resistance to kinking (Elliott et al. 1994a). The most popular materials are polymers, such as polyethylene, Teflon (a fluoropolymer), polyvinyl chloride (PVC),
Figure 1.9  The Dialock device (Biolink). This device consists of two 11.5Fr catheters, which extend into the venous system from the implanted titanium port.
Chapter One

silicon, elastomeric hydrogel and polyurethane (Canaud 1999, Orr and Ryder 1993). All have advantages and disadvantages: for example, silicon is generally thought to be the most biocompatible, however it has a poor tolerance to pressure, whereas polyethylene has a high inherent strength but it can easily bend (kink) within the patient.

All catheter materials are thrombogenic to some degree, however polyurethane, and silicone are less thrombogenic than Teflon and polyvinyl chloride (Orr and Ryder 1993). Catheters that have irregular surfaces have also been associated with increased thrombogenicity (Hecker 1981). In some cases it is the physico-chemical properties of the catheter surface that influence bacterial attachment in vitro (Jansen and Peters 1991).

1.3 Complications associated with CVCs

Despite the many benefits that CVCs offer, complications arising from their use have been noted since their introduction (Collins 1991). CVC related complications can occur during the insertion procedure and at any time whilst the patient is catheterised (post-catheter placement). The many improvements that have been made in catheter design, manufacture and materials science, together with improvements in insertion techniques, have not led to the reduction of complications. Reports to the Food and Drug Administration (FDA) in the USA indicated that 55% of the complications between 1984 to 1986 were related to the management of CVCs by healthcare professionals (Scott 1988). This data was based upon device malfunction but did not include complications related to infection.

1.3.1 Complications associated with insertion of CVCs

Complication rates associated with the insertion of CVCs vary according to chosen insertion site, technique, catheter type (Cobb et al. 1992, DeJong et al. 1985, Mansfield et al. 1994, Michel et al. 1988 and Newsome et al. 1984) and the skill and experience of the operative (Rothschild 2001). However, the type and relative frequency of complication is similar in most studies. In decreasing order of frequency the complications associated with insertion are: failure of access, misplacement of the catheter, laceration of adjacent arteries, pneumothorax (collection of air within the pleural cavity), air embolism (bolus of air within the venous circulation), hydromediastinum (water collecting in the space
separating the lungs), haemothorax (collection of blood within the pleural cavity) and perforation of the myocardium. Other less common complications include cardiac tamponade (excessive fluid in the pericardial space leading to circulatory abnormalities), catheter embolus/rupture, cardiac dysrhythmia and nerve injury (Elliott et al. 1994a).

1.3.2 Post-insertion complications

Following successful placement of a CVC, approximately 10% of patients will experience a complication that is secondary to catheter insertion or use (Collins 1991). These complications generally fall into one of three categories, namely occlusion, thrombosis and infection (Elliott et al. 1994a, Orr and Ryder 1993). Other less common post-insertion complications include hydrothorax due to vessel erosion and mechanical complications not related to occlusion such as CVC leakage (Scott 1988).

a) Catheter occlusion

Catheter occlusion is evident when blood cannot be withdrawn nor solutions infused through the catheter (Wickham et al. 1992). Loss of patency (a reduction in flow rate through the catheter) or a complete blockage of the CVC can result from a number of scenarios. These include the CVC being positioned incorrectly in the vein, the CVC kinking (where the CVC has partially folded over itself resulting in restricted flow), a build up of a fibrin resulting in a sheath on the CVC or clot formation (thrombosis) within the CVC (Raad et al. 1994a). Precipitated TPN, drugs or IV solutions can also cause an obstruction (Wickham et al. 1992).

b) Catheter thrombosis

Thrombosis formation occurs when a CVC is inserted into the bloodstream and the device surface is immediately coated with plasma proteins. Two types of thrombosis are associated with CVCs. These are known as fibrin sheath thrombosis and vascular occlusive thrombosis (Raad et al. 1994a). Fibrin sheath thrombosis occurs on the extraluminal surface of the catheter and can occur on the whole length of the catheter, whereas vascular occlusive thrombosis occurs when clots of material form and come together to block the passage of solutions from the CVC through the vein – this usually occurs at the tip of the catheter (Davenport 2000).
Generally, thrombosis can be related to the characteristics of the catheter itself, for example, polyvinyl chloride is more thrombogenic than silicone (Bozzetti et al. 1983, Orr and Ryder 1993). However, thrombosis can also be induced by the infusate and the low rate of blood flow at the catheter tip (Orr and Ryder 1993). In some cases thrombolytic agents such as urokinase can be used to break down thrombus and therefore, restore patency to a blocked CVC (Orr and Ryder 1993). In cases where there is a kink or a mechanical blockage then catheter removal can often be the only solution available.

c) Catheter associated infections

Catheter-related bloodstream infections (CRBSI) or catheter-related bacteraemia are the most frequent and serious complication associated with successfully inserted CVCs (Norwood et al. 2000). Bacteraemia is defined as the presence of bacteria in the circulating blood as demonstrated by blood culture (Norwood et al. 1991). It is asymptomatic, but can lead to septicaemia, which is a bacteraemia with clinical manifestations of the pathogenic activity of the bacteria in blood. Septicaemia can result in widespread damage to the host tissues and cells. Bentley and Lepper (1968) reported from a study in their hospital that CVCs were responsible for almost 50% of nosocomial septicaemias (bloodstream infections that have occurred as a result of hospitalisation and treatment). However, device-related infections are perhaps the least frequently recognised of all nosocomial infection (Maki 1991).

By the end of the 1990’s the infection rate in successfully placed CVCs was accepted to be about 4%, with a range 1-10% (Bozzetti et al. 1982, 1983, Goldman and Maki 1973, Kite et al. 1997, Nehme 1980, Padberg et al. 1981, Pettigrew et al. 1985, Snydman et al. 1982). This figure is, however, thought to be considerably underestimated (Maki 1991).

It has also been observed that microbial colonisation occurs in 6-40% of indwelling catheters (Bozzetti et al. 1982, 1983, Goldman and Maki 1973, Kite et al. 1997, Nehme 1980, Padberg et al. 1981, Pettigrew et al. 1985, Snydman et al. 1982). This reported variation is due, in part, to the use of different diagnostic criteria, diverse study populations (from ICU patients to those receiving TPN) and varying practices, such as the length of time the catheter dwells within the patient (Dobbins et al. 1999).
In a review article by Crump and Collignon (2000) it is suggested that over 500,000 cases of catheter-associated bloodstream infections occur annually in Western Europe and the USA; and these may be associated with up to 100,000 deaths.

Catheter-associated infections are reviewed in greater detail in Section 1.4.

1.3.3 Catheter removal

a) Conventional CVC removal

The procedures undertaken for catheter removal will vary depending on the type of CVC that is in place and the reason for its removal. Temporary CVCs are in situ for a relatively short period of time (less than ten days) and can be easily 'pulled' when CVC removal is necessary.

However, long-term CVCs, such as the Hickman® catheters are more difficult to remove (Adal and Farr 1996). This is because the patient's tissue has grown into the cuff and therefore surgical intervention is required.

Traditionally CVCs used in intensive care units (generally short-term non-cuffed CVCs) are routinely removed and replaced (Cyna et al. 1998). This is based upon the premise that routine replacement will lower infection rates, as it is generally believed that the longer a CVC is in place then the higher the risk of infection. However, routine replacement of CVCs does not, per se, prevent infection (Adal and Farr 1996) and there is no evidence to support this general belief (Cyna et al. 1998, O'Leary and Bihari 1998, Timsit 2000). Additionally, CVC replacement and reinsertion increases the risk of mechanical complications associated with CVC insertion (Bozzetti et al. 1983). Cobb et al. (1992) stated that CVCs should remain in place until there are clinical indications requiring a CVC change. Long-term tunnelled CVCs such as Broviac® and Hickman® catheters can, however, be used almost indefinitely as some infections can be treated in situ using an antibiotic lock (a concentrated dose of antibiotic instilled into the catheter for 12 hours) technique rather than catheter removal (Messing et al. 1990, Norwood et al. 1991).
b) Catheter replacement by guide wire exchange

Guide wire exchange (GWX) involves a guide wire being fed through one lumen of the CVC. The CVC is then pulled out over the guide wire leaving the guide wire in place in the patient’s vein and a new CVC is then fed over the guide wire (Bozzetti et al. 1983, Michel et al. 1988). The tip is cut from the removed CVC and this is sent to the laboratory for analysis. If significant levels of microorganisms are found, the newly inserted CVC is removed and a new CVC is inserted at another site (Bozzetti et al. 1983, Norwood et al. 1991, Pratt et al. 2001). GWX is therefore, only useful if there is not a significant number of microorganisms colonising the exhausted CVC. This technique is best employed in situations where the number of available new sites is limited, especially in the chronic haemodialysis population who are dependent on long-term CVC use (Shaffer 1995). GWX is also a favoured technique if the CVC has undergone some sort of mechanical damage, such as bending or kinking or the CVC needs to be repositioned within the patient. In such cases GWX reduces morbidity as there is no infection and the complications associated with insertion of a CVC at a new site are avoided (Michel et al. 1988, Orr and Ryder 1993). The use of GWX versus insertion at a new site gives a fourfold reduction in the risk of insertion complication (Cobb et al. 1992). However, exchanging an infected or heavily colonised CVC over a guide wire invariably leads to the need for yet a further replacement as a consequence of contaminating the exchanged CVC (Cobb et al. 1992, Olson et al. 1992, Pettigrew et al. 1985, Roberts 1993).

1.4 Catheter-associated infections

1.4.1 Background

Over 90% of diagnosed CRBSIs are associated with CVCs (Maki 1991), with the remainder being associated with peripheral catheters. The extent at which CRBSI affect the hospitalised population is increasing steadily, with Safdar and Maki (2002) reporting that noncuffed short-term CVCs cause at least 250,000 bloodstream infections in United States hospitals each year.

Infections associated with catheters have been referred to by different terms over the past 30 years. Some authors (Dobbins et al. 1999, Kite et al. 1997) have referred to this type of infection as catheter-related sepsis (CRS), whilst others prefer terms such as catheter-
related bacteraemia (Reed et al. 1995) or CRSBI (Fletcher and Bodenham 1999a, Gowardmann et al. 1998, Raad 1998).

Pulmonary artery catheters have a similar sepsis rate to CVCs (Fletcher and Bodenham 1999a). On the other hand PICCs are reported to have a much lower incidence of sepsis (Maki 1991), with Collingnon (1994) reporting a level of 0.036%. The reason for lower infection rates with PICCs is that PICCs are inserted at sites such as the arms whilst CVCs are placed in the neck and chest (Ryder 1993). Generally the cooler and drier extremities, such as the arms, have lower levels of bacterial colonisation than the moist neck region. Another possible reason is that PICCs are located further away from mouth and nose - which are often the source of organisms through oral and nasal secretions - than CVCs which are placed in the neck or chest (Ryder 1993).

1.4.2 Defining catheter-associated infections

Historically, no definition of a catheter-associated infection has found universal approval and agreement. The reasons for this emanate from the number of methods that have been developed to culture catheters (Siegman-Igra et al. 1997) and the large variation in what is considered to be an "infected catheter" (Dobbins et al. 1999, Elliott et al. 1994a). In addition, the variable definition of terms such as "catheter-related infection" and "catheter-associated infections" when used by different investigators has led to confusion and difficulties in comparing results of various clinical studies (Raad and Bodey 1992). What was accepted was the fact that proof could only be obtained by culture of the removed catheter (Johnson and Oppenheim 1992, Maki et al. 1977). Moreover, there was no true definition as to what constitutes an infected catheter (Orr and Ryder 1993). This difficulty leads to many catheters being incorrectly suspected as the source of infection and being inappropriately removed. Currently, suspected catheters are removed with approximately 80% (Padberg et al. 1981, Ryan et al. 1974) of these proving to have been removed unnecessarily. More recent studies (Kite et al. 1997, Pettigrew et al. 1985) have shown a similar high rate of inappropriate catheter removal suggesting that accepted clinical methods for diagnosing CRS have not improved in 25 years.

It is also important to remember that often the patients who require a CVC are frequently those at greatest risk of acquiring infection at other sites. Therefore, it is often difficult to
determine whether the CVC is the source of a given systemic infection (Raucher et al. 1984). Elliott et al. (1994a), as part of a joint working group (Hospital Infection Society and Research Unit of the Royal College of Physicians) published guidelines for good practice in central venous catheterisation. Within these guidelines “infection” associated with catheterisation is defined as either localised infection or systemic infection: these definitions are shown in Table 1.2.

No-one has defined levels at which microorganisms may be said to contribute to infection or colonisation, nor indeed what level may result due to contamination (the presence, in the specimen taken for culture, of organisms introduced by the person collecting the specimen during the course of obtaining the sample (Norwood et al. 1991)). It should be noted that the occurrence of bacteraemia is much less frequent than the level of colonisation seen in CVCs (Raad 1998). Nevertheless the degree of colonisation does vary and a high level of colonisation is generally thought to be a precursor to bacteraemia (Cleri et al. 1980, Dittmer et al. 1999, Kite et al. 1997, Maki et al. 1977). Dittmer et al. (1999) demonstrated that post-dialysis transient bacteraemia is very common and the duration of catheterisation and the degree of colonisation increase the risk of bacteraemia. Fortunately, from a diagnostic point of view, most cases of clinically proven CRS produce colony counts of much greater than 1000 colony forming units (CFUs) on the catheter tip (Cleri et al. 1980, Kite et al. 1997, Maki et al. 1977).

The most accepted definition of a catheter-associated infection is that provided by the members of the Hospital Infection Control Practices Advisory Committee (HICPAC) members, shown in Table 1.3 (Pearson et al. 1996).

1.4.3 Implications and effects of sepsis

a) Cost implications to the hospital

CRS is the most frequent cause of nosocomial bacteraemia in the critically ill (Waghorn 1994) and complicates up to 16% of catheters (Segura et al. 1996) leading to increased morbidity, mortality and cost. From the beginning of 1992 to end of 1993 the direct mortality rate in one UK hospital was approximately 16%, where 6 patients died from 37
<table>
<thead>
<tr>
<th>Infection</th>
<th>Definition</th>
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<tr>
<td><strong>Systemic</strong></td>
<td>Systemic infections or septicaemias are not always easily diagnosed. Often the patient has a raised temperature, which is unresponsive to broad-spectrum antibiotics. There are no other symptoms or signs that suggest an alternative site of infection.</td>
</tr>
<tr>
<td><strong>Localised</strong></td>
<td>May occur at the insertion site or along the track of a tunnelled device. Clinical evidence may include oedema, erythema and thrombophlebitis. The patient may be pyrexial, although this is usually insignificant. There may be irritation and pain at the insertion site.</td>
</tr>
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that were diagnosed as suffering with CRS (Waghorn 1994). On average CRS prolongs an ICU stay by 6.5 days, with the excess cost per survivor, in the USA, put at $28,690 (Pittet 1994a, Pittet et al. 1994b). Arnow et al. (1993) estimated that it costs US$3,707 to treat a CRS patient and US$6,064 for CRS caused by the microorganism Staphylococcus aureus. These figures were calculated by combining the total costs for laboratory tests, therapy and the hospital room. During 1991 there were 3,883 cases of CRS (or line-associated bacteraemia) in England and Wales reported to the Communicable Disease Surveillance Centre (Elliott 1993). However, Elliott (1993) comments that this figure is probably a conservative estimate and suggests that if CRS occurs in 4% of placed CVCs (as observed in many clinical studies) then the true figure would be around 8,000 patients per year.

b) Treatment of CRS

The treatment of CRS varies depending on the catheter type and underlying pathology of the patient. In some cases CRS is initially treated by removal of the CVC, however this is dictated by the availability of alternative access. Following this, the clinician can administer antibiotics or, in uncomplicated cases, antibiotic therapy may be withheld. The antibiotic of choice should reflect the most likely organism to be present. Vancomycin should be considered if coagulase-negative staphylococci are suspected to be present. Initial antibiotic therapy, such as cephalosporin or aminoglycoside, should also attack Gram-negative organisms. Antifungal therapy, such as Amphotericin B could be considered for Candida albicans infection (Fletcher and Bodenham 1999b).

c) Consequences of untreated sepsis

Sepsis has the same consequences no matter whether it originates from a post-operative wound, an abscess, peritonitis or an implanted device such as a catheter. It is the body’s systemic response to infection (Martin 1998). When there is a bloodstream invasion by an infecting organism, various mediators are released within the body. The cell walls of invading bacteria and/or fungi contain endotoxins such as lipopolysaccharide, which trigger the activation of host blood cells such as macrophages (Bullard and Dunn 1996).
Table 1.3  Accepted definitions of a catheter-associated infection provided by HICPAC members (Pearson et al. 1996).

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Catheter-related bloodstream infection (CRBSI)</td>
<td>Isolation of the same organism (identical species, antibiogram) from a semiquantitative or quantitative culture* of a catheter segment and from the blood drawn from a peripheral vein of a patient with accompanying clinical symptoms of BSI and no other apparent source of infection. In the absence of laboratory confirmation, defervescence** after device removal may be considered indirect evidence of CRBSI.</td>
</tr>
<tr>
<td>Colonised catheter</td>
<td>Growth of ≥15 CFUs*** in a semiquantitative culture or &gt;1000CFU in quantitative culture from the distal catheter segment in the absence of accompanying clinical symptoms.</td>
</tr>
</tbody>
</table>

* Semiquantitative and quantitative culture are defined in Section 1.7
** Disappearance of a fever
*** Colony forming units or CFUs are defined as discrete populations of microorganisms which are considered to have developed from a single parent cell. For example, if there are 35 distinct colonies growing on a blood agar plate then there were originally assumed to be 35 CFUs present.
The macrophages then synthesise and secrete cytokines such as Interleukin-1, Interleukin-2 and Tumour Necrosis Factor (TNF-α) (Bullard and Dunn 1996), with Interleukin-1 and TNF-α often being over-produced (Kuby 1994a). These mediators stimulate a number of responses in the body, including vasoconstriction and other vascular effects, as well as direct myocardial effects. If the patient reaches this stage without intervention, then there is little that can be done. Severe multiple organ system dysfunction ensues, followed by multiple system organ failure and death (Kuby 1994a).

1.4.4 Factors influencing the risk of catheter-associated infections

A catheter-associated infection will ultimately have resulted from microbial contact with the CVC. The actual bacteria and yeasts that are involved in CVC colonisation are discussed in detail in Section 1.5. However, it is important to highlight the factors that can lead to microbial colonisation and infection of a CVC. Some of these factors are considered in the following sections.

a) Underlying health status of the patient

The risk of catheter colonisation leading to infection and ultimately CRS is higher in certain hospitalised patient groups. There is a higher incidence of CRS in those patients that have immune deficiencies such as, AIDS (Acquired Immune Deficiency Syndrome) or neutropenia - a low level of neutrophils or white blood cells (Fletcher and Bodenham 1999a). However, even though CRS rates are 3-7% and higher in neutropenic patients, the positive aspects of catheterisation far outweigh any alternative (Johnson and Oppenheim 1992).

In the critically ill there is also a higher risk of the patients suffering CRS. This is due to their immune systems having defective surveillance, the underlying disease processes, such as trauma or burns, and the type of surgery that the patient has undergone.

The basic microbial environment in which the patients are nursed is also important as this can render the patient more susceptible to colonisation and infection (Fletcher and Bodenham 1999a). Moreover, the hospital environment can act like a reservoir for microorganisms, especially antibiotic resistant strains, such as MRSA - Methicillin-resistant S. aureus (Herwaldt and Wenzel 1995), with the result that there has been an
ever increasing problem with hospital related or nosocomial infections (Cheung and Fischetti 1990).

b) Catheter insertion

The skill of the person carrying out the CVC insertion is critical (Rothschild 2001) and studies have shown that CVC insertion carried out by a well-trained team can lead to improvements in catheter site care (Elliott et al. 1994a). Generally, CVCs are inserted, by either, an anaesthetist, a surgeon or an interventional radiologists, with PICCs insertion carried out by each of these as well as clinical nurse specialists (Ryder 1993). It is important that the insertion site is well swabbed and that the environment where the procedure is carried out is sterile (Collins 1991). This is why long-term tunnelled catheters are inserted in theatre. However many short-term CVCs are often inserted at the bedside in the hospital ward. Carrying-out an insertion procedure in this type of non-sterile environment would increase the risk of exposing the CVC and patient to a potentially pathogenic organism.

The site of CVC insertion may also be important, with placement of a CVC in the internal jugular vein or the femoral vein resulting in higher incidence of CRS than subclavian catheterisation (Adal and Farr 1996). However, much of the work done on site of placement has been observational and there is little data available that compares CRS rates of femoral, subclavian and jugular vein insertion sites.

c) Catheter use

The administration of TPN through a CVC may increase the incidence of catheter-associated infections (Snydman et al. 1982). Estrada (2002) reports that the presence of a CVC and TPN administration were identified as significant risk factors for BSI in the neonatal population. However, further analysis has shown that it may not be TPN administration alone, that is the problem but the poor routine care of the catheter by the hospital staff (Adal and Farr 1996). The issue here is that a balance needs to be sustained between the period of time one session of TPN administration should last and the number of catheter manipulations that are carried for adequate TPN administration. If a large volume of TPN is continuously administered to the patient over a long period of time, then the risks of this TPN getting contaminated increases as each day passes. However, if
a TPN administration set is changed daily then there is an increased risk that the patient or member of hospital staff may inadvertently contaminate the hub of the CVC thus increasing the potential for infection. In an attempt to solve this dilemma, studies into catheter hub contamination show that 72 hours is the optimum time period between changing the TPN administration sets. (Fletcher and Bodenham 1999a).

d) **Duration of catheterisation**

Increased duration of catheterisation has been thought to correlate with increases in CRS (Cobb *et al.* 1992). Thus some clinicians recommend routine replacement of CVCs, but this has never been shown to reduce CRS rates (Cyna *et al.* 1998, Fletcher and Bodenham 1999a). Nonetheless, there is conflict as to whether the risk per catheter per day (risk incidence) increases with duration of catheterisation (Adal and Farr 1996). The Centre for Disease Control (CDC) in the USA recommend that the incidence of CRS should be expressed in terms of episodes per 1000 days of catheterisation to reflect the variation in duration of catheter-residence (Fletcher and Bodenham 1999a). Using this definition it has been observed that in oncology out-patients there is an incidence of CRS in 0.08 days per 1000, compared to 19 days per 1000 days in the hospitalised critically ill (Fletcher and Bodenham 1999a).

e) **Post-insertion care**

Strict aseptic procedures are necessary for both the insertion and post-insertion care of the CVCs. By using maximal sterile barrier procedures during insertion Raad *et al.* (1994b) significantly reduced the risk of subsequent catheter-associated infections.

The choice of dressing that is used to cover the CVC, after it has been inserted, is important. Ideally the dressing needs to be water permeable as those that accumulate moisture have higher levels of microflora. Maki *et al.* (1994) found that gauze had low cutaneous colonisation, but there was no difference in catheter colonisation and CRS.

f) **Catheter removal**

The use of a guidewire exchange procedure to replace an infected CVC may lead to more rapid colonisation of the new CVC followed by CRS if the insertion site of the previous catheter was significantly colonised (Cobb *et al.* 1992, Olson *et al.* 1992). However, this
procedure does reduce the incidence of insertion complications through avoiding a new site replacement (Orr and Ryder 1993).

g) Catheter manufacture

Since 1997 all manufacturer of CVCs who want to sell CVCs with Europe, have had to meet the requirements of the European Union Directive 93/42/EEC concerning medical devices. This means that all aspects of catheter material and component selection, assembly, packaging, labelling and sterilisation, have to be approved and are fully traceable. The “CE mark” symbol is displayed on medical device packaging to demonstrate that that medical device meets with the directive and therefore is "fit for purpose". This directive has helped to ensure that the CVC itself will not pose a risk to the patient, however in countries outside Europe and the United States there are often no such regulations and therefore inadequate CVCs can be an issue.

1.5 Pathogenesis of catheter-associated infections

The pathogenesis of catheter-associated infections is complex and not fully understood (Crump and Collignon 2000). Nevertheless, what is accepted is that for microorganisms to cause catheter-associated infections they first must gain access to the internal and/or external lumens of the catheter where adherence and subsequent colonisation can occur (Crnich and Maki 2002a, 2002b). Raad (1998) suggests that adherence of microorganisms to the catheter surface depends on the physical characteristics of the catheter surface, the surface characteristics of the adherent bacteria, the presence of host-derived proteins and phenotypic changes of the adhering organisms. This section will discuss how various microorganisms gain access to CVCs and their subsequent implication in catheter colonisation and catheter-associated infections.

1.5.1 Microorganisms implicated in catheter-associated infections

Fletcher and Bodenham (1999a) grouped 35 studies together to determine which microorganisms were implicated as the causative organisms in CRS. Figure 1.10 shows coagulase negative staphylococci (CNS) to be the most common (37%) source of CRS
Figure 1.10  Causative organisms from 35 studies of CRS (Fletcher and Bodenham 1999a).
followed by *Staphylococcus aureus* (22%). Indeed *S. aureus* and CNS, such as *Staphylococcus epidermidis*, are generally the most common cause of infections associated with all types of implantable medical devices (Klimmer et al. 1999).

*a) Staphylococcus species*

Members of the genus *Staphylococcus* are Gram-positive, non-motile, non-spore forming cocci, which are 0.5 to 1.5\(\mu\)m in diameter and occur in “bunch of grape-like” clusters as well as individually and in pairs. They are ubiquitous in nature, but are mainly found living on the skin, skin glands and mucous membranes of mammals and birds (Kloos and Bannerman 1995).


*S. aureus* is coagulase-positive and has been well documented as an opportunistic pathogen in humans (Kloos 1998). *S. aureus* infections have been a major cause of morbidity and mortality in hospitals for many years. MRSA has become a major clinical and epidemiologic problem in hospitals since the 1970’s. From 1975 to 1991 the percentage of *S. aureus* isolates that were resistant to methicillin increased from 2.4% to 29% (Herwaldt and Wenzel 1995). The increased use of CVCs will only increase the level of CRS due to *Staphylococcus* species (Cheung and Fischetti 1990).

*b) Pseudomonas species*

These are aerobic, motile, non-spore forming, gram-negative rods, approximately 1\(\mu\)m to 5\(\mu\)m long and 0.5\(\mu\)m to 1\(\mu\)m wide. They are usually resident in water and soil, but are also found in the bowel microflora of healthy humans (Gilligan 1995). In addition to catheter-associated infections, *Ps. aeruginosa* is a leading cause of nosocomial respiratory tract infection and nosocomial urinary tract infections (Gilligan 1995). *Ps. aeruginosa* is a nosocomial pathogen that is very difficult to treat. It has been found in a variety of fluids (disinfectants, soaps, ointments) and equipment that is in contact with water, such as shower heads and anaesthetic equipment. Additionally, *Ps. fluorescens* -
Chapter One

another opportunistic pathogen - has been isolated from dental chair unit output water (Tuttlebee et al. 2002). Its presence in this environment creates a potential risk both to dental staff and patients, particularly those that are immunocompromised.

c) Candida species

*Candida* species are yeasts that are found in all areas of the human gastrointestinal tract. *Candida* species are responsible for at least 9% of nosocomial bloodstream infections (Fletcher and Bodenham 1999a) and the incidence of *Candida* fungaemia represents a serious problem in this clinical situation. Moreover, fungal infections are extremely difficult to treat and often the treatments, such as Amphotericin B (Bullard and Dunn 1996), can be highly toxic to the patient.

In hospitals, *Candida* species cause bloodstream infections especially in immunocompromised patients (Warren and Hazen 1995). Historically, *C. albicans* is the species most commonly isolated from patients with candidiasis and evidence for person-to-person transmission of this organism in hospital wards has been put forward (Fanello et al. 2001). Moreover, the recently discovered species, *Candida dubliniensis* (Sullivan et al. 1995), has been implicated in a growing number of cases of fungemia around the world (Brandt et al. 2000, Marriott et al. 2001, Meis et al. 1999). The clinical identification of *C. dubliniensis* as the causative pathogen in cases of candida fungemia is important, as concerns have been expressed that resistance strains of *C. dubliniensis* may develop in HIV-infected patients treated with fluconazole (Moran et al. 1997). This suggests that the widespread use of fluconazole, may have contributed to the emergence of *C. dubliniensis* as an opportunistic pathogen.

d) Other organisms

Other organisms that have been implicated in bacteraemia include, gram-negative bacilli (Fletcher and Bodenham 1999a, Kite et al. 1997, Turnbull and Kramer 1995), coliforms, corynebacteria and various gram-positive organisms (Fletcher and Bodenham 1999a).

1.5.2 Modes of colonisation

Five major paths or routes have been identified whereby microorganisms can gain access to the catheter and colonise it (Bouza et al. 2002, Elliott 1993, Linares et al. 1985). The
schematic diagram (Figure 1.11) from Elliott (1993) displays these various routes of colonisation and also suggests contamination from hospital staff as well as the patient’s skin as possible sources of the organisms. Table 1.4 shows these five mechanisms and definitions for four of them as suggested by Linares et al. (1985).

Of the five mechanisms, it is unlikely that a remote infection could produce bacteraemia and seed the catheter (Anaissie et al. 1995). However, in non-central venous catheters this route has been implicated in over 50% of yeast infections seen in hospitals and some other infections that involved *Escherichia coli* and *Klebsiella* species. The risk of a catheter-associated infection resulting from contaminated commercially prepared infusion fluid - a problem in the past - is now very remote due to much improved manufacturing procedures and regulations (Norwood et al. 1991).

There has, however, been much debate in recent years about which of the remaining mechanisms are the most important in the development of catheter-associated infections.

*a) Extraluminal spread*

Maki (1989) proposed that cutaneous organisms contaminate the catheter during insertion or migrate along the outside of the catheter into the patient, post-insertion. Some studies have shown that there is a good correlation between organisms isolated from skin cultures (swabs from the insertion site), the catheter and blood cultures taken from the patient (Raad et al. 1995, Maki et al. 1997). Further work has demonstrated that the use of silver impregnated cuffs and the technique of tunnelling the catheter may also reduce CRS rates by impeding the migration of organisms along the subcutaneous track (Timsit et al. 1996). Other evidence comes from studies where an antiseptic solution used to clean the site prior to catheter insertion reduced CRS rates by removing cutaneous microorganisms (Maki et al. 1991).

In recent years Maki, an original advocate of the skin hypothesis, has concluded that hub colonisation was the cause of a significant proportion of CRS (Maki et al. 1991, 1994, 1997). Furthermore studies by Dobbins and Kite (1999) indicate that endoluminal colonisation of the catheter is more important in the development of CRS and that bacterial counts in these cases exceed extraluminal counts by up to 100 fold.
Table 1.4 The route taken by the microorganisms colonising a CVC and the criteria used to define each route.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Criteria on which each is based</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraluminal Spread*</td>
<td>The hub and inside of the CVCs are colonised. The same organism is recovered from the hub, the tip and from a blood culture. There is a negative skin culture for that organism.</td>
</tr>
<tr>
<td>Extraluminal Spread *</td>
<td>The same organism is isolated from the skin, subcutaneous segment, the tip and blood. There is a negative hub culture.</td>
</tr>
<tr>
<td>Haematogenous seeding *</td>
<td>The same organism is recovered from the hub, the tip and a blood culture.</td>
</tr>
<tr>
<td>Contamination of infusion fluid *</td>
<td>The same organism is isolated from the TPN, the hub, the tip and blood. There is a negative skin culture.</td>
</tr>
<tr>
<td>Contamination of the tip on insertion **</td>
<td>N/a</td>
</tr>
</tbody>
</table>

* These definitions are taken from (Linares et al. 1985).

** This was not included as a mechanism by Linares et al. (1985), but was subsequently added by Elliott (1993).
Figure 1.11 Sources and routes taken of infecting microorganisms in central venous catheters (Elliott 1993). Reproduced with permission of the PHLS Communicable Disease Surveillance Centre © PHLS.
Using Scanning Electron Microscopy (SEM), Raad et al. (1993) have shown that there are more organisms on the inside of the CVC lumen than on the external part of the CVC when it has been inserted for longer than 10 days. It is now generally accepted that in short-term catheters (those dwelling for less than 10 days) extraluminal spread is the most likely mechanism of pathogenesis and in long-term tunnelled catheters (generally in place for >10 days) intraluminal spread is more relevant (Bouza et al. 2002, Crnich and Maki 2002a, 2002b).

b) Intraluminal spread

The intraluminal theory is based upon the fact that the same organisms have been recovered from the hub of the CVC and the blood of patients with CRS. It is thought that the hub is colonised by organisms from the patient’s skin or health care worker’s hands (Sitges-Serra et al. 1983). Microorganisms then migrate down the internal lumen of the catheter to colonise it (Sitges-Serra et al. 1985a, 1985b). If there is endoluminal colonisation of the CVC, then the infusate that is introduced into the CVC will flow over the planktonic and sessile microorganisms in the biofilm. The planktonic organisms inside the CVC can then enter the infusate, as it travels into the patient’s systemic circulation (Dobbins et al. 1999) and this can result in systemic infection. Linares et al. (1985) tried to determine the route taken by the organisms causing CRS in patients receiving total parenteral nutrition. Twenty of 135 catheterised patients were diagnosed with CRS (14.8%). Of these 20 CRS cases, 14 resulted from a colonised hub and only two from skin infection. They concluded that the catheter hub is the most common site of origin of organisms causing catheter tip infection and bacteraemia.

Kite et al. (1997), using a sampling tool called the FASEB, showed that sampling and culturing biofilm from the internal lumen of a catheter predicted CRS with greater specificity and sensitivity than extraluminal techniques like the Maki roll technique. In addition, the controlled study of a “new hub” containing 3% iodinated alcohol, led to a reduction in the incidence of CRS. In this study Segura et al. (1996) showed that the number of true CRS cases was lower in the “new hub” group (3 of 78) than the control group (12 of 73). Furthermore, it was demonstrated that the number of days of catheterisation prior to CRS diagnosis was greater in the “new hub” group (19 days) than the control group (12.3 days). Evaluations that have involved antimicrobially coated
catheters have shown the catheters that are coated both endoluminally and extraluminally are more effective than those coated only on the outside (Darouiche et al. 1999).

1.5.3 Biofilms

Microorganisms can adhere to and colonise surfaces found within industrial systems, natural aquatic environments and medical devices implanted in the body (Potera 1998). Some of the implantable devices that are commonly affected are hip and knee replacements, continuous ambulatory peritoneal dialysis (CAPD) catheters, shunts and CVCs (Bayston 1999). Additionally, biofilm colonisation has been evident in other medical devices, such as prosthetic heart valves, urinary catheters, contact lenses and intrauterine devices (Donlan and Costerton 2002). Indeed urinary catheters can rapidly become contaminated with microorganisms and this generally leads to colonisation with biofilm. Strickler (1996) notes that all patients undergoing long-term (>28 days) urinary catheterisation will develop urinary tract infections due to the presence of biofilms.

Clinical isolates of \textit{S. aureus} and \textit{S. epidermidis} can adhere to catheter material by the generation of thick multilayered biofilms consisting of bacteria and extracellular polysaccharides (Krimmer \textit{et al.} 1999). Biofilms can also form on devices and tubing which are associated with medical instrumentation, such as the silicon tubing attaching a dialysis machine to the dialysis patient (Marion-Ferey \textit{et al.} 2003) or dental unit waterlines (Tuttlebee \textit{et al.} 2002).

As with any biofilm structure, microorganisms on catheter surfaces can be found growing in one of two different environments (Dickinson and Bisno 1989, Potera 1998, Raad 1998). The "sessile bacteria" grow on the surface of the catheter inside a biofilm, whilst the "planktonic bacteria" grow freely in the surrounding fluids, where they live at the surface of the biofilm and fluid interface (Raad 1998). There are a number of differences between organisms of the same species, when they exist in either the planktonic or sessile state. These phenotypic differences are discussed in the sections below.

a) \textit{Definition of a biofilm}

The definition of a biofilm has evolved over the last 25 years as more research has been performed on the subject. Donlan and Costerton (2002) have provided an updated
definition of a biofilm, which is pertinent in that the biofilm cells have to exhibit both the observable and phenotypic traits of a true biofilm.

“....a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription” (Donlan and Costerton 2002).

b) Biofilm formation

Generally, the adherence of microorganisms to the biomaterial surface depends on the physical characteristics of the biomaterial and the surface characteristics of the adhering bacteria (Raad 1998). Adhesion of bacteria to a surface is thought to occur in a sequential manner and the most common theory is that it involves the four phases shown in Table 1.5 (Tunney et al. 1996). This is based on the DLVO theory of colloid stability, named after Derjaguin, Landau, Verway and Overbeek (Dickinson and Bisno 1989). This theory describes the electromagnetic forces that attract and repulse colloidal particles.

It is accepted that three components are important in the initial adhesion process of bacteria to a biomaterial: namely the bacteria themselves, the suspending fluids and the substrata (Raad 1998, Tunney et al. 1996).

Bacterial cell-surface components, such as filamentous structures extending from the bacterial cell surface (fimbriae and polysaccharides), are thought to promote adhesion to surfaces (Tunney et al. 1996). Exopolysaccharide production is important in phases two to four (Table 1.5), where bacteria, such as S. epidermidis, are known to secrete a glycocalyx coating (Norwood et al. 1991).

When medical devices, such as a CVC, are implanted into the body they become coated with fibrin, thrombin, fibronectin and immunoglobulins (Dickinson and Bisno 1989). The resulting film of plasma, which coats the device, can then itself promote adhesion (Cheung and Fischetti 1990) through hydrophobic adsorption. Bacteria and fungi, such as Candida sp., adhere well to this protein coating or “sleeve” (Raad et al. 1994a) as they
<table>
<thead>
<tr>
<th>Phase</th>
<th>Description</th>
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<tbody>
<tr>
<td><strong>Phase One</strong></td>
<td>The bacteria move towards the device surface by either Brownian motion or bacterial chemotaxis and motility.</td>
</tr>
<tr>
<td><strong>Phase Two</strong></td>
<td>Within 50nm of the surface van der Waals forces draw the bacterium towards the surface and repulsive electrostatic forces are overcome. Bacterial structures (bridging polymers) such as fimbriae make adhesion to the surface irreversible.</td>
</tr>
<tr>
<td><strong>Phase Three</strong></td>
<td>Covalent, ionic and hydrogen bonds link the bacterium to the surface.</td>
</tr>
<tr>
<td><strong>Phase Four</strong></td>
<td>The attached bacteria synthesise° and secrete exopolymeric substances which encase the bacteria. Further bacterial cell division gives microcolonies within the matrix, which can lead to colonisation and a continuous bacterial biofilm.</td>
</tr>
</tbody>
</table>
have a great affinity for the plasma proteins. However, different microorganisms adhere to different proteins, for example, *S. aureus* adheres tightly to fibronectin, fibrinogen and laminin, whilst *S. epidermidis* only adheres to fibronectin (Raad 1998).

As CVCs can be manufactured from a various materials such as silicone or polyurethane, the properties of these materials can influence bacterial and host protein adhesion as well as biofilm formation. Norwood *et al.* (1991) demonstrated that *S. epidermidis* adhered to Teflon, silicon and some polyurethane catheters. Mermel and Maki (1994) also showed that Teflon™ and polyurethane are more resistant to colonisation by CNS than polyethylene, polyvinyl chloride or silicon. The colonisation process has been shown to be enhanced by components of the catheter material that can impair polymorphonuclear leucocyte activity (Lopez-Lopez *et al.* 1990). Biomaterial surfaces, for example the surface of a CVC, can in many cases be uneven and have numerous irregularities and these defects can provide niches for bacterial adhesion (Tunney *et al.* 1996). The microrugosity or roughness of the surface can also increase over time, post-insertion. This was observed by Gorman *et al.* (1993) in a study of CAPD catheters, where the adherence of *S. epidermidis* to the rough implanted catheters was significantly greater than to unused (control) catheters. The factors that may be involved in the production of increased roughness during medical device implantation may include shear forces arising from fluid dynamics and bacterial or host degradation of the device biomaterial (Tunney *et al.* 1996).

c) **Biofilm growth and maturation**

Once the bacteria have attached and are actively colonising a surface, they synthesise and secrete exopolymeric substances such as extracellular polysaccharides. It has been observed that extracellular polysaccharide adhesins have a major role to play in the formation of *S. epidermidis* biofilms on plastic surfaces. *S. epidermidis* produces the capsular polysaccharide adhesin (PSA) which mediates the initial adherence of cells to the surface and polysaccharide intercellular adhesin (PIA) which is responsible for the accumulation of cells (O’Gara and Humphreys 2001). The combination of these exopolymeric substances forms a matrix which is known as a “glycocalyx” (Tunney *et al.* 1996). Further bacterial cell division leads to the formation of microcolonies within the
matrix and in appropriate conditions a continuous biofilm can rapidly become established (Douglas 2003). The biofilm can also grow larger due to the further attachment of bacteria from the surrounding fluid as well as additional exopolysaccharide production (Tunney et al. 1996). Living, fully hydrated biofilms are composed of cells (±15% by volume) and matrix material (±85% by volume) with open water channels interspersed between the microcolonies containing the sessile cells (Donlan and Costerton 2002).

Biofilm populations exhibit highly co-ordinated 'social behaviour' with respect to population density under the control of quorum sensing regulatory systems (McKeegan et al. 1999). Quorum sensing is defined by Allison et al. (1999) as the accumulation of a low molecular weight signalling molecules beyond a critical threshold concentration, enabling individual cells to 'sense' when a minimum population unit of bacteria has been achieved in order to initiate a co-ordinated population response. Quorum sensing may play a specific role in the development of biofilms through the accumulation of extracellular signalling molecules that modulate the transcription of target operons. Accumulation of the signal might be brought about through achieving a localised high cellular density or "trapping" of both the signal molecule and the cells at a common interface (McKeegan et al. 1999). Signalling molecules associated with biofilm formation include the N-acyl homoserine lactones (HSLs) of Gram-negative bacteria (Davies et al. 1998) and small cyclic hydrophobic peptides in Gram-positives (Kleerebezem et al. 1997).

In Gram-negative bacteria HSLs are thought to be involved in different aspects of biofilm differentiation and development. As quorum sensing requires a sufficient density of cells to be present it is generally accepted that HSL signals are unlikely to be involved in the initial stages of biofilm proliferation (Allison et al. 1999). Nevertheless, the involvement of HSLs in the maturation of biofilms has been observed, with a twelve carbon (dodecanoyl) HSL being implicated in the maturation of Ps. aeruginosa biofilms through modulation of exopolymer production (Davies et al. 1998).

d) **Biofilms associated with CVCs**

Passerini et al. (1992) demonstrated, using SEM, that there was extensive biofilm formation on all the 42 arterial catheters and 26 CVCs, inserted in ICU patients, that they
examined. These catheters dwelt within the patients for between 1 and 14 days and bacteria were observed within the biofilms of 69% (29/42) of arterial catheters and 88% (23 of 26) of the CVCs. Three of the four single lumen CVCs that were in situ for only one day showed coccoid bacterial cells on the surface.

