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GRAM-POSITIVE INFECTIONS IN PATIENTS WITH END-STAGE RENAL DISEASE

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Doctor of Philosophy

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November 2004

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THE UNIVERSITY OF ASTON IN BIRMINGHAM

Gram-positive infections in patients with end-stage renal disease.

A thesis submitted by Mercia Kathleen Spare BSc (Hons) RGN

For the degree of Doctor of Philosophy

2004

SUMMARY

Gram-positive microorganisms, specifically coagulase-negative staphylococci are the most common species recovered from clinical culture specimens of patients with end-stage renal disease. The propensity of coagulase-negative staphylococci (CNS) to cause infection in this patient group has been widely debated. However, it is still unclear how this usually avirulent commensal microorganism produces infection that contributes to high rates of morbidity and mortality in patients with end-stage renal disease. The aim of this thesis was to investigate the rate, geographical distribution, molecular and phenotypic mechanisms of Gram-positive microorganisms associated with infection in renal dialysis patients. In addition, it sought to assess the value of early serological diagnosis of dialysis catheter-associated infection and the effect of antimicrobial treatment regimens on the faecal carriage of enteric microorganisms.

In this study, the incidence of haemodialysis catheter-associated infection was established with the Meditrend audit tool. This tool was used to assess the infection outcomes of catheter insertion and management procedures until the catheter was explanted. Introduction of a catheter management protocol decreased the incidence of catheter-related infection. Staphylococcal species recovered from episodes of haemodialysis catheter-associated infection and continuous ambulatory peritoneal dialysis (CAPD)-associated peritonitis were genotyped by determination of macrorestriction profiles with pulsed-field gel electrophoresis. This highlighted horizontal transfer of microorganisms between different patients and the environment. The phenotypic characteristics of these strains were also investigated to determine characteristics that could be used as markers for dialysis catheter-associated infection. The expression of elastase, lipase and esterase by CNS was significantly associated with infection. A rapid enzyme-linked immunosorbent assay incorporating a novel staphylococcal antigen (lipid S) was used to evaluate the early detection of anti-staphylococcal immunoglobulin gamma in patient sera. The comparison of culture positive and culture negative patients demonstrated a steady state of immune activation in both groups. However anti-lipid S serum antibody titres >1000 were found to be a predictor of infection. The effect on faecal carriage of vancomycin resistant enterococci (VRE) and *Clostridium difficile* toxins in patients treated with CAPD when empiric cephalosporin therapy was substituted for piperacillin/tazobactam was investigated. The introduction of piperacillin/tazobactam demonstrated a decrease in the faecal carriage of VRE.

Key words: Dialysis catheter infection, Pulsed-field gel electrophoresis, Serodiagnosis, Phenotypic characteristics, Surveillance.

DEDICATION

This body of work is dedicated to my dad

Albert David Spare 1923-2004

ACKNOWLEDGEMENTS

A would firstly like to extend very special thanks to every person who has touched my life; You have helped shape my future.

I would like to thank Professor Tom Elliott, Dr. Peter Lambert and Dr. Tony Worthington for the opportunity to progress and for their faith in me to get the job done.

Dr. Sarah Tebbs without whom I would not be submitting this thesis. You made everything feel possible.

Dr. Sue Lang for her endless help and support. You are the 'Master chef'. Thank you so much.

Renal consultants Graham Lipkin, Simon Ball, Paul Cockwell and Dwomoa Adu, without whose commitment to bettering the lives of their patients, I could not have done it. Thank you for your continual support.

Tracey Prescott and her fantastic team of nurses whose never-ending patience and dedication helped keep it all going. Thank you.

All of the patients accessing the renal service at UHB who have inspired me on a daily basis.

The Microbiology department at the UHB, who have helped me more than they will ever know. You are amazing.

The microbiology department at Aston University who have accommodated my every need. Thank you for the glassware. With special thanks Roy McKenzie, Lynda Birt and Rita Chohan.

Anna Taylor for her magical skill with citation and her long-time support. I'm sorry it took so long.

Clair Phillips for her humour, calmness and her ability to find a silver lining!

Andrew Traube, Karen Jones, Dr. Alan Perris, Dr. Ann Vernallis and Anna Casey; You have been my most precious support network, thank you for all you have given me. I have been blessed to have you as my friends.

Dr. Mike Allen at Wyeth Pharmaceuticals whose commitment to and enthusiasm for my work has driven me forward especially through difficult times. You continually restore my faith in human nature. Thank you to Wyeth Pharmaceuticals who supported this work with an educational grant.

Last but by no means least; I would like to thank my family for their love and support. Thank you for believing in me.

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ABBREVIATIONS

Å	Angstrom
AAP	Accumulation associated protein
ANOVA	One-way analysis of variance
AOLC	Acridine orange leucocyte cytospin
API	Analytical profile index
ARF	Acute renal failure
AtlE	Autolysing Enzyme
ATP	Adenosine triphosphate
AVF	Arteriovenous fistula
BA	Blood agar
BHI	Brain heart infusion broth
BNF	British national formulary
BSAC	British Society of Antimicrobial Chemotherapy
BSI	Bloodstream infection
CANUSA	Canada and United States of America
CAPD	Continuous ambulatory peritoneal dialysis
CDAD	*Clostridium difficile* associated diarrhoea
CDC	Centre for disease control
CHEF	Contour-clamped homogenous electric field
CNS	Coagulase-negative staphylococci
CRF	Chronic renal failure
CRP	C reactive protein
CRI	Catheter-related infection
CVC	Central venous catheter
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DOH	Department of health
DSN	Dialysis surveillance network
EAP	Extracellular adherence protein
ECM	Extacellular matrix
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPIC	Evidence-based practice infection control group

ES	EDTA, sarcosyl
ESI	Exit site infection
ESP	EDTA, sarcosyl, proteinases K
ESR	Erythrocyte sedimentation rate
ESRD	End stage renal disease
ESS	Extracellular slime substance
FAME	Fatty acid modifying enzyme
FDA	Food and Drug Administration
FPLC	Fast protein liquid chromatography
GFR	Glomerular filtration rate
GOV	Government
GRE	Glycopeptide resistant enterococci
h	Hour
H_2O_2	Hydrogen peroxide
HAI	Hospital Acquired Infection
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HICPAC	Hospital Infection Control Practices Advisory Committee
HLR	High level resistance
HPA	Health Protection Agency
HPLC	High performance liquid chromatography
IgA	Immunoglobulin alpha
IgD	Immunoglobulin delta
IgE	Immunoglobulin epsilon
IgG	Immunoglobulin gamma
IgM	Immunoglobulin mui
IP	Intraperitoneal
IV	Intravenous
Kb	kilo-base pairs
KDa	Kilodaltons
KDOQI	Kidney Dialysis Outcomes Quality Initiative
LLOQ	Lower limit of Quantification

LTA	Lipoteichoic acid
M	Molar
mg	Milligram
µg	Microgram
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
mM	Millimolar
MMWR	Morbidity and Mortality Weekly Report
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesion matrix molecules
MSSA	Methicillin sensitive *Staphylococcus aureus*
N	Normal
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogenase
NAO	National Audit Office
NCTC	National Collection of Type Cultures
NET-100	Tris-buffered saline EDTA
NHS	National Health Service
NICE	National Institute for Clinical Excellence
nm	Nanometers
NSAIDS	Nonsteroidal anti-inflammatory drugs
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PD	Peritoneal dialysis
PFGE	Pulsed-field gel electrophoresis
PHLS	Public Health Laboratory Services
PIA	Polysaccharide intercellular adhesion
PMN	Polymorphonuclear neutrophils
PS/A	Polysaccharide adhesin
QCH	Quality control high
QCL	Quality control low
QCM	Quality control medium
QEH	Queen Elizabeth Hospital

RAPD	Random amplification of polymorphic DNA
RBC	Red blood cells
RE	Restriction endonuclease
RNA	Ribose nucleic acid
RRT	Renal replacement therapy
SAA	Slime-associated antigen
Sarcosyl	N-lauroyl-sarcosine
SSP-1	Staphylococcal surface protein 1
SSP-2	Staphylococcal surface protein 2
TBE	Tris borate ETDA
TBS	Tris buffered saline
TBST	Tris buffered saline and Tween 20
TE MED	N,N,N'N'-tetramethyl-ethylenediamine
TE	Tris EDTA
Tris	Tris [hydroxymethyl] aminomethane
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UHB	University Hospital Birmingham
UK	United Kingdom
UPGMA	Unweighted pair group method of arithmetic averages
USA	United States of America
USRDS	United States renal data system
UV	Ultra violet
v/v	volume for volume
VRE	Vancomycin resistant enterococci
VRSA	Vancomycin resistant *staphylococcus aureus*
w/v	Weight for volume
WCC	White cell count
WHO	World Health Organisation
WWW	World Wide Web

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Spare MK., Adu D., Lipkin G., Ball S., Worthington T., Lambert PA., Elliott TSJ. The Relationship of Antibiotic Use on Carriage and Infection with Vancomycin Resistant Enterococci and *Clostridium difficile* in Renal Patients. Poster presented at the 35th Annual Meeting and Scientific Exposition of The American Society of Nephrology. Philadelphia, USA. November 1st – 4th 2002.

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CHAPTER 1: INTRODUCTION

1.1 RENAL DISEASE

The kidneys perform multiple roles that include metabolic, endocrinologic and fluid and electrolyte balance. The index of functioning renal mass is measured by the glomerular filtration rate (GFR). This represents the rate at which fluid is filtered from the glomerular capillaries across endothelial-capsular membranes, into the capsular space (figure 1.1). The GFR of a healthy kidney is approximately 180 litres per day (125mL/min) (Tortora and Grabowski, 1993).

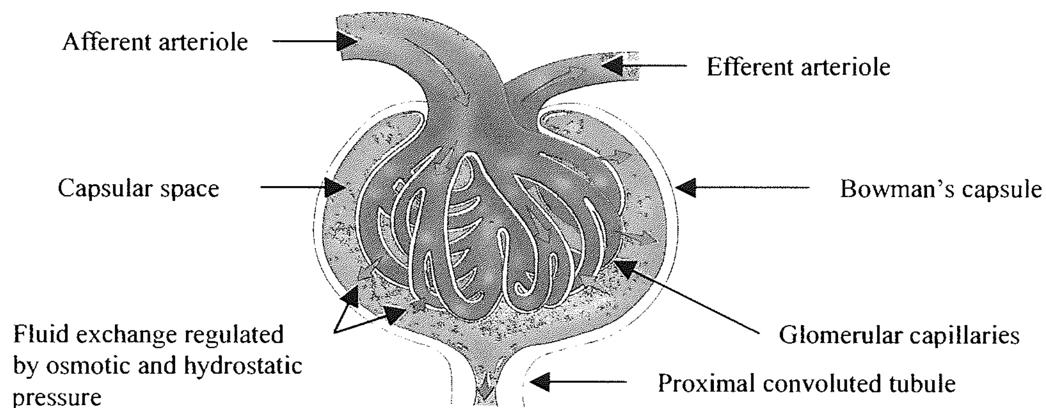


Figure 1.1 Schematic of glomerular filtration (adapted from Tortora and Grabowski, 1993)

Acute renal failure (ARF) is defined as precipitous and significant (>50%) decrease in GFR over a period of hours to days, with an accompanying accumulation of nitrogenous wastes in the body (Sinert, 2002). Causes of ARF include decreased renal perfusion, diseases of the glomerulus or tubule associated with release of afferent vasoconstrictors and obstruction. A consequence of ARF is reduced renal blood flow. Depressed renal blood flow leads to ischaemia and cell death. The ischaemic insult triggers the production of oxygen free radicals and enzymes that continue to cause injury even after the restoration of renal blood flow. Tubular cellular damage results in disruption of tight junctions between cells allowing the 'back leak' of glomerular filtrate and promotes depression the GFR. In addition, dying cells slough off into the tubules, forming obstructions that further decrease the GFR, and lead to oliguria. Recovery from ARF is primarily dependent upon the rapidity of restoration of renal blood flow and removal of tubular obstructions to increase GFR. Total recovery is dependent upon the size of the remaining nephron pool. Early normalisation of renal blood flow predicts

better recovery of renal function. There is currently no known methodology to regain lost kidney function.

Chronic renal failure (CRF) is a consequence of progressive disease that includes immunoglobulin A nephropathy, hypertension, diabetes and systemic lupus erythematosus. Renal failure that requires long-term renal replacement therapy (dialysis or transplantation) is known as end-stage renal disease (ESRD). The term 'end-stage renal disease' is used to describe the gradual erosion of kidney function to a point where GFR falls to less than 20% of normal (25mL/min). In the United Kingdom (UK), 333,363 people were receiving renal replacement therapy in 2001 (Renal Registry, 2002) of which approximately 800 people access dialysis services at University Hospital Birmingham NHS Trust (UHB).

In the USA diabetic nephropathy, hypertension and glomerulonephritis cause approximately 75% of all adult cases of ESRD (Krause, *et al.*, 2002). In the UK these factors accounted for <40% of the conditions leading to consultation by a renal physician (Renal Registry, 2002). Diabetic nephropathy as a cause of renal failure is seen in 18% of new patients, which is lower than in the United States of America (USA) and much of Europe (Renal Registry, 2002).

Patients with ESRD often have a complex array of comorbid processes that reduce the survival rate and impede the management of their renal disease; such processes include ischaemic heart disease, diabetes and immunodeficiency. Management of patients with ESRD is therefore a complicated process. The long-term goal, where possible, is transplantation, however this method of treatment presents with its own complications due firstly to a lack of organ donors and secondly to organ rejection. In 2001 only 46.6% of ESRD patients in the UK benefited from transplantation (Renal Registry, 2002), of the remaining patients 37.1% were managed by means of haemodialysis and 16.3% by peritoneal dialysis. It is estimated that there are approximately 15,000 people in the UK being treated with dialysis (Smith, 2002). The National Renal Register (2002) reported that the number of patients being treated with dialysis is increasing annually by 566 per million of the population (7%). This is attributed primarily to the ageing population, increasing life expectancy of patients with a high risk of ESRD and the growth of population subsets with a greater risk of ESRD. Without dialysis, the patient is unable to regulate electrolyte, fluid and acid-base balance. This results in an accumulation of toxic products from amino acid metabolism in the serum. Presenting clinically with a range of symptoms that include anorexia, nausea, peripheral neuropathy,

anaemia and abnormalities in white cell and platelet function, leading to increased susceptibility to infection. The 2002 annual report from the Renal Registry announced a 33% increase in infection related deaths in renal patients (Renal Registry, 2002).

1.2 RENAL REPLACEMENT THERAPY

There are two main methods of renal dialysis: haemodialysis, which achieves filtration of metabolic by-products from the blood via an external artificial semi-permeable membrane and peritoneal dialysis that employs the peritoneal membrane to achieve haemofiltration via the peritoneal capillaries.

1.2.1 Haemodialysis

Haemodialysis is generally performed three times per week (alternate days); each session lasts approximately 4 hours. Dietary and fluid intake can be extremely restricted and compliance to the regime dictates the volume of fluid that is removed at each dialysis session.

Haemodialysis requires the cycling of blood via a dialysis membrane (dialyser). The dialyser consists of thousands of hollow-fibre capillaries bundled tightly together. When blood enters the dialyser, it is forced to flow through the centre of the capillaries while the dialysate is forced to flow around the outside of the capillaries.

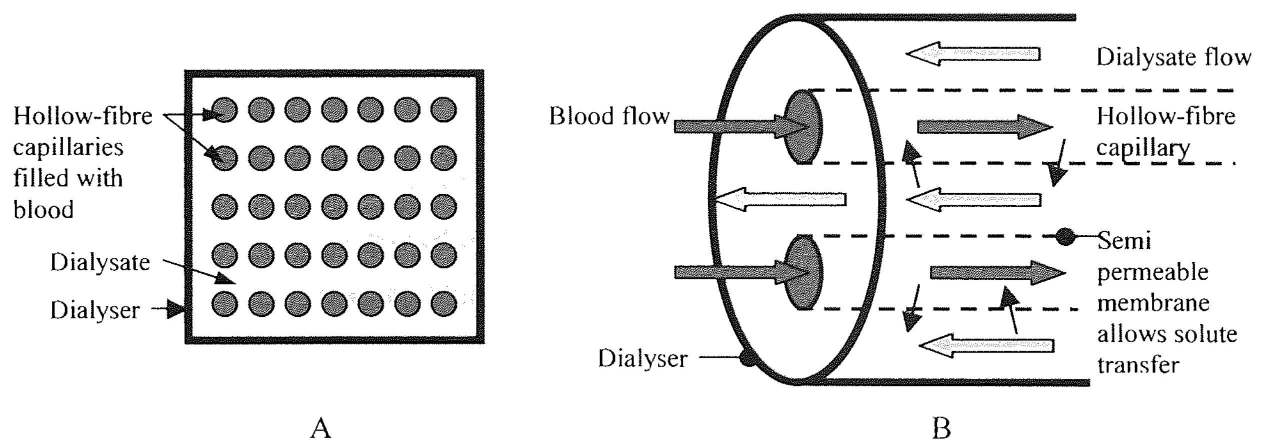


Figure 1.2 Schematic diagrams of dialyser A, composition and B, dialyser function.

Blood is drawn from the patient, circulated via a pump at 35-37°C through the dialyser and returned to the patient. At the same time, dialysate containing pre-determined concentrations of electrolytes is pumped through the dialyser in the opposite direction forming a

concentration gradient. This facilitates the transfer of solutes through the dialyser membrane by means of diffusion and ultrafiltration. Arterial and venous blood pressure is monitored at strategic points of the cycle and anticoagulants are added to prevent blood clotting (figure 1.3).

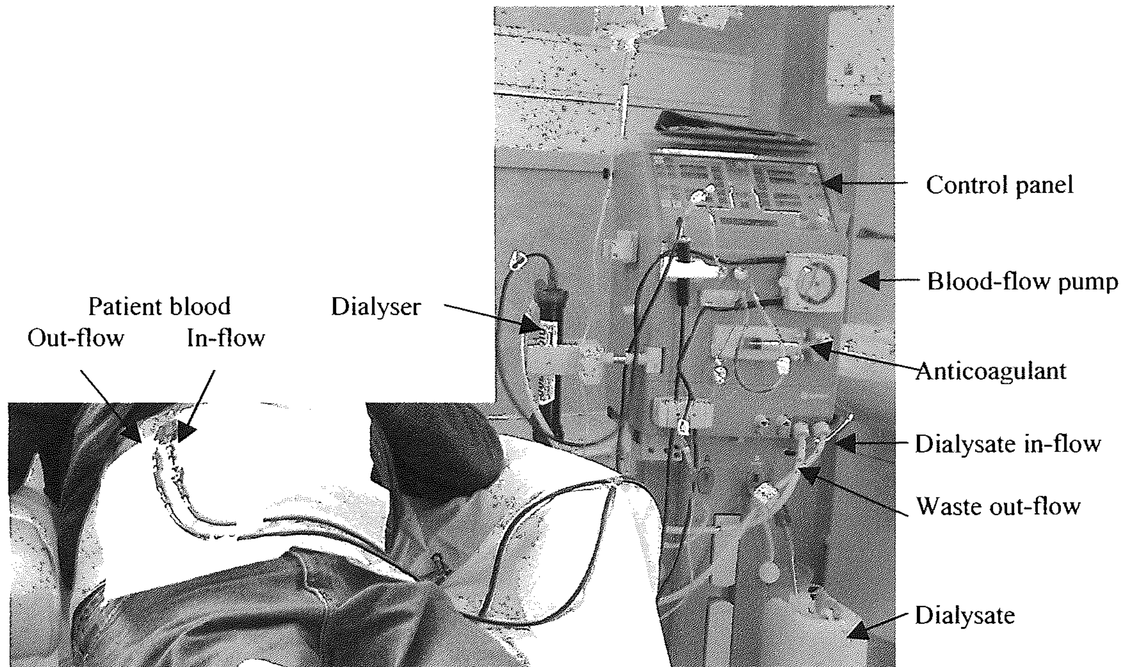


Figure 1.3 Patient receiving haemodialysis via tesio® line.

Vascular access is the most important factor affecting successful haemodialysis treatment and can mean the difference between life and death for patients with ESRD. There are three types of vascular access commonly used for haemodialysis; these are arteriovenous fistula (AVF), arteriovenous graft and polyurethane dialysis catheter. The gold standard method for long-term access is the formation of an AVF. It is considered the safest and the longer lasting permanent vascular access. The advantages of the AVF are lower complication rates, increased patency and lower morbidity associated with its formation. The disadvantages are that it requires a surgical procedure not appropriate for all patients, time for maturation and the development of adequate blood flow. These factors, combined with comorbid disease processes and waiting lists for permanent vascular access, have maintained a dependence on synthetic grafts and intravascular catheters (Butterly and Schwab, 2000).

1.2.1.1 Arteriovenous fistula

The formation of the AVF requires the anastomosis of an artery and a vein. Common sites are the radiocephalic (wrist) and brachiocephalic (elbow). This constitutes anastomosis of the

radial or brachial artery with the cephalic vein (figure 1.4). Arteriovenous grafts are constructed by means of synthetic polytetrafluoroethylene tubes that join an artery to a vein.

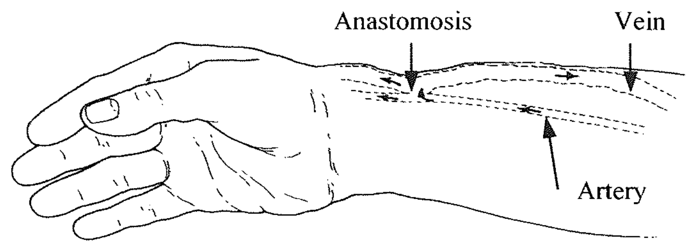


Figure 1.4 Schematic representation of radiocephalic fistula formation

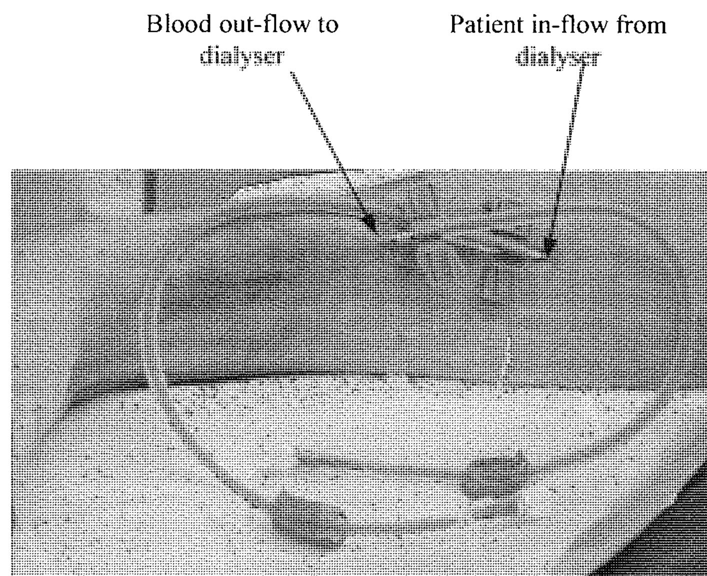


Figure 1.5 Placement of access needles in fistula during haemodialysis

The arterial dialysis needle is inserted into the vein superior to the anastomosis; the blood is drawn from the patient to the dialysis machine and returned via the venous needle, which is placed in the vein superior to the arterial needle. This is shown in figure 1.5.

1.2.1.2 Polyurethane dialysis catheter

The length of the time that the catheter may remain *in situ* varies from 4h to longer than a year. Patients requiring insertion of a dialysis catheter for an individual dialysis treatment or short-term treatment would have a temporary catheter implanted, whereas patients who are waiting for an AVF to mature or who are not considered anatomically suitable for fistula formation would have a long-term catheter inserted. The most common sites for temporary and long-term catheter placement are the internal jugular vein or the femoral vein. The

differences between short and long-term catheters are the catheter type, size and subcutaneous tunnelling of the proximal portion of the catheter through the skin.

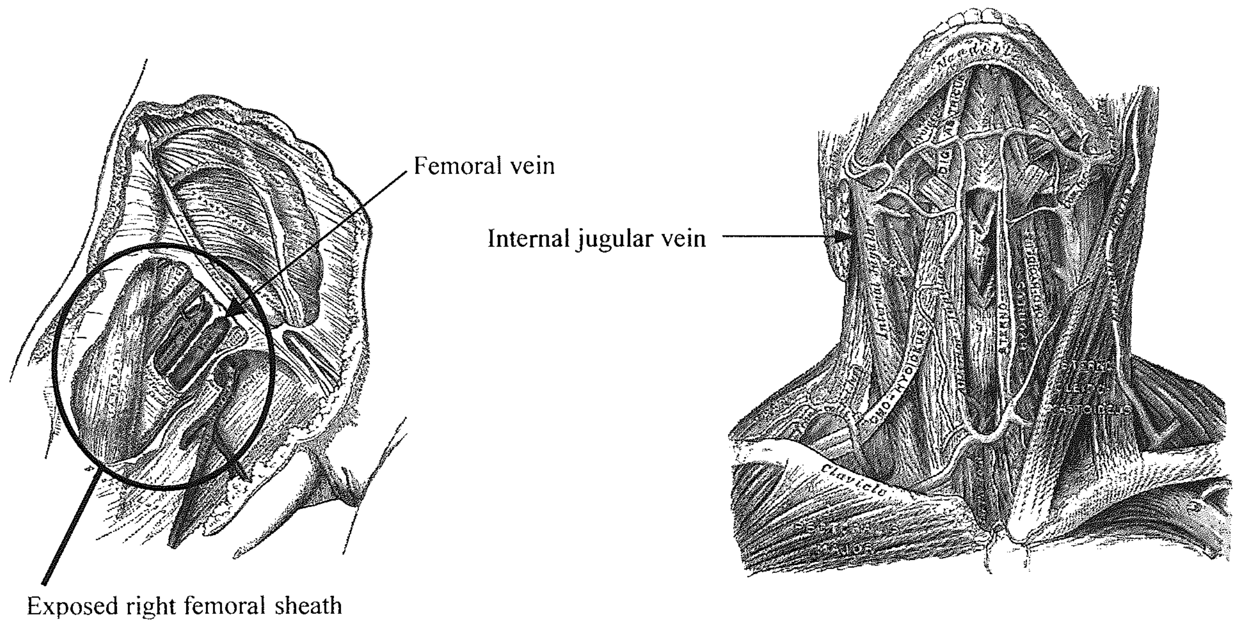


Figure 1.6 Diagrams of common anatomical placement sites of central venous dialysis catheters (adapted from Gray's Anatomy, 2000).

1.2.1.2.1 Short-term dialysis catheters

Short-term dialysis catheters generally have two lumens and the entry site is via a direct subcutaneous approach into the vein (figure 1.7).

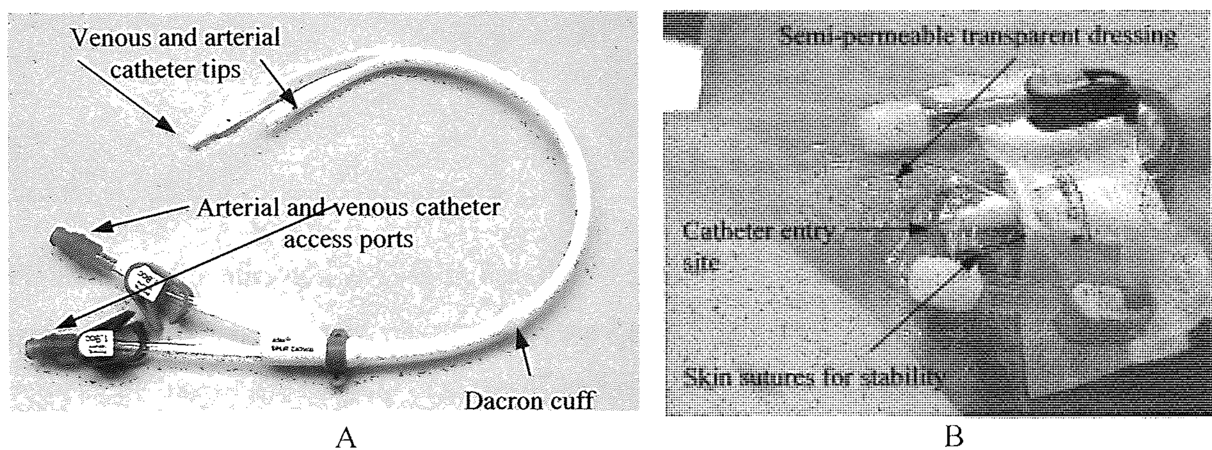


Figure 1.7 A, short-term dialysis catheter and B, anatomical placement of short-term dialysis catheter.

1.2.1.2.2 Long-term dialysis catheters

Long-term catheters commonly consist of two single lumens inserted individually into the vein and tunnelled through the subcutaneous tissue in order that the exit site of the proximal portion is a distance from the site of entry to the vessel (figure 1.8C). The placement of the Dacron cuff within the subcutaneous layer of the skin provokes a local inflammatory response, which progresses to form fibrous and granulation tissue within one month of implantation. This functions to fix the catheter in place and prevent the migration of microorganisms into the subcutaneous tunnel.

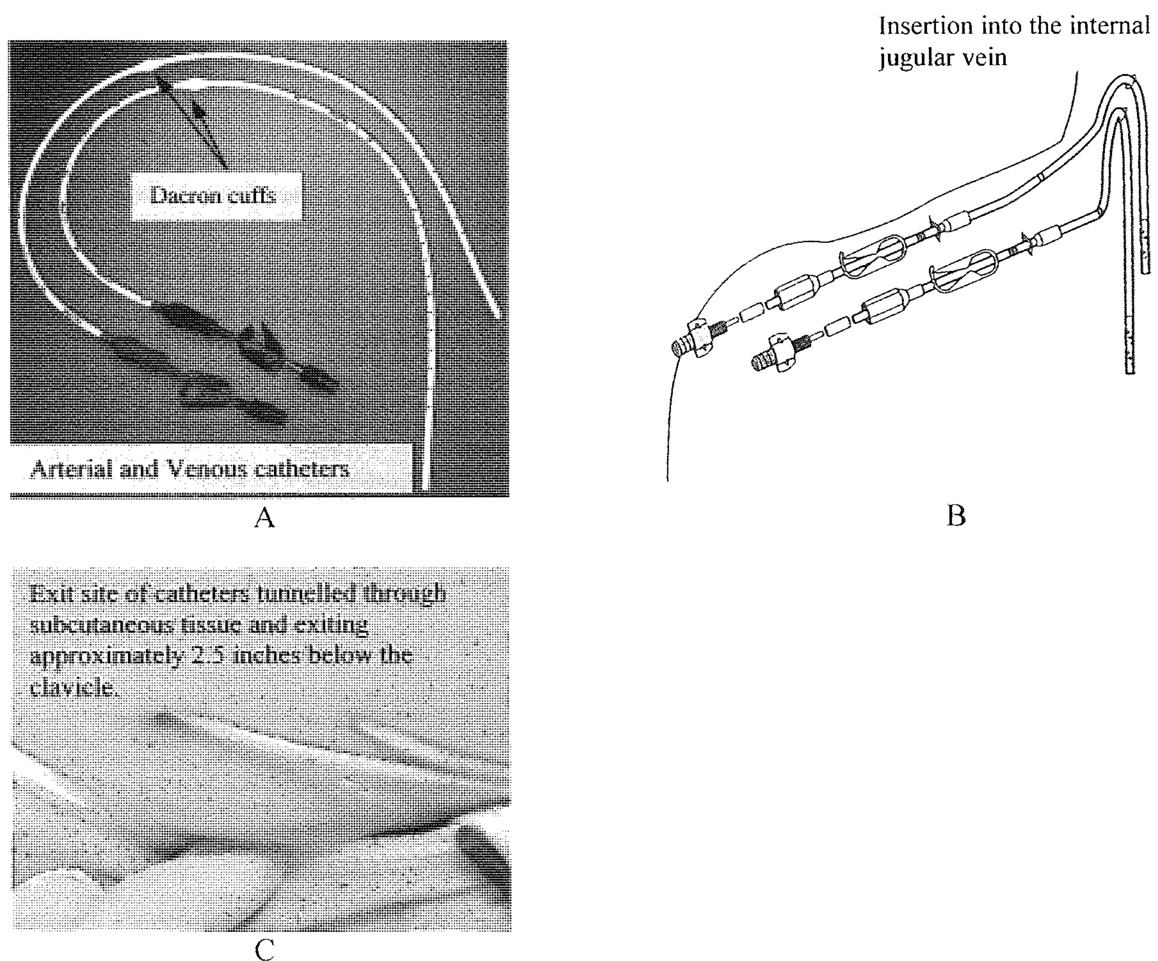


Figure 1.8 A, picture of a long-term Bio-flex™ Tesio® catheter (adapted from Medcomp® product catalogue, 2002). B, placement of the Tesio® catheter in the right internal jugular vein. C, picture of the exit site of a Tesio® catheter (adapted from Handbook of Dialysis, 2001).

1.2.2 Continuous ambulatory peritoneal dialysis (CAPD)

CAPD relies on the peritoneal membrane to function as an artificial kidney. The peritoneum is the serosal membrane that forms the peritoneal cavity. It comprises of a visceral and

parietal layer and has a surface area of 1-2m^2 in an adult. The visceral peritoneum accounts for approximately 80% of the total peritoneal surface area and is supplied with blood via the superior mesenteric artery. Venous drainage is via the portal system. The blood supply to the parietal peritoneum is from the lumbar, intercostal and epigastric arteries and drainage is into the inferior vena cava. The peritoneal cavity is the potential space between the parietal and visceral membranes. Under normal circumstances, the peritoneal cavity contains between 50-100 mL of fluid that acts as a lubricant.

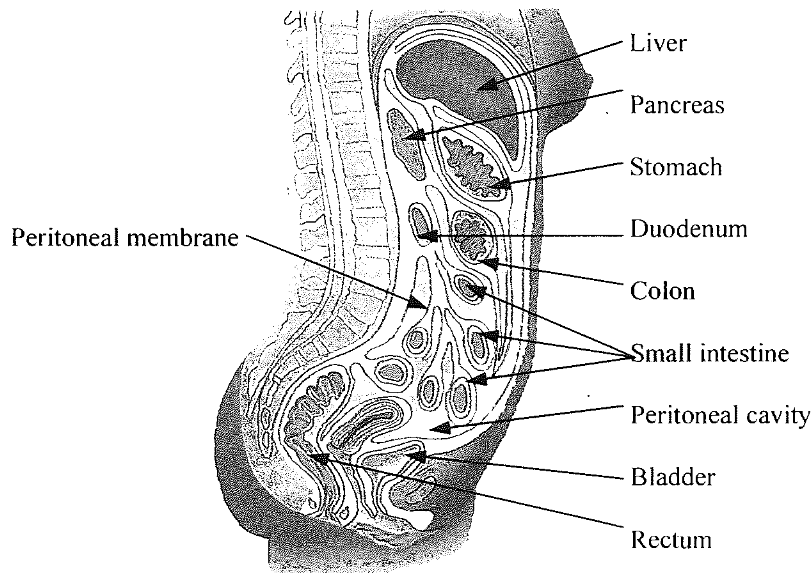


Figure 1.9 The peritoneal membrane lining the organs within the abdominal cavity.

The peritoneal membrane comprises three layers:

- Mesothelium - microvilli on the luminal side of the cells increase the surface area.
- Peritoneal interstitium - formed by fibres and bundles of collagen.
- Capillary endothelium – forms a complex branching system.

The membrane also has lymphatic drainage that acts as a one-way system that returns excess fluid and proteins into the systemic circulation and removes foreign bodies from the peritoneal cavity. The flow rate of lymphatic drainage may be affected by the respiratory rate, posture and peritonitis.

The passage of solutes and fluid between the blood and the peritoneal cavity occurs across the blood capillary wall, through the interstitium and across the mesothelium. The role of the peritoneal membrane as a dialyser has been described by the three-pore model (Rippe, 1993).

It suggests that there are three different sized pores contained within the wall of the peritoneal capillary endothelium and that it is these, which are the critical barrier to peritoneal transport. The 'pores' are thought to be interendothelial clefts and intramembrane proteins. Solute movement and exchange is thought to occur by three methods and pathways:

- Osmosis - movement of water is through 'ultrasmall pores' (radius 2-4 Å).
- Diffusion - transfer of water and small solutes are via 'small pores' (radius 40-50 Å) dictated by a concentration gradient and molecular weight of the solute.
- Convection - movement of water 'drags' solute across the membrane. Large molecules such as proteins are transported across the membrane via 'large pores' (radius 200-300 Å). In addition to convection, large solutes move via diffusion dictated by a concentration gradient and molecular weight.

Peritoneal dialysis employs the area within the peritoneal cavity and exploits the membranes capacity to perform ultrafiltration. This is made possible by the placement of a peritoneal dialysis catheter within the peritoneal cavity, located in the left iliac portion of the lower abdomen.

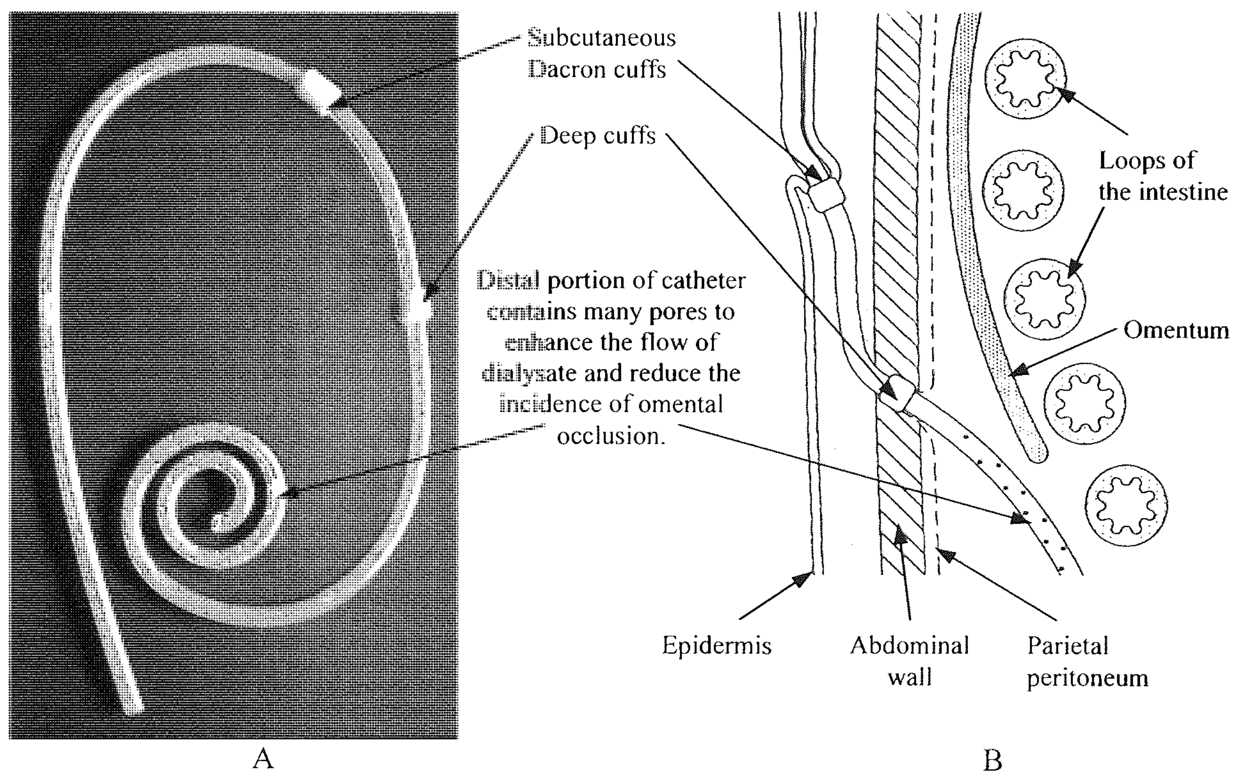


Figure 1.10 A, picture of a Tenckhoff peritoneal catheter. B, diagram to illustrate the positioning of the dialysis catheter within the peritoneal cavity (Handbook of Dialysis, 2001).