Although most CVCs are colonised with microorganisms (Dittmer et al. 1999, Passerini et al. 1992, Raad et al. 1993), only a small percentage of these catheters cause bloodstream infections (Raad 1998). SEM performed on removed CVCs by Raad et al. (1993) showed that almost all CVCs have visible adherent microorganisms on their surface, but only about 3% in this study led to CRBSI. It may be that infection depends upon whether or not the organisms on the catheter surface, exceed a certain quantitative threshold (Raad 1998) and/or the length of time the CVC is left in situ within the patient (Dittmer et al. 1999).

Figure 1.12 is a SEM of a longitudinal cross section of a CVC tip. On the right lumen a small build up of biofilm can be seen (within the blue box). Irregularities in the catheter surface can also be seen on the left lumen. Figure 1.13 is a SEM of the transverse cross section of a CVC tip showing both the extraluminal biofilm (blue box) and intraluminal biofilm (red box) formation. Both of these SEMs were taken as part of the current study and details of preparation are contained within Appendix 1.

e) Resistance to antimicrobial agents

The nature of biofilm structure and physiology gives biofilms an inherent resistance to antimicrobial agents (Fletcher and Bodenham 1999a, Potera 1998, Vorachit et al. 1993). An example are the biofilms of Candida albicans, which can consist of matrix-enclosed microcolonies of yeasts and hyphae, arranged in a bilayer structure resulting in resistance to antifungal agents such as amphotericin B and fluconazole (Douglas 2003).

Often there are dramatic differences in susceptibility of planktonic and biofilm organisms to microbial agents. For example is the action of imipenem on Ps. aeruginosa (ATCC 27853) where 1μg/ml of the antibiotic was adequate to treat the planktonic phenotype, but >1,024μg/ml was required to eradicate the biofilm phenotype (Donlan and Costerton 2002). The mechanisms responsible for this resistance may be due to one or more of the following:
Figure 1.12 A scanning electron micrograph (SEM) of a longitudinal cross section of a double lumen CVC. Source: work carried out by the author as part of this study.
Figure 1.13 A SEM of a CVC lumen showing the biofilm on the extraluminal and intraluminal surfaces. Source: work carried out by the author as part of this study.
The biofilm matrix could act as a simple diffusion barrier to delay penetration of the antimicrobial agent (Wilcox et al. 2001).

Negatively charged glycocalyx would bind positively charged antibiotic molecules; however, this does not account for the resistance to neutral and negatively charged antibiotics (Potera 1998).

Production of extracellular enzymes such ß-lactamase, which would inactivate antibiotics diffusing into the biofilm thereby reducing the concentration reaching the bacteria in the biofilm (Tunney et al. 1996).

The altered growth rate of the biofilm organisms and other physical changes associated with the biofilm state (Bayston 1999). This has been observed where planktonic and young (2 days old) biofilm cells have been inactivated by an antibiotic but older (10 day old) biofilm cell counts were reduced by only 20% with the same dose of antibiotic (Donlan and Costerton 2002).

**f) Relationship between biofilm production and infection**

The exact process by which biofilm-associated organisms elicit an infection is poorly understood (Donlan and Costerton 2002). Some of the suggested mechanisms include:

- Detachment or sloughing off of cells or cell aggregates from the implanted device resulting in a bloodstream infection.

- The production of endotoxins by gram-negative bacteria, which may elicit an immune response.

- The biofilm cells are in some ways resistant to the host immune system; an example is where the macrophage's (a white blood cell) normal phagocytic activity is ineffective against S. epidermidis cells in the biofilm state (Donlan and Costerton 2002).

It has been observed, however, that there is no clear association between biofilm production and virulence. In an in vitro study by Vogel et al. (2000), the association between biofilm production and virulence was investigated by comparing the biofilm production between 22 S. epidermidis isolates associated with CRBSI and 31 nasal-
colonising isolates from healthy individuals. Of the 22 catheter-related isolates only seven had significantly higher levels of biofilm production compared to all other catheter-related and nasal isolates. This suggests that biofilm production may be an adaptive response of some strains of microorganisms to the presence of an indwelling device rather than a necessary factor for virulence (Vogel et al. 2000).

g) Phenotypic changes and organism detection

As a biofilm matures phenotypic modification of the cells in the deeper layers can often take place (Bayston 1999). They include a slow-down and change in the metabolism of the cells, as a response to nutrition deprivation, resulting in virtually no cell wall material being synthesised. Therefore these cells grow very slowly and this “small-colony phenotype” is often difficult to detect using conventional microbiological techniques (Krimmer et al. 1999). These small colony variants (SCVs) may be why the microbiological diagnosis of device-associated infections frequently remains uncertain, since some staphylococci have the capacity to reduce their growth rate considerably (Bayston 1999, Krimmer et al. 1999).

Improved detection and identification of *S. aureus* and *S. epidermidis* by an *in situ* hybridization method with fluorescence-labelled oligonucleotide probes specific for staphylococcal 16S rRNA has been reported (Krimmer et al. 1999). In their study of hip replacement patients, Krimmer et al. (1999) successfully used the technique for the detection of intracellularly persisting bacteria, including SCVs of *S. aureus*, and the differentiation of *S. epidermidis* from other clinically relevant staphylococci even when they were embedded in biofilms. The authors suggest that the 16S rRNA hybridization technique may be useful for the detection and differentiation of many other microorganisms.

1.6 Measures to reduce catheter-associated infection rates

There are a number of improvements in clinical practice currently being investigated, which could lower the rates of infection in catheterised patients. These include improvements to insertion techniques, better aseptic technique, modifications to the catheters, hub modifications and the introduction of intravenous therapy teams. Guidelines for preventing infections associated with the insertion and maintenance of
CVCs have been developed (Pratt *et al.* 2001). These are outlined in Table 1.6 and have been developed by a nurse-led, multi-professional team composed of clinical microbiologists, infection control practitioners and researchers amongst others. The guidelines cover all interventions from catheter selection through to strategies for removing and replacing catheters.

1.6.1 *Insertion technique*

The use of maximal aseptic precautions whilst inserting a CVC will reduce the incidence of CRS (Raad *et al.* 1994b). For insertion of all CVCs it is recommended that the surgeon uses a cap, mask, sterile gown, sterile gloves and a wide sterile drape (Collins 1991). As the skin is the main source of many of the microorganisms that are implicated in CRS, the use of a disinfectant or antiseptic solution to reduce the microbial bioburden and thus reduce the risk of contamination during insertion is necessary. In the United States solutions such as 10% povidone-iodine are the most widely used antiseptic for the disinfection of the catheter insertion site (Crnich and Maki 2002a). However even lower rates of colonisation have been observed when a 2% aqueous solution of chlorhexidine was compared with 10% povidone-iodine solution and 70% alcohol in a randomised trial (Maki *et al.* 1991). Chlorhexidine-containing solutions are now recommended as the first choice for vascular access (Crnich and Maki 2002a).

1.6.2 *Catheter modifications*

a) *CVCs with anti-infective surfaces*

One recent advance in catheter design has been the introduction of catheters with antimicrobial and antiseptic properties. Antimicrobial bonding of catheters was first demonstrated in a rat model, where microbial colonisation was lower in penicillin-bonded polyethylene catheters when compared to control catheters (Trooskin *et al.* 1985). Since then several other antimicrobial compounds have been incorporated into catheters to give a number of different so called “coated catheters”.
<table>
<thead>
<tr>
<th>Intervention</th>
<th>Guidelines associated with intervention</th>
</tr>
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</table>
| Selection of catheter type | a) Use a single-lumen catheter unless multiple lumens are essential for patient management.  
b) Use one lumen exclusively for the administration of TPN.  
c) If the duration of catheterisation is anticipated to be >30 days, use a tunnelled catheter or an implantable VAD.  
d) Consider the use of antimicrobial impregnated catheters in adults who require a short term central venous catheterisation (<10 days) and are at high risk of CRBSI. |
| Selection of catheter insertion site | e) Assess the risk of infection against the risks of mechanical complication.  
f) When possible use subclavian site in preference to the jugular or femoral sites for non tunnelled catheter placements.  
g) Consider the use of PICCs as an alternative to subclavian or jugular vein catheter placements. |
| Optimum aseptic technique during catheter insertion | h) During the insertion of CVCs use optimum aseptic technique, including gloves, a sterile gown and a sterile drape. |
| Cutaneous antisepsis | i) Prior to CVC insertion, clean the skin site with a alcoholic chlorhexidine gluconate solution and allow to dry. In cases where patients are known to be chlorhexidine sensitive use an alcoholic povidone-iodine solution.  
j) Do not apply organic solvents (acetone or ether) or antimicrobial ointment to the skin prior to catheter insertion. |
| Catheter and catheter site care | k) Prior to accessing the system disinfect the external surfaces of the catheter hub and connection ports with an aqueous solution of chlorhexidine gluconate or povidone-iodine, unless contraindicated by the manufacturer.  
l) Use either a sterile gauze or transparent dressing to cover the catheter site. Where gauze and tape catheter site dressing is used, it must be replaced when the dressing becomes damp, soiled or inspection of the insertion site is necessary.  
m) Do not apply antimicrobial ointment to CVC insertion sites as part of routine catheter site care.  
n) Routinely flush indwelling CVCs with an anticoagulant unless contraindicated by the manufacturer. |
| Replacement strategies | o) Do not routinely replace non-tunnelled CVCs as a method to prevent catheter-related infections.  
p) Use GWX method to replace a malfunctioning catheter only if the signs of infection at the catheter site are not present. If subsequent catheter-associated infection is detected the catheter should be removed and a new catheter, placed at a new site.  
q) Do not use GWX for patients with CRl. The catheter should be removed and a new catheter, if required, placed at a new site.  
r) Replace all tubing when the CVC is replaced, otherwise intravenous tubing should be replaced no more frequently than every 72 hours unless clinically indicated or if used to administer blood, blood products or lipid emulsions (replace every 24 hours). |
| Antibiotic prophylaxis | s) Do not administer systemic antimicrobials routinely before insertion or during use of a CVC to prevent catheter colonisation. |
Catheters coated on both the internal and external surface with benzalkonium chloride have demonstrated a significant decrease in colonisation (Elliott et al. 1994b) but no reported improvements have been observed in CRBSI rates in any of the trials using this type of catheter (Crnich and Maki 2002a).

The chlorhexidine - silver sulphadiazine catheter, where the combination of the two compounds has a synergistic activity against species such as Staphylococcus aureus, and the minocycline – rifampicin catheter, which has a synergistic effect on Staphylococcus species, gram negative bacteria and yeasts. Results from trials have, however, been conflicting (Civetta et al. 1996, Logghe et al. 1997, Mabubula et al. 2000, Pemberton et al. 1996, Raad et al. 1997, Saint et al. 2000, Veenstra et al. 1999a). Both Civetta et al. (1996) and Veenstra et al. (1999a) demonstrated that catheters impregnated with silver sulfadiazine and chlorhexidine reduce catheter-associated infections. However, Pemberton et al. (1996) found no difference in CRS rates between the antimicrobial-impregnated CVCs and a standard triple lumen CVC (7.3% and 7.5% respectively) in their study of 72 CVCs.

A meta-analysis of eleven studies, totalling some 2603 catheters, showed that CVCs impregnated with a combination of chlorhexidine and silver sulfadiazine are effective in reducing the incidence of both catheter colonisation and CRBSI in patients at high risk for catheter-associated infections (Veenstra et al. 1999b). However Schierholz et al. (2000a) questioned the exclusion of the largest clinical trial by Logghe et al. (1997) which showed that there was no significant difference between the incidence of catheter-related infection for impregnated and non-impregnated catheters (5.0% and 4.4% respectively) in long-term catheterised patients. Veenstra et al. (1999b) excluded this trial because the definition of CRS was based on the culture of blood taken both through the CVC and from a peripheral site, and the CVC was not always removed in suspected cases. Schierholz et al. (2000b) conclude that chlorhexidine and silver sulfadiazine coated catheters may be of little benefit for long-term use (>10 days) as there is no intraluminal protection.

A study by Darouiche et al. (1999) comparing the minocycline - rifampicin catheter and the chlorhexidine - silver sulphadiazine catheter, demonstrated the minocycline - rifampicin catheter to be more effective than the chlorhexidine - silver sulphadiazine
catheter against microbial colonisation of the catheter. However, the explanation for this may depend as much on the lack of coating on the inside of the chlorhexidine - silver sulphadiazine catheter, as on the compounds coated on each CVC (Wilcox et al. 1998).

A further more recent study by Ranucci et al. (2003) also demonstrated that an oligon-treated (polyurethane combined with silver, carbon, and platinum) catheter was effective in limiting the rate of microbial colonisation. However there was no significant difference between oligon-treated catheters and control catheters in terms of the rate of CRBSI.

Some concerns about the use of both of these antimicrobial-impregnated CVCs have been voiced and are highlighted below:

- The chlorhexidine - silver sulphadiazine CVC is coated on the external surface only, therefore it has no effect against endoluminal colonisation (Fletcher and Bodenham 1999b, Schierholz et al. 2000b). To be effective this type of CVC requires antimicrobial coating on both the internal and external lumen surfaces, including the extension pieces at the manifold (Wilcox et al. 1998).

- Even-though chlorhexidine is associated with a lower rate of catheter colonisation and CRS (Orr and Ryder 1993, Norwood et al. 2000), it can cause allergic or anaphylactic responses in some individuals. It has been observed that the native Japanese are particularly prone to anaphylaxis caused by chlorhexidine (Burlington 1998). As a result, the chlorhexidine - silver sulphadiazine CVC was withdrawn in Japan after twelve reported cases of severe anaphylaxis and at least one death (Yasukawa et al. 1999).

- There are issues with the use of antibiotics in this situation; for example Rifampicin which is a first line drug against tuberculosis, as this may create resistant strains of organisms (Fletcher and Bodenham 1999b).

- There may be problems with sterilisation of chlorhexidine-coated catheters. Sherertz et al. (1996) demonstrated that a chlorhexidine-coated catheter loses its antimicrobial activity after gamma irradiation is used for sterilisation. The ethylene oxide method is recommended as an alternative for these catheters.
Tacconelli et al. (2000) have raised concerns about the efficacy of the chlorhexidine-silver sulphadiazine CVC against Candida species, as the combination of chlorhexidine-sulphadiazine will only affect bacterial growth and yeasts, such as Candida species, may not be adversely affected.

b) Active iontophoresis

Active iontophoresis is the newest strategy to reduce infection rates associated with CVCs (Crnich and Maki 2002a). In an in vitro study, Liu et al. (1993) found that when a constant electric current of low amperage (10μA) is applied to a catheter, there is a reduction in microorganism adherence to the CVC. Liu et al. (1993) then concluded that this low amperage current may be useful in reducing bacterial colonisation of indwelling intravascular catheters. Although promising, this technology has yet to be evaluated in a clinical trial (Crnich and Maki 2002a).

1.6.3 Hub modifications

The new type of hub, evaluated by Segura et al. (1996), has given a fourfold reduction in the incidence of CRBSI when compared to controls. This hub contains iodinated alcohol and a needle must be passed through a chamber containing iodinated alcohol, before it can be used.

Needleless connectors have been used in conjunction with IV catheters to reduce the time spent manipulating intravenous connections and prevent the occurrence of needlestick injury. They have also been shown to reduce significantly the likelihood of microbial colonisation via the internal route (Brown et al. 1997). However their net benefit has been questioned by reports of increased rates of bloodstream infection (Crnich and Maki 2002a). This may however be due to the contamination of flush solutions (Worthington et al. 2001) and a combination of pre-filled sterile syringes (and therefore sterile flush solution) and needless connectors may lead to further reduced contamination rates (Casey et al. 2002).

1.6.4 Therapy teams

Studies have shown that the use of a well-trained team, responsible for insertion and maintenance of VADs within the hospital can lead to a dramatic improvement in catheter
site care (Elliott et al. 1994a). An overall reduction in the level of CRS, especially in patients receiving TPN, has also been observed (Fletcher and Bodenham 1999b, Nehme 1980). In the study by Nehme (1980) it was observed that CRS rates varied from 1.3% (5 of 211) in the group of patients that were managed exclusively by a nutrition support team to 26.2% (43 of 164) in the control group that were managed by a variety of physicians.

1.7 Diagnosis of catheter-associated infections

A case of true CRBSI is almost always accompanied by clinical symptoms (Dobbins et al. 1999). CRBSI is suspected in a catheterised patient when fever or other signs of sepsis are observed even when that patient is otherwise well. Should the symptoms abate after catheter removal, further confirmatory testing may not be required. However, there may be an alternative source of the sepsis, therefore, good clinical practise would demand that further tests be carried out (Fletcher and Bodenham 1999a). When CRBSI is suspected and/or diagnosed conventional wisdom would deem that it should be treated, either by antibiotic therapy and/or catheter replacement. However, no data exists to predict whether intervention on suspicion is better than intervention on data.

A number of laboratory-based techniques have been developed specifically to aid in the diagnosis of catheter-associated infections (Collignon and Munro 1989, Fletcher and Bodenham 1999a, 1999b, Siegman-Igra et al. 1997). Indeed, Siegman-Igra et al. (1997) conducted a meta-analysis of published studies which involved some sixteen methods for diagnosing CRBSI. These techniques use a number of different types of samples, ranging from culture of the removed distal tip of the suspected catheter (Brun-Buisson et al. 1987, Cleri et al. 1980, Maki et al. 1977), to blood withdrawn directly from the catheter lumen (Wing et al. 1979). In the following section some of these techniques and methods are described in detail.

1.7.1 Central Venous Catheter tip culture

a) Qualitative catheter tip culture

Druskin and Siegel (1963) introduced a technique that was to be known as a qualitative catheter tip culture. This involved the immersion in nutrient broth of the distal catheter
tip after removal from the patient. Using this technique any microbial growth is significant so consequently it has a high sensitivity for diagnosing catheter-associated infections. However a potentially contaminated catheter or catheter with a low level of colonisation would also give a positive result, therefore this method has a poor specificity. This was highlighted in a study by Norwood et al. (1991) where 50% of the tips were falsely diagnosed as positive for CRS when in actual fact they were simply contaminated or colonised. For the same reasons Nahass and Weinstein (1990) found that the qualitative culture of catheter tips was not predictive of catheter-related bacteraemia because they calculated a positive predictive value of 17% in their study.

Therefore, a problem with this technique is that there is no distinction between true infection leading to sepsis and the non-pathogenic levels of colonisation and contamination. Other quantitative and semi-quantitative methods have since been developed, so as to improve upon this technique (Brun-Buisson et al. 1987, Cleri et al. 1980, Maki et al. 1977); this are reviewed below.

b) Semi-quantitative catheter tip culture - the Maki roll technique

Maki et al. (1977) described a catheter culture technique where they evaluated 250 catheters that were removed from the peripheral veins of 198 patients. They found that they could distinguish between colonisation associated with localised inflammation or systemic infection and contamination (the procedure for removal of a catheter creates the opportunity for contamination) by using a cut off of 15 CFU or more on a blood agar plate culture of the catheter tip.

The technique itself involves removal of the catheter from the patient when CRBSI is suspected. The distal tip (approximately 6cm long) is then severed from the rest of the catheter. In the laboratory the tip is rolled on a blood agar plate which is then incubated for 24 hours. The colonies are counted and if there are $\geq 15$ CFUs the count is considered significant as the infection is distinguished from contamination at this level (Maki et al. 1977). Collignon et al. (1986) used the same technique but found that five or more colonies was a more appropriate cut-off for a positive result. Using this new cut-off they determined the sensitivity and specificity to be 92% and 83% respectively, in a cohort of 780 catheter tips.
This semi-quantitative method based on ≥15 CFUs has become the gold standard in catheter analysis in the past twenty years (Dobbins and Kite 1999). Initially it was thought that this technique was an accurate method of catheter culture as well as being inexpensive and simple. Unfortunately it does have a number of inherent flaws (Sitges-Serra and Linares 1988). Like the qualitative broth culture described in Section 1.7.1a, it involves the sacrifice of the catheter, which renders it a retrospective test as the CVC has been replaced. The results gained from both techniques can also be influenced by possible contamination of the external catheter tip surface by organisms on the skin.

Moreover in the case of the Maki et al. (1977) technique the outside of the catheter is the only portion that is cultured, with possible internal colonisation going undetected. This coupled with its low positive (≥15 CFUs) criteria, and the potential for heavy contamination from leakage of endoluminal fluid during specimen transit, are reason enough to exclude the method as a ‘gold standard’ (Dobbins and Kite 1999).

c) Quantitative catheter tip culture - the Cleri flush technique

Cleri et al. (1980) developed a technique to culture a sample from the internal lumen of the catheter. This study looked at 189 intravenous and intravascular catheters and determined that all catheters associated with bacteraemia had catheter cultures of at least 1000 CFUs. The technique, now known as the "Cleri method" involves flushing and culturing of the contents of the removed CVC tip on blood agar. Those catheters with >1000 CFUs are defined as infected (Cleri et al. 1980). Those catheters with <1000 CFUs are described as either colonised or contaminated during removal.

Using 1000 CFUs as a cut off level, this method was used successfully in a study involving catheterised patients receiving TPN (Pettigrew et al. 1985). It was also successfully used in a study by Linares et al. (1985), who compared the method used by Cleri et al. (1980) with the semiquantitative method (Maki et al. 1977). Kristinsson et al. (1989) evaluated three methods for culturing intravascular catheters. The methods used were the semiquantitative method (Maki et al. 1977), followed by the quantitative flush method (Cleri et al. 1980) and finally a sonication method involving 10ml of phosphate buffered saline (PBS). They found that the culture of the inside of the catheter (Cleri et al. 1980) was the best predictor for infection at a level of 100 CFUs rather than 1000
CFUs which was the level set by Cleri et al. (1980). They also found that almost all infected catheters were colonised both on the inside and the outside (Kristinsson et al. 1989).

The technique described by Cleri et al. (1980) suffers similar problems to the Maki et al. (1977) technique. This is because the CVC also needs to be removed in order for a diagnosis to be made, although it is the microbial composition of the outside of the catheter that is ignored in this case. Rello et al. (1992) suggested that semi-quantitative culture of the external surface (Maki et al. 1977) is adequate for CRS diagnosis in the critically ill and intraluminal methods, such as the Cleri et al. (1980) technique be restricted to long-term CVCs used for the administration of TPN.

d) Quantitative tip culture – other methods

Brun-Buisson et al. (1987) examined 331 CVCs from 232 patients. They used a similar method as Cleri et al. (1980) with the additional step of vortex mixing the CVC tip in order to obtain a sample from the outside of the CVC. Those catheters with >1000 CFUs per ml after culture on a blood agar plate were defined as clinically significant and this cut-off level was 97.5% sensitive and 88% specific for the diagnosis of CRS in this study (Brun-Buisson et al. 1987).

Sherertz et al. (1990) conducted a three-year study using a similar quantitative sonication method, where 1,681 consecutive catheters were cultured and a total of 46% of these were positive. They concluded that sonication is an effective means of removing organisms from CVCs and it greatly increases the number of organisms that can be counted compared to the Maki roll technique. The level of significance for CRS was set at >100 CFUs in the sample matched by positive blood cultures for the same organism.

e) Disadvantages associated with CVC tip culture

There are a number of problems associated with each of the techniques described in Sections 1.7.1(a) to 1.7.1(d). The main problem that they all share is the requirement for CVC removal so that the laboratory diagnosis can be made. This is inappropriate and unnecessary when studies have shown that approximately 80% of CVCs that are removed in this situation are actually not the source of the suspected infection (Kite et al. 1997,
Pettigrew et al. 1985, Ryan et al. 1974). Catheter removal is generally based on the clinician’s judgement as to whether the clinical signs of fever are related to the CVC. To improve this, the clinician needs to be more certain that it is the CVC that is the source of infection. The only way that this can be done is to use methods that can determine the number of organisms in the CVC whilst the CVC remains within the patient. Thus, the requirement is for techniques that do not involve CVC removal.

### 1.7.2 In situ CVC sampling techniques

The assessment of the microbial status of a CVC whilst it remains in situ can be done using catheter blood culture, exit-site culture, hub culture, or culture of a sample retrieved by a FASEB (Fan et al. 1988, Kite et al. 1997, Wing et al. 1979). The advantage of these "in situ" methods is that the unnecessary sacrifice of catheters not associated with infection is avoided.

**a) Peripheral blood culture**

If a patient is showing signs of suffering a catheter-associated infection, a peripheral blood sample can be taken from the patient whilst the CVC is still in place. The presence of a systemic infection or bacteraemia can then be determined, however it should be noted that, on its own, this technique is not useful in determining if a CVC is the source of the bacteraemia or infection. Nevertheless, the confirmation of a peripheral bacteraemia is crucial to the diagnosis a catheter-associated infection when used in conjunction with other tests (Dobbins et al. 1999).

**b) Quantitative blood cultures**

It is possible to draw blood through the CVC and then culture this blood to determine if any microorganisms are present. This technique of culturing blood samples aspirated from the CVC has been used by a number of authors in various studies (Paya et al. 1989, Snydman et al. 1982, Wing et al. 1979). A paired quantitative blood culture (QBC) is based on the principle that blood is withdrawn from both the catheter and a peripheral vein and the two isolates compared (Paya et al. 1989, Wing et al. 1979).

If blood is drawn through a colonised catheter then planktonic microorganisms will enter the blood. This blood sample will then contain organisms from the CVC, and therefore
can be used to determine if these are the same organisms as those in the peripheral blood sample. The blood samples are cultured and if the culture from the CVC yields a 4 to 10 times greater colony count per millilitre than peripheral blood, then the CVC is the most likely source of sepsis. Wing et al. (1979) found blood withdrawn through the peripheral vein grew 25 colonies per ml whereas blood drawn through the catheter had greater than 10,000 colonies per ml. On this basis the catheter was removed and the tip cultured to confirm infection. If a patient is bacteraemic then culture of blood drawn through the catheter will give a positive result, but this does not necessarily implicate the catheter as the source of sepsis (Fletcher and Bodenham 1999b). This is because the bacteraemia could have its source in another region of the patient’s body, such as a post-operative wound.

The blood culture technique cannot, however, be employed when it is not possible to aspirate blood from the CVC (Dobbins and Kite 1999, Moyer et al. 1983). Moyer et al. (1983) were unable to obtain blood samples from 34 of the 101 catheters in their comparative study of diagnostic techniques. Other studies have reported that in up to 50% of catheters blood cannot be aspirated (Dobbins and Kite 1999).

Whilst QBC may be useful in making a decision as to whether or not to remove a CVC, negative CVC cultures cannot be assumed to rule out infection (Dobbins et al. 1999). Paya et al. (1989) found that blood drawn through a catheter gives high rates of both false-negative and false-positive results. They concluded that differential quantitative blood cultures drawn simultaneously from a suspected CVC and a peripheral vein are not useful for predicting a CRBSI. Blot et al. (1998, 1999) and Rogers and Oppenheim (1998) suggested alternatives to traditional plate culture of the blood. The continuous monitoring blood culture system and differential positivity times for blood cultures are described in Section 1.8.2.

c) **Hub culture**

Swabs can be taken from the hub of the catheter to provide a sample for analysis (Atela et al. 1997, Fan et al. 1988, Sitges-Serra and Linares 1988). In the past hub culture was generally used as a means of assessing the route taken by microorganisms which travel to the catheter tip rather than as a sample for CRS diagnosis (Sitges-Serra and Linares
Swabs of the CVC hub have been cultured in very few controlled studies and therefore data on the usefulness of the results provided by such cultures are limited (Goldmann and Pier 1993). In a study by Fan et al. (1988), 29 of 142 (20.4%) catheters studied were found to have resulted in CRS, however only 10 of the 29 were detected through the hub cultures to give a sensitivity of 34.5%.

The catheter hub has frequently been treated as a separate part of the CVC as it is regularly cleaned, often with an antiseptic. Thus a result could prove to be falsely negative, because the hub would not be a true reflection of the remainder of the CVC as it is cleaned more often (Dobbins et al. 1999).

d) Exit-site swabs

The site where the CVC exits the skin can be swabbed and this sample cultured (Atela et al. 1997, Bjornson et al. 1982, Fan et al. 1988). Exit-site cultures can go some way in predicting and diagnosing CVC infections and correlations between positive skin cultures and catheter tip culture have been reported (Bjornson et al. 1982, Fan et al. 1988). However, this good correlation could have resulted from contamination of catheter tip by organisms at the exit site upon removal (Dobbins et al. 1999).

It should be noted though, that clinical signs of wound infections, such as suppuration (pus formation) and tract erythema (reddening) do not necessarily correlate with CRBSI as they may be a separate, local, infective complication (Dobbins et al. 1999, Elliott et al. 1994a, Safdar and Maki 2002) as outlined in Table 1.2.

Both the hub and the exit site have been used in surveillance cultures where swabs can be taken to detect organisms at the sites most likely to be the source of a CRBSI. Fan et al. (1988) recommend that a combination of skin and hub cultures are required for satisfactory surveillance of catheters in order to detect colonisation and possible CRS.

e) FAS Endoluminal Brush

The FASEB is designed to retrieve a sample of fibrinous material from the internal lumen wall of a CVC that is suspected as being infected (Markus and Buday 1989). The sample can then undergo microbiological examination. The objective of FASEB use is to prevent the unnecessary removal of catheters wrongly suspected as the source of sepsis (Kite et al. 1988).
al. 1997, Tighe et al. 1996a). Information on the microbial status of a sample, obtained using the FASEB, would allow the clinician to make an informed judgement as to the continued management of the patient. Endoluminal brushing has been investigated by a number of authors and has been shown to be an adequate tool for the retrieval of a sample from a CVC, whilst it remains in situ (Dittmer et al. 1996, Dobbins and Kite 1999, Kite et al. 1997, 1999, 2001, Llancaqueo et al. 1996, van Heerden et al. 1996). In addition, use of the FASEB, in conjunction with plate culture methods, has been shown to be the most sensitive and specific technique available to determine if a CVC is infected whilst it remains in situ (Dobbins and Kite 1999, Kite et al. 1997, 1999, Tighe et al. 1996a). Kite et al. (1997) reported sensitivity and specificity levels for the FASEB to be greater than those for catheter tip cultures – assessed by the Maki roll technique (Maki et al. 1977) and a modified Cleri-flush technique. Dobbins and Kite (1999) have since reported that the FASEB technique is 95% sensitive and 96% specific for detecting CRBSI. Further work by Kite et al. (2001) has shown that the FASEB method has a sensitivity of 100% for CRBSI in a patient population with double lumen haemodialysis catheters.

The FASEB, shown in Figure 1.14 consists of a wire handle, a brush and a protective microbially impervious bag disposed over the handle and brush. It is a sterile device that is connected to a CVC via a Luer lock. As the FASEB is advanced through the catheter it is simultaneously removed from the protective bag. Then it is withdrawn from the catheter, back into the bag. The tip of the FASEB can then be cut off (approximately 7cm) and placed in a plastic tube. In the microbiology laboratory, the FASEB head is immersed in 1ml of buffered phosphate. Following vortex mixing 10\mu l and / or 100\mu l aliquotes of the resulting suspension are spread on each of two blood agar plates, which are left for 24 hours at 37°C. A significant result is >100 CFUs (Kite et al. 1997).

Advantages of in situ techniques over the traditional tip culture methods

Unnecessary insertion procedures, induced by unnecessary removal of viable catheters cause significant morbidity. Therefore, it is critical that in situ methods of CVC analysis are used more frequently so that functional CVCs are not removed and replaced unnecessarily, when they are wrongly suspected as infected. The risk of complication in the management of suspected catheter-associated infection by the use of in situ sampling
Figure 1.14  The FAS Endoluminal Brush and CVC hub (Tranter and Donoghue 2000).
techniques such as the FASEB is significantly less than the risk associated with current techniques requiring the removal and replacement of suspected CVCs. In addition to the reduction in risk afforded by the accurate in-situ identification of non-infected CVCs there can also be considerable savings in the cost associated with patient management (Archis et al. 2000, Dobbins and Kite 1999, Farmer et al. 1997, Tranter and Donoghue 2000).

1.8 Rapid diagnosis of catheter-associated infections

Traditional sample culture methods require at least 24 to 48 hours before a result can be obtained (Cleri et al. 1980, Kite et al. 1997, Maki et al. 1977). More rapid alternatives to this overnight culture have been developed and evaluated (Blot et al. 1999, Cooper et al. 1985, Kite et al. 1999, Rogers and Oppenhiem 1998, Rushforth et al. 1993, Tighe et al. 1996b, Worthington et al. 2002a, Zufferey et al. 1988). A review of these techniques is below.

1.8.1 Staining techniques

Gram staining and the use of metachromatic dyes, such as acridine orange, have been applied to the rapid diagnosis of CRBSI (Cooper et al. 1985, Kite et al. 1999, Tighe et al. 1996b, Zufferey et al. 1988). Staining has been used on both catheter tip samples (Cooper et al. 1985) and blood samples aspirated from that CVC (Tighe et al. 1996b).

Acridine orange is a fluorochrome, which can differentially stain double-stranded and single-stranded nucleic acids (Chapin 1995). When acridine orange intercalates into DNA (double-stranded) it emits green fluorescence upon excitation at 480-490 nm. On the contrary, it emits red-orange it when interacts with RNA and indeed single stranded DNA (Chapin 1995). This intercalated into either RNA or DNA can take place both in the native and denatured state. Acridine orange is particularly useful in situations where the Gram stain is not interpretable, for example in blood specimens. Consequently it has been used as a stain in the rapid detection of organisms in blood aspirated from CVCs in both neonatal (Rushforth et al. 1993) and adult (Tighe et al. 1996b) patient groups.

However, as acridine orange stains nucleic acids in all bacteria and fungi, including both living and dead cells, it may be a less sensitive method of cell enumeration than viable
cell counts. One way in which the sensitivity of the technique can be improved, is to use acridine orange in conjunction with fluorescent redox probes. These lead to the direct visualisation of actively respiring bacteria only (Heidelberg et al. 1997). One such fluorescent redox probe is p-iodonitrotetrazolium violet (INT), which has been used in conjunction with acridine orange to detect viable but non-culturable gram-negative bacteria (Heidelberg et al. 1997).

Another problem that can occur in fluorescence microscopy is the decomposition of the fluorescent molecules, due to the presence of molecular oxygen. This, fortunately, can now be avoided through the use of anti-fade agents, which enhance the sample and make visualisation better (Pawley 1995). One such agent is diazabi-cyclo-octane (DABCO), which can be added to the sample prior to staining with the fluorescent molecule (Jolly et al. 1997).

a) Staining samples derived from the removed catheter

Cooper et al. (1985) reported the use of a Gram stain method for diagnosis of CRBSI. The catheter was stained and then examined under oil immersion light microscopy. Using Gram staining the authors detected organisms on all 41 catheter tips that were associated with infection. However the anticipated high sensitivity and specificity of the assay was premature when it was subsequently found that seven samples that were reported as Gram positive were infected by yeast, and not Gram positive bacteria.

This technique was repeated by Coutlee et al. (1988) who looked at the staining of the catheter tip, using both Gram stain and an acridine orange direct stain. Coutlee et al. (1988) found acridine orange easier to use than Gram staining, but generally they were not satisfied with the overall correlation with true infection. Moreover they observed that the direct staining techniques were laborious and the results from some catheters could not be interpreted. They did suggest, however, that this technique could be useful in detecting fungal infections, especially when normal culture is producing negative results.

Another study concluded that acridine orange staining was easier to perform than Gram-staining, where direct acridine orange staining of the catheter tips was found to be 84% sensitive and 99% specific (Zufferey et al. 1988).
Collignon et al. (1987) have carried out some work on Gram staining impression smears of catheters, and it was shown to be useful, however, the patient group had a low rate of CRBSI.

Although these techniques are useful, rapid, inexpensive and relatively simple to perform, they are flawed by the fact that they rely on the removal of the CVC in order to provide a result. Because of this the only real benefit of using these techniques is to get a rapid result which would lead to the provision of appropriate treatment, such as antibiotic therapy, at an earlier stage (Dobbins et al. 1999).

b) Staining of "in situ" samples

The usefulness of staining blood drawn from the catheter has been evaluated with varying results. Moonens et al. (1994) concluded that Gram-stained blood was useful in cases where there was a definitive positive result, but that a negative Gram stain could not rule out catheter infection. Kleiman et al. (1984) concluded that acridine orange, although not a differential stain like the Gram-stain, was useful in providing information on the presence of microorganisms and this may be useful in the selection of antibiotics.

c) The acridine orange leucocyte cytospin (AOLC) test

The AOLC test was found to be a sensitive, rapid (<1 hour) in situ test for the detection of infected intravenous catheters in neonates, using a small volume of blood (Rushforth et al. 1993). A positive result in the AOLC test is the presence of one or more viable or denatured bacteria (Tighe et al. 1996b).

Four studies involving the AOLC method in conjunction with blood drawn through adult CVCs have been reported (Gowardman et al. 1998, Kite et al. 1999, Tighe et al. 1996b and von Baum et al. 1998). Tighe et al. (1996b) concluded that the use of the AOLC method with blood drawn through the catheter was not sensitive enough to detect CRS in adults. However, when used in combination with the FASEB, the AOLC method has been shown to be much more sensitive (Tighe et al. 1996b). von Baum et al. (1998) reported that the AOLC method used in conjunction with blood was not useful and they concluded that it should not be used as a routine method for the CRS in adults. In another study of 499 CVCs placed in 400 patients, where there were 12 proven cases of CRS, the
AOLC test was negative in all 12 cases (Gowardman et al. 1998). The studies by von Baum et al. (1998) and Gowardman et al. (1998) involved ICU patients and the poor results may have been due to the short duration of catheterisation of the patients (generally <10 days).

d) Combination of AOLC and Gram staining

Kite et al. (1999) published a technique that used Gram-stain and AOLC in combination to detect organisms in blood drawn through the catheter. They found this technique to be an accurate approach to diagnosing CRS as it correlated well with other methods such as the FASEB culture and the Maki roll plate technique. The assay had a sensitivity of 96% and specificity of 92%. However, in this study, blood could not be obtained from 16 of 128 patients (Kite et al. 1999). However, in their review of intravascular catheter-associated infections Crump and Collignon (2000) commented that this technique is labour intensive and smaller laboratories may not have all the facilities needed to perform a test. They also felt that the technique should be used selectively in patient populations with a high pre-test probability of sepsis due to the catheter.

1.8.2 Rapid blood culture techniques

a) A continuous monitoring blood culture system

Rogers and Oppenheim (1998) suggested an alternative to quantitative blood cultures. This technique involves the use of a continuous monitoring blood culture system, for example the BacT/Alert system (Organon Teknica, USA). They found that the time it takes to culture a blood sample containing microorganisms (in addition to length of lag phase) and therefore obtain a positive result, is directly related to the initial inoculum of microorganism. Therefore the higher the concentration of microorganisms in the initial sample then the more quickly a positive result will be obtained. Rogers and Oppenheim (1998) found that the time to positivity of blood withdrawn from a peripheral vein was on average 4.1 hours longer than blood aspirated from the CVC of patients that were subsequently diagnosed with CRS.
b) Differential positivity times for blood cultures

Blot et al. (1998) evaluated the differential time to positivity (DTTP) of cultures of blood drawn simultaneously from CVC and peripheral sites. This technique involves the use of a blood culture system, for example the Vital-Duo (bioMerieux, France), which detects microbial growth in blood cultures. In the case of this technique peripheral and through the CVC blood samples are taken. If the DTTP between peripheral and catheter blood samples is short then it is unlikely that the patient has CRS, however if the blood obtained through the CVC gives a positive result and there is a long period (hours to days) before the peripherally obtained sample becomes positive then CRS is likely.

By measuring the time to blood culture positivity Blot et al. (1998) found a cut-off limit of >120 minutes had a specificity of 100% and a sensitivity of 96.4% for the diagnosis of CRS in this retrospective study. In a further prospective study Blot et al. (1999) demonstrated 91% specificity and 94% sensitivity for the diagnosis of catheter-related infection using the same cut off of 120 minutes.

However Rijnders et al. (2001) found that the DTTP of hub-blood versus nonhub-blood cultures was not useful for the diagnosis of CRBSI in their prospective study of 100 critically ill patients. This may have been due to the short length of time the CVCs were in place (Farr 1999).

1.8.3 A serological test

Elliott et al. (2000) described an enzyme linked immunosorbent assay (ELISA) for the diagnosis of CRS which takes 24 hours to perform. Optimisation of this assay led to an ELISA for the diagnosis of intravascular CRS specifically caused by CNS, which can be completed within four hours (Worthington et al. 2002a). This is a sensitive (70%) and specific (100%) indirect ELISA, which is based on the detection of specific IgG antibodies in the serum of patients. The serum IgG are present in response to a glycerophospholipid (lipid S) antigen, which is produced by CNS. Worthington et al. (2002a) found that there was a significant difference in serum IgG to lipid S between patients with CRS and controls who had no clinical symptoms of sepsis. In the control group the blood was withdrawn immediately after CVC insertion.
CHAPTER TWO

Method Selection
2.1 Background

The major objectives of this section of the study were to choose a sample, sampling procedure and method of sample analysis that could be used to detect microorganisms, which have colonised a CVC. Firstly a literature search of published studies involving various catheter-sampling procedures, used in the detection of catheter-associated infections, was carried out. From this, one sampling procedure was chosen and a rationale for its selection, based on its performance in various publish studies was given. A second literature search of scientific publications and corporate technical information was subsequently performed to identify a list of technologies that may have the capability of rapidly detecting the presence of microorganisms in clinical and non-clinical samples. Each of these technologies were then reviewed and an assessment was made, based upon published studies and technical performance data. In addition, three technologies were evaluated in detail and this involved conversations directly with the manufactures as well as brief equipment tests to determine how user-friendly each technique would be. One was finally chosen and put forward as a possible method, which could routinely be used to detect microorganisms in conjunction with the sampling procedure already selected.

2.2 Choice of CVC sampling technique

The assessment of the microbial status of a CVC whilst it remains in-situ has been carried out using a number of different types of sample, which have been described in Section 1.7. All of these technologies have been shown to be of varying use in the detection of microorganisms associated with possible catheter-associated infections and the merits of each of these sample types are reviewed below.

2.2.1 Hub and skin cultures

When evaluating hub culture results, it should be remembered that the hub is regularly cleaned before and after CVC manipulation. Hence the hub may not reflect the extent of colonisation of the remainder of the CVC, thus giving false negative cultures (Dobbins et al. 1999). A combination of both hub and skin cultures has been shown to have a high negative predictive value (Atela et al. 1997, Fan et al. 1988). However the positive predictive values were less than 45% in both of these studies. Fan et al. (1988) recommend that a combination of skin and hub cultures are required for satisfactory
surveillance of catheters in order to detect microbial colonisation and possible CRS. However neither has been used solely to aid in the diagnosis of catheter-associated infections. On the basis of this neither of these two techniques were thought to be suitable for use in this study.

2.2.2 Blood aspirated versus FASEB sampling

Blood drawn through the CVC has been used by a number of groups in an attempt to aid in the detection of CRBSI (Blot et al. 1999, Paya et al. 1989, Wing et al. 1979). Blot et al. (1999) demonstrated a 91% specificity and 94% sensitivity for the diagnosis of catheter-related infection using the DTTP technique in conjunction with blood aspirated from the CVC and a peripheral blood sample. However when Rijnders et al. (2001) aspirated blood from the CVCs of 100 patients with suspected CRBSI, they were unable to detect CRS without associated bacteraemia in five patients using the DTTP technique. Rijnders et al. (2001) did comment that 78 of the 100 patients were receiving antibiotics at the time of catheter removal and this may be why more patients had CRS than had CRBSI. However this does raise an issue that if patients are undergoing systemic antibiotic therapy then they may not have bacteria in the blood sample, even though they have the clinical indications of sepsis, and therefore a false negative result would be reported. In addition to this possible issue, blood drawn through the CVC could potentially contain microorganisms from another septic focus within the body. Thus the fact that microorganisms are cultured from a blood sample does not necessarily mean that the bacteria originated from the CVC (Rijnders et al. 2001).

Another difficulty is that in up to 50% of cases blood cannot be aspirated from the CVC due to blockages within the catheter (Dobbins and Kite 1999). Paya et al. (1989) found in their prospective study that quantitative blood cultures failed to predict intravascular device related infection in over 50% of cases where infection was subsequently diagnosed. Infection was defined in this particular study as >15 CFU of the same organism from catheter tip culture as grown from peripheral blood.

The FASEB has been identified as the only device that can provide an uncontaminated sample that is definitively from the inside of the CVC, without removal of the CVC from the patient (Kite et al. 1997). The technique has been found to be have a higher sensitivity and specificity (95% and 96% respectively) than other laboratory techniques
for the diagnosis of clinically and microbiologically proven CRS (Dobbins and Kite 1999) and it is the only \textit{in situ} method capable of retrieving a sample in all suspected CVCs (Dobbins and Kite 1999).

A study by Tighe \textit{et al.} (1996b) demonstrated that only 12\% of blood samples drawn through infected CVCs gave a positive result using the AOLC technique. In contrast 83\% of blood samples drawn through the CVC after prior passage of the FASEB through the catheter were positive using the AOLC technique. Therefore, blood aspirated through the CVC may contain planktonic organisms that have originated from the CVC but few sessile biofilm organisms will form part of the sample. By using the FASEB to disturb the biofilm, more of the sessile biofilm associated cells are sampled and this may be critical when a clinical decision is made to treat on the basis of the sample (Donlan and Costerton 2002).

Donlan and Costerton (2002) in a recent review article on biofilms suggest that use of the FASEB could be extremely useful in determining the biofilm cell count within CVCs as it does not involve CVC removal. However they point out that the recovery efficiency of the technique (percentage of cells that are actually removed from the CVC lumen) needs to be determined. Recent \textit{in vitro} studies carried out by Worthington \textit{et al.} (2002b) have in part addressed this point by suggesting that 25\% of the bioload of the internal lumen of a CVC is removed through brushing. Furthermore it was observed that a combination of FASEB use and withdrawal of 10ml of blood through the CVC post brushing may lead to the removal of up to 70\% of the microbial bioload (Worthington \textit{et al.} 2002b).

From the evidence above it can be seen that there are a number of problems associated with using blood aspirated from the CVC. Often blood cannot be aspirated from the CVC and when it is there is no guarantee that it will contain organisms associated with the CVC. However, even if it does these are generally planktonic organisms and are not phenotypically similar to the sessile biofilm organisms (Donlan and Costerton 2002). The FASEB on the other hand provides a sample that is definitely from the inside of the CVC and contains both planktonic and sessile bacteria (Dobbins and Kite 1999). Therefore it was decided that the FASEB technique should be chosen as the sampling technique for use in this study.
2.2.3 Safety concerns of using the FASEB

A number of groups (Bouza et al. 2002, Donlan and Costerton 2002, Raad 1998, Rogers and Oppenheim 1998) have questioned the safety of using the FASEB technique, suggesting that the introduction of a brush into a CVC could potentially cause any number of complications. However any complications that have arisen, such as cardiac arrhythmias, embolisation or transient bacteraemia, have done so in very low frequency where none have required clinical intervention (Bouza et al. 2002, Tighe et al. 1996a) or not at all (Archis et al. 2000, Dittmer et al. 1996, Dobbins et al. 1997, Dobbins and Kite 1999, Farmer et al. 1997, Kite et al. 1997, McLure et al. 1997, Tighe et al. 1996a, Tranter and Donoghue 2000, van Heerden et al. 1996). Generally complications have arisen in cases where the FASEB has not been used in accordance with the manufacturers current instructions (Bouza et al. 2002, Tighe et al. 1996a, 1996b). Moreover, FASEB use inherently avoids the complications associated with catheter replacement and the technique is no more hazardous than the analogues procedure of GWX, which is accepted as a safe clinical practice (Dobbins and Kite 1999, Fletcher and Bodennham 1999b).

The FASEB is an approved medical device and has been designed with safety in mind. The bristles on the brush are spirally wound such that the bristle ends are orientated to the rear of the brush and therefore do not abrade the surface of the biofilm in a forward motion. This can be observed in Figure 2.1 where the FASEB has been passed through the hub into the leader tube of a CVC. The bristles flattened when the FASEB is travelling in a forward motion. The advantage of this is that material within the catheter lumen is not pushed forward in front of the brush. When the motion is reversed and the FASEB is withdrawn from the catheter, all of the bristle ends are “turned” to abrade the biofilm and thus remove material, which is then retained by the body of the bristles.

There are fifteen types of FASEBs available for use in conjunction with the various types of commercially available catheters; depending on the length and lumen diameter of the catheter. Therefore, if the correct brush is used with a particular CVC then the technique is both effective and safe (Dobbins and Kite 1999). Worthington et al. (2002b) confirmed that the FASEB was an adequate tool for sampling CVC and they demonstrated that no microorganisms are expelled from the CVC if brushing did not include the 2cm distal tip of the CVC. This supported the findings of Dobbins et al. (1997) where FASEB use was a clinically safe technique in 226 patients.
Figure 2.1  
A FASEB travelling through the leader tube of a CVC on its way into the CVC lumen. The bristles compact when the FASEB is travelling in a forward motion and these bristles will compact further when the brush enters the smaller diameter CVC lumen.
2.3 Choice of technology

2.3.1 Rapid tests currently in use

Some novel techniques, based on samples retrieved in situ, have been used to detect microorganisms in an attempt to reduce the number of CVCs that are unnecessarily removed. These have been described in Section 1.8 (Blot et al. 1998 and 1999, Kite et al. 1999, Rogers and Oppenhiem 1998, Tighe et al. 1996b, Worthington et al. 2002a). They are all laboratory-based techniques and provide a result well within the 24-hour period that traditional culture methods usually require. These “rapid tests” with a total assay time from as little as 30 minutes to as long as 6 hours have been developed for use in conjunction with in situ retrieved samples (Blot et al. 1998 and 1999, Kite et al. 1999, Kleiman et al. 1994, Moonens et al. 1994, Rogers and Oppenheim 1998, Tighe et al. 1996b, Worthington et al. 2002a). Many of these techniques involve the use of staining and microscopy, and all require blood samples drawn through the CVC.

The problems with these rapid techniques lie both in the sample that is used and in the technique used to evaluate the sample. Staining of samples can be time consuming and requires specialised training for a result to be determined. In addition, many of these use blood samples, which cannot be obtained from all patients. An example of this is the study by Kite et al. (1999) where blood could not be obtained from 16 of 128 patients. This was, however, not taken into consideration when the authors determined the sensitivity and specificity values, which were based on 112 patients and not 128. Therefore the true specificity for the assay should be reported as 73% and not 92%.

2.3.2 Preliminary requirements of a rapid technique

A number of different technologies were identified as suitable rapid direct counting procedures. These included, fluorescence microscopy, ELISA, ATP bioluminescence and particle counting.

For a technology to be useful in this particular application it is critical that any microorganisms that may be present in a sample are actually detected, meaning that the positive and negative predictive values of a detection system need to be high. The terms positive and negative predictive values are often used to measure the extent to which the
result of the assay reflects the true status of the patient. Positive predictive values (PV^+\text{+}) are determined by using the total number of samples that give positive results (false positives and true positives when compared to the reference method) in the patient population. Negative predictive values (PV^-) are determined by using the total number of samples that give negative results (false negatives and true negatives when compared to the reference method) in the patient population. The closer both of these are to 100% then the more specific the assay and the greater the chance of obtaining a correct result which will lead to appropriate clinical action.

It is critical that a CVC that is associated with a bloodstream infection is identified and quickly removed from the catheterised patient. This would indicate that a high PV^+\text{+} is important and therefore an assay with high specificity is required. However, it may be equally as important clinically, that a CVC which has been diagnosed as negative is a true negative because this CVC will allowed to remain in situ within the patient. Should a CVC that has tested negative actually be positive (a false negative by definition) it may cause a CRBSI which could result in mortality from a full-blown sepsis. Therefore, it is critical that such false negative results are kept to a minimum and the PV^- should be as close to 100% as possible.

A positive sample retrieved by the FASEB is reported as >100 CFU/ml and a negative sample reported <100 CFU/ml (Kite et al. 1997). Therefore, the selected technology will need to have the capability of detecting this level of microbial colonisation. If this level cannot be detected using a specific technique then there may be cases where low levels of colonisation or even infection are not detected therefore leading to a lower PV^- and a less sensitive and specific test.

2.3.3 Technologies that met the initial requirements

The three technologies listed in Table 2.1 were selected because they met the preliminary requirement of having the potential of detecting as few as 100 CFU/ml. Additionally, each have been shown to be useful in the rapid detection of microorganisms in other clinical and non-clinical applications, such as routine urine screening, food microbiology or environmental monitoring and testing. In addition all three technologies have been
<table>
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<th>Technology</th>
<th>Instruments and reagents</th>
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<tr>
<td>ATP bioluminescence</td>
<td>• The <em>Bioprobe</em>™ luminometer and reagents system.</td>
<td>Hughes Whitlock Limited, UK</td>
</tr>
<tr>
<td></td>
<td>• The <em>L1</em> luminometer and reagents system.</td>
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</tr>
<tr>
<td>Particle counting</td>
<td>• The <em>Questor</em>™ (variable orifice probe technology) used in combination with reverse agglutination.</td>
<td>Micromed Limited, UK</td>
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<tr>
<td>Staining-Microscopy</td>
<td>• The <em>StatSpin</em>™ and an ultra-violet microscope (Acridine Orange Leucocyte Cytospin).</td>
<td>StatSpin Technologies, USA</td>
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Table 2.1 A list of the technologies to be evaluated, as well as associated instrumentation and manufacturers.
used in clinical settings in the past (Lundin 1989, Medcraft et al. 1993, Tighe et al. 1996b) with the Questor, L1 and Bioprobe used in conjunction with urine samples and the Statspin used with blood samples.

Each of these technologies, as well as instrumentation based upon these are discussed in detail in Section 2.4.

2.3.4 Technologies that did not meet the initial requirements

Some other technologies were examined but these did not meet with the preliminary requirements set out above. These included techniques based on technologies such as immunoassay and fluorescence microscopy.

The major disadvantage that could be foreseen when an ELISA-based assay is used to rapidly detect a catheter-associated infection is that there no single antibody that would specifically detect all the microbial species that could be implicated in an episode of catheter-associated infection. To accurately detect a particular organism, approximately ten different assays would need to be performed so that each of the microbial species in Figure 1.10 could be detected or ruled out. Additionally, as up to 80% of CVC’s that are removed on suspicion of causing pyrexia are in fact negative, it would be unnecessary and expensive to perform a number of assays on a sample that could actually be negative.

The method described by Worthington et al. (2002a) is an ELISA based on the detection of specific IgG antibodies that are present in response to a glycerophospholipid (lipid S) antigen, which is produced by CNS. Even-though such an assay is useful for the detection of infection caused by CNS, it will not be useful in detecting infection caused by other microorganisms (up to 63%). Therefore, a negative result obtained using this ELISA cannot be assumed to mean that a catheter-associated infection has not occurred.

The use of a specific molecular probe dye (Molecular Probes Inc., USA) was also thought to be of potential use for the differential staining of microorganisms that may be present in CVC samples. However, it was pointed out by the manufacturer that many of the dyes that may be most appropriate for this application, are approved only for use in development work, and are much too toxic for use in a routine diagnostic test. Therefore their application was not investigated further.
2.4 Microbial detection technologies

This section contains details on each of the three technologies listed in Table 2.1. The principle behind each of the three technologies is given, in addition to applications and examples of equipment that are based on the technologies.

2.4.1 Staining and Microscopy

a) Acridine orange

Staining, such as the Gram stain, has been used in conjunction with microscopy to aid in clinical diagnostics for many years. Other stains such as acridine orange have also been useful tools in many aspects of clinical and environmental microbiology (Heidelberg et al. 1997, Tighe et al. 1996b). Kleiman et al. (1984) concluded that acridine orange was useful in providing information on the presence of cocci and rods in blood samples, drawn through a CVC. Since then the application of acridine orange in the detection of catheter-associated infections has been attempted in other studies (Collignon et al. 1987, Coutlee et al. 1988, Gowardman et al. 1998, Rushforth et al. 1993, Tighe et al. 1996b, von Baum et al. 1998, Zufferey et al. 1988). However, problems related to the use of acridine orange have been identified and these have been outlined in Section 1.8.

b) The AOLC method

The acridine orange leucocyte cytospin (AOLC) test is performed using a method that requires two major pieces of equipment, a cytology centrifuge and an UV microscope. In most routine pathology or microbiology laboratories access to a UV microscope is possible. However the presence of a cytology centrifuge, which can be used to concentrate cells, is less frequent.

The AOLC test uses approximately 1ml of blood which has been aspirated from a CVC in situ. Hypotonic formol saline (lyses red blood cells and fixes leucocytes) is added to the blood and then hypertonic saline is added and the suspension centrifuged. The resultant pellet is re-suspended and then transferred to a cupule, which is placed in a cytology centrifuge where the cells are dispersed onto a glass slide forming a layer of cells. The slide is then stained with acridine orange (~1:10,000 dilution) and left to dry in air. The preparation is then immersed in oil and examined under an ultraviolet
microscope (x1000). As stated in Section 1.8, acridine orange fluoresces when it binds to either RNA or DNA. In their study of samples retrieved from CVCs, Tighe et al. reported that a positive result was the presence of one or more viable or denatured bacteria (Tighe et al. 1996b) on the microscope slide. Other studies where AOLC has been used have been described in Section 1.8.

c) Statspin Cytofuge

In this study the value of the Statspin Cytofuge (Figure 2.2) when used within the procedure for the AOLC technique was determined. The Statspin Cytofuge is smaller (163mm in length and 120mm tall) and more lightweight (2.5kg) than conventional dedicated cytology systems. During centrifugation a specially designed cell concentrator is used to spread a “film” of cells on a specific region of a microscope slide. This slide can then be stained as appropriate and the slide viewed under a microscope.

2.4.2 Particle counting

a) Background

Walter Coulter developed the “Coulter Principle” in 1953 and it has since become the accepted reference method for particle size analysis (Hobson and Mann 1973). The principle involves an electro-zoning technique to detect the number of particles passing through an orifice (Medcraft 1993). This method of counting and sizing particles is based on the movement of a liquid, containing particles in suspension, though a small opening or aperture (Hobson and Mann 1973). The aperture is situated between electrodes and this is collectively known as the sensing zone. As the liquid is an electrolyte, a current can be passed between the electrodes on either side of the aperture. If the aperture contains only the solution then the circuit has a minimum resistance and this is readily measured. When a non-conducting particle moves through the hole it obstructs the path of the current and causes the measured resistance to increase. The change in resistance is proportional to the size of the particle and is closely related to the size of the aperture (Hobson and Mann 1973). The quantity of suspension drawn through the aperture is precisely controlled, thus giving the most accurate possible result.
Figure 2.2  The Statspin Cytofuge microcentrifuge (Statspin, USA).
This technique enables both the size distribution and the concentration of particles to be estimated, but does not distinguish between particle shape, colour and density (Hobson and Mann 1973). Therefore, coccoid or rod-shaped bacteria are counted and their size estimated as if they were spherical in shape. It is only possible to count organisms in a mixed culture if there is a sufficient differentiation in size between each type of organism (Hobson and Mann 1973).

The sensitivity of the technique depends upon matching the aperture and diameter of the particles under analysis. If the particle is much smaller than the aperture then the sensitivity will be reduced and it may not be possible to distinguish the measured resistance signal from the background ‘noise’ of the detector. Thus samples which have a widespread range of particle sizes require more than one orifice for examination. This problem has been addressed by using apertures of different size diameter, for example 10µm to 60µm, which are tailored for specific particle magnitude. This can be useful in the screening of urine samples so that there is good differentiation between bacterial and human cells (Stevens et al. 1993).

b) The variable orifice probe (VOP)

One solution to the problem of sensitivity mentioned above is to use one aperture which can be automatically varied in diameter. An apparatus with a variable orifice has been developed (Holley 1987, Stevens et al. 1993) in which a tapered spear is located within a fixed orifice. The orifice size may be effectively varied by adjusting the degree of insertion of the spear into the aperture. This provides the advantage of increased dynamic range compared to the open (fixed) orifice and therefore the size and number of smaller particles can be determined much more accurately (Holley 1987).

The Electro-Zoning Variable Orifice Probe (VOP) is used in the sizing and counting of particles of between 0.8µm to 40µm in diameter (Holley 1987). A small orifice is located at one end of the probe, where internal and external electrodes are situated. When a sample is introduced into the probe an electrical field is established between the two electrodes; a vacuum draws the sample through the orifice and any particles present in the sample will displace the electrolyte and change the impedance of the circuit. These changes are detected as pulses, which are measured and recorded. Larger particles cannot cause a blockage of the VOP as these particles are repelled from the orifice (Holley
1987). This is known as the "filter effect" (Stevens et al. 1993) and it happens because the flow pattern sets up a standing wave at the point where the probe enters the orifice (this is referred to as an annulus) and this wave sweeps larger particles away from the annulus but allows smaller particles to go through (Stevens et al. 1993).

MicroMed Limited have taken the VOP technology and combined it with an automatic pipetting system to form the Questor™ instrument (Figure 2.3). The Questor™ system is currently used as an automated, rapid (60 seconds per sample) urine analysis system, which counts white blood cells, red blood cells, epithelial cells and bacteria (Medcraft 1993, Report 1993 and Stevens et al. 1993). This relatively large instrument (870mm x 480mm x 435mm; weighing 25kg) is computer-controlled and this aids in the precision necessary for the tapered glass spear's insertion into the orifice, thus giving the probe the accuracy to analyse particles of various sizes from 0.8µm to 40µm.

Numerous evaluations and trials have been performed on the Questor system (Medcraft 1993, MicroMed Limited unpublished data 1989-95, Stevens et al. 1993). The Questor has been successfully evaluated for use in routine of screening urine samples and the potential applications of the VOP technology outside the urine analysis sector are enormous. In the Reading Public Health Laboratory study, 2273 urine samples were examined both on the Questor system and by routine methods. The Questor system was found to be a satisfactory method for screening large numbers of urine samples, as it had a high (96%) PV (Medcraft 1993). The Leicester Public Health Laboratory study used 1023 urine samples and showed the Questor system to have a PV of 98% (Stevens et al. 1993).

c) Agglutination

Agglutination is the clumping or aggregating together of particulate antigens after primary reaction with antibody (Kuby 1994b). In some types of agglutination assays a specific antibody is attached to the surface of a larger particle e.g. a latex bead. When a sample containing a specific analyte, for example Escherichia coli O157, is added to a solution containing antibody-bead suspension, the specific antibodies will bind to antigenic determinant sites on the analyte. If there are many of these antigenic
Figure 2.3  The Questor (MicroMed Limited, UK).
determinant sites then more than one antibody may bind to the analyte - this is called “cross-linking”. When agglutination has taken place the resulting clump of material is larger than the latex beads themselves. The clumps are sometimes so large that they are visible (Kuby 1994b).

If a known number of beads are added to a urine sample, for example a concentration of $10^4$ per ml, and agglutination takes place between the analyte and the antibodies, then clumps will form. These clumps of beads and analyte are too large to pass through the orifice and are repelled due to the “filter effect”. A decrease in the number of beads counted will indicate that the analyte was present (positive result). Thus there is an inverse relationship between the number of beads counted and the concentration of the analyte. This technique has been successfully used in detection of rheumatoid factor as well as other analytes in the body (Medcraft 1993, MicroMed Limited unpublished data 1993-95).

It is the application of the VOP technology with reverse agglutination that could be most applicable to this study. By combining these technologies there is an opportunity to rapidly detect the organisms, at clinically significant concentrations, which could be implicated in the suspected catheter-associated infection.

2.4.3 ATP bioluminescence

a) Introduction

The generation of light by a biological process is called bioluminescence and it was McElroy (1947) who first analysed the method by which a firefly, Photinus pyralis produces a flash of light. McElroy found that there is a specific enzyme reaction, which involves the breakdown of adenosine triphosphate (ATP) and the amount of light emitted is directly proportional to the amount of ATP that is present.

ATP which is universally found in all living cells is now being used as a measure of the viability of living cells. The purification of the components of the bioluminescence reaction (luciferin and luciferase) has led to the development of an assay that is useful in the enumeration of bacteria (Stanley 1989). By using a luminometer the detection of relative light units (RLUs) indicates that the sample contains ATP. Furthermore, the higher the number of RLUs that are detected then the higher the ATP concentration. This
is an extremely rapid and sensitive enzyme reaction, with light detection taking place within milliseconds (Stanley 1989).

ATP bioluminescence is now a well-established technology that is used in the rapid estimation of cell numbers, both microbial and somatic. It is widely used in the food industry as a rapid method of detecting if work surfaces are clean as well as in food hygiene monitoring for rapid determination of the microbial content of food-stuffs (Hawronskyj and Holah 1997, Siragusa et al. 1996). The popularity of this technology in the food industry is due to the fact that it is such a rapid alternative to traditional microbiology techniques. The use of ATP bioluminescence for monitoring the various stages in food production allows information to be provided in time for corrective action to be taken, thus avoiding recall campaigns and food scares (Hawronskyj and Holah 1997).

ATP bioluminescence is also a useful tool in research applications within biomedical sciences, such as its use in conjunction with reporter genes (Kricka 2000). It has however, not been as popular in routine clinical settings, even-though it has been shown to be of use in bacteriuria testing (Lundin 1989, Thore et al. 1975), with >2 million assays per year (Lundin 2000), as well as having possible applications for rapid drug susceptibility testing in TB patients (Hoffner et al. 1999).

b) Adenosine triphosphate (ATP)

ATP is a molecule found in all living organisms and is the primary energy source within the cells of these organisms. Its structure is displayed in Figure 2.4. It is a carrier molecule of high-energy bonds consisting of an adenosine region, made up of adenine and ribose, and three phosphate units (each of which has one phosphorous atom and four oxygen atoms). When the high-energy bonds between the phosphate groups are broken, ATP is converted first to ADP (adenosine diphosphate) and then to AMP (adenosine monophosphate) and inorganic phosphate (P_i) is released. The energy that is liberated during these reactions (equations 1 & 2) is used in cellular reactions such as cell division, protein synthesis, nerve signal transmission and muscle movement (Cohen 1999).

\[
\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{Pi} + \text{Energy} \quad (1)
\]

\[
\text{ADP} + \text{H}_2\text{O} \rightarrow \text{AMP} + \text{Pi} + \text{Energy} \quad (2)
\]
Figure 2.4 Structure of adenosine triphosphate (ATP).
Much of the ADP is immediately converted back to ATP (equation 3) through an enzyme based process involving the conversion of glucose to pyruvate ions (Cohen 1999).

\[ \text{ADP} + \text{P}_1 + \text{Energy} \rightarrow \text{ATP} + \text{H}_2\text{O} \]  

(3)

c) **ATP concentration**

The concentration of ATP within a living cell varies depending on the type of cell. In terms of human cells, neutrophils will have a large concentration of ATP, followed by the smaller red blood cells and the even smaller platelets (Lundin 1997). In terms of microbial cells yeasts are larger than bacterial cells and they too will have a larger concentration of ATP than bacterial cells. Lundin (2000) suggests that most bacterial cells contain $2 \times 10^{-18}$ moles of ATP, however this is questionable as microorganisms such as *Campylobacter jejuni* have been shown to have reduced ATP levels in response to adverse environmental conditions (Beumer *et al*. 1992).

d) **ATP in the firefly reaction**

The reaction that occurs during bioluminescence (equation 4) involves the following (De Wet *et al*. 1986).

\[ \text{Luciferin} + \text{ATP} + \text{O}_2 \xrightarrow{\text{Luciferase and Mg}^{2+}} \text{Oxyluciferin} + \text{CO}_2 + \text{AMP} + \text{pyrophosphate} + \text{light} \]  

(4)

Luciferin in the presence of the enzyme luciferase and ATP is converted to oxyluciferin, with a by-product of this reaction being light. One photon of light is released as one molecule of ATP is broken down, thus the number of photons of light that are detected provides a direct correlation with the number of molecules of ATP that are broken down.

e) **Luminometers**

A luminometer typically contains a photo-detector to detect the number of photons of light that are emitted from a sample, a chamber to hold the sample and an LED to display the number of photons of light emitted as relative light unit (RLU) values (Stanley 2000). There are many luminometer manufacturers in the world today, with the result that many types of commercial luminometers are available. Indeed, Stanley (2000) has published a
### Table 2.2  Luminometer specifications.

<table>
<thead>
<tr>
<th></th>
<th>Bioprobe</th>
<th>L1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dimensions</strong></td>
<td>250mm x 170mm x 155mm</td>
<td>220mm x 220mm x 170mm</td>
</tr>
<tr>
<td><strong>Weight</strong></td>
<td>2.0kg</td>
<td>2.5kg</td>
</tr>
<tr>
<td><strong>Display</strong></td>
<td>2 lines of alphanumeric characters</td>
<td>2 lines of alphanumeric characters</td>
</tr>
<tr>
<td><strong>Power</strong></td>
<td>Internal rechargeable battery. AC mains adapter 230 V AC 50/60 Hz</td>
<td>12V, 400mA supplied through mains adaptor</td>
</tr>
<tr>
<td><strong>Operating software</strong></td>
<td>Via four soft-keys</td>
<td>Via three soft-keys</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>Several orders of magnitude</td>
<td>Several orders of magnitude</td>
</tr>
<tr>
<td><strong>Maximum Count</strong></td>
<td>20,000,000 RLU</td>
<td>24,000,000 RLU</td>
</tr>
<tr>
<td><strong>Sample formats</strong></td>
<td>Direct surface measurement as well as concentration filters up to 90mm diameter, liquid volumes of up to 10ml</td>
<td>Samples contained within tubes up to 16mm in diameter, micro-centrifuge tubes, 35mm culture dishes.</td>
</tr>
</tbody>
</table>

* The ATP detection limit is determined by the quality of the luciferin-luciferase reagent in addition to the maximum count.
list of all luminometer manufacturers and the type of luminometer that is manufactured by each, from microtitre plate luminometers to specialist portable luminometers. From this list two luminometers were selected for evaluation. The first was the L1 luminometer (Mediators, Austria) and the second was the more portable Bioprobe (Hughes-Whitlock, UK). These particular luminometers were chosen because they are relatively smaller in size and weight than other larger more dedicated systems. In addition, they are also relatively low cost (<£6,000) and have a relatively high sensitivity. The specifications of each of these two instruments are shown in Table 2.2, where it can be seen that there is little difference between the luminometers in terms of dimensions, weight, sensitivity and maximum RLU count. However the two instruments do differ in terms of what type, volume and format of sample can be used in conjunction with each luminometer. They also differ in the way the light is detected by the photo-detector.

The Mediators L1 is a sensitive single sample luminometer, which has a number of features that differ from the Bioprobe. The sample can be contained within a standard 12 x 75mm tube or a micro-centrifuge tube where it is introduced into the reading chamber by way of a sliding drawer that pulls out from the main body of the instrument (Figure 2.5). In order to determine the ATP concentration of the sample the drawer is gently closed and the luminometer automatically determines (integrates) the RLUs over a 10 second period after a 2 second delay (this can be adjusted) and the results given on an LED display on the instrument. When the drawer is closed the sample is 3mm away from the photo-detector, which is located beneath the drawer. If the sample is contained within a standard tube then the photo-detector can easily detect all the light emitted. After detection of the RLUs the sample can be discarded by simply opening the drawer and removing the tube.

The Bioprobe (Hughes-Whitlock Limited, UK) is a luminometer used mainly in the food industry for assessing the cleanliness of work surfaces (Report 1995). The Bioprobe can be seen in Figure 2.6 and consists of a stainless steel test plate, which fits to the base of the plastic cased luminometer. During operation the sample to be analysed in conjunction with the appropriate reagents is placed onto the stainless steel test plate. The sample can be on a plastic disk or an aluminium tray (otherwise known as a coupon), in a 1.5ml centrifuge tube or inside a syringe filter. The luminometer part is placed and gently
Figure 2.5  The L1 luminometer (Mediators, Austria). The drawer to the right of the luminometer is in the open position and a sample is contained within a 12 x 75mm glass tube held in position in the reading chamber. In order to determine the ATP concentration in the sample the drawer needs to be closed and the RLU's are determined.
Figure 2.6  The Bioprobe luminometer (Hughes Whitlock Limited, UK).
pressed down onto the stainless steel test plate. A vacuum automatically seals the space between the top and the bottom steel plate, creating a “light-tight” environment where the detector only measures light emitted from the sample. The luminometer then detects the number of photons of light and expresses these as RLUs on the LED display, with readings usually taken after 10 seconds. This instrument differs greatly from the L1 in that the photo-detector is located over 20mm above the sample. In general this is adequate, however the photons of light need to travel further to be detected and this can cause sensitivity issues if there is a low concentration of ATP to be assayed (Dr A. Lundin, personal communication).

f) Reagents used in conjunction with the luminometers

In order to detect microbial ATP, there are a number of steps, which need to be followed prior to the determination of the ATP concentration. These involve the extraction and degradation of ATP from the non-microbial cells and the subsequent extraction of ATP from the microbial cells.

Besides the Bioprobe, Hughes-Whitlock Limited are also manufacturers of specialised bioluminescence reagents, which are optimised for use with the Bioprobe. These reagents include specific somatic and microbial cell lysis systems, ATPase (to degrade non-microbial ATP) and a luciferin-luciferase reagent. These reagents have been optimised for use specifically with the Bioprobe. On the other hand, Mediators do not supply reagents with the L1 luminometer. However they do recommend the Biothema AB range of luciferin-luciferase reagents to be used in conjunction with their instrument. Unfortunately Biothema do not supply cell lysis agents or ATP removal reagents.

2.5 Selection Criteria

2.5.1 Investigations and evaluations

The value and feasibility of the Bioprobe and L1 luminometers, the Questor and the Statspin cytofuge were investigated. Firstly the manufacturers of each of the instruments were approached and the specification of each was discussed. This was followed by an initial trial period with each of the instruments, where the author was allowed to test each system and determine how applicable that system would be in a hospital laboratory
environment. Through this initial testing and the conversations with the manufacturers an assessment was made on how useful each instrument could be, should it be used to analyse a FASEB-retrieved sample.

The feasibility of the Bioprobe and L1, the Questor and the Statspin was assessed using a scoring system based on the three parameters described below.

a) Cost

Each of the three technologies were assessed on the basis of cost. The two areas that related specifically to cost in each case were:

- The initial cost of set up; which would include the capital investment for equipment and reagents.
- Subsequent running cost of the techniques on a day-to-day basis. The running costs include; the cost per test of the reagents required, the cost of personnel required to carry out the test (based on the time required to perform the assay) and the requirement for special facilities, such as safety cabinets or special waste disposal.

b) Time

The traditional method of culturing a FASEB sample takes at least 24 hours before the result is obtained. However, the time taken to process the sample and then determine the result after the incubation period is relatively short (<10 minutes in total). Thus the total assay time should include all incubation periods that are necessary.

Using a rapid assay will undoubtedly reduce the total length of time required to complete the assay but it may become more labour intensive. In addition the total time required to perform an assay will be influenced by set up times such as the thawing, reconstitution and/or dilution of reagents. Moreover, the total time between sampling and returning the result will be dependent upon the time required to transport of the sample to the laboratory and the procedures involved in reporting back the result.

Each of the technologies will be assessed in terms of how quickly a sample could be processed from the time it is received in the laboratory to the result being reported. Ideally the total turn-around time from the retrieval of the patient’s sample to the reporting of the result should be within the working day. This is because the clinician
will need to make a decision on whether to give antibiotics to the patient, remove the CVC or look for an alternative source for the infection.

c) **Ease of use**

Techniques that are difficult to perform, require a high level of operator skill or have a high number of time-consuming individual procedures are of little use as rapid tests in a routine clinical laboratory. It is important that the developed technique should have as few individual procedures as possible and each of these steps is not time consuming. It may be better to have two individual steps separated by a 15 minute long incubation time, rather than six short manipulations (vortex mixing, heating, washing) during a 10 minute period. In a routine clinical laboratory a rapid test should have a simple enough method to allow it to be routinely carried out by both specialist staff and “on call” staff. An example of such a test would be the use of the Staphytecf™ kit for identification of staphylococci. This kit is based on the principle of agglutination, is complete within five minutes and minimal steps are required to obtain a result.

### 2.5.2 The scoring system

In order to evaluate each technology a scoring system was devised to determine their feasibility, as well as show the difference between each technology. This was thought to be the least complex method of translating the subjective into the objective and quantitative. The assessment parameters, shown in Table 2.3, were broken down into the three groups (cost, time and ease of use) outlined in Section 2.5.1. Each parameter scored between 1, 2 or 3, with the better and more appropriate technology receiving a higher score for each section where it would perform well. The technology with the highest score was then selected as the most suitable technology to be used in conjunction with the FASEB. Each of the three parameters has been deemed to be equally important for the purposes of this investigation.
2.6 Scoring and selection

2.6.1 Advantages and disadvantages of each technology

The advantages and disadvantages of each of the three technologies were summarised and these can be seen in Table 2.3. These advantages and disadvantages are again based upon the three selection criteria identified in Section 2.5.

2.6.2 Scoring each technology

Using the devised scoring method based on the three criteria described above, a total score was calculated for each of the technologies. Table 2.4 shows the values obtained by each of the technologies for each of the three criteria, as well as the total scores.

a) Staining and microscopy – the AOLC method

The combination of the Statspin cytofuge and the AOLC method achieved a score of 5 out of a possible 9. This scored well in the areas of cost and assay specificity. The initial capital outlay would be relatively low as an UV microscope is generally found in most clinical laboratories and a Statspin cytofuge costs less than £3,000. Moreover, on a day-to-day basis this would be a low cost detection system to use as it does not require any specific reagents with a short shelf-life or expensive components. Even-though the technology could achieve a result in approximately 20-30 minutes, the method does involve a number of individual steps and some of these are time consuming to perform.

Another problem is that a number of fields in the microscope view need to be examined (possibly up to 100) in order to obtain an accurate result. To do this a high level of specialised training may be required. Therefore it is important that the operator is well trained and can distinguish between microorganisms and somatic cell debris. However, it needs to be remembered that “on call” staff may need to use this method and they might not have the necessary expertise. This is especially important when put into the context of Gowardman et al. (1998) conclusions, that in their experience the AOLC test was labour intensive and demanding of skilled personnel for accurate performance and interpretation.
Table 2.3  Advantages and disadvantages of each technology in terms of the three section criteria.

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Cost</th>
<th>Ease of use</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Advantage</td>
<td>Disadvantage</td>
<td>Advantage</td>
</tr>
<tr>
<td>ATP bioluminescence</td>
<td>Sample analysis usually takes &lt;1 minute.</td>
<td>Sample preparation could take ~30 minutes, and therefore would be time consuming.</td>
<td>Luminometers such as the L1 or Bioprobe are low cost (&lt;£6,000).</td>
</tr>
<tr>
<td>VOP</td>
<td>Sample analysis usually takes &lt;1 minute and batch testing of samples will lead to a great time saving.</td>
<td>Sample preparation may be time consuming and instrument will require a &quot;warm up time&quot;.</td>
<td>Low running cost of samples in batch testing.</td>
</tr>
<tr>
<td>AOLC</td>
<td>A positive result can be determined quickly.</td>
<td>Sample preparation is time consuming (&gt;10 minutes) and negative samples may take longer to analyse.</td>
<td>Basic reagents required. Statspin costs &lt;£3,000.</td>
</tr>
</tbody>
</table>
Table 2.4  Scores achieved by each technology.

<table>
<thead>
<tr>
<th>Technology</th>
<th>Parameter</th>
<th>Score</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine Orange Leucocyte Cytospin</td>
<td>Cost</td>
<td>3</td>
<td>Basic reagents required and the consumables are relatively cheap. Microscope already available. Statspin £3,000.</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>1</td>
<td>Sample preparation is time consuming (&gt;10 minutes) and negative samples may take longer to analyse.</td>
</tr>
<tr>
<td></td>
<td>Ease of use</td>
<td>1</td>
<td>A significant amount of training would be necessary. On call staff may not find it simple to use.</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Variable Orifice Probe</td>
<td>Cost</td>
<td>1</td>
<td>Instrument costs £39,950. High cost of reverse agglutination beads and other consumable costs are relatively high.</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>A result can be obtained within 30 minutes depending on the level of sample preparation that is required.</td>
</tr>
<tr>
<td></td>
<td>Ease of use</td>
<td>3</td>
<td>It is an automated instrument so only the sample preparation could potentially be difficult.</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>ATP Bioluminescence</td>
<td>Cost</td>
<td>2</td>
<td>Instrumentation costs &lt;£6,000. Reagents can be expensive and issues with the shelf lives after reconstitution.</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>3</td>
<td>Sample preparation could take ~30 minutes. Sample analysis usually takes &lt;1 minute.</td>
</tr>
<tr>
<td></td>
<td>Ease of use</td>
<td>2</td>
<td>Training is necessary, however using a luminometer is straightforward. Sample preparation is more labour intensive.</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
b) **Particle counting – the VOP technology in the Questor™**

The VOP technology in the form of the Questor instrument and in combination with reverse agglutination achieved a score of 6 out of 9. One of the main issues with this technology is the cost of the instrumentation. A Questor would not normally be a standard piece of equipment in a clinical laboratory and therefore there would be an initial capital outlay of up to £39,950 (Stevens *et al.* 1993). In some hospitals the particle counting based VOP technology is routinely used to screen urine samples (Medcraft 1993, Stevens *et al.* 1993). However, the number of urine samples received in a typical hospital laboratory is much greater than the potential number of CVC tip samples. Therefore, it is difficult to justify the expense of purchasing the Questor instrument for catheter sample screening. Additionally, the fact that antibody based agglutination assays may be required would increase the cost still further. This is because a panel of tests may be required so as to identify the species of organism that is present.

c) **ATP bioluminescence – the Bioprobe and L1 luminometers**

ATP bioluminescence based instrumentation and reagents scored 7 out of 9. Using ATP bioluminescence there is the capability of not only detecting the ATP in all the microbial cells present in the sample, but also determining the concentration of ATP present and therefore easily quantifying the number of microorganisms in the sample.

In terms of costs luminometers are not usually available in a routine microbiology laboratory and therefore there is an initial capital outlay. Luminometers vary in price and specification but an adequate luminometer for routine clinical use would cost approximately £6,000 (Report 1995). The day-to-day running of the system could be high as reagents tend to be expensive, due to their high enzyme content and the need to present them as freeze dried solids due to their short stability in liquid form. ATP bioluminescence is, nevertheless, a very straightforward system to use and the time required to perform an assay, including sample preparation, would be less than one hour (Lundin 2000).

### 2.6.3 Selection

As ATP bioluminescence scored the highest number of points it was chosen as a platform for the development work to be carried out in the next section. Although the nature of the
selection process means that the points awarded (5, 6 and 7) are not statistically
dissimilar, ATP bioluminescence technology does have a number of advantages over the
other two techniques.

- ATP bioluminescence can be used to determine (indirectly) the total number of
  microorganisms in a sample. Particle counting in combination with agglutination can
  also be used to do this, however a number of specific agglutination assays many be
  required before a satisfactory result is obtained.

- The ATP bioluminescence reaction also occurs very quickly and the potential turn
  around time from sampling to reporting of the result could be well within the
  working day.

- The difficulty with using the AOLC as a screening method is that it takes a lot
  longer to obtain a negative result than a positive result using microscopy. This is
  because a minimum number of fields need to be analysed in order to say with
  confidence that there are no organisms present. The potential number of negative
  sample (~80%) would mean that this method would be time consuming.

A method based on a combination of the ATP bioluminescence and a sample retrieved
with a FASEB was proposed for further development. With the use of appropriate “front
end” sample preparation and a specific reagent system then microbial and non-microbial
ATP can be differentiated and the microbial ATP measured. There is, however, a
significant amount of development that needs to be done on various aspects of the whole
system before obtaining an easy to use all-inclusive system.

2.7 Assay development using ATP Bioluminescence

Both the Bioprobe and the L1 luminometer were investigated in the initial development
stage and details of the proposed development work using the both the Bioprobe and
reagent system and the L1 and reagents follow in Chapter 4. However, before looking at
this work it is important to identify and discuss the potential difficulties that are
associated with using ATP bioluminescence as the basis for a rapid test system.
2.7.1 ATP extraction

Although ATP bioluminescence is one of the most sensitive and rapid methods available for the enumeration of bacteria (Lundin 2000) it is necessary to follow a series of steps in order to determine how many bacteria are present within a clinical sample. These steps involve the extraction and degradation of ATP from the non-microbial cells, followed by the independent extraction of ATP from the microbial cells.

For the \textit{in vitro} ATP bioluminescence reaction to occur ATP must be extracted from the cell. This ATP extraction must take place within milli-seconds, because ATP has a rapid turnover inside the cell. In addition, some intracellular enzymes may try to use the free ATP for repair of the ruptured cell wall, therefore, the extractant needs to have chaotropic properties which allow inactivation of these intracellular enzymes (Lundin \textit{et al.} 1994).

In many cases and especially with clinical samples, microbial ATP needs to be distinguished from non-microbial ATP. As yeast and mammalian cells have ATP concentrations of up to 1000 times higher than bacterial cells, it is important that there is no contamination of the final sample with non-microbial ATP. To overcome this situation extractants have been developed which selectively extract ATP from non-microbial cells but do not affect the microbial cells.

\textbf{a) Extraction and degradation of ATP from somatic or non-microbial cells}

Somatic or non-microbial cells can generally be lysed quite easily. Exposure to a non-ionic detergent such as Triton X-100 for a period of up to 1 minute is usually sufficient to lyse most somatic cells (Lundin and Thore 1975). However, when clumps of cells or tissues exist, the Triton X-100 may not fully penetrate the mass and some ATP may still remain within the structures (Stanley 1989).

Once the non-microbial cell has been lysed and the intracellular ATP released it needs to be removed from the solution where the intact microbial cells remain. It is important that this contaminating ATP is degraded before the microbial ATP is released since contaminating ATP often makes up over 99\% of the total ATP present (Schram and Witzenburg 1989, Stanley 1989).

Removal of unwanted ATP can be carried out by addition of an ATP dephosphorylating enzyme, such as apyrase (Stanley 1989). Apyrase (EC 3.6.1.5) is basically an adenosine
$5'$-triphosphatase (ATPase), which is naturally present in living cells where it regulates the levels of ATP within the cell. When apyrase is added to an ATP containing solution it begins to degrade the ATP into ADP, as shown in Equation 1 in Section 2.4.3.