In peritoneal dialysis catheters the deep Dacron cuff is positioned within the abdominal wall or rectus muscle (dependent on insertion procedure) and functions to reduce leakage from the peritoneal cavity as well as to fix the catheter's position.

Peritoneal dialysis can be performed manually or via an automated process. When undertaken manually the patient needs to perform 4 'exchanges' per day. An exchange constitutes the influx of a prescribed volume of dialysate into the peritoneal cavity and its subsequent efflux after an appointed amount of time. The dialysate is introduced and removed with the aid of gravity. The dialysate volume used most commonly is 2L, but can vary from 1.5-2.5L.

When peritoneal dialysis is automated, patients undertake a programme of continuous dialysis during the night. The advantages of automated peritoneal dialysis over manual exchanges are enhanced solute clearance (due the increased volumes of dialysate cycled through the peritoneal cavity and therefore increased ultrafiltration) and less infringement on time. In comparison to haemodialysis, the patient is able to perform treatment at home and is only required to attend the dialysis unit when complications occur or to be seen in the outpatients clinic.

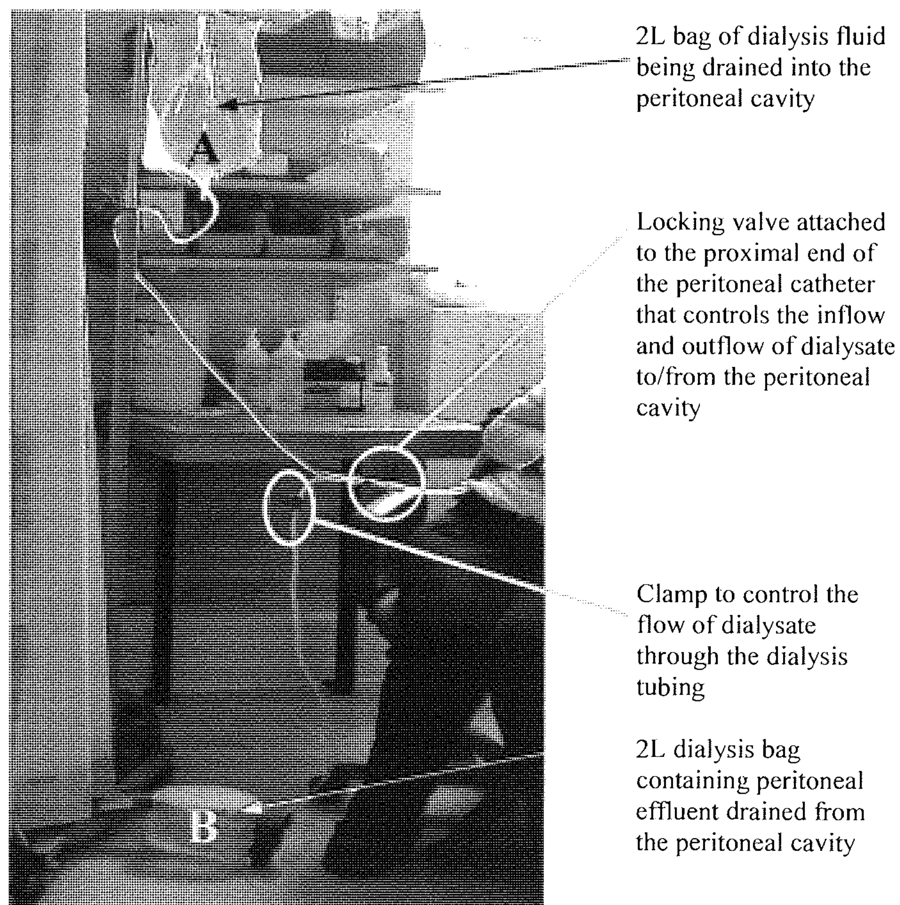


Figure 1.11 Patient performing a peritoneal dialysis exchange.

In figure 1.11 the patient concerned is experiencing peritonitis. Dialysis effluent has been drained from the peritoneal cavity into the bag marked B and fresh dialysate is being introduced into the peritoneal cavity from the bag marked A (note the discoloration of the solution in bag B compared to that in bag A, this is an indication of infection within the peritoneal cavity).

1.3 DIALYSIS CATHETERS

Dialysis has been made possible by the development of intravascular and peritoneal catheters. The development of catheters for use in dialysis began with the introduction of the Uldall subclavian catheter in 1979 (Uldall, *et al.,* 1979). In modern clinical practice, intravascular catheters have become essential in providing vascular haemodialysis access (Pearson, 1996). Biomaterials developed for use in the production of catheters include polyvinyl chloride, polyethylene, silicone and polyurethane. The catheter material is important as it carries varying degrees of risk associated with microbial colonisation. Polyvinyl chloride and polyethylene catheters are less resistant to the adherence mechanisms of microorganisms such as coagulase negative staphylococci than those made for polyurethane or silicone elastomer (Ashkenazi, *et al.,* 1986). Other catheter features that play an important role in the colonisation with microorganisms are irregularities of the catheter surface and how thrombogenic the biomaterial is (Locci, *et al.,* 1981); (Nachnani, *et al.,* 1972); (Stillman, *et al.,* 1977).

Commercially available catheters impregnated with antimicrobial agents such as chlorhexidine-silver sulfadiazine, minocycline-rifampin or benzalkonium chloride have been shown to reduce the risk of microbial adhesion (Saint, *et al.,* 2000); (Moss, *et al.,* 2000). The majority of catheters inserted for haemodialysis at UHB are made of polyurethane.

Complications associated with intravascular catheters include thrombosis, vascular stenosis and vascular perforation (Tapson and Uldall, 1983); (Tapson and Uldall, 1984); (Barton, *et al.,* 1983); (Brady, *et al.,* 1989). However, infectious complications of vascular and peritoneal access remain a major cause of morbidity and mortality in the dialysis population. They have the highest rate of infection of all access types and are often associated with serious metastatic complications (Butterly and Schwab, 2000). In the USA, healthcare institutions purchase millions of intravascular catheters each year. The incidence of catheter-related infection in the USA is reported as 250 000 per year (Kluger and Maki, 1999) with an associated mortality of

12-25% and attributable cost of $25000 per infection (Kluger and Maki, 1999); (Maki and Mermel, 1998); (Mermel, 2000). The annual cost of caring for patients with catheter-related blood stream infections in the USA is estimated at $296 million to $2.3 billion (Mermel, 2000). In the UK, Moss and Elliott reported mean costs for CRI in long-term catheters to be £2.5 million per year (Moss and Elliott, 1997).

1.4 TYPES OF MICROORGANISMS ASSOCIATED WITH PATIENTS TREATED WITH RENAL REPLACEMENT THERAPY.

Renal replacement therapy, especially chronic haemodialysis, supports the acquisition and spread of infection within the renal patient population. This can be attributed to several factors that include the prolonged need for vascular access and treatment within an environment where multiple patients receive dialysis concurrently. This presents continual opportunities for horizontal transfer of infectious agents between patients and clinical personnel, either by direct person-to-person contact or indirect contact with contaminated devices, equipment and supplies or environmental surfaces (Centres Disease Control (CDC), 2001b). In addition, haemodialysis patients are immunosuppressed (Hörl, 1999) due to both their disease state. The clinical management of this patient group requires frequent hospital attendance/admission and this increases the risk of exposure to nosocomial pathogens and contraction infection.

The types of microorganisms that most commonly cause hospital-acquired blood stream infections have changed over time. It has been reported that since the mid 1980's the number of Gram-positive bacteria causing nosocomial bloodstream infections has increased significantly (Banerjee, *et al.*, 1991) (table1.0). Infections associated with CAPD-associated peritonitis are predominantly due to Gram-positive microorganisms however; Gram-negative microorganisms have been implicated in 25% of cases (Gokal and Mallick, 1999).

Table 1.1 Most common pathogens isolated from blood cultures

Microorganism	1986-1989 (%)	1992-1999 (%)
Coagulase-negative staphylococci	27	37
S. aureus	16	13
Enterococci	8	13
Gram-negative rods	19	14
E. coli	6	2
Enterobacter	5	5
P. aeruginosa	4	4
K. pneumoniae	4	3
Candida Species	8	8

(Adapted from O'Grady, CDC 2002)

Currently the four most common pathogens associated with nosocomial bloodstream infections are coagulase-negative staphylococci (CNS), *Candida* species, enterococci and *Staphylococcus aureus* (Fridkin, *et al.*, 1997).

1.4.1. Staphylococci

The genus *Staphylococcus* is defined as: Gram-positive cocci (0.5 to 1.5μm in diameter); usually arranged in clusters; non-motile; non-sporing; grown at a temperature range of 10 to 42°C with an optimum of 37°C; aerobic and facultatively anaerobic (Elliott, *et al.*, 1998). The staphylococci naturally form part of the skin and mucosal surfaces normal commensal flora. They are responsible for a wide variety of diseases in man and other animals, which range from relatively minor skin infections and food poisoning to potentially life-threatening conditions such as toxic shock syndrome and septicaemia (Kloos and Bannerman, 1994). Staphylococci are increasingly associated with hospital-acquired infection following surgery or other invasive medical procedures and indwelling medical devices. At UHB, the majority of microorganisms isolated from blood culture of renal patients diagnosed with catheter-related infection are staphylococci (Spare, renal audit data, 2003).

1.4.1.1 Coagulase-negative staphylococci

CNS includes more than 20 unique species, one of which is *Staphylococcus epidermidis* (Kloos, *et al.*, 1994). They are predominant members of the normal skin flora and are generally considered non-virulent (Dowell and James, 2001). However, in the last two decades it has become clear that CNS are important nosocomial pathogens who are adept at colonising indwelling medical devices (Worthington, *et al.*, 2000a); (Banerjee, *et al.*, 1991), prosthetic heart valves and prosthetic joints (Peters, *et al.*, 1982); (Baddour, *et al.*, 1986).

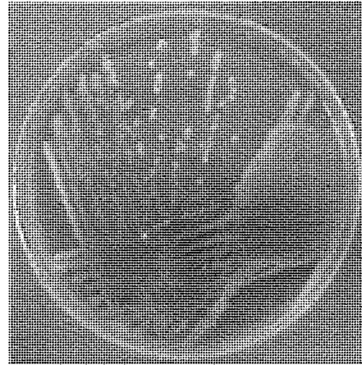


Figure 1.12 Colonial morphology of coagulase-negative staphylococci grown on blood agar.

CNS, in particular *Staphylococcus epidermidis*, has become the most frequently isolated pathogen in catheter-related infection (CRI) accounting for 50 to 70% of CRI (VonEiff, *et al.*, 2001). Mortality from CNS is low but morbidity is high, resulting in prolonged hospitalisation and increased treatment costs (Martin, *et al.*, 1989); (Moss and Elliott, 1997).

1.4.1.2 *Staphylococcus aureus*

S. aureus is a common human commensal accounting for 5 to 10% of skin flora; nasal carriage occurs in approximately 30 to 50% of healthy adults and faecal carriage in about 20% (Elliott, *et al.*, 1998). Asymptomatic colonisation is more common than infection (Chambers, 2001), however, unlike CNS, *S. aureus* is widely reported to be a pathogen. In 1999, it accounted for 12.6% of hospital-acquired bloodstream infections in the USA (CDC, 1999). In 2001, the PHLS Communicable Diseases Surveillance Centre received 13,085 reports of *S. aureus* bacteraemia from laboratories in England and Wales; this accounted for approximately 20.5% of the total number of bacteraemias reported that year (PHLS Communicable Diseases Surveillance Centre, 2002).

S. aureus disease can be broadly grouped into two types (Parker, 1984):

- Acute inflammation - this begins at or near the point of entry of the organism into the tissue. It remains mild and localised but it can extend, leading to generalised infection.
- Acute toxaemia - results from the absorption of extracellular products formed by staphylococci multiplying within a local lesion.

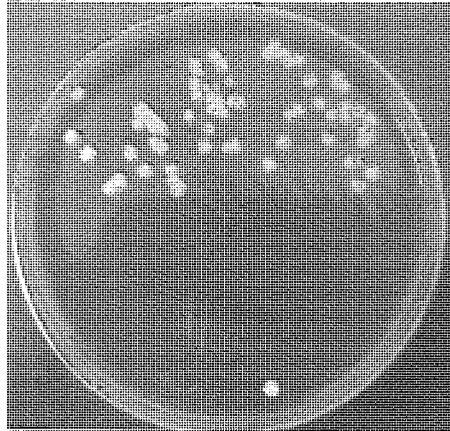


Figure 1.13 Colonial morphology of *Staphylococcus aureus* grown on blood agar

1.4.1.3 Methicillin resistant *Staphylococcus aureus* (MRSA)

Microbiologists have recognised MRSA since the 1960's. It was considered not to be a serious problem in the UK until 1990 when an outbreak of an epidemic strain occurred in a Kettering hospital (Cox, *et al.,* 1995). This specific outbreak led to the emergence of MRSA in virtually all hospitals in the UK. The Central Public Health Laboratory has reported that the incidence of MRSA as a proportion of all *Staphylococcus aureus* blood stream infections has risen from 2% in 1990 to 40% in 2000 (Health Protection Agency (HPA), 2003). The Nosocomial National Infection Surveillance Scheme (NNISS) report for 1997 to 1999 stated that, in the UK, 47% of microorganisms causing surgical site infections were staphylococci, of which 81% were *S. aureus* and 61% of these were MRSA (DOH, 2002).

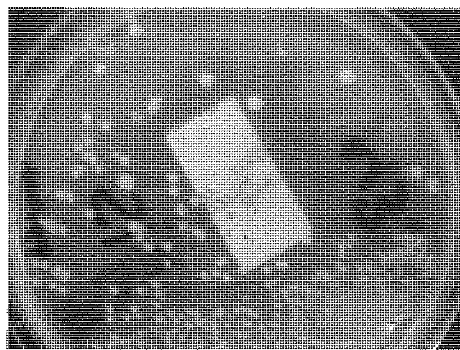


Figure 1.14 Colonial morphology of MRSA grown on Isosensitest agar.

1.4.2. Enterococci

Enterococci are a major bacterial group in the intestinal flora of humans. Over the last 20 years, they have emerged as important nosocomial pathogens (Schaberg, *et al.*, 1991); (Emori and Gaynes, 1993) reflected as an increase in the number of infections in hospitalised patients (Jensen, *et al.*, 1998). The two main species of enterococci recovered from clinical isolates are *Enterococcus faecalis* (80 to 90%) and *Enterococcus faecium* (10 to 15%) (Murray, 1990). The types of infections caused by enterococci include urinary tract infections, bacteraemia, endocarditis, intra-abdominal and pelvic infections (Lewis and Zervos, 1990).

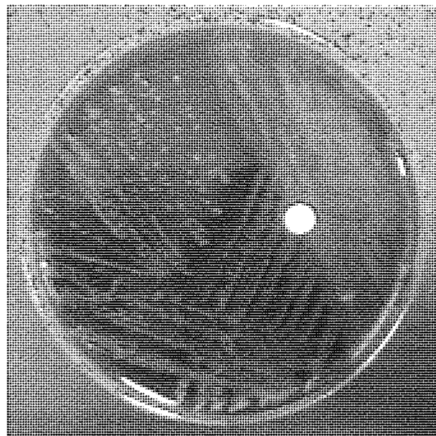


Figure 1.15 Colonial morphology of enterococci grown on blood agar, paper disc contains vancomycin (5μg/mL).

1.4.3 Clostridium species

Clostridia are spore-forming anaerobic Gram-positive bacilli. The clinically significant species are *Clostridium difficile*, *Clostridium botulinum*, *Clostridium perfringens* and *Clostridium tetani*. Spore formation facilitates survival in hostile environments. In addition, it enables the microorganism to withstand many forms of heat and chemical disinfection (Elliott, *et al.*, 1998). They are found in the gastrointestinal tract of mammals and are responsible for infections including toxaemia associated with gas gangrene, anaerobic cellulitis, food poisoning, tetanus, botulism and pseudomembranous colitis.

Clostridium difficile has become a significant cause of nosocomial infection that is responsible for nearly all cases of pseudomembranous colitis and 20% of cases of antibiotic-associated diarrhoea without colitis (Jobe, *et al.*, 1995). Faecal carriage of *C. difficile* is 3% in

the general population (Bartlett, 1994) however, it has been reported that 10 to 21% of hospitalised patients are colonised (Elliott, *et al.,* 1998); (McFarland, *et al.,* 1989) *C. difficile* infection accounts for an average 3-week increase in hospital stay (Settle and Wilcox, 1996).

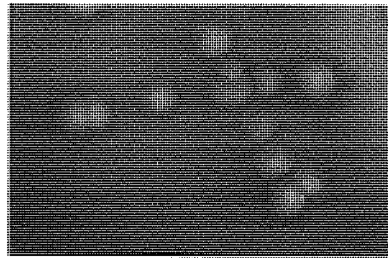


Figure 1.16 Colonial morphology of *Clostridium difficile* (Elliott, *et al.,* 1998)

1.4.4 Hepatitis

Historically, surveillance of infections associated with chronic haemodialysis was concentrated on viral hepatitis, specifically the hepatitis B virus (HBV). The CDC began conducting surveillance of haemodialysis-associated hepatitis in 1972, eventually widening the focus of the surveillance over time to include hepatitis C virus (HCV), vascular access associated infections and various practice-related issues (CDC, 2001b).

Table 1.2 Types of hepatitis

Type of hepatitis	Known as	Long-term effects
A	HAV	None - 15% of people have prolonged or relapsing symptoms over a 6 to 9 month period
B	HBV	Chronic infection occurs in: 90% of infants infected at birth 30% of children infected at age 1 to 5 years 6% of persons infected after age 5 years Death from chronic liver disease occurs in: 15 to 25% of chronically infected people
C	HCV	Chronic infection occurs in: 75 to 80% of infected people Chronic liver disease occurs in: 70% of chronically infected people Deaths from chronic liver disease occur in: >3% of people
D	HDV	Needs HBV to exist. Co-infection: Low risk of chronic infection Super-infection: Usually develop chronic HDV infection High risk of sever chronic liver disease
E	HEV	None - severity increases with age Overall fatality 1 to 3% 15 to 25% in pregnant women

(Adapted from information at www.cdc.gov/hepatitis)

The two most common forms of hepatitis in haemodialysis patients are HBV and HCV.

1.4.4.1 Hepatitis B virus Infection

Hepatitis B virus is a partially double-stranded DNA virus contracted through blood, body fluids containing blood and sexual contact. It is reported to have infected 2 billion people worldwide, of which 350 million have chronic infections (World Health Organisation (WHO), 2000). In patients with ESRD HBV is largely asymptomatic (Daugirdas, *et al.*, 2001). The virus is relatively stable in the environment and remains viable for at least 7 days on surfaces at room temperature (Bond, *et al.*, 1981). In studies conducted in haemodialysis units in the USA hepatitis B surface antigen has been isolated on clamps, scissors, dialysis

machine control buttons and door knobs (Favero, *et al.,* 1973). The majority of HBV infection outbreaks among haemodialysis patients have been attributed to cross infection via contaminated equipment, clinical environments used for preparation of intravenous medications and manipulation of blood samples and clinical personnel simultaneously caring for HBV infected patients and patients susceptible to infection (CDC, 2001b); (Snydman, *et al.,* 1976); (Carl, *et al.,* 1983); (Niu, *et al.,* 1989). In the USA, HBV infection in haemodialysis patients has decreased from 6.2% (1974) to 0.06% in 1999 (CDC, 2001b). This is a reflection of the implementation of infection control recommendations, serological surveillance of patients and the introduction of HBV vaccination programmes. However, HBV remains a significant problem in the haemodialysis population.

1.4.4.2 Hepatitis C virus infection

The hepatitis C virus is an enveloped single-stranded RNA virus (Harper, 1994) that is contracted through infected blood (CDC, 2001a). WHO estimates that 170 million (3%) people worldwide are infected with HCV (WHO, 1998). Risk factors for HCV infection in haemodialysis patients include history of blood transfusions, the volume of blood transfused and the number of years on dialysis (Moyer and Alter, 1994). Studies have shown that patients who have been on dialysis < 5 years have on average a 12% prevalence of HCV infection, however this increases to 37% in patients who have been dialysing for ≥ 5 years (Niu, *et al.,* 1993); (Hardy, *et al.,* 1992); (Selgas, *et al.,* 1992). In the USA, the Notifiable National Disease Surveillance System (NNDSS) reported a decrease in the incidence of HCV infection in haemodialysis patients between 1982 to 1997 from 1.7% to 0.2% (Tokars, *et al.,* 2000). This decrease is partially explained by the introduction of blood screening in 1985. In addition, implementation of HCV serological screening programmes and improved infection control practices has helped reduce the incidence of HCV infection in the USA.

1.5 COMMON INFECTIONS IN PATIENTS TREATED WITH RENAL REPLACEMENT THERAPY

1.5.1 Infections associated with implantable intravascular devices

Risk factors associated with the development of CRI include the length of time the catheter remains *in situ*; use of effective skin and connection disinfection; anatomical positioning;

application of tunnelled or percutaneous insertion and clinical management of the catheter site (Tokars, *et al.*, 1999).

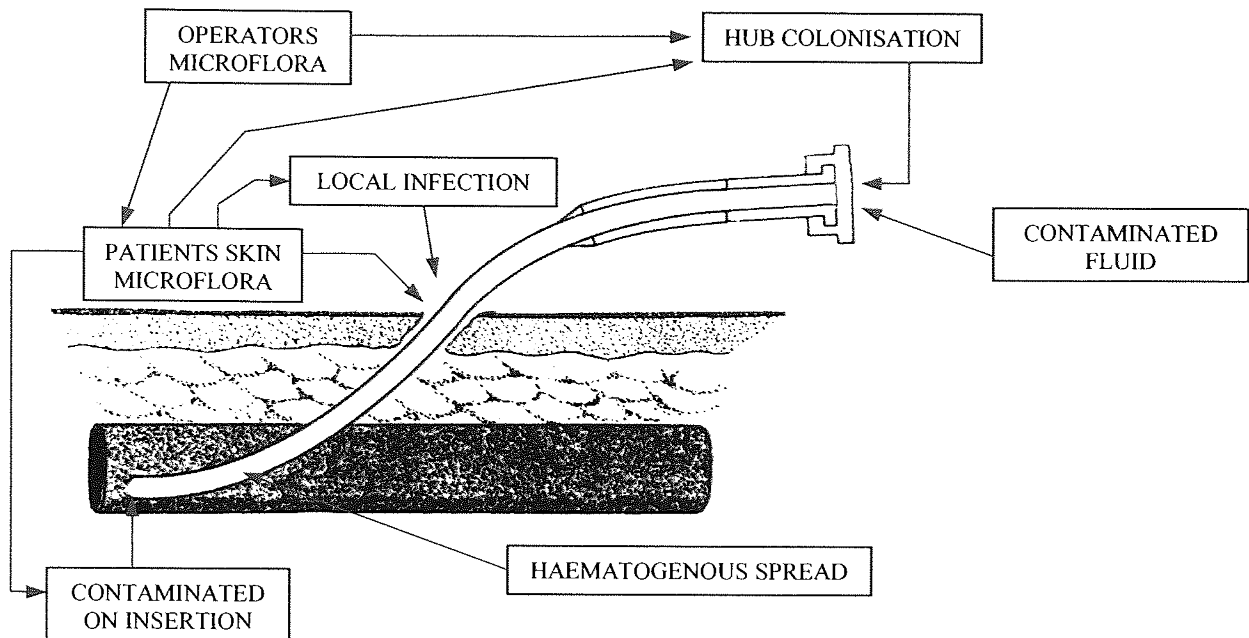


Figure 1.17 Microbial sources of intravascular catheter-related infection

In haemodialysis catheter-related bloodstream infection and CAPD-associated peritonitis, access of CNS can occur via the skin insertion site/epidermal skin tunnel, catheter connections, blood (haematological spread) or the peritoneum for CAPD catheters. As shown if figure 1.17, infection can be localised at the catheter/skin insertion site or systemic (Elliott, *et al.*, 1995).

1.5.1.1 Localised infection

A localised catheter-related infection or exit site infection is defined as erythema, tenderness, induration or purulent discharge within 2 cm of the skin at the exit site of the catheter (Pearson, 1996) (figure 1.18 A & B). It results from the migration of skin organisms at the exit site into the cutaneous catheter tract. The type of microorganism, the density of the microbial population on the skin and the phenotypic characteristics of microorganisms involved influence the extent of the infection (CDC, 2002b). The incidence of exit site infection in the dialysis population is hard to determine as localised infection is often reported under the umbrella of vascular access infection, which includes both localised and systemic manifestations. However, in the USA initial results from a new surveillance system for haemodialysis-associated infection indicated that, in the period 1999 to 2001, there were 1082

local access infections accounting for 45% of the total number of access related infections (Tokars, *et al.*, 2002).

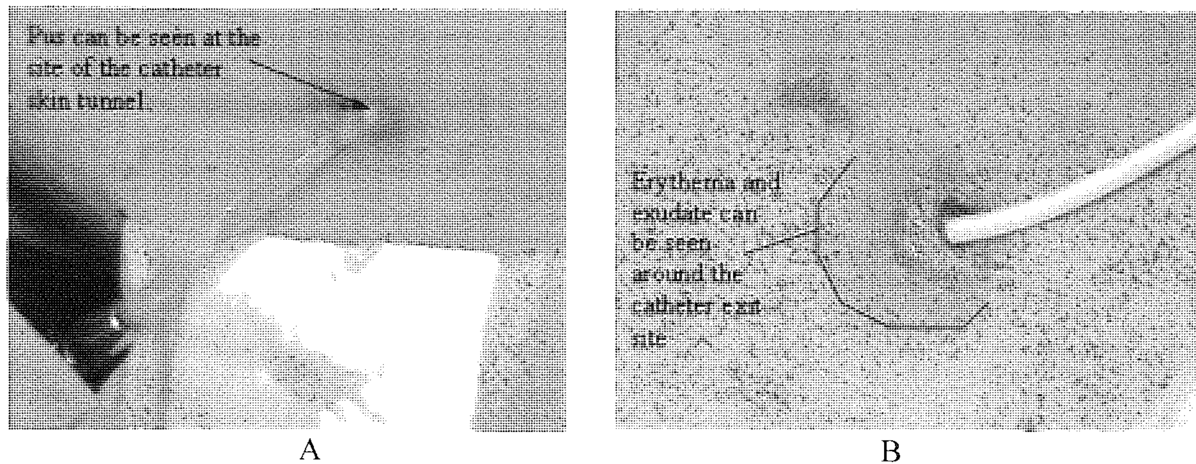


Figure 1.18 localised catheter site infection: A. CAPD catheter B. haemodialysis catheter

<u>1.5.1.2 Systemic infection</u>

Catheter-related systemic infection is defined as isolation of the same species of microorganism from the catheter tip and from the blood culture (preferably taken via a peripheral vein) from a symptomatic patient with no other focus of infection. Alternatively, defervescence after removal of the implicated catheter without laboratory conformation is accepted as indirect evidence (Department Of Health And Human Services, 1995). In the USA initial results from the new surveillance system for haemodialysis-associated infection reported 1347 access-related bacteraemias in the period 1999 to 2001, accounting for 55% of the total number of access-related infections (Tokars, *et al.*, 2002).

<u>1.5.1.3 Peritonitis</u>

Peritonitis is the most frequent serious complication of CAPD and the leading clinical cause of technique failure leading to a switch to haemodialysis (CANUSA - Peritoneal Dialysis Study Group, 1996). It is defined as an inflammation of the peritoneum and is caused by invasion of bacterial agents or a foreign matter irritant (Kasner and Tindall, 1985). The rate of peritonitis varies between dialysis units and geographical areas. In the USA patients experienced 1 episode of peritonitis every 24 treatment months (Keane, 2000).

For catheter-related infection to develop the catheter must first become colonised with microorganisms (Elliott, 1999). Microbial colonisation of the catheter can occur at the time of

catheter insertion as the device passes through the skin (Elliott, *et al.*, 1997), or subsequently following catheter manipulation (Elliott, 1988). The colonisation of vascular devices is a relatively fast process. During the first 48 hours after catheter insertion, a number of physico-chemical reactions take place between the bacterial cell surface and the catheter biomaterial that result in adherence to the foreign substratum.

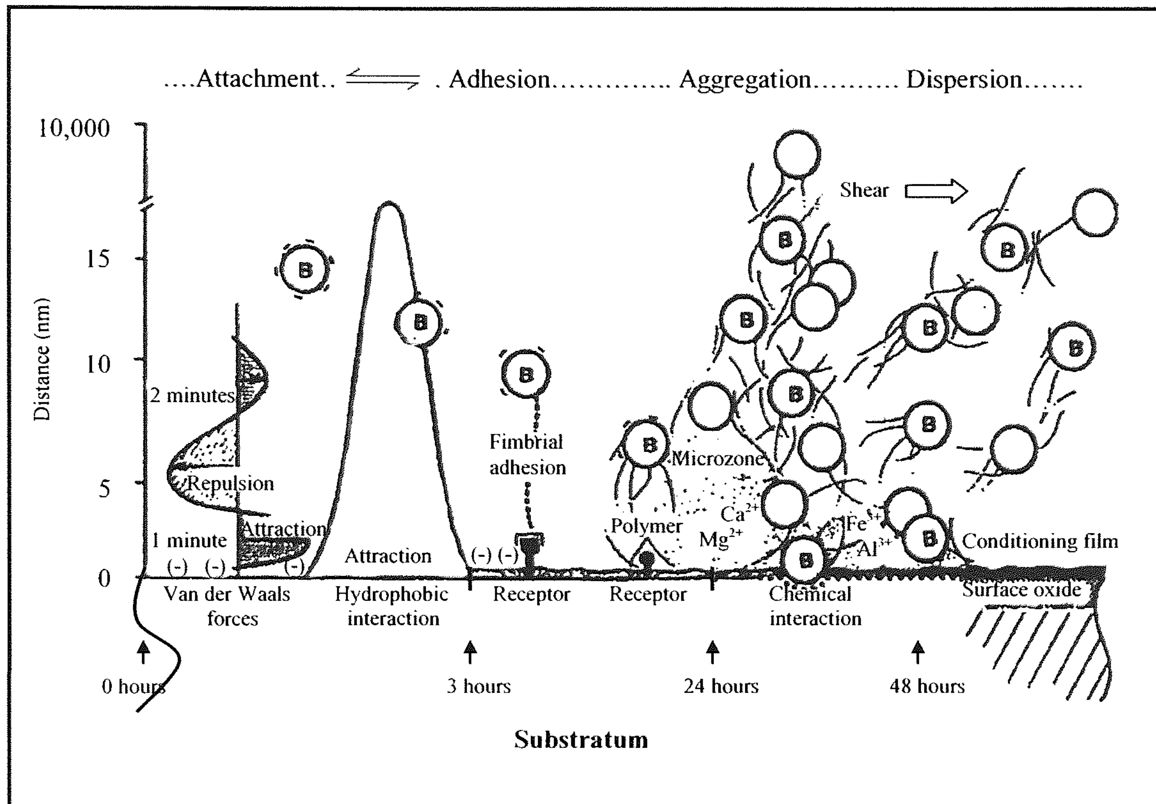


Figure 1.19 Physico-chemical mechanisms of microbial adherence to polymer surfaces (Adapted from Gristina, 1987)

Bacterial cells come into close contact with polymer surfaces via Van der Waals, hydrophobicity and electrostatic forces. This interaction results in irreversible adhesion to the substratum (Allison, 1993); (Rupp, 1996). Microbial surface components recognising adhesion matrix molecules (MSCRAMMS) and extracellular polysaccharide slime components including AtlE, PS/A, SSP-1 and SSP-2 have been reported to facilitate attachment to polymer surfaces (McKenny, *et al.*, 1998); (Heilmann, *et al.*, 1997); (Veenstra, *et al.*, 1996); (Patti and Hook, 1994). Following this initial attachment of the bacterial cells to the polymer surface, biofilm formation ensues. This creates a multi-layered film of bacterial cells held together by an intracellular polysaccharide antigen, embedded within an amorphous extracellular layer (Rupp, 1996). Figure 1.19 illustrates the development of the bacterial biofilm in relation to time, distance from the polymer surface and the stage of adherence.

Biofilms associated with peritoneal catheters for example, enable staphylococci to grow as micro-colonies on the polymeric silicone materials which facilitates the development of peritonitis (Marrie, *et al.,* 1983). Scanning electron microscopy studies have also revealed the extensive production of extracellular slime by staphylococci, especially *S. epidermidis*, ultimately showing that multiple layers of CNS can become encased in the biofilm (Peters, *et al.,* 1982).

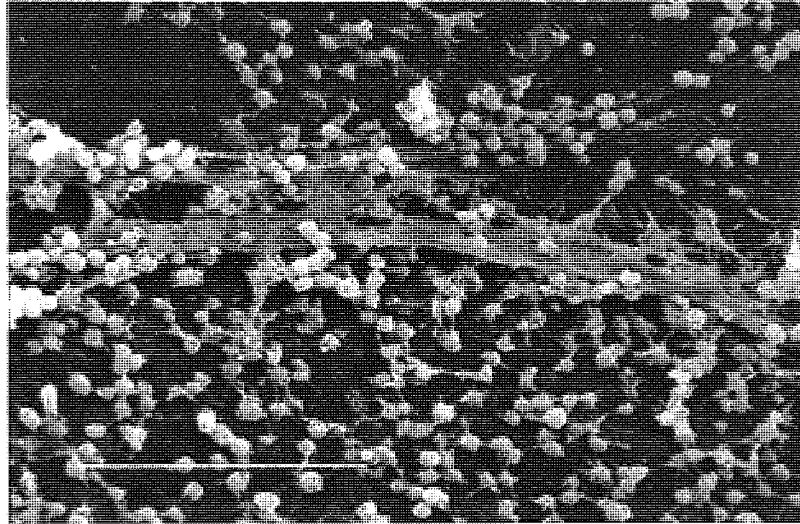


Figure 1.20 Electron micrograph of staphylococcal biofilm on the internal surface of an indwelling medical device (adapted from Donlan, 2002).

Microorganisms, especially staphylococci, express a range of phenotypic characteristics that enable them to establish infection once they have gained access to a prosthetic device. These characteristics are outlined in table 1.2 and some are discussed in more detail in section 1.6.2.2.

Table 1.3 Phenotypic characteristics produced by microorganisms to exploit the host

Phenotypic characteristic	**Reference**
Factors potentially associated with attachment	
Fibronectin binding receptor	Valentin-Weigand *et al.*, 1993 Paulsson *et al.*, 1992 Switalski *et al.*, 1983
Fibrinogen binding receptor	Pei *et al.*, 1999 Nilsson *et al.*, 1998
Vitronectin binding receptor	Paulsson *et al.*, 1992 Paulsson & Wadström, 1990
Laminin binding receptor	Paulsson *et al.*, 1992 Paulsson & Wadström, 1990
Collagen binding receptor	Paulsson *et al.*, 1992 Paulsson & Wadström, 1990 Watts *et al.*, 1990
Biofilm/adhesion/polysaccharide slime	Hussain *et al.*, 1997 Heilmann *et al.*, 1997 Veenstra *et al.*, 1997 Mack *et al.*, 1992 Christensen *et al.*, 1990 Tojo *et al.*, 1988 Christensen *et al.*, 1982
Von Willebrand factor	Li *et al.*, 2000b
Haemagglutinin	Rupp *et al.*, 1995 Rupp & Archer, 1992
Heparin/heparinised surfaces	Paulsson *et al.*, 1994
Factors potentially associated with evasion of host defences	
Enterotoxin A, B or C	Udo *et al.*, 1999 Crass & Bergdoll, 1986
Lipase (including esterase)	Götz *et al.*, 1998 Molnàr *et al.*, 1994 Farrell *et al.*, 1993 Lambe *et al.*, 1990
Iron restriction (transferring-binding proteins and environmental signal)	Modun *et al.*, 1994 Deighton & Borland, 1993
Factors potentially associated with tissue invasion	
Proteinase activity (non-specific proteinase and elastase)	Zhang & Maddox, 2000 Watts *et al.*, 1990 Janda, 1986 Varadi & Saqueton, 1968
DNase	Molnàr *et al.*, 1994 Lambe *et al.*, 1990
Urease	Schäfer & Kaltwasser, 1994 Gatermann *et al.*, 1989
Cytolytic toxins (haemolysins δ and β)	Butt *et al.*, 1998 Molnàr *et al.*, 1994 Lambe *et al.*, 1990 Hébert, 1990 Scheifele & Bjornson, 1988 Gemmell, 1987

Although the phenotypic characteristics in table 1.2 have been placed in to categories, this does not limit a particular characteristic to a specific function. Expression of extracellular polysaccharide slime substance is a characteristic that facilitates adherence to polymer surfaces but also functions to impede phagocytosis and promote antimicrobial resistance (Christensen, *et al.*, 1994)

1.5.2 Infections associated with antimicrobial therapy

The quantity of antibiotics prescribed within the renal service at UHB is a reflection of the increased infection risk associated with end stage renal disease. However, the use of antimicrobial therapy disrupts the natural balance of commensal flora and applies selective pressure on the body's microbial population, suppressing some flora and allowing others to proliferate. This is visible during outbreaks of antibiotic associated diarrhoea and the emergence of antimicrobial resistance.