There are, however, problems associated with using apyrase to degrade the non-microbial ATP (Stanley 1989). If insufficient apyrase is used, the removal of the non-microbial ATP may take longer, resulting in unwanted non-microbial ATP remaining to contaminate the sample. Conversely, the addition of a high concentration of apyrase could lead to the rapid removal of the non-microbial ATP, but this excess apyrase will be present to degrade the microbial ATP subsequent to its extraction from the cell.

In many situations a relatively high concentration of apyrase is added and after a specific period of time, during which the ATP is degraded, an inhibitor is added to denature the apyrase. Unfortunately, the use of such inhibitors can lead to the simultaneous inhibition of the luciferase enzyme. Therefore, an effective and selective inhibitor of apyrase is required that does not have an adverse affect on luciferase (Stanley 1989).

Alternatives to apyrase have been sought. Many of these are also ATP dephosphorylating enzymes (Sakakibara et al. 1997) such as adenosine triphosphatase (EC 3.6.1.3) and hexokinase (EC 2.7.1.1). These enzymes have been used individually and in combination with various other enzymes, such as AMP deaminase (EC 3.4.5.6), adenosine deaminase (EC 3.5.4.4) and alkaline phosphatase (EC 3.1.3.1). Sakakibara et al. (1997) demonstrated that a combination of apyrase and adenosine phosphate deaminase (EC 3.5.4.17) was extremely effective in removal of non-microbial background ATP from a culture broth.

In order to degrade further the background ATP present in blood samples, apyrase has also been used in combination with Percoll density gradients (Molin et al. 1983, Nilsson et al. 1989). This involves a centrifugation step to separate blood cell debris from bacteria.

b) Extraction of ATP from microbial cells

In order to lyse microbial cells, a more vigorous extractant is required, because microbial cell walls and membranes are much more robust than somatic cells. It is important that this extractant is both aggressive enough to release ATP from a broad range of microorganisms and has the capability to inactivate intracellular enzymes that would
normally breakdown the ATP (Stanley 1989). Three criteria that an extractant or microbial cell lysis agent needs to fulfil, are listed below (Lundin et al. 1994, Meighan et al. 1994).

1. Complete release of the entire ATP pool from the cell,
2. Complete, irreversible and immediate inactivation of all ATP converting enzymes,
3. No analytical interference from the extractant with the ATP assay.

The problem is, however, that criteria 2 and 3 are contradictory in that a chemical that would inactivate all ATP converting enzyme would intrinsically inactivate luciferase (Meighan et al. 1994).

A number of different types of extractants have been described (Hoffner et al. 1999, Stanley 1989). Some of the more popular extractants include trichloroacetic acid (TCA) and dimethyl sulphoxide (DMSO), but these adversely affect the luciferase assay. The types of microbial ATP extractants have been divided into four groups (Stanley 1989) and these are shown in Table 2.5 along with the problems that are associated with each. TCA is thought to be the most reliable extractant and, at a final concentration of 2.5% (w/v), it is used as a reference extraction method (Lundin 2000).

In addition to the type of extractant it is also important to determine how long the microbial cell should be exposed to the extractant. Selan et al. (1992) observed that higher RLUs values were detected when a stock of *Ps. aeruginosa* was exposed to a commercial ATP extractant for 30 seconds rather than 10 seconds.

As many of the extractants in Table 2.5 are potent inhibitors of luciferase, their application to this technology is difficult. Nevertheless, a number of ways to limit their effects on the luciferin-luciferase reaction have been assessed. Traditionally, the inhibition has been overcome by diluting the extract prior to assay with the luciferase (Lundin et al. 1994), but this leads to a great loss in sensitivity. An alternative has been to use neutralising agents, which bind the chemicals leading to a neutralisation of the whole solution. Lundin et al. (1994) described the use of α-cyclodextrin to neutralise the effects of dodecyl trimethyl ammonium bromide (DTAB). Cyclodextrins are “doughnut shaped” molecules made up of 6 to 8 glucose molecules with a hydrophobic interior.
<table>
<thead>
<tr>
<th>Microbial ATP Extractant</th>
<th>Problem with Extractant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic compounds (ethanol, acetone or dimethyl sulphoxide)</td>
<td>Very potent inhibitors of luciferase</td>
</tr>
<tr>
<td>Acids (sulphuric or trichloroacetic)</td>
<td>Very potent inhibitors of luciferase</td>
</tr>
<tr>
<td>Boiling buffers (Tris-EDTA) and steam</td>
<td>Sensitivity is reduced due to dilution of sample</td>
</tr>
<tr>
<td>Cationic detergents</td>
<td>Very potent inhibitors of luciferase</td>
</tr>
</tbody>
</table>
Lundin et al. (1994) found that the addition of α-cyclodextrin to a solution containing microbial ATP extracted by DTAB, led to complete neutralisation of the DTAB and therefore no subsequent interference, or inhibition of the luciferase based ATP assay. However, it is noted that cyclodextrins also form complexes with α-luciferin and this needs to be considered when optimising the system (Lundin 2000). Therefore, during development, the cyclodextrin should only be present in a small excess over the DTAB.

2.7.2 Measurement of ATP

a) Assay conditions

Once the non-microbial ATP has been extracted and degraded and the microbial ATP has been released, the concentration of microbial ATP can be determined. When a luciferin-luciferase reagent is added to the sample an almost immediate reaction will take place, as in Equation 4 in Section 2.4.3, with light being produced. The quantum yield is 0.88, therefore, almost one photon of light is emitted per ATP molecule (Lundin 2000). The assay can be calibrated using a known amount of ATP standard where no more than 1% of the final volume should be added. However, volumes of <10μl are generally avoided (as these are less accurate to measure). Thus, in addition to an RLU value being obtained, the amount of ATP can be expressed as moles of ATP. This is important so that samples can be compared and it compensates for variations such as those listed below (Lundin 2000).

- Luminometer to luminometer variations (even between the same model), as well as deterioration in the sensitivity of a single luminometer over time.
- Batch to batch variations between the same luciferin-luciferase reagent.
- The inhibition from sample components. It has been noted that biological samples can contain substances that are inhibitory to the luciferase reaction. In addition, emitted light can be partially quenched by cellular debris or proteins in the sample to give a falsely low RLU value.
- Inhibition of the luciferase reaction by the compounds used to extract the ATP from the microbial cells (concentration dependent).
It is also important that the ATP standard should not exceed the upper end of the linear range of the luminometer, which is generally set at <1 μM final ATP concentration in the reaction tube.

The most sensitive luciferin-luciferase reagents allow the detection of approximately $10^{-18}$ moles (or 1 Attamole) of ATP (Lundin 2000). This is equivalent to the ATP present in half of one bacterial cell. However, this is not practical, as there are issues with cross contamination of equipment and consumables at these levels.

There are a number of variables that can affect the luciferase reaction. These include the D-luciferin – which should be as pure as possible, excess magnesium ions and the type, concentration and pH of the buffer used. The optimal buffer usually contains 50 or 100 mM Tris and 0.5 to 2 mM EDTA all adjusted to 7.75 with acetic acid (Nichols et al. 1981, Webster et al. 1980).

b) ATP reagents

Three specific types of ATP reagent have been described by Lundin (1997, 2000). Further details on each of these are displayed in Table 2.6.

1. The stable light-emitting reagent has a very low decay rate and is useful for ATP monitoring. Measurements can be taken at leisure as there is a stable light emitted over time. However, its detection limit is not adequate for low numbers of bacteria.

2. The slow decay reagent has a higher decay rate (10% / minute). This is useful in that this type of reagent can be formulated to degrade its own ATP background during preparation. The detection limit is higher than in the stable light-emitting reagent and it is useful for detecting low levels of bacteria ATP.

3. The flash reagents have very high decay rates and consequently have a very sensitive detection limit. They are useful in the detection of very low levels of ATP (1 amol) but injector systems are required to add the reagents whilst the sample is in the luminometer and ATP free containers are required to avoid issues of contamination.

2.7.3 Correlation between ATP concentration and CFU/ml

Another issue that needs to be remembered is that often ATP cut-off limits for positive assay generally correspond to higher cell numbers than would be expected from cut-off
limit in terms of CFU/ml (Lundin 2000). The reasons for this can be due to incomplete removal of non-microbial ATP or that the average CFU originates from more than one bacterial cell. This has been observed in bacteriuria testing, however few patients with urinary tract infections produce samples with bacterial levels close to the cut-off limits (Lundin 2000).

2.7.4 Crucial areas in the development process

Figure 2.7 illustrates critical steps that have been identified and needed to be addressed in order to develop a successful technique based on ATP bioluminescence. The first major complication was that a sample retrieved by the FASEB would possibly contain both microbial and non-microbial cells, therefore it was necessary to determine how to effectively lyse non-microbial cells and remove their ATP whilst leaving the microbial cells intact. Secondly, it was necessary to lyse the microbial cells and simultaneously inhibition all ATP degrading enzymes so that the released ATP was not degraded. In the final step the released microbial ATP needed to be quantified so that the level of microbial colonisation could be determined.
Table 2.6  Properties and applications of different types of ATP reagents (Lundin 2000).

<table>
<thead>
<tr>
<th></th>
<th>Stable light reagent</th>
<th>Slow decay reagent</th>
<th>Flash reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical rate constant</td>
<td>0.005 min⁻¹</td>
<td>0.10 min⁻¹</td>
<td>2.35 min⁻¹</td>
</tr>
<tr>
<td>$t_{1/2}$ of decay</td>
<td>139 min</td>
<td>6.9 min</td>
<td>0.3 min</td>
</tr>
<tr>
<td>$t_{9/10}$ of decay</td>
<td>461 min</td>
<td>23 min</td>
<td>1 min</td>
</tr>
<tr>
<td>Reagent blank per ml</td>
<td>&gt;1000 amol</td>
<td>10-100 amol</td>
<td>&lt;1 amol</td>
</tr>
<tr>
<td>Detection limit</td>
<td>&gt;500 bacterial cells</td>
<td>5-50 bacterial cells</td>
<td>&lt;0.5 bacterial cells</td>
</tr>
<tr>
<td>Light measurement</td>
<td>Intensity</td>
<td>Intensity</td>
<td>Total integrated light</td>
</tr>
<tr>
<td>Commercial name</td>
<td>SL</td>
<td>HS</td>
<td>SS</td>
</tr>
</tbody>
</table>

* $t_{1/2}$ of decay means that 50% of the ATP and the light intensity remains; $t_{9/10}$ of decay means that 90% of the ATP is consumed and that 10% of the light intensity remains.
STEP ONE
Determine how to effectively lyse non-microbial cells so that their intracellular ATP is exposed. Then determine how to remove (degrade) this ATP so that intact microbial cells (containing their ATP) remain.

STEP TWO
Lyse the microbial cells so that their ATP is released and simultaneously inhibit all ATP degrading enzymes.

STEP THREE
Detect the microbial ATP using appropriate reagents and a luminometer.

Figure 2.7 A flow chart showing the major steps that need to be followed in order to develop a system based on ATP bioluminescence.
CHAPTER THREE
Methods & Materials
3.1 General introduction

This chapter details the materials and methods that were used in the laboratory. Details of all the materials used are given; these include enzymes, commercially available reagents, laboratory-formulated reagents and buffers, as well as the equipment and consumables. Information is also given on the samples used and the approvals gained in order to obtain these clinical samples. General methods for reagent formulation are also detailed as well as methods for operating various equipment. Finally methods that were development as part of this study are detailed, including the extraction and detection of microbial ATP from the clinical samples.

3.2 Materials

3.2.1 Chemicals

Table 3.1 lists all the chemicals that were formulated. All were dissolved in sterile distilled water to form stock solutions. The storage conditions and the supplier details are also listed in the table.

3.2.2 Enzymes

a) Apyrase (100U/ml)

Apyrase (EC 3.6.1.5) was purchased from Sigma Aldrich, UK, as a partially purified lyophilised powder containing adenosine 5'-triphosphatase and adenosine 5'-diphosphatase. Grade III apyrase (catalogue number A7646) was used in batches of 2000 Units per vial. The contents of the vial were dissolved in 5ml of sterile distilled water and then filtered through a 0.22μm, 25mm syringe filter (Syrtec, USA). The filter was then flushed with 15ml of sterile distilled water to give a 20ml solution containing 100 Units/ml apyrase. This stock was then aliquoted (1ml each) into micro-centrifuge tubes and these were stored at -20°C until required.
Table 3.1 Chemicals formulated for use during the development phase.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Concentration</th>
<th>Storage conditions</th>
<th>Supplier and catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>α - Cyclodextrin *</td>
<td>10% (w/v)</td>
<td>Between 2°C and 8°C</td>
<td>Sigma, C4642</td>
</tr>
<tr>
<td>Dodecyltrimethyl ammonium bromide (DTAB)</td>
<td>2% (w/v)</td>
<td>Room temperature</td>
<td>Sigma, D-8638</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl₂ •6H₂O)</td>
<td>1 Mol/L</td>
<td>Between 2°C and 8°C</td>
<td>BDH Chemicals, 29096</td>
</tr>
<tr>
<td>Suramin</td>
<td>0.1mg/ml</td>
<td>Between 2°C and 8°C</td>
<td>Sigma, S-2671</td>
</tr>
<tr>
<td>Thimerosal</td>
<td>0.04% (w/v)</td>
<td>Room temperature</td>
<td>Sigma, T-8784</td>
</tr>
<tr>
<td>Trichloroacetic acid (TCA)</td>
<td>10% (w/v)</td>
<td>Room temperature</td>
<td>Sigma, T-9159</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1% (w/v)</td>
<td>Between 2°C and 8°C</td>
<td>Sigma, X-100</td>
</tr>
<tr>
<td>Tween</td>
<td>1% (w/v)</td>
<td>Between 2°C and 8°C</td>
<td>Sigma, P-1379</td>
</tr>
</tbody>
</table>

* Stock of 10% (w/v) α-Cyclodextrin was prepared by dissolving 500mg of α-Cyclodextrin in 5ml of sterile water
b) 5' - adenylic acid deaminase (20 Units/ml)

One vial of 5' - adenylic acid deaminase (EC 3.5.4.6) from *Aspergillus* Species contains 1000 Units. This was supplied in diatomaceous earth (total weight of 9.4g) by Sigma Aldrich, UK. To prepare a solution containing 100 Units/ml, 940mg was removed and suspended in 3ml of water. The resultant suspension was vortex-mixed (30 seconds) and then filtered through a 0.22μm, 25mm syringe filter (Syrtec, USA). This filter was then flushed with 2ml of sterile distilled water giving 5' - adenylic acid deaminase at a final concentration of 20 Units/ml. This solution was stored at 2-8 °C until required.

c) Hexokinase (1000 units/ml)

Hexokinase (Sigma Aldrich, UK) was supplied in a vial containing 1000 Units and this was reconstituted in 1ml of sterile distilled water. This solution was stored at 2-8 °C until required.

3.2.3 Commercially available reagents

a) Reagents used in conjunction with the Bioprobe luminometer

The Isostat isolator reagent (Oxoid, UK) was supplied in pre-aliquoted (700μl) tubes. This reagent was developed by Dorn and Smith (1978), and it contains the lytic agent saponin, which lysed red and white blood cells. It also contains anti-foaming and anti-coagulating agents and when used as intended, blood is added to the tube to give a final volume of approximately 10ml.

Hughes-Whitlock Limited supplied the ATPase, the Luciferin-Luciferase reagent with diluent buffer and the Bacterial Lysis Agent. The exact constituents and formulation of these reagents are unknown, as they were supplied as part of a commercial kit. All three of the reagents were dispensed from plastic reagent bottles, with tips that deliver 50μl (±5μl) of liquid per drop.

The Luciferin-Luciferase reagent (Hughes-Whitlock Limited, UK) was supplied as a freeze-dried solid and it was reconstituted in a single vial of the diluent buffer when required. As a powder it has a shelf-life of two years. When reconstituted, it is useful for
7 days if stored between 2°C and 8°C. In all investigations involving the Hughes-Whitlock Bioprobe the “High Sensitivity” Luciferin -Luciferase kit was used.

The ATPase was supplied as a standard reagent and it was found that increasing volumes were required to achieve high levels of ATP degradation. On request, Hughes Whitlock supplied a 10 times concentrated ATPase, which was subsequently used in the method development. Due to the manufacturer’s confidentiality concerns the contents and concentrations of both of these reagents were unknown but they were assumed to contain apyrase and MgCl₂.

The Bacterial Lysis Agent (Hughes-Whitlock Limited, UK) has a shelf-life of two years if stored at between 2°C and 8°C and was used in the liquid form in which it was supplied.

b) Reagents used in conjunction with the L1 luminometer

The contents of the ATP Kit SL (Biothema AB, Sweden) are shown in Table 3.2. The formulation of the reagents within this kit is unknown (see the SL kit instructions for use in Appendix 2). When required, the SL reagent was removed from the freezer and brought up to room temperature. It was then reconstituted in a single vial of “Diluent C”. In its lyophilised form the SL reagent has a shelf-life of two years when frozen. When reconstituted it remains effective for 7 days if stored between 2°C and 8°C. Each reagent was dispensed using ATP-free pipette tips.

Biothema AB also supplied the HS reagent as a lyophilised reagent containing D-luciferin and luciferase. The HS reagent contains higher concentrations of both D-luciferin and luciferase, resulting in a more sensitive measurement of ATP level. One vial of HS reagent was reconstituted in a single vial of “Diluent E” and the shelf-life is similar to that of the SL reagent.

The ATP standards of choice for use in conjunction with the HS reagent are vials containing 5ml of 10⁻⁸ moles/L ATP or 5ml of 10⁻⁷ moles/L ATP (Biothema AB, Sweden).

Bactolyse (LumiTech, UK) is a mixture of cationic detergents in an aqueous buffer. This was supplied in a 50ml bottle from which it was dispensed in 50μl drops. It has a shelf-
life of 2 years when stored between 2-8°C. A nucleotide-releasing agent (LumiTech, UK) was also supplied in a 50ml bottle and dispensed in 50μl drops. The shelf-life is again 2 years when stored between 2-8°C.

3.2.4 ATP degradation reagent

The ATP degradation reagent was made up freshly each day using the components listed in Table 3.3. By adding each component to 6.57ml of sterile distilled water a final volume of 10ml was achieved. This 10ml solution was then filtered through a 0.22μm, 25mm syringe filter, which was subsequently flushed with 4ml of sterile distilled water giving a final volume of 14ml and the final component concentrations shown in Table 3.3. In situations, where greater than 14ml of the ATP degradation reagent were required, the volume of each of the components was doubled to give 28ml final volume.

3.2.5 Staphytect Plus kit

Staphytect Plus (Oxoid, UK) is a latex agglutination test for the differentiation of S. aureus by detection of a clumping factor. The Staphytect Plus kit contains blue latex particles coated with porcine fibrinogen and specific antibodies raised against capsular polysaccharides of S. aureus. It also contains a control reagent, which contains blue unsensitised latex particles and reaction cards, which provide a surface on which to carry out the assay. Positive (S. aureus strain ATCC 25923) and negative (S. epidermidis strain ATCC 12228) controls, supplied by Oxoid UK, were also used.

3.2.6 Microbank beads

Microbank beads (Pro-Lab Diagnostics, USA) were used to store microorganisms (below -20°C) for extended periods. These porous beads are acid washed and allow microorganisms to adhere readily to their surface. The beads (approximately 25 in total) are contained within a cryovial full of cryo-preserve.
Table 3.2 Components of the Biothera ATP Kit SL.

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Vials / bottles</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL reagent</td>
<td>Lyophilised reagent containing D-luciferin and luciferase</td>
<td>4 vials</td>
</tr>
<tr>
<td>Diluent C</td>
<td>ATP-free distilled water used to reconstitute the lyophilised D-luciferin</td>
<td>4 vials of 10ml each</td>
</tr>
<tr>
<td></td>
<td>and luciferase</td>
<td></td>
</tr>
<tr>
<td>ATP standard</td>
<td>$10^5$ moles/L of ATP *</td>
<td>4 vials of 5ml each</td>
</tr>
<tr>
<td>Tris-EDTA buffer</td>
<td>0.1mol/L Tris (hydroxymethyl) aminomethane, 2mmol/L EDTA and adjusted to pH 7.75 with acetic acid</td>
<td>4 bottles of 50ml each</td>
</tr>
</tbody>
</table>

* In many cases $10^5$ moles/L of ATP has been used instead of $10^6$ moles/L of ATP. This was a dilution (1:10) of the $10^5$ moles/L of ATP in sterile distilled water. This was because the linearity of the L1 luminometer is better within this range (Lundin 2000).

Table 3.3 Components of the ATP degradation reagent as formulated for this study.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100 (1% w/v)</td>
<td>1.4ml</td>
<td>0.1%</td>
</tr>
<tr>
<td>Apyrase (100Units/ml)</td>
<td>1.4ml</td>
<td>10Units/ml</td>
</tr>
<tr>
<td>Magnesium chloride (1mol/L)</td>
<td>280ul</td>
<td>0.02mol/L</td>
</tr>
<tr>
<td>5'-adenylic acid deaminase (20Units/ml)</td>
<td>350ul</td>
<td>0.5Units/ml</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>6.57ml</td>
<td>N/a</td>
</tr>
<tr>
<td>Sterile distilled water (used in recovery from filter)</td>
<td>4ml</td>
<td>N/a</td>
</tr>
</tbody>
</table>
3.2.7 Other solutions, buffers and media

a) Sterile distilled water
Distilled water was autoclaved at 121°C for 15 minutes, then stored in airtight 500ml bottles at room temperature prior to use. Once a bottle was opened for use the remaining contents were discarded after one day as the water was deemed to be no longer sterile.

b) Tris buffer (0.5 Mol/L)
Trizma HCl (Sigma Aldrich, UK) (10.64g) was added to Trizma Base (Sigma Aldrich, UK) (3.94g) and then dissolved in 200ml of sterile distilled water. This solution (pH 7.8) was stored at room temperature for up to one month prior to use.

c) Phosphate buffered water (PBW)
One phosphate tablet (Sigma Aldrich, UK), was dissolved in 500ml of distilled water and sterilised (autoclaved) at 121°C for 15 minutes.

d) Blood agar plates (7%)
Blood Agar Base No.2 (Oxoid, UK) (40g), was dissolved in 1 litre of distilled water and then autoclaved at 121°C for 15 minutes. After cooling to just under 50°C, 70ml of defibrinated horse blood (Oxoid, UK) was gently stirred in. The solution (approximately 25ml per plate) was immediately poured into sterile plastic petri dishes (Sterlin, UK). These blood agar plates were then left to set overnight before being stored at 2-8°C (for up to one month) until required.

e) Brain Heart Infusion Broth (BHI broth)
BHI Broth (Oxoid, UK) (18.5g) was added to 500ml of distilled water. This was thoroughly mixed and dispensed into glass vials (approximately 20ml each). These glass vials were then sealed and autoclaved at 121°C for 15 minutes. The vials were left to cool and stored at room temperature until required.
3.2.8 Consumables

a) Syringe filter units
A number of syringe filter units were evaluated for use in this study. The filters ranged in diameter from 13mm to 25mm. The pore size of the filters was either, 0.2μm or 0.45μm. The filters were supplied by Syrtec, USA; Millipore, USA; Sartorius, USA and Gelman, UK.

b) Syringes
Single use 1ml, 2ml and 5ml Steripak plastic syringes (Becton Dickinson, Ireland) were used.

c) ATP-free pipette tips
Other groups have noted that there are potential ATP contamination problem resulting from the use of normal autoclaved pipette tips (Lundin 2000). As a result special “ATP-free” (Biopur) tips (Eppendorf, UK) were used to transfer the luciferin-luciferase reagents to the luminometer. These particular pipette tips were supplied in tip boxes each containing 96 tips and were guaranteed to have an ATP level of less than 10⁻¹⁸ Mol/L ATP.

d) Micro-centrifuge tubes
To avoid potential contamination special ATP-free micro-centrifuge tubes (Eppendorf, UK) were used when the sample was mixed with the luciferin-luciferase reagent. These were supplied in boxes of 50 and were guaranteed to have an ATP level of less than 10⁻¹⁸ Mol/L ATP.

e) Silver sample dishes (coupons)
These silver coloured sample trays, known as “coupons”, were supplied by Hughes Whitlock for use in conjunction with the Bioprobe luminometer.
3.2.9 Microbial and non-microbial samples

a) Human blood

Whole blood provides a rich source of a somatic ATP (background ATP). By using blood, the efficacy of the reagents and method in releasing and degrading such ATP could be determined.

In order to get fresh blood samples the Campus Surgery at the University of Surrey was approached. Approval was obtained for the duty nurse to remove up 10ml of blood from healthy volunteers as required. The sampling of blood is described in Section 3.4.1.

b) CVC tips removed from catheterised patients

Due to ethical constraints it was not possible to sample the CVCs whilst they remained in situ within the patient. Therefore sampling of the CVC tip was carried out in the laboratory after the CVC was removed.

Generally, CVCs are removed from hospitalised patients, if CRS was suspected, or if the use of the access device is no longer required. After removal of a CVC that is suspected as infected, the tip is severed from the rest of the CVC and sent to the hospital microbiology laboratory for analysis. This analysis involved application of the technique developed by Maki et al (1977) and the tip is discarded. After overnight culture any growth was documented. Organisms were identified using conventional microbiology techniques where growth was observed.

The Medical Microbiology Department in St George’s Hospital, London was approached to collect the CVC tips for investigation in this study. Approval was granted from St George’s Hospital for the personnel in the Medical Microbiology Department to collect CVC tips for this study as long as the following restrictions were adhered to:

- No clinical information would be given except for the data filled out on the report sheets supplied (see Appendix 3).

- In no way should the sample be traced to the patient, therefore a label (see Appendix 3) was supplied to cover existing information already on the sample tube.
These restrictions created a major constraint within the study in that it was not possible to get further information on the underlying condition of the patient, as well as the regime of treatment. Therefore it was unknown whether antibiotics were infused through the CVC lumen. Moreover, there was no information on how many of the patients actually had an episode of CRBSI.

After the "Maki" technique was carried out by the laboratory staff the CVC tips were stored in individual plastic containers at 4°C until collection, in batches of 15 to 20, for use in this study. In addition to collecting the CVC tips, accompanying information was supplied on data sheets (Appendix 3). This additional information included the date of CVC removal, the results of the Maki roll investigation and the microorganism isolated if there was any growth.

It should be noted that even-though a wide variety of CVC tips were collected, such as those from long-term CVCs (Hickman and PermCaths) and short-term CVCs, only the tips taken from Arrow catheters (Figure 1.1) were analysed in this study.

c) Microorganisms

One ampoule of \textit{S. epidermidis} (NCTC 11047) was cultured and colonies stored using the Microbank beads at -20°C. Other microorganisms that were used in this study were an environmental isolate of \textit{E. coli} (Dr K. Powell personal communication) and clinical isolates of CNS and \textit{S. aureus} cultured from CVCs and stored using the Microbank beads at -20°C.

### 3.3 Equipment used

#### 3.3.1 Luminometers

The detection of relative light units (RLUs) that are emitted from the sample involved a combination of either the Bioprobe luminometer (Hughes-Whitlock Ltd, UK) and its specific reagents or the L1 luminometer (Mediators, Austria) and Biotherma AB reagents. More details on each of these luminometers was given in Section 2.4.3.
Table 3.4 Equipment used in the laboratory.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclave</td>
<td>All sterilisation was carried out using a Rodwell MP24 control Autoclave. The two major cycles that were used involve sterilisation at 121°C for 15 minutes for reagents and media, and 121°C for 30 minutes for sterilisation of waste.</td>
</tr>
<tr>
<td>Incubator</td>
<td>All incubations were carried out at 37°C in a 97D241 incubator (Genlab, UK).</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>All centrifugation was carried out using a Micro-centrifuge (Eppendorf, UK).</td>
</tr>
<tr>
<td>Biological safety cabinet</td>
<td>All work involving the transfer of microorganisms and the counting of colonies on blood agar plates was carried out in an InterMed MDH biological safety cabinet.</td>
</tr>
<tr>
<td>Balances</td>
<td>Measurement of weight up to 50g was carried out on an Oertling R20 balance. Measurements of between 50g and 500g were carried out using a Mettler PJ 600 balance.</td>
</tr>
<tr>
<td>pH meter</td>
<td>All pH measurements were carried out using a Corning 240 pH meter which was calibrated using pH 4, 7 and 10 buffers prior to use.</td>
</tr>
</tbody>
</table>
3.3.2 Other instrumentation

The other instruments used in this study are listed in Table 3.4.

3.4 General methods

This section details the methods that were common to all phases of this study. They include basic sample preparation for both stock solutions of microorganisms and dilutions of blood samples as well as the sampling of CVC tips.

3.4.1 Blood collection

Peripheral blood (10ml) was drawn each day from healthy human volunteers. Blood that was required for clotting experiments was drawn into a red-capped Vacutainer tube (Becton Dickinson, UK) containing no additives. Blood for routine work (where the blood was required un-clotted) was drawn into a Vacutainer tube containing lithium-heparin gel (green capped) only.

In addition, blood (10ml) was drawn through the CVC in the hospital wards by the hospital staff. The method used was similar to that described by Wing et al (1979).

3.4.2 Platelet rich plasma

Blood was freshly drawn from a healthy individual into a red capped Vacutainer tube (Becton Dickinson, UK) with no exposure to EDTA or heparin. It was then immediately transferred into a sterile glass tube where it was allowed to clot. After one hour the tube was gently mixed by inverting twice and 1ml of the supernatant removed – leaving the blood clot in the tube. This supernatant (plasma) was red and white cell-depleted, but was a rich source of platelets, therefore it was termed “platelet-rich plasma” for the purposes of this study.

3.4.3 Serial dilutions of bacteria

a) Dilution of overnight culture

One colony was picked from a blood agar plate using a sterile loop and put into a glass vial containing 20ml nutrient broth. After incubation (overnight) at 37°C, 100μl of the
"overnight culture" was taken and added to 900μl of sterile PBS to give a ten-fold dilution of the stock. Serial dilutions were then continued for a further five transfers so that a dilution of 10^6 was achieved.

b) Washing cells by centrifugation

One colony was picked from a blood agar plate using a sterile loop and put into a glass vial containing 20ml nutrient broth. After incubation (overnight) at 37°C, 1ml of the "overnight culture" was transferred into a centrifuge tube and centrifuged at 2500 rpm for 5 minutes. The supernatant was then removed from this to leave a pellet, which was reconstituted in 1ml of PBW. The centrifugation process was repeated a further two times so that a final volume of 1ml of "washed" microorganisms was obtained.

3.4.4 Sampling and culture of a CVC

Although the FASEB is intended for use while catheters are in-situ, it may also be used in the laboratory (in-vitro) to sample CVC tips that have already been removed from patients (Koch et al. 2002) or CVCs that have been colonised in vitro (Worthington et al. (2002b).

A new FASEB was taken and removed from its plastic sheath, then cut to a length of approximately 10cm. The CVC tip was held using forceps and the FASEB advanced into the lumen, unless more than one lumen was sampled. Generally the longest (distal) lumen was brushed. The FASEB was inserted all the way through the lumen before being removed and placed in a test tube.

Once the sample was retrieved it was placed in a tube. The solution containing the FASEB acquired sample (generally a final volume of 1ml) was vortex-mixed for 1 minute and then 100μl was removed and spread on a blood agar plate. This was incubated overnight at 37°C and the number of colonies estimated the next day.

3.4.5 Volume of sample removed using the FASEB

Fresh heparinised blood (1ml) was weighed and then a FASEB was dipped into the blood. The brush was then removed and the blood remaining subsequently weighed again. The difference in the two weights was recorded as the volume attached to the brush. This was repeated a further nine times and the mean value calculated.
3.4.6 Using Microbank beads for storage of microorganisms

**a) Preparation for storage**
Approximately 3 to 5 colonies were taken from a fresh culture of organisms (on a blood agar plate). These were used to inoculate the cryopreservation fluid, which surrounds the beads in the cryovial. The lid was closed on the cryovial and the vial inverted five times. All the excess cryopreservation fluid was removed, using a sterile pipette, to leave the vial containing the beads with the microorganisms attached. The vial was closed tightly and stored below -20°C.

**b) Recovery of microorganisms**
The cryovial was opened and a bead removed using sterile forceps. The vial was then returned to the freezer as quickly as possible. The bead was dropped into a vial containing 20ml of nutrient broth. This was then cultured at 37°C overnight. An aliquot of this was taken to inoculate another 20ml of nutrient broth.

3.4.7 Using the Staphytect Plus kit
The Staphytect Plus kit (Oxoid, UK) was brought to room temperature. One drop of the test latex was dropped onto one of the circles on the reaction card. One drop of control latex was dropped on to another circle. Using a loop, up to five colonies were removed from a blood agar plate and added to the test latex in the circle. This was repeated for the control latex. In both cases the latex was spread all over the circle. The card was then gently swirled (for up to 1 minute) so that there was mixing within the contents of each circle.

The presence of *S. aureus* was indicated by agglutination of the blue latex particles in the test circle and absence of agglutination in the control circle. If no agglutination occurred then the contents of the blue test circle remained a smooth blue suspension.

3.4.8 Determination of the “hold-up volume” of a syringe filter
Distilled water (1ml) was drawn into a plastic syringe, which was subsequently weighed as were a micro-centrifuge tube and a syringe filter. The water was then flushed through the filter and the filtrate collected in the micro-centrifuge tube. All three were re-weighed
before air (1ml) was then flushed through the filter and any liquid displaced from the filter was collected. All three were again re-weighed a further five times and the average volumes were recorded for both hold-up volume before air-purging and after air-purging.

3.4.9 Determination of the ATP content of a syringe filter

The ATP was released by passing 100µl of TCA (10%) through each filter and flushing this with Tris-EDTA (2ml). The ATP concentration was then determined using 50µl extract in the SL reaction. This was repeated three times using separate filter units.

3.4.10 Environmental conditions and reagent storage

Unless otherwise stated all experiments were carried out at room temperature. This was defined as between 20°C and 25°C. The room temperature was regularly recorded using a min./max. thermometer in the laboratory and experiments were not carried out if the temperature was outside these limits.

All work that was carried out in conjunction the luminometers was conducted in the area of the laboratory that was the furthest from the window. This limited the amount of sunlight that would be exposed to the photo-detector of the luminometer. In addition, on days of particularly bright sunlight, the blinds were drawn to block out as much of light as possible.

All luciferin-luciferase reagents and other light sensitive reagents were stored in light proof containers in the refrigerator.

3.5 Methods used in conjunction with the Bioprobe luminometer

3.5.1 Determination of RLU's using the Bioprobe luminometer

The sample (either on one of the silver coupons or inside a whole syringe filter unit) was placed onto the stainless steel base plate. The luminometer part of the Bioprobe was then placed onto the steel base and gently pressed down (Figure 2.6). A vacuum automatically sealed the space between the instrument and the bottom (steel plate), creating a "light-tight" environment where the detector measures only light emitted from the sample. The luminometer then detected the number of photons of light emitted and expressed these as RLU's on the LED display (measuring over a 10 second period). A second reading was
taken by gently pressing down on the Bioprobe to repeat the measuring process. The luminometer portion was then lifted off and the sample removed and discarded.

3.5.2 **Samples contained within syringe filters**

Using a syringe, 500μl of “treated” sample (sample that has been exposed to reagents that degrade contaminating ATP) was filtered through a 25mm (diameter) 0.45μm Millipore filter. This filter was then immediately flushed with 5ml of phosphate buffered water. Luciferin-Luciferase reagent (50μl) was added to the filter using a sterile 1ml syringe. The filter was immediately placed on the steel base-plate and the RLUs determined as in Section 3.5.1. Then using a new syringe, 100μl of bacterial extractant was added to the filter and the RLUs determined again. One further measurement was taken immediately before the filter was discarded.

In addition, 100μl of the remaining 500μl of sample was spread onto a blood agar plate and incubated overnight at 37°C.

3.5.3 **Samples on the silver coupons**

The serial dilution (100μl) of a particular microorganism was placed on a coupon and the Luciferin-Luciferase reagent (50μl) added. The dish was gently shaken and placed on the reading area of the Bioprobe where the RLUs were subsequently determined to give the background ATP level. Bacterial extractant (50μl) was added and the RLUs determined again.

The active concentration of the bacterial lysis agent was 25% (v/v) of the original supplied concentration. The Luciferin-Luciferase reagent was also used at 25% (v/v) of the original concentration. This is due to dilution of the final concentration of each reagent as they are added sequentially to the dish; for example, the bacterial extractant (50μl) was added to give a final volume of solution of 200μl on the dish.

3.5.4 **Preliminary work with 10x ATPase**

The *S. aureus* serial dilution (100μl) was placed on a coupon and the 10x ATPase added (50μl). This was then followed by the Luciferin-Luciferase reagent (50μl) and subsequently read using the Bioprobe (background). Bacterial extractant (50μl) was then
added and the filter read again. In controls where ATPase was not added, PBW was added instead to account for dilution effects.

The 10x ATPase (50µl) was requested from the manufacturer so that dilution effects resulting from the addition of normal apyrase (500µl) could be avoided.

3.5.5 Analysis of blood samples which have been exposed to ATPase

A 1ml solution containing Isostat isolator reagent (1:50) and 10x ATPase (1:20) in PBW had fresh heparinised blood added to give a final blood concentration of 1.5%. The tube was vortex-mixed for 30 seconds before being incubated for 60 minutes. Using a 1ml plastic syringe, 500µl of sample was removed and filtered through a 25mm (diameter) 0.45µm Millipore filter. This was flushed, using a 5ml plastic syringe, containing 5ml of PBW and the ATP level determined as before.

3.5.6 Method used to evaluate clinical samples

Nine blood samples were taken through the CVC of patients who were receiving total parenteral nutrition (TPN) through the CVC. These were all out-patients and all samples were collected on the same day. They were stored at 4°C overnight prior to assay. Each sample was taken and 15µl added to 1ml of Isostat isolator reagent (0.0014% (v/v)) to give a final concentration of 1:66.7). The tubes were vortex-mixed for one minute before 10X ATPase was then (final concentration 1:10 v/v) added and the tube vortex-mixed for 5 seconds. Using a 1ml plastic syringe, 500µl of sample was removed and filtered through a 25mm diameter, 0.45µm pore size Millipore filter. Following a flush with 5ml of PBW, one drop (50µl) of luciferin-luciferase reagent was added to the filter using a 1ml syringe. The whole filter was immediately placed in the Bioprobe and the RLUs determined. Two drops of bacterial extractant (100µl) were then added to the filter and the filter RLUs determined again with a further reading immediately taken to show whether luminescence was occurring. Additionally all nine samples were spread onto blood agar plates and incubated overnight at 37°C.
3.6 Methods used in conjunction with the LI luminometer

3.6.1 Determination of RLUs using the Mediators L1 luminometer

When measuring the ATP concentration of a sample in the Mediators L1, two methods were used depending upon which luciferin-luciferase reagent was selected.

a) Determination of ATP concentration using the SL kit

Various volumes (between 700μl and 790μl) of Tris-EDTA buffer (Biothema AB, Sweden) were transferred to clean ATP-free Eppendorf centrifuge tubes. Each tube was placed in the Mediators L1 luminometer reading chamber and the SL reagent (200μl) added and the RLUs determined over a ten-second period. The sample drawer was then re-opened (see Figure 2.5) and the sample (between 10μl and 100μl) added to give a final volume of 1ml. Again the RLU values were automatically determined when the drawer was re-closed. The drawer was opened for a third time and ATP standard (10μl) added. The RLUs were again determined and recorded.

b) Determination of ATP concentration using the HS reagent

The HS reagent (400μl) was transferred to a clean ATP-free Eppendorf centrifuge tube, which was then placed in the Mediators L1. Following this “blank RLU level” determination, between 50μl and 500μl of the vortex-mixed sample was added and the RLUs determined again. The volume of sample measured depends upon the extractant used; TCA was added in small amounts (<100μl) and DTAB extracts were added in larger amounts (<500μl). Following this 10μl of an ATP standard was added and the RLUs determined and recorded.

c) Determination of ATP concentration

To determine the concentration of ATP in a sample, equation 5 was used:

\[
\frac{\text{(Sample - Blank)}}{\text{(Standard - Sample)}} \times \text{Concentration of Standard} \times \frac{\text{Extraction volume}}{\text{Sample volume}} \tag{5}
\]

where the sample, standard and blank values are reported as RLU. The concentration of the standard is usually 1μMol/L and the extraction volume is 1.1ml for the DTAB
method and 2.8ml for the TCA method, and the sample volume measured is between 10μl and 100μl for the TCA method and up to 400μl for the DTAB method.

### 3.6.2 Method development

**a) Concentration of Triton X-100 required to lyse somatic cells**

Various concentrations of Triton X-100 (0 to 4% final concentration) were added to a solution containing apyrase (10U/ml) in excess magnesium ions. After a 30 minute incubation to degrade the released ATP, TCA (final concentration 2.5%) was added to inactivate the apyrase and release any further ATP. The subsequent ATP level of each solution was determined using SL method.

**b) Filter flush volume**

A solution containing heparinised blood (1:50) in ATP degradation reagent was left for 60 minutes at room temperature. Aliquots (800μl) were filtered through each of four 25mm diameter (0.2μm) syringe filters. These were then flushed with either 2.5ml, 5ml, 7.5ml or 10ml sterile water before 300μl of TCA (10%) was added and then flushed using 2.5ml sterile water. The ATP concentrations were then determined using the SL method. This was repeated a further three times.

**c) Incubation period required for the ATP degradation reagent**

ATP was added to the ATP degradation reagent to give a final ATP concentration of 1μMol/L in a 5ml solution. This solution was vortex-mixed for 5 seconds and after a 2 minute incubation 550μl was removed and added to 10% TCA (185μl) to stop the ATP degradation reagent reaction with ATP. Another 800μl was removed and filtered through a 25mm, 0.22μm syringe filter.

This was repeated at intervals of 5, 10, 20, 30, 60 and 120 minutes and these exposed immediately to TCA and filtered in the same way. Each sample that was filtered was subsequently flushed through with sterile water (7.5ml) and 10% TCA (300μl) added. The ATP in each was assayed using the SL reaction.
d) **Incubation time required for TCA**

Serial dilutions of stocks of *E. coli*, *S. epidermidis*, and *C. albicans* (to $10^6$) were exposed to a final TCA concentration of 2.5% for either a 1, 2, 3, 5 or 10 minute period. At the end of each incubation a sample was immediately taken and the concentration of ATP quantified using the SL method. Each was left for one hour and the concentration of ATP determined again (control).

This was repeated using the 25mm diameter, 0.2μm pore size syringe filters. The organisms were added to the filter and 300μl of TCA (10%) added to each filter for the same incubation periods before being flushed from the filter with 2.5ml Tris-EDTA and quantified using the SL reaction.

### 3.6.3 Methods used specifically with TCA

a) **Detection of microbial serial dilutions in a pure system (TCA extracted)**

A ten-fold dilution of the microorganism stock was made from a stock to $10^6$. Then 750μl of each microorganism dilution was added to 10% TCA (250μl) and following a vortex-mix for 5 seconds, each of these was left to stand for 20 minutes at room temperature. The extract (between 20μl and 50μl) was removed and transferred to an ATP-free micro-centrifuge tube (Eppendorf, UK) containing Tris-EDTA buffer (between 750μl and 780μl) and SL reagent (200μl). The RLUs were determined using the SL method. In order to determine the number of organisms in each dilution, 100μl of the $10^5$ dilution was spread on a blood agar plate (in triplicate) and the readings were compared after 24 hours.