Clostridium difficile is not normally present in the human gastrointestinal tract and is acquired from the environment. It can be carried asymptomatically however, when the normal gut flora become disrupted by antimicrobial therapy *C. difficile* is able to proliferate and cause infection. It is the principal pathogen causing antibiotic associated diarrhoea, and responsible for outbreaks of *C. difficile*-associated diarrhoea (CDAD) within the hospital environment (Kato, *et al.*, 2001). Yousuf *et al*, reported that although chronic renal insufficiency was not a risk factor for CDAD, patients with chronic renal insufficiency and CDAD had significantly higher mortality and recurrence than those who had normal renal function (Yousuf, *et al.*, 2002).

Enterococci (*E. faecalis* and *E. faecium*) are normal components of the intestinal flora. They are fast becoming prominent nosocomial pathogens causing infections in hospitalised patients. Exposure of enterococci to prolonged antibiotic therapy by the glycopeptide class of agents is being reflected in the emergence of glycopeptide resistant enterococci (GRE). Asymptomatic faecal carriage rates of vancomycin resistant enterococci (VRE) in the dialysis population has been reported as 70% (Mark Hastings Consultant Microbiologist QEH, personal communication). This limits the therapeutic options available to treat infections caused by VRE.

1.6 DIAGNOSIS OF INFECTION IN RENAL PATIENTS

1.6.1 Clinical diagnosis

Common clinical signs of infection include fever, chills and general malaise. In patients with ESRD, the clinical signs of infection can be confounded by treatment modality. For example, haemodialysis can cause some patients to experience chills, fever, hypotension and extreme tiredness. In addition, the most common microorganism associated with infection in this patient group are CNS; infections related to this group of microorganisms usually present with non-specific signs and symptoms, including malaise and low-grade fever. Patients rarely appear toxic or septic (Schmidt, *et al.,* 1987). In addition, in 50% of haemodialysis patients, the predialysis body temperature is subnormal (Daugirdas, *et al.,* 2001). This suggests that without overt signs of infection, i.e. purulent discharge from a wound or productive cough, timely clinical diagnosis of infection in this patient group may be more difficult than in the general population.

Patients presenting with systemic infection or bacteraemia would be characterised by the general symptoms outlined above. The clinical signs and symptoms of localised infection were outlined in section 1.5.1.1.

CAPD-associated peritonitis is indicated by cloudy dialysis effluent ± abdominal pain and fever, and a white cell count of greater than 100 cells per mm^3 of blood of which >50% are polymorphonuclear neutrophils (PMN) (Keane, *et al.,* 2000). Evidence of these clinical signs and symptoms would result in samples of peritoneal dialysate being sent to the laboratory for cell count, culture, identification and antimicrobial sensitivity testing.

C. difficile-associated diarrhoea presents with symptoms that include mild diarrhoea, pseudomembranous colitis, toxic megacolon, electrolyte imbalance and bowel perforation (Urbán, *et al.,* 2002). Faeces appears unformed, watery or muciod and may be accompanied by abdominal pain and fever. The majority of patients who present with *C. difficile* diarrhoea are taking, or have recently been given, antibiotic therapy. In most patients, the illness is mild and full recovery is usual, however elderly patients may become seriously ill with dehydration due to diarrhoea (Association of Medical Microbiologists, 1998). Any patient who is or has recently received antimicrobial therapy and who develops diarrhoea would be considered for

C. difficile infection. Faecal samples would be sent to the enteric laboratory for *C. difficile* toxin assay.

1.6.2 Laboratory diagnosis

Laboratory analysis of specimens underpins the clinical diagnosis of infection. Preliminary clinical diagnosis of infection would be made based on the symptoms of the patient however definitive diagnosis would not be made until the microbiology results are available. The types of specimens sent for laboratory diagnosis are outlined in table 1.3.

Table 1.4 Common clinical specimens processed for the diagnosis of infection in renal patients

Specimen	Anatomical site	To support diagnosis of	Laboratory tests
Swab	Catheter exit	Localised catheter infection	
	Wound	Surgical site infection	Microbial culture and sensitivity
	Nose Groin	MSSA/MRSA carriage	
Blood	Via catheter or graft Peripheral vein	Infection associated with indwelling medical devices	Microbial culture and sensitivity
		Systemic infection	
Peritoneal dialysate	Peritoneum	CAPD-associated peritonitis	Microbial culture and sensitivity
Faeces	Rectum	VRE and *C. difficile* associated diarrhoea/carriage	Microbial culture *C. difficile* toxin assay

1.6.3 Catheter-associated infection

The clinical diagnosis of infection associated with intravascular catheters is often difficult to make due to the associated non-specific symptoms and signs (Elliott and Tebbs, 1998). Emmerson *et al.*, (1996) reported that the degree of certainty in diagnosing infection related to central venous catheters (CVC) was 55%. Indeed, confirmation of the diagnosis often necessitates removal of the device (Maki, *et al.*, 1977). This results in between 75 to 85% of catheters being removed unnecessarily (Ryan, *et al.*, 1974).

A clinical criterion for the diagnosis of catheter-related infection was developed by Elliott *et al.* (1998). A patient who had a central venous catheter *in situ* and who exhibited two or more of the following clinical symptoms was investigated for catheter-related infection. These were a low grade pyrexia (37 °C to 38.5 °C) with no other underlying cause; rigors immediately post-flushing of the catheter; erythema, oedema and purulent exudates at the insertion site; the pyrexia being unresponsive to broad spectrum antibiotics but settling on catheter removal. The diagnosis of a catheter-related infection was supported by the isolation of the same microorganism from one or more blood culture samples either via the catheter, a separate peripheral venepuncture, or both.

1.6.4 Identification of phenotypic characteristics

Microorganisms exhibit a variety of phenotypic characteristics that have been associated with the ability to cause infection (table 1.2). The detection and study of these characteristics allows the identification of virulent nosocomial endemic microorganisms that have a greater propensity to cause infection.

1.6.4.1 Production of proteinase

The ability of a microorganism to invade a host is dependent upon its ability to overcome the host's defence mechanisms. Proteinases such as elastin mediate tissue degradation and modulation of the immune system (Travis, *et al.,* 1995). They are therefore pivotal to the survival, growth and pathogenicity of the microorganism within the host (Kawano, *et al.*, 2001); (Finlay and Falkow, 1997). The role played by proteinases in pathogenesis is shown in figure 1.21.

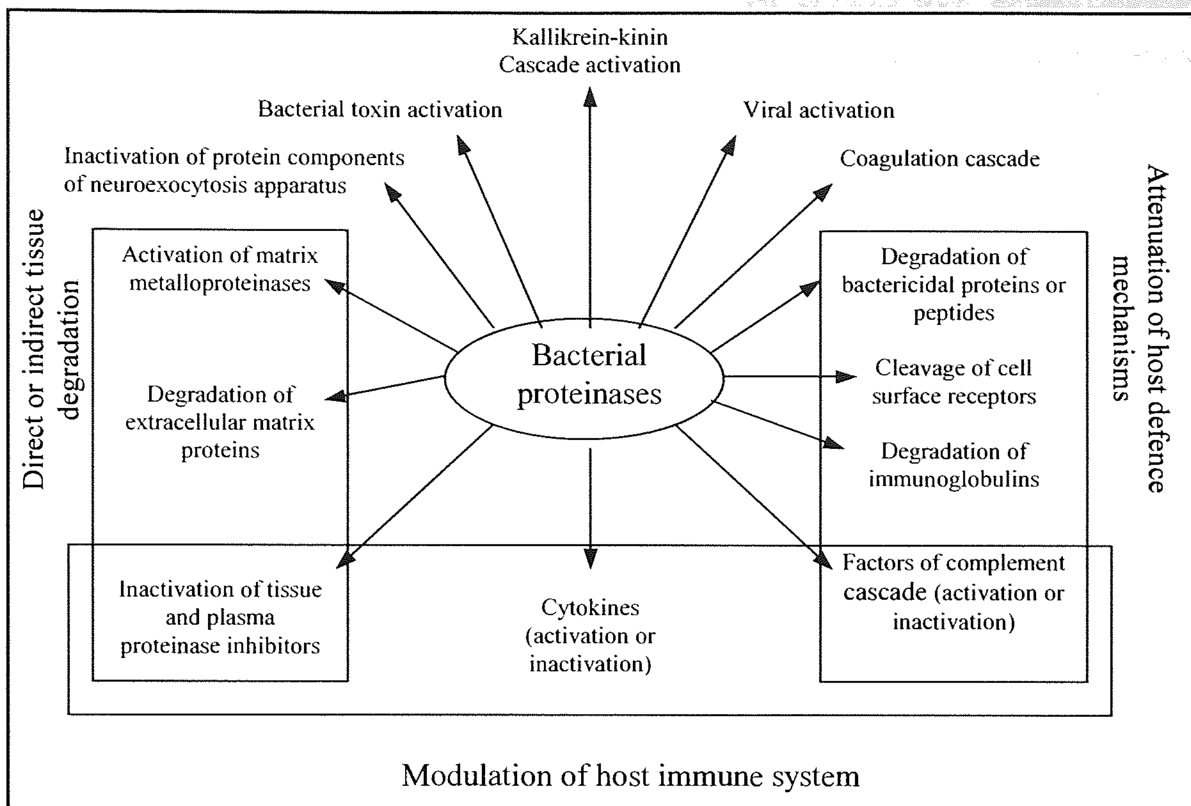


Figure 1.21 The role of bacterial proteinases in infection (adapted from Travis, Potempa and Meada, 1995).

1.6.4.2 Production of lipase and esterase

Lipase (glycerol ester hydrolase) and esterases (lipolytic activity on water-soluble substrates) are common for both environmental and pathogenic microorganisms (Jaeger, *et al.,* 1994); (Smeltzer, *et al.,* 1992). Staphylococci have been reported to produce lipases and esterases that express no substrate specificity. The function of staphylococcal lipases and esterases is not fully understood. Bowden *et al.* (2002), reported that the GehD lipase secreted by *S. epidermidis* binds type I, II and IV collagen and may therefore mediate the adherence of *S. epidermidis* to immobilized collagens. In addition, the lipase molecule is reported to be bifunctional as it acts as a glycerol ester hydrolase and a collagen adhesin (Bowden, *et al.,* 2002).

1.6.4.3 Production of extracellular polysaccharide slime and adherence to polymer surfaces

Jones, Deibel and Niven first noted a muciod substance (slime) produced by bacteria in 1963. Bayston and Penny (1972) published theories concerning slime and the pathogenesis of bacteria when their clinical observations confirmed the bacterial colonisation and infection of

Holter shunts in patients with hydrocephalus (Christensen, *et al.,* 1994). Studies concerning the purification of slime did not begin until the early 1980's and to date the composition of the extracellular slime substance (ESS) produced by *Staphylococci* has not yet been fully characterised; however much scientific work is focused in this area.

The major macromolecules of bacterial extracellular slime have been a source of some debate. Christensen *et al.* reported it to be a polysaccharide, after polysaccharide-specific staining with Alcian blue (Christensen, *et al.,* 1982). Further work showed the nature of the polysaccharide to be mannose and galactose rich (Ludwicka, *et al.,* 1984); (Peters, *et al.,* 1987) and to have the capacity to inhibit phagocytosis (Johnson, *et al.,* 1986), clotting (Bykowska, *et al.,* 1985) and treatment with vancomycin (Farber, et al. 1990}. Subsequently, studies have demonstrated that the high galactose concentration obtained from slime culture, is derived from the medium rather than ESS (Hussain, *et al.,* 1991b); (Drewry, *et al.,* 1990). Culture on defined media containing low molecular weight constituents confirmed that recovery of high molecular weight products are indeed derived from the ESS as opposed to the agar medium (Hussain, *et al.,* 1991b). Hussain and co-workers also identified concentrations of glycerol phosphate, alainine, glucose and glucosamine in the slime material, suggesting slime consists mainly of cell wall factors and is similar to staphylococcal teichoic acid (Hussain, *et al.,* 1991b).

Functional analysis of ESS by Muller and co-workers led to the discovery of a polysaccharide adhesin (PS/A) molecule (1993). PS/A is a reported to be important in the initial attachment of bacteria to polymer surfaces, whereas slime is implicated in bacterial accumulation (Tojo, *et al.,* 1988). Subsequent work by Mack *et al.* suggests that PS/A mediates cell adherence to biomaterials and another antigen, termed polysaccharide intercellular adhesin (PIA) (Mack, *et al.,* 1992); (McKenny, *et al.,* 1998). Chemical characterisation of PIA revealed two structurally related homoglycans composed 130 residues of β-1,6-linked glucosamine with succinate or acetate on amino groups. Their structure was un-branched and appeared to favour long-range contact and interaction between polysaccharide strands and cell wall/lectin-like proteins leading to bacterial accumulation on polymer surfaces (Mack, *et al.,* 1996). The *ica* gene locus encodes production of PIA and is able to synthesise PS/A, therefore the *ica* locus encodes the production of PS/A mediating initial biofilm formation, bacterial adherence and subsequent accumulation of cells into the biofilm via the synthesis of PIA (McKenny, *et al.,* 1998). Ziebuhr *et al.* reported that isolated cells produce very little PIA compared to cells that were involved in clusters. In addition, biofilm production by bacteria recovered from patients

with plastic medical device–associated infections, was greater than in strains isolated from the skin. This suggests PIA is important in the accumulation stage of biofilm formation (Ziebuhr, *et al.,* 1997).

Studies of ESS by Christensen *et al.* described a slime-associated antigen (SAA) that was only expressed in slime-producing strains of CNS (1994). SAA was found to be immunologically distinct from PS/A and when strains were cultured on synthetic media (Hussain, *et al.,* 1991a), characterisation showed similarities to the slime extract described by Hussain *et al.* (Hussain, *et al.,* 1992); (Christensen, *et al.,* 1994). Baldassarri *et al.* (1996) have shown that SAA is chemically identical to PIA.

Kolonitsiou *et al.* (2001), have described a 20-kDa acidic polysaccharide (20-kDa PS) comprising 60% of carbohydrate-containing slime macromolecules, a peptidoglycan with average molecular size of 80-kDa (30% slime dry weight) and cell wall teichoic acid like substance. In addition, Kolonitsiou and coworkers suggest that the 20-kDa PS is distributed on the surface of the slime and forms the major antigenic component.

The contribution of other polymers and proteins in the adherence of bacteria to synthetic surfaces and the development of biofilm has been widely reported. Veenstra and coworkers described staphylococcal surface proteins (SSP-1 and SSP-2) present on organised fimbria-like appendages that protrude from the cell surface. It is reported that these proteins contribute to *S. epidermidis* adherence to polystyrene (Veenstra, *et al.,* 1996). Hussain *et al.* have reported the presence of an 140-kDa accumulation-associated protein (AAP) that is speculated to mediate *S. epidermidis* accumulation and growth, bind PIA and PSA to the staphylococcal cell surface or function as a signal transduction factor (Hussain, *et al.,* 1997). More recently, this group have reported the role of teichoic acids as bridging molecules between microorganisms and immobilized fibronectin, enabling polymer colonisation in early *S. epidermidis* pathogenesis (Hussain et al, 2001). In 2002, they reported a 60-kDa cell-secreted extracellular adherence protein (Eap) that has broad-spectrum binding characteristics (Hussain, *et al.,* 2002); (Palma, *et al.,* 1999). Eap is an analog of the major histocompatibility complex class II analog protein (Map) and is thought to contribute to pathogenesis by adhesion of whole staphylococcal cells to complex eukaryotic substrates. Eap binds fibrinogen, fibronectin and prothrombin (Palma, *et al.,* 1999). It forms oligomers and rebinds itself to the staphylococcal cell surface mediating bacterial agglutination. In addition, it has been shown to increase binding to epithelial cells and fibroblasts due to dual affinity for

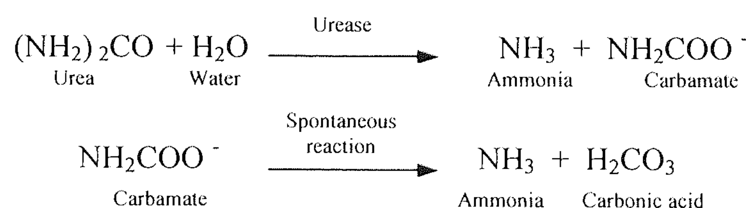
eukaryotic components and the *S. aureus* cell surface (Palma, *et al.*, 1999); (Hussain, *et al.*, 2002); (Haggar, *et al.*, 2003).

Heilmann *et al.* described an autolysin (At1E) that mediates attachment of bacterial cells to polymer surface, a prerequisite for biofilm formation. At1E has two bacteriolytically active domains a 60-kDa amidase and a 52-kDa glucosaminidase generated by proteolytic processing. The autolysin exhibits vitronectin binding activity suggesting a role in binding cells to naked polystyrene in early adherence and plasma coated polymer surfaces in the later stages of adherence (1997).

The trademark of biofilm formation appears to be the production of slime, however its composition remains elusive. Attachment and colonisation of prosthetic devices is a stepwise process though the generic and molecular basis of biofilm formation in staphylococci is multifaceted (Götz, 2002b).

1.6.4.4 Production of urease

Urease (urea amidohydrolase) is a heteromultimer nickel-containing metalloenzyme (Mobley, *et al.*, 1995). It is produced by a variety of bacteria including, *Proteus mirabilis*, *Helicobacter pylori*, *Escherichia coli* and staphylococcal species. The role of this enzyme is to catalyse the hydrolysis of urea to generate ammonia and carbamate. This results in the spontaneous decomposition of carbamate to ammonia and carbonic acid.



The synthesis of urease is pivotal to the pathogenicity of *P. mirabilis* and *H. pylori* allowing the generation of stone formation, acute pyelonephritis by *P. mirabilis* and infiltration of the gut mucosa and indirect neutralisation of gastric acid by *H. pylori* (Mobley, *et al.*, 1995); (McGee, *et al.*, 1999). In *Staphylococcus saprophyticus* urease production is reported to contribute to cystopathogenicity in urinary tract infections (Gatermann and Marre, 1989) (Gatermann, *et al.*, 1989). Urease expression is evident in a variety of bacteria and the mechanisms of urease regulation therefore vary according to the bacterial species involved. It is suggested that in *P. mirabilis* the mechanism responsible for urease regulation is the UreR

transcriptional regulator that, in the presence or urea activates the expression of the *ure* gene cluster (Dattlebaum, *et al.,* 2003). In *H. pylori* the mechanism involves the *flbA* gene cluster and in the presence of acidic conditions nickel has been shown to activate urease transcriptionally (McGee, *et al.,* 2002).

1.6.4.5 Production of DNase

Coagulase-positive staphylococci, i.e. *Staphylococcus aureus* are widely reported to produce DNases, however CNS, with the exception of *S. caprae* does not (Shuttleworth, *et al.,* 1997). The function of nuclease active enzymes is thought to aid tissue penetration by release of hyaluronidase, which degrades hyaluronic acid in cell membranes. In addition, this enzyme reduces the viscosity of DNA released from white blood cells in the form of viscous pus, and supports the proliferation of infection (Patrick and Larkin, 1995).

1.6.4.6 Production of cytolytic toxins

The production of cytolytic toxins is common to many bacterial species. They have a variety of functions that include acquisition of iron, tissue invasion and degradation of immune cells (Lebek and Gruenig, 1985); (Mempel, *et al.,* 2002); (Szmigielski, *et al.,* 1999); (Jonas, *et al.,* 1994).

S. aureus is reported to produce alpha, beta, gamma and delta cytolytic toxins/haemolysins (Jordens, *et al.,* 1989). These support the proliferation of bacterial infection in the following way:

- Alpha toxin — Depletes ATP levels in T lymphocytes and makes cell membranes permeable to monovalent ions. This pore formation may trigger programmed cell death in lymphocytes (Jonas, *et al.,* 1994).
- Beta toxin — Cytotoxicity and apoptotic cell damage enable tissue invasion (Mempel, *et al.,* 2002).
- Gamma toxin — Acts on cell membranes to trigger lysis of phagocytic cells allowing evasion of host immune defences (Szmigielski, *et al.,* 1999).
- Delta toxin — Membrane pore formation leads to cell lysis and allows penetration and spread of microorganisms (Titball, 1993).

1.6.5 Biotyping and Antibiograms

The identification of bacterial strains causing clinically significant infection is undertaken using commercial kits such as the analytical profile index (API) (BioMérieux, France). These kits exploit the reactions between the chemicals secreted by the microorganism and their substrates. Each test is scored according to its positive/negative reaction and generates a 7 to 9 digit profile number (dependent upon the test strip used) that identifies the bacterial species.

api Staph

REF.: Sample XIIXV

Origine / Source / Herkunft / Origen / Prelievo :

bioMérieux

O	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL	NIT	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE	LSTR
-	+	+	-	+	+	-	-	-	-	+	+	+	-	-	+	-	-	+	+	
1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
6			6			0			6			1			1			3		

Autres tests / Other tests / Weitere Tests / Altri tests / Otros tests :

Ident. : *S. epidermidis*

Letter triplets indicate biochemical tests contained in cupule.

Figure 1.22 Analytical profile index score sheet

Clinically significant microorganisms recovered from patient specimens are tested against a panel of antibiotics to establish a pattern of sensitivity or antibiogram. This antibiogram can be used to identify microorganisms with similar characteristics and aid with the diagnosis of endemic strains.

Previous work investigating identification methods has shown that traditional typing methods such as biotyping, antibiogram and bacteriophage typing have low discrimination for CNS. Strains of *S. epidermidis* exhibit too few distinct biotypes to have any significant discriminatory power (Geary, *et al.*, 1997); (Hébert, *et al.*, 1988). The epidemiological value of antibiograms as tools for typing is low for nosocomial strains of CNS as they have been cultivated from an endemic environment that promotes antibiotic resistance (Lang, *et al.*, 1999). Antibiograms may be able to identify strains, which are different but are not reliable for identification of strains that are the same. Similarly, bacteriophage typing is of limited value since many clinical strains of CNS are non-typable (Geary, *et al.*, 1997).

1.6.6 Pulsed-field gel electrophoresis

Molecular methods such as restriction endonuclease (RE) analysis of genomic DNA, plasmid analysis, random amplification of polymorphic DNA (RAPD) and DNA-DNA hybridisation are, in comparison to biotyping and antibiograms, sensitive tools for identifying different types of staphylococcal isolates (Riou, *et al.,* 1997), (Kloos and Bannerman, 1994); (Lina, *et al.,* 1992). Pulsed field gel electrophoresis (PFGE) of the staphylococcal chromosome following cleavage with rare-cutting restriction enzymes generates a profile of approximately 18 bands and allows comparison of isolates via analysis of macrorestriction patterns (Tenover, *et al.,* 1995). PFGE is currently the most sensitive technique available for epidemiological investigation of clonal relatedness between Gram-positive isolates (Lina, *et al.,* 1992); (Snopkovà, *et al.,* 1994); (Poddar and McClelland, 1991); (Livesely, *et al.,* 1998); (Kluytmans, *et al.,* 1998). The benefit of this method of molecular technique is that the complete bacterial genome is analysed as opposed to a proportion (Lang *et al.,* 1999). The choice of RE affects the number of bands generated in the profile; *Sma*I (cutting at CCC↓GGG) has been reported to be the most suitable RE for use in PFGE as it provides an adequate number of clearly separated fragments (Snopkovà, *et al.,* 1994).

PFGE allows the identification of the source of infection (Lang, *et al.,* 1999); (Lina, *et al.,* 1992) by allowing the relatedness of strains from the patient, clinical staff and the environment. In addition, it is an invaluable tool for the differentiation of relapse, recurrence and reinfection in patients with indwelling medical devices such as dialysis catheters (Chang, *et al.,* 2000)

1.6.7 Serodiagnosis of infection

The contribution of the Clinical Laboratory Service to the diagnosis of infection is not exclusively the remit of the Microbiology Department. Haematological investigation of blood samples also aids the clinical diagnosis of infection. A variety of biological factors can be used as markers of the immune response to infection; routinely these include white cell count (WCC), erythrocyte sedimentation rate (ESR) and C reactive protein (CRP).

In the event of bacteria entering the body the immune systems first line of defence is non-specific, that is to say that the recognition system used by immune cells such as phagocytes is innate. Monocytes, macrophages and polymorphonuclear neutrophils are able to bind to a

variety of microbial products allowing internalisation and killing of the bacteria to occur. In addition to this innate immune response, a more specific immune response is triggered. This involves lymphocytes that are able to recognise specific pathogens and is known as the adaptive immune response. Lymphocytes fall into two broad categories; B lymphocytes that produce antibody and eliminate extracellular pathogens and T lymphocytes that have a wide range of roles including control of B cell production, interaction with phagocytes to enhance bacterial killing and intracellular virus recognition.

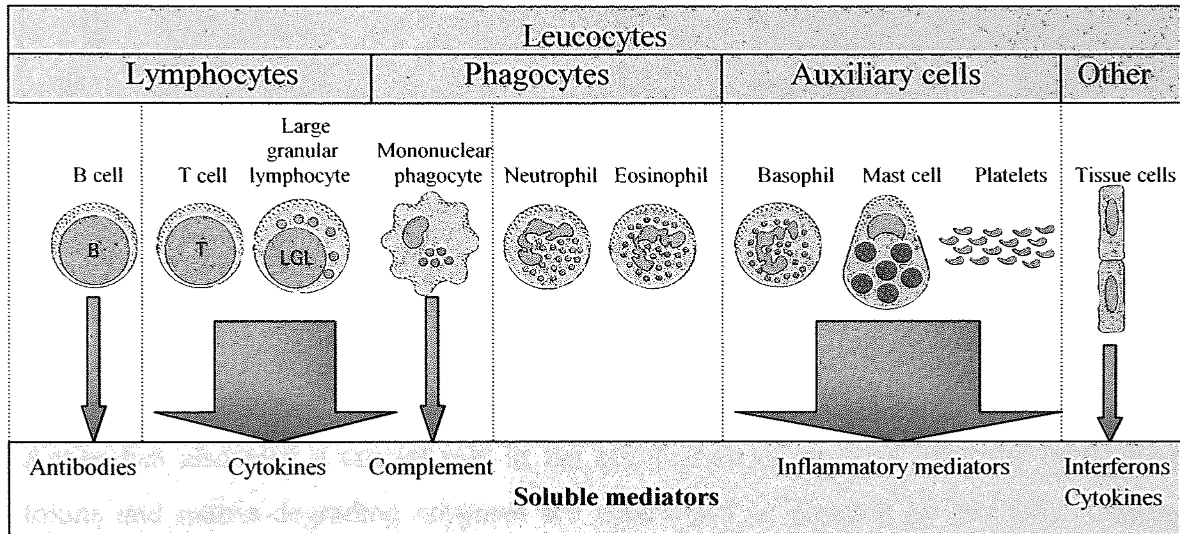


Figure 1.23 Components of the immune system and their soluble mediators (adapted from Roitt, Brostoff and Male, 1998).

In recognition of virulent microorganisms, the immune system is triggered by a number of molecules present in the serum and by receptors on cells, resulting in the activation of the alternative complement pathway. This recognition pathway signals the release of factors C3, B, D, P with subsequent release of C3a and C5a and activation of neutrophils, macrophages and natural killer cells. In addition, the triggering of cytokine release and mast cell degranulation leads to increased blood flow in the local capillary network and increased adhesion of cells and fibrin to endothelial cells (Roitt, *et al.*, 1998).

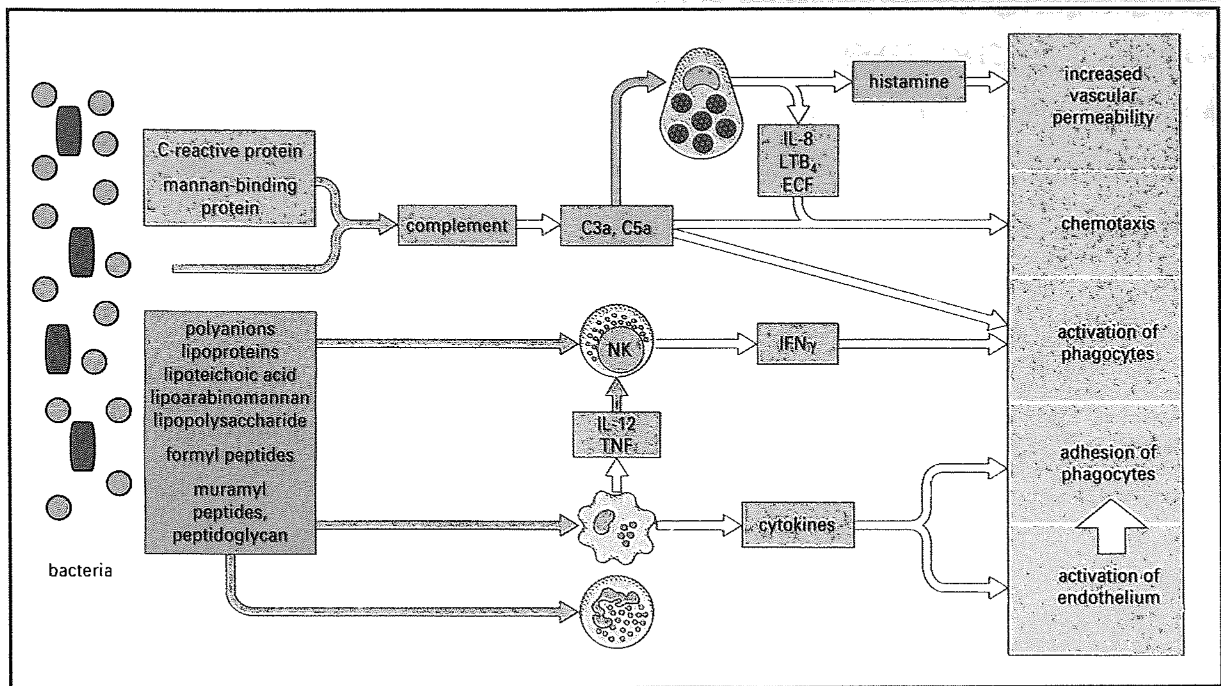


Figure 1.24 Bacterial components recognised by molecules present in serum and cell receptors (adapted from Roitt, Brostoff and Male, 1998).

Antibodies also play a crucial role in the elimination of bacteria from the body. Bacterial toxins and matrix-degrading enzymes are neutralised or blocked by antibody, limiting the bacteria's ability to spread. Immunoglobulin A (IgA) stops bacteria binding to epithelial cells and it is suggested that other immunoglobulins bind to the bacterial cell surface and interfere with iron-chelating compounds and nutrient uptake (Roitt, *et al.,* 1998). In non-toxigenic bacteria, antibodies ensure bacterial opsonization with complement. This increases the uptake of bacteria by phagocytes. The most efficient complement-fixing antibodies are IgG1, IgG3 and IgM (Roitt, *et al.,* 1998).

Figure 1.25 Antibacterial effect of antibody during infection (Roitt, Brostoff and Male, 1998).

There are five distinct classes of immunoglobulins or antibodies namely IgA, IgD, IgE, IgG, and IgM and in addition, IgG has a subclass of 4 molecules (IgG1, IgG2, IgG3 and IgG4). The different antibody classes vary in size, charge, amino acid and carbohydrate composition. In response to the initial exposure to a bacterial pathogen, the majority of immunoglobulin produced is IgM whereas subsequent exposure to the same pathogen results in an immunoglobulin class switch to predominantly IgG expression.

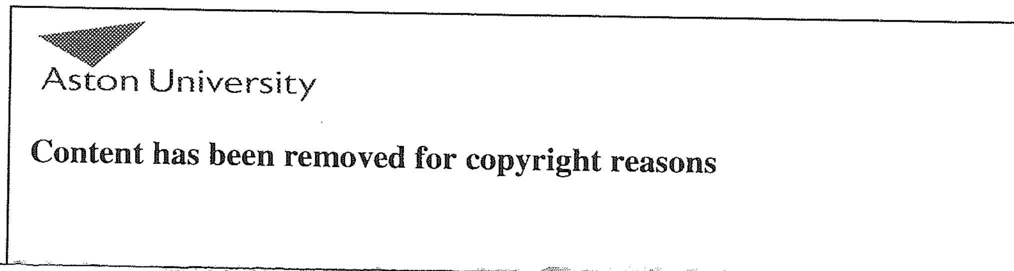

Aston University
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Figure 1.26 Primary and secondary immunoglobulin response to antigen stimulus (Roitt, *et al.,* 1998).

Phylogenetically CRP is a crude early immunoglobulin that initiates the inflammatory reaction. It forms complexes with antigen and facilitates the fixation of Clq. In addition, it triggers the complement cascade in response to tissue damage or antigen (Hoffbrand, *et al.,* 2001). After tissue injury, an increase in CRP can be detected in the blood within 6 to 10h. It is routinely used to detect early acute inflammation, tissue injury and the response of infection to antimicrobial treatment.

Erythrocyte sedimentation rate is a non-specific test routinely used to measure the speed of sedimentation of red cells in plasma over a period of 1h. The speed is dependent on the plasma concentration of large proteins e.g. fibrinogen and immunoglobulins. An increased ESR can be indicative of a number of systemic inflammatory conditions including pregnancy. At a rate of >100mm/h the ESR has a 90% predictive value for serious disease including infection and malignancy (Hoffbrand, *et al.,* 2001)

Haematological methods of the diagnosis of infection are not specific; however, during episodes of infection the number of certain types of immune cells and their products increase. Conversely, it is worth noting that some patients with ESRD experience impairment of phagocytosis and chemotaxis by polymorphonuclear leucocytes, depressed natural killer cell

activity, defective B and T lymphocyte function, impaired macrophage FCγ function, absolute lymphopenia and in addition, iron overload (Onyekachi, 1988). Although these immune insults may be partially reversed by dialysis, haematological inflammation/infection indicators may be of limited use in some patients with ESRD. However, in conjunction with microbiology culture these data give clinicians a quantitative analysis in order to establish a wider picture of the patient's condition.

1.6.7.1 *In situ* serodiagnosis of catheter-related infection

Methods to assist in the early diagnosis of catheter colonisation and related infection, whilst the device remains *in situ,* have been developed. These methods include a serodiagnostic test based on a novel short-chain lipoteichoic acid antigen isolated from staphylococci termed lipid S (Worthington, *et al.,* 2000b) and an acridine orange leucocyte cytospin (AOLC) (Kite, *et al.,* 1999); (Tighe, *et al.,* 1996). Indeed, these *in situ* diagnostic assays may aid clinicians in diagnosing CRI without unnecessary removal of the catheter. Accurate identification of CRI by these methods may not only improve patient outcomes but substantial savings could also be achieved by avoiding the removal of catheters that are not associated with infection (Moss and Elliott, 1997).

1.6.7.1.1 Acridine orange leucocyte cytospin

Methods of diagnosing catheter-related infection are slow and often require the removal and culture of the catheter to make an accurate diagnosis. The acridine orange leucocyte cytospin assay has been developed to enable rapid (30 min) diagnosis of catheter-related infection without catheter removal (Kite, *et al.,* 1999); (Tighe, *et al.,* 1996). The test relies on blood taken through the catheter to determine the presence or absence of microorganisms within the sample. Kite *et al.* (1999) reported a 96% sensitivity and 92% specificity for the test with a positive and negative predictive value of 91% and 97% respectively. One disadvantage of this method is the necessity to withdraw blood through the catheter hub, a recognised source of microbial contamination. Great care is therefore required with cleaning of the hub prior to the withdrawal of the sample.

1.6.7.1.2 Enzyme-linked immunosorbent assay

Verbrugh *et al.,* first described the enzyme-linked immunosorbent assay (ELISA) for the detection of staphylococcal antigens in the early 1980's (Verbrugh, *et al.,* 1983). They

demonstrated that ELISA detected significant levels of IgG antibodies to peptidoglycan. In addition, they reported that peptidoglycan was more antigenic than teichoic acids, alpha-toxin and nuclease, however, cross reactivity occurred with peptidoglycan but not with the other antigens tested (Verbrugh, *et al.,* 1983). Other staphylococcal products used to detect IgM and IgG antibodies have included α toxin, lipoteichoic acid (LTA) (Wergeland, *et al.,* 1984), staphylococcal lipase and protein A. These studies reported that IgM was of limited value in the detection of staphylococcal infection however; measurement of IgG was able to differentiate between infection types and severity (Julander, *et al.,* 1983). IgG antibodies to staphylococcal lipase were also reported to differentiate between infection types but were considered to be a supplementary test to support diagnosis by other means (Christensson, *et al.,* 1985) Protein A was reported to differentiate between infected and non-infected patients but no difference was demonstrated between types of infection (Greenberg, *et al.,* 1990)

More recently, antibody to a number of *S. aureus* antigens was reported to differentiate *S. aureus* osteomyelitis from bone infections (Lambert, *et al.,* 1996); (Lambert, *et al.,* 1992); (Krikler and Lambert, 1992). Subsequently, the modification of this assay based on the detection of a short-chain exocellular form of lipoteichoic acid (lipid S) has been reported (Elliott, *et al.,* 2000); (Worthington, *et al.,* 2002).

1.6.7.1.2.1 The Lipid S ELISA

Lipid S has been characterised as a short chain length exocellular form of the cellular lipoteichoic acid and shares common antigenic determinants with it. In contrast to LTA lipid S contains only six glycerophosphate units compared to the 40 to 42 units in LTA. This staphylococcal exocellular antigen can be recovered from the supernatant of liquid culture medium and has been reported to produce significant IgG titres in patients with catheter-related infection (Lambert, *et al.,* 2000); (Elliott, *et al.,* 2000), prosthetic joint infection (Rafiq, *et al.,* 2000) and infective endocarditis (Connaughton, *et al.,* 2001). Modification of this method has reduced the waiting time for results to less than 24 hours. This permits improved targeting of antimicrobial therapy and treatment outcomes in patients with Gram-positive infection (Worthington, *et al.,* 2002).

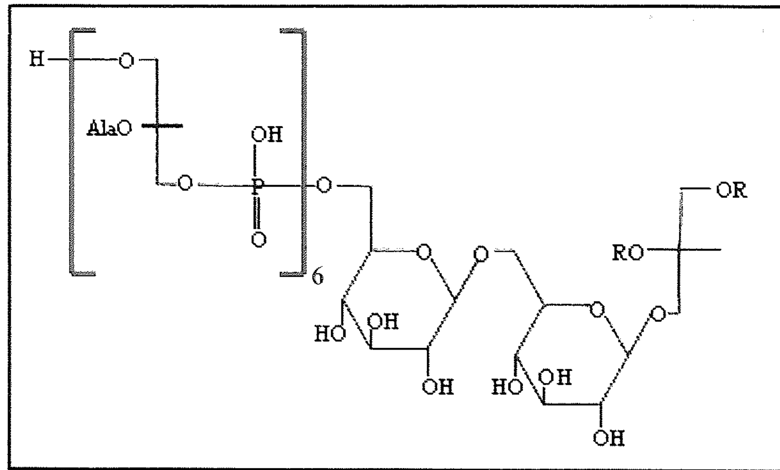


Figure 1.27 Structure of Lipid S from *S. epidermidis*. R, ester-linked fatty acids; Ala, ester-linked D-alainine (adapted from Lambert *et al.*, 2000).