The above was also repeated using 20μl of extract in HS reagent (400μl), in conjunction with 10μl of 100nMol/L.

b) **Degradation of ATP from an ATP standard**

Various concentrations of ATP standard were exposed to the ATP degradation reagent for approximately 20 minutes (room temperature). 150μl of each was removed and added to 50μl TCA (10%). Further solutions were set up as above using 1ml of distilled water instead of the ATP degradation reagent.
The ATP bioluminescence assay (SL reagent) was carried out using 20μl of extract removed from each of the tubes.

c) **Effect of TCA on S. epidermidis (NCTC 11047)**

Various concentrations of TCA were added to serial dilutions of a stock of *S. epidermidis* (NCTC 11047) to achieve final TCA concentrations of between 0 and 2%. These were left to incubate for 10 minutes at room temperature and the ATP concentration determined using the SL method.

d) **Effect of TCA on E. coli**

Various concentrations of TCA were added to serial dilutions of a stock of *E. coli* to achieve final TCA concentrations of between 0 and 2%. These were left to incubate for 10 minutes at room temperature and the ATP concentration determined using the SL method.

e) **Effect of TCA on apyrase and 5′-adenylic acid deaminase**

Tubes containing 500μl of one of the following were set up:

- MgCl₂ (0.01M) and apyrase (10U/ml)
- MgCl₂ (0.01M) and 5′-adenylic acid deaminase (0.7U/ml)
- MgCl₂ (0.01M) and apyrase (10U/ml) and 5′-adenylic acid deaminase (0.7U/ml)

TCA was added to each to achieve various concentrations (0 to 1.2%). These were vortex-mixed and then left for 10 minutes at room temperature. A final concentration of 5μmol/L ATP standard was added to each. Again these were left for 10 minutes before TCA (10%) was added to each to achieve a final concentration of 4% TCA (to stop the reaction). Water was then added to correct the volumes before the bioluminescence assay was carried out using the SL reagent.

f) **Buffering ability of Tris on TCA**

Various concentrations of TCA were added to give a final TCA concentration of 2.5% (w/v) in final Tris concentrations of 0.1mol/L to 0.4mol/L. The pH of each was then determined. This was repeated using lower concentrations of TCA (0 - 2.5%).
3.6.4 Methods using DTAB

a) Detection of microbial serial dilutions in a pure system (DTAB extracted)

A ten-fold dilution of the microorganism stock was made to $10^{-6}$. Then 50μl of each of these was then transferred to an Eppendorf micro-centrifuge tube (ATP-free) and 0.2% DTAB (50μl) added. After a vortex-mix for 5 seconds, each of these was left to stand for 20 minutes at room temperature. Sterile distilled water (400μl) was then added to each to give a final volume of 500μl. In addition, 100μl of the $10^{-5}$ dilution was spread on a blood agar plate (in triplicate).

The DTAB extract was vortex-mixed for two seconds before 400μl of the Biothema HS reagent was added to the Eppendorf micro-centrifuge tube. The number of RLUs was determined using the Mediators L1 luminometer (settings are a 10 second delay and a 5 second read time). A subsequent reading was taken and then 10μl of 100nMol/L standard was added to the solution and again the ATP level (RLUs) determined. The readings were compared with the plate culture results from the blood agar plates after 24 hour incubation.

b) Detection limit of the DTAB extraction (six clinical isolates)

Clinical isolates were recovered from CVC tips (St George’s Hospital, London, UK) and stored using the “Microbead” (Prolab, USA) system. When required, stock cultures were grown in nutrient broth and then the broth cultures were serially diluted. The HS reagent detection system was used to determine how sensitive the ATP bioluminescence method was in detecting low levels of each of the microorganisms. 50μl of the $5 \times 10^{-5}$/ml stock was added to 50μl of DTAB (2%). After vortex-mixing for 5 seconds, each of the extracts was left to stand for 20 minutes before sterile distilled water (400μl) was then added to each giving a final volume of 500μl. The RLU level was determined using the HS reagent.
3.6.5 Formulation of ATP-free α-Cyclodextrin (2.5%w/v)

a) Formulation of 10% α-Cyclodextrin

α-Cyclodextrin (Sigma C4642) (500mg) was taken and dissolved in 5ml of sterile distilled water. 4ml of this preparation was added to 4ml of vigorously shaken “washed” Q Sepharose (slurry). The suspension was vortex-mixed for ten seconds and then allowed to sediment (15 minutes). The supernatant (6mls) was removed and added to 6mls of shaken “washed” Q-Sepharose. Again the suspension was vortex-mixed for ten seconds and then allowed to sediment (15 minutes). The supernatant was then aliquoted and stored.

b) Washing the Q Sepharose

Washing of the Q Sepharose Fast Flow (Amersham Pharmacia Biotech AB) is necessary as it is suspended in a solution containing 40% ethanol. This ethanol needs to be removed as it interferes with the HS reagent.

The Q Sepharose was vigorously shaken and approximately 20ml poured off. After allowing the slurry to sediment (15 minutes) 6ml of the ethanol supernatant was removed and replaced with 6ml of sterile distilled water. The suspension was vortex-mixed for ten seconds and then allowed to sediment (15 minutes). A further 8ml was removed and replaced with 8ml water. After vortex-mixing and sedimentation this was repeated using a further 8ml of water.

3.6.6 Methods used to determine ATP levels on CVC tips

Figure 3.2 illustrates the details of how the FASEB-retrieved samples were tested using either a TCA- or DTAB-based method. In both cases the FASEB-retrieved sample was added to 1ml of the ATP degradation reagent. This was vortex-mixed for 60 seconds before being left to incubate for 60 minutes at room temperature. Following incubation, 100μl was removed from the solution and spread on a blood agar plate. These plates were incubated at 37°C overnight. After this initial step the treatment of each sample differed and this is described below.
a) Evaluation of the "TCA method"

Using a sterile 1ml syringe 800μl of the solution was filtered through a 0.2μm pore size, 25mm diameter Syrtec filter. The filtrate was discarded and the filter was immediately washed by flushing through 7.5ml of sterile distilled water (3 x 2.5ml washes). 10% TCA (300μl) was then added to the syringe filter via another sterile 1ml syringe. This time the filtrate was collected in a sterile ATP-free plastic container. After two minutes, 2.5ml sterile water was flushed through the filter from syringe and the filtrate collected in the same container, giving a final volume of 2.8ml. Seventy-five microlitres of this extract was then used in the HS reaction and readings were compared with the plate culture results from the blood agar plates after 24 hours.

Using the equation in Section 3.6.1(c) the concentration of ATP in the sample was determined. In this case 2.8ml was extracted and flushed to form the sample. However only 75μl of the sample was actually used in the reaction. This equates to approximately 2.7% of the extracted sample and 2.1% of the original sample.

b) Evaluation of the DTAB method

Using a sterile 1ml syringe 800μl of the solution was filtered through a 0.2μm pore size, 13mm Nalgene filter. The filtrate was discarded and the filter was immediately washed by flushing through 2.5ml of sterile distilled water. 2% DTAB (100μl) was then added to the top of the syringe filter using a pipette. The DTAB was allowed to slowly (approximately 10 minutes) fall through the filter membrane and the filtrate collected in a sterile (ATP-free) plastic container. Sterile distilled water (1ml) was flushed through the filter from syringe and the filtrate collected in the same container, giving a final volume of 1.1ml.

The DTAB extract was vortex-mixed for 5 seconds before 400μl was added to 100μl of 2.5% α-cyclodextrin solution in an ATP-free Eppendorf micro-centrifuge tube. This was vortex-mixed for 10 seconds and left to stand for 2 minutes before the background ATP level (RLUs) was determined. 400μl of the HS reagent was added to the same micro-centrifuge tube and the ATP level (RLUs) determined, twice.

Using equation 5 in section 3.6.1(c) the concentration of ATP in the sample was determined. Here 1100μl were extracted as sample and 400μl of the sample was used in
the reaction. This equates to approximately 35% of the extracted sample, which is 28% of the total sample. Readings were compared with the plate culture results from the blood agar plates after 24 hours incubation.

3.7 Cut-off limits and assay performance

The sensitivity and specificity of the technique based upon the ATP bioluminescence were determined as shown in Equations 6 and 7:

$$\text{Sensitivity} \, (%) = \left(\frac{a}{a+c}\right) \times 100 \tag{6}$$

$$\text{Specificity} \, (%) = \left(\frac{d}{b+d}\right) \times 100 \tag{7}$$

Where:

- a True positive
- b False positive
- c False negative
- d True negative

This was based on the assumption that >100CFU/ml from a FASEB-retrieved sample was positive and <100CFU/ml from a FASEB-retrieved sample was negative (Kite et al. 1997).

In addition the $PV^+$ and the $PV^-$ were determined using Equations 8 and 9.

$$PV^+ \, (%) = \left(\frac{a}{a+b}\right) \times 100 \tag{8}$$

$$PV^- \, (%) = \left(\frac{d}{c+d}\right) \times 100 \tag{9}$$
Transfer FASEB retrieved sample into 1ml of the "ATP degradation reagent", vortex-mix (60 seconds) followed by an incubation (60 minutes) at room temperature.

Remove 100μl and spread on a blood agar plate.

### TCA extraction method

- Filter 800μl of the solution through a 0.2μm, 25mm filter and then flush 7.5ml of sterile water (3 x 2.5ml) through the filter.
- Add 300μl of 10% TCA to the syringe filter whilst placed on a sterile (ATP-free) plastic container.
- After a 2 minute incubation flush 2.5ml sterile water through the filter whilst collecting the filtrate in the sterile plastic container.
- Vortex-mix this TCA-extracted filtrate for two seconds before removing either 75μl for addition to 400μl HS reagent, or 100μl for addition to 900μl of SL (in Tris EDTA buffer).

### DTAB extraction method

- Filter 800μl of the solution through a 0.2μm, 13mm filter and then flush 2.5ml of sterile water through the filter.
- Add 100μl of 2% DTAB to the syringe filter whilst placed on a sterile (ATP-free) plastic container.
- After a 2 minute incubation flush 1.0ml sterile water through the filter whilst collecting the filtrate in the sterile plastic container.
- Vortex-mix the DTAB extract (5 seconds) before removing 400μl and adding it to 100μl of 2.5% α-cyclodextrin.
- Vortex-mixed this solution (10 seconds) then leave to stand for 2 minutes before adding 400μl of the Biothema HS reagent.

The number of RLU's are then determined using the L1 (settings here are a 10 second delay and a 5 second read time).

Add 10μl of 10nMol/L standard to the solution and determine the RLU value. Use this value to determine the concentration of ATP in the sample (mol/L).

Compare this concentration of ATP with the CFU count from the blood agar plates.

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**Figure 3.1** Protocols used when evaluating FASEB-retrieved samples (DTAB and TCA methods) in conjunction with the L1 luminometer at room temperature.
RESULTS
Structure of the results section

The results section of this thesis is made up of three chapters (Chapters 4, 5 and 6). This approach was selected because three specific sections of research were carried out.

Initial research was conducted using two luminometers, the Bioprobe in conjunction with its reagent system and the LI with the recommended Biothema AB reagents. Details of this preliminary research is reported in Chapter 4, where the off-the-shelf system (Bioprobe) was found to be unsuitable as few parameters could be varied and therefore optimisation of the technique was difficult. Chapter 5 details research carried out using the tube luminometer (LI) and a combination of commercial reagents and laboratory-developed reagents. A method was developed and evaluated using clinical samples, however improvements were identified to improve the performance of the technique. In Chapter 6 the further development and optimisation of the technique evaluated in Chapter 5, is reported. This work was mainly centred around a new microbial lysis agent, resulting in the development of a modified technique, which was evaluated using clinical samples and the results analysed to determine the potential usefulness of the assay.

Display of results

Results are displayed as bar graphs, or occasionally as line graphs, showing the mean ± the standard deviation (SD) of a series of four experiments. Values plotted are either the original RLU values or in cases where a standard was used, as the ATP concentration (in mol/L). In cases where a small change in ATP level is displayed, results are displayed as a percentage of a control.
CHAPTER FOUR
Chapter Four

4.1 Background

In Chapter 2 ATP bioluminescence was identified as a useful technology that could potentially form the basis of a rapid test system for the detection of catheter-associated infection. Two luminometers were chosen for further evaluation: the Bioprobe luminometer and its associated reagent system (Hughes-Whitlock, UK) and the LI luminometer (Mediators, Austria) in conjunction with the recommended Biothema AB luciferase reagents.

The objective of this section was to determine which one of these two ATP bioluminescence detection systems is most appropriate for further use in this study. Experiments based on both for the systems are reported in this chapter. Initially the sensitivity of the systems were determined, with subsequent investigations involving the development of strategies to remove non-microbial contaminating ATP from the sample, before the release and detection of microbial ATP.

4.2 The Bioprobe and associated reagents

The Bioprobe is supplied as an off-the-shelf instrument with the manufacturer also supplying all the reagents that are necessary for determining ATP levels. Therefore, reagents such as the bacterial lysis agent and the ATPase have been optimised, by the manufacturer, for use with the luciferase-based reagents.

In this section preliminary experiments were conducted to determine how sensitive the system is and if further optimisation of the reagents were necessary.

4.2.1 Release and detection of microbial ATP

A bacterial lysis agent (Hughes Whitlock, UK) was used to lyse microorganisms so that intracellular ATP could be released. The manufacturers suggested that a final concentration 1:5(v/v) of the supplied concentration would be adequate to lyse most bacteria and yeast species. This was confirmed when serial dilutions (to 10^6) of stocks of S. aureus, E. coli and C. albicans were exposed to various final concentrations of the bacterial lysis agent (1:5 to 1:2.5) and subsequent spread plate incubations reported <1 CFU/ml present.
A lucerin-luciferase reagent (Hughes Whitlock, UK) was used to detect and quantify the ATP that was released. The luciferin-luciferase reagent was used at a concentration of 1:5 (v/v) of that supplied, as suggested by the manufacturers.

### 4.2.2 Sensitivity

The sensitivity of the Hughes-Whitlock reagents and Biprobe luminometer was determined using the bioluminescence assay method described in Section 3.5. Figures 4.1, 4.2 and 4.3 show the detection limit for each of the three serial dilutions of stock microorganisms. In Figure 4.1 it can be seen that for *S. aureus* there is only good differentiation between the contaminating and microbial ATP at a level equivalent to 10,000 CFU/ml. Figure 4.2 displays a level of between 100,000 and 1,000,000 CFUs for a serial dilution of *E. coli* and in Figure 4.3 there is good differentiation for *C. albicans* at the 1000 CFU/ml level.

In each of Figures 4.1, 4.2 and 4.3 it was observed that the limit of detection is dependent upon the level of background ATP present within the sample. This non-specific, contaminating ATP is naturally present within the broth and is only reduced through dilution of the broth; thus the higher the concentration of organisms then the higher the concentration of contaminating ATP.

In an attempt to reduce this level of contaminating ATP, the stock cultures were “washed” in PBW using a centrifugation method described in Section 3.4.3. However, this was found to be ineffective because even though the concentration of contaminating ATP was reduced, the ATP released from the microorganisms was much lower than expected. This was presumably due to the level of stress the microorganisms were put under during the centrifugation process, which led to depletion of their intracellular ATP.

### 4.2.3 Removal of contaminating ATP

In Section 4.2 it was observed that relatively high levels of contaminating ATP remain in the sample. In order to improve on sensitivity and specificity this contaminating ATP needs to be removed. To do this an ATPase (Hughes Whitlock, UK) was evaluated.
Figure 4.1  Relative light units detected before (blue) and after (yellow) lysis of a serial dilution of *S. aureus*. Each bar represents the mean ± SD of four experiments.
Figure 4.2 Relative light units detected before (blue) and after (yellow) lysis of a serial dilution of *E. coli*. Each bar represents the mean ± SD of four experiments.
Figure 4.3  Relative light units detected before (blue) and after (yellow) lysis of a serial dilution of *C. albicans*. Each bar represents the mean ± SD of four experiments.
a) ATPase

The manufacturer suggested that the ATPase should be used at a concentration of 1:10 (v/v) of that supplied. However, it was found that concentrations of 1:2 (v/v) were necessary to degrade the high levels of contaminating ATP present in serial dilutions of broth cultures. This created a problem in that adding more ATPase to an aliquot of a solution containing microorganisms resulted in a large dilution of the sample leading to the requirement for larger volumes of bacterial lysis reagent and luciferase reagents.

After consultation with the manufacturer, a ten times more concentrated ATPase (Hughes Whitlock, UK) was then obtained and Figure 4.4 shows that a 10% (v/v) final concentration of this 10x ATPase was much more effective at degrading the contaminating ATP. It can be clearly seen that the level of contaminating ATP is reduced to a low background level that is independent of the concentration of *S. aureus*. It can also be observed that the detection limit for *S. aureus* has improved from that observed in Figure 4.1 and is now <10,000 CFU/ml.

b) Release and degradation of ATP from somatic cells

In order to remove contaminating ATP from a sample it is less than adequate to simply add ATPase. A solution containing somatic cells, such as red blood cells and platelets, will require cell lysis so that all the intracellular ATP is released for subsequent degradation by the 10x ATPase.

Fresh heparinised blood was used as a source of non-microbial ATP. Preliminary experiments used a 1:66.7 (v/v) final concentration in 1ml. This particular volume was chosen because the FASEB collects between 10 and 15mg of material from a CVC and if this were dissolved in 1ml it would also give a concentration of approximately 1.5%.

The Isostat isolator reagent (Oxoid, UK) described in Section 3.2.3, was chosen as a possible somatic cell lysis agent. This particular reagent is routinely used in clinical microbiology laboratories for the lysis of red and white blood cells, prior to microbial cell concentration by centrifugation. Investigations were carried out to determine if this reagent could effectively lyse somatic cells. Various concentrations of the isolator
Figure 4.4  Relative light units detected in serial dilutions of *S. aureus*. Each bar represents the mean ± SD of four experiments where RLU values were determined before ATPase was added, after ATPase (10% v/v) was added and after bacterial lysis agent was added.
reagent (0 to 0.02% v/v) were used in combination with 10x ATPase to degrade the released ATP. Subsequent addition of bacterial lysis agent lysed any intact cells and the remaining ATP was subsequently assayed.

Figure 4.5 shows that the optimum concentration at which somatic cell lysis occurs is between a final concentration of 0.001 and 0.002% of the isolator reagent.

The isolator reagent is supplied in a tube containing 700μl of liquid. Its final working concentration is 7.0% (v/v) when blood is added to make a final volume of 10ml. A concentration of 2% of the 7% working solution was found to give optimum cell lysis. Therefore 0.0014% (v/v) isolator reagent in PBW was used in further experiments.

4.2.4 Filtration of sample

In section 4.2.3 it was observed that the use of the isolator reagent (0.0014% v/v) in combination with ATPase (10%) effectively releases and degrades the ATP released from a heparinised blood sample (1:66.7v/v). However, the level of ATP remaining is still relatively high and this reduces the potential sensitivity and specificity of the assay. To lower the concentration of ATP remaining, syringe filtration was evaluated in combination with the isolator reagent.

a) Filter characteristics

Two standard 25mm diameter syringe filters (Millipore, USA) were chosen for evaluation. One had a pore size of 0.2μm whilst the other has a pore size of 0.45μm. The 25mm diameter gives a relatively large surface area for the sample to spread over, with the result that any microorganisms that are present could collect on the filter membrane without causing a blockage of the membrane pores.

Serial dilutions (to \(10^{-6}\)) of stock solution of \(S. aureus\), \(E. coli\) and \(C. albicans\) were passed through each of the two syringe filters and it was determined after subsequent culture of the filtrate that no microorganisms passed through either the 0.2μm or 0.45μm filters. However, it was observed that increasing physical force was required to pass a solution containing the high concentrations of microorganisms through the 0.2μm filter. This was presumably due to microorganisms blocking the smaller pores of the 0.2μm filter. It was also determined that both had a hold-up volume of approximately 400μl and a post-air-purge hold-up volume of 220μl.
**Figure 4.5** Effect of a combination of 10x ATPase and various concentrations of isolator reagent on the ATP concentration remaining in fresh heparinised blood (1:66.7 v/v). Each bar represents the mean ± SD of four experiments. The control was the ATP concentration observed in a blood sample that was not exposed to isolator reagent.
b) **Determination of microbial ATP concentration**

The manufacturer of the Bioprobe luminometer suggested that ATP from the sample contained within a syringe filter could be determined if the luciferase was added to the filter and the whole unit placed in the reading area of the luminometer, where the RLUs would be determined through the filter. In order to do this however, an appropriate volume of the luciferase reagent needs to be added. Therefore, it is important to take into account the hold-up volume in the syringe filter as any luciferase added to the filter will be diluted by liquid already present.

In Section 4.2.1 it was observed that RLUs could be determined by adding 50µl of luciferase to the sample to achieve a final luciferase concentration of 1:5 (v/v). As the hold-up volume here is ~220µl a decision was made to again use 50µl of luciferase as this would give a final concentration of just under 1:5 (v/v) luciferin-luciferase.

c) **Volume of bacterial lysis agent required**

A similar situation exists with the bacterial lysis agent where once the microorganisms are isolated onto the filter membrane, it is necessary to lyse them so that the ATP is released. Previous experiments on the silver coupons demonstrated that the bacterial lysis agent could be added to a large volume of sample as long as a final concentration of >1.5 (v/v) was achieved.

Although 50µl should be adequate, an evaluation was conducted to determine if bacterial lysis agent (100µl) should be added to the filter in order to achieve complete lysis. Figure 4.6 illustrates that there is little difference between the addition of 50µl or 100µl (to achieve a final concentrations of 18.5% and 31.1% v/v respectively) of bacterial lysis agent to various concentrations of *E. coli* in terms of the final RLU signal.

d) **Sample volume added to filter**

A sample volume of 500µl was passed through each filter. This represented 50% of the original sample. It was observed that addition of sample volumes of >500µl led to increased pressure being exerted on the filter, which may be due to the filter pores getting blocked.
Figure 4.6  The RLU values detected from a serial dilution of *E. coli*, where the ATP is extracted using either 50μl or 100μl (18.5% or 31.1%) of bacterial lysis agent. Each bar represents the mean ± SD of four experiments.
e) Flush volume

After the sample has been added to the filter it needs to be flushed through the filter, otherwise it will remain there forming part of the hold-up volume. Various volumes (1-10ml) of PBW where flushed through both the 0.2μm and 0.45μm filters. It was found that at least 5ml of PBW was required to remove any material that could be seen with the naked eye on the syringe filter membrane.

f) Analysis of blood samples taken through CVCs

The basic extraction method was challenged with blood samples drawn through the CVCs of nine home TPN patients in an outpatient clinic (Leeds General Infirmary). An aliquot of each of the blood samples was added to the isolator reagent and 10x apyrase added to give final concentrations of blood (1:66.7) and apyrase (1:10). These then had their ATP assayed as in Section 3.5 and the RLU values are reported for each in Table 4.1.

In seven of the nine samples an RLU value of greater than the background was observed. This suggested that these seven samples had significant levels of microorganisms. However, when these RLU values were compared to the CFUs obtained in plate culture it was observed that eight of the nine samples grew no colonies. Sample number four did grow 50 colonies but it was one of the two samples that had an RLU value lower than its background.

4.3 The L1 and Biothema AB reagents

The L1 luminometer, supplied by Mediators, is a stand-alone instrument. Mediators do not supply reagents for use with this luminometer, however, representatives from Mediators did recommend the Biothema AB luciferin-based reagents. Unfortunately, these only comprise just the luciferase and luciferin reagents, with associated buffers. Through conversations with Mediators and Biothema recommendations for microbial lysis agents were chosen (Lundin 2000, Lundin 2000 personal communication) and these were evaluated.
Table 4.1  ATP content of whole blood samples (500μl filtered) drawn through the CVC from patients receiving TPN at Leeds General Infirmary. Values shown for the samples are the mean RLU values obtained from four sequential readings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLU (Background)</td>
<td>3748</td>
<td>4578</td>
<td>3223</td>
<td>2643</td>
<td>4514</td>
<td>1746</td>
<td>4656</td>
<td>1772</td>
<td>3644</td>
</tr>
<tr>
<td>RLUs (Sample)</td>
<td>4459</td>
<td>5558</td>
<td>3823</td>
<td>2279</td>
<td>7073</td>
<td>2178</td>
<td>2662</td>
<td>2051</td>
<td>3932</td>
</tr>
<tr>
<td>CFUs</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
<td>50</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
</tbody>
</table>
4.3.1 Detection limit of SL and HS reagents

Using serial dilutions of an overnight stock of *S. epidermidis* (NCTC 11047) the detection limit of the ATP bioluminescence assay using the Mediators L1 luminometer was determined in combination with two types of luciferin-luciferase reagent (HS and SL) supplied by Biothema AB, and a TCA based extraction method.

Figure 4.7 shows the calibration curves determined using each of the two reagent systems, where 20μl of extract is measured in both. In each case the plotted values are the mean for four replicates of the *S. epidermidis* serial dilution. The CFU values were determined using a mean of triplicate spread plate CFU values (10^4 to 10^7 dilutions).

The CFU values represent the number of cells that are present within the reaction tube, which is approximately 1.5% of the original sample.

Figure 4.7 demonstrates that the HS reagent is the more sensitive of the two reagents with its detection limit in the 10 to 100 CFU region. As for the SL reagent there is poor differentiation at the 10 to 100 CFU level but it does improve between 100 and 1000 CFU. However, the RLU levels report with the SL reagent are much lower than those observed with the HS reagent.

4.3.2 Lysis of the microbial cells and release of ATP - TCA

As stated in Section 2.7, the use of TCA is a standard method to lyse microbial cells, and by which all other microbial lysis agents are compared. Its chaotropic properties at a final concentration of 2.5% w/v are well documented (Lundin 2000).

a) Effect of TCA on *S. epidermidis* (NCTC 11047) and *E. coli*

After exposure of a known concentration of *S. epidermidis* (5.5 x 10^6 cells) to various concentrations of TCA, it was observed that a final TCA concentration of ≥ 0.1% led to complete lysis of all *S. epidermidis* cells. Similar results were observed with *E. coli* cells, where complete lysis of all the cells occurred with TCA (0.25%).
Figure 4.7  A standard curve showing the relationship between the RLUs and S. epidermidis cells (CFU) using the SL and HS reagents (Biothema AB, Sweden). Each point represents the mean ± SD of four experiments.
b) **Effect of TCA concentration on the luciferin-luciferase reaction (SL Reagent)**

The instructions for use supplied with the SL kit by Biothema AB (Appendix 2) suggested that the sample volume added to the Tris-EDTA buffer and SL reagent should not be such that it leads to >50% inhibition of the light emission. In Figure 4.8 it is demonstrated that if the sample contains a final concentration of approximately 0.1% TCA, then there is 50% inhibition of the signal that would normally be observed when no TCA is present (control). In this case if the extracted sample has a final concentration of 2.5% TCA, only 20μl can be removed and measured in a 1ml reaction containing SL reagent buffered in Tris-EDTA.

c) **Effect of TCA on the HS reagent**

Increasing the TCA concentration lowers the RLU signal from the HS and standard reaction. Figure 4.9 shows the gradual decrease in RLU values as the concentration of TCA is increased from 0 and 0.2%. It can be seen that at a final concentration of 0.2% TCA approximately 60% of the control signal is observed.

d) **Effect of various concentrations of Tris buffer on TCA**

In order to suppress the inhibitory effects of the TCA on the luciferase reagent an attempt was made to buffer the TCA. It was observed that TCA (<0.25% w/v) had a pH of 7.8 in 0.5mol/L Tris and that TCA concentrations of <0.1% have a pH of 7.8 in 0.1mol/L Tris. However, at the final working concentration of TCA (2.5%) that are likely to be observed in this study, Tris (0.5 mol/L) was unable to buffer the TCA and the pH remained at 1.7. Moreover, as Figure 4.10 illustrates, increasing the concentration of Tris from 0.1mol/L to 0.4mol/L also attenuates the RLU signal from the HS and standard. Therefore, if TCA is used then the most effective strategy for maintaining an optimum pH of 7.75 for the luciferase to function, is to reduce the TCA concentration through dilution of the sample in Tris-EDTA buffer.
Figure 4.8  Effect of TCA on the signal emitted by the SL reagent and an ATP standard in Tris-EDTA buffer. Each bar represents the mean ± SD of four experiments.
Figure 4.9 Effect of TCA on the signal emitted by the HS reagent and an ATP standard in Tris-EDTA buffer. Each bar represents the mean ± SD of four experiments. Note that six concentrations were originally selected (between 0 and 0.125%(v/v)) as in the SL work above in section 4.3.2b, however an extra three concentrations (0.15, 0.175 and 0.20%(v/v)) were required investigated as <60% had not been achieved at 0.15%(v/v).
Figure 4.10 Effect of Tris concentration in the buffer used in the reaction between the HS reagent and an ATP standard. Each bar represents the mean ± SD of four experiments.
4.3.3 Lysis of the microbial cells and release of ATP - Bactolyse

a) Concentration required for bacterial cell lysis

The use of a commercially available microbial cell lysis agent called “Bactolyse” (Lumitech, UK) was evaluated (Section 3.2.3). Preliminary investigations using serial dilutions of stocks of *E. coli*, *S. epidermidis* and *C. albicans* ($10^{-3}$ to $10^{-6}$) in Bactolyse (concentration of 50% (v/v) of that supplied) demonstrated that Bactolyse does indeed lyse microbial cells as none of the stocks reported any CFUs after subsequent plate culture.

b) Effects of Bactolyse on luciferase

Figure 4.11 demonstrates that using increased volumes of Bactolyse extracted sample, subsequently leading to an increase in the concentration of Bactolyse in the final reaction tube, reduces the signal emitted from the standard - SL reaction. This is a similar effect as observed with TCA in Section 4.3.2, where the microbial lysis agents are inhibiting the luciferase reaction in a concentration-dependent manner. In this case the use of Bactolyse is of no real advantage over TCA. Moreover Bactolyse is a commercially available reagent of unknown formulation and this too makes it unattractive for further evaluation in this study. Moreover the chaotropic properties of the Bactolyse were investigated and it was observed that Bactolyse, at its supplied concentration, had little inhibitory effect on the activity of apyrase (1 to 20 Units/ml) in excess MgCl$_2$ (>0.02 Mol/L).

4.3.4 Lysis of the microbial cells and release of ATP - Triton X-100

Triton X-100 is a detergent that has been evaluated with clinical samples (Lundin *et al.* 1975). Its potential usefulness in this investigation was evaluated using blood cells obtained from freshly drawn blood samples.

a) Concentration required to lyse somatic cells

Using fresh heparinised blood (1:100) the optimum concentration of Triton X-100 required for lysis of non-microbial cells was determined. It was observed that a final concentration of between 0.1 and 0.2% Triton X-100 was required for the highest level of
Figure 4.11  Effects of various concentrations of Bactolyse-extracted sample on the signal produced by the SL reagent (9.5% v/v) and an ATP standard. The control does not contain any Bactolyse-extracted sample (just Tris-EDTA, SL and ATP standard). Each bar represents the mean ± SD of four experiments.
cell lysis. This is illustrated in Figure 4.12 where the concentrations of ATP remaining after lysis of blood cells with various concentrations of Triton X-100 (0.02 to 0.8% v/v) are shown. Post-lysis, apyrase (10U/ml) was added to degrade the ATP and TCA (2.5% final concentration) added to inhibit the apyrase and lyse any remaining cells. The graph shows that low concentrations of Triton X-100 (<0.1% v/v) do not result in high levels of somatic cell lysis, therefore a higher concentration is required. However, concentrations of Triton X-100 (>0.2% v/v) also give poor cell lysis. As a result a concentration of 0.1% Triton X-100 was adopted for use in this study.

b) Effect of Triton X-100 on microbial cells

Triton X-100 (0 to 2%) was found to have no adverse effects on stock serial dilutions (10^-4 to 10^-6) of S. aureus and E. coli (in triplicate) after the organisms were exposed (30 minutes) to the Triton X-100 prior to overnight culture. The data is not shown here but there was basically no difference in CFU counts between organisms exposed to Triton X-100 and controls in PBW.

4.3.5 Degradation of released ATP - apyrase

ATP released from non-microbial cells can be degraded using enzymatic or physical means. The use of ATP degrading enzymes, such as apyrase, is popular (Sakakibara et al. 1997) and investigations using such enzymes are detailed below.

a) Grade Selection

Preliminary experiments involved apyrase grade I (A-6132, Sigma, UK), which is partially purified with a low adenosine 5'-triphosphatase (ATPase) activity and variable adenosine 5'-diphosphatase (ADPase) activity. This particular grade was found to be of little use as very high concentrations (~100 U/ml) were required for effective ATP degradation. Experiments using apyrase grade I (<20 U/ml final concentration) were found to be ineffective at reducing the relatively high concentrations of ATP released from fresh heparinised blood cells (1:50).

Following this initial work a grade III apyrase (A-7646, Sigma, UK) was chosen for use in this study. It is partially purified with ten times higher ATPase activity than the grade I apyrase and it too has variable ADPase activity.
Figure 4.12 The concentration of ATP remaining in blood cells (diluted 1:100) after lysis with various concentrations of Triton X-100 and subsequent treatment with apyrase and TCA. Each point represents the mean ± SD of four experiments.
b) **Preparation of apyrase stock**

It became necessary to filter the reconstituted apyrase prior to storage and/or use, as the level of contaminating ATP in the reconstituted solution was high (>50 pmol/L). The method that was elucidated to prepare ATP free apyrase solutions (100U/ml) is detailed in Section 3.2.2. This method involves the filtration and washing of a highly concentrated solution of apyrase through a 0.22μm, 25mm syringe filter.

c) **Effective apyrase (grade III) and magnesium ion concentration**

In order for apyrase to function properly, the presence of a divalent metal ion, such as magnesium, is required. Preliminary experiments to determine a suitable apyrase concentration for the degradation of ATP were carried out in excess magnesium chloride (0.05 mol/L) using sterile distilled water. The optimum working concentration for apyrase was found to be in the range of 5 to 20 U/ml. Figure 4.13 shows that after a 10 minute incubation in excess magnesium ions, apyrase (10U/ml) will degrade a high level of ATP to leave <600 pmol/L of ATP remaining.

Further investigations using apyrase (10U/ml) in the presence of various concentrations of magnesium chloride (0 to 0.2 mol/L) are shown in Figure 4.14. Here it can be seen that there is little difference in the activity of the apyrase (10U/ml) in the presence of ≥0.02 mol/L magnesium chloride. This led to the selection of a magnesium ion concentration of 0.02 mol/L for use in all future experiments involving apyrase (10U/ml).

Additionally, it was demonstrated that magnesium chloride (0.02 mol/L), in the presence of apyrase (10U/ml), had no adverse affects on the ATP content of *S. epidermidis* and *E. coli* or the function of 0.1% Triton X-100.

d) **Apyrase in combination with magnesium chloride and Triton X-100**

The effect of a combination of apyrase (10U/ml), magnesium chloride (0.02 mol/L) and Triton X-100 (0.1% v/v) on a standard level of pure ATP as well as ATP released from blood cells was investigated with the results shown in Table 4.2. It was demonstrated that incubation of fresh heparinised blood (1:50) in a solution containing apyrase (10U/ml), magnesium chloride (0.02 mol/L) and Triton X-100 (0.1% v/v) gave similar levels of
Figure 4.13  Effect of various concentrations of apyrase (grade III) on the degradation of a known concentration of ATP in excess magnesium chloride. Each bar represents the mean ± SD of four experiments.
Figure 4.14 Effect of various concentrations of magnesium chloride on the activity of apyrase (10U/ml). The control contains apyrase (grade III) only. Each bar represents the mean ± SD of four experiments.
Table 4.2  Effect of a combination of apyrase (10U/ml), magnesium chloride (0.02 M Ol/L) and Triton X-100 (0.1% v/v) on ATP concentrations (n/Mol/L) from ATP standards and from ATP released from blood cells. Results shown are the mean of four (±SD) four separate experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequential addition * nMol/L (SD)</th>
<th>Simultaneous incubation nMol/L (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP standard (50nmol/L)</td>
<td>0.54 (0.13)</td>
<td>0.59 (0.19)</td>
</tr>
<tr>
<td>Heparinised blood (1:50)</td>
<td>9.61 (1.57)</td>
<td>9.28 (0.99)</td>
</tr>
<tr>
<td>Platelet rich plasma (1:50)</td>
<td>5.1 (0.68)</td>
<td>5.6 (1.17)</td>
</tr>
</tbody>
</table>

* Sample exposed to Triton X-100 for 10 minutes before apyrase and Mg²⁺ added.
degradation as incubation of fresh heparinised blood (1:50) in a solution containing Triton X-100, followed by the sequential addition of apyrase. It was also observed that this combination of chemicals had no adverse effect on serial dilutions of *E. coli* and *S. epidermidis*.

### 4.4 The L1 system versus the Bioprobe system

When using the Bioprobe system to lyse blood cells and degrade the released ATP, it was observed that the Isostat isolator reagent in combination with the ATPase was partially effective. However, a relatively high level of contaminating non-microbial ATP still remained within each sample. The addition of filtration to the method was evaluated, but in Section 4.2.4(f) it was observed that the results obtained using the Bioprobe system were of no clinical benefit and therefore the method based on this instrument and reagents was of no clinical use. In order to detect microbial ATP, all non-microbial ATP needs to be removed from the sample. This sample preparation is crucial, with ATP from white and red blood cells as well as platelets needing to be extracted and removed. If this is not achieved, contaminating ATP would form a significant proportion of the sample and therefore the ATP released from the microbial cells would be lost in this contaminating background ATP.

The major reason for the poor performance of the Bioprobe and associated reagents is that they constitute an “off-the-shelf” system and it was difficult to vary the concentrations of component reagents and, therefore, effectively vary parameters during the development phase. In addition, the fact that some of the component chemicals that make up reagents are unknown further complicates the situation. Figure 2.7 in Section 2.7 illustrated the three main obstacles that needed to be overcome in order to develop a method based upon ATP Bioluminescence. The first two steps cannot adequately be dealt with without knowing the constituent components of the reagents used to lyse cells and degrade ATP.

By using the L1 luminometer in combination with Biothema luciferase reagents and other chemicals it was possible to vary parameters much more freely. Background RLU levels could be reduced using a combination of chemicals as shown in Table 4.2.

Thus the L1 luminometer, Biothema reagents and various chemicals were selected for use in further development. The main reasons for this selection are summarised below:
1. The concentrations of components such as apyrase, Triton X-100 and TCA can be easily varied, so that optimisation of a method is more straightforward.

2. The luciferase reagents supplied by Biothema worked well with the L1 luminometer. In addition the Biothema reagents include an ATP standard which means that ATP concentrations can be determined from the detected RLU values are designed.

3. The L1 luminometer is more sensitive than the Bioprobe in that low levels of ATP can be detected.

Further investigation and optimisation of the technique based upon the Mediators L1 is required so that a sample containing intact microbial cells with no non-microbial ATP could be obtained. This further development is described in Chapter 5, where the Mediators L1 luminometer and luciferin-luciferase based kits supplied by Biothema AB (Sweden) are exclusively used. Chapter 5 also details the development of a reagent and method, based on a number of enzymes and physical techniques, which could be used to remove non-microbial ATP and release microbial ATP for detection and quantification.
CHAPTER FIVE
5.1 Background

In Chapter 4 it was reported that the use of an off-the-shelf luminometer (the Bioprobe) and reagent system was of no clinical benefit. However, the Mediators L1 luminometer used in conjunction with laboratory-formulated reagents did provide a system that could potentially degrade substantial levels of background ATP prior to extraction and detection of microbial ATP. Further development using the Mediators L1 luminometer and luciferin-luciferase based kits supplied by Biothema, was carried out and is described in this chapter. This includes the development of a reagent and method that could be used to remove non-microbial ATP and release microbial ATP for detection and quantification.

5.2 Optimisation of the L1-based method

5.2.1 Improving the level of degradation

In Section 4.3 it was determined that apyrase alone was ineffective at removing high levels of contaminating ATP. The possibility of using alternative enzymes or a combination of enzymes to improve the level of ATP degradation was investigated. Two enzymes were identified as potentially beneficial: adenosine phosphate deaminase and 5'-adenylic acid deaminase. The combination of adenosine phosphate deaminase and apyrase has previously been investigated by Sakakibara et al. (1997) where contaminating ATP concentration was successfully degraded to ~1pMol/L. This level was set as a target in the present study, but unfortunately a source for adenosine phosphate deaminase could not be found in the UK and shipping costs from Japan were extremely high. Therefore, the usefulness of this enzyme was not investigated. However, 5'-adenylic acid deaminase (A1907, Sigma, UK) was obtained. This enzyme breaks down 5'-AMP to 5'-IMP (Inosine monophosphate) through a deamination reaction. Its usefulness in combination with apyrase in magnesium ions (0.02mol/L) was evaluated.

a) Apyrase in combination with 5'-adenylic acid deaminase

The effect of 5’ adenylic acid deaminase (concentrations of 0.1U/ml to 10U/ml) in conjunction with apyrase (10U/ml), magnesium chloride (0.02 mol/L) and Triton X-100 (0.1% v/v) on the ATP concentration of platelet-rich plasma (1:50) is shown in Table 5.1.
Table 5.1  Concentration of ATP remaining after platelet-rich plasma (1:50) is exposed to various concentrations of 5’ adenylic acid deaminase in conjunction with apyrase (10U/ml), magnesium chloride (0.02 Mol/L) and Triton X-100 (0.1% v/v). Values shown are the mean (±SD) of four experiments.

<table>
<thead>
<tr>
<th>5’ adenylic acid deaminase concentration (U/ml)</th>
<th>Mean (±SD) ATP concentration (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control *</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>0</td>
<td>4632 (166.1)</td>
</tr>
<tr>
<td>0.1</td>
<td>1547 (42.3)</td>
</tr>
<tr>
<td>0.2</td>
<td>964 (29.4)</td>
</tr>
<tr>
<td>0.5</td>
<td>582 (21.8)</td>
</tr>
<tr>
<td>1</td>
<td>596 (19.7)</td>
</tr>
<tr>
<td>5</td>
<td>573 (18.5)</td>
</tr>
<tr>
<td>10</td>
<td>618 (22.9)</td>
</tr>
</tbody>
</table>

* The control was the ATP concentration of the platelet-rich plasma in Triton X-100 (0.1%) only.

Table 5.2  Degradation of various concentrations of ATP by the ATP degradation reagent. Values shown are the mean of four experiments.

<table>
<thead>
<tr>
<th>ATP concentration (nMol/L)</th>
<th>ATP concentration (nMol/L) after exposure to the ATP degradation reagent</th>
<th>% ATP remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>69.48</td>
<td>0.050</td>
<td>0.072</td>
</tr>
<tr>
<td>109.10</td>
<td>0.053</td>
<td>0.048</td>
</tr>
<tr>
<td>195.42</td>
<td>0.053</td>
<td>0.027</td>
</tr>
<tr>
<td>258.24</td>
<td>0.062</td>
<td>0.024</td>
</tr>
<tr>
<td>350.86</td>
<td>0.082</td>
<td>0.023</td>
</tr>
<tr>
<td>558.87</td>
<td>0.057</td>
<td>0.010</td>
</tr>
</tbody>
</table>
In Figure 5.1 it can be seen that a high level of degradation occurs with the addition of ≥ 0.5U/ml 5' adenylic acid deaminase.