1.6.8 Laboratory diagnosis of the faecal carriage of *C. difficile* and VRE

In the laboratory, the most reliable way of confirming the diagnosis is by the detection of toxin produced by *C. difficile* in the faeces of patients. Such tests are not routinely performed on all faeces specimens but are recommended for specimens from patients at increased risk of *C. difficile* infection, such as those on antibiotics and elderly patients in hospital. Although the bacterium may be grown on special culture medium, its isolation alone does not conclusively prove that the diarrhoea is due to this organism, as strains of *C. difficile* that do not produce toxin are unlikely to cause disease (Association of Medical Microbiologists, 1998).

Detection of the faecal carriage of VRE is confirmed by faeces culture on Slanetz and Bartley agar (Oxoid) containing a 5μg paper vancomycin disc (Oxoid). VRE is shown on a blood agar plate in section 1.4.2 figure 1.15.

1.7 TREATMENT OF INFECTION IN RENAL PATIENTS

1.7.1 Antimicrobial therapy

Improvements in dialysis treatment have resulted in patients with ESRD being dialysed more frequently, for longer periods with dialysers that are more efficient. Consequently, re-evaluation of antimicrobial administration regimes has been required. Antimicrobial agents that are completely renally excreted will necessitate more frequent dosing than those that are not. The difficulty in the choice of treatment for infection in patients with ESRD is

accentuated by the increased half-life experienced with decreasing renal function. The majority of antibiotics require varying degrees of adjustment in the dose given in patients with ESRD. For example, in non-ureamic patients the half-life of 1g of vancomycin is 5.6h whereas in a patient on dialysis it increases to 200h (Daugirdas, *et al.,* 2001), requiring an adjusted dose of 10% of that administered in non-ureamic patients. This has obvious advantages in terms of the reduction in the dose and frequency of administration however; the risk of toxicity increases greatly requiring careful monitoring of plasma levels. This is especially important with agents known to be nephrotoxic or those that increase nephrotoxicity when given in combination with other compounds, such as gentamicin when given with vancomycin or teicoplanin (British Medical Association / British National Formulary (BNF), 2003).

Management of patients receiving haemodialysis is largely undertaken in an out-patient setting with tri-weekly visits to independent dialysis units. Patients treated on CAPD manage themselves at home under the guidance of a hospital based CAPD unit. This poses further limitations of the choice of treatment for infection, as it needs to be flexible enough for administration on a tri-weekly basis while maintaining a concentration > 50% time above the minimum inhibitory concentration (MIC) of the causative microorganism and simple enough to be administered by the patient in the community. Furthermore, management of dialysis units are nurse led and as such, antimicrobial therapy is often protocol driven. This has advantages as dose administration is crudely based on the patients weight and the duration of treatment is uniformly dictated, thus making it easy to understand. However, this method of management does not allow for differences in patient symptoms and therefore restricts specific dosing based on the individual.

Antimicrobial therapy is commenced prior to the conformation of infection by microbiology culture results. This requires empirical treatment to encompass both Gram-positive and Gram-negative microorganisms. Until recently the established empirical antibiotic therapy for CRI and CAPD-associated peritonitis at the UHB, was vancomycin and ceftazidime (intravenous (IV) and intraperitoneal (IP) respectively). Vancomycin was given for the treatment of Gram-positive infection and ceftazidime for the treatment of Gram-negative microorganisms.

Vancomycin is a glycopeptide first isolated from *Streptococcus orientails* in the 1950's. It is an effective drug against *S. aureus*, methicillin resistant *S. aureus*, CNS, *E. faecalis*, *C. difficile* and Gram-positive cocci. Vancomycin exerts a bactericidal effect by inhibition of

peptidoglycan synthesis in the bacterial cell wall; moreover, it can be administered weekly when the patient presents for dialysis (Hugo and Russell, 1998). Equally, the liberal use of vancomycin in renal patients has played a role in the epidemic of vancomycin resistant enterococci and limited its usefulness in the control of serious staphylococcal disease (Speller, *et al.*, 1997); (CDC, 1997).

In 1988, a renal unit in London reported the first ever case of a vancomycin resistant *Enterococcus* (VRE) (Uttley, *et al.*, 1989). Furthermore, of the first four patients worldwide who were colonised with strains of *S. aureus* with intermediate susceptibility to vancomycin, one was receiving temporary dialysis and two were receiving chronic dialysis. In 2002, the first case of clinical infection with vancomycin resistant *S. aureus* (VRSA) was reported in a haemodialysis patient in Michigan USA. The patient had received long-term vancomycin therapy for a chronic *S. aureus* foot ulcer. VRE was also isolated from the ulcer and lateral transfer of resistance genes from *E. faecalis* to *S. aureus* was demonstrated (CDC, 2002).

A *Cephalosporium* species was first isolated from a sewage outlet off the Sardinian coast in the 1950's was found to have antimicrobial activity attributed to cephalosporin P and cephalosporin N. The latter compound giving rise to cephalosporin C, which was the basis for the first class of cephalosporin antimicrobial agents. As a group, the cephalosporins have low toxic and allergenic risks, which have allowed them to become an integral part of antimicrobial therapy in the hospital environment (Dancer, 2001). Ceftazidime is a third generation cephalosporin that is active against a wide variety of both Gram-positive and Gram-negative microorganisms. However, it is this broad-spectrum of activity that has applied selective pressure to certain microbial groups resulting in the emergence of resistance and treatment-associated disease (Dancer, 2001).

The use of ceftazidime has been implicated in the increased incidence of glycopeptide resistant enterococci (GRE), resistant to both vancomycin and teicoplanin (Nourse, *et al.*, 1998); (Bradley, 2000). The emergence of these glycopeptide resistant microorganisms is of particular concern as it leaves few therapeutic options. Studies have also shown that third generation cephalosporins including ceftazidime are particularly related with *C. difficile*-associated diarrhoea (CDAD) (Spencer, 1998); (Barbut and Petit, 2001); (Dahms, *et al.*, 1998); (Settle and Wilcox, 1996). Cephalosporin treatment disrupts the normal ecology of the bacteria in the gut, allowing *C. difficile* to proliferate in the absence of other flora. This results in the development of diarrhoea (Dancer, 2001).

The mounting evidence reported in the scientific press regarding the use of cephalosporins has prompted a change in empirical antimicrobial therapy within the renal service at the UHB. This has resulted in the withdrawal of cephalosporins from clinical use within this specialty. In the same way, the recognized consequence of widespread vancomycin use has encouraged the implementation of a 'consultant only' prescription, in an attempt to subvert the reliance on vancomycin as a first line treatment in hospitalised patients.

Haemodialysis patients who have a dialysis line *in situ* and who present with bacteraemia are currently treated with IV vancomycin and oral levofloxacin or rifampicin. If the patient is acutely unwell, the first line treatment is IV vancomycin and a single dose of gentamicin, then IV piperacillin/tazobactam. Patients who do not have a dialysis line in situ are treated with oral flucloxacillin and levofloxacin unless acutely unwell and then oral flucloxacillin and IV piperacillin/tazobactam. This is continued until microbiology results are available.

The protocol for the treatment of CAPD-associated peritonitis in patients who are not acutely unwell is oral levofloxacin and IP vancomycin and in those who are acutely unwell, IP vancomycin and piperacillin/tazobactam. This is continued until microbiology results are available.

Levofloxacin is a synthetic broad-spectrum antibiotic belonging to the fluoroquinolone class of agents. It acts as an inhibitor of bacterial topoisomerase IV and DNA gyrase; enzymes required for bacterial DNA replication, transcription, repair and recombination. Bacterial killing is concentration dependent and occurs via a bactericidal action at concentrations 1 to 4 fold greater than the microorganism's MIC. Levofloxacin is active against a range of Gram-positive and aerobic Gram-negative microorganisms however, methicillin-resistant strains of *S. aureus* and *S. epidermidis* are not susceptible and *E. faecalis* exhibits only moderate susceptibility to this agent (Ortho-McNeil, 2000). Resistance has been reported to develop rapidly during treatment, especially in *Pseudomonas* and cross-resistance among the fluoroquinolone class is common (Soussy, *et al.,* 1999).

The contribution of fluoroquinolones to CDAD was previously thought to be low however; recent studies have shown an increasing risk of CDAD associated with the newer fluoroquinolones (McCusker, *et al.,* 2003); (Ozama and Valadez, 2002); (Yip, *et al.,* 2001). In addition, one study reported a 95% resistance to levofloxacin by *C. difficile* (Alonso, *et al.,* 2001). This suggests that while the bioavailability for this class of agents is good and the

advantage of no additional post-dialysis dosing being required is convenient, the risk of serious complications due to this therapy is high and therefore the limitation of its use may be required in the future.

Piperacillin/tazobactam has recently been introduced into clinical practice in the UHB renal service as part of the empirical antimicrobial regime. Piperacillin is a member of the β-lactam class of antimicrobial agents with a broad-spectrum of activity, however the increased prevalence of β-lactamase producing microorganisms over the past few years has led to an increase in resistance to this class of agents resulting in their limited usefulness in the clinical setting. Tazobactam is a β-lactamase inhibitor that shows good inhibitory activity against plasmid-mediated β-lactamases, staphylococcal penicillinase and chromosomal 2e β-lactamases. This combination of compounds gives a broad-spectrum of antimicrobial activity against most Gram-positive and Gram-negative aerobic bacteria and anaerobic species. Piperacillin/tazobactam does not have activity against MRSA but has consistently demonstrated excellent activity against *C. difficile* (Spangler, *et al.,* 1994); (Namavar *et al.*, 1994), *E. faecalis*, and some strains of *E. faecium*. The action of piperacillin/tazobactam is bactericidal and it achieves its effect by attaching to penicillin-binding proteins and inhibiting cell wall synthesis (Marra and Jewesson, 1997). Excretion is largely via the renal route therefore dose adjustment is required in patients with renal impairment and as up to 50% is removed by haemodialysis, additional doses are required post treatment; this is not necessary in patients treated with CAPD (Perry and Markham, 1999).

Piperacillin/tazobactam has been shown to have good activity against *C. difficile* and enterococcal species and therefore does not contribute to antimicrobial associated complications. Bradley *et al.* (1999) reported that the substitution of ceftazidime with piperacillin/tazobactam in conjunction with the introduction of infection control measures has virtually eradicated GRE in some units. In addition, a subsequent return to ceftazidime use resulted in the re-acquisition of GRE (Bradley, *et al.,* 1999). Intravenous piperacillin/tazobactam has previously been used in patients undergoing CAPD, however, few studies have been published where the drug is administered via IP route.

Treatment of *C. difficile* associated diarrhoea is metronidazole. This agent belongs to a large and diverse group of compounds that include antibacterial, antifungal and antihelmintic agents (Hugo and Russell, 1998). It acts by interfering with anaerobic metabolism causing the generation of reactive products that breaks the bacterial DNA strands. Resistance to

metronidazole is uncommon therefore making it a useful agent in the treatment of *C. difficile* infection. Vancomycin is also active against *C. difficile* and can be used in cases where treatment outcomes with metronidazole are unsatisfactory.

1.7.2 Prophylactic therapy

Nationally, the Renal Association governs all renal service providers. This body was founded in 1950 and functions to improve the quality of treatment provided for people with kidney disease. In this role, the association develops evidence-based practice standards to ensure clinicians achieve an adequate quality of care. The prevention of infection in patients with ESRD is one component of these clinical standards and consequently forms a major part of clinical management at UHB. Many protocols have been developed and implemented within the renal service to reduce the incidence of infection in this patient group, especially in those patients requiring a dialysis catheter.

In accordance with 2002 the renal standards, the application of 2% mupirocin cream to the exit site of all newly implanted long-term dialysis catheters has recently been implemented. The cream is applied to the exit site tri-weekly at dialysis, for a period of 4 weeks post insertion. It is reported that by employing this practice the incidence of both exit site infection and catheter-related bacteraemia can be significantly reduced (Johnson, *et al.,* 2002). However, inspection of the catheter at the skin exit site should be carried out at each dialysis as 2% mupirocin cream contains polyethelene glycol, which is known to degrade polyurethane catheters (Tsuchida, *et al.*, 1998).

There has been a plethora of evidence reporting the emergence of mupirocin resistance in staphylococci, with the introduction of the application of mupirocin cream to the exit site of dialysis catheters (Pérez-Fontán, *et al.*, 2002); (Annigeri, *et al.*, 2001); (Skellum, *et al.*, 1998). This suggests that while incidence of infection is reduced the careful surveillance of staphylococci should also be introduced to elucidate the emergence of resistance.

Mupirocin ointment is used for the eradication of nasal carriage of *S. aureus* and MRSA in patients at UHB. Patients are screened prior to line insertion to establish their 'carrier' status. Patients who are nasally colonised with *S. aureus*/MRSA are treated with 2% nasal mupirocin 4 times a day for 10 days. Post treatment the patient is re-screened to establish eradication has taken place. If the screen is clear then line insertion can take place. In addition, cutaneous

application of chlorhexidine scrub is undertaken to minimise hairline and skin carriage of *S. aureus*/MRSA in all patients prior to line insertion. Equally, studies have shown that eradication of nasal carriage of MRSA is effective up to 2 weeks, however after 22 weeks 50% were carriers again, although it appeared to be a different phage type, rather than a true relapse (Casewell and Hill, 1986). Fernandez *et al.* (1995) reported post-treatment *S. aureus* recolonisation rates of 43%, 56% and 67% after 1 month, 2 to 4 and 6 months respectively. In addition, recolonisation with the same strain of *S. aureus* that had been isolated before treatment was reported as 32%, 40% and 48% (Fernandez, *et al.,* 1995).

Screening for *S. aureus*/MRSA at UHB is focused on swabs taken from the anterior naries and the groin however; Balfour and colleagues have suggested that throat carriage is of importance in the reaquisition of nasal *S. aureus* and therefore should be included in screening protocols (Balfour, *et al.*, 1997).

Maximum aseptic precautions including cap, mask, gown, gloves and drapes have been adopted for all line insertions and where possible all catheters are inserted in a designated procedure room. For each new catheter inserted, a calculated volume of heparin is injected (locked) into each catheter lumen to maintain the patency of the line and reduce the build-up of fibrin on the internal surface of the catheter. It is removed prior to line use. Antibiotic/anticoagulant locks have also been employed for the prevention of catheter-related infection and where indicated, this technique can be employed to treat colonised/infected haemodialysis catheters (Boorgu, *et al.,* 2000). Conversely, this is not appropriate for all antimicrobial agents, as incompatibilities with anticoagulants have been reported, for example, gentamicin is contraindicated for use with anticoagulants (BNF, 2003).

The rational behind antibiotic lock therapy is to improve the rates of catheter salvage and reduce the risks of antibiotic side effects (Berrington and Gould, 2001). This is achieved by the instillation of a concentrated antibiotic solution into each lumen of a colonised/infected line, at a volume that will fill the line but not escape into the circulation. This is then left locked in place for a designated period before being removed. This technique allows higher concentrations of antibiotic to be delivered directly to the site of infection and left for longer periods than would be possible by systemic administration. At UHB, teicoplanin is the agent of choice for use in this technique.

As previously discussed in section 1.3 new technological advances such as antibacterial coatings have made a difference in the development of indwelling catheters, however these devices have not yet found their place in routine practice (Berrington and Gould, 2001).

1.7.3 Infection control

With the national spotlight currently on the acquisition of nosocomial infection and the emergence of antibiotic resistance, the introduction of prophylactic management of infection and the enhanced profile of infection control provision is high on the agenda in all NHS trusts in the UK. Infection control is no longer exclusively the domain of the infection control team; it is the responsibility of everyone including domestic staff, the patient and the chief executive. Therefore, the control of infection is an integral part of the management of patients accessing the renal service at UHB.

One area of particular importance is the control of horizontal transfer of microorganisms between patients, staff and different clinical environments. This type of infection is often described as hospital acquired infection (HAI) and according to the National Audit Office (NAO) it costs the NHS an estimated £1000 million per year in treatment and extended hospital stay (NAO, 2000).

The environment in which patients receive haemodialysis lends itself to the horizontal transfer of infectious agents. This is a result of multiple patients receiving treatment concurrently therefore; guidelines have been developed to reduce the incidence of transmission of infection among haemodialysis patients and the patient population in general. These practice guidelines have been provided by the Hospital Infection Control Practices Advisory Committee (Hospital Infection Control Practices Advisory Committee (HICPAC), 2001) and Evidence-based Practice in Infection Control group (Evidence-based Practice in Infection Control group (EPIC), 2001).

Outbreaks of *C. difficile* infection have been described in many hospitals (Wilcox and Smuth, 1998). Patients with diarrhoea may unintentionally spread the infection to other patients. In addition, the ability of this bacterium to form spores enables it to survive for long periods in the environment, e.g. on bed tables, dialysis machine controls, floors and around toilets (Urbán, *et al.,* 2002); (Worthington, *et al.,* 2001). At the UHB, patients with diarrhoea are segregated from non-affected patients. Ideally, they are placed in a single room however if

this is not possible affected patients are cohorted. Staff wear disposable gloves and aprons when caring for infected patients. Rigorous cleaning regimes are adopted with warm water and chlorine detergent to removing spores from the contaminated environment. However, the most efficient control measure in preventing person-to-person spread of this infection is the thorough washing of hands by healthcare staff before and after patient contact and the education of staff and patients (Barbut and Petit, 2001).

1.7.4 Surveillance and audit of infection

Surveillance of CRI in dialysis catheters is poorly documented in the UK at present. In the USA the CDC has a Dialysis Surveillance Network (DSN) which aims to provide a method for individual haemodialysis centres to record and track rates of vascular access infections and other bacterial infections. These data will be used for 'benchmarking' rates between units and to motivate practice changes that may lead to a decrease in infection and antibiotic resistance (Hospital Infections Program, 2000).

Over the last few years, there has been a multitude of literature produced by the government to address the need for surveillance of HAI. In February 2000, the British government issued a report outlining the introduction of NNISS, clinical governance and controls assurance initiatives. These proposals would focus attention on ways of improving the management and control of HAI. July 2000 saw the government and the NHS plan announce a £61 million campaign to clean up our hospitals (HMSO, 2000). In October of the same year the minister for health declared that surveillance of HAI was to become compulsory and that the figures would be published (PHLS Communicable Diseases Surveillance Centre, 2000). In addition, another document entitled 'The path of least resistance' had been published which would look at antibiotic use and resistance (DOH, 1998). Since that time the Chief Medical Officer (CMO) has published 'Getting ahead of the curve' a strategy for combating infectious diseases (CMO, 2002). Collectively these documents emphasised the need for clinical audit of infection especially in those clinical areas where patients are at greater risk of acquiring nosocomial infection.

The UK Renal Registry does have a national surveillance scheme to determine among other things, the incidence of infection in renal centres in the UK, however not all centres participate. The renal service at the UHB is in the process of joining the scheme (Renal

Registry, 2002). Surveillance at a local level through clinical audit is important to maintain the progression of clinical effectiveness in individual units.

In the USA, the DSN reported on a new national surveillance system for haemodialysis-associated infections. Data entry was via the Internet or by paper submission. They reported that the data provided could be used for quality improvements at individual centres and help evaluate the efficacy of infection control measures (Tokars, *et al.,* 2002). With the introduction of evidence based practice guidelines the necessity for clinical audit is ever-increasing. Audit is a fundamental part of clinical management of patients with ESRD at UHB. An audit tool for the surveillance of CRI has been designed and is being piloted within the renal service at the UHB. The aim of the audit is to establish infection rates in catheters used for haemodialysis and highlight areas of clinical practice that can be improved in order to reduce CRI and improve clinical effectiveness.

Medical technology is advancing at an exponential rate. This provides clinicians with the tools to carry out procedures that are more intricate and prolong the life of patients who would previously have died however, such rapid advancement brings its own set of complex challenges. The reliance on indwelling vascular devices has increased the risk of infection; the excessive use of antimicrobial agents has spawned a worldwide epidemic or antimicrobial resistant microorganisms; HAI has made the hospital environment a dangerous place to be. Government initiatives have begun to address the problem, but the capacity to meet these challenges head on, lies in the education of clinical personnel at a local level. The mandatory reporting of infection requires surveillance to be effective in providing useful data, and as such, should be timely, comprehensive, meet local needs and provide information on the magnitude, causes and impact of disease (Wilcox, 2003). The provision of good surveillance systems is the key to infection control and the maintenance of clinical effectiveness.

1.8 AIMS AND OBJECTIVES

Patients with ESRD are a high-risk group for the acquisition of infection. The most frequent microorganism isolated from clinical samples in these patients is Gram-positive cocci, specifically staphylococci. Much work has been published in the scientific press suggesting the nature of opportunistic infection in patients with ESRD treated with RRT and strategies for its control. However, while guidance based on the experience of others is essential, infection surveillance and its subsequent control should be pertinent to the local environment and the endemic microorganisms that proliferate within it.

The aims of this study were to:

- 1a. Determine the rate of infection associated with renal dialysis catheters by means of a novel clinical audit tool and to identify common causative microorganisms.
- 1b. Establish genotypic macrorestriction profiles of CNS strains associated with infection in dialysis catheters.
- 1c. Identify specific CNS genotypes that may predict the likelihood of infection in renal dialysis catheters.
- 1d. Identify the phenotypic characteristics associated with staphylococcal strains associated with infection in dialysis catheters.
- 2a. Investigate the value of haematological and serological diagnosis of infection in renal dialysis patients.
- 2b. Assess any identified phenotypic characteristics as markers of infection in renal patients with dialysis catheters *in situ*.
- 3a. Determine the rate of faecal carriage of vancomycin-resistant enterococci and *Clostridium difficile* toxin in renal patients treated with intraperitoneal ceftazidime and vancomycin for CAPD-associated peritonitis.
- 3b. Determine the rate of faecal carriage of vancomycin-resistant enterococci and *Clostridium difficile* toxin in renal patients treated with piperacillin/tazobactam and vancomycin for CAPD-associated peritonitis.

CHAPTER 2: ETHICAL APPROVAL, MEDIA, REAGENTS, BACTERIAL STRAINS AND SERA

2.1 ETHICAL AND TRUST APPROVAL FOR RESEARCH

Local Research Ethical Committee and University Hospital Birmingham NHS Trust approval was obtained for all aspects of the work undertaken as part of this doctorate.

2.2 MEDIA

All growth media components were purchased from Oxoid Ltd, UK unless otherwise stated. During preparation of agar plates constituents were agitated by magnetic stirrer and autoclaved for 15 min at 121°C.

2.3 REAGENTS

All reagents used in this study were standard laboratory grade chemicals purchased from Sigma Chemical Company, USA, unless otherwise stated.

2.4 BACTERIAL STRAINS

A total of 305 clinically significant microbial isolates were recovered from 100 patients who presentated with infection and/or nasal carriage of *S. aureus*/MRSA. The clinical significance of isolates was determined by the number of similar colony forming units (CFU) observed following culture (cinical significance was determined if >15 CFU were observed) and Gram's stain. All plates were examined at 24 and 48 hours to allow for the emergence of colonial variants. Patient infection groups were divided in to haemodialysis infection (n=42) and those with CAPD-associated peritonitis (n=58). Three patients had been treated by both methods during the period of sample collection. All isolates were recovered from clinical specimens received by the Clinical Microbiology Laboratory, UHB NHS Trust. Clinical samples used for the recovery of microorganisms included blood cultures, peritoneal dialysate cultures, exit site swabs, MRSA screens and the distal tip of explanted catheters.

Table 2.1 Origin of Gram-positive microorganisms included in the study

Clinical specimen	Number of isolates recovered from CAPD patients	Number of isolates recovered from Haemodialysis patients
Blood culture	1	83
Peritoneal dialysis effluent	87	0
Haemodialysis catheter tip	0	36
Peritoneal dialysis catheter tip	6	0
Catheter exit swab	14	28
MRSA screen	3	12
Wound swab	1	14
Skin swab	8	12

Table 2.2 Types of microorganisms recovered from each patient group

Microorganism	**Patient Infection Groups**	
	Number of isolates from patients treated with CAPD	**Number of isolates from patients treated with haemodialysis**
CNS	37	35
Staphylococcus aureus	22	18
MRSA	4	4
Polymicrobial (Staphylococcal)	5	13
Enterococcal species	26	7

2.5 ROUTINE CULTURE METHODS FOR PERITONEAL DIALYSIS (PD) EFFLUENT AND BLOOD CULTURES

All PD and blood samples were cultured for the presence of microorganisms using the Bactec 240 blood culture system (Becton Dickinson, France). Positive PD and blood samples were detected by a colour change system located within the culture bottles. Positive samples were then inoculated onto Columbia agar containing 7% defibrinated horse blood supplemented with 20mg/L NAD (blood agar), cysteine, lactose, electrolyte deficient agar (CLED) and chocolate agar. These were then incubated at 37°C in 5% CO_2 for 48h. A second blood agar plate was also inoculated and cultured anaerobically at 37°C for 48h. After the incubation period the culture plates were inspected for microbial growth.

2.6 PD CELL COUNTS

The quantification of cells contained in PD fluid were determined by the following method: Two drops of the PD fluid were added to a test tube containing dehydrated 0.1% Toluidine blue stain and left to reconstitute for 1 minute. The solution was then mixed and loaded into a counting chamber (Fast Read 10) where the numbers of polymorphonuclear and mononuclear cells were counted. The following calculation was then used to find the number of cells per mm^3:

Total volume of counting chamber (ten 3x3 counting squares) $=10^{-3}$ mL volume of 3x3 counting square = 10^{-4}mL

Therefore: $$\text{Total cell count/mm}^3 = \frac{\text{Number of cells counted}}{\text{Number of 3x3 squares counted}}$$

2.7 CHARACTERISATION OF BACTERIAL STRAINS

2.7.1 Gram's stain

Morphology and Gram's reaction were evaluated by the standard microbiological Gram's stain.

2.7.2 Catalase production

A bacterial colony was transferred to a glass slide and 50 μl of 6% (v/v) hydrogen peroxide was placed over the colony. The liberation of oxygen from hydrogen peroxide, denoted by the immediate release of bubbles, indicated the presence of the bacterial catalase enzyme.

2.7.3 Tube-coagulase test

A bijou bottle containing 0.5mL of brain-heart infusion broth (BHI) was inoculated with three colonies of the test strain and combined with an equal volume of human plasma (UHB Blood Bank UK). The suspension was incubated at 37°C for 4h and then transferred to 20°C for a further 16 h. At incubation times 1, 4, and 20h the broth-plasma suspension was observed for coagulation: indicated by the formation of a plasma clot (Duguid, 1989). *S. epidermidis* NCTC 11047 and a fresh broth-plasma solution were included as negative controls. *S. aureus* NCTC 6571 provided a positive control.

2.7.4 Vancomycin sensitivity of enterococcal strains recovered from faeces

Vancomycin resistant enterococci were identified by culture on a Slanetz and Bartley agar plate containing a 5µg/mL vancomycin disc placed over the secondary inoculation streak.

2.7.5 Determination of *Clostridium difficile* toxins in faecal samples

All faecal samples were subjected to enzyme-linked immunosorbent assay (Meridian Bioscience Inc. USA) employing toxin specific monoclonal and polyclonal antibodies to determine the presence of *C. difficile* toxins A and B.

2.8 SPECIATION OF BACTERIAL STRAINS

2.8.1 Staphylococci

Coagulase negative staphylococci and *Staphylococcus aureus* were biotyped by API-Staph 20E Analytical Profile Index (bioMérieux, France), following the manufacturer's instructions and accordingly assigned to species. MRSA strains were further subjected to antimicrobial sensitivity testing with a panel of antimicrobial agents (table 2.3) and culture on Isosensitest agar.

Table 2.3. Antimicrobial agents tested against strains of methicillin resistant *staphylococcus aureus*.

Antimicrobial agent	Concentration of antimicrobial disc (µg)
Clindamycin	2
Erythromycin	5
Fucidin	10
Gentamicin	10
Linezolid	10
Mupirocin	5
Mupirocin	200
Rifampicin	2
Synercid	15
Tetracycline	10
Trimethoprim	5
Vancomycin	5

2.8.2 Enterococci

Enterococcal species were biotyped by BBL Crystal™ and vancomycin sensitivity was determined as outlined in section 2.2.4.

2.9 ADDITIONAL STRAINS

- *Staphylococcus*: the type strain *S. epidermidis* NCTC 11047; *S. epidermidis* (NU1); *S. epidermidis* 8965; *S. epidermidis* 023; *S. lugdunensis* (LON2); *S. sciuri* (CAPD 17); *S. simulans* (CAPD 24) and *S. aureus* NCTC 6571 (Oxford Strain) were supplied by Dr. S Lang, Aston University, United Kingdom.
- *Pseudomonas aeruginosa* (PAO1) was supplied by the Clinical Microbiology Laboratory, University Hospital NHS Trust, United Kingdom.

2.10 MAINTENANCE AND CULTURE OF BACTERIAL STRAINS

During periods of investigation, all bacterial strains were maintained on Columbia agar base supplemented with 5% (v/v) defibrinated horse blood. Strains were subcultured every 4 to 6 weeks. When isolates were not in use, they were stored on polystyrene beads at –70°C. Isolates were cultured in TSB or BHI broth at 37°C for 18h with aeration at 200rpm.

2.11 SERA

One thousand five hundred and twenty serum samples were collected from 623 renal patients over a period of 2 years. Serum samples were collected from haemodialysis patients with signs of infection and CAPD-associated peritonitis at the onset of infection and then monthly whenever possible. In addition, serum samples were collected monthly from patients who had no symptoms of infection, this is outlined in table 2.4

Table 2.4 Source of sera samples

Microbiological status	Number of serum samples		Number of patients	
	CAPD	Haemodialysis	CAPD	Haemodialysis
Infected patients	237	717	94	200
Non infected patients	166	400	90	240

CHAPTER 3: CLINICAL AUDIT AND THE SURVEILLANCE OF NOSOCOMIAL CATHETER-RELATED INFECTION

3.1 INTRODUCTION

Attitudes towards the quality of care provision within the NHS have changed in recent years. This has been illustrated by a conscious shift in policy that places emphasis on continual improvement of healthcare through life long learning and Clinical Governance. The pivotal ideology at the heart of these policies is the integration of clinical audit into the everyday practice of all healthcare professionals and service users.

Clinical audit is defined as a quality improvement process that seeks to improve patient care and outcomes through systematic review of care against explicit criteria and the implementation of change. Aspects of the structure, process and outcomes of care are selected and systematically evaluated against explicit criteria. Where indicated, changes are implemented at an individual, team or service level and further monitoring is used to confirm improvement in healthcare delivery (National Institute for Clinical Excellence (NICE), 2002).

Clinical audit is an effective way of measuring treatment outcomes, especially in connection with infection rates associated with indwelling medical devices. The increase in the incidence of hospital-acquired infection has led to the implementation of improved surveillance for nosocomial infections. The NAO stated in their 2000 report that at least 15% of hospital acquired infections are preventable (NAO, 2000). It is thought that improved infection control measures and instigation of clinical audit is the way to reduce the incidence of HAI. At the UHB, a clinical audit tool has been developed in conjunction with BD (Becton Dickinson, UK Ltd), to monitor the incidence of central venous catheter-related infection and recommend best practice guidelines.

3.2 MATERIALS AND METHODS

3.2.1 Patients

Two hundred and nine patients requiring a dialysis catheter as part of their clinical management were entered into the audit and followed-up until the catheter was explanted. Patients were entered into the audit as they presented for dialysis catheter insertion and over time, were divided into three cohorts based on periods of data collection.

3.2.2 Clinical practice outlines for dialysis catheter insertion and management

Over a period of three and a half years practice change was introduced following which, further audit was performed. These changes are shown schematically in figure 3.1

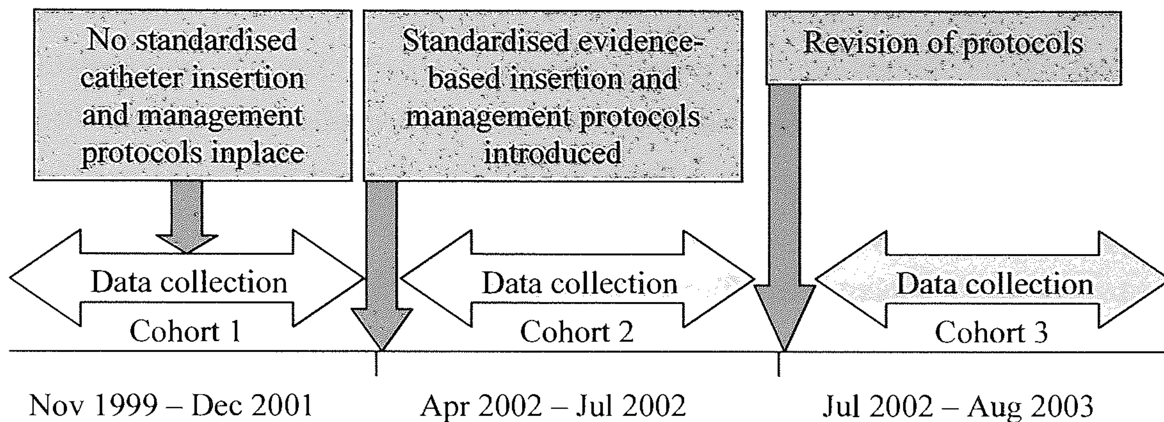


Figure 3.1 Schematic diagram to illustrate practice change and periods of data collection

3.2.2.1 Cohort 1 - November 1999 to December 2001

When the first cohort of patients were recruited to the study no standardised catheter insertion or management protocol was in operation, however all catheters were inserted under aseptic conditions and where possible in a dedicated procedure room. Catheter insertion was performed by a limited number of medical personnel who used the same insertion technique. Protective clothing that comprised of sterile gloves and gown were worn. Chlorhexidine gluconate 0.5% (w/v) in 70% isopropyl alcohol (v/v) was used to clean the skin site and sterile drapes were used to flank the insertion site.

Catheter management relied on individual dialysis nurses and the patient to assess when the dressing required changing. Commonly a Mepore dressing (Mölnlycke Health Care AB, Sweden) was applied to the catheter exit site and changed at each dialysis. The catheter exit site was cleaned at each dialysis with either 0.9% normal saline or chlorhexidine gluconate 0.5% (w/v) in 70% isopropyl alcohol (v/v). Catheter hubs were sealed with sterile Luer caps and long-term catheter limbs wrapped in sterile gauze between dialysis treatments. The hubs of long-term catheter limbs were not cleaned prior to attachment to the dialysis tubing at the next dialysis treatment.

3.2.2.2 Cohort 2 - April 2002 to July 2002

In April 2002, standardised evidence-based dialysis catheter insertion and management protocols were implemented within the renal services. These clinical practice guidelines stipulated all dialysis catheter insertions should take place in a dedicated procedure room and outlined infection control practices that were to be employed to maintain an appropriate environment. Patients were asked to shower and wash their hair with Hibiscrub (chlorhexidine gluconate 4% (w/v)) on the morning of the procedure. Patient screening for MRSA carriage was introduced prior to catheter insertion and only patients who were MRSA free were allowed to proceed. In addition, medical personnel inserting catheters were also screened for MRSA and were required to be MRSA free before performing catheter insertions. Medical personnel were required to be sufficiently trained for the procedure prior to performing catheter insertions. Masks and caps were introduced as part of the protective clothing worn during catheter insertion and an OpSite dressing (Smith and Nephew, UK) was applied to the catheter exit site post insertion.

An extensive dialysis catheter management protocol was introduced to all dialysis units managing UHB patients. It consisted of a 33 step procedural guide that covered preparation of the required equipment, protective clothing and hand disinfection, preparation and protection of the patient and their catheter lumens, hub disinfection, exit site cleaning and connection/disconnection to the dialysis tubing using chlorhexidine gluconate 4% (w/v). Catheter exit site dressings were restricted to Tielle (Johnson and Johnson Medical Limited, UK) or IV3000 (Smith and Nephew, UK). Prior to the manipulation of any dialysis catheter, all nursing personnel working in a dialysis unit were assessed against the catheter management protocol. Dailysis personnel were required to competently perform procedures before being able to undertake the catheter management protocol independently.

3.2.2.3 Cohort 3 - July 2002 to August 2003

Revision of the dialysis catheter insertion and management protocols in July 2002 resulted in the implimentation of 2% mupirocin cream (Glaxo SmithKline, UK) being applied to the exit site of all newly implanted dialysis catheters. This was introduced in accordance with the practice guidlines of the National Kidney Foundation Kidney Dialysis Outcomes Quality Initiative (2000) and the Renal Association practice standards (2002). Mupirocin (2%) was applied immediately post insertion and then three times per week at dialysis for three weeks.

In addition, all patients requiring a long-term dialysis catheter were screened for *Staphylococcus aureus* nasal carriage and if positive treated with a course of Naseptin® (chlorhexidine hydrochloride 0.1%, neomycin sulphate 3250 units/g, Alliance Pharmaceuticals, UK), then re-screened. They were required to be *S. aureus* free prior to catheter insertion.

3.2.3 Meditrend audit tool

The Meditrend audit tool consisted of a questionnaire and a computer software package. The questionnaire comprised of 64 questions (appendix 1) that related to catheter insertion and management. The initial section was completed at the time of catheter insertion and a follow-up period was used to monitor the development of local or systemic bloodstream infection associated with the catheter. The questionnaire was completed when the catheter was explanted. These data were then entered onto the computerised database.

3.2.4 Diagnosis of catheter-related infection

Diagnosis of catheter-related infection was determined based on the criteria of Elliott *et al.* (1998). This criteria is outlined in section 1.6.3 of the introduction.

3.2.5 Statistical analysis

Statistical analysis was undertaken using a Microsoft Excel spreadsheet (Microsoft Corporation, USA) and GraphPad InStat software (GraphPad Software, Inc., USA).

3.3 RESULTS

3.3.1 Patients

Two hundred and nine renal medical patients were studied over a period of three years and nine months. The breakdown of patient cohorts are shown in table 3.1

Table 3.1 Patient demographics

Patient Group	Number of patients	Gender ratio (M:F)	Age range (Mean)
C1	77	42:35	19-94 (57)
C2	75	39:36	22-89 (62)
C3	57	29:28	24-84 (62)

3.3.2 Dialysis catheter insertion

During the study period, 305 dialysis catheters were inserted. Ninety seven percent of catheter placements were undertaken in a dedicated procedure room situated on one of the renal medical wards. The other 3% were undertaken in the radiology department by a radiologist or in the acute renal care unit by a registrar level doctor. In both locations, dialysis catheters were inserted under strict aseptic conditions. The number and types of catheters studied are shown in table 3.2

Table 3.2 Types of dialysis catheters inserted in each cohort of patients

Patient Group	Total number of dialysis catheters inserted	Number of temporary non-tunnelled catheters	Number of long-term tunnelled catheters
C1	81	22	59
C2	162	120	42
C3	62	0	62

In the first cohort of patients catheter placement was carried out by medical registrars and senior house officers; in the second and third cohort of patients, catheter placement could only be undertaken by medical staff who were registrar level or above and who had been assessed by a senior medical practitioner and certified competent to carry out the role.

Chlorhexidine gluconate 0.5% (w/v) in 70% isopropyl alcohol (v/v) with pink colouring (Adams Healthcare, UK) was used to clean the skin prior to catheter insertion in all patient

cohorts. In addition, all catheters were inserted aseptically using the Seldinger technique and the insertion site was enlarged using a dilator and a scalpel. All of the catheters used within the renal services were made of polyurethane and did not contain any antimicrobial impregnation/coatings. Once the catheter was inserted, Luer lock caps were routinely attached to the catheter hubs.

A breakdown of anatomical placement is shown in figure 3.2

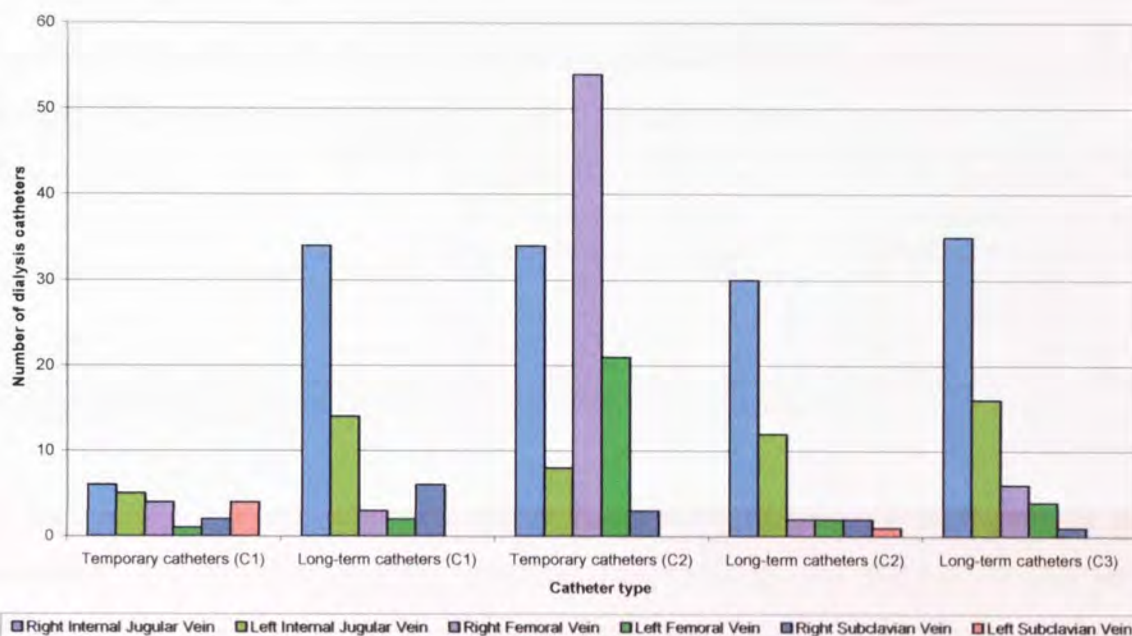


Figure 3.2 Anatomical placement of dialysis catheters

3.3.3 Dressings applied to the catheter exit site

In the first cohort of patients a variety of sterile dressing types were applied to the catheter exit site following insertion. These ranged from plain gauze to semi-permeable transparent dressings. In the following two patient cohorts, the dressings were restricted to one of three makes of semi-permeable dressing, OpSite (Smith and Nephew, UK) immediately post insertion, Tielle (Johnson and Johnson Medical Limited, UK) or IV3000 (Smith and Nephew, UK) for general catheter management.

3.3.4 Time to catheter removal and incidence of catheter-related bloodstream infection

The length of time the catheters remained *in situ* is shown in table 3.3.

Table 3.3 The number of days dialysis catheters remained *in situ*

Patient Group	Number of days temporary catheters remained *in situ* (Mean)	Number of days long-term catheters remained *in situ* (Mean)
C1	2-163 (49.7)	10-398 (161.7)
C2	1-232 (11.4)	1-430 (82.6)
C3	N/A*	2-359 (125.4)

* No temporary catheters were inserted at the time of data collection.

The numbers of bloodstream infections (BSI) per 1000 catheter days are shown in table 3.4

Table 3.4 The effect of implementation of standardised practice protocols on the number of BSI per 1000 catheter days in temporary and long-term catheters.

Patient Group	Total number of BSI per 1000 catheter days in temporary catheters	Total number of BSI per 1000 catheter days in long-term catheters
C1	8.0	2.8
C2	7.7	2.4
C3	N/A*	2.0

*No temporary catheters were inserted at the time of data collection.

A two-tailed t test was used to compare the number of BSI per 1000 catheter days in temporary and long-term dialysis catheters. The result showed the rate of BSI per 1000 catheter days to be significantly lower in long-term dialysis catheters than in temporary dialysis catheters ($p=0.0061$).

The number of days post insertion to onset of BSI is shown in table 3.5

Table 3.5 The number of days post catheter insertion to onset of BSI

Patient Group	Mean number of days to onset of BSI in temporary catheters (Range)	Mean number of days to onset of BSI in long-term catheters (Range)
C1	62 (2-136)	149 (2-348)
C2	10 (0-71)	50 (4-181)
C3	N/A*	51 (1-209)

* No temporary catheters were inserted at the time of data collection.

The rate of catheter exit-site infection (ESI) per 1000 catheter days can be seen in table 3.6

Table 3.6 The effect of implementation of standardised practice protocols on the number of catheter ESI's per 1000 catheter days in temporary and long-term catheters.

Patient Group	Total number of catheter ESI per 1000 catheter days in temporary catheters	Total number of catheter ESI per 1000 catheter days in long-term catheters
C1	2.0	1.3
C2	2.3	0.9
C3	N/A*	0.8

* No temporary catheters were inserted at the time of data collection.

The number of days post insertion to onset of ESI is shown in table 3.7

Table 3.7 The number of days post catheter insertion to onset of ESI

Patient Group	Mean number of days to onset of ESI in temporary catheters (Range)	Mean number of days to onset of ESI in long-term catheters (Range)
C1	79 (9-120)	162 (2-458)
C2	7 (4-11)	29 (4-53)
C3	N/A	51 (11-208)

3.3.5 Long-term dialysis catheter survival

Dialysis catheter survival is shown in figure 3.3

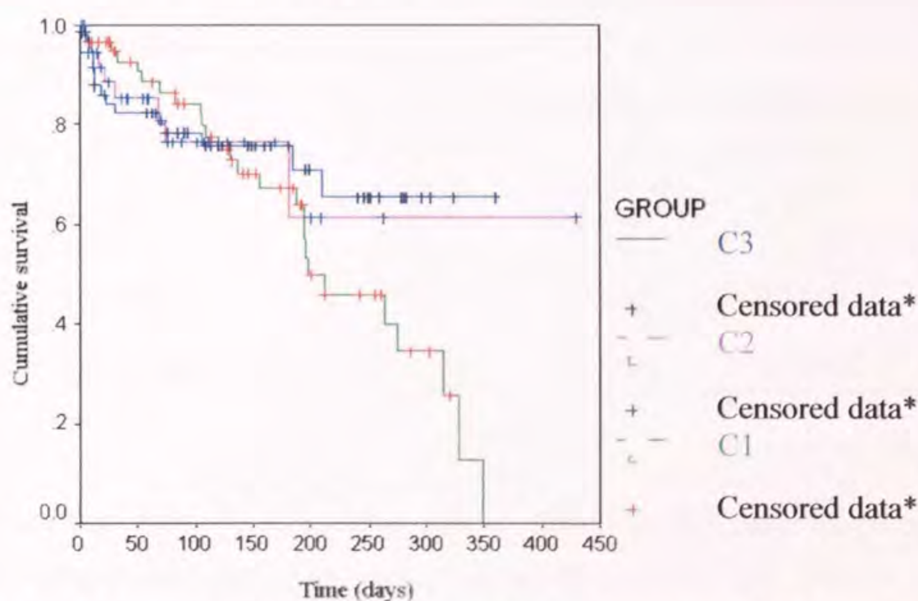


Figure 3.3 Kaplan-Meier curves to compare the survival of long-term dialysis catheters with the implementation of standardised insertion and management protocols

* Censored data reflects patients who had their catheters removed without developing BSI.

Statistical analysis using Log Rank, Breslow and Tarone-Ware tests returned p values of 0.3829, 0.9407 and 0.9507 respectively. This indicates that the difference in dialysis catheter survival over time with the implementation of standardised insertion and management protocols was not statistically significant.

3.3.6 Patient risk factors at the time of catheter insertion

Patient risk factors for infection at dialysis catheter insertion are shown in table 3.8

Table 3.8 Patient risk factors for acquisition of infection at time of catheter insertion

Risk factor	C1	C2	C3
Concurrent central line	5	1	0
Anticoagulant therapy	39	43	35
Colonisation by resistant microorganisms	1	1	1
Diagnosed Immune-deficiency	6	10	8
Diabetic	26	23	31

The relative risk of BSI in patients who had one of the above risk factors (table 3.8) was analysed with a 2 X 2 contingency table. A two-sided p value was calculated by Fisher's Exact Test; the relative risk (95% confidence interval) with the approximation of Katz. These data are shown in table 3.9.

Table 3.9 Statistical significance and relative risk of infection associated with patient risk factors at the time of catheter insertion.

Risk factor	Temporary non-tunnelled catheters		Long-term tunnelled catheters	
	P value	Relative risk	P value	Relative risk
Concurrent central line	0.0231	7.000	0.5546	0.000
Anticoagulant therapy	1.0000	0.4204	1.0000	0.9521
Colonisation by resistant microorganisms	N/A*	N/A*	0.5546	0.000
Diagnosed immune-deficiency	1.0000	0.3574	0.5536	0.6793

*Zero values obtained

Patients who already had one catheter *in situ* at the time of further temporary catheter insertion were significantly more likely to develop a BSI (p=0.0231, RR 7.000, 4.665-10.504) than those patients who did not. There was no significant difference between the total number of risk factors for each patient and the likelihood of BSI in any of the patient cohorts (C1 Chi^2=2.529 p=0.4701, C2 Chi^2=0.7877 p=0.8524, C3 Chi^2=0.4594 p=0.9277).

3.3.7 Comorbidity and risk of BSI in patients with dialysis catheters

Patient comorbidity (more than one diagnosed medical condition) was analysed to establish if an increased risk of developing BSI existed given certain medical conditions. These data can be seen in table 3.10.

Table 3.10 Statistical significance and relative risk of BSI associated with comorbidity in patients with dialysis catheters.

Medical condition	**Temporary non-tunnelled catheters**		**Long-term tunnelled catheters**	
	P value	**Relative risk**	**P value**	**Relative risk**
Asthma	**0.0686**	**2.646**	1.0000	0.2185
Autoimmune disease	0.6919	0.5159	0.7235	0.4384
Cardiovascular disease	1.0000	0.4128	0.4639	0.7681
Cerebrovascular disease	0.3766	0.6573	0.7726	**1.121**
Diabetes	0.5716	**1.375**	**0.0002**	0.180
Epilepsy	N/A*	N/A*	0.5122	**1.677**
Gout	**0.0231**	**7.000**	1.0000	0.2203
Hypertension	0.1542	0.2515	0.3047	0.7754
Hypoparathyroidism	0.6537	0.3583	1.0000	0.1492
Malignancy	1.0000	0.7037	0.4302	0.5972
Rheumatoid arthritis	1.0000	0.000	1.0000	0.1492

* Zero values obtained. Figures in bold indicate statistically significant result and an increased relative risk.

Patients who had gout and had a temporary dialysis catheter inserted were significantly more likely to develop a BSI (p=0.0231, RR 7.000, CI 95%, 4.665-10.504). Patients with asthma who had a temporary dialysis catheter inserted, were twice as likely to develop BSI (RR 2.646) than patients without asthma. Patients who had a long-term dialysis catheter inserted had a slightly increased risk of developing BSI if they had cerebrovascular disease or epilepsy (RR 1.121, CI 95%, 0.5257-2.392 and RR 1.677, CI 95%, 0.4109-6.844 respectively). In addition, diabetic patients with long-term catheters were significantly more likely to develop BSI (p=0.0002). In temporary catheters, diabetic patients were shown to have a slightly increased risk of developing BSI (RR 1.3750, CI 95%, 0.5957-3.175). There was no significant difference between the accumulative number of comorbid processes each patient had and the incidence of BSI in any of the patient cohorts (C1 Chi^2=23.26, p=0.5075, C2 Chi^2=2.537, p=0.4686, C3 Chi^2=1.635, p=0.6515).

3.3.8 Microorganisms recovered from clinical specimens

The microorganisms recovered from blood culture specimens are shown in figure 3.4

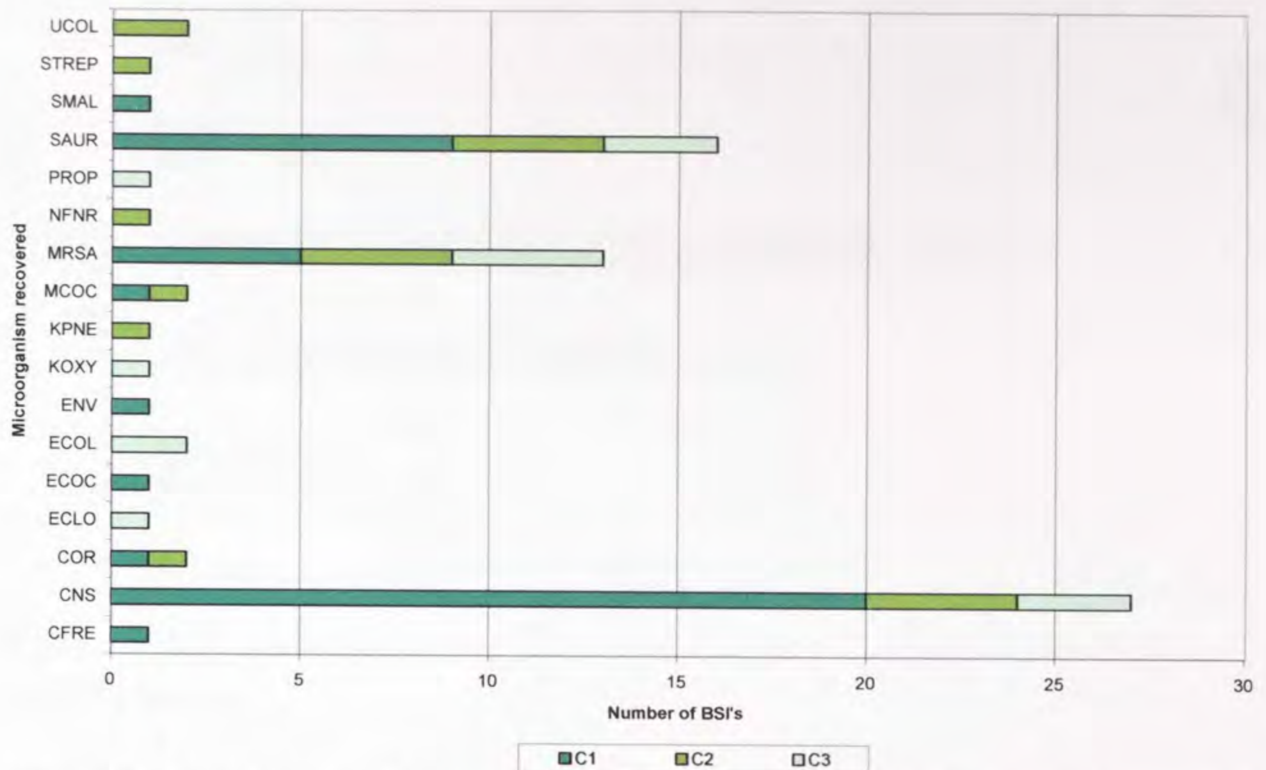


Figure 3.4 Microorganisms recovered from clinical specimens of patients with suspected catheter-related bloodstream infection.

Key to microorganisms: UCOL unidentified Coliform, STREP - *Streptococcus* species, SMAL – *Stenotrophomonas maltophilia*, SAUR - *Staphylococcus aureus*, PROP - *Propionibacterium*, NFGNR – non-fermenting Gram-negative rod, MRSA - methicillin-resistant *Staphylococcus aureus*, MCOC - *Micrococcus* species, KPNE - *Klebsiella pneumoniae*, KOXY - *Klebsiella oxytoca*, ENV - environmental Gram-negative, ECOL - *Escherishia coli*, ECOC - *Enterococcus* species, ECLO - *Enterobacter cloacae*, COR - *Corynebacterium*, CNS - coagulase-negative staphylococci, CFRE - *Citrobacter freundii*.

Gram-positive microorganisms were recovered from blood culture specimens most often in all patient cohorts (C1 92%, C2 78%, C3 73%) with the predominant genus being *Staphylococcus*. However, the rate of recovery of Gram-negative microorganisms increased with each patient cohort (C1 8%, C2 22%, C3 27%).

The microorganisms recovered from skin swabs taken from the catheter exit site are shown in figure 3.5

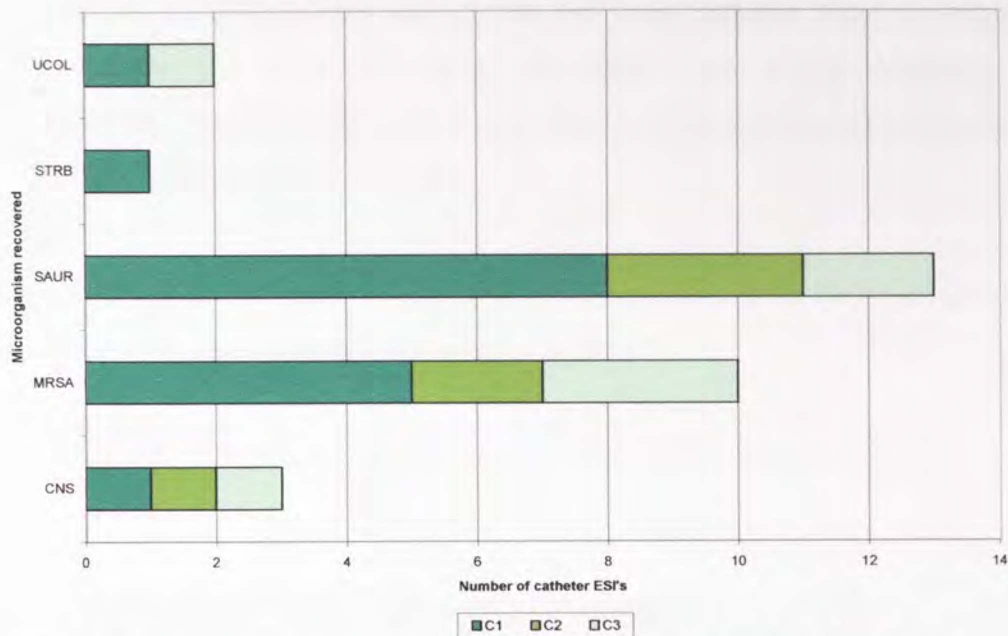


Figure 3.5 Microorganisms recovered from catheter exit sites of patients with suspected localised infection

Gram-positive microorganisms were recovered from catheter exit site swabs most often in all patient cohorts (C1 93%, C2 100%, C3 85%) with the predominant genus being *Staphylococcus*. However, the rate of recovery of Gram-negative microorganisms increased over time (C1 7%, C2 0%, C3 15%).

The microorganisms recovered from the distal tip of dialysis catheter specimens are shown in figure 3.6

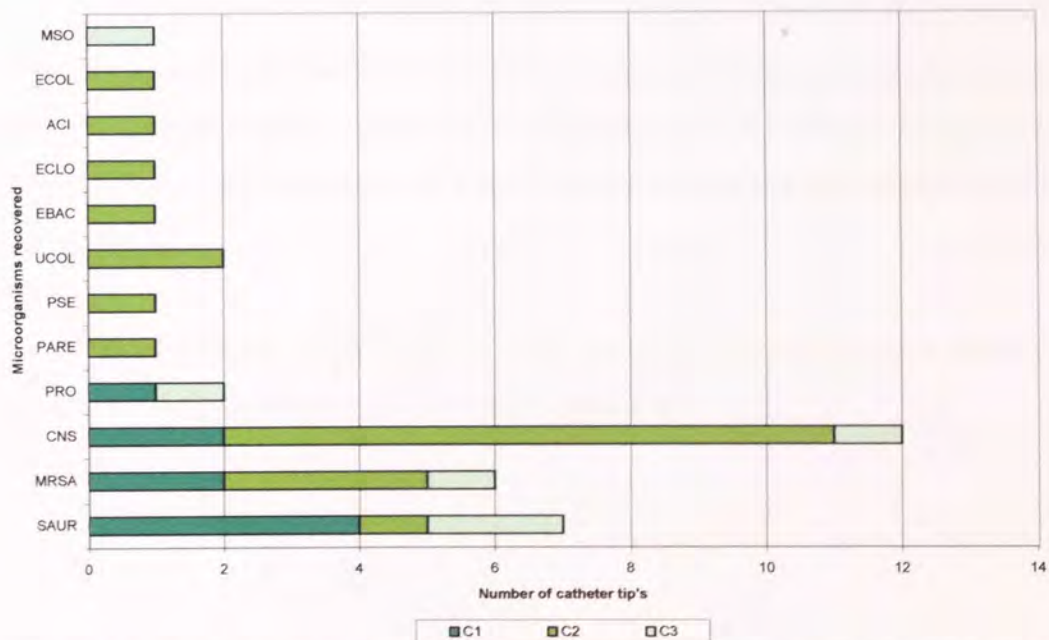


Figure 3.6 Microorganisms recovered from the distal tip of dialysis catheters in patients with suspected catheter-related bloodstream infection

Gram-positive microorganisms were recovered from catheter distal tip most often in all patient cohorts (C1 88%, C2 61%, C3 66%) with the predominant genus being *Staphylococcus*. However, the rate of recovery of Gram-negative microorganisms increased over time (C1 12%, C2 39%, C3 34%).

The incidence of the same microorganisms being recovered from the blood culture and the distal tip of the dialysis catheter is shown in figure 3.7

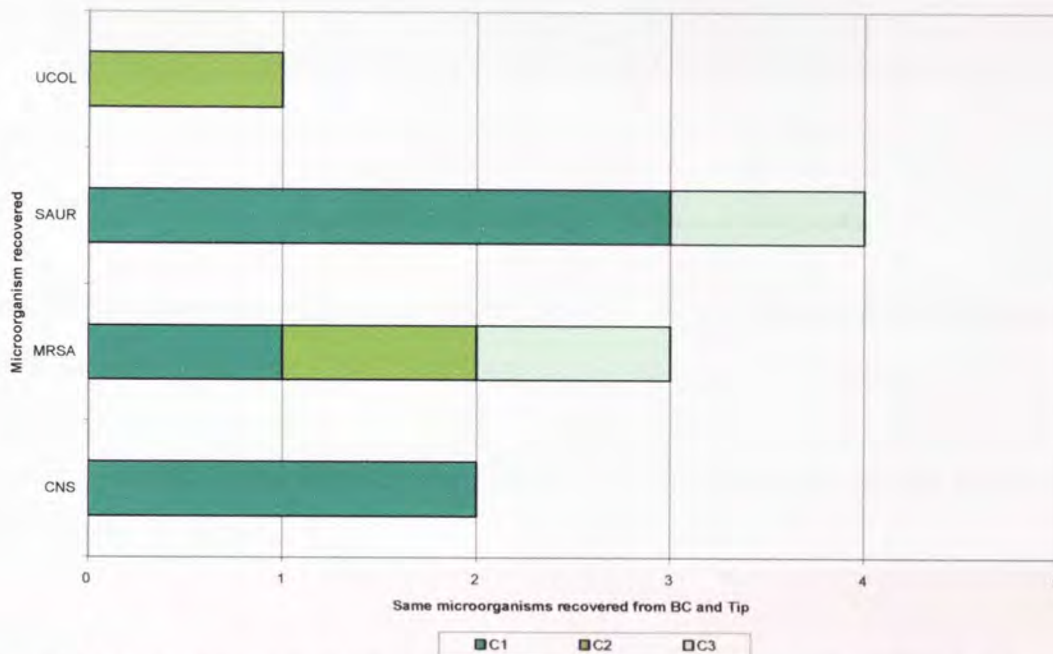


Figure 3.7 The incidence of the same microorganisms being recovered from the blood culture and the distal tip of the dialysis catheter

The same microorganisms recovered from blood culture and catheter exit site swabs were predominantly Gram-positive (90%) and of the genus *Staphylococcus*. There was a higher incidence of recovering staphylococci from the blood culture and the catheter distal tip in C1 than in C2 and C3.

The incidence of the same microorganism being recovered from the catheter exit site and the distal tip of the dialysis catheter are shown in figure 3.8

Figure 3.8 The incidence of the same microorganisms being recovered from the catheter exit site and the distal tip of the dialysis catheter

The incidence of the same microorganism being recovered from the exit site and the blood culture are shown in figure 3.9

Figure 3.9 The incidence of the same microorganism being recovered from the exit site and the blood culture

Staphylococci accounted for all incidences where the same microorganism was isolated from the catheter exit site swab and the blood culture.

3.4 DISCUSSION

3.4.1 Dialysis catheter insertion and management

Three hundred and five renal dialysis catheters were investigated during the study period. In C2, many of the lines inserted were temporary femoral catheters whereas in C3 no temporary lines were inserted during periods of recruitment. This was due to the clinical condition of patients who presented for catheter insertion during these periods of audit.

Patient demographics were similar in all cohorts. End stage renal disease is more common in men than women (UK Renal Registry, 2002); (United States Renal Data System (USRDS), 2004)) and this was reflected in the number of each gender requiring dialysis catheter insertion.

Bloodstream infections per 1000 catheter days were the highest in C1 for both temporary and long-term catheters (8.0 and 2.8 respectively). Reported dialysis catheter infection rates vary from 3.8-6.5 per 1000 catheter days for temporary non-tunelled catheters and 1.6-5.5 per 1000 catheter days for long-term tunnelled catheters (Kairaitis and Gottlieb, 1999); (Saad, 1999) (Oliver, *et al.,* 2000); (Little, *et al.,* 2001); (Beathard, 1999). It has been suggested that the variance in reported infection rates may relate to differing definitions of complications and a diversity of catheter management protocols in individual renal units (Lund, *et al.,* 1996). In this study the standard insertion technique and skin disinfection was the same for all catheters however the infection control practices and catheter management protocols changed over time. The incidence of BSI in those patients with temporary dialysis catheters was higher than reported rates.

Weijmer and colleagues (2004) suggest that inserting long-term tunnelled catheters requires the operator to have more experience and special skills compared to the insertion of temporary non-tunelled catheters. In this study, medical personnel were educated and assessed in catheter insertion procedures as part of their general medical training, however this may have taken place in a non-dialysis setting. In addition, in C1 more than one level of medical personnel undertook the role of dialysis catheter insertion. These factors may have contributed to inconsistencies in technique and experience. At this time, dialysis catheters were inserted in a range of clinical environments; making it difficult to standardised infection control procedures. Furthermore, the level of protective clothing adopted during catheter

insertion was insufficient to minimise the risk of the horizontal transfer of microorganisms from operator to patient. The dispersion of commensal flora such as *S. aureus* from the anterior nares of the operator onto the insertion site could contribute to the incidence of BSI and exit site infection. In addition, chronic haemodialysis patients are at increased risk of *S. aureus* nasal carriage; the literature reports a 50% to 60% carriage rate in haemodialysis patients (Yu, *et al.,* 1986). This may result in the seeding of the skin with endogenous *S. aureus* leading to increased incidence of BSI and exit site infection.

The National Kidney Foundation Disease Outcomes Quality Inititive (National Kidney Foundation Kidney Dialysis Outcomes Quality Initiative (K/DOQI), 2000) recommend that long-term tunnelled catheters are inserted in any patients who require a dialysis catheter for longer than 3 weeks and those with maturing AVF. They also state that temporary non-tunnelled catheters should be used for no more than 3 weeks as they are associated with a greater incidence of infectious complications (Bander and Schwab, 1992); (Fan, 1994). In C1 temporary non-tunnelled dialysis catheters remained *in situ* for a mean of 49.7 days thus contributing to a higher than expected rate of BSI.

Establishing the incidence of ESI in haemodialysis catheters is difficult as there is a paucity of studies specifically addressing ESI. Tokars, Miller and Stein (2002) reported local access infection rates of 1.19 per 1000 catheter days for long-term catheters and 1.08 per 1000 catheter days for temporary catheters. In C1 of this study, the incidence of ESI in temporary and long-term dialysis catheters was 2.0 and 1.3 respectively. A contributing factor to these higher ESI rates was the inconsistency in catheter management.

It has been reported that infection rates increase when catheter dressing changes or manipulations are performed by inadequately trained staff (Vanherweghem, *et al.,* 1986); (Department Of Health And Human Services, 1995); (Keohane, *et al.,* 1983). In C1 of this study, catheter management was a subjective process. Following observation of practice in several UHB dialysis units, it was demonstrated that solutions used to clean the catheter exit site were selected based on the personal preference. The most common solution used was 0.9% normal saline, which contains no preservatives and therefore had no bactericidal activity.

Anatomical placement of dialysis catheters in the internal jugular vein or femoral vein makes dressing maintenance of the catheter exit site difficult as patient movement puts continual

pressure on the catheter. In C1 of this study, the dressings used at the exit site were not opaque and therefore the site could not be observed without removing the dressing. Common practice was to lift the corner of the dressing to examine the exit site; this often resulted in inadequate coverage of the exit site, as once lifted the dressing would not re-adhere to the patients skin, allowing entry of microorganisms. Furthermore, the application of non-waterproof dressings restricted the ability of the patients to shower/bathe.

As previously discussed, the high incidence of *S. aureus* nasal carriage in haemodialysis patients contributes to the risk of ESI and as such K/DOQI guidelines recommend that both the patient and the clinician wear a surgical mask every time the catheter is accessed (National Kidney Foundation Kidney Dialysis Outcomes Quality Initiative (K/DOQI), 2000).

In April 2002, the QEH Renal Unit introduced evidence based practice guidelines, which dictated the standards to be employed for insertion and management of all dialysis catheters. Catheter insertion was only undertaken by medical registrars or consultants following a competence assessment based on adherence to the protocol. This standardisation of catheter management resulted in a decrease in the incidence of BSI per 1000 catheter days in both long-term and temporary dialysis catheters in patient cohort 2 (2.4 and 7.7 respectively). In addition, the onset of BSI in this patient cohort was faster. In temporary catheters (C2) onset of BSI had a mean of 10 days and for long-term catheters a mean of 50 days. It has been suggested that catheter-related BSI associated with catheters that have been in place for less than 10 days are due to contamination at the skin insertion site with subsequent migration of microorganisms down the extraluminal surface of the catheter lumen (Crnich and Maki, 2002); (Mermel, *et al.*, 1991); (Raad, *et al.*, 1998).

Factors contributing to the decrease in BSI in this patient cohort were the addition of a cap and mask to the protective clothing worn at the time of catheter insertion. This reduced the risk of horizontal transfer of microorganisms, especially *S. aureus* from the operator to the patient. All catheters were inserted in a dedicated procedure room, which enabled the introduction of standardised infection control procedures. An opaque semi-permeable dressing was applied to the exit site of all catheters immediately post insertion which allowed inspection of the site without removing the dressing.

The mean number of days temporary and long-term dialysis catheters remained *in situ* was reduced in these patients (11.4 and 82.6 days respectively). This brought temporary dialysis catheter use within the recommended K/DOQI practice guidelines.

In C2, the incidence of ESI in temporary catheters increased to 2.3 per 1000 patient days. In addition, the time to onset was much faster with a mean of 7 days. This may be due to the increased manipulation of the catheter and exit site when the standardised protocol was introduced. It may also support contamination at the skin insertion site with subsequent migration of microorganisms down the extraluminal surface of the catheter lumen (Crnich and Maki, 2002); (Mermel, *et al.,* 1991); (Raad, *et al.,* 1998). However, the same microorganism recovered from the exit site swab and the blood culture was found in only two temporary catheters in this patient cohort (figure 3.6).

The introduction of screening for MRSA nasal carriage in patients and clinical personnel involved in catheter manipulation addressed both endogenous and horizontal spread of MRSA further reducing the risk of BSI and ESI in this patient cohort.

The catheter management protocol required all nursing personnel to be assessed as competent to manipulate dialysis catheters; based on adherence to the protocol and understanding of the rationale underpinning clinical practice. This removed subjective catheter management and replaced it with evidence-based practice. In addition, catheter hubs were wrapped in sterile gauze soaked in chlorhexidine gluconate 4% (w/v) for two minutes prior to connection and disconnection to the dialysis tubing. Chlorhexidine has been shown to significantly reduce the microbial contamination of catheter hubs (Casey, *et al.,* 2003); (Chaiyakunapruk, *et al.,* 2002). The catheter exit site was dressed with one of two dressings, both of which have been shown to reliably secure the catheter and permit moisture to escape from beneath the dressing, thus reducing the risk of dialysis catheter associated infection (Department Of Health (DOH), 2001). Furthermore, the dressings were shower proof, enhancing the patients ability to maintain a high standard of personal hygiene. Both dressing types re-adhered to the skin if manipulated to inspect the catheter exit site.

In August 2003, audit of dialysis catheter associated infection following the implementation of further protocol initiatives demonstrated a further reduction in BSI and ESI in long-term dialysis catheters (2.0 and 0.8 per 1000 catheter days respectively). The main practice changes in this patient cohort were screening and treatment of *S. aureus* nasal carriage with

Bactroban® and the application of 2% mupirocin cream to the exit site of newly implanted dialysis catheters. As previously discussed eradication of *S. aureus* from the anterior nares reduced the risk of both BSI and ESI.

In accordance with the Renal Association standards (2002), the UHB renal service implemented the application of 2% mupirocin cream to the exit site of all newly implanted long-term haemodialysis catheters. The cream was applied around the catheter exit site immediately post insertion and then three times a week at dialysis for 4-8 weeks.

3.4.2 Catheter survival

Dialysis catheter survival was improved with the implementation of standardised insertion and management protocols. In C1, all dialysis catheters were non-functioning 350 days after insertion whereas in C2 60% of the catheters were still functioning at 350 days and in C3, catheter survival at 350 days was 65%. These data were not significant due to small numbers of catheters surviving over time.

3.4.3 Risk factors and comorbid processes

Patient risk factors at the time of dialysis catheter insertion were recorded. Of the risk factors outlined in the questionnaire, only the ones relevant to dialysis patients were analysed. These were concurrent central venous catheter, anticoagulant therapy, diagnosed immunodeficiency and colonisation of antimicrobial resistant microorganisms. Hoen *et al* (1998) reported that the relative risk of BSI in haemodialysis patients with a dialysis catheter was sevenfold compared to patients with an AVF. In this study, the relative risk of BSI in haemodialysis patients who had a concurrent temporary dialysis catheter was sevenfold compared to haemodialysis patients who had only one catheter *in situ* (p=0.0231, RR=7.000 95% CI, 4.665-10.504). None of the other risk factors studied increased the likelihood of developing BSI in haemodialysis patients with a dialysis catheter *in situ*.

All the patients studied were diagnosed as having ESRD; this is reported to increase the risk of infection due to the effect of uraemia on the immune system. All patients were therefore acknowledged to have a baseline immune dysfunction prior to the analysis of other comorbid factors. Diabetic patients dialysing via a long-term catheter were significantly more likely to develop BSI than were their non-diabetic counterparts (p=0.0002). These findings are

supported by Jean *et al.* (2002) who reported a significant increase in the incidence of catheter bacteraemia in diabetic patients (p=0.03). One reason for this is the impact of diabetes mellitus on phagocytic function. Hyperglycaemia reduces oxidative killing capacity as increased glucose metabolism reduces the availability of NADPH to generate superoxide radicals (Diabetes Forum, 2004). In addition, abnormalities of granulocytes adhesion, chemotaxis and intracellular killing have been linked to increased glucose levels (Micek, *et al.,* 2003). This demonstrates the inability of diabetic dialysis patients to display an innate immune response and therefore increases the risk of infection. These data were not examined by treatment compliance or how well the patients diabetes was controlled. Further study of these data may demonstrate an increased risk of infection in those patients that experienced poorly controlled blood glucose levels compared to those patients who's treatment regimen maintained their blood glucose within normal limits (Umpierrez and Kitabchi, 2003).

Asthma can be classed as allergic or non-allergic, however the effect of the physiological stress response on the immune system is similar. Neuroendocrine hormones triggered during stress may lead to immune dysregulation, altered or amplified cytokine production resulting in decreased host defences (Frieri 2003). In addition, anti-inflammatory medicines commonly used to treat patients with asthma, especially glucocorticoids, are known to depress the immune system by destruction of lymphocytes within lymphoid tissue and reduction of antibody production (Sherwood, 1993). In this study, asthmatic patients who had a temporary dialysis catheter in situ were twice as likely to develop a BSI than their non-asthmatic counterparts (RR 2.646, CI 95%, 1.085-6.457).

Patients with chronic kidney disease have a predisposition to the development of gout. This manifests as a deposition of monosodium urate crystals in and around the joints that invokes an inflammatory response. In this study, patients with gout who had a temporary dialysis catheter in situ were significantly more likely to develop a BSI than patients without gout (p=0.0231, RR 7.000, CI 95%, 4.665-10.504). Treatment of gout includes nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, which inhibit cyclo-oxygenase and the synthesis of prostaglandin's and thromboxanes. This leads to the inhibition of interleukin-2 and subsequent lymphocyte activation (Katzung, 1995).

Patients with epilepsy that had a long-term dialysis catheter *in situ* had an increased risk of developing BSI than their non-epileptic counterparts (RR 1.677, CI 95%, 0.4109-6.844). This may be due to the medications used to control epileptic seizures. Phenytoin is a common drug

used for the treatment of epilepsy that has a profound effect on the immune system. These effects include leukopenia, granulocytopenia, thrombocytopenia, pancytopenia and the depression of IgA production in mucosal surfaces (British Medical Association / British National Formulary (BNF), 2003).