Figure 5.1 demonstrates that the combination of 5' adenylic acid deaminase (0.5U/ml and apyrase (10U/ml) produces a higher level of degradation from a pure ATP sample than the use of either of these enzymes individually.

**b) Effect of magnesium concentration**

Further experiments were conducted using heparinised blood (1:25) to determine the optimum magnesium ion concentration required when both 5' adenylic acid deaminase and apyrase are present together. It was reported that there was little difference between results with 0.02mol/L (38,453 RLUs) and 0.2mol/L (37,237 RLUs). Therefore, the optimum magnesium ion concentration, when used with heparinised blood (<1:25), remains 0.02mol/L magnesium chloride.

c) **Effect of 5' adenylic acid deaminase on microorganisms**

It was also observed that *S. epidermidis* cells were not affected by 5' adenylic acid deaminase (up to 5U/ml), when exposed for 30 minutes prior to overnight culture.

### 5.2.2 Effectiveness of the ATP-degrading reagent

In Section 5.2.1 the combination of 5' adenylic acid deaminase (0.5U/ml), apyrase (10U/ml), magnesium chloride (0.02 mol/L) and Triton X-100 (0.1% v/v) degraded high levels of ATP. Further experiments were carried out using these reagents in combination and for simplicity this combination of enzymes and chemicals was termed “ATP degradation reagent”.

Table 5.2 shows that when various concentrations of ATP standard are exposed to the ATP degradation reagent, >99% of the ATP is removed. Samples reporting between 69.48 to 558.87 nmol/L were reduced to <0.1 nmol/L. Further experiments were carried out and in Table 5.3 platelet-rich plasma which had an ATP concentration of 60.32 nmol/L, was degraded to 0.058 nmol/L after exposure to the ATP degradation reagent. Similar levels of degradation were evident with heparinised blood (393.56 nmol/L to 0.584 nmol/L) although the concentration of ATP was initially higher - due to the more
Figure 5.1  Effect of various enzyme combinations within a solution containing magnesium chloride (0.02 mol/L) and Triton X-100 (0.1% v/v) on the degradation of a known concentration of ATP. Each bar represents the mean (±SD) of four experiments.
Table 5.3  Effect of the ATP degradation reagent on clotted and heparinised blood. Data is displayed as % remaining of the control which has not been exposed to the degrading enzymes. Values shown are mean of four experiments.

<table>
<thead>
<tr>
<th></th>
<th>Platelet-rich plasma</th>
<th>Heparinised blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1:50</td>
</tr>
<tr>
<td>RLU(^{s})</td>
<td>2758253</td>
<td>3047</td>
</tr>
<tr>
<td>nmol/L</td>
<td>60.32</td>
<td>0.058</td>
</tr>
<tr>
<td>% ATP remaining</td>
<td>100</td>
<td>0.110</td>
</tr>
</tbody>
</table>
homogeneous distribution of white and red blood cells, in addition to platelets. From this it was evident that in samples with a high number of blood cells there will be a problem degrading contaminating ATP.

The ATP degradation reagent was shown to have no adverse affects on the ATP content of serial dilutions of *S. epidermidis*, *C. albicans* and *E. coli* when exposed for 30 minutes prior to overnight culture.

### 5.2.3 Further improvements to the ATP degradation reagent

In Sections 5.2.2 it was demonstrated that the ATP degradation reagent improved upon the release and degradation of ATP associated with non-microbial cells without adversely affecting the microbial cells. The formulation of this working reagent is shown in Table 3.3 in Chapter 3. However, despite this level of degradation, somatic ATP concentrations could still be higher than the concentrations of ATP, which would be present in some CVC samples. Therefore, other enzymes and techniques were investigated in order to achieve the highest level of ATP degradation that is possible.

**a) Hexokinase**

Exposure of fresh heparinised blood (dilutions from 1:16.7 to 1:50) to the ATP degradation reagent in the presence of hexokinase (1 to 10U/ml) did not lead to an increase in the level of ATP degradation already observed.

**b) Incubation periods**

An attempt was made to improve the level of degradation by increasing the time that the sample was exposed to the ATP degradation reagent at room temperature. Figure 5.2 illustrates the effects of various incubation times and it was observed that a minimum of 10 minutes was required to achieve a high level of degradation. However, as optimal degradation of ATP occurred between a 30 to 60 minute incubation period, a 60-minute incubation period at room temperature was chosen as the most appropriate for sample exposure to the ATP degradation reagent. Further experiments using this incubation period but conducted at 30°C and 37°C did not improve upon the level of ATP degradation.
Figure 5.2  Determination of the optimum incubation time required for the ATP degradation reagent to degrade ATP from an ATP standard. Each point represents the mean ± SD of four experiments.
5.2.4 Inhibition of the ATP-degrading enzymes

In Section 4.3 the concentration of TCA required to lyse microorganisms was determined to be 0.25%. Further experiments were conducted to determine if this concentration of TCA would be adequate to simultaneously inhibit the ATP-degrading enzymes that are released from the microbial cells and those that are present due to the ATP degradation reagent.

a) Effect of TCA on ATP-degrading enzymes

In Table 5.4 it can be seen that as the concentration of TCA exposed to a particular ATP-degrading enzyme is reduced, there is a reduction in the signal given out by the SL reaction with the sample. TCA concentration of >0.8% is required to inhibit both apyrase (10U/ml) in excess MgCl₂ and 5'-adenylic acid deaminase (0.5U/ml), individually and in combination with each other. At levels of <0.8% TCA the apyrase and 5'-adenylic acid deaminase were not inhibited and these enzymes were subsequently capable of degrading ATP.

Therefore the concentration of TCA required for inhibition of the ATP-degrading enzymes (1%) is much higher than that required for microbial cell lysis (0.25%). This creates an issue in that the high concentration of TCA would also affect the luciferase enzyme. Alternative strategies to inhibit the enzymes were investigated so that this high final TCA concentration would not be necessary and the adverse effects on the luciferase would be avoided.

b) Effect of heat on apyrase and 5'-adenylic acid deaminase

Individually and as part of the ATP degradation reagent, it was demonstrated that apyrase (10 U/ml) and 5'-adenylic acid deaminase (0.5U/ml) are still active at temperatures <60°C, but inactivated at 70°C. Experiments were carried out by adding the sample to sterile distilled water at the selected temperature (for example 70°C). Unfortunately, due to evaporation, a volume of at least 5ml of water was required so that adequate inhibition could take place. This dilution effect of boiling buffers was found to be too great and it created issues with the sensitivity of the assay, therefore boiling buffers were not explored further.
Table 5.4 Effect of TCA concentration on the activity of various combinations of ATP-degrading enzymes. Values shown are the mean of four experiments.

<table>
<thead>
<tr>
<th>TCA Concentration (%v/v)</th>
<th>Enzyme combination</th>
<th>RLU value</th>
<th>% of control</th>
<th>Enzyme combination</th>
<th>RLU value</th>
<th>% of control</th>
<th>Enzyme combination</th>
<th>RLU value</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control *</td>
<td>Apyrase &amp; MgCl₂</td>
<td>7710818</td>
<td>100</td>
<td>5’-adenylic acid deaminase</td>
<td>6781980</td>
<td>100</td>
<td>Apyrase, MgCl₂ &amp; 5’-adenylic acid deaminase</td>
<td>6419635</td>
<td>100</td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td>8111859</td>
<td>105.20</td>
<td></td>
<td>6767732</td>
<td>99.79</td>
<td></td>
<td>6588994</td>
<td>102.64</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>7356782</td>
<td>95.41</td>
<td></td>
<td>6404104</td>
<td>94.43</td>
<td></td>
<td>6397716</td>
<td>99.66</td>
</tr>
<tr>
<td>0.8</td>
<td></td>
<td>7149703</td>
<td>92.72</td>
<td></td>
<td>6071305</td>
<td>89.52</td>
<td></td>
<td>5362462</td>
<td>83.84</td>
</tr>
<tr>
<td>0.6</td>
<td></td>
<td>4553546</td>
<td>59.05</td>
<td></td>
<td>3926868</td>
<td>57.90</td>
<td></td>
<td>263309</td>
<td>4.10</td>
</tr>
<tr>
<td>0.4</td>
<td></td>
<td>1579</td>
<td>0.0205</td>
<td></td>
<td>4978</td>
<td>0.0734</td>
<td></td>
<td>517</td>
<td>0.0081</td>
</tr>
<tr>
<td>0.2</td>
<td></td>
<td>1481</td>
<td>0.0192</td>
<td></td>
<td>4710</td>
<td>0.0694</td>
<td></td>
<td>439</td>
<td>0.0068</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>1415</td>
<td>0.0184</td>
<td></td>
<td>4410</td>
<td>0.0850</td>
<td></td>
<td>450</td>
<td>0.0070</td>
</tr>
</tbody>
</table>

* It is assumed that the control is 100% (that is the maximum signal given out by the reaction between the luciferin-luciferase and the standard). The controls contain 4% TCA which is the final concentration of TCA in each of the other samples (stopped reaction).
c) Effect of Suramin and Thimerosal on apyrase

The possible inhibitory effect of Suramin \([8-(3\text{-benzoamido}-4\text{-methylbenzamido})\,\text{naphalene-1,3,5,-trisulfonic acid}]\) was investigated and it was observed that concentrations of 0.02mg/ml Suramin had no effect on the activity of apyrase (10U/ml) in excess MgCl\(_2\). In similar experiments concentrations of up to \(10^{-4}\) mol/L Thimerosal had little effect on apyrase (10U/ml) in excess MgCl\(_2\).

Additionally, it was observed that the SL reagent reaction with an ATP standard (100nmol/L) was affected by Suramin concentrations of >0.00015mg/ml.

No alternative to TCA was found therefore it was used as an inhibitor of the ATP-degrading enzymes as well as a microbial cell lysis agent.

Although it was observed that TCA (1.0%) was adequate for degrading enzymes and microbial cell lysis it was decided that it was still necessary to use TCA (2.5%) as recommended by Lundin (2000). This is because the effectiveness of TCA (1.0%) has not been investigated with all microbial species that may be encountered.

5.3 Filtration

In Section 5.2 it was reported that high levels of ATP could be released and degraded from non-microbial cells using the developed ATP degradation reagent. It was also observed that this reagent does not affect \(S.\,epidermidis, C.\,albicans\) and \(E.\,coli\) and it is inhibited by TCA. However, there is still a significant level of contaminating ATP (~0.2%) remaining and this has potentially detrimental effects on the sensitivity of the assay. In order to improve on the removal of this contaminating ATP, the potential benefits of combining ATP-degrading enzymes with syringe filtration was evaluated.

5.3.1 Syringe filter selection

Syringe filters used in combination with the ATP degradation reagent provide a possible solution to the ineffectiveness of the degradation process observed thus far. A number of different syringe filters were evaluated and results are shown in Table 5.5. Three 0.45μm pore size syringe filters were evaluated and the Sartorius syringe filter was found to be contaminated with ATP (>5pmol/L). Both the Millipore and the Gelman filters, however were basically ATP free (<1pmol/L). The two 0.2μm filters were also ATP free but the
Table 5.5  
Syringe filters (25mm diameter) that were evaluated and the parameters investigated. Values are the mean ± SD of five replicates.

<table>
<thead>
<tr>
<th>Manufacturer and pore size</th>
<th>Hold-up volume</th>
<th>Contaminating ATP content</th>
<th>Pressure build up</th>
<th>Retention of organisms ***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before air purge *</td>
<td>After air purge **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelman (0.45μm)</td>
<td>430μl ± 45μl</td>
<td>210μl ± 20μl</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
<tr>
<td>Millipore (0.2μm)</td>
<td>400μl ± 30μl</td>
<td>220μl ± 10μl</td>
<td>Negligible</td>
<td>Yes</td>
</tr>
<tr>
<td>Millipore (0.45μm)</td>
<td>400μl ± 40μl</td>
<td>220μl ± 10μl</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
<tr>
<td>Sartorius (0.45μm)</td>
<td>600μl ± 60μl</td>
<td>210μl ± 20μl</td>
<td>Contaminated</td>
<td>Negligible</td>
</tr>
<tr>
<td>Syrtec (0.2μm)</td>
<td>420μl ± 50μl</td>
<td>260μl ± 20μl</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

*  Total volume of water retained by a syringe filter  
** Volume of water retained by the membrane after air was flushed through  
*** Serial dilutions of S. epidermidis, E. coli and C. albicans filtered, with the filtrate cultured giving no growth in all cases
Millipore (0.2µm) filter was difficult to use as pressure would build up when fluid was passed through it. There were no such problems with the Syrtec (0.2µm) 25mm syringe filter and consequently it was chosen for further evaluation.

Serial dilutions (to 10^{-6}) of stocks of all E. coli, S. epidermidis, S. aureus and C. albicans were passed through each of the two syringe filters and it was determined after subsequent culture of the filtrate that no microorganisms passed through either the 0.2µm or 0.45µm filters.

The following Sections detail the assay development using a combination of the ATP degradation reagent with 25mm diameter, 0.2µm pore size Syrtec syringe filters.

In order to improve upon the levels of degradation already observed in Section 5.2, various physical parameters were evaluated. These included the determination of the length of incubation period required at the point the sample is introduced to the ATP degradation reagent and the point where TCA is added to the syringe filter.

5.3.2 TCA optimisation

Three specific areas of optimisation were identified with regard to the use of TCA to extract ATP from microorganisms that are trapped in a syringe filter. These are described below, in three separate Sections, although much of the work overlapped.

a) Incubation period

Studies carried out to determine how fast TCA lyses microbial cells and inactivates ATP-degrading enzymes demonstrated that at a final concentration of TCA (2.5%) lysis and degradation are complete within one minute. Further experiments carried out using 25mm diameter, 0.2µm pore size syringe filters indicated that less than two minutes was required for the TCA (final concentration >2.5%) to penetrate all areas of the filter membrane and lyse all the microbial cells.

b) Volume of TCA to be added

TCA needs to be added at a higher concentration so that a final concentration of 2.5% can be achieved. With syringe filters it is slightly more complicated in that the true volume of liquid in the syringe post-washing is difficult to determine. Therefore in order to achieve a final concentration of TCA (2.5%) within the syringe filter unit a number of
factors needed to be taken into consideration. The assumption is that the hold up volume within the Syrtec syringe filter is 420µl and the post-air-purge hold up volume is 260µl (see Table 5.5 for further details). As a final concentration of 2.5% is required it is necessary to add a higher concentration of TCA in order to compensate for the liquid already retained in the filter. It is assumed therefore that there is 420µl of liquid in the filter and the addition of 140µl of TCA (10%) will give a final TCA concentration of 2.5%. However, as can be seen in Table 5.6 this was not the case. The optimum volume of TCA (10%) which was necessary in this group of experiments was between 250 to 350µl, which in a “closed system” should give a final concentration of >3.5% TCA. However, this is not closed and liquid is being lost through the bottom of the filter housing as liquid is introduced at the top. It is possible therefore that much of the 10% TCA flows straight through the filter and does not mix quickly enough with the residual liquid in the filter. As a result of this work it was decided that a minimum of 300µl of 10% TCA should be added to the filter in future experiments.

c) Retrieving ATP after TCA extraction

After the appropriate concentration of TCA has been added to a syringe filter, the released ATP needs to be collected for assay. The simplest way to perform this is to flush the TCA-extracted ATP from the filter. Experiments were carried out to determine how much sterile water or Tris-EDTA was required to extract the highest ratio of ATP. As stated above there is already 420µl of TCA-extracted ATP in the Syrtec filter. If this was air-purged then approximately 160µl (<40% of sample) could be collected as the remaining TCA-extracted sample would remain within the syringe filter, absorbed in the membrane as part of the 260µl “post-air-purge hold up volume”. In order to get a higher recovery it was necessary to flush a high volume of liquid through the filter. A volume of 2.5ml was selected because it resulted in a final TCA concentration of just over 1% in the final extract. The use of 100µl of this sample in the bioluminescence reaction (SL) meant that there was a final TCA concentration of 0.107% in the bioluminescence reaction involving the SL reagent in Tris-EDTA buffer. This is based upon the observations in Section 4.3.2, where 0.1% TCA gives 50% inhibition of the signal that is normally observed when no TCA is present.
Table 5.6 Volume of TCA (10%) that needs to be added to a 25mm diameter syringe filter in order to extract the highest concentration of ATP from a *S. epidermidis* stock solution. Values shown are the mean (±SD) of four experiments.

<table>
<thead>
<tr>
<th>TCA (10%) volumes added (µl)</th>
<th>Assumed final TCA concentration in syringe filter (% v/v) *</th>
<th>Mean (±SD) ATP concentration (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.92</td>
<td>756.32 (71.2)</td>
</tr>
<tr>
<td>120</td>
<td>2.22</td>
<td>825.14 (65.4)</td>
</tr>
<tr>
<td>150</td>
<td>2.63</td>
<td>840.25 (62.9)</td>
</tr>
<tr>
<td>200</td>
<td>3.23</td>
<td>923.63 (81.4)</td>
</tr>
<tr>
<td>250</td>
<td>3.73</td>
<td>1126.47 (124.6)</td>
</tr>
<tr>
<td>300</td>
<td>4.17</td>
<td>1234.87 (131.8)</td>
</tr>
<tr>
<td>350</td>
<td>4.55</td>
<td>1248.12 (130.7)</td>
</tr>
<tr>
<td>400</td>
<td>4.88</td>
<td>1054.16 (129.7)</td>
</tr>
</tbody>
</table>

* Assumes a closed system and is based on the volume added plus the hold up volume of the filter (420µl)
5.3.3 ATP degradation reagent optimisation

a) Incubation of sample with the ATP degradation reagent
In Section 5.2.3(b) experiments were conducted to determine the effect of having various incubation times for the reaction between the sample and the ATP degradation reagent. In Figure 5.3 this data is displayed again but also on this graph is the effect of filtration after the incubation. Similar trends were observed between the filtered and unfiltered samples, and as expected, lower concentrations of contaminating ATP remained in the filtered samples. Using the data in Figure 5.3 it was determined that optimal degradation of ATP using this combination of reagents occurred at 60 minutes.

b) Volume of sample to be filtered
The sample is added to 1ml of the ATP degradation reagent. For the purposes of sensitivity it is necessary to assay as much of this sample as possible. However, in addition to the bioluminescence assay, some of the sample needs to be cultured. It was therefore decided that 800μl of the sample should be filtered and finally be assayed whilst 100μl of the sample be used in plate culture.

c) Filter flush volume after sample addition
It was important to flush the syringe filter after addition of the solution containing sample and ATP-degrading enzymes, to remove cellular debris that could clog the filter or lead to quenching of the bioluminescence signal and to remove any remaining ATP-degrading enzymes that might be non-specifically bound to the filter.

Studies were carried out to determine how useful it would be to wash with a solution (2.5ml) containing one of each of the following: Triton X-100 (0.02%), a 1:10 dilution of the ATP degradation reagent, Tris-EDTA or sterile water. It was observed that there was no difference between each, with the result that sterile water was selected for use in the step.

In further work it was demonstrated that exposing heparinised blood (1:50) to the ATP degradation reagent, followed by filtration through a 25mm diameter (0.2μm) syringe filter and then flushing with various volumes of sterile water, led to the detection of
Figure 5.3 Determination of the optimum incubation period required for the ATP degradation reagent to degrade ATP from an ATP standard (1 μmol/L). Each point represents the mean ± SD of four experiments, with the pink points representing samples that have been filtered after ATP degradation and the blue points have not been filtered.
various concentrations of ATP after TCA extraction. Figure 5.4 illustrates that a flush volume of greater than 5ml was required in situations where heparinised blood (1:50) was used. Further experiments suggested that when a higher concentration of blood (1:10) was used a flush volume of at least 7.5ml was required. Therefore, 7.5ml flush volumes were adopted for use in conjunction with 25mm diameter (0.2μm) syringe filters.

5.3.4 Evaluation of method

Even with the addition of a filtration step contaminating ATP still remained. The possible usefulness of hexokinase (<10U/ml) was investigated again and as in Section 5.2.3(a), the addition of hexokinase (2.25U/ml) to the ATP degradation reagent in combination with filtration had no additional effect on the level of ATP degradation (Table 5.7).

Further experiments with hexokinase (2 to 20U/ml) did not enhance the degradation of ATP from platelet-rich plasma (1:100 dilution), when used in combination with the ATP degradation reagent and filtration.

In Table 5.8, the combination of apyrase and 5’ adenylic acid deaminase with filtration achieved the highest level of degradation (5pmol/L). Levels of >500pmol/L remained after apyrase (10U/ml) and Triton X-100 in the presence of magnesium ions had been used to degrade the ATP. The use of filtration improves the technique, with levels of <20pMol/L observed. When 5’adenylic acid deaminase is added to the apyrase (10U/ml) and Triton X-100 the level of ATP degradation improves in both filtered and unfiltered samples. The use of 5’adenylic acid deaminase in the absence of apyrase was not as effective as was the use of hexokinase on its own.

5.3.5 Volume of sample measured in the luciferase reaction

a) Effect of TCA extractant volume on the SL reagent

When 300μl of TCA (10%) is added to the filter and subsequently flushed with Tris-EDTA buffer (2.5ml) to give a final volume of 2.8ml, the final TCA concentration is 1.07%. Based on the observations in Section 4.3.2, a final concentration of approximately 0.1% TCA gives 50% inhibition of the signal that would normally be observed when no
Figure 5.4  Effect on the concentration of ATP remaining on syringe filters (25mm, 0.2μm) after flushing with various volumes of sterile water prior to extraction with TCA. Each bar represents the mean ± SD of four experiments.
Table 5.7  Degradation of ATP (6 μmol/L) using enzyme combinations, with or without filtration. Each value represents the mean ± SD of four replicates.

<table>
<thead>
<tr>
<th>Final concentrations (1ml reaction)</th>
<th>[ATP] (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No filters</td>
</tr>
<tr>
<td>Triton X-100 (0.1%) and MgCl₂ (0.02M); Apyrase (10U/ml)</td>
<td>563.2 ± 16.2</td>
</tr>
<tr>
<td>Triton X-100 (0.1%) and MgCl₂ (0.02M); Apyrase (10U/ml); 5' adenylic acid deaminase (0.5U/ml)</td>
<td>244.6 ± 17.5</td>
</tr>
<tr>
<td>Triton X-100 (0.1%) and MgCl₂ (0.02M); Apyrase (10U/ml); 5' adenylic acid deaminase (0.5U/ml); Hexokinase (2.25U/ml)</td>
<td>299.4 ± 7.0</td>
</tr>
<tr>
<td>Triton X-100 (0.1%) and MgCl₂ (0.02M); 5' adenylic acid deaminase (0.5U/ml)</td>
<td>1295 ± 15.9</td>
</tr>
<tr>
<td>Triton X-100 (0.1%) and MgCl₂ (0.02M); 5' adenylic acid deaminase (0.5U/ml); Hexokinase (2.25U/ml)</td>
<td>1870 ± 12.8</td>
</tr>
<tr>
<td>Triton X-100 (0.1%) and MgCl₂ (0.02M); Apyrase (10U/ml); Hexokinase (2.25U/ml)</td>
<td>616 ± 14.8</td>
</tr>
<tr>
<td>Triton X-100 (0.1%) and MgCl₂ (0.02M); Hexokinase (2.25U/ml)</td>
<td>&gt;100,000</td>
</tr>
</tbody>
</table>
TCA is present. Therefore, 100μl of the TCA (1.07%) extract was used as the sample in the bioluminescence reaction involving the SL reagent in Tris-EDTA buffer (total volume 1ml), as this gives a final TCA concentration of 0.107%.

b) Effect of TCA extractant volume on the HS reagent

Using the HS reagent the final volume of the bioluminescence reaction contains 400μl of HS reagent plus the sample volume. In Section 4.3.2 it was observed that a final TCA concentration of 0.2% reduced the signal to ~60% of normal. As there are 400μl of HS this equates to the addition of <100μl of the TCA (1.07%) extract.

Using a laboratory-prepared extract it was observed that volumes of sample of <20μl reported lower than expected RLU values. Predictably, there are problems with the addition of >100μl of sample, as this leads to a concentration-dependent inhibition of the luciferase observed through the RLUs produced by the luciferase-ATP standard reaction. Values shown in Table 5.8 demonstrate that a final TCA concentration of 0.051% to 0.357% gave a reduction in signal of 40% of the control. Based on these results, it was decided that a sample volume of 75μl would be the most appropriate for addition to the 400μl of HS reagent. However, 75μl is still only 2.68% (75/2800 x 100) of the original extracted volume, which in turn is 2.14% of the original sample ((75/2800) x (800/1000) x 100).

5.4 Analysis of CVC tip samples

The method developed and described in the Sections 5.1 through to 5.3 was challenged with FASEB samples retrieved from CVC tips that were removed from catheterised hospital patients. The methodology used has been described in detail in Section 3.6.6 of Chapter Three.
Table 5.8 The effect of various volumes of TCA-extracted sample (therefore TCA concentration) on the signal emitted by the HS reagent (400µl). Values shown are the mean of four experiments.

<table>
<thead>
<tr>
<th>Volume of sample added (µl)</th>
<th>% of extract in final reaction</th>
<th>[TCA] in reaction tube (% w/v) *</th>
<th>Sample RLU values</th>
<th>RLU value after ATP standard added</th>
<th>[ATP] in Sample (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.0</td>
<td>4.8</td>
<td>0.051</td>
<td>3131</td>
<td>117505</td>
<td>379.6</td>
</tr>
<tr>
<td>40.0</td>
<td>9.1</td>
<td>0.097</td>
<td>6388</td>
<td>110355</td>
<td>428.3</td>
</tr>
<tr>
<td>50.0</td>
<td>11.1</td>
<td>0.119</td>
<td>7465</td>
<td>102402</td>
<td>439.2</td>
</tr>
<tr>
<td>75.0</td>
<td>15.8</td>
<td>0.169</td>
<td>10399</td>
<td>103093</td>
<td>417.7</td>
</tr>
<tr>
<td>100.0</td>
<td>20.0</td>
<td>0.214</td>
<td>12314</td>
<td>93878</td>
<td>421.9</td>
</tr>
<tr>
<td>200.0</td>
<td>33.3</td>
<td>0.357</td>
<td>15811</td>
<td>66851</td>
<td>433.2</td>
</tr>
<tr>
<td>Blank **</td>
<td>N/a</td>
<td>N/a</td>
<td>N/a</td>
<td>112056</td>
<td>N/a</td>
</tr>
</tbody>
</table>

* Assumption is that 300µl of TCA (10%) is added to a filter, then flushed through with 2.5ml water to give total volume of 2.8ml and a TCA concentration of \([300/2800 \times 10] = 1.07\%\).  
** This is the RLU value obtained from the HS reagent with no sample added.
5.4.1 Evaluation of clinical samples using the bioluminescence assay

A total of 114 Arrow Triple lumen, 7Fr, 20cm CVCs (as shown in Figure 1.1) were removed from patients in a local hospital (St George’s Hospital, London). The tips were cut from the remainder of the CVC and each tip was subjected to semi-quantitative extraluminal culture using the method described by Maki et al. (1977). The results of the Maki technique were reported in CFUs and where a sample was too numerous to count (TNTC) an estimation of the number of cells was made (for example >500). Each of the 114 tips were subsequently stored at 2-8°C until the internal lumen of the tip was sampled using the FASEB. The CVC tips were collected in batches each week from the hospital and this resulted in a variation in time between CVC removal and examination of the internal lumen. A portion of the FASEB-retrieved sample was cultured to determine the number of organisms present inside the CVC, whilst the remainder of this sample was taken and used in the bioluminescence assay. All 114 were exposed to the ATP degradation reagent and filtration through a 0.2μm 25mm syringe filter. The ATP concentration was determined in 65 samples using the SL reagent (Table 5.9) and in the remaining 49 samples using the HS reagent (Table 5.10). These tables also display the identity of microorganisms that were isolated from the samples, as well as the RLUs and the ATP concentration obtained from the FASEB samples. Initially, organisms were not identified in the FASEB cultures of the first 65 samples (Table 5.9).

From Tables 5.9 and 5.10 it can be seen that there are FASEB-retrieved samples that reported no growth and others that reported low to high levels of colonisation with various species of microorganisms. Sixteen (24.6%) samples reported no growth in Table 5.9 and 10 (20.4%) in Table 5.10.
Table 5.9  CVC tips tested using the SL reagent.

<table>
<thead>
<tr>
<th>Storage (Days)</th>
<th>CFU's on the tip</th>
<th>Microorganism(s) on the CVC tip</th>
<th>CFU's on brush</th>
<th>RLU's from the brush sample</th>
<th>[ATP] (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>~800 CNS &amp; Diphtheroids</td>
<td>10000</td>
<td>9073</td>
<td>2067.72</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>&gt;1000 CNS &amp; Diphtheroids</td>
<td>30</td>
<td>248</td>
<td>22.37</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>&gt;500 C. albicans</td>
<td>110</td>
<td>250</td>
<td>10.44</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>300 Mixed CNS</td>
<td>No growth</td>
<td>374</td>
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**MRSA**  Methicillin resistant *S. aureus*

**CNS**  Coagulase negative staphylococci
Table 5.10 CVC tips tested using the HS reagent.

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<td>7</td>
<td>No growth</td>
<td>N/a 90 CNS</td>
<td>CNS</td>
<td>2027</td>
<td>224.12</td>
</tr>
<tr>
<td>40</td>
<td>7</td>
<td>1 CNS</td>
<td>No growth</td>
<td>N/a</td>
<td>2878</td>
<td>51.32</td>
</tr>
<tr>
<td>41</td>
<td>6</td>
<td>No growth</td>
<td>N/a 10 CNS</td>
<td>CNS</td>
<td>995</td>
<td>29.02</td>
</tr>
<tr>
<td>42</td>
<td>5</td>
<td>&gt;500 Mixed CNS</td>
<td>170 CNS</td>
<td>CNS</td>
<td>309</td>
<td>20.01</td>
</tr>
<tr>
<td>43</td>
<td>5</td>
<td>&gt;500 Mixed CNS &amp; MRSA</td>
<td>No growth</td>
<td>N/a</td>
<td>10135</td>
<td>4.86</td>
</tr>
<tr>
<td>44</td>
<td>4</td>
<td>&gt;500 Coliform</td>
<td>5000 Not identified</td>
<td>CNS</td>
<td>12613</td>
<td>74.58</td>
</tr>
<tr>
<td>45</td>
<td>15</td>
<td>&gt;500 Mixed CNS</td>
<td>1120 CNS</td>
<td>CNS</td>
<td>4968</td>
<td>223.01</td>
</tr>
<tr>
<td>46</td>
<td>13</td>
<td>&gt;500 CNS</td>
<td>2030 CNS</td>
<td>CNS</td>
<td>2227</td>
<td>111.43</td>
</tr>
<tr>
<td>47</td>
<td>11</td>
<td>&gt;500 S. aureus</td>
<td>3100 S. aureus</td>
<td>S. aureus</td>
<td>18720</td>
<td>866.50</td>
</tr>
<tr>
<td>48</td>
<td>8</td>
<td>80 CNS</td>
<td>100000 CNS</td>
<td>CNS</td>
<td>919271</td>
<td>15610.72</td>
</tr>
<tr>
<td>49</td>
<td>8</td>
<td>&gt;500 MRSA</td>
<td>10000 S. aureus</td>
<td>MRSA</td>
<td>126052</td>
<td>6056.60</td>
</tr>
</tbody>
</table>

MRSA = Methicillin resistant S. aureus  
CNS = Coagulase negative staphylococci
5.4.2 Correlation between ATP concentration and CFU

An attempt was made to determine if there was any correlation between the ATP concentrations measured for each of the FASEB samples and the CFU/ml reported from culture of the same FASEB sample (Figures 5.5 and 5.6). In both graphs the "no growth" samples have been converted to "1" to allow logarithmic plot.

A strong correlation was not evident in the case of the samples detected using the SL reagent (Figure 5.5). However there was evidence that samples which reported 1000 CFU/ml or less reported ATP concentrations of approximately 1000 pMol/L or less.

A slightly better correlation was observed in the samples detected using the HS reagent (Figure 5.6). Samples which reported 1000 CFU/ml or more reported ATP concentrations of approximately 100 pMol/L or greater. This suggested that the application of cut-off values might be useful.

5.4.3 Cut-off limit based on CFU/ml values

To investigate further the possibility of applying a cut-off limit in terms of the CFU/ml, graphs were plotted which categorised samples by whether they had greater or less than 100CFU/ml or 1000CFU/ml. The cut-off of 1000 CFU/ml was selected because of work carried out by Brun-Buisson et al. (1987) and Cleri et al. (1980) on quantitative tip culture, and the cut-off of 100 CFU/ml based on a study by Kite et al. (1997) on the FASEB.

a) SL reagent

Figures 5.7 and 5.8 illustrate the cut-off points at >1000 CFU/ml and >100 CFU/ml respectively for ATP concentrations determined using the SL reagent.

In Figure 5.7, thirteen (20%) of the 65 CVC tips have colony counts of >1000 CFU/ml. The mean ATP concentration for samples reporting <1000 CFU/ml is 208.25 pMol/L (range 1.98 to 1397.0 pMol/L) and the mean ATP concentration for a sample with >1000 CFU/ml is 83547.34 pMol/L (range 2.39 to 1040000 pMol/L). Nine (69.2%) of the 13 samples reporting >1000 CFU/ml gave values that overlap with the ATP concentrations of the samples that reported <1000 CFU/ml. Thus there is poor distinction between positive and negative results based on this cut-off.
Figure 5.5 Correlation between the ATP concentrations measured for each of the FASEB samples using the SL reagent and the CFU/ml reported from culture of the same FASEB sample (n=65). The "no growth" samples have been converted to "1" to allow logarithmic plot.
Figure 5.6  Correlation between the ATP concentrations measured for each of the FASEB samples using the HS reagent and the CFU/ml reported from culture of the same FASEB sample (n=49). The “no growth” samples have been converted to “1” to allow logarithmic plot.
In Figure 5.8, 41 (63.1%) of the 65 CVC tips have colony counts of >100 CFU/ml. The mean ATP concentration for samples reporting <100 CFU/ml is 289.81 pMol/L (range 1.98 to 1397.0 pMol/L) and the mean ATP concentration for a sample with >100 CFU/ml is 26503.83 pMol/L (range 2.39 to 1040000 pMol/L). 37 (90.2%) of the 41 samples reporting >100 CFU/ml gave values that overlap with the ATP concentrations of the samples that reported <100 CFU/ml. Again poor distinction was observed between positive and negative results as defined by this 100 CFU/ml cut-off.

b) HS reagent

Figures 5.9 and 5.10 illustrate the cut-off points at >1000 CFU/ml and >100 CFU/ml respectively for ATP concentrations determined using the HS reagent.

In Figure 5.9, 14 (28.6%) of the 49 CVC tips have colony counts of >1000 CFU/ml. The mean ATP concentration for samples reporting <1000 CFU/ml is 220.67 pMol/L (range 4.86 to 1484.37 pMol/L) and the mean ATP concentration for a sample with >1000 CFU/ml is 4618.10 pMol/L (range 8.98 to 15610.72 pMol/L). 6 (42.9%) of the 14 samples reporting >1000 CFU/ml gave values that overlap with the ATP concentrations of the samples that reported <1000 CFU/ml.

In Figure 5.10, 26 (53.1%) of the 49 CVC tips have colony counts of >100 CFU/ml. The mean ATP concentration for samples reporting <100 CFU/ml is 238.77 pMol/L (range 4.86 to 1484.37 pMol/L) and the mean ATP concentration for a sample with >100 CFU/ml is 2572.50 pMol/L (range 8.98 to 15610.72 pMol/L). 18 (69.2%) of the 26 samples reporting >100 CFU gave values that overlap with the ATP concentrations of the samples that reported <100 CFU/ml. Again there is poor distinction between positive and negative results for both the 100 and 1000 CFU/ml cut-off limits.

c) Cut-off selection

Distinction observed between positive and negative results is poor but it seems to be slightly better with samples assayed with the HS reagent than with the SL reagent. Basing a CFU count on 100 CFU/ml gives high levels of overlap between samples measured with the SL (90.2%) and HS (69.2%) reagents. Using >1000 CFU/ml the levels of overlap between samples measured with the SL (69.2%) and HS (42.9%) are improved, but still do not provide a test of clinical utility.
Figure 5.7  The ATP concentrations (pMol/L) determined using the SL reagent in CVC tips that have recorded >1000 CFUs or <1000 CFUs in culture using the FASEB technique (n=65).
Figure 5.8 The ATP concentrations (pMol/L) determined using the SL reagent in CVC tips that have recorded >100 CFUs or <100 CFUs in culture using the FASEB technique (n=65).
Figure 5.9 The ATP concentrations (pMol/L) determined using the HS reagent in CVC tips that have recorded >1000 CFUs or <1000 CFUs in culture using the FASEB technique (n=49).
Figure 5.10  The ATP concentrations (pMol/L) determined using the HS reagent in CVC tips that have recorded >100 CFUs or <100 CFUs in culture using the FASEB technique (n=49).
The poor differentiation between positive and negative samples based on these cut of values may be due to the small percentage of the extracted sample (<5%) that is used in the final ATP determination. This creates a great sensitivity problem as 95% of the sample is not measured. The amount of sample that is eluted from the filter is 2.8ml and as 75μl and 100μl were used in the HS and SL reactions respectively then only 2.7% and 3.6% of the sample is measured. However, as 800μl of the original sample is filtered then this equates to 2.2% and 2.9% respectively of the original sample.

5.4.4 Cut-off based on ATP concentration

Kite et al. (1997) defined significant counts after culture of a FASEB-retrieved sample as ≥100 CFU/ml. This was used as the level of significance for cultured samples in this study. The sensitivity and specificity of the bioluminescence assay to detect positive catheter tip samples (>100CFU/ml) as a function of the ATP limit defining a positive FASEB sample was determined. The sensitivity and specificity of the SL reagent to detect positive FASEB samples at various ATP concentration cut-off (pMol/L) levels is displayed in Figure 5.11. The FASEB samples detected using the HS reagent are displayed in Figure 5.12.

If for example a cut-off limit of 10 pMol/L was chosen the bioluminescence assay using the SL reagent detected 20 (83.3%) of the positive CVC tips (sensitivity) and 37 of the samples were classified as false positive to give a specificity of 9.8%. In Figure 5.11 it can been seen that if the cut-off limit was increased the sensitivity decreases but the specificity increases. This is observed when at a cut-off limit of 100 pMol/L the bioluminescence assay detected 9 (37.5%) of the positive CVC tips and 12 of the samples were classified as false positive to give a specificity of 70.7%.

By using the HS reagent (Figure 5.12) it was observed that at a cut-off limit of 100 pMol/L the bioluminescence assay detected 16 (61.5%) of the positive CVC tips and 12 samples were classified as false positive to give a specificity of 47.8%.
Figure 5.11 The sensitivity and specificity of the bioluminescence assay to detect positive catheter samples (>100 CFU/ml) as a function of the ATP limit defining a positive catheter sample using FASEB culture as the reference method. This graph is based upon 65 FASEB samples detected using the SL reagent.
Figure 5.12 The sensitivity and specificity of the bioluminescence assay to detect positive catheter samples (>100CFU/ml) as a function of the ATP limit defining a positive catheter sample using FASEB culture as the reference method. This graph is based upon 49 FASEB samples detected using the HS reagent.
5.4.5 *Strategies to improve the cut-off based on ATP concentration*

In order to improve clinical utility and introduce a greater separation between positive and negative samples, three additional factors were identified and investigated, with results reported in Chapter Six. These were, use of an alternative microbial cell lysis agent that is more specific than TCA, ways to increase the volume of sample, measured in the luciferase reaction and improvement in the separation of microbial and non-microbial ATP.
CHAPTER SIX
6.1 Background and rationale

In Chapter 5 it was observed that an assay based on syringe filtration and TCA extraction was inadequate, as there was poor differentiation between positive and negative samples. One of the reasons for this poor definition was that less than 5% of the TCA-extracted ATP sample was used in the bioluminescence assay. To improve upon the sensitivity level, the extracted ATP should form a larger part of the final sample. This chapter details the introduction and use of 13mm diameter syringe filters instead of the 25mm diameter syringe filters used in experiments reported in Chapter Five. The application of DTAB as an alternative lysis agent to TCA is also reported here. Finally as in Chapter Five the modified methodology was challenged with CVC tips that have been removed from catheterised hospital patients.

6.2 Application of 13mm diameter syringe filters

6.2.1 Syringe filter selection

In Chapter 5, a final volume of 2.8ml was extracted from the 2.5mm diameter syringe filters to provide a TCA-extracted ATP sample. The addition of 2.5ml of sterile water is necessary to extract the majority of ATP from the filter (Section 5.3). Further work was carried out to determine if the use of a syringe filter with a diameter of 13mm diameter and 0.2μm pore size (Nalgene, USA) would lower the volume of liquid required to flush the ATP and thus increase the concentration of ATP in the final sample.

6.2.2 Characteristics of the 13mm diameter Syringe filter

The 13mm diameter syringe filter had a hold-up volume of 210μl and a post-air-purge hold-up volume is 50μl and it was observed that each filter had a low ATP content (<10pmol/L). It was demonstrated that the syringe filter successfully trapped all E. coli, S. epidermidis, and C. albicans cells contained within solutions that were flushed through the filter, as there were no colonies present after culture of the filtrate.

6.2.3 Method development

This section details the assay development using a combination of the ATP degradation reagent with 13mm diameter, 0.2μm pore size Nalgene syringe filters.
a) **Volume of sample to be filtered**

As with the 25mm diameter, 0.2μm pore size syringe filters, 800μl of the solution containing sample in ATP degradation reagent was added to the filter before being flushed with 5ml of liquid. Previously 7.5ml had been used in conjunction with 25mm diameter syringe filters.

b) **TCA optimisation**

As these syringe filters are smaller than the 25mm diameter filters used in Chapter 5 it was necessary to carry out some optimisation of the assay.

Again TCA needed to be added so that a final concentration of >2.5% was achieved. As the hold-up volume is 210μl, a volume of at least 70μl of TCA (10%) was thought to be necessary. However, as can be seen in Table 6.1 this was not the case as the optimum volume of TCA (10%) which was necessary was 100μl.

Addition of the 100μl of TCA (10%) left 210μl of TCA-extracted ATP remaining within the syringe filter. In the 25mm filters a volume of 2.5ml was used to flush the TCA-extracted TCA from the filter. Based upon this it was decided to add a volume of 1.5ml to the 13mm diameter filters in order to flush out the TCA-extracted ATP and give a final volume of 1.6ml, and a final TCA concentration of <0.65% (v/v).

c) **Volume of sample measured in the HS reagent**

Based on the observations in Section 5.3.1, a final concentration of approximately 0.2% TCA gives approximately 50% inhibition of the HS reagent signal that would normally be observed when no TCA is present. Therefore, 75μl of the TCA (0.65%) extract was used as the sample in the bioluminescence reaction involving the HS reagent (total volume 475μl), as this gives a final TCA concentration of 0.1% (v/v).