3.4.4 Microorganisms

The microorganisms recovered most often from all types of clinical specimens were staphylococci. These findings are supported by many studies reported in the literature (Berns, 2003); (Rodriguez, *et al.*, 2003). Wheat and co-workers (1981) reported MRSA recolonisation rates of 63 and 100% even after therapy with topical vancomycin (Wheat, *et al.,* 1981).

The ability of staphylococci to cause infection and proliferate at distant sites is illustrated by the frequency with which species with the same antibiogram profiles were found at the exit site and/or distal tip and/or in the blood. This suggests that the phenotypic characteristics of some staphylococci are such that these microorganisms are able to adapt and survive irrespective of the procedures put in place to prevent them (this is discussed fully in chapter 6).

Haemodialysis patients often require multiple dialysis catheter insertions as part of their clinical management. The initial impact of the introduction of topical exit site prophylaxis with 2% mupirocin cream was demonstrated by a reduction in the overall incidence of BSI. However, an increase in Gram-negative microorganisms was observed over time suggesting that prophylactic use of 2% mupirocin cream at the exit site of newly implanted dialysis catheters may place selective pressure on commensal flora supporting the proliferation of Gram-negative microorganism and thus Gram-negative BSI. Furthermore, this treatment requires constant vigilance for the emergence of antimicrobial resistance. Prolonged use and multiple courses of mupirocin are reported to be the factors most frequently associated with the development of mupirocin resistance (Cookson, 1998). Long-term use of mupirocin was first reported to lead to mupirocin resistance in coagulase-negative staphylococci 11 years ago (Connolly, *et al.,* 1993). In contrast, high-level resistance (HLR) to mupirocin (defined by an MIC of $\geq$ 512mg/L) in *S. aureus* strains has been uncommon in dialysis patients ((Boelaert, *et al.,* 1989); (Mupirocin Study Group, 1996); (Kluytmans, *et al.,* 1996)). However, the emergence of HLR to mupirocin with the introduction of prophylactic mupirocin application to the catheter exit site has been reported (Pérez-Fontán *et al.,* 2002; Annigeri *et al.,* 2001).

Furthermore, a dramatic increase in HLR in patients who were *S. aureus* carriers has been noted. No initial HLR to intranasal mupirocin and then exit site application of mupirocin was detected, however, after one year they reported HLR to mupirocin in 8.3% of *S. aureus* isolates and after a further year, this rate had increased to 12.4%. In addition, the MIC_{90} had increased from 0.125 μg/ml to 1024 μg/ml over a three-year period.

Harbarth and colleagues (1999) reported that the emergence of low level resistance (LLR) to mupirocin (defined by an MIC of 8 to 256 mg/L) was acquired during therapy and that patients colonised with strains of MRSA exhibiting LLR at the time of entry to the study, were associated with higher incidence of treatment failure (Harbarth *et al.*, 1999). They suggested that where MRSA is endemic, mupirocin should only be used after careful consideration as most studies reported have been carried out during an outbreak situation and in conjunction with multiple control measures, making it difficult to identify the role of mupirocin in these studies. Cookson (1998) reported that the emergence of high-level mupirocin resistance in staphylococci may limit the effectiveness of this agent in the future, especially for the control of MRSA (Cookson 1998). In contrast, Conly and Vas (2002) suggest that while there is increasing evidence of high-level mupirocin resistance it does not preclude the beneficial effect of prophylactic treatment of *S. aureus* (Conly and Vas, 2002).

In an attempt to stem the increase in resistant microorganisms, the British National Formulary (BNF) recommends that the application of nasal mupirocin be held in reserve for MRSA cases. In hospital, mupirocin nasal ointment should be used only for the treatment of MRSA (three times daily for 5 days). To avoid mupirocin resistance, the course of treatment should not exceed 7 days and/or be repeated on more than one occasion. If MRSA strain is mupirocin resistant or does not respond to two courses, alternative treatment should be sought. For the treatment of skin infections the BNF recommend that mupirocin be avoided in the hospital setting and only used for a maximum of 10 days in the community (British Medical Association / British National Formulary (BNF), 2000)

3.4.5 Meditrend audit tool

The Meditrend audit tool was developed to monitor the incidence of infection associated with central venous catheters. In this section of study, the audit tool proved useful as a means of collecting certain types of data such as the number of catheters inserted and incidence of infection. However, for statistical analysis the tool demonstrated insufficient power to provide

meaningful results. Statistical analysis of the data required export into an Excel spreadsheet and the use of computerised statistical software.

The Meditrend questionnaire was extensive and labour intensive. The tool was developed for monitoring infection in short-term central venous catheters and as such, aspects of clinical management associated with dialysis catheters were excluded from the tool. This meant that in some categories the available responses were modified to accommodate standard renal requirements. In addition, the ability of the tool to generate a protocol for best clinical practice was redundant within this particular patient group. This was due to a combination of the need to modify the questionnaire to collect data specific to dialysis catheters, which conversely could not be modified on the database and the use of standardised procedures for catheter insertion; a situation that further confounded the ability of the database to provide meaningful results.

Patients receiving renal replacement therapy often require several dialysis catheters over the period of their disease. During episodes of clinical instability, patients may require several successive temporary dialysis catheters within a short period or indeed, require concurrent catheters while long-term catheter access is being evaluated. In its present form, Meditrend does not support this, as there is no way to enter successive catheters for individual patients. The inclusion of this facility would allow clinicians to monitor recurrence or relapse in infection. In addition, the ability to enter specific dates for catheter insertion and removal would allow the generation of catheter survival data.

In conclusion, haemodialysis patients are predisposed to infection firstly due to the nature of ESRD and secondly as a consequence of inflammatory comorbid disease. Dialysis catheter associated BSI can be controlled in this high-risk patient group by using standardised catheter insertion and management protocols based on stringent infection control procedures. Clinical practice should be reflective so optimum clinical effectiveness can be maintained. The implementation of new treatments may have adverse outcomes that are not initially evident, or may select for the proliferation of different microorganisms, therefore on going clinical audit of patient outcomes has been shown to be of great importance. To generate accurate data the audit tool must be sufficiently sensitive and robust. In its present form, Meditrend is better suited to data collection for short-term or temporary catheters implanted in patients who will be entered onto the database only once; for example, cardiac surgical patients who receive a central venous catheter as part of their coronary artery bypass procedure and then have it

explanted post operatively within 72 hours. In its current format, this audit tool would work very well in this clinical environment. Furthermore, it could be viewed as a starting point from which to refine a tool that could be used more generally in the healthcare setting.

CHAPTER 4: HAEMATOLOGICAL DETECTION OF INFECTION

4.1 INTRODUCTION

As previously discussed in chapter 3 patients with ESRD have an increased risk of developing catheter associated BSI as a consequence of their renal disease state, comorbid processes and clinical reliance on polyurethane catheters. Vascular access is one of the most important aspects of treatment in this patient group as RRT means the difference between life and death. Infection is one of the biggest mortality risks associated with RRT and therefore early detection and diagnosis saves lives.

Techniques for the diagnosis of catheter-related infection have historically relied upon culture of the distal tip following removal of the catheter, however negative culture results are common despite clinical suspicion. In the absence of visible signs of infection or positive microbiology, clinicians often rely upon changes in biochemical and haematological blood results to predict the activity of the immune system and thus likelihood of infection. The tests most commonly observed include C-reactive protein (CRP), white cell count (WCC), serum albumin and erythrocyte sedimentation rate (ESR).

In renal patients, the continuing performance of the dialysis catheter is of optimum importance and therefore methods of diagnosing CRI without catheter removal are invaluable. A great deal of work has been done in the area of serodiagnosis of infection that has included the development of assays to detect anti-α-toxin antibody (anti-staphylolysin), anti-wall teichoic antibodies (Crowder and White, 1972), peptidoglycan and lipoteichoic acid (Wergeland, *et al.,* 1984), (Verbrugh, *et al.,* 1983). These methods have had varying degrees of success in the diagnosis of Gram-positive infection. Assays that detect exocellular antigens have been developed that distinguish serious staphylococcal infection from uncomplicated septicaemia (Christensson, *et al.,* 1985), (Espersen, *et al.,* 1986). Antibody to a number of exocellular *S. aureus* antigens has been demonstrated (Lambert, *et al.,* 1996), (Lambert, *et al.,* 1992), (Krikler and Lambert, 1992). As a continuation of this work, an enzyme-linked immunosorbent assay (ELISA) based on the detection of anti-lipid S (a secreted short chain form of lipoteichoic acid) has been developed at Aston University and the University Hospital Birmingham NHS Trust (Lambert, *et al.,* 2000), (Elliott, *et al.,* 2000).

The ELISA technique uses an enzyme labelled conjugate to quantitate a specific antigen-antibody reaction (Wood and Wreighitt, 1990). A heterogeneous indirect ELISA is a method for the quantitation of immunoglobulin specific to a particular antigen. The various reagents (serum sample, enzyme-labelled conjugate, chromogenic substrate) are added sequentially to solid phase bound antigen with an incubation period and washing step between each stage. The coloured product generated by the enzyme on addition of the substrate, and quantitation using a spectrophotometer, is directly related to the amount of antibody in the sample. This method is routinely used in microbiology laboratories for the diagnosis of infectious diseases including *Chlamydia* species, *Helicobacter pylori*, hepatitis and HIV.

This section of work aims to determine the efficiency of the anti-lipid S ELISA in detecting early onset of Gram-positive infection in renal dialysis patients and compare its predictive value with routine haematological and biochemical tests in infected and non-infected renal patients.

4.2 MATERIALS AND METHODS

4.2.1 Patients

One thousand five hundred and twenty serum samples were collected from 624 renal patients over a two-year period. All patient samples were subjected to analysis by lipid S ELISA irrespective of their treatment modality and infection status.

Infection status was determined from microbiology culture results. The patient was considered to have been infected if a they had been treated for a significant positive culture result within the previous six months of the blood sample being taken. The significance of the culture was based on the isolation of >15 cfu and Gram's stain. All clinically significant positive culture results were telephoned through to the appropriate medical staff for action by the microbiologist.

4.2.2 Lipid S ELISA

4.2.2.1 Preparation of antigen

The antigen was prepared from seven strains of coagulase negative staphylococci isolated from patients with proven CVC sepsis and identified by the API-Staph identification system (bioMérieux, France).

Fast protein liquid chromatography (FPLC) on a superose 12 gel permeated column was used to produce the antigen as previously described (Elliott, *et al.,* 2000). The antigen was subsequently identified as a short chain length form of lipoteichoic acid having the chemical structure of six glycerol phosphate units linked to a glycolipid molecule (Lambert, *et al.,* 1996).

4.2.2.2 Preparation of the ELISA plates

The purified antigen diluted in 0.05M carbonate buffer pH 9.6 was used to coat the microtitre plates, (Immulon 2, Dynex Laboratories, Chantilly, USA) with 100µL and incubated at 4°C for 18h. After removal of excess antigen, the wells were washed with TBS-Tween (0.01M Tris-HCl, pH 7.4, NaCl 0.9% (w/v), Tween-20 0.3% (v/v). Unbound sites were blocked by incubation at 4°C for 1h, in the same buffer.

4.2.2.3 Measurement of IgG in patient sera

Patients sera was diluted 1 in 6400 in TBS-Tween and 100µL added to wells in duplicate on a microtitre plate. After incubation at 37°C for 2h, excess serum was removed and the plates were washed with TBS-Tween. Bound IgG was detected by the addition of 100µL of anti-human IgG conjugate (0.5µg/mL in TBS/Tween) and further incubation for 1h at 37°C. After removal of conjugate by washing with TBS-Tween, 100µL of chromogenic substrate was added to each well. The substrate contained 10mg 3,3',5,5'-tetramethylbenzidine dissolved in 1mL dimethyl sulphoxide and diluted into 100mL of sodium citrate/acetate buffer (0.1M, pH 6.0) containing 50µL of H_2O_2 (5% (v/v)). After 25 min at 37°C the reaction was stopped by the addition of 100µL sulphuric acid (1M) and the optical density at 450nm was measured. Control wells containing TBS-Tween 20 only produced no colour.

A positive control with a titre of 100,000 and a negative control of 10,000 were used in assays of IgG. Titres were calculated using the following equation:

$$\frac{\text{absorbance}_{450}\text{ test sample - absorbance}_{450}\text{ negative control}}{\text{absorbance}_{450}\text{ positive control} - \text{absorbance}_{450}\text{ negative control}} \quad \text{x } 100\,000$$

4.2.3 Routine sample analysis

All haematological and biochemical tests were performed by trained personnel in accordance with departmental standard operating procedures. Hepatitis antibody titre was determined by AxSYM® immunoassay system (Abbott laboratories, UK) and carried out by microbiology personnel in accordance with the manufacturers instructions and departmental standard operating procedures.

4.2.4 Acridine orange leukocyte cytospin (AOLC)

4.2.4.1 Patients

Ninety renal patients were studied over a one-year period.

4.2.4.2 Preparation of blood for analysis

A 100μl aliquot of blood was spread on a 7% blood agar plate and incubated for 24 to 48h to determine presence of microorganisms.

A 50μl sample was taken from 2mL of blood, which was aspirated from the CVC in a heparinised blood tube. The 50μl of blood was then mixed with 1.2mL of hypotonic formol saline (0.146% NaCl in 4% formalin) for 30 seconds to lyse the erythrocytes and fix leucocytes. 2.8mL of a 1.168% hypertonic saline was added and the sample mixed at 2000rpm for 5 minutes. The supernatant was decanted and the resuspended deposit transferred to a cupule for cytospin at 1200rpm for 5 minutes in a Shandon II cytospin (Shandon Inc, Pittsburgh, PA).

The cytospin showered cells from the supernatant onto a glass slide forming a leucocyte monolayer. The slide was then stained with acridine orange (1:25,000 w/v) for 30 seconds,

air-dried and examined using oil immersion ultraviolet microscopy at a magnification of 40 times. Non-viable bacteria were seen as bright-orange structures within and around the leucocytes. Viable bacteria were seen as bright-green structures. A positive result for CRI is defined by the presence of one or more viable bacteria on the slide. Method taken from Tighe *et al.* (1996).

4.3 RESULTS

4.3.1 Patient demographics

Six hundred and twenty-four renal patients were allocated into two patient groups dependent upon their positive microbiology results. A patient was considered to have been infected if a they had been treated for a significant positive culture result within the previous six months of the blood sample being taken. The significance of the culture was based on the isolation of >15 cfu and Gram's stain. Those patients who had culture positive clinical specimens within the preceding 6 months from the date of the blood sample tested for anti-lipid S antibodies, were allocated to the 'culture positive' patient group. Those patients that had no culture positive clinical specimens within the preceding 6 months from the date of the blood sample tested for anti-lipid S antibodies were allocated to the 'culture negative' category (table 4.1).

Table 4.1 Patient demographics

Patient status	**Patient Modality**		**Ratio M:F**	**Age years (mean)**	**Age years (range)**
	CAPD	**haemodialysis**			
Culture positive	94	200	177:117	55	17-89
Culture negative	90	240	200:130	56	18-90

The number of serum samples from culture positive and culture negative renal patients tested for anti-lipid S antibodies can be seen in table 4.2.

Table 4.2 Origin of patient serum samples

Patient status	**Number of serum samples from CAPD patients**	**Number of serum samples from haemodialysis patients**
Culture positive	237	717
Culture negative	166	400

The number of Gram-positive, Gram-negative and yeast/fungal species recovered from a range of clinically significant specimens of the patients who were allocated to the culture positive group are shown in table 4.3.

Table 4.3 Microorganisms recovered from clinical specimens of culture positive renal patients

Total number of culture positive isolates	Number of Gram-positive isolates	Number of Gram-negative isolates	Number of Yeast/Fungal isolates
1703	1300	339	64

4.3.2 Anti-lipid S antibody recovery

The range of anti-lipid S IgG antibody titres recovered from renal patients was between 0 and 119326, to adequately assess the significance of the titre levels with regards to infection the range of titres was categorised, for example 0, 1-100, 101-1000, 1001-10000, 10001-100000 and >100000. The amount of anti-lipid S antibody recovered from blood specimens of renal patients who were culture positive and culture negative was compared (figure 4.1).

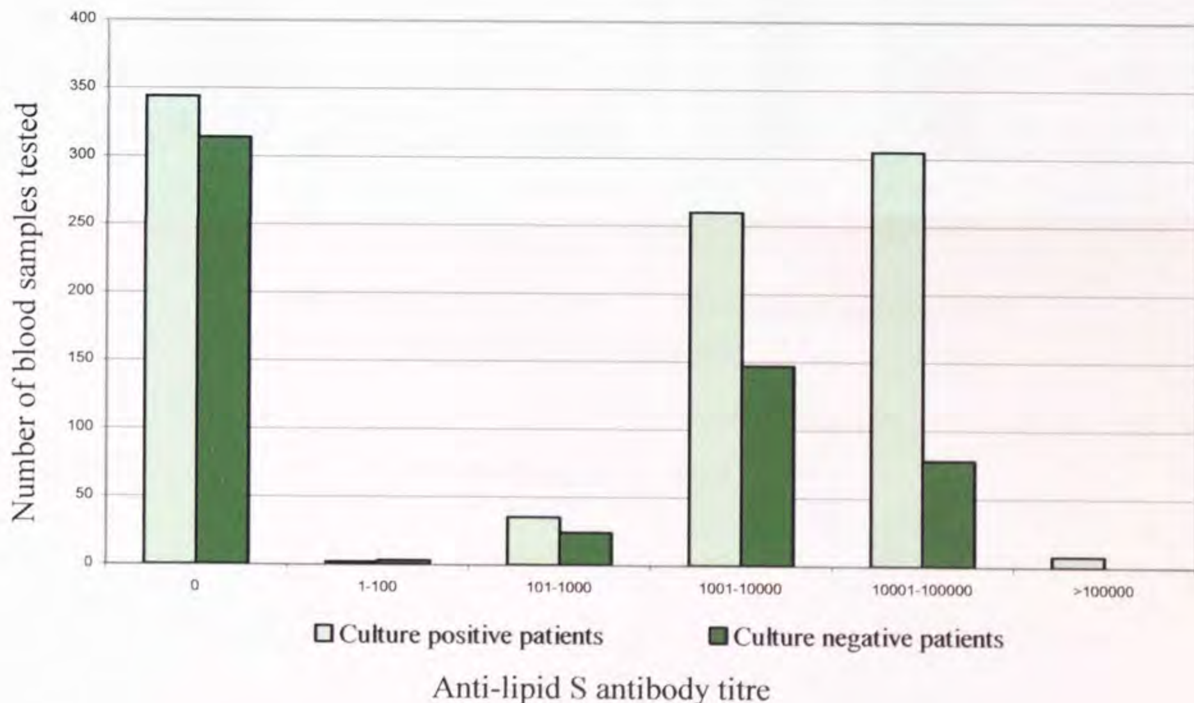


Figure 4.1 Comparison of the amount of anti-lipid S antibody recovered from the serum of culture positive and culture negative renal patients.

The mean anti-lipid S antibody titre for culture positive patients was 11754 and for culture negative patients 4934. The range of values in each anti-lipid S antibody titre category is shown in table 4.4.

Table 4.4 Range of anti-lipid S antibody titres in culture positive and culture negative renal patients

Patient status	1-100 (Mean)	101-1000 (Mean)	1001-10000 (Mean)	10001-100000 (Mean)	> 100000 (Mean)
Culture positive	33-61 (47)	134-965 (537)	1009-9896 (4747)	10064-99191 (29762)	101382-119326 (110355)
Culture negative	56-90 (79)	108-960 (615)	1181-9830 (4864)	10154-78743 (26446)	N/A

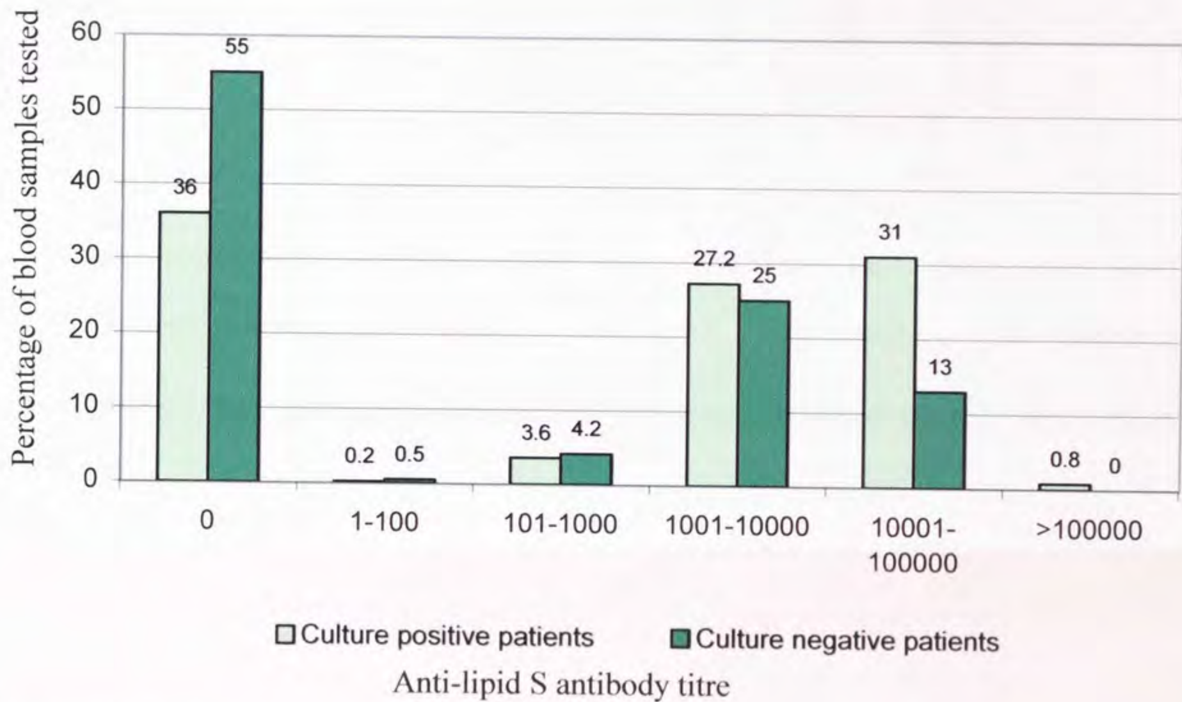


Figure 4.2 Comparison of the percentage of anti-lipid S antibody recovered in each titre category from culture positive and culture negative renal patients.

4.3.3 Anti-lipid S antibody titres in renal patients over time

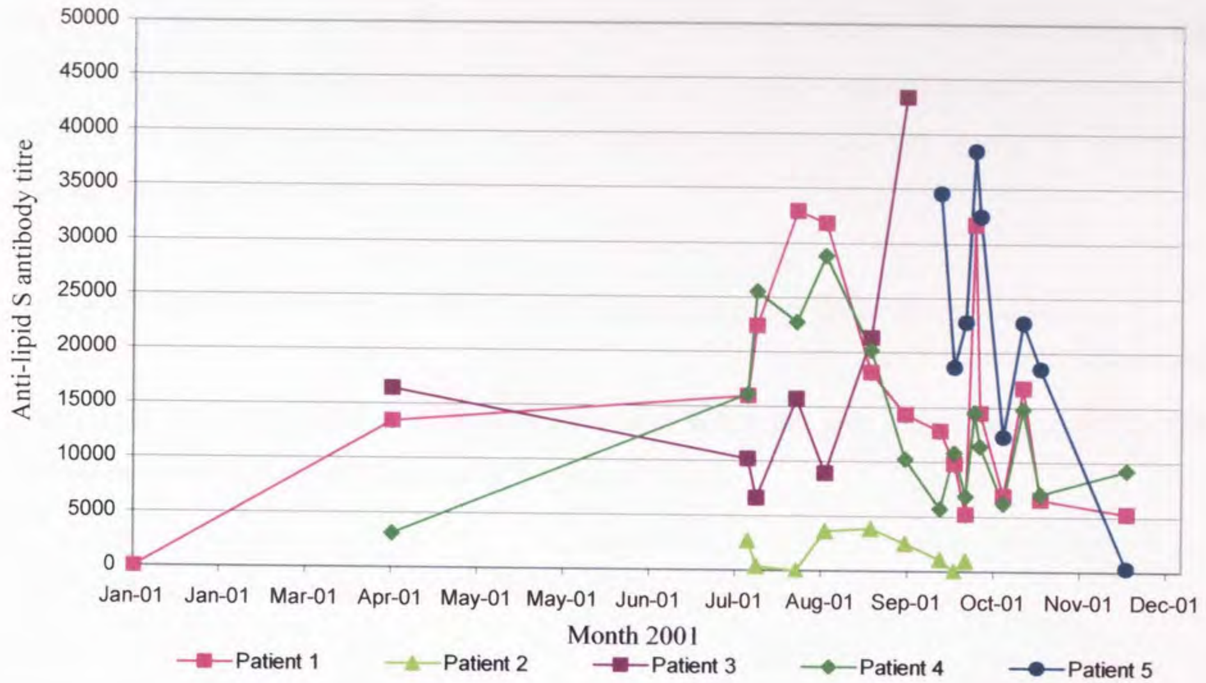


Figure 4.3 Anti-lipid S antibody titres of five culture positive renal patients over a one-year period

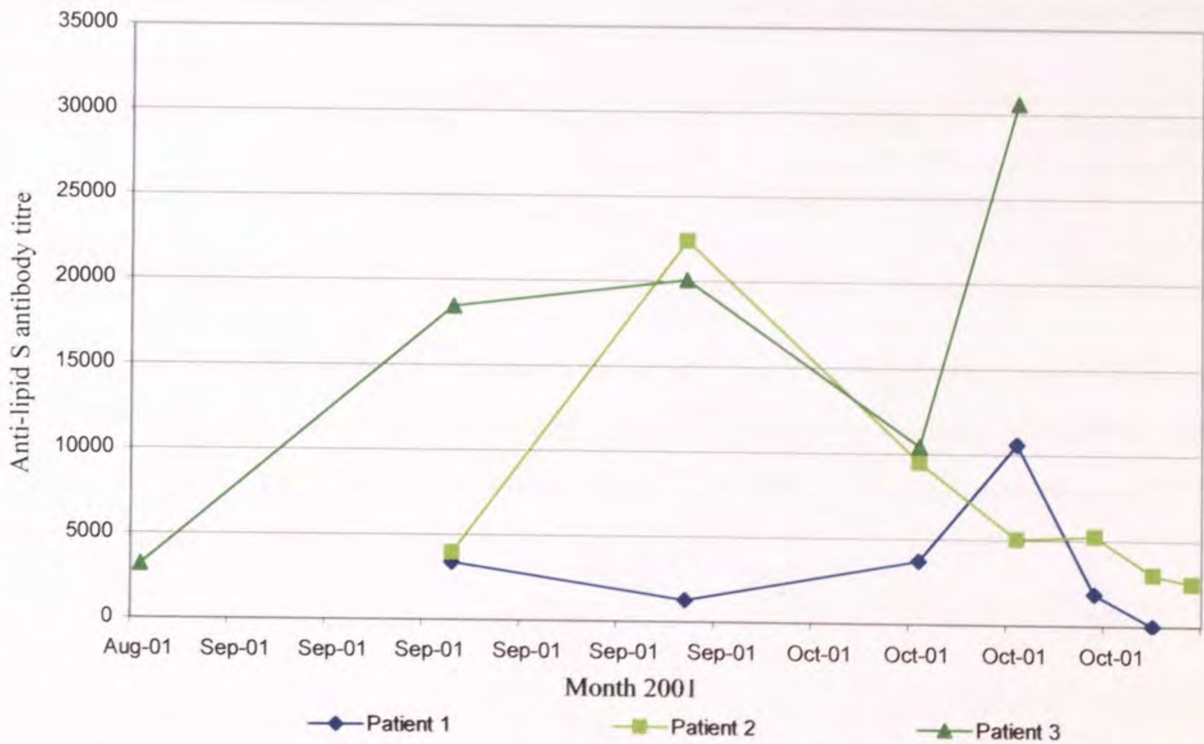


Figure 4.4 Anti-lipid S antibody titres of three culture negative renal patients over a one-year period

The peak anti-lipid S antibody titres in culture positive renal patients were found to be higher than in culture negative patients. In addition, the patterns of anti-lipid S antibody flux over time were different. All of the culture positive patients studied over time had an initial increase in antibody that declined rapidly, however it was closely followed by a second peak equal to or higher than the first. Culture negative patients did not appear to produce the second increase in antibody.

4.3.4 Anti-lipid S antibody production compared to other immune markers

The value of the anti-lipid S ELISA in detecting Gram-positive infection was compared to routine haematological and biochemical tests.

4.3.4.1 Comparison of CRP and anti-lipid S titres

A total of 518 blood samples were analysed for CRP from culture positive (n=364) and culture negative (n=154) renal patients. The normal range for CRP is less than 10. These data are shown in table 4.5.

Table 4.5 CRP concentrations recovered from blood samples of culture positive and culture negative renal patients

Patient status	Number of patients with a CRP of <10mg/L	Range mg/L (Mean)	Number of patients with a CRP of >=10mg/L	Range mg/L (Mean)
Culture positive	63	1-9 (5)	301	10-440 (104)
Culture negative	96	1-9 (4)	122	10-260 (65)

Culture positive renal patients were less likely to have a CRP concentration within a normal range than culture negative patients. In addition, culture negative patients had a lower mean CRP concentration when outside the normal range. Anti-lipid S antibody production and CRP concentration is compared in table 4.6.

Table 4.6 Comparison of mean CRP and anti-lipid S values from culture positive and culture negative renal patients

Patient status	**0**		**1-100**		**101-1000**		**1001-10000**		**10001-100000**		**> 100000**	
CRP	**<10**	**>=10**	**<10**	**>=10**	**<10**	**>=10**	**<10**	**>=10**	**<10**	**>=10**	**<10**	**>=10**
Culture positive	5	96	0	0	4	73	5	102	5	120	0	68
Culture negative	5	74	0	0	5	34	4	54	6	90	0	0

4.3.4.2 Comparison of ESR and anti-lipid S titres

A total of 140 blood samples were analysed to establish the ESR (mm/h) in culture positive (n=97) and culture negative (n=43) renal patients. These data are shown in table 4.7. Many variables contribute to the interpretation of ESR results including gender, therefore these data have been sub categorised into male (M) and female (F). The normal range in women is considered to be 5-15mm/h and in men 1-5mm/h.

Table 4.7 ESR in culture positive and culture negative renal patients

Patient status	Number of male patients with an ESR of 1-5 mm/h (Mean rate)	Number of male patients with an ESR of >5 mm/h (Mean rate)	Number of male patients with an ESR of >100 mm/h (Mean rate)	Number of female patients with an ESR of <5 mm/h (Mean rate)	Number of female patients with an ESR of 5-15 mm/h (Mean rate)	Number of female patients with an ESR of >15 mm/h (Mean rate)	Number of female patients with an ESR of >100 mm/h (Mean rate)
Culture positive	0	35 (59)	20 (121)	0	1 (13)	24 (60)	17 (124)
Culture negative	0	22 (47)	2 (118)	0	1 (7)	18 (65)	2 (118)

Table 4.8 Comparison of mean ESR and anti-lipid S values from culture positive and culture negative male renal patients

Anti-lipid S →	**0**			**1-100**			**101-1000**			**1001-10000**			**10001-100000**			**> 100000**		
ESR	1-5	>5	>100	1-5	>5	>100	1-5	>5	>100	1-5	>5	>100	1-5	>5	>100	1-5	>5	>100
Patient status																		
Culture positive	0	16	8	0	0	0	0	0	2	0	12	2	0	7	8	0	0	0
Culture negative	0	11	1	0	0	0	0	5	3	0	2	0	0	0	0	0	0	0

Table 4.9 Comparison of mean ESR and anti-lipid S values from culture positive and culture negative female renal patients

Anti-lipid S →	**0**				**1-100**				**101-1000**				**1001-10000**				**10001-100000**				**> 100000**			
ESR	<5	5-15	>15	>100	<5	5-15	>15	>100	<5	5-15	>15	>100	<5	5-15	>15	>100	<5	5-15	>15	>100	<5	5-15	>15	>100
Patient status																								
Culture positive	0	1	13	1	0	0	0	0	0	0	0	1	0	0	3	6	0	0	7	9	0	0	1	0
Culture negative	0	1	9	0	0	0	0	0	0	0	0	0	0	0	6	1	0	0	3	1	0	0	0	0

4.3.4.3 Comparison of WCC and anti-lipid S titres

One thousand and forty-four blood samples were analysed to establish the WCC in culture positive (n=692) and culture negative (n=352) renal patients. These data are shown in table 4.10. The normal WCC is considered is 4-11 x10^9/L.

Table 4.10 WCC in culture positive and culture negative renal patients

Patient status	Number of patients with an WCC of <4 x 10^9/L	Range (Mean)	Number of patients with an WCC of 4-11 x 10^9/L	Range (Mean)	Number of patients with an WCC of >11 x 10^9/L	Range (Mean)
Culture positive	39	1.8-3.9 (3.2)	506	4.0-11.9 (7.4)	123	12.0-37.7 (15.6)
Culture negative	24	1.4-3.9 (3.4)	288	4.0-11.8 (7.0)	40	12.1-25.4 (15.6)

A comparison of WCC and anti-lipid S antibody ranges is shown in table 4.11

Table 4.11 Comparison of mean WCC and lipid S values from culture positive and culture negative renal patients

Patient status	**0**			**1-100**			**101-1000**			**1001-10000**			**10001-100000**			**> 100000**		
	<4	**4-11**	>11	<4	**4-11**	>11	<4	**4-11**	>11	<4	**4-11**	>11	<4	**4-11**	>11	<4	**4-11**	>11
Culture positive	3.2	7.4	15.4	0	8.4	0	2.9	7.8	0	3.1	7.6	15.4	3.5	7.3	16.1	3.2	7.4	15.4
Culture negative	3.3	6.9	15.4	3.4	7.6	0	3.2	8.1	13.5	3.5	7.0	18.7	3.6	6.6	15.8	3.3	6.9	15.4

4.3.4.4 Anti-lipid S antibody production compared to routine immune markers

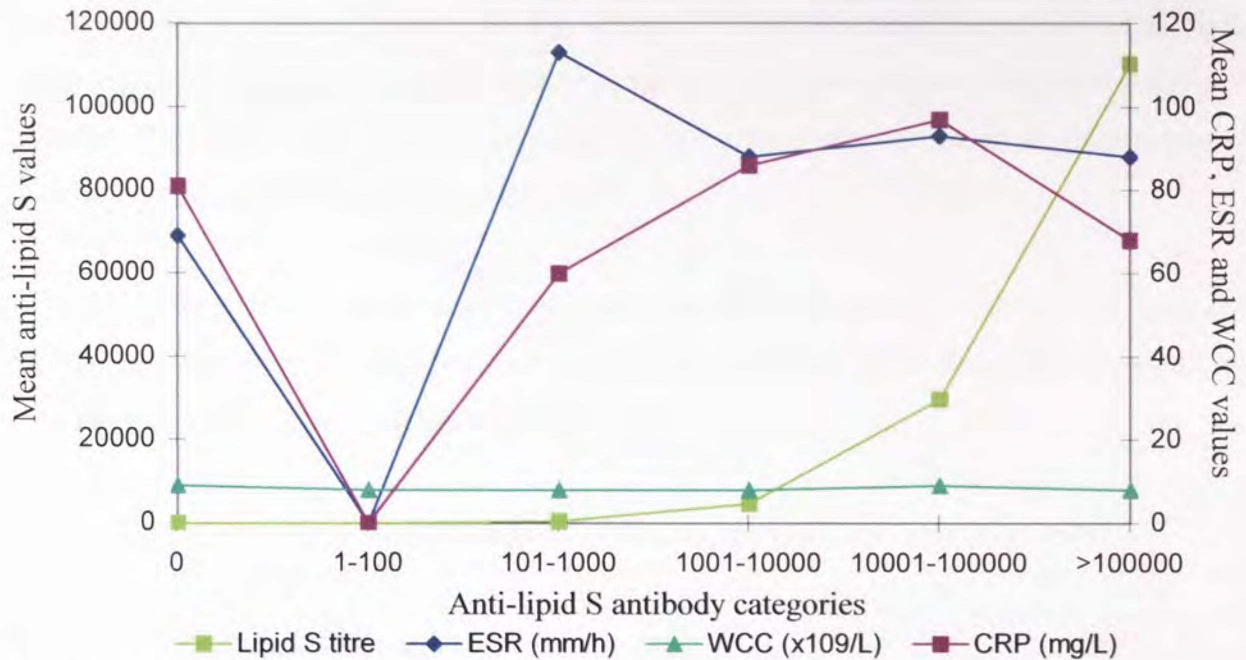


Figure 4.5 Comparison of mean immune marker patterns in culture positive renal patients

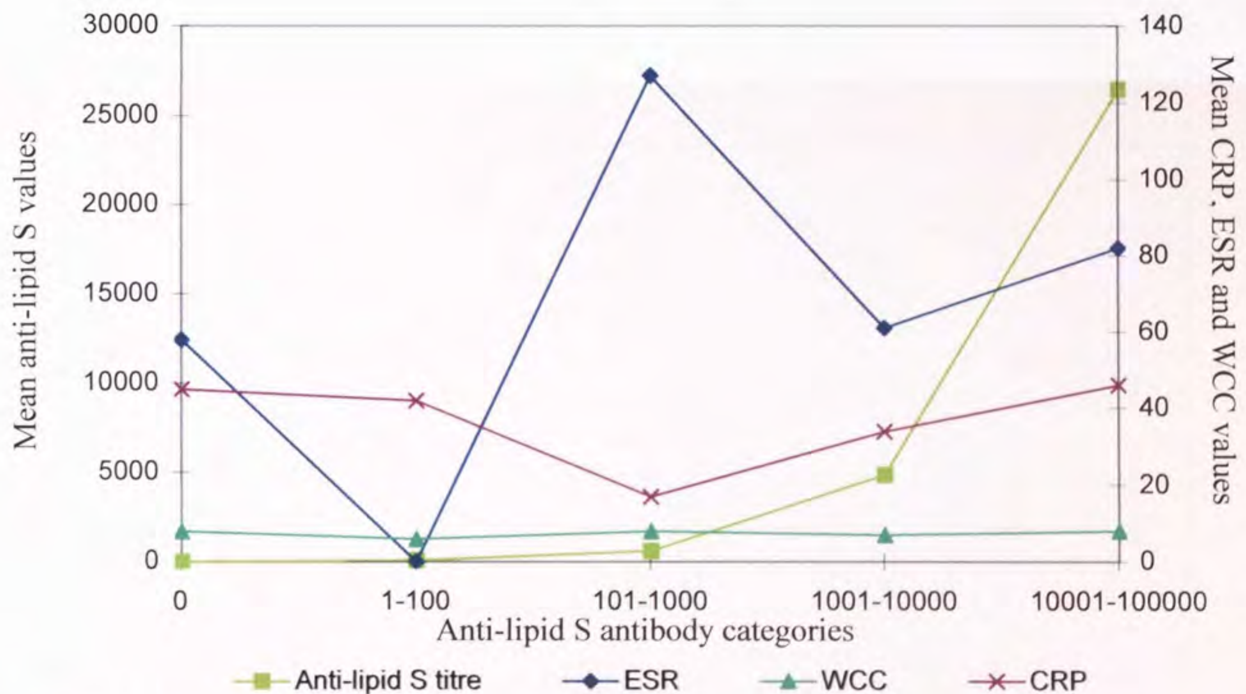


Figure 4.6 Comparison of mean immune marker patterns in culture negative renal patients

With the exception of CRP, there was no observed difference in the comparison of anti-lipid S and other immune markers in culture positive and culture negative patients.

4.3.5 Comparison of anti-hepatitis B and anti-lipid S antibody production

Renal dialysis patients are treated with HB-Vaxpro™ II 40 (Pasteur Mérieux, UK) hepatitis B vaccine prior to commencing RRT. One hundred and fifty-five patients who provided blood samples to determine their immune response to the vaccine were compared for their response to lipid S antigen using the anti-lipid S ELISA.

Analysis of hepatitis B blood results revealed that 75% of renal patients treated with the vaccine did not have a protective level of HbSAb, however, 57% were able to mount an immune response to Gram-positive microorganisms.

Table 4.12 Comparison of hepatitis B antibody production and anti-lipid S IgG titre

Hepatitis antibody titre	>10	10-100	>100
Mean Lipid S antibody titre	6944.50	6045.06	12579.77

The spread of individual anti-lipid S titres are compared to the response of renal patients to the anti-hepatitis B vaccine in figure 4.7

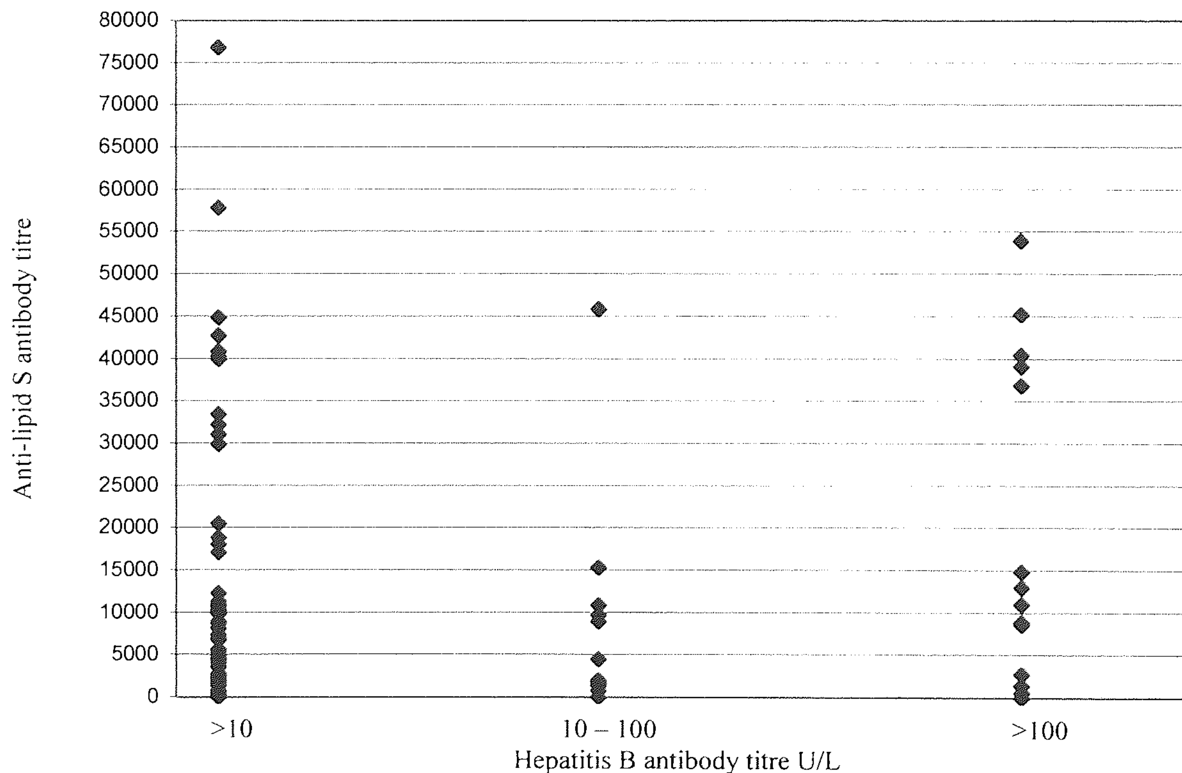


Figure 4.7 Comparison of anti-lipid S and anti-hepatitis B antibody response

Figure 4.7 suggests that the response to antigenic stimulation by renal patients is diverse. The production of antibodies to the hepatitis B vaccine is limited however; the immune response to Gram-positive microorganisms is vigorous.

4.3.6 Acridine orange leucocyte cytospin

Ninety unclotted blood specimens from culture positive and culture negative renal patients were assessed with the acridine orange leucocyte cytospin (AOLC) (Kite *et al.*, 1999) to determine the presence of Gram-positive microorganisms.

Sixty-eight out of 90 (75%) patients had microorganisms in their blood sample as identified by ultraviolet microscopy.

4.3.6.1 Comparison of anti-lipid S ELISA and the acridine orange leucocyte cytospin test

Blood samples for AOLC and anti-lipid S antibody detection were received from renal patients in a pair wise fashion and test results compared. These data are shown in table 4.13.

Table 4.13 Comparison of anti-lipid S antibodies and the presence of microorganisms identified by ultraviolet microscopy.

Comparison	Number of patients
Positive anti-lipid S result and positive AOLC result	38 (42%)
Positive anti-lipid S result and negative AOLC result	16 (17%)
Negative anti-lipid S result and positive AOLC result	30 (33%)
Negative anti-lipid S result and negative AOLC result	6 (6%)

The AOLC method was shown to be valuable in determining infection in patients who do not respond to the lipid S antigen.

4.4 DISCUSSION

4.4.1 Anti-lipid S ELISA

Lipid S has been characterised as a short chain length exocellular form of the cellular lipoteichoic acid and shares common antigenic determinants with it. In contrast to LTA lipid S contains only six glycerophosphate units compared to the 40 to 42 units in LTA. This staphylococcal exocellular antigen can be recovered from the supernatant of liquid culture medium and has been reported to produce significant IgG titres in patients with catheter-related infection (Lambert, *et al.,* 2000); (Elliott, *et al.,* 2000), prosthetic joint infection (Rafiq, *et al.,* 2000) and infective endocarditis (Connaughton, *et al.,* 2001).

In this section of study, 1520 serum samples from 624 patients were investigated for anti-lipid S IgG antibodies. Serum samples were collected monthly whenever possible for a period of 2 years. On examination of microbiology data, more serum samples were received from culture positive patients than culture negative patients (figure 4.1). Analysis of mean anti-lipid S IgG titres indicates that the concentration of antibody recovered from culture positive patients was double that of culture negative patients (11754 and 4934 respectively). This suggests that Gram-positive antigenic stimuli is persistent within the renal environment and culture negative patients exhibited a wide range of antibody concentrations with no evidence of infection. The use of polyurethane catheters may support the positive immune response to lipid S antigen because colonisation of the internal lumen of the catheter combined with the hydrostatic pressure of dialysis flows through the catheter may steadily dislodge microorganisms from the internal catheter surface thus maintaining a steady antigenic stimulus. In addition, the use of arteriovenous fistula for dialysis requires inserting needles through the skin three times per week. This may contribute to a low level of Gram-positive antigenic stimuli as commensal skin flora on the surface and in the epidermal layers of the skin which can be transferred to the bloodstream. This may explain the positive anti-lipid S tires in culture negative renal patients and the concentrations recovered from culture negative renal patients compared to those recovered from other patient groups (Lambert, *et al.,* 2000); (Elliott, *et al.,* 2000); (Rafiq, *et al.,* 2000); (Connaughton, *et al.,* 2001).

The range of anti-lipid S titres recovered from culture positive and culture negative renal patients suggest that concentrations of 1001-10000 could be used as a marker for infection as this is the point at which culture positive patients have consistently higher titres than culture

negative patients (figure 4.2). In addition, ESR begins to fall at this concentration of anti-lipid S antibodies supporting the presence of infection (section 4.4.2.2).

Sequential serum samples received from renal patients over a one-year period demonstrated a 'double peak concentration' pattern. Culture positive patients produced a steady concentration of anti-lipid S antibodies over a period of weeks to months prior to the production of high levels of antibody. This steady response to stimuli may be explained by the high incidence of exposure to Gram-positive antigens within the renal environment as discussed in the previous paragraph. The sudden increase in antibody production may indicate the onset of infection and the immune activation by the compliment cascade. The second peak demonstrated in culture positive patients may represent the recognition of antigen by memory cells and the activation of specific anti-lipid S antibodies.

In culture negative patients, a baseline concentration of anti-lipid S antibodies was demonstrated however, no second peak occurs and this may support the theory of activation of specific anti-lipid S antibodies by the cell-mediated immune system.

In both patient groups, anti-lipid S antibody concentrations remain elevated for many months this also supports the theory of persistent low-level Gram-positive stimuli within the renal environment.

4.4.2 Comparison of anti-lipid S antibody secretion to other inflammatory markers.

4.4.2.1 CRP

C reactive protein is a crude early immunoglobulin, which initiates an inflammatory reaction. CRP-antigen complexes can substitute for antibody fixation and trigger the inflammatory response to antigen or tissue damage, subsequent binding of C3b to the surface of microorganisms opsonizes them for phagocytosis.

The mean CRP concentrations in culture positive and culture negative renal patients suggest that culture negative patients are more likely to have a CRP within the normal range than those that are culture positive. Furthermore, culture negative patients who do not have a CRP within normal parameters demonstrate a high mean CRP (122mg/L) suggesting reaction of

acute phase proteins to antigenic stimulation. This may be again due to the antigenic incidence within the environment.

4.4.2.2 ESR

The results show that renal patients rarely have an ESR within normal limits; this may be a consequence of their disease progression. Normochromic anaemia is present in most patients with chronic renal failure. Generally, there is a 2g/dL fall in haemoglobin level for every 10mmol/L rise in blood urea. Red cell production is impaired because of inadequate erythropoietin secretion. In addition, there is iron deficiency anaemia following blood loss on dialysis, defective platelet function and folate deficiency in some patients. Anaemia is corrected in renal patients by the administration of erythropoietin and intravenous iron sucrose.

A raised ESR is indicative of a wide variety of systemic inflammatory and neoplastic diseases. ESR values >100mm/h are considered to have a 90% predictive value for serious diseases including infection (Hoffbrand *et al.,* 2001). In renal patients, the ESR was commonly >100mm/h in both males and females in the culture positive group as compared to the culture negative group. This supports the diagnosis of infection in renal patients despite the overall increase in ESR because of their disease state.

When comparing ESR with anti-lipid S IgG, the ESR is most elevated when anti-lipid S antibodies begin to increase (101-1000) then it falls (figures 4.5 and 4.6). This suggests that prior to a detectable level of anti-lipid S antibodies in the bloodstream, the presence of Gram-positive microorganisms may contribute to the depletion of circulating erythrocytes due to haemolysis. Once anti-lipid S antibodies are secreted, this effect is reduced and the ESR falls. However, the dialysis schedule of the patient should be considered as the ESR may increase when iron therapy is due to be administered. This may suggest that even though this test is routinely observed as a marker of infection, the increase in variables affecting its value in renal patients makes it less reliable.

4.4.2.3 WCC

A consequence of ESRD is that the immune response to infection is impaired and this was supported by the lack of variation expressed in the WCC in both culture positive and culture

negative renal patients (figures 4.5 and 4.6). The mean WCC was very similar in both patient groups (tables 4.9 and 4.10). This suggests that the immune system may experience a low level of persistent activation, almost certainly due to undergoing dialysis three times per week for 4 hours. This combined with the immunocompromised state of the host supports the reduced state of the immune response to infection.

4.4.3 Comparison of anti-lipid S antibody production and the AOLC

As previously stated in section 4.4.2.3 the immune response in renal patients is subdued and therefore in the absence of positive microbiology culture limits the value of immune markers as a predictor of the infection status. AOLC is a rapid method for the diagnosis of haemodialysis catheter-related infection as it relies on the visualisation of microorganisms by ultraviolet microscopy. In addition, it can be performed without the need for catheter explantation and results are available in 30 minutes. One disadvantage of this method is that blood is withdrawn through the catheter hub, which is a common source of microbial contamination; therefore, stringent cleaning of the hub prior to and after withdrawal of the sample must be implemented. In this section of study blood samples taken through the catheter of 90 renal patients revealed positive microbiology in 30 patients (33%) where no anti-lipid S antibodies were produced. This suggests that this technique is of clinical value in the diagnosis of haemodialysis catheter-related infection in patients who are unable to illicit an immune response.

4.4.4 Comparison of anti-hepatitis B and anti-lipid S antibody production in renal patients.

Comparison of immune response to the anti-hepatitis B vaccine and production of anti-lipid S antibodies demonstrated that 75% of patients were not considered to have protection against hepatitis B following a course of vaccination. Conversely, over 50% of those patients produced antibodies to lipid S antigen. This suggests that in renal patients suppressed immune response in not universal, that is to say, that while antibodies are not secreted to one antigenic stimuli other antigens may illicit an immune response due to the specificity of the antibody/antigen interaction.

In conclusion, the variety of methods available to monitor the immune status of patients is broad, however their value in predicting the onset of infection in renal patients is limited. This is due to the nature of ESRD and the comorbid processes that accompany this disease. The effects on the immune system are varied and immune response is therefore limited to specific antigenic challenge. The environment in which renal patients are treated, the mechanisms of dialysis and the frequency with which the patient is exposed to both contribute to the acquisition of antigen. The development of early detection methods are of paramount importance therefore the anti-lipid S ELISA along with the AOLC are valuable tools, however, of equal importance is the reduction in the reliance of polyurethane lines and the increase in infection control policies to reduce the opportunity of exposure to antigen.

CHAPTER 5: PULSED-FIELD GEL ELECTROPHORESIS

5.1 INTRODUCTION

The hospital environment has long been recognised as a reservoir for microorganisms and one that supports the horizontal transfer of infection between patients, staff and the environment. Pulsed field gel electrophoresis (PFGE) is currently the most sensitive technique available for epidemiological investigation of clonal relatedness between Gram-positive isolates (Lina, *et al.,* 1992); (Snopkovà, *et al.,* 1994); (Poddar and McClelland, 1991); (Livesely, *et al.,* 1998); (Kluytmans, *et al.,* 1998). The benefit of this molecular method is that the complete bacterial genome is analysed as opposed to a proportion (Lang *et al.,* 1999). The choice of restriction enzyme (RE) affects the number of bands generated in the profile; *Sma*I (cutting at CCCGGG) has been reported to be the most suitable RE for use in PFGE as it provides an adequate number of clearly separated fragments (Snopkovà, *et al.,* 1994). PFGE supports the identification of the source of infection (Lang, *et al.,* 1999); (Lina, *et al.,* 1992) by allowing the relatedness of strains from the patient, clinical staff and the environment to be examined. In addition, it is an invaluable tool for the differentiation of relapse, recurrence and reinfection in patients with indwelling medical devices such as dialysis catheters (Chang, *et al.,* 2000). This section of study aims to identify common banding patterns of staphylococcal species and investigate the extent to which staphylococcal infection is transferred between different anatomical sites of individuals, different renal dialysis patients and dialysis modalities. In addition, the efficacy of antimicrobial treatment protocols will be evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial isolates

Three hundred and five staphylococcal isolates from 100 renal dialysis patients that presented at UHB or one of its satellite centres with clinical signs of infection, were examined by PFGE. The patients were grouped according to dialysis modality. The types of staphylococcal infections experienced by these patients were CAPD-associated peritonitis, CAPD-associated catheter infection, haemodialysis catheter-associated infection, wound infections and MRSA carriage. All specimens were collected in accordance with Trust protocols and processed using microbiology standard operating procedures.

Following PFGE, the relatedness of 60 of the 120 CNS isolates were determined by means of the Phoretix ID Advanced gel analysis computer program in accordance with the Tenover criteria (Tenover, *et al.,* 1995). The relatedness of the other CNS, *S. aureus* and MRSA isolates was determined by the Tenover criteria alone.

5.2.2 Preparation of chromosomal DNA

Chromosomal DNA was prepared by the method of Lina *et al.* (1992). Bacterial cells were cultivated in 20mL of BHI broth (Oxoid) at 37°C with aeration at 200rpm for 18h. The cells were recovered from 1mL of the culture by centrifugation (11,400g for 5min) and resuspended to a concentration of 20mg wet weight cells/ml in NET-100 (0.1M Na_2EDTA (pH 8.0), 0.1M NaCl, 0.01M Tris (Tris [hydroxymethyl] aminomethane)-HCl (pH 8.0)). An equal volume of molten low melting point chromosomal grade agarose (BioRad, USA) (0.9% (w/v) chromosomal grade agarose in NET-100) cooled to 65°C, was gently mixed with the cell suspension and the molten agarose-bacterial mixture dispensed into an ice cooled block mould (BioRad). The agarose blocks (three blocks (8mm x 15mm) were prepared for each bacterial strain) were placed in a bijou bottle containing 3mL of lysis solution (6mM Tris-HCl (pH 7.6), 100mM Na_2EDTA (pH 8.0), 1M NaCl, 0.5% (w/v) N-lauroyl-sarcosine (sarcosyl), 1mg/mL lysozyme, 6.6 units/mL lysostaphin) and incubated at 37°C for 24h. The lysis solution was replaced by 3mL of ESP solution (0.5M Na_2EDTA (pH 9.0), 1% (w/v) sarcosyl, 0.15% (w/v) protinase K) and incubated at 50°C for 48h to remove cellular debris. The blocks, drained of ESP, were washed in TE buffer (10mM Tris-HCl (pH 8.0), 1mM Na_2EDTA (pH 8.0)) (Maslow, *et al.,* 1993) four times with continuous agitation over an 8h period. The prepared agarose blocks, containing intact bacterial chromosomes, were stored in TE buffer at 4°C (Birren and Lai, 1993).

5.2.3 Restriction endonuclease digestion of chromosomal DNA

Slivers of the agarose blocks (1x1x9mm) containing chromosomal DNA were suspended in 0.2ml of restriction endonuclease (RE) buffer (prepared with sterile double distilled water) and equiliberated at 4°C for 15min. The buffer was replaced with a fresh RE buffer supplemented with 50 units of the RE *Sma*I (Boehringer Mannheim, Germany). The restriction digest was incubated at 4°C for 15min before transfer to 25°C for 18h. The RE suspension was replaced with 0.2mL ES solution (0.5M Na_2EDTA (pH9.0) supplemented with 1% (w/v) sarcosyl) and incubated at 50°C for 15min to inactivate residual enzyme

activity. The sliver was then held in 1ml of TE buffer for 15min to ensure the removal of ES solution. Once drained, the RE digested agarose sliver was subjected to PFGE directly or maintained without drying at 4°C for a maximum of 48h.

5.2.4 Resolution of macrorestriction fragments by pulsed-field gel electrophoresis

The RE digested DNA slivers were loaded into a 1% (w/v) agarose gel (molecular biology grade agarose, BioRad) prepared with 0.5 x TBE buffer (0.45mM Tris-borate, 1mM Na_2EDTA (pH8.0)). An equal sized sliver of a bacteriophage lambda (λ) concatamer ladder (BioRad) was incorporated as a DNA size standard. The slivers were sealed into the wells with 0.5% (w/v) molecular grade agarose prepared with 0.5 x TBE. The macrorestriction fragments were separated by PFGE performed in a contour-clamped homogeneous electric field (CHEF) electrophoresis system (CHEF-DR III system, BioRad). The following conditions were applied to the gel: a 1-50s ramped switch interval with a voltage gradient of 6V/cm at an angle of 120° was applied over a 24 h period. A Tris-borate running buffer (0.5 x TBE) was maintained at 10°C.

5.2.5 Visualisation of resolved DNA fragments

After electrophoresis, the gel was stained with 0.5µg/mL ethidium bromide with gentle agitation for 30 min followed by destaining in distilled water for 1h and the DNA visualised using a ultra violet (UV) light scanner (UVP products, UK) (Maslow, *et al.*, 1993).

5.2.6 Analysis of PFGE profile reproducibility

A restriction digest of the reference strain *S. epidermidis* NCTC 11047 was included on 12 individual gels to assess the reproducibility of the method. Statistical analysis of the reference strain profiles with the Phoretix ID Advanced gel analysis computer program (Phoretix International, UK) and the data evaluated using a one-way analysis of variance (one-way ANOVA) (InStat, GraphPad Software Inc.).

5.2.7 Patient isolates

The criteria of Tenover *et al.* (1995) for the analysis of macrorestriction profiles were developed for use in the outbreak situation but may be applied to isolates recovered from a single patient over time, as described in this study.

Multiple isolates from individual patients were subjected separately to PFGE to identify clones or indicate where two isolates were dissimilar according to the guidelines of Tenover *et al.* (1995). The criteria suggested for the interpretation of PFGE macrorestriction patterns:

- No difference in banding patterns was considered indistinguishable isolates.
- Two or three band differences within the profile indicated that the isolates were closely related.
- Four to six band differences suggested that the two isolates were possibly related.
- Seven or more band deviations indicated that the two strains were not related (Tenover, *et al.*, 1995).

5.2.8 Dendrogram analysis

Interpretation of the macrorestriction fragment profiles was performed using the Phoretix ID Advanced gel analysis computer programme (Phoretix International, UK) together with supporting visual assessment. The size of the profile bands was evaluated by comparing against the bands of the λ phage DNA standard. Only bands $\geq$30kb were included in the analysis. Completed gel analysis data was transferred to the Phoretix ID Database programme (Phoretix International) to allow strain comparison by calculation of the Dice correlation coefficient:

$$\text{Dice coefficient} = \frac{2h}{a + b}$$

where a is the total number of bands in isolate A, b is the total number of bands in isolate B and h is the total number of bands shared by A and B (Dice, 1945).

The Dice coefficient is a measure of the amount of association between both of the two isolates. Isolates were clustered by the unweighted pair group method of arithmetic averages (UPGMA) to permit the construction of a dendrogram.

5.3 RESULTS

5.3.1 Patient demographics

Table 5.1 Patient demographics

Patient group	Number of patients	Male to Female ratio	Age range (Mean age)
CAPD	58	32:26	18-88 (53)
Haemodialysis	42	20:22	21-89 (56)

The types of staphylococcal species responsible for infection in these two renal patient groups are shown in section 5.3.2.

5.3.2 Microorganism demographics

Table 5.2 Microorganism demographics

Patient group	Number of patients with CNS infection	Number of patients with SAUR infection	Number of patients with MRSA infection	Number of patients with staphylococcal polymicrobial Infection*
CAPD	37	22	4	5
Haemodialysis	35	18	4	13**

* Polymicrobial refers to patients who had subsequent infections over the study period caused by different staphylococcal species.

** Two patients were infected with CNS, SAUR and MRSA over the study period.

The majority of isolates recovered from haemodialysis patients were from blood cultures and in CAPD patients from peritoneal dialysis effluent. Isolates were recovered from other clinical specimens and these are outlined in table 5.3.

Table 5.3 Site of microorganism collection

Clinical specimen	Number of isolates recovered from CAPD patients	Number of isolates recovered from Haemodialysis patients
Blood culture	1	83
Peritoneal dialysis effluent	87	0
Haemodialysis catheter tip	0	36
Peritoneal dialysis catheter tip	6	0
Catheter exit swab	14	28
MRSA screen	3	12
Wound swab	1	14
Skin swab	8	12

The types of staphylococcal species recovered from different clinical specimens are shown in table 5.4.

Table 5.4 Staphylococcal species recovered from different clinical specimens

Clinical specimen	Number of isolates recovered from CAPD patients			Number of isolates recovered from Haemodialysis patients		
	CNS	SAUR	MRSA	CNS	SAUR	MRSA
Blood culture	0	1	0	51	23	9
Peritoneal dialysis effluent	52	32	3	0	0	0
Haemodialysis catheter tip	0	0	0	12	19	5
Peritoneal dialysis catheter tip	4	2	0	0	0	0
Catheter exit swab	0	13	1	2	19	7
MRSA screen	0	0	3	1	1	10
Wound swab	0	0	1	1	5	8
Skin swab	7	0	1	4	5	3

5.3.3 Macrorestriction profiles

5.3.3.1 Reproducibility

This analysis showed that there was no significant variance between the gels when restriction with the enzyme *Sma*I ($P > 0.99$). *Sma*I restriction digestion yielded profiles of between 5 and 18 bands ranging in size from 30-694kb.

The size of the macrorestriction profile bands generated with *Sma*I restriction endonuclease was evaluated by comparing against the bands of the lambda phage DNA standard (λ). The standard consistently generated 13 bands representing a range of 48.5-630.5 kb (figure 5.0).

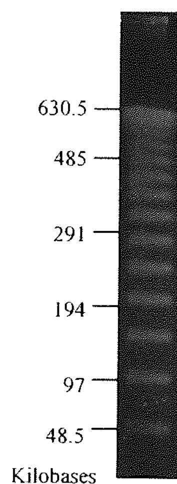


Figure 5.1 Profile of Lambda DNA concatamer standard.

Three hundred and five macrorestriction profiles were compared. The number of different staphylococcal profiles is shown in table 5.5.

Table 5.5 Analysis of macrorestriction profiles

Patient group	Total number of macrorestriction profiles compared	Number of CNS macrorestriction profiles compared	Number of SAUR macrorestriction profiles compared	Number of MRSA macrorestriction profiles compared
CAPD	120	63	48	9
Haemodialysis	185	71	72	42

Genotypic analysis of the macrorestriction profiles in accordance with the Tenover criteria (Tenover, *et al.*, 1995) generated a large number of different genotypes in each patient group. These are shown in table 5.6.

Table 5.6 Genotypic analyses of macrorestriction profiles

Patient group	Total number of genotypes	Number of CNS genotypes	Number of SAUR genotypes	Number of MRSA genotypes
CAPD	78	57	19	2
Haemodialysis	88	49	28	11

<u>5.3.3.2 CNS</u>

A total of 72 patients, 37 CAPD and 35 haemodialysis, generated 63 and 71 CNS macrorestriction profiles respectively, from a range of clinical specimens. These profiles were heterogeneous and grouped into 106 genotypes that exhibited between 6 and 18 bands. One distinct profile was generated from a peritoneal dialysis effluent sample (figure 5.2).

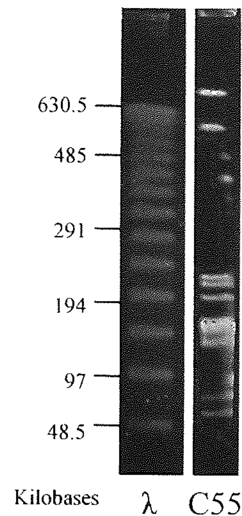


Figure 5.2 A distinct macrorestriction profile for a CNS isolate from peritoneal dialysis effluent.

One hundred and thirty-four CNS profiles were compared. Common bands were located at 291kb, 145.5kb and 97kb representing 56%, 77% and 67% of profiles respectively.

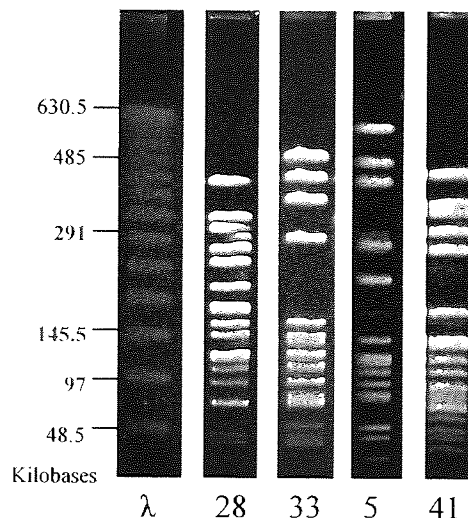


Figure 5.3 Common macrorestriction banding patterns in CNS

Common band sizes are shown in red. Profiles 28 and 33 were recovered from two CAPD patients and profiles 5 and 41 from two haemodialysis patients.

5.3.3.3 *S. aureus*

One hundred and twenty *S. aureus* macrorestriction profiles from 40 patients, 22 CAPD and 18 haemodialysis were compared. These profiles were also heterogeneous and grouped into 47 genotypes that exhibited between 6 and 18 bands. No distinct profile was generated. Common bands were located at 388kb, 339.5kb, 194kb, 145.5kb and 97kb (figure 5.4) representing 63%, 61%, 76%, 80% and 72% of *S. aureus* isolates respectively.

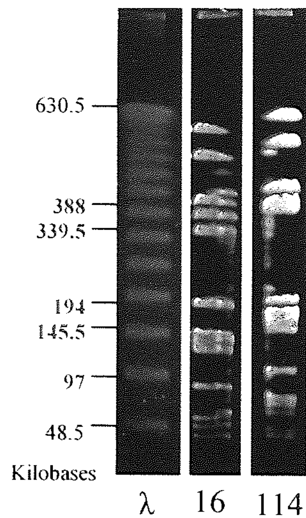


Figure 5.4 Common macrorestriction banding patterns in *S. aureus*

Common band sizes are shown in red. Profile 114 was recovered from a CAPD patients and profile 16 from a haemodialysis patient.

5.3.3.4 MRSA

Fifty-three MRSA macrorestriction profiles from 8 patients, 4 CAPD and 4 haemodialysis were compared. These profiles were grouped into 13 genotypes that exhibited between 11 and 15 bands. No distinct MRSA profile was generated. Common bands were located at 630kb, 533.5kb, 339.5kb, 291kb, 194kb, 145.5kb and 97kb (figure 5.5) representing 92%, 61%, 76%, 76%, 84%, 92% and 84% of MRSA profiles respectively.

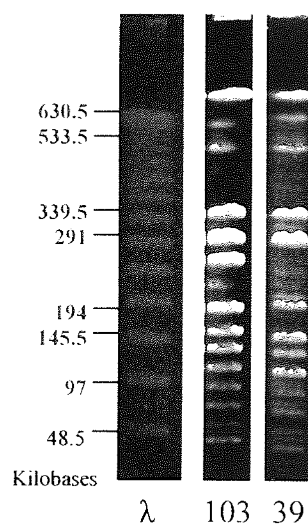


Figure 5.5 Common macrorestriction banding patterns in MRSA

Common band sizes are shown in red. Profile 103 was recovered from a CAPD patient and profile 39 from a haemodialysis patient.

Common banding patterns in CNS, *S. aureus* and MRSA were located at 339.5kb, 194kb, 145.5kb and 97kb (figure 5.6).

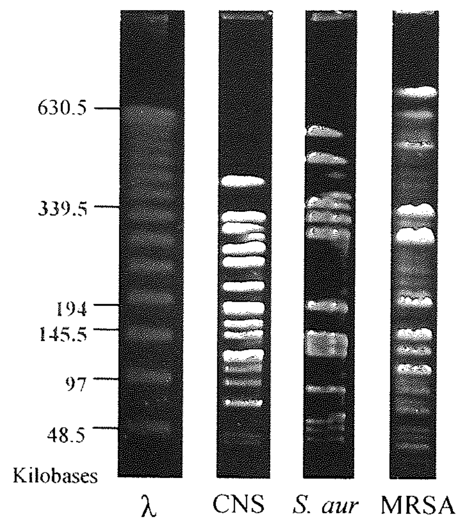


Figure 5.6 Macrorestriction banding patterns common in CNS, *S. aureus* and MRSA

5.3.4 Strain relatedness

5.3.4.1 CNS

The relatedness of 60 CNS isolates was compared with Phoretix ID Advanced gel analysis computer programme and in accordance with the Tenover criteria (Tenover, *et al.*, 1995).

5.3.4.1.1 Phoretix ID Advanced gel analysis computer programme.

The macrorestriction profile analysis demonstrated genetic heterogeneity amongst the 60 strains of CNS recovered from renal patients with haemodialysis-CRI or CAPD-associated peritonitis. A dendrogram representing the relatedness of these strains can be seen in figure 5.7.

S.epidermidis isolates recovered from patients with haemodialysis-CRI were clustered into 16 genotypic profiles and *S. epidermidis* isolates recovered from patients with CAPD-associated peritonitis were clustered into 18. Hospitalised patients (n=30) accounted for 76% of the *S. epidermidis* isolates recovered compared to 24% in those patients treated in the community (n=9). Furthermore, 56% of the *S. epidermidis* isolates recovered from hospitalised patients had spent greater than 4 days on the same ward (data not shown).

Coefficient: Dice Algorithm: UPGMA

CAPD *S.epidermidis*
HAEMODIALYSIS *S.hominis*
CAPD *S.hominis*
HAEMODIALYSIS *S.saprophyticus*
CAPD *S.hominis*
CAPD *S.hominis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
CAPD *S.lugdunensis*
HAEMODIALYSIS *S.cohnii*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
HAEMODIALYSIS *S.hominis*
CAPD *S.hominis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
CAPD *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.haemolyticus*
HAEMODIALYSIS *S.warneri*
CAPD *S.haemolyticus*
HAEMODIALYSIS *S.haemolyticus*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.hominis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
CAPD *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
CAPD *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
CAPD *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
CAPD *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
CAPD *S.haemolyticus*
HAEMODIALYSIS *S.haemolyticus*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.epidermidis*
HAEMODIALYSIS *S.capitis*
CAPD *S.lugdunensis*
CAPD *S.xylosus*
CAPD *S.haemolyticus*

0.28

0.3 0.4 0.5 0.6 0.7 0.8 0.9

Figure 5.7 Dendrogram constructed using unweighted pair group method of arithmetic averages (UPGMA) and Dice correlation coefficient, based on *Sma*I macrorestriction fragment profiles for CNS.

In figure 5.7, CNS species were grouped according to the number of restriction fragments that they had in common when compared to one another. The dendrogram shows 14 clusters of isolates with varying degrees of relatedness based on the Dice coefficient for that cluster. The closer the Dice coefficient is to 1.0 the more closely related the macrorestriction profiles are. CNS isolates recovered from CAPD-associated peritonitis and haemodialysis-CRI were shown to be closely related with Dice coefficients between 6.0 and 9.0.

5.3.4.1.2 Tenover criteria

The relationship of 60 CNS isolates recovered from patients with haemodialysis-CRI or CAPD-associated peritonitis was determined according to the guidelines of Tenover *et al.*, (1995) (Tenover, *et al.*, 1995). This method compared each macrorestriction profile to each of the other macrorestriction profiles and identified the CNS isolates that were indistinguishable (I), closely related (CR), possibly related (PR) or unrelated (UR).

The percentage relatedness of these macrorestriction profiles is shown in table 5.7.

Table 5.7 Percentage relatedness of CNS macrorestriction profiles

Total number of CNS macrorestriction profiles	Total number of profile comparisons	Percentage identical profiles	Percentage closely related profiles	Percentage possibly related profiles	Percentage unrelated profiles
60	3540	0.7	1.9	9.3	88.1

Eighty-eight percent of CNS isolates were unrelated demonstrating heterogeneity amongst isolates recovered from patients with CAPD-associated peritonitis and haemodialysis-catheter related sepsis. However, 12% of isolates were related; these isolates were recovered from different patients and different infection groups. These data suggest the horizontal transfer of *S. epidermidis* strains between patients with haemodialysis-CRI and CAPD-associated peritonitis.

The relatedness of each of the CNS macrorestriction profiles can be seen in table 5.8.