### 6.2.4 Evaluation of the method using CVC tips

The method that has been developed and described in the Sections 6.1 through to 6.2.3 was used to determine if microbial ATP could be detected in CVC tips removed from catheterised hospital patients. A total of 6 CVC were removed and treated as already
Table 6.1  Volume of TCA (10%) that needs to be added to a 13mm diameter, 0.2μm pore size syringe filter in order to extract the highest concentration of ATP from a *S. aureus* stock solution. Values shown are the mean of four experiments.

<table>
<thead>
<tr>
<th>TCA (10%) volumes added (μl)</th>
<th>Assumed final TCA concentration in syringe filter (% v/v) *</th>
<th>ATP concentration (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.92</td>
<td>352.57</td>
</tr>
<tr>
<td>60</td>
<td>2.22</td>
<td>407.11</td>
</tr>
<tr>
<td>70</td>
<td>2.50</td>
<td>418.35</td>
</tr>
<tr>
<td>80</td>
<td>2.76</td>
<td>442.35</td>
</tr>
<tr>
<td>90</td>
<td>3.00</td>
<td>458.79</td>
</tr>
<tr>
<td>100</td>
<td>3.23</td>
<td>487.63</td>
</tr>
<tr>
<td>150</td>
<td>4.17</td>
<td>398.54</td>
</tr>
</tbody>
</table>

* Assumes a closed system and is based on the volume added plus the hold-up volume of the filter (210μl)
outlined in Section 5.4. All six were exposed to the ATP degradation reagent and filtration through a 0.2μm pore size 13mm syringe filter; the ATP concentration was determined using the HS reagent.

Table 6.2 shows results from CVC tips tested using the HS reagent. However the sensitivity is poor, resulting in poor differentiation between positive and negative results. Again there is still a low volume of TCA-extracted sample (<4.7%) used in the final assay and perhaps an alternative microbial cell lysis agent to replace TCA may be appropriate as this could lead to a higher volume of the sample being used.

### 6.3 An alternative microbial ATP extractant to TCA

Alternative microbial cell extractants have been reported in a Chapter 2 where it was detailed that all microbial cell extractants intrinsically inhibit the luciferase enzyme. A way around this problem is to use an extractant that can be neutralised after it has irreversibly inhibited the ATP degrading enzymes. An example is the chemical DTAB, which can be neutralised by α-cyclodextrin (Lundin et al. 1994). Experiments involving the development of a method using this extractant are described below.

#### 6.3.1 Concentration of DTAB necessary to extract microbial ATP

DTAB is less inhibitory to the HS reagent than TCA when used at similar concentrations (1.0-1.5%). In addition DTAB (0.01-0.5%) has a pH of 5.2, unlike TCA, which has a pH of 1.5.

A stock culture of *S. epidermidis* (approximately 10^7 cells/ml) was exposed to various concentrations of DTAB (0 to 0.1%). A final concentration of 0.5% TCA was used as a control to show 100% lysis. The detection of RLUs involved the SL reagent system in combination with the L1 luminometer. Figure 6.1 demonstrates that at concentrations of ≥ 0.01% DTAB, the percentage of ATP released is similar to that of the control.
Table 6.2  CVC tips tested using the HS reagent and after extraction from 13mm diameter, 0.2μm pore size syringe filters.

<table>
<thead>
<tr>
<th>Storage (Days)</th>
<th>CFUs on the tip</th>
<th>Microorganism(s) on the CVC tip</th>
<th>CFUs on brush</th>
<th>Microorganism(s) on the brush sample</th>
<th>RLU's from the brush sample</th>
<th>[ATP] (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>No growth</td>
<td>N/a</td>
<td>No growth</td>
<td>N/a</td>
<td>4857</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>No growth</td>
<td>N/a</td>
<td>No growth</td>
<td>N/a</td>
<td>1311</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>&gt;500</td>
<td>CNS</td>
<td>10</td>
<td>CNS</td>
<td>641</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>&gt;1000</td>
<td>CNS</td>
<td>1930</td>
<td>CNS</td>
<td>469</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>50</td>
<td>CNS</td>
<td>10</td>
<td>CNS</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>&gt;500</td>
<td>CNS &amp; Diphtheroids</td>
<td>20</td>
<td>CNS</td>
<td>1644</td>
</tr>
</tbody>
</table>
These results were further verified by culture of each extract using the spread plate technique. This showed that there are \textit{S. epidermidis} colonies present at the 0.005% (>1000 CFU/ml) and the 0.01% (15 CFU/ml) concentrations of DTAB. However at concentrations of >0.01% DTAB there are no colonies. This would suggest that all of the \textit{S. epidermidis} have been lysed. Therefore a DTAB concentration of greater than 0.01% is required to ensure complete lysis of a solution of \textit{S. epidermidis} (NCTC 11047).

A similar investigation, with a stock solution of \textit{E. coli}, showed that at concentrations of <0.02% DTAB there were \textit{E. coli} colonies present after culture. However at concentrations of ≥0.02% DTAB there were no colonies present. Therefore a DTAB concentration of >0.02% DTAB is required to ensure complete lysis of a solution of \textit{E. coli}.

Using a final concentration of 0.02% DTAB in a serial dilution to 10^{-6} in 1ml of \textit{S. epidermidis} (NCTC 11047) the detection limit of the HS reagent was found to be 188 \textit{S. epidermidis} in a final volume of 900\mu l. RLU readings were compared with the CFU counts obtained for each dilution and these are plotted on Figure 6.2. The RLU values plotted are taken from one experiment that showed similar results to that of four replicates. Here 100% of the DTAB-extracted sample is measured.

\subsection*{6.3.2 Effect of DTAB on apyrase and MgCl$_2$}

Using the HS reagent system (Biothema AB) in combination with the Mediators L1 luminometer, the effects of various concentrations of DTAB (0 to 1%) on the activity of apyrase (10 Units/ml) in the presence of MgCl$_2$ (0.02M) was investigated. Apyrase (10 Units/ml) in the presence of the excess MgCl$_2$ is completely inhibited by ≥ 0.4% DTAB. This level of inhibition is similar to that of the control, that is, the RLUs emitted by a standard that has not been exposed to the combination of apyrase and MgCl$_2$. Figure 6.3 shows the percentage inhibition at each concentration compared to the control.
Figure 6.1  Effects of various concentrations of DTAB on the release of ATP from a stock culture of *S. epidermidis*. The control has a final concentration of 0.5% TCA only and it is assumed that 100% lysis has occurred using this concentration of TCA. Each bar is the mean±SD of four experiments.
Figure 6.2 A standard curve showing the relationship between the RLU's and the number of cells of *S. epidermidis* using the HS reagent system (Biothema AB) in combination with the Mediators LI luminometer after extraction with DTAB. Each point is the mean±SD of four experiments.
Figure 6.3  Effect of various concentrations of DTAB on the activity of apyrase (10 units/ml) in an excess of magnesium chloride (0.02mol/L). Each bar is the mean±SD of four experiments.
6.3.3 **Effect of DTAB on the ATP degradation reagent**

Using the HS reagent system in combination with the Mediators L1 luminometer, it was demonstrated that the activity of the ATP degradation reagent (containing apyrase (10 Units/ml), 5'-adenylic acid deaminase (0.2U/ml), 0.02M MgCl$_2$ and Triton X-100 (0.1%w/v)) was completely inhibited by ≥ 0.8% DTAB. This level of inhibition is similar to that of the control. The percentage inhibition of various concentrations of DTAB (0 to 1%) is shown in Figure 6.4.

Results show that a minimum of 0.8% DTAB is required to effectively extract the microbial ATP from a microbial cell and inhibit the ATP degrading enzymes.

6.3.4 **Detection limit of assay using the DTAB extraction method**

Five clinical isolates from colonised CVC tips were cultured. Serial dilutions (to 5 x 10$^{-5}$) were made from each stock of isolate. Serial dilutions of each stock were also cultured and a count (CFU/ml) determined for the original stock solution. The sensitivity of the ATP bioluminescence assay (HS reagent method) was determined for each. Table 6.3 shows that levels of <500 organisms could be detected in four of the five samples. The organisms were identified using Gram staining and the Oxoid Staphytect Plus assay for staphylococci.

In a further study, ATP was quantified at all the serial dilutions so that calibration curves could be determined. Figures 6.5, 6.6 and 6.7 show the calibration curves that can be obtained by lysing various microorganisms with DTAB (0.01%) and detecting the RLU emitted from each at the various dilutions using the HS reagent. The CFU values are based upon culture results of 100µl of the 10$^{-5}$ dilution, which was carried out in triplicate, in each case.

Based on these results, the sensitivity of the assay (when measuring 100% of the sample) is < 500 CFU/ml for the staphylococci, but it is > 1,000 CFU/ml for *E coli*. 
Figure 6.4 The effect of various concentrations of DTAB on the activity of the ATP degradation reagent. Each bar is the mean±SD of four experiments.
Table 6.3 Detection limit of the ATP Bioluminescence method in a pure system using five isolates (numbered 1 to 5) from CVC tips and two known laboratory strains of *S. epidermidis* and *E. coli* (A and B respectively).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Identification of microorganism</th>
<th>CFUs *</th>
<th>RLU reported</th>
<th>ATP concentration pmoL/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CNS</td>
<td>310</td>
<td>431</td>
<td>300.59</td>
</tr>
<tr>
<td>2</td>
<td><em>E. coli</em></td>
<td>TNTC</td>
<td>1415</td>
<td>1101.0</td>
</tr>
<tr>
<td>3</td>
<td><em>S. aureus</em></td>
<td>198</td>
<td>218</td>
<td>123.42</td>
</tr>
<tr>
<td>4</td>
<td><em>S. aureus</em> (possibly MRSA)</td>
<td>428</td>
<td>1053</td>
<td>914.70</td>
</tr>
<tr>
<td>5</td>
<td>CNS</td>
<td>445</td>
<td>1130</td>
<td>954.34</td>
</tr>
<tr>
<td>A</td>
<td><em>S. epidermidis</em></td>
<td>228</td>
<td>1064</td>
<td>891.16</td>
</tr>
<tr>
<td>B</td>
<td><em>E. coli</em></td>
<td>TNTC</td>
<td>14838</td>
<td>14158.35</td>
</tr>
</tbody>
</table>

* The CFU/ml values reported here are the number of organisms that correspond to the RLU value that is quoted in the adjacent column. Both of these refer to 50μl of the 5 x 10^-5 dilution (a final concentration of 2.5 x 10^-7 / ml)
Figure 6.5 A calibration curve showing the detection limit of the DTAB (0.1%)-extracted ATP from the *S. epidermidis* cells. Each point is the mean±SD of four experiments.
Figure 6.6  A calibration curve showing the detection limit of the DTAB (0.1%)-extracted ATP from the *E. coli* cells. Each point is the mean±SD of four experiments.
Figure 6.7  A calibration curve showing the detection limit of the DTAB (0.1%)-extracted ATP from the MRSA cells. Each point is the mean±SD of four experiments.
6.3.5 Effect of DTAB on luciferin-luciferase reaction with ATP

The effect of increasing the concentration of DTAB on the reaction between the HS reagent and an ATP standard was investigated. Figure 6.8 shows the effect of cumulative additions of DTAB and water was added as a control so that dilution effects were minimised. From the graph, it can be seen that the addition of increasing concentrations of DTAB does lead to an inhibition of the HS induced signal (RLUs) and this is a concern as the relatively dilute solution of DTAB (0.057%) leads to a great reduction in the signal.

When TCA was used, as reported in Section 6.2, the final concentration of TCA was diluted by the volume of sterile water flushed through the filter. However, as a concentration of DTAB (0.8%) is necessary to lyse the cells and inhibit the enzymes, then a 1:10 dilution of the extract will only give a concentration of 0.08%, which according to these results will still be inhibitory to the HS reagent.

6.4 Neutralisation of the inhibitory effects of DTAB

6.4.1 α-cyclodextrin

In Section 6.3 it was observed that the reaction between the HS reagent and an ATP standard was inhibited in the presence of DTAB (0.057%). In Chapter 2 α-cyclodextrin was introduced as a possible agent to neutralise the effects of DTAB. The use of α-cyclodextrin was investigated in this study and in some preliminary studies it was observed that the inhibitory effects of DTAB were reversed in the presence of α-cyclodextrin (0.5%).

Further studies showed that DTAB (0.2%) was neutralised by α-cyclodextrin (0.75%). It was also observed that the use of concentrations of α-cyclodextrin of >0.75% leads to quenching of the light given off and the use of <0.75% leads to the DTAB-induced inhibition of the luciferase reaction.

6.4.2 Effect of α-cyclodextrin on the DTAB (0.046%) induced inhibition

In Figure 6.8 it was observed that there was a dramatic effect on the signal given out by the HS reagent in the presence of DTAB (0.046%). Further work was conducted
Figure 6.8  The effect of increasing the concentration of DTAB (0 to 0.057% w/v) on the reaction between the HS reagent and the ATP standard (10⁻³ mol/L). Each point is the mean of four experiments.
where DTAB (0.046%) was exposed to various concentrations α-cyclodextrin (0 to 0.76%). Figure 6.9 shows the effect of these various concentrations of α-cyclodextrin on the DTAB-induced inhibition of the HS plus standard reaction. It is clear that increasing the concentration of α-cyclodextrin leads to an attenuation of the DTAB-induced inhibition of the HS standard reaction. Therefore, in the presence of α-cyclodextrin the effect of DTAB is partially neutralised. The dramatic drop in RLU signal after the addition of the DTAB was due to the dilution effect of adding extra liquid.

6.4.3 Addition of α-cyclodextrin to DTAB-extracted ATP

Figure 6.9 demonstrates that by increasing the concentration of α-cyclodextrin to 0.76% (w/v) there is better neutralisation of DTAB (0.046%). Further work was carried out using samples extracted from syringe filters using DTAB (2%). The manufacturer of the HS reagent claim that up to 600μl of liquid can be added to 400μl of the HS reagent. Therefore with this in mind, various volumes (100μl to 600μL) were evaluated. Optimum results were observed with a final volume of 500μl of sample, which comprised of 400μl (36.4%) of the DTAB extract and 100μl of α-cyclodextrin (2.5% w/v) to give a final α-cyclodextrin of 0.5% and a final DTAB concentration of <0.091%. Then this 500μl of sample was added to the HS reagent and the RLUs determined.

6.5 Removal of contaminating ATP from α-cyclodextrin

The α-cyclodextrin supplied by Sigma was found to contain a relatively high concentration of ATP. This had to be removed in order for the α-cyclodextrin to be of any use in this assay. The method that was developed involved the use of Q Sepharose Fast Flow (Amersham Pharmacia Biotech AB) and it is detailed in Section 3.6.5. During the development of this method it was found that the Q Sepharose needed to be washed, as it was supplied in ethanol. A washing procedure was also developed and this too is detailed in Section 3.6.5.

Analysis of the final ATP content of the resultant "ATP free" α-cyclodextrin showed that the RLU value reduced from levels of approximately 2,800 to <50 RLUs.
Figure 6.9 Effect of various concentrations of α-cyclodextrin (0 to 0.76%) on the DTAB (0.046%) induced inhibition of the HS - standard reaction. Each point is the mean (±SD) of four experiments.
6.6 Detection of microbial ATP in CVC tips

The method developed and described in the Sections 6.1 to 6.3 was challenged with FASEB samples retrieved from CVC tips that were removed from catheterised hospital patients. The methodology used has been described in detail in Section 3.6.6 of Chapter 3.

6.6.1 Analysis of clinical samples

A total of 46 Arrow Triple lumen, 7Fr, 20cm CVCs were removed from patients in St George’s Hospital, London after they were suspected to be implicated in catheter-associated infection. After removal the tips were cut from the remainder of the CVC and each tip was subjected to semi-quantitative extraluminal culture using the method described by Maki et al. (1977). Each tip was then stored at 2-8°C until the internal lumen of the tip was sampled using the FASEB. The CVC tips were collected in batches from the hospital and this resulted in a variation in time between CVC removal and examination of the internal lumen. Of the 46 samples the range of sample storing time varied from 3 to 37 days (mean = 13.34 days).

The FASEB was used to sample the distal lumen of each of these CVC tips. A FASEB was also used to sample the intermediate lumen of one of the triple lumen CVCs (number 44), thus giving 47 results from 46 CVCs.

The results in Table 6.4 are taken from 47 samples where the CFU values from cultures using both the Maki technique and FASEB-retrieved sample are displayed. The RLUs (determined using the HS reagent) and the ATP concentration obtained from the FASEB sample are also displayed. In addition the identity of microorganisms that were present have been listed. The ATP concentrations shown in Table 6.4 range from 0.47 to 9230.33 pMol/L (mean 232.69, mean excluding the outlying result =37.09).

In determining the ATP concentration 400µl of a total of 1100µl extract was used as the final sample, which equates to 36.4% of the total extract. However as 800µl of the original FASEB-retrieved sample is filtered and subsequently extracted then the proportion of sample forming the final sample is 29.1% of the original sample.
Table 6.4 CVC tip samples assayed using the DTAB extractant.

<table>
<thead>
<tr>
<th>Storage (Days)</th>
<th>CFUs on the tip</th>
<th>Microorganism(s) on the CVC tip</th>
<th>CFUs on brush</th>
<th>Microorganism(s) on the brush sample</th>
<th>RLU's from the brush sample</th>
<th>[ATP] (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>&gt;500</td>
<td>330</td>
<td>CNS &amp; <em>S. aureus</em></td>
<td>963</td>
<td>3.85</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>25</td>
<td>No growth</td>
<td>CNS</td>
<td>980</td>
<td>3.69</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>100</td>
<td>10</td>
<td>CNS</td>
<td>585</td>
<td>2.23</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>&gt;300</td>
<td>&gt;5000</td>
<td>CNS</td>
<td>15709</td>
<td>60.69</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>200</td>
<td>20</td>
<td>CNS</td>
<td>945</td>
<td>3.63</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>10</td>
<td>No growth</td>
<td>N/a</td>
<td>431</td>
<td>1.71</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>80</td>
<td>&gt;5000</td>
<td>Not identified</td>
<td>914</td>
<td>3.51</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>&gt;500</td>
<td>&gt;10000</td>
<td><em>Candida</em> spp &amp; CNS</td>
<td>14936</td>
<td>54.30</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>No growth</td>
<td>N/a</td>
<td>No growth</td>
<td>1454</td>
<td>4.87</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>&gt;500</td>
<td>No growth</td>
<td>N/a</td>
<td>181</td>
<td>0.51</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>&gt;500</td>
<td>10</td>
<td>CNS</td>
<td>3358</td>
<td>11.33</td>
</tr>
<tr>
<td>12</td>
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<td>&gt;500</td>
<td>No growth</td>
<td>N/a</td>
<td>6755</td>
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</tr>
<tr>
<td>13</td>
<td>17</td>
<td>&gt;500</td>
<td>&gt;100000</td>
<td><em>Pseudomonas</em> sp.</td>
<td>26907</td>
<td>88.44</td>
</tr>
<tr>
<td>14</td>
<td>13</td>
<td>&gt;500</td>
<td>&gt;100000</td>
<td>CNS</td>
<td>29531</td>
<td>101.91</td>
</tr>
<tr>
<td>15</td>
<td>12</td>
<td>&gt;500</td>
<td>30</td>
<td>CNS</td>
<td>740</td>
<td>2.11</td>
</tr>
<tr>
<td>16</td>
<td>37</td>
<td>No growth</td>
<td>N/a</td>
<td>No growth</td>
<td>151</td>
<td>0.47</td>
</tr>
<tr>
<td>17</td>
<td>36</td>
<td>No growth</td>
<td>N/a</td>
<td>No growth</td>
<td>8895</td>
<td>33.87</td>
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</table>

Continued
<table>
<thead>
<tr>
<th>Storage (Days)</th>
<th>CFUs on the tip</th>
<th>Microorganism(s) on the CVC tip</th>
<th>CFUs on brush</th>
<th>Microorganism(s) on the brush sample</th>
<th>RLU from the brush sample</th>
<th>[ATP] (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>35</td>
<td>&gt;300 Coliforms &amp; Diphtheroids</td>
<td>No growth</td>
<td>n/a</td>
<td>17645</td>
<td>105.65</td>
</tr>
<tr>
<td>19</td>
<td>32</td>
<td>100 CNS</td>
<td>20</td>
<td>Yeast</td>
<td>57219</td>
<td>230.90</td>
</tr>
<tr>
<td>20</td>
<td>28</td>
<td>No growth</td>
<td>10</td>
<td>CNS</td>
<td>13313</td>
<td>52.40</td>
</tr>
<tr>
<td>21</td>
<td>25</td>
<td>&gt;100 CNS</td>
<td>No growth</td>
<td>N/a</td>
<td>625</td>
<td>2.23</td>
</tr>
<tr>
<td>22</td>
<td>25</td>
<td>&gt;500 S. aureus</td>
<td>&gt;5000</td>
<td>C. albicans</td>
<td>1692773</td>
<td>9230.33</td>
</tr>
<tr>
<td>23</td>
<td>13</td>
<td>5 CNS</td>
<td>No growth</td>
<td>N/a</td>
<td>10052</td>
<td>41.49</td>
</tr>
<tr>
<td>24</td>
<td>16</td>
<td>5 CNS</td>
<td>3100</td>
<td>CNS</td>
<td>1406</td>
<td>14.37</td>
</tr>
<tr>
<td>25</td>
<td>12</td>
<td>&gt;500 Coliforms &amp; CNS</td>
<td>5000</td>
<td>Coliforms</td>
<td>9678</td>
<td>83.53</td>
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<tr>
<td>26</td>
<td>11</td>
<td>50 Acinetobacter sp.</td>
<td>50</td>
<td>Not identified</td>
<td>6564</td>
<td>68.35</td>
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<td>27</td>
<td>10</td>
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<td>N/a</td>
<td>5575</td>
<td>49.69</td>
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<tr>
<td>28</td>
<td>7</td>
<td>&gt;500 CNS &amp; S aureus</td>
<td>3000</td>
<td>CNS</td>
<td>9120</td>
<td>102.87</td>
</tr>
<tr>
<td>29</td>
<td>7</td>
<td>&gt;500 S aureus &amp; Diphtheroids</td>
<td>20</td>
<td>S. aureus</td>
<td>3220</td>
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</tr>
<tr>
<td>30</td>
<td>6</td>
<td>&gt;500 CNS</td>
<td>350</td>
<td>CNS</td>
<td>1659</td>
<td>17.27</td>
</tr>
<tr>
<td>31</td>
<td>6</td>
<td>10 CNS</td>
<td>100</td>
<td>CNS</td>
<td>1968</td>
<td>21.39</td>
</tr>
<tr>
<td>32</td>
<td>5</td>
<td>No growth</td>
<td>N/a</td>
<td>No growth</td>
<td>704</td>
<td>8.81</td>
</tr>
<tr>
<td>33</td>
<td>3</td>
<td>No growth</td>
<td>N/a</td>
<td>980</td>
<td>10939</td>
<td>364.32</td>
</tr>
<tr>
<td>34</td>
<td>3</td>
<td>&gt;300 CNS &amp; MRSA</td>
<td>30</td>
<td>CNS</td>
<td>1751</td>
<td>19.94</td>
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</table>

Continued
<table>
<thead>
<tr>
<th>Storage (Days)</th>
<th>CFUs on the tip</th>
<th>Microorganism(s) on the CVC tip</th>
<th>CFUs on brush</th>
<th>Microorganism(s) on the brush sample</th>
<th>RLU from the brush sample</th>
<th>[ATP] (pMol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>3</td>
<td>No growth</td>
<td>20</td>
<td>CNS</td>
<td>450</td>
<td>3.68</td>
</tr>
<tr>
<td>36</td>
<td>14</td>
<td>~300</td>
<td>30</td>
<td>S. aureus</td>
<td>641</td>
<td>2.54</td>
</tr>
<tr>
<td>37</td>
<td>14</td>
<td>&gt;500</td>
<td>300</td>
<td>Coliforms &amp; CNS</td>
<td>258</td>
<td>1.00</td>
</tr>
<tr>
<td>38</td>
<td>7</td>
<td>5</td>
<td>No growth</td>
<td>N/a</td>
<td>4795</td>
<td>26.10</td>
</tr>
<tr>
<td>39</td>
<td>7</td>
<td>No growth</td>
<td>No growth</td>
<td>N/a</td>
<td>554</td>
<td>2.65</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>No growth</td>
<td>No growth</td>
<td>N/a</td>
<td>1246</td>
<td>5.54</td>
</tr>
<tr>
<td>41</td>
<td>5</td>
<td>30</td>
<td>No growth</td>
<td>N/a</td>
<td>421</td>
<td>1.57</td>
</tr>
<tr>
<td>42</td>
<td>4</td>
<td>&gt;300</td>
<td>60</td>
<td>Bacilli &amp; yeast</td>
<td>2681</td>
<td>13.24</td>
</tr>
<tr>
<td>43</td>
<td>4</td>
<td>~400</td>
<td>40</td>
<td>Enterococci &amp; CNS</td>
<td>287</td>
<td>1.13</td>
</tr>
<tr>
<td>44</td>
<td>4</td>
<td>~400</td>
<td>No growth</td>
<td>N/a</td>
<td>1098</td>
<td>5.45</td>
</tr>
<tr>
<td>45</td>
<td>4</td>
<td>&gt;500</td>
<td>530</td>
<td>Yeast &amp; S. aureus</td>
<td>1011</td>
<td>5.34</td>
</tr>
<tr>
<td>46</td>
<td>4</td>
<td>No growth</td>
<td>220</td>
<td>Yeast</td>
<td>2787</td>
<td>12.64</td>
</tr>
<tr>
<td>47</td>
<td>4</td>
<td>&gt;300</td>
<td>820</td>
<td>CNS</td>
<td>1755</td>
<td>7.58</td>
</tr>
</tbody>
</table>

MRSA  Methicillin resistant S. aureus
CNS  Coagulase negative staphylococci
6.6.2 Correlation between internal and external colonisation.

From Table 6.4 it can be seen that in 17 (36.2\%) cases the same predominant species of microorganism was detected using the Maki technique and the FASEB. Of these twelve samples grew >100 CFU/ml. A further six samples reported no growth using both the Maki technique and the FASEB technique. In the remaining 24 samples there was growth detected using one technique and not the other in 15 cases and 9 samples grew different predominant species using both techniques. Mixed growth was observed in 11 samples analysed using the Maki technique and 9 using the FASEB.

Table 6.5 details the predominant microorganisms identified using the Maki and FASEB techniques. It can be seen that CNS are the most common species isolated from both the intra-luminal and extra-luminal portions of the CVC tip (Table 6.5).

6.6.3 Correlation between ATP concentration and CFU/ml

It was observed that 6 samples reported no growth using the Maki technique and the FASEB technique. Moreover 5 (83.3\%) of these reported ATP concentrations of <10 pMol/L ATP. A further 6 samples reported growth of <100 CFU/ml using both techniques and 5 (83.3\%) of these reported ATP concentrations of <50 pMol/L ATP. An attempt was made to determine if there was any correlation between the ATP concentrations measured for each of the FASEB samples and the CFU/ml reported from culture of the same FASEB sample (Figure 6.10). A strong correlation was not evident, however there was an indication that samples reporting <1000 CFU/ml generally reported ATP concentrations of approximately 200 pMol/L ATP or less.

6.6.4 Cut-off limits based on CFU counts

The utility of cut-off limits was investigated using the same cut-off criteria applied in Section 5.4. The mean ATP concentration for samples reporting <1000 CFU/ml was 31.49 pMol/L ATP (range 0.47 to 364.32 pMol/L ATP) and the mean ATP concentration for samples with >1000 CFU/ml was 1082.22 pMol/L ATP (range 3.51 to 9230.33 pMol/L ATP). It was observed that 9 (14.9\%) of the CVC tips had colony counts of >1000 CFU/ml, however 8 (88.9\%) of these reported ATP concentrations which
Table 6.5 Microorganisms implicated in CVC colonisation as detected using either the technique developed by Maki et al. (1977) or the FASEB technique.

<table>
<thead>
<tr>
<th>Microorganism *</th>
<th>Number (%) detected using Maki et al. technique (n=37) No.</th>
<th>%</th>
<th>In vitro FASEB technique (n=30) No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase negative staphylococci</td>
<td>23</td>
<td>62.2</td>
<td>16</td>
<td>53.3</td>
</tr>
<tr>
<td>S. aureus</td>
<td>5</td>
<td>13.5</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>Yeasts</td>
<td>2</td>
<td>5.4</td>
<td>4</td>
<td>13.3</td>
</tr>
<tr>
<td>Others</td>
<td>7</td>
<td>18.9</td>
<td>8</td>
<td>26.7</td>
</tr>
<tr>
<td>(Mixed growth)</td>
<td>11</td>
<td>N/a</td>
<td>9</td>
<td>N/a</td>
</tr>
</tbody>
</table>

* Predominant microorganism in the case of mixed growth
Figure 6.10  Correlation between the ATP concentrations measured for each of the FASEB samples and the CFU/ml reported from culture of the same FASEB sample (n=47). The "no growth" samples have been converted to “1” to allow logarithmic plot.
overlapped with the ATP concentrations of the samples reporting <1000 CFU/ml (Figure 6.11). In Figure 6.12, seventeen (36.2%) of the CVC tips had colony counts of >100 CFU/ml and 15 (88.2%) reported ATP concentrations which overlapped with the ATP concentrations of the samples that reporting <100 CFU/ml. The mean ATP concentration for samples reporting <100 CFU/ml was 25.44 pMol/L ATP (range 0.47 to 230.9 pMol/L ATP) and the mean ATP concentration for samples with >100 CFU/ml was 598.43 pMol/L ATP (range 1.00 to 9230.33 pMol/L ATP).

Basing a CFU count on 100 CFU/ml gives high levels of overlap between the samples (88.2%). There was no difference if a level of >1000 CFU/ml was chosen (88.9%). Thus there is poor distinction between positive and negative results as defined by this 100 CFU or 1000 CFU cut-off limits.

6.6.5 Cut-off based on ATP concentration

Thirty (63.8%) of the samples were reported as negative and 17 (36.2%) as positive, based upon a level of significance of ≥100 CFU/ml for cultured samples. The sensitivity and specificity of the bioluminescence assay to detect positive catheter tip samples (≥100 CFU/ml) as a function of the ATP limit defining a positive catheter sample is shown in Figure 6.13.

By taking a nominal cut-off limit of 10 pMol/L ATP as an example, it can be determined that the bioluminescence assay detected 12 (70.6%) of the positive CVC tips (sensitivity), yet 13 of the samples were classified as false positive, using this cut-off limit, to give a specificity of 56.7% (Figure 6.13). If the cut-off limit was increased then the sensitivity decreased but the specificity increased. Therefore, an increase in the cut-off limit to 50 pMol/L ATP meant that 26 (86.7%) of the negative samples which had ATP concentrations of less than 50 pMol/L were true negatives. Indeed 16 (94.1%) of the samples that had no growth had an ATP concentration of <50 pMol/L ATP. Furthermore, 8 (47.1%) of the samples that reported >100 CFU/ml had ATP concentrations in excess of 50 pMol/L ATP. However, 9 (52.9%) samples were reported as false negative using this cut-off limit.
Figure 6.11 The ATP concentrations (pMol/L) determined using the HS reagent in CVC tips that have recorded >1000 CFUs or <1000 CFUs in culture using the FASEB technique (n=47).
Figure 6.12  The ATP concentrations (pMol/L) determined using the HS reagent in CVC tips that have recorded >100 CFUs or <100 CFUs in culture using the FASEB technique (n=47).
Figure 6.13 The sensitivity and specificity of the bioluminescence assay to detect positive catheter tip samples (>100 CFU/ml) as a function of the ATP limit defining a positive catheter sample using FASEB culture as the reference method. This is based on all 47 samples.
6.6.6 Positive and negative predictive value

In order to determine a suitable cut-off limit the predictive value of a positive result (PV⁺) and the predictive value of the negative result (PV⁻) as a function of the ATP cut-off limit for a positive CVC tip were determined (Figure 6.14).

At a concentration 1 pMol/L ATP the PV⁺ and PV⁻ are 37.8% and 100% respectively. By increasing the cut-off limit of these predictive values (Figure 6.14) the PV⁺ was increased and the PV⁻ decreased to give 100% and 65.2% respectively at a cut-off limit of 1000 pMol/L ATP.

6.6.7 Effect of sample storage prior to analysis

Of the 47 samples that were analysed 18 (38.3%) were stored for 7 days or less prior to sampling by the FASEB technique (Table 6.6). The remainder of the tips were stored for between 8 and 37 days (mean duration was 18.8 days). Those tips stored for <7 days produced results with a higher specificity (81.8%) at a cut-off level of 20 pMol/L ATP than those tips stored for >7 days. However, the length of storage time had little effect on the sensitivity of the assay at this cut-off level of 20 pMol/L ATP.

6.6.8 Selection of a cut-off level

It is a requirement that this assay has a high predictive value for a negative result; that is the PV⁻ should be as close to 100% as possible. By combining the results in Figure 6.13 with those from Figure 6.14 it is possible to determine the optimal cut-off limit. A suitable cut-off limit would have to fall between 1 and 10 pMol/L ATP so that a PV⁻ of >70% could be achieved. However the problem with a cut-off limit at this ATP concentration is that the specificity and PV⁺ of the method are low (<60%). Nevertheless, a low ATP concentration cut-off is necessary so that false negative results are avoided. At 10 pMol/L ATP cut-off limit 17 (56.7%) of the negative samples had ATP concentrations of less than 10 pMol/L ATP, but five (29.4%) false negatives results were also reported using this cut-off limit. As a consequence the cut-off limit for the assay would need to be set at an ATP concentration closer to 1 pMol/L ATP, which would ensure that all positive results are determined and as few false negative results as possible are achieved.
Figure 6.14  The predictive values of positive and of negative assay as a function of the ATP limit for a positive CVC sample using FASEB culture as the reference method (n=47).
Table 6.6  Effect of sample storage at 2-8°C, for greater or less than seven days, on the sensitivity and specificity of the bioluminescence assay using a cut-off level of 20pMol/L ATP (n=47).

<table>
<thead>
<tr>
<th></th>
<th>&lt;7 days</th>
<th>&gt;7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of tips (%)</td>
<td>18 (38.3%)</td>
<td>29 (61.7%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>60%</td>
<td>57.9%</td>
</tr>
<tr>
<td>Specificity</td>
<td>42.9%</td>
<td>81.8%</td>
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</table>
CHAPTER SEVEN

Discussion
Despite the many benefits that CVCs offer, complications arising from their use have been noted since their introduction (Collins 1991). It is now estimated that over 500,000 cases of CRBSI occur annually in Western Europe and the USA (Crump and Collignon 2000). This creates a tremendous burden on the healthcare system with the average excess cost per survivor estimated to be $28,690, which is partly due to an additional 6.5 days required in ICU (Pittet 1994a, Pittet et al. 1994b).

Infections involving CVCs derive from a complex interaction between the host, the CVC and the microorganism (Mermal and Maki 1994, Raad 1998). There are no defined levels at which microorganisms may be said to contribute to infection or colonisation, however the occurrence of bacteraemia is less frequent than the level of observed device colonisation (Kite et al. 1997, Raad 1998). Nevertheless, a high level of colonisation is generally thought to be a precursor to bacteraemia (Cleri et al. 1980, Dittmer et al. 1999, Kite et al. 1997, Maki et al. 1977).

In order to determine if a CVC is the source of an infection, common practice involves the removal of the CVC (Johnson and Oppenheim 1992, Maki et al. 1977). However, such retrospective analysis results in the unnecessary removal of approximately 80% of CVCs as they are subsequently found not to be the source of the infection (Kite et al. 1997, Padberg et al. 1981, Pettigrew et al. 1985, Ryan et al. 1974). The accurate diagnosis of a catheter-associated infection is a challenge in that clinical symptoms are often non-specific (Dobbins et al. 1999, Elliott et al. 1994a). Moreover, the results of microbiological investigations are often difficult to interpret (Elliott et al. 2000, Worthington et al. 2002a). Methods are available by which the CVC can be sampled whilst it remains in situ within the patient (Dobbins et al. 1999, Siegman-Igra et al. 1997). However, clinicians are generally not in favour of leaving a suspected CVC in situ whilst waiting up to 24 hours for a culture result. Therefore, there is a requirement for a more rapid method of determining the microbial status of the CVC whilst it remains in situ.

Many of the rapid techniques that have been evaluated for use in the diagnosis of CRBSI have used blood aspirated through the CVC and have not required catheter removal (Blot et al. 1999, Gowardman et al. 1998, Kite et al. 1999, Kleiman et al. 1984, Moonens et al. 1994, Rogers and Oppenhiem 1998, Rushforth et al. 1993, Tighe et al. 1996b, von Baum
et al. 1998, Worthington et al. 2002a). However, none of these techniques has been adopted for routine use in the determination of the microbial status of a CVC. In a meta-analysis by Siegman-Igra et al. (1997) of sixteen methods used for the diagnosis of catheter-associated infection only three were rapid techniques and two of these (the Gram staining and the AOLC method) allowed the CVC to remain in situ.

The development of a technique that would aid in the rapid diagnosis of catheter-associated infections without CVC removal was attempted in this study. This chapter discusses the results of this investigation both in the context of the development of the technique and with reference to literature reviewed in Chapter 1. The development and evaluation processes are presented as well as major findings and the recommendations for future work.

Assay Development

The FASEB was used as the sampling device in this study. It has been shown to be the only device that can provide a sample from the inside of the CVC without the risk of contamination. Indeed, use of the FASEB in combination with conventional culture methods has been reported to have a higher sensitivity and specificity for the diagnosis of catheter-associated infections than traditional tip culture methods (Dobbins and Kite 1999, Kite et al. 1997, 1999). However, no studies have been conducted that have evaluated the feasibility of using a FASEB-retrieved sample in conjunction with a rapid test to aid in the diagnosis of catheter-associated infections. Perhaps Tighe et al. (1996b) came closest with the use of the FASEB to dislodge material from the catheter lumen prior to the aspiration of blood for use in the AOLC test.

Rapid detection of the presence of microorganisms in various clinical and non-clinical samples has been conducted using different types of detection technologies, such as particle counting (Medcraft 1993, Stevens et al. 1993), microscopy (Tighe et al. 1996b) and ATP bioluminescence (Lundin et al. 1989). In this study three such technology platforms were evaluated and using a scoring system, ATP bioluminescence was selected. One of the advantages of ATP bioluminescence was that it has a detection limit that allows measurement of as few as 100 microorganisms within a clinical sample (Lundin 2000). Indeed ATP bioluminescence has been successfully used in conjunction with other

Preliminary investigations into the possible application of ATP bioluminescence to a rapid test for the detection of microorganisms associated with CVCs involved the attempted development of a method using the Bioprobe luminometer and its dedicated reagent system. Contrary to the manufacturer's claims that the detector was sensitive enough to detect the equivalent of between 100 and 1000 CFU/ml (Report 1995), it was demonstrated that the sensitivity of the ATP bioluminescence assay was inadequate for detecting microbial ATP at this level. This was most likely to be due to the distance from the sample to the photo-detector (~20mm), which would mean that not all the light is detected. In attempting to distinguish between contaminating and microbial ATP, a commercially available ATPase was evaluated and it was observed (Figure 4.4) that a relatively high concentration of the ATPase could reduce contaminating ATP levels prior to release and determination of the microbial ATP. Further development using an isolator reagent and syringe filtration in combination with the ATPase demonstrated that further release and degradation of ATP from fresh blood cells was possible. However, a relatively high concentration of ATP still remained to contaminate samples. This was demonstrated during an evaluation with nine blood samples drawn through the CVC where the RLU values obtained did not correlate with subsequent CFU/ml counts.

As the background RLU level could not be reduced using the combination of this off-the-shelf instrument and reagent system it was considered necessary to develop and formulate a reagent system that would facilitate a reduction in the background ATP level leading to the successful extraction and detection of microbial ATP. Figure 2.7 in Section 2.7 illustrated the three main steps that needed to be overcome in order to develop an adequate method for detection of microorganisms based upon ATP bioluminescence. These steps involve the lysis and degradation of non-microbial ATP, followed by the lysis of microbial cells and the simultaneous inhibition of ATP converting enzymes and thirdly the detection of the released microbial ATP. In order to satisfy the Steps 1 and 2 of Figure 2.7 the Mediators L1 luminometer and recommended Biothema AB reagents were evaluated in conjunction with laboratory formulated reagents. This system was more appropriate than the Bioprobe system in that the sample could be contained within a tube
that could be located close to the photo-detector during the bioluminescence reaction. Additionally, using the L1 system in conjunction with laboratory formulated reagents it was observed that more of the contaminating, non-microbial ATP could be removed from the sample, thus leaving microbial ATP to be detected.

The development of a system based upon laboratory-formulated degradation reagents in conjunction with commercially available luciferase detection reagents and the L1 luminometer was described in Chapter 5 after preliminary development was reported in Chapter 4. To fulfil the requirements of Step 1 of Figure 2.7, a combination of chemicals and enzymes were evaluated to determine if it would be possible to exclusively lyse non-microbial cells and degrade the released ATP, whilst leaving microbial cells intact. The use of Triton X-100 in the lysis of somatic cells has previously been documented (Lundin and Thore 1975). Triton X-100 (0.1%v/v) was found to be the optimum concentration for lysing somatic cells such as those in a fresh heparinised blood sample (Figure 4.12). Additionally the combination of Triton X-100 with apyrase in excess Mg$^{2+}$ led to the release and degradation of >90.0% of the ATP in fresh heparinised blood samples and platelet-rich plasma. This was an improvement on the level of degradation previously reported by Molin et al. (1983) with blood samples, however Molin et al. used a grade I apyrase which contained a low ATPase activity. The grade III apyrase used in the present study has ten times higher ATPase activity than the grade I apyrase, therefore more ATP is converted to ADP over a much shorter period of time. Nevertheless, with this level of degradation approximately 600 pMol/L of ATP is left remaining, which may be equivalent to as many as 300,000 bacterial cells (Lundin 2000). Further methods of degrading this remaining ATP were investigated. Sakakibara et al. (1997) have previously demonstrated high levels of non-microbial ATP degradation by using a combination of apyrase and adenosine phosphate deaminase to achieve a residual ATP concentration of 1 pMol/L. Unfortunately a source for adenosine phosphate deaminase could not be found for use in this study. However, it was possible to obtain 5' adenylic acid deaminase, which like adenosine phosphate deaminase breaks down AMP to IMP. Using a combination of 5' adenylic acid deaminase and apyrase in excess of Mg$^{2+}$ with Triton X-100 (known in the present study as the ATP degradation reagent) led to the release and degradation of >99.0% of the ATP from platelet-rich plasma, to leave <250 pMol/L ATP remaining (Figure 5.1). As the aim was to reduce the ATP concentration to
approximately 1 pMol/L, a significant concentration of potentially contaminating ATP was still remaining. Further additional enzymatic alternatives were investigated with no additional success. Other strategies to aid in the removal of this residual ATP were investigated. Syringe filtration has been suggested by Lundin (2000) as a suitable method for isolating microbial cells and maximising their separation from contaminating ATP. A washing technique involving syringe filters was developed and evaluated in this study. Syringe filters with a 25mm diameter and 0.22μm pore size were used in conjunction with platelet-rich plasma (1:50), which was pre-treated with the ATP degradation reagent. An improved level of ATP removal was observed which resulted in <0.2% of the original ATP concentration remaining (Table 5.3). This equated to ~ 50 pMol/L of ATP remaining from platelet-rich plasma (1:50) and ~500 pMol/L of ATP remaining from heparinised blood (1:50). By degrading this level of ATP from such a rich source of free ATP, it was accepted that the criteria set in Step 1 of the flow chart (Figure 2.7) were fulfilled. Additionally, this washing procedure was found to have no adverse effect on the microbial cells and their intracellular ATP concentration. This is in agreement with comments made by Lundin (2000) who prefers this type of washing and cell concentration to that of centrifugation, which can lead to non-physiological conditions that could result in depleted microbial ATP levels.