Table 5.8 Relatedness of sixty CNS strains according to the Tenover criteria

	38	69	115	50	34	108	65	62	66	61	41	63	17	18	1	6	8	19	20	24	35	15	13	23	11	104	118	103	37	77	87	75	81	89	33	88	79	106	114	117	91	45	54	83	73	113	60	101	111	31	100	107	43	57	28	70	22	116	39	40
38		UR	PR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
69	UR		PR	UR	PR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
115	PR	PR		UR	UR	CR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR
50	UR	UR	UR		UR	UR	PR	PR	PR	PR	UR	UR	UR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	PR	PR	PR	UR	UR	I	PR	PR	PR	I	CR	UR	I	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR
34	CR	PR	UR	UR		PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
108	UR	CR	CR	UR	PR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR
65	UR	UR	UR	PR	UR	UR		PR	PR	PR	UR	PR	UR	PR	PR	PR	UR	UR	UR	PR	PR	PR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	PR	PR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR
62	UR	UR	PR	PR	UR	UR	PR		PR	PR	UR	UR	UR	UR	PR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	PR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR
66	UR	UR	UR	PR	UR	UR	PR	PR		PR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	PR	PR	PR	UR	PR	UR	UR	PR	PR	PR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
61	UR	UR	UR	PR	UR	UR	PR	PR	PR		UR	UR	UR	UR	PR	UR	UR	UR	PR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	PR	PR	PR	PR	UR	UR	PR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
41	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	PR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
63	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
17	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR		CR	UR	UR	UR	PR	UR	UR	PR	CR	CR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR
18	UR	UR	UR	PR	UR	UR	PR	UR	UR	UR	UR	UR	CR		UR	UR	UR	UR	UR	UR	PR	PR	PR	PR	CR	UR	UR	UR	UR	UR	PR	UR	UR	CR	CR	UR	UR	UR	UR	UR	PR	PR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
1	UR	UR	UR	PR	UR	UR	PR	PR	PR	PR	UR	UR	UR	UR		PR	PR	PR	PR	PR	UR	UR	UR	CR	UR	UR	UR	UR	UR	UR	PR	UR	UR	PR	PR	CR	UR	PR	UR	UR	I	CR	UR	UR	CR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
6	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	PR		CR	UR	PR	UR	UR	UR	UR	CR	PR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	CR	UR
8	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	CR		UR	PR	UR	PR	UR	UR	UR	PR	UR	UR	UR	UR	UR	PR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	PR	UR	UR	UR	UR	PR	UR	UR	CR	PR	UR
19	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	PR	UR	UR		CR	CR	PR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR
20	UR	UR	UR	UR	UR	UR	UR	PR	UR	PR	UR	UR	UR	UR	PR	PR	PR	CR		PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
24	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	CR	PR		UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	CR	UR	PR	UR	UR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR
35	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	PR	PR	PR	PR	UR	UR	PR	PR	UR	UR		PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
15	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	CR	PR	UR	UR	UR	PR	UR	UR	PR		CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
13	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	CR	PR	UR	UR	UR	PR	UR	UR	UR	CR		UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
23	UR	UR	UR	PR	UR	UR	PR	UR	PR	PR	UR	UR	UR	PR	CR	CR	UR	UR	UR	PR	UR	UR	UR		PR	UR	UR	UR	UR	UR	UR	UR	UR	CR	CR	CR	PR	I	PR	UR	CR	I	CR	UR	I	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR
11	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	CR	UR	PR	PR	UR	UR	UR	UR	UR	UR	PR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
104	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
118	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
103	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
37	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
77	UR	UR	PR	UR	UR	PR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR		UR	PR	I	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
87	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	PR	PR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
75	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR		PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
81	UR	UR	PR	PR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	I	UR	PR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
89	UR	UR	UR	PR	UR	UR	PR	PR	PR	PR	UR	UR	UR	CR	PR	UR	UR	PR	UR	PR	UR	PR	UR	CR	UR	UR	UR	UR	UR	PR	UR	UR	UR		UR	PR	UR	PR	UR	UR	PR	PR	UR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
33	UR	UR	UR	PR	UR	UR	UR	UR	PR	PR	UR	UR	UR	CR	PR	UR	PR	PR	UR	UR	UR	UR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	CR	CR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
88	UR	UR	UR	UR	UR	UR	UR	PR	UR	PR	UR	UR	UR	UR	CR	UR	UR	UR	PR	CR	UR	UR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR		UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
79	UR	UR	UR	UR	UR	UR	UR	UR	PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		PR	UR	UR	UR	UR	UR	I	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
106	UR	UR	UR	I	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	PR	UR	UR	UR	UR	PR	UR	UR	UR	I	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	PR		UR	UR	UR	CR	I	UR	I	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
114	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	PR	UR	UR	PR	PR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
117	UR	UR	UR	PR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR
91	UR	UR	UR	PR	UR	UR	PR	UR	PR	PR	UR	UR	UR	PR	I	UR	UR	UR	UR	PR	UR	UR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	PR	UR	UR	UR	UR		CR	PR	UR	I	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
45	UR	UR	UR	I	UR	UR	PR	UR	PR	UR	UR	UR	UR	PR	CR	UR	UR	UR	UR	PR	UR	UR	UR	I	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	CR	UR	UR	CR	PR	UR	CR		UR	UR	I	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
54	UR	UR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	CR	UR	UR	I	UR	UR	PR	UR		UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR
83	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	I	UR	UR	PR	UR	UR	UR		PR	PR	UR	UR	UR	UR	UR	UR	UR	UR	PR	PR	UR	UR	UR	UR
73	UR	UR	UR	I	UR	UR	PR	UR	UR	PR	UR	UR	UR	PR	CR	PR	UR	UR	UR	UR	UR	UR	UR	I	UR	UR	UR	UR	UR	UR	UR	UR	UR	CR	UR	UR	PR	I	PR	UR	I	I	UR	PR		PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
113	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	CR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	PR	PR	UR	PR	UR	PR	PR	PR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR
60	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR
101	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
111	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR
31	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		PR	UR	UR	UR	UR	UR	UR	UR	UR	UR
100	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR		UR	UR	UR	UR	UR	UR	UR	UR	UR
107	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR	UR	UR	UR
43	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR		CR	UR	UR	UR	UR	UR	UR
57	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	CR		UR	UR	UR	UR	UR	UR
28	UR	UR	PR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR	UR
70	UR	UR	UR	PR	UR	UR	PR	PR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR	UR
22	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR	UR
116	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	CR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR	UR
39	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	CR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	PR	UR	UR	PR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR		UR
40	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	UR	

Key to table 5.8:

I Indistinguishable | CR Closely Related | PR Possibly Related | UR Unrelated

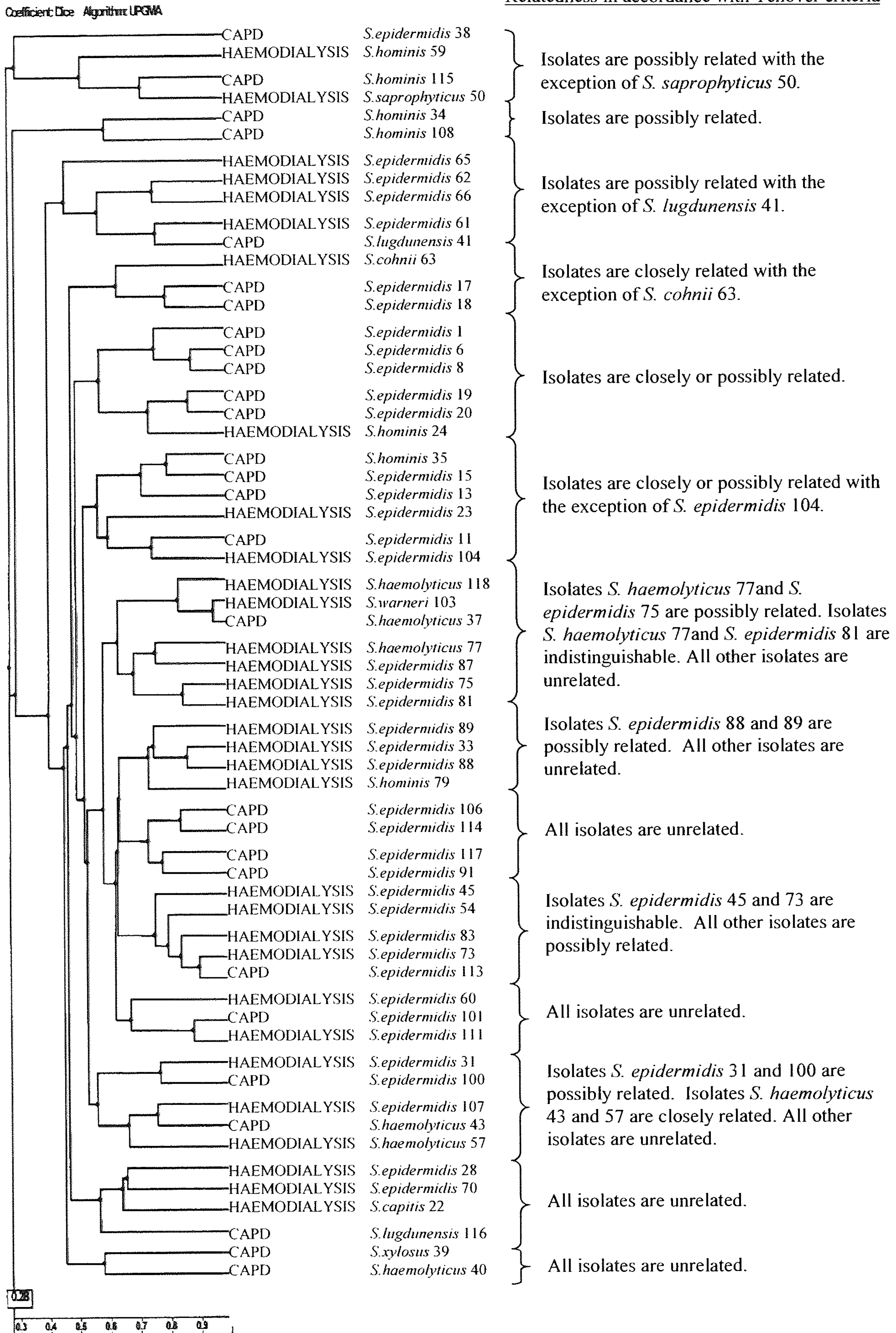


Figure 5.8 Comparison of methods for identifying the relatedness of CNS isolates

5.3.4.1.2.1 Relatedness of all CNS isolates recovered

The relationship of different CNS isolates recovered from a variety of clinical samples was determined according to the guidelines of Tenover *et al.* (1995).

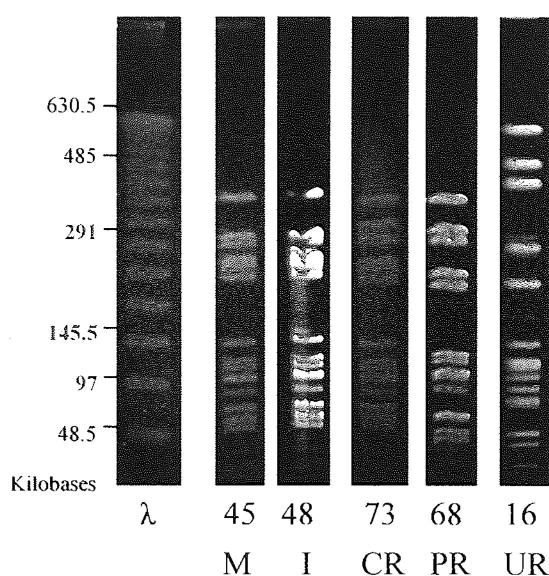


Figure 5.9 Macrorestriction profiles demonstrating the relatedness of CNS isolates.

Profile 45 was used as the marker (M) isolate with which the other four profiles were compared. All profiles were recovered from different patients, 45 (blood culture), 73 (blood culture), 68 (blood culture) and 16 (nose swab) were recovered from haemodialysis patients and profile 48 (peritoneal dialysis effluent) from a CAPD patient. The percentage relatedness of CNS isolates can be seen in table 5.9.

Table 5.9 Percentage relatedness of all CNS isolates

Total number of CNS macrorestriction profiles	Total number of profile comparisons	Percentage identical profiles	Percentage closely related profiles	Percentage possibly related profiles	Percentage unrelated profiles
134	11842	2.7	3.2	10	83

5.3.4.1.3 *S. aureus*

Macrorestriction profiles of *S. aureus* were compared according to the Tenover criteria (Tenover, *et al.*, 1995).

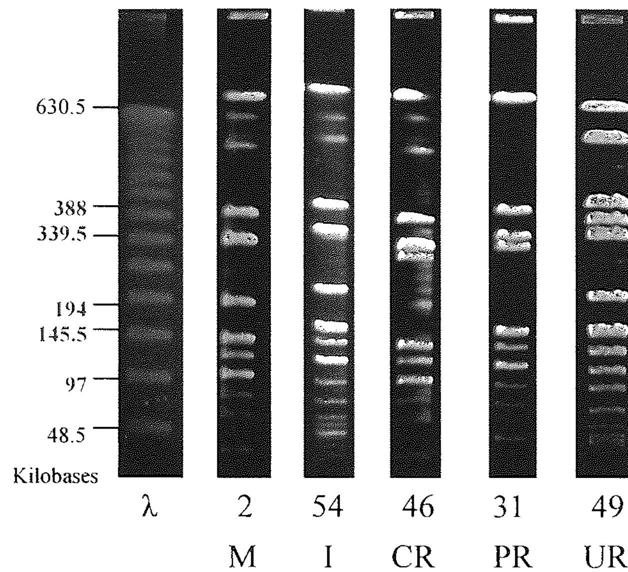


Figure 5.10 Macrorestriction profiles demonstrating the relatedness of *S. aureus* isolates.

Profile 2 (blood culture) was used as the marker (M) isolate with which the other four profiles were compared. All profiles were recovered from different patients, 54 (peritoneal dialysis effluent) from a CAPD patient, 46 (haemodialysis catheter tip), 31 (wound swab) and 49 (blood culture) were recovered from haemodialysis patients.

The percentage relatedness of *S. aureus* isolates can be seen in table 5.10.

Table 5.10 Percentage relatedness of *S. aureus* macrorestriction profiles

Total number of CNS macrorestriction profiles	Total number of profile comparisons	Percentage identical profiles	Percentage closely related profiles	Percentage possibly related profiles	Percentage unrelated profiles
120	6850	1.8	4.7	8.5	84

5.3.4.1.4 MRSA

Macrorestriction profiles of MRSA were compared according to the Tenover criteria (Tenover, *et al.*, 1995).

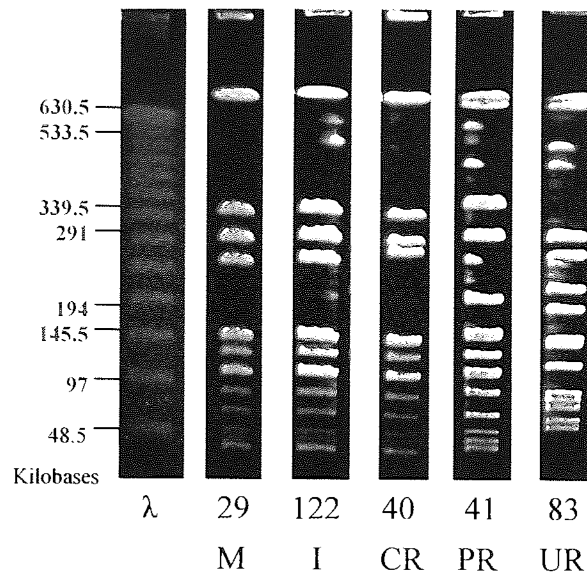


Figure 5.11 Macrorestriction profiles demonstrating the relatedness of MRSA isolates.

Profile 29 (swab) was used as the marker (M) isolate with which the other four profiles were compared. All profiles were recovered from different patients, 122 (peritoneal dialysis effluent) from a CAPD patient, 40, 41 and 83 were recovered from the MRSA screening swabs of haemodialysis patients.

The percentage relatedness of MRSA isolates can be seen in table 5.11.

Table 5.11 Percentage relatedness of MRSA macrorestriction profiles

Total number of CNS macrorestriction profiles	Total number of profile comparisons	Percentage identical profiles	Percentage closely related profiles	Percentage possibly related profiles	Percentage unrelated profiles
51	1086	0	28	4.4	67

5.3.5 Endemic strains

5.3.5.1 Endemic CNS strains

The number of related CNS isolates recovered from different patients and those who are treated by different modalities is shown in table 5.12.

Table 5.12 Transfer of CNS strains between patients

Number of related isolates recovered from different CAPD patients			Number of related isolates recovered from different haemodialysis patients			Number of related isolates recovered from patients treated with different modalities		
I	CR	PR	I	CR	PR	I	CR	PR
162	240	827	110	56	258	188	196	608

The number of profiles that were matched as indistinguishable, closely related or possibly related for each dialysis modality were divided by total number of comparisons and multiplied by 100 to show the percentage relatedness for CNS isolates. Isolates recovered from patients treated with CAPD revealed 10.7% relatedness and isolates recovered from patients treated with haemodialysis 6.6% relatedness. The relatedness of CNS isolates recovered from patients treated by different dialysis modalities was 16.5%.

5.3.5.2 Endemic *S. aureus* strains

The number of related *S. aureus* isolates recovered from different patients and those who are treated by different modalities is shown in table 5.13.

Table 5.13 Transfer of *S. aureus* strains between patients

Number of related isolates recovered from different CAPD patients			Number of related isolates recovered from different haemodialysis patients			Number of related isolates recovered from patients treated with different modalities		
I	CR	PR	I	CR	PR	I	CR	PR
20	72	208	74	143	367	83	223	367

The number of profiles that were matched as indistinguishable, closely related or possibly related for each dialysis modality were divided by total number of comparisons and multiplied by 100 to show the percentage relatedness for *S. aureus* isolates. Isolates recovered from patients treated with CAPD revealed 15% relatedness and isolates recovered from patients treated with haemodialysis 11.7% relatedness. The relatedness of *S. aureus* isolates recovered from patients treated by different dialysis modalities was 19.7%.

5.3.5.3 Endemic MRSA strains

The number of related MRSA isolates recovered from different patients and those who are treated by different modalities is shown in table 5.14.

Table 5.14 Transfer of MRSA strains between patients

Number of related isolates recovered from different CAPD patients			Number of related isolates recovered from different haemodialysis patients			Number of related isolates recovered from patients treated with different modalities		
I	CR	PR	I	CR	PR	I	CR	PR
0	0	28	0	522	20	0	49	24

The number of profiles that were matched as indistinguishable, closely related or possibly related for each dialysis modality were divided by total number of comparisons and multiplied by 100 to show the percentage relatedness for MRSA isolates. Isolates recovered from patients treated with CAPD revealed 100% relatedness and isolates recovered from patients treated with haemodialysis 39.1% relatedness. The relatedness of MRSA isolates recovered from patients treated by different dialysis modalities was 19.2%.

5.3.6 Patient infection over time

Seven patients were studied over time to look at the efficacy of antimicrobial treatment and migration of microorganisms to different anatomical sites.

5.3.6.1 Efficacy of antimicrobial treatment

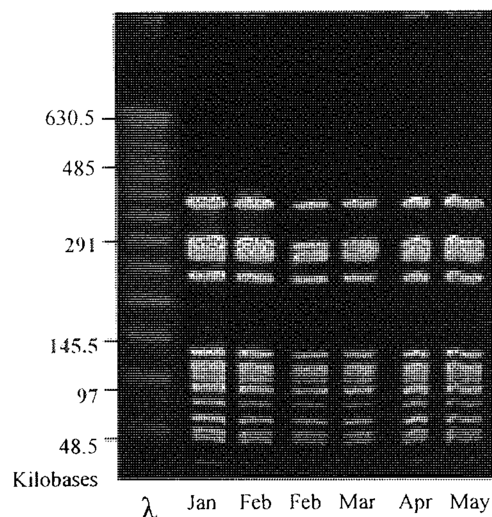


Figure 5.12 CNS isolates recovered from the peritoneal dialysis effluent of one patient over a five-month period.

The patient in figure 5.12 received intraperitoneal vancomycin and levofloxacin orally in January and intraperitoneal vancomycin in February, March, April and May of the same year however, an indistinguishable CNS isolate was recovered from peritoneal dialysis effluent each time the patient presented with symptoms of CAPD-associated peritonitis. The CAPD catheter was removed in May.

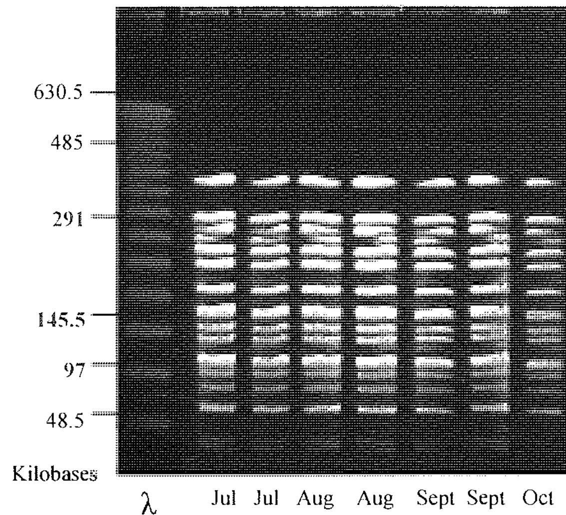


Figure 5.13 CNS isolates recovered from the peritoneal dialysis effluent of one patient over a four-month period.

The patient in figure 5.13 received intraperitoneal vancomycin and levofloxacin orally in July and intraperitoneal vancomycin in August, September and October however, an indistinguishable CNS isolate was recovered from peritoneal dialysis effluent each time the patient presented with symptoms of CAPD-associated peritonitis. The CAPD catheter was removed in October.

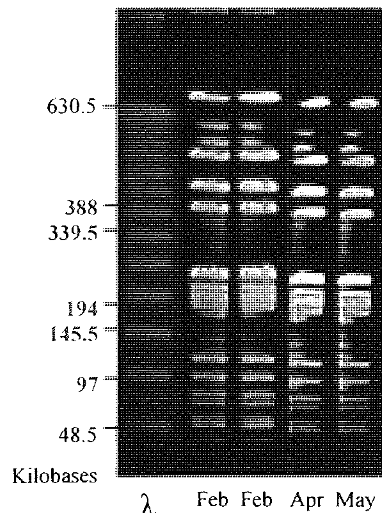


Figure 5.14 *S. aureus* isolates recovered from swabs of a haemodialysis catheter exit site of one patient over a three-month period.

The patient in figure 5.14 was treated with oral flucloxacillin however, an idistinguishable *S. aureus* isolate was recovered from subsequent catheter exit site swabs for three-months.

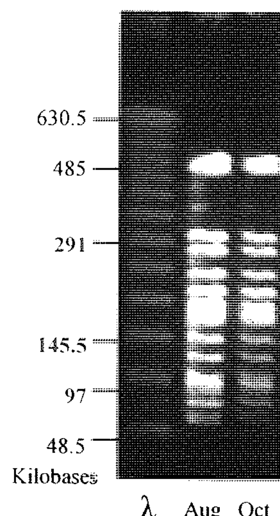


Figure 5.15 CNS isolates recovered from the blood cultures of one patient over a three-month period.

5.3.6.2 Migration of microorganisms over time

Haemodialysis patients that had multiple positive microbiology specimens within a ten-month period were studied to determine if migration of microorganisms occurs from one site to another.

One haemodialysis patient had 49 positive samples during the study period (figures 5.16.1-5.16.3)

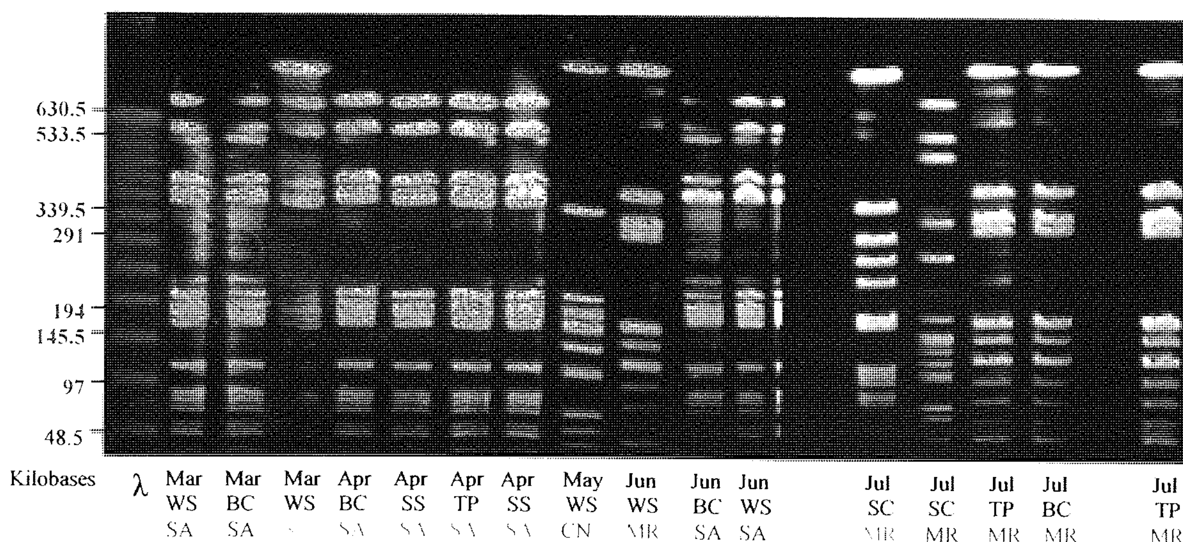


Figure 5.16.1 Macrorestriction profiles of isolates recovered from one patient over 5 months.

BC refers to blood culture, WS to wound swab, SS to catheter exit site swab and TP to haemodialysis catheter tip. SA refers to *S. aureus*, CN to CNS and MR to MRSA. Text colour denotes indistinguishable marcrorestriction profiles.

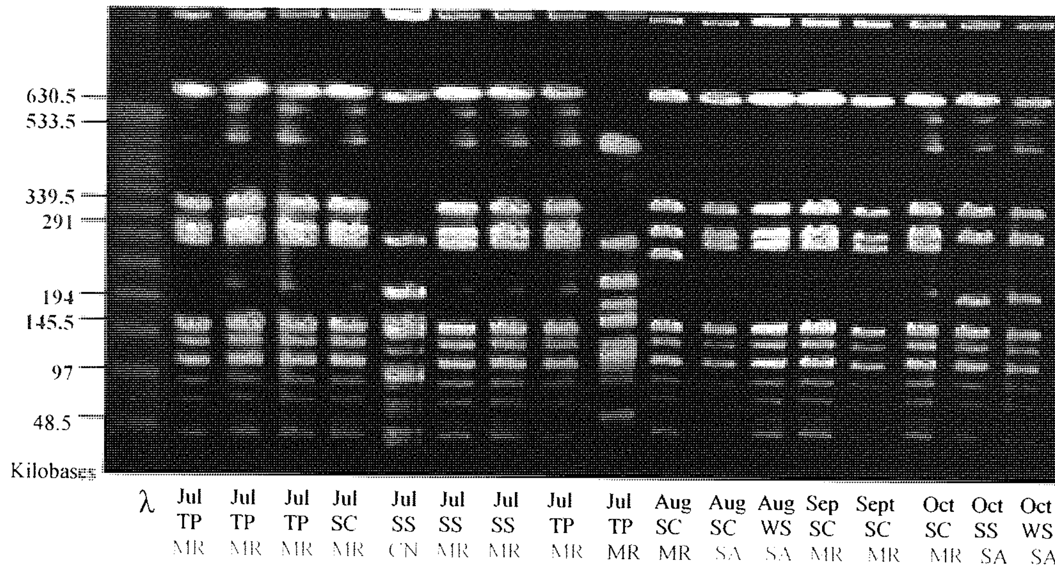


Figure 5.16.2 Macrorestriction profiles of isolates recovered from one patient over 4 months.

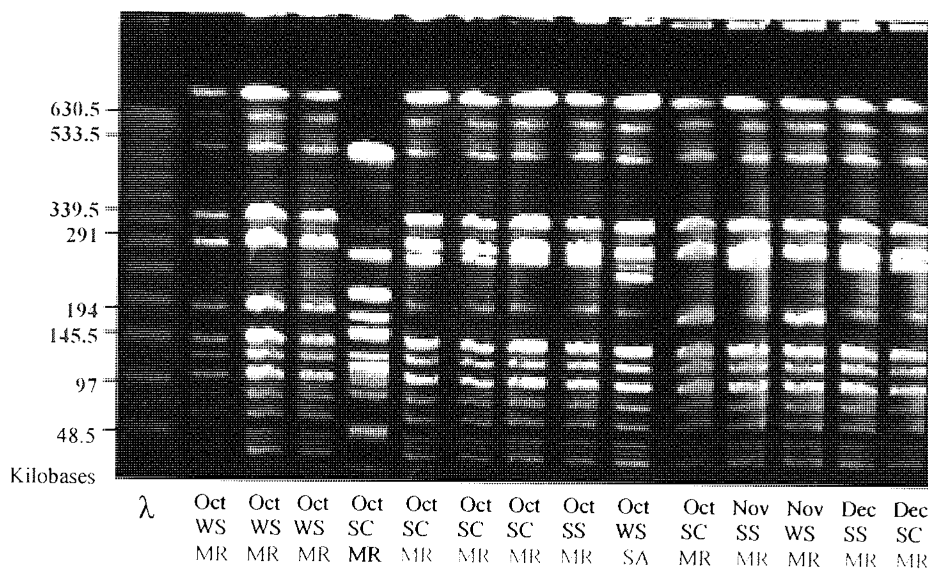


Figure 5.16.3 Macrorestriction profiles of isolates recovered from one patient over 3 months.

The patient represented in figures 5.16.1 to 5.16.3 was infected by CNS, *S. aureus* and MRSA over the study period. There were 12 different genotypes, two CNS unrelated, five *S. aureus* all closely related and seven MRSA 3 of which were closely related and four that were distinct. Two *S. aureus* and MRSA genotypes were indistinguishable (figures 5.16.2 and

5.16.3). *S. aureus* and MRSA strains were shown to migrate to different anatomical sites over time.

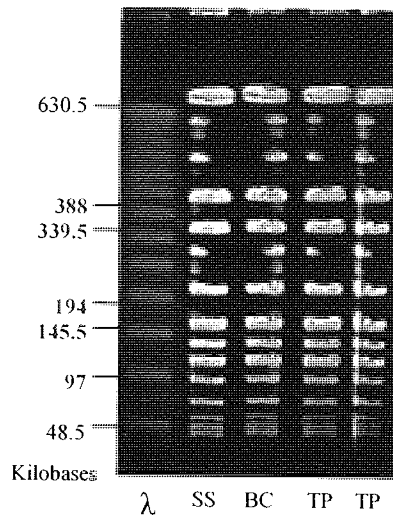


Figure 5.17 Macrorestriction profiles of *S. aureus* isolates recovered sequentially from one haemodialysis patient over a three-week period.

The macrorestriction profiles in figure 5.17 demonstrated the migration of *S. aureus* from the catheter exit site into the systemic circulation via the external surface of the catheter.

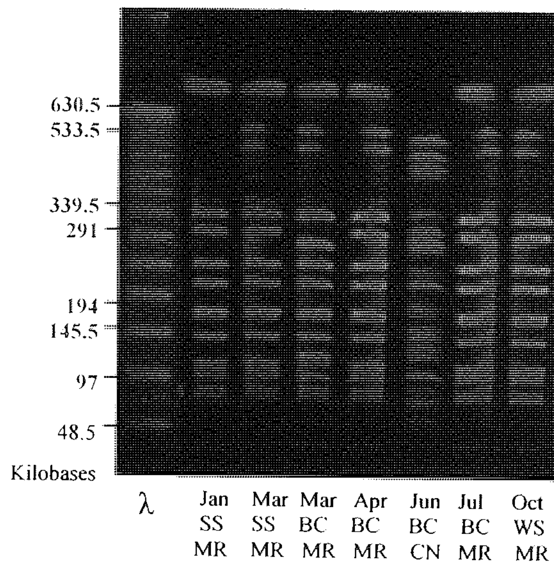


Figure 5.18 Macrorestriction profiles of isolates recovered sequentially from one haemodialysis patient over a ten-month period.

The patient represented in figure 5.18 presented with an MRSA infection at the haemodialysis catheter exit site. An indistinguishable microorganism was recovered from the catheter exit site 2-months later and repeatedly recovered from blood culture specimens over the following 5 months. The same microorganism was recovered from a wound on the patients hip after a further three-months.

5.4 DISCUSSION

5.4.1 Patient and strain recovery

Clinical audit data confirms that staphylococci are the most frequently recovered species from the clinical specimens of renal patients. This includes pathogenic microorganisms and those that are considered contaminants. The most common of the staphylococci to be recovered from the clinical specimens of this patient group are CNS. They are ever-present within the environment and on the skin of both patients and attending clinical staff and therefore frequently contaminate specimens. Assessing the clinical significance of CNS isolates recovered from single patient samples can be difficult (Hedin, 1996); (Zaidi, *et al.*, 1996) therefore the availability of multiple specimens from a single patient improves the interpretation of results, as repeated recovery of the same microorganism aids the accurate diagnosis of infection.

Within the clinical microbiology department, the identification of strains is made based on biotyping, antibiogram patterns and a limited number of biochemical reaction tests such as latex agglutination and tube coagulation. These tests will identify strains that are different at genus level, however they will not identify the genotypic relatedness of pathogenic strains. In order to classify an infection as current, concurrent or recurrent genotypic analysis of the causative microorganism is required.

5.4.2 Reproducibility

In order for any technique that aims to measure genetic variability to be successful, there must be genetic homogeneity within the isolates under investigation. The genetic diversity within and between staphylococcal species lends itself to macrorestriction pattern analysis. The relatedness of strains was measured by comparison of the distribution of rare cutting restriction endonuclease sites within the genome. The GC nucleotide content of the staphylococcal genome is low (35% to 37%) (Iandolo, *et al.*, 1996) therefore, cleavage with a restriction endonuclease such as *Sma*I that cuts at GC rich sites, will produce a sufficient number of bands to allow interpretation whilst maintaining a high level of discrimination. In this study cleavage of the staphylococcal genome with *Sma*I produced between 6 and 18 bands and was consistent with other studies (Snopkovà, *et al.*, 1994); (Lina, *et al.*, 1992); (Poddar and McClelland, 1991).

Macrorestriction pattern analysis of CNS, *S. aureus* and MRSA isolates identified common bands in each group (figures 5.3 to 5.5). CNS isolates revealed 2 common bands that were present in >60% of the CNS isolates compared. In *S. aureus* isolates 5 common bands were found in >60% of the *S. aureus* isolates compared and macrorestriction pattern analysis of MRSA isolates revealed 7 common bands that were present in >60% of MRSA isolates. These data are supported by previous work (Lina, *et al.*, 1992); (Snopkovà, *et al.*, 1994). This suggests that while there is heterogeneity in and between the staphylococci, there are characteristic macrorestriction banding patterns that could be used to identify *S. aureus* species.

5.4.3 Strain relatedness

Macrorestriction patterns are an estimate of the genetic relatedness of isolates, and can be equated to the molecular evolution of strains within a taxon. The combined use of macrorestriction profiling and Tenover's criteria for the genotyping of staphylococci can increase the accuracy of diagnosis. The ability of macrorestriction pattern analysis to define strain relatedness was evaluated in CNS, *S. aureus* and MRSA isolates.

Macrorestriction profile analysis of 60 CNS isolates allowed the relatedness of the strains to be visualized in the form of a dendrogram (figure 5.7). This demonstrated that isolates were segregated into 14 clusters of related strains. Within each cluster, the strains that were closely related were positioned in close proximity, for example *S. epidermidis*. In some cases different strains were clustered together, for example, *S. warneri* and *S. haemolyticus*. This may be the result of a lack of genetic diversity within the taxon therefore the similarity in the genotype of unrelated strains is increased.

The clusters of isolates that were generated in the dendrogram were compared by the Tenover criteria. This method also confirmed the heterogeneity of the CNS strains with only 12% of the isolates that were compared being related. In addition, this method identified different strains as unrelated for example, *S. epidermidis* and *S. lugdunensis* however, it also identified isolates of the same species that were unrelated (figure 5.8). This suggests that while computer aided macrorestriction analysis reduces bias, manual analysis of band patterns demonstrates equally effective discrimination of CNS isolates.

The relatedness of staphylococcal strains recovered from different patients was examined (figures 5.9 to 5.11). Comparison of macrorestriction patterns of CNS, *S. aureus* and MRSA identified strains recovered from different types of clinical specimens and different patients that were from a common ancestor. This suggests that horizontal transfer of staphylococcal strains between patients is occurring.

5.4.4 Endemic strains

No specific endemic strain was identified however there were closely related strains in different clinical areas. In addition, the transfer of CNS, *S. aureus* and MRSA strains between different patients within the same unit and between different patients who dialyse in different units was demonstrated. In figure 5.7 and 5.8, CNS isolates that where indistinguishable and/or related were recovered from different patients that were treated by different modalities.

The management of patients with end stage renal disease occasionally requires the transfer of patients from peritoneal dialysis to haemodialysis over time and similarly hospital admission of haemodialysis patients from the dialysis unit. This may explain the proliferation of clusters of endemic virulent strains, which are horizontally transferred to other patients.

The transfer of related staphylococcal isolates between patients treated by CAPD (CNS 10.7%, *S. aureus* 15% and MRSA 100%) was higher than in patients treated by haemodialysis (CNS 6.6%, *S. aureus* 11.7% and MRSA 39.1%). This was an unexpected finding as patients who dialyse with CAPD are mainly self-treated in the community, whereas patients treated with haemodialysis attend a specific unit and have more contact with clinical staff. This suggests that the hospital CAPD unit may be more suited to promoting the horizontal transfer of staphylococcal species. In addition, the use of intraperitoneal antimicrobial therapy will have reduced systemic distribution therefore not affecting the concentration of staphylococcal skin flora. Conversely, the introduction of a haemodialysis catheter management protocol may have improved the infection control practice within the haemodialysis setting reducing the horizontal transfer of microorganisms.

The transfer of staphylococci between patients treated by different dialysis modalities was higher than that of microorganisms transferred between patients in the same treatment group. This suggests that the transfer of patients from one modality to another increases the risk of

horizontal transfer of staphylococci between patients and clinical environments and supports the theory that reservoirs of endemic staphylococcal strains are present in the dialysis units.

The treatment of infection in renal patients is protocol driven. The use of broad-spectrum antimicrobial regimens may place selective pressure on local bacterial strains encouraging the development of antimicrobial resistant clones and may select for the production of specific phenotypic characteristics, for example, extracellular polysaccharide slime. This will promote the proliferation of endemic strains within different clinical areas.

5.4.5 Infection over time

The majority of patients treated with CAPD undergo a general anaesthetic and invasive surgical procedure for the implantation of their dialysis catheter. The patient risk involved and the financial cost to the Trust for this procedure, dictate that the sustained function of the catheter is of paramount importance. CAPD-associated peritonitis is the major complication of this dialysis modality therefore patient and staff education on infection control practices is imperative. Equally, the use of appropriate antimicrobial therapy to treat CAPD-associated peritonitis is also important.

The patients represented in figures 5.12 and 5.13 had recurrent peritonitis with CNS. Both patients were treated with broad-spectrum antibiotics however, an indistinguishable CNS isolate was recovered from their peritoneal dialysis effluent each time they became symptomatic. This may suggests either a) the antimicrobial therapy was at sub-therapeutic concentration b) the catheter was colonised with CNS or c) the antimicrobial agents were unable to penetrate the biofilm.

Patients with CAPD-associated peritonitis may present with extremely dense dialysate effluent. When sensitivity testing is carried out in the microbiology laboratory a microbial suspension with a density of 0.5 McFarland is used. The sensitivities of the microorganisms to a panel of antimicrobial agents are determined based on this concentration. Within the peritoneum, the concentration of microorganisms may be far greater preventing the effective bactericidal activity of antimicrobial agents. In addition, the time between the administrations of subsequent doses may also affect the efficiency of antimicrobial therapy. Patients with CNS CAPD-associated peritonitis will be treated with intraperitoneal vancomycin on presentation at the unit (day 1), on day 4 and then again on day 7. If the concentration of

microorganisms in the peritoneum is high, then this may result in the proliferation of microorganisms between doses and therefore only a bacteriostatic action is achieved.

If infection is not successfully cleared from the peritoneum, colonisation of the internal lumen of the catheter will ensue. Once attachment of CNS to the catheter biomaterial has occurred, phenotypic characteristics such as polysaccharide slime production may be selected, further reducing the bactericidal activity of antimicrobial agents. This is discussed in more detail in chapter 6.

The patient represented in figures 5.14 experienced repeated skin infection with an indistinguishable *S. aureus* strain, at the exit site of their haemodialysis catheter. This suggests that the causative strain may have been part of the natural micro flora of the patient and thus was able to reinfect the exit site on completion of antimicrobial treatment. Conversely, the *S. aureus* strain may have been transferred from a specific dialysis nurse at dressing change. This could be confirmed by comparing the macrorestriction profiles of staphylococci recovered from the anterior nares of attending clinical staff and those recovered from the patient.

The migration of microorganisms from one site to another was illustrated in figures 5.16.1 to 5.16.3 by one patient who had 49 positive microbiology specimens over a ten-month period. The patient presented with a *S. aureus* infection in a suture line after a graft insertion. Two weeks later, an indistinguishable *S. aureus* strain was recovered from a blood culture. This suggests the transfer of the strain from the skin into the systemic circulation. A closely related *S. aureus* strain with the addition of one band was then recovered from the infected graft