In order to release ATP from microbial cells and inhibit all ATP converting enzymes, so that the requirements of Step 2 in Figure 2.7 could be fulfilled, TCA was evaluated in conjunction with the syringe filter technique. Lysis of microbial cells trapped on the filter membrane was achieved by the addition of TCA to obtain a final concentration of >2.5%. This TCA concentration was suggested by Lundin (2000) and was found in the present study to be adequate for rapid cell lysis and simultaneous inhibition of all ATP degrading enzymes. It was observed that apyrase (10 Units/ml) and 5'-adenylic acid deaminase (0.5U/ml) were both inhibited by TCA (>0.8%).

The ATP released from the microorganisms after lysis by TCA was collected by flushing the syringe filter with over five times the hold-up volume. This led to a significant dilution of the ATP pool in the sample, as the final volume was 2.8ml. Nevertheless, the TCA concentration was simultaneously diluted from 2.5% to just over 1.0%, therefore reducing its inhibitory effects on the luciferase reaction. Further optimisation of the assay
led to the finding that no more than 100μl of this TCA extracted sample could be measured in a final 1ml volume of the luciferin-luciferase reagent (SL) in Tris-EDTA. Indeed a similar situation was observed with the HS reaction where no more than 75μl could be assayed in a final volume of 475μl. These observation were consistent with those identified by Lundin (1997) and in the previous development work carried out in this study (Figures 4.8 and 4.9) were a final concentration of 0.1% TCA gives a 50% inhibition of the luciferase reaction. At this level of inhibition the detection of RLUs is still possible as addition of an internal standard will compensate for the observed inhibition, however further inhibition leading to a signal of <50% of the maximum would lead to unreliable results (Lundin 1997, 2000).

The combination of the ATP degradation reagent in conjunction with a syringe filtration method and TCA was evaluated using 114 FASEB-retrieved samples and found to be of little clinical utility as there was little correlation between CFU and ATP concentration. The poor differentiation may be due, in part, to the small percentage of extracted sample (<5%) that is used in the final ATP determination. In all cases the volume of sample eluted from the filter was 2.8ml, however only 75μl and 100μl were assayed in the HS and SL reactions respectively. Therefore only 2.7% and 3.6% of the sample is measured. However, as only 800μl of the original sample is filtered (with the remainder used in plate culture) this equates to just 2.2% and 2.9% respectively of the original sample. This creates a great sensitivity problem where >95% of the sample is not measured and low levels of microbial colonisation and infection will not be distinguishable from background levels of ATP. Unfortunately, this is one of the issues with chemicals used to lyse microbial cells and inhibit ATP converting enzymes. Other chemicals, which can be used as an alternative to TCA extraction have been reported in Section 2.7, but all have problems associated with their use (Table 2.4). Nevertheless, in this study the quaternary ammonium compound, DTAB, which is less hazardous than TCA (Lundin 2000) was investigated. By combining the DTAB extraction with 13mm diameter, 0.2μm pore size syringe filters - instead of 25mm diameter syringe filters - an improved method was developed. It was observed that DTAB (≥0.8%) was adequate for rapid, simultaneous cell lysis and inhibition of all ATP degrading enzymes, thus fulfilling the requirements of Step 2 in Figure 2.7. Further development showed that just 1.0ml of sterile water was required to elute the ATP solution (extract) from the 13mm syringe filter.
The advantage of using DTAB was observed when its inhibitory effects could be neutralised by α-cyclodextrin (Lundin et al. 1994). Further development of the assay led to optimum neutralisation of a final DTAB concentration (~0.08%) with a final concentration of 0.5% α-cyclodextrin. This is consistent with the findings of Lundin et al. (1994) who suggest that cyclodextrins should be present in a small excess over the quaternary ammonium compound.

Using α-cyclodextrin to neutralise the DTAB contained within the ATP extracted from the syringe filter in combination with the HS reagent led to 29.1% of the eluted sample being used in the assay. This was much improved upon the 2.7% observed when the TCA extract was used. This 10-fold increase in sample volume led to better assay performance when compared to the TCA extraction method.

**Assay Evaluation**

The DTAB extraction method was outlined in Figure 3.1 and was used to determine the microbial ATP content of 47 FASEB samples retrieved from CVC tips that were removed from hospitalised patients after a catheter-associated infection was suspected. In Table 6.4 the microorganisms isolated intraluminally and extraluminally were reported and these were consistent with those found in other studies (Dobbins et al. 1999, Fletcher and Bodenham 1999a). Organisms were isolated from the extraluminal surface of 37 CVCs and from the internal lumen of 30 CVCs. CNS was the predominant microorganism isolated from both the internal and external lumens (Table 6.5) and this is again consistent with previous studies, as illustrated in Figure 1.10 in Chapter 1. The high percentage of CNS isolated from both surfaces of the CVC is in agreement with the findings of a study by Kite et al. (1997) who reported CNS in 83% of colonised CVCs. Six samples reported no growth using both the Maki technique and the FASEB technique. This suggests that these CVCs may have been inserted for a short period of time (<24 hours) because microbial colonisation of a CVC surface often occurs just 24 hours after insertion (Raad 1998).

The results of FASEB culture were the basis for establishing true positive (>100 CFU/ml) and true negative (<100 CFU/ml) results for catheter colonisation (Kite et al. 1997). Unfortunately, in this study no clinical evaluation was carried out due to ethical
restrictions. This meant that details on the underlying condition of the patient, the
treatment being undertaken and whether antibiotics were infused through the CVC
lumen, were all unknown. Therefore information on which CVCs actually were
associated with bacteraemia or septicaemia was not available. This was a major
constraint and it is recognised as so by the author. Further work in this area will require
more patient information to be collected. Such information would be useful in
understanding the selective pressures that certain biofilm-forming microorganisms may
have been influenced by.

Using the DTAB extraction method, many of the samples reported ATP concentrations of
>1 pMol/L. If it were assumed that one bacterial cell has an ATP concentration of 2 x 10^{-18}
Mol/L (Lundin 2000) then 10^{12} Mol/L (or 1 pMol/L) would correspond to 500
microorganisms in 1ml of sample. However, in 33 (70.2%) samples where <500 CFU/ml
were observed >1pMol/L ATP was detected. Indeed within this study ATP concentrations
are generally in the pMol/L to nMol/L region rather than the fMol/L to the pMol/L range,
even-though 80.9% of samples reported less than 1000 CFU/ml after culture of the
FASEB-retrieved sample. This finding is consistent with the observation by Lundin
(2000) who suggests the ATP concentration can often correspond to higher cell numbers
than would be expected from CFU/ml count. Moreover, Nilsson et al. (1989) have
reported a similar situation where ATP concentrations determined for microorganisms in
aerobic blood cultures were much higher than the actual number of bacteria that appeared
to be present. A possible explanation for this may be that the removal of non-microbial
ATP from the FASEB-retrieved sample was incomplete because the ATP degradation
reagent was unable to access each individual somatic cell and/or unable to degrade all the
ATP present (Nilsson et al. 1989). A second possible explanation may be that a single
CFU is not necessarily originating from one distinct cell (Lundin 2000). Indeed
organisms such as staphylococci, exist in clusters and, therefore, a single CFU may
equate to many cells. A third reason may have been that the DTAB (0.8%) may not have
been at a high enough concentration to penetrate the glycocalyx of some of the biofilm-
forming microorganisms. Future work in this area should allow enough sample for direct
counting procedures such as microscopy so that more accurate counts can be obtained.
Due to the difficulty in relating ATP concentration to the CFU count, the application of a cut-off based upon ATP concentration and using the 100 CFU/ml FASEB significance level was evaluated. Thirty (63.8%) of the CVCs that were removed on suspicion of causing infection were subsequently categorised as negative following culture of the FASEB-retrieved sample (<100 CFU/ml). These samples consisted of 17 CVC tips that gave no growth and 13 that were colonised with low numbers of microorganisms. This proportion of negative CVCs is similar to that seen in other studies (Dobbins et al. 1999).

Of these 30 that would have been saved by FASEB culture, the ATP bioluminescence assay detected 26 (86.7%) if a cut-off limit of 50 pMol/L was applied between positive and negative results as determined by the ATP bioluminescence assay (Figure 6.13). However, only 8 (47.1%) of the samples that were reported positive by the FASEB culture method were detected at this cut-off limit. By using a lower cut-off limit of 10 pMol/L, 17 (56.7%) of the negative samples were detected using the ATP bioluminescence assay. Nevertheless, 12 (70.6%) of the positive CVCs were detected at this cut-off limit. A further reduction of the cut-off limit to 1 pMol/L ATP would ensure that all positive results were determined as the sensitivity of the assay would be high (close to 100%) resulting in as few false negatives as possible being achieved. However, the specificity at a cut-off limit of 1 pMol/L ATP would be low (<10%) resulting in many positive results (both true positives and false positives) which would ultimately lead to unnecessary CVC removals. Nevertheless, a low ATP concentration cut-off may be necessary so that false negative results are avoided. A possible solution to this dilemma of reducing the level of false positives without increasing the level of false negatives would be to combine the ATP bioluminescence assay with subsequent confirmatory plate culture. This method has been successfully used by Nilsson et al. (1989) when distinguishing between positive and negative blood cultures. In their particular study ATP concentrations were categorised as positive, borderline or negative, with follow up culture only on the borderline samples. Using this strategy a high level of sensitivity and specificity was achieved (Nilsson et al. 1989). By employing this method in the present study it may be possible to identify ATP concentrations at which the FASEB-retrieved sample is positive, borderline or negative. In the case of positive samples the CVC would be removed immediately and in the case of a negative result the CVC would be allowed to remain *in situ* within the patient. For results that would fall into the borderline category
the CVC would be left in situ but the patient would be monitored closely for any further symptoms of bacteraemia or septicaemia, before the confirmatory plate cultures are received. It would be envisaged, however that plate cultures would be carried out for all samples so that any microorganisms are identified and that appropriate antibiotic therapy or other clinical action is recommended.

In the present study, it may be possible to classify results, in terms of ATP concentration, as negative (<10 pMol/L), borderline (10 pMol/L to 50 pMol/L) and positive (>50 pMol/L). By classifying the results in this manner it was observed that 22 of the samples are negative and 12 are positive, with 13 (27.7%) borderline results. Of the 12 samples reporting an ATP concentration of >50 pMol/L ATP, four (33.3%) gave colony counts of <100 CFU/ml and are therefore false positive results. Five (22.7%) of the 22 negative results reported >100 CFU/ml and are false negative results. Of the 13 borderline results 9 reported <100 CFU/ml after plate culture. Therefore of the 30 that had <100 CFU/ml by FASEB culture the ATP bioluminescence assay detected 17 (56.7%) immediately and a further 9 after the borderline results were cultured to give a total of 26 and a specificity of 86.7%. Five of the CVCs were reported as false negative to give a specificity of 70.6%. Based upon this cut-off limit the PV+ of the assay is 75% and PV− of the assay is 83.9%.

Nine (19%) CVCs were identified incorrectly using the ATP bioluminescence assay and specified cut-off limits. There were four false positive results where the ATP concentration reported was high (>50 pMol/L) but <100 CFU/ml were cultured. CNS (10 CFU/ml) was cultured from one sample, whilst the other had no growth and another had 50 CFU/ml of an organism that was not identified. Yeast (20 CFU/ml) was cultured from the fourth sample, where an ATP concentration of 230.9 pMol/L was also reported. The high ATP concentrations in these samples may be explained by one or a combination of the following:

1. The most likely explanation is that all the contaminating non-microbial ATP was not removed from the sample prior to addition of the DTAB. This could be due to possible clumping of non-microbial cells within the sample and the inability of the Triton X-100 and/or the ATP degrading enzymes to fully penetrate these cells and consequently ATP remained within cells and/or debris, until DTAB is added. Sonication may have added value in this situation, but it was not evaluated as part of
this study. Future work should investigate the possible advantages of sonication for these types of sample.

2. There is also a possibility that microorganisms were present but could not be cultured. This has been observed before where Passerini et al. (1992) demonstrated that 76% (52/76) of catheters studied using SEM had bacterial cells present within the biofilm but only 32% of the catheters yielded significant numbers of bacteria when a proven scraping/sonication recovery method was employed. If this were the case then it is possible that the ATP bioluminescence assay is detecting viable microorganisms that cannot be cultured. Therefore the possible existence of SCVs cannot be ruled out as these would contain ATP which would be detected, but the SCV may be difficult to culture using conventional microbiological techniques (Bayston 1999, Krimmer et al. 1999). Bayston (1999) suggests that ATP generation is reduced in these cells, however the actual ATP turnover may be lower but the ATP concentration remains the same (Lundin, personal communication 2001). More research in this field will determine if the measurement of ATP is appropriate in these clinical samples. This is because the samples may contain mature biofilms, which have inherently lower ATP concentrations than would normally, be observed in planktonic cells (Bayston 1999).

3. It may be the case that the CFUs are not originating from one distinct cell. Indeed, as stated before, organisms such as staphylococci, exist in clusters and, therefore, a single CFU may equate to many cells (Lundin 2000). One of the samples contained 10 CFU/ml of CNS and it may be possible that more cells were present in clusters thus giving few CFU/ml. Further work that utilises total counting techniques (microscopy) would be useful for these samples.

4. In the case of the yeast sample these are much larger than bacterial cells and therefore have a greater volume of ATP. Consequently, a low number of yeast cells could give a relatively high concentration of ATP. Nevertheless, it is useful that ATP was detected in this sample as a CVC that is colonised by yeast, even at low levels, should be removed (Raad 1998, Wickham et al. 1992). Additionally some yeast are difficult to culture and therefore high ATP levels may be observed in cases where there are no growth.
Of the 22 negatives that reported <10 pMol/L ATP, 5 (22.7%) reported >100 CFU/ml after culture. These are false negative results, where mixed growth was observed in three of the samples, colonies of CNS were observed in the fourth and an unidentified organism was reported in the fifth sample. A concern with these samples is that significant levels of yeast, *S. aureus* and coliforms were cultured, and often when these organisms colonise a CVC there is a much higher risk of the colonisation leading to infection (Kite *et al.* 1997). In contrast CNS frequently colonise CVCs, as has been observed in this study, but are less likely to be implicated in septicemia. These 5 false negative results are more difficult to explain than the false positive results discussed above, because if viable cells are present in significant quantities then it would be expected that their intracellular ATP could be detectable. Possible reasons for this anomaly may be:

1. It is possible that the DTAB (>0.8%) was not concentrated enough to lyse the microorganisms that were present in these samples. Moreover, the DTAB may not have effectively broken down the glycocalyx that may have been present within these biofilm samples. A similar situation may have occurred here as has occurred with antimicrobial agents (Douglas 2003, Fletcher and Bodenham 1999a, Potera 1998, Vorachit *et al.* 1993) and a higher concentration of DTAB may be required. More work is necessary to determine if this concentration of DTAB is appropriate and effective in all possible FASEB-retrieved samples. The effectiveness of DTAB may be dependant on the maturity of the biofilm in addition to the species of microorganism involved in the colonisation of the CVC.

2. The signal generated during the ATP bioluminescence assay may have been quenched thus giving a low RLU value (Lundin 2000). This is unlikely, however, because an ATP standard was added at the end of each of the assays and there was no significant reduction observed in RLU count from the reaction between the bioluminescence reagents and the standard.

3. The ATP content of the microorganisms may have been affected by the components of the ATP degradation reagent. However, as the ATP degradation reagent was exposed to the whole sample and the fact that subsequent plate culture was possible would suggest that this is unlikely.
4. The filtration process may have in some way affected the concentration of ATP by stressing the organisms. Beumer et al. (1992) has observed that microorganisms such as *Campylobacter jejuni* have reduced ATP levels in response to adverse environmental conditions and stress. More development is necessary to determine the effects on ATP level of flushing microorganisms through a filter membrane.

5. The ATP content of SCV cells may vary with the length of time the CVC is in place and the maturity of the biofilm, with older biofilm cells possibly containing lower levels of ATP (Bayston 1999). It was not noted whether the cells that were cultured were actually SCV that may have reverted back to the normal phenotype when cultured on the blood agar plate. Further work in the area of biofilms and the physiology of biofilm cells may provide further evidence on the ATP content of these cells.

*Comparison of present technique with other rapid techniques*

*Sensitivity and specificity*

A sensitivity and specificity of 70.6% and 86.7% respectively, is comparable with studies that have sought to develop rapid techniques to detect catheter-associated infections. Elliott et al. (2000) reported a sensitivity and specificity of 75% and 90% respectively for an antibody assay test for diagnosis of CRI due to CNS. Further optimisation of this assay by Worthington et al. (2002a) resulted in an ELISA assay with a sensitivity of 70% and a specificity of 100%. However, a major disadvantage of this assay is the fact that only patients with catheter-associated infections caused by CNS can be diagnosed. This is because the antibody used in the detection system is specific for CNS. Therefore, based on Figure 1.10 in Chapter 1, this would suggest that infections caused by other organisms (up to 63%) would go undetected.

Other studies that have sought to develop rapid techniques have reported varying performance during evaluation. Five studies involving the AOLC method in conjunction with blood drawn through the CVC have been reported (Gowardman et al. 1998, Kite et al. 1999, Rushforth et al. 1993, Tighe et al. 1996b and von Baum et al. 1998). The study by Rushforth et al. (1993) suggested that the AOLC test had a high sensitivity and specificity (87% and 94% respectively) for the diagnosis of CRS using blood aspirated
through the CVC. However, the success of the test in this neonatal population could not be translated in adult populations (Gowardman et al. 1998, Tighe et al. 1996b, von Baum et al. 1998). Gowardman et al. (1998) reported that all twelve CVCs associated with microorganisms that were confirmed as the cause of CRBSI were not detected using the ALOC method (false negative results). These included 5 species of CNS, one yeast and one *S. aureus* amongst others. von Baum et al. (1998) demonstrated that the AOLC method was positive in 2 of 4 cases of CRBSI and the AOLC method detected organism is only 20% of all colonised catheters. Tighe et al. (1996b) reported 15 false negative results, however a higher level of sensitivity was reported when the AOLC method was used in combination with the FASEB. The combination of these techniques reduced the level of false negative result to three, where CNS was subsequently cultured from two CVC tips and *Streptococcus pneumoniae* from the other. Kite et al. (1999) demonstrated a higher level of specificity (92%) and sensitivity (96%) of the AOLC method when used in combination with Gram staining. Nevertheless, in this study of 128 samples five results were reported as false positive and two as false negative. It was observed that three of the false positive samples contained yeast that could subsequently not be cultured and it was reported that these yeast cells had the morphological appearance of *Malassezia* spp, which would require specialist culture conditions (Kite et al. 1999). The reason for differences in results within these studies may not only be due to the assay which was conducted but also the types of patients and duration of CVC placement. The studies by von Baum et al. (1998) and Gowardman et al. (1998) involved ICU patients, whereas Tighe et al. (1996b) and Kite et al. (1999) involved surgical patients and those receiving TPN. On the face of it, this may not seem to be an issue, however CVCs are generally in place for a longer period of time in the TPN patients than the ICU patients, therefore they are more likely to be colonised endoluminally (Cnich and Maki 2002b, Raad 1998). The mean duration of catheter placement in the Kite et al. (1999) study was 16 days, whereas in the Gowardman et al. (1998) study it was 6 days. Current thinking suggests that catheters in place for <10 days are less likely to be colonised on the internal lumen than CVCs in place for >10 days (Raad 1998). Therefore the results obtained by Gowardman et al. (1998) may not be unexpected.

Unfortunately in the present study, the duration of catheterisation was unknown, however as the catheters were all Arrow triple lumen catheters, these are generally placed for
periods of no more than 10 to 30 days. This could lead to a situation where colonisation would be more likely on the outside rather than the inside of the CVC lumen. This was observed in this study where microorganisms were isolated from the extraluminal surface of 37 CVCs and from the internal lumen of 30 CVCs. In future work this will be important information that should to be collected as CVCs which have been inserted for <10 days may report more CFU extraluminally than endoluminally.

Other studies that do not involve microscopy, but do require blood aspirated from the CVC have reported varying results. A prospective study by Blot et al. (1999) demonstrated that a cut-off DTTP value of 120 minutes had a 91% specificity and 94% sensitivity for the diagnosis of CRI. However, Rijnders et al. (2001) found that the DTTP was not useful for diagnosis of CRBSI in a mixed medical-surgical ICU. Four false positives and 1 false negative were reported leading to a sensitivity of only 25%. These patients had short-term multi-lumen CVCs and the mean duration of catheter placement was 12 days. This again suggests that the duration of catheter placement is crucial to the success of any rapid test involving blood aspirated from the CVC. Additionally, Rijnders et al. (2001) comments that 78 of the 100 patients were receiving antibiotics at the time of catheter removal. This is a possible reason for the fact that more patients had CRS without associated bacteraemia (5) than CRBSI (3). This too may have an effect of the efficacy of an assay based upon blood aspirated from the CVC. Therefore the sampling of planktonic cells may lead to false negative results especially if these cells have been exposed to bactericidal concentrations of a specific antibiotic. This again suggests that techniques which sample sessile organisms in a biofilm are more relevant to the detection of catheter-associated infection causing organisms (Wilcox et al. 2001). Additionally, the ATP bioluminescence assay may be better applied to CVCs that are in place for >10 to 30 days rather than CVCs in place for <10 days where extraluminal colonisation is more prevalent.

Total assay time

The ATP bioluminescence assay has a laboratory processing time to result of approximately 75 minutes from receipt of the FASEB-retrieved sample. This is much longer than the routine methods currently used in pathology laboratories (Cleri et al. 1980, Kite et al. 1997, Maki et al. 1977). However, these techniques require overnight
culture before a result is obtained. The present technique compares well with other rapid techniques that do not require CVC removal. The technique described by Blot et al. (1999) requires a laboratory processing time of at least 2 hours and the techniques described by Elliott et al. (2000), Worthington et al. (2002a) and Tighe et al. (1996b) require 12 hours, 4 hours and 1 hour respectively. More rapid techniques such as the Gram stain and AOLC test described by Kite et al. (1999) can be completed in approximately 30 minutes. The 75 minutes required by the ATP bioluminescence assay could be reduced by testing multiple samples simultaneously (in practice up to six samples could be processed at one time). Additionally, through further optimisation of the assay it may be possible to reduce the total assay time. This may be achieved by using other ATP degrading enzymes either in combination with, or instead of, the current dephosphorylation enzymes, or the addition of chemicals that could disrupt fibrin and thrombus so that all somatic cells are exposed to the Triton X-100. The use of Percoll density gradients or indeed the customisation of columns used in DNA preparation work may aid the process.

The possibility of supplying a tube containing the ATP degradation reagent to the hospital ward should also be investigated. In this way a FASEB-retrieved sample could be added to this reagent and much of the initial 60 minute incubation currently required could be completed before the sample reaches the pathology laboratory. However this may be difficult to control if the incubation is temperature sensitive.

Ease of use

The present assay is somewhat labour intensive in comparison to the routine conventional methods currently used and the DTTP method described by (Blot et al. 1998, 1999). However, many of the other rapid assays are labour intensive and require specialist training (Tighe et al. 1996b, Worthington et al. 2002a). Kite et al. (1999) comment that the Gram stain and AOLC technique is "easily learnt", which suggests that specialist training is required and that it is a technique that may not be familiar to all "on call" staff who provide emergency cover outside the normal 9 to 5pm working day. Moreover, Gowardman et al. (1998) found the AOLC test to be labour-intensive and demanding on skilled personnel for accurate performance and interpretation. The assay based on ATP bioluminescence has a number of individual steps, which makes it slightly
cumbersome, however through further development and optimisation it may be possible to merge certain steps so that less manipulations are required.

**Cost of assay**

Although the application of ATP bioluminescence technology is relatively expensive (~£6,000 for the luminometer and approximately a further £2.00 per test thereafter) when compared with an average of £0.35 for conventional microbiology culture (Kite, personal communication 2002), it is similar in expense to the other rapid methods that have been evaluated for use in CRBSI detection. Techniques such as the DTTP method (Blot et al. 1999), the ELISA method (Worthington et al. 2002a) and the AOLC method (Kite et al. 1999, Tighe et al. 1996b) all require specialist equipment and if these are not readily available in the routine pathology laboratory then initial capital outlay could be as high as £30,000. The accuracy and cost-effectiveness of the DTTP method and the method based upon Gram stain and AOLC have been discussed by Farr (1999) in a commentary in the Lancet. Farr (1999) suggests that further studies be carried out by other investigators, within other clinical settings, before either method is recommended for use. Farr (1999) also suggests that of the two the DTTP method would be easier to set up as the continually monitoring blood-culture systems are used in most clinical microbiology laboratories. However, like the ELISA technique (Worthington et al. 2002a) the ATP bioluminescence assay would require specialist equipment and reagents, which could be expensive. Both are more suited to batch testing as control samples (positive and negative) may be needed. Additionally, the current assay requires various consumables, such as disposable syringes and syringe filters. These could again be removed through optimisation of the assay or through automation of some of the steps.

**Overall performance**

The ATP bioluminescence technique in combination with a FASEB-retrieved sample compares favourably with other rapid techniques that are currently available. Its sensitivity and specificity, of 70.6% and 86.7% respectively, are adequate, in comparison to some of the techniques that have been evaluated. However, further improvements are required in terms of ease-of-use, cost and total assay time. This technique has a number of advantages and disadvantages, with one of the major issues that needs to be addressed
being the number of individual steps that are required in order to prepare the sample prior to ATP quantification.

Conclusions

As a result of this study a number of conclusions can be made. These have been separated into those associated with assay development and those made as a result of the evaluation of the developed method.

Assay Development

- The ATP degradation reagent, comprising of apyrase (grade III, 10 Units/ml), 5' adenyllic acid deaminase (0.5U/ml), Triton X-100 (0.1% v/v) in excess magnesium ions can be used to lyse blood cells and degrade up to 99% of ATP that is subsequently released.

- Use of the ATP degradation reagent in combination with syringe filtration increases the level of ATP removal from blood cells in the sample to beyond 99%. The remaining ATP equates to approximately 50 pMol/L in platelet-rich plasma (1:50) samples and 500 pMol/L in heparinised blood (1:50) samples.

- The use of DTAB at a final concentration of >0.8% to extract ATP from selected clinical isolates associated with catheter infections, was equally as effective as the standard TCA (2.5%) method recommended by Lundin (2000).

- Both TCA (2.5%) and DTAB (>0.8%) will irreversibly inhibit the activities of apyrase (10 Units/ml) and 5'-adenyllic acid deaminase (0.5U/ml).

- Neutralisation of the final DTAB concentration (0.08%) was achieved with a final concentration of 0.5% α-cyclodextrin.

- The use of α-cyclodextrin in combination with DTAB increased the volume of microbial ATP extract that could be assayed by 10-fold, when compared with the TCA extraction method.
• By using 13mm syringe filters the volume of buffer required to elute microbial ATP from the filter is reduced from 2.5ml (required by 25mm filters) to 1.0ml.

• The Mediators L1 luminometer was found to be a more appropriate luminometer than the Bioprobe for this application. This was mainly due to the close proximity of the sample to the photo-detector. The L1 also had a sufficient linear range to allow the detection of low levels of microbial ATP in combination with the HS luciferase reagent.

Assay Evaluation

• The ATP bioluminescence technique (outlined in Figure 3.1 – the DTAB extraction technique) in combination with a FASEB-retrieved sample compares favourably with other rapid techniques that are currently available, in terms of sensitivity, specificity, ease-of-use, cost and total assay time.

• By using the cut-off limits for negative (<10 pMol/L), borderline (10 pMol/L to 50 pMol/L) and positive (>50 pMol/L) it was observed that the ATP bioluminescence assay has a sensitivity and specificity of 70.6% and 86.7% respectively, with a PV+ of 75% and a PV− of 83.9% for CVCs associated with significant colonisation (>100 CFU/ml).

• Even though four false positive and five false negative results were reported, this assay based on the cut-off limits described provided accurate information on 26 CVCs that were true negatives and 12 that were colonised (true positive results).

• The use of this ATP bioluminescence technique in combination with a FASEB-retrieved sample may assist in the rapid detection of CVCs associated with suspected bacteraemia in catheterised patients.

Overall Conclusions

This study indicates that ATP bioluminescence is a useful technique that may be used in conjunction with FASEB-retrieved samples to detect or eliminate catheter-associated infections without removing the CVC. It is the first time that ATP bioluminescence has
been applied as a technique to the area of catheter-associated infection diagnosis. In addition, this is the first study, which has investigated directly whether a FASEB-retrieved sample can be used in combination with rapid detection technologies.

Other novel features that were successfully developed and may add to the literature already available in the field of ATP bioluminescence include, the development of the ATP degradation reagent and its use in combination with the 13mm syringe filters, which provided a novel approach to degrading high levels of contaminating ATP prior to microbial cell lysis. Degradation of relatively high concentrations of ATP in blood samples has been achieved in this study, with previous studies degrading much lower ATP concentrations in laboratory-spiked broth samples (Sakakibara et al. 1997). Moreover, the application of a DTAB-based extraction method, instead of the TCA-based microbial cell extraction method, was successfully demonstrated and the subsequent neutralisation of DTAB by α-cyclodextrin was successfully achieved and applied to samples in this study. This again is a new approach that had not been applied to clinical samples.

Further work will be required to improve upon the sensitivity and specificity of the final proposed testing method (Figure 3.1– the DTAB extraction technique), with the main emphasis being on the sample preparation so that all contaminating ATP is degraded and all microbial cells are exposed to the microbial lysis agent prior to ATP quantification. Suggestions for further development and optimisation are detailed in the following section.

**Further work**

This study demonstrates that ATP bioluminescence is a useful technique for detecting low numbers of microbial cells. The application of this technique to a FASEB sample retrieved from a CVC tip that has been removed from a catheterised patient has also shown some potential but a number of issues have been raised. These issues are best discussed in reference to Figure 2.7 where the critical steps to method development where identified. The present study has demonstrated that Step 3 can be achieved through
technology that already exists, however, a number of issues remain unresolved within Steps 1 and 2.

In Step 1, the goal was to lyse non-microbial cells and release their intracellular ATP for subsequent degradation without affecting the ATP concentration of the microbial cells. The combination of the ATP degradation reagent and syringe filtration was adequate to some degree but contaminating ATP remained. Further work is necessary to reduce and/or completely degrade this contaminating ATP. Some possible ways of doing this have been identified and are listed below.

- Use of micro-centrifuge filters instead of syringe filters, where more sample could be forced through the filter membrane in a controlled manner, and leaving intact cells imbedded in the membrane. However, the effect of the stress of the filtration process needs investigation.

- Use of Percoll® density gradients in conjunction with a centrifugation step to separate blood cell debris from microorganisms. Some work using this technique to degrade background ATP present in blood samples has been attempted in the past (Molin et al. 1983, Nilsson et al. 1989). Additionally, the application of techniques such as the use of DNA preparation columns should be investigated.

- Identification of other ATP-degrading enzymes which could be used in combination with, or instead of, the current constituents of the ATP degradation reagent, for example adenosine phosphate deaminase (Sakakibara et al. 1997).

- The addition of chemicals or enzymes that could disrupt fibrin and thrombus so that all somatic and microbial cells are exposed and no clumps of cells exist. The use of sonication may be advantageous.

The aim in Step 2 was to develop a method for microbial cell lysis so that all the intracellular ATP is released and all ATP-degrading enzymes are simultaneously and irreversibly inhibited. In the present study two such compounds, namely TCA and DTAB were found to be useful in that, at an appropriate concentration, all microbial cells were lysed and the ATP-degrading enzymes were inhibited. However, as pointed out in Table 2.5, these, like many microbial ATP extractants, are potent inhibitors of luciferase, thus they need to be diluted or neutralised prior to ATP detection. Further work here would be
beneficial as the more extracted sample that can be used in the final ATP determination then the greater the sensitivity of the technique. Some possible improvements are detailed below.

- Investigate further if DTAB has the capability of releasing ATP from actual biofilm (that is, the capability of penetrating glycocalyx) rather than planktonic microorganisms.

- Optimise the reaction between α-cyclodextrin and DTAB.

- Determine if there are other compounds available that could be used to lyse microbial cells and simultaneously inhibit ATP degrading enzymes before being completely neutralised so as not to affect the luciferase reaction; for example another member of the cyclodextrin family (Lundin et al. 1994).

- Use boiling buffers and allow most of the liquid to evaporate prior to ATP concentration determination, so that the effects of dilution are not a factor.

There is a possibility of improving the method through at least one of the points raised above or through a combination of the points, identified for both Steps 2 and 3 from Figure 2.7.

In addition to these, another area where improvements could be made is through the use of samples that are more representative of the internal lumen of a CVC. For example, attempting to grow a biofilm within a CVC in vitro and then using this as a standard sample. This could be more appropriate than using blood.

In summary, this work does indicate that ATP Bioluminescence technology can be used with this type of CVC sample. Further work will be required in some of the areas identified above, in order to improve sample preparation so that an adequate sample is obtained for accurate ATP concentration determination. A future evaluation should involve FASEB samples retrieved for patients whilst the CVC remains in situ so that the effects of variables such as CVC tip storage duration are avoided.

The Mediators L1 luminometer was seen to be an extremely useful instrument, which had a good linear range and was sensitive at the low end. The detection reagents supplied by Biothema AB were also seen to be useful, with the HS reagent being more sensitive than
the SL reagent. Both this instrument and the HS detection reagent are recommended for use in further development.
REFERENCES


References


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References


Appendix 1
Scanning Electron Microscopy (SEM)

Scanning Electron Microscope
Samples were observed using a Hitachi S-4000 scanning electron microscope.

Materials

Sodium cacodylate (1M)
A stock solution of sodium cacodylate (1M) (Sigma Aldrich, UK) was prepared and buffered to pH 7.2 using HCl (1M). This was stored at room temperature until required.

Solution containing glutaraldehyde (3% v/v) and Sodium cacodylate (0.1M)
A stock of glutaraldehyde (3% v/v) and sodium cacodylate (1M) was prepared by dissolving 10ml of glutaraldehyde (25%) (Sigma Aldrich, UK) in 65ml of sterile water and 8.33ml of the sodium cacodylate (1M).

Preparation of CVC tips
The CVC tips were selected and these were sectioned longitudinally and transversely using a sterilised double-edged razor blade (which was sterilised with alcohol).

The sections were then fixed in a solution containing glutaraldehyde (3% v/v) in sodium cacodylate (1M) for 3 hours. The samples were then exposed to the following series:

• Washed in sodium cacodylate (0.1M) (3 x 10 minutes),
• Washed in distilled water (3 x 10 minutes),
• Dehydrated in an ethanol series of 20%, 40%, 60%, 80%, 90% and 100% (v/v) ethanol for 10 minutes each,

The samples were then placed in acetone and stored at room temperature.

Further processing of the sample
The samples were removed from the acetone and mounted on aluminium stubs. These were then coated with gold using an Edwards sputter coating device (S150B). The samples were then observed using a Hitachi S-4000 scanning electron microscope at an accelerating voltage of 20.0kV. The software package was set up so that the scale bar was automatically placed on the image. The images were saved to zip disc on a Dell computer.
Appendix 2
ATP Kit SL 144-041

Instructions for use

Intended use
ATP Kit SL is intended for monitoring of adenosine triphosphate (ATP) over the range of $10^{-12}$ to $10^{-6}$ mol/L. The low decay rate of the light emission (around 0.5%/min) is due to a low consumption of ATP in the firefly luciferase reaction and a luciferase activity that is unchanged during the time of the measurement. Under these circumstances measurement of the intensity of the light emission allows continuous monitoring of formation or degradation of ATP in enzymatic reactions or release of ATP from cells. Thus assays can be performed in much the same way as NAD(P)H is spectrophotometrically or fluorometrically monitored in assays based on dehydrogenase reactions. Furthermore the stable light makes assays even of a constant ATP level more convenient and reliable, particularly when using manual luminometers. If a higher sensitivity is required BioThema has two other reagents allowing the detection of $10^{-17}$ mol and $10^{-19}$ mol, resp. Please cf. our Website: www.biothema.com.

Assay principles
Firefly luciferase catalyses the following reaction:

$$\text{luciferase} \ \ ATP + D-\text{luciferin} + O_2 \rightarrow AMP + PPI + \text{oxy Luciferin} + CO_2 + \text{light}$$

The assay has been optimised to give a stable light at all ATP levels up to $10^{-6}$ mol/L. The reagent contains D-luciferin, magnesium ions, PPI (inorganic pyrophosphate) and BSA (bovine serum albumin).

Kit contents
1. ATP Reagent SL. 4 vials of lyophilised reagent containing D-luciferin and luciferase.
2. Diluent C 10 mL. 4 vials containing ATP free distilled water.
3. Tris-EDTA Buffer 50 mL. 4 bottles containing 0.1 mol/L Tris(hydroxymethyl) aminomethane, 2 mmol/L EDTA and adjusted to pH 7.75 with acetic acid.
4. ATP Standard 5 mL. 4 vials containing $10^{-17}$ moles/L of ATP.

The kit should be stored at -18 °C. However, during normal transport times the kit is not affected by ambient temperature. If used within a week the kit may be stored at +4 °C. The kit and the individual components are labelled with expiry date assuming storage at -18 °C.

Reagent reconstitution
The ATP Reagent is reconstituted by adding the entire content of the Diluent C vial (10 mL) to the reagent vial. It is very important to avoid contamination. Please use a clean pair of tweezers to remove the rubber stopper from the ATP Reagent vial. Pour the diluent gently into the reagent vial to dissolve the lyophilised reagent. Subsequently pour the solution back into the Diluent C vial. Once reconstituted the reagent must be protected from light. In reconstituted ATP Reagent the luciferase activity slowly decays. However, calibrating the assays with ATP Standard makes it possible to use the ATP reagent for several days at +4 °C or for 1 month at -18 °C. Reagents should be allowed to attain room temperature before the assay (the optimum temperature for the luciferase reaction is around 25 °C).

Instrumentation
The stable light makes it possible to use manual single tube luminometers, automatic tube luminometers or microplate luminometers. The detection limit obviously depends on the luminometer. With most luminometers $10^{-17}$ moles of ATP can be detected.
**Assay procedure using internal ATP Standard**

In this procedure a known amount of ATP is added in the assay of each individual sample. This strongly increases the reliability of the assay and makes it possible to express ATP results in moles rather than fM or other non-chemical units. The assay can also be performed using external ATP Standard. However, this is a less reliable procedure in most cases. Please consult BioThema for advice, if you are considering using external ATP Standard.

A. Tube luminometer

1. Add sample and Tris-EDTA Buffer to give a total volume of 0.8 mL in the cuvette.
2. Add 0.2 mL ATP Reagent SL.
3. Measure the light emission corresponding to sample ATP, \( I_{\text{exp}} \).
4. Add 10 \( \mu \)L of ATP Standard.
5. Measure the light emission corresponding to sample plus standard ATP, \( I_{\text{exp, std}} \).
6. Calculate the sample ATP concentration in the cuvette by the following equation:

\[
\text{ATP}_{\text{m, p}} = 10^7 \times \frac{I_{\text{exp}}}{(I_{\text{exp, std}} - I_{\text{exp}})}
\]

The factor \( 10^7 \) is the concentration of ATP Standard in the cuvette (10 \( \mu \)L \( 10^{-5} \) mol/L in a total reaction volume of 1 mL).

B. Microplate luminometer

1. Add sample and Tris-EDTA Buffer to give a total volume of 160 \( \mu \)L in the cuvette.
2. Add 40 \( \mu \)L ATP Reagent SL.
3. Measure the light emission corresponding to sample ATP, \( I_{\text{exp}} \).
4. Add 10 \( \mu \)L of ATP Standard diluted 1/5 in Tris-EDTA buffer.
5. Measure the light emission corresponding to sample plus standard ATP, \( I_{\text{exp, std}} \).
6. Calculate the sample ATP concentration in the cuvette by the following equation:

\[
\text{ATP}_{\text{m, p}} = 10^7 \times \frac{I_{\text{exp}}}{(I_{\text{exp, std}} - I_{\text{exp}})}
\]

The factor \( 10^7 \) is the concentration of ATP Standard in the cuvette (10 \( \mu \)L \( 0.2 \times 10^{-5} \) mol/L in a total reaction volume of 0.2 mL).

**Procedural notes**

1. The sample volume should be chosen to give an ATP level in the cuvette in the interval \( 10^{-12} - 10^{-6} \) mol/L (lower limit somewhat dependent on luminometer sensitivity). For maximum precision a sample volume should be chosen to give an ATP level in the interval \( 10^{-10} - 10^{-8} \) mol/L. High sample ATP levels approaching the concentration obtained from the ATP Standard tend to give too low values on \( I_{\text{exp, std}} \), resulting in an overestimation of ATP\(_{\text{m, p}}\). The sample volume should also be chosen to avoid unnecessary inhibition of light emission from sample components (e.g. extractants). An inhibition of up to 50% is normally not a problem, since the effect is compensated for by the internal ATP Standard.

2. The ATP Standard volume should be as low as possible with a retained precision. Ideally the volume should be <1% of the total reaction volume, since the dilution effect coming from the addition of ATP Standard otherwise cannot be neglected. With a total reaction volume of 1 mL it is acceptable to use 10 \( \mu \)L. With a total reaction volume of 200 \( \mu \)L an ATP Standard volume of 10 \( \mu \)L is somewhat too high, but has to be accepted since lower volumes of ATP Standard can not normally be added with high precision.

3. The light emission should be measured as soon as possible after addition of reagents (ATP Reagent and ATP Standard, resp.). Maximum light emission is normally obtained within a few seconds and will then start to decay by 0.5 %/min.

4. Every series of assays should include at least two blanks in the beginning and at the end. Blanks should contain all reagents added in preparation of samples (e.g. extractants used for releasing ATP from cells) and should be assayed and calculated exactly as normal samples. Blanks calculated by the formula above are subtracted from sample ATP values calculated in the same way. The ATP concentration in the original biological sample is then calculated by multiplying with the dilution in the assay and in the preparation of the sample for the assay.
Appendix 3
Data sheet for CVC tips

Copies of the sheet on the following page were supplied to St George’s Hospital (London) Medical Microbiology Department. The details were filled out for each CVC tip retained for this study. The corresponding number (1 to 20) from the sheet was written on the lid of the sample pot containing the CVC tip. The label below was placed on the sample pot and the relevant details recorded.

For CRS Research Project

For One Catheter Tip Only

Patient Number: __________
Date Received: __________
Store at 4°C

The laboratory number corresponded to the department’s code given to each sample received in the laboratory. This was recorded so that clinical data could be traced on certain patients, however it was not required as ethical approval was not granted to access patient details.

The catheter type was recorded as either, CVC or PICC. On occasion, further details were recorded, such as the source of the CVC (femoral, subclavian or IJ). Other CVC tips were investigated.

The results of the Maki culture were also recorded, as well as the predominant species identified. In cases of mixed growth as many isolates as possible were identified.
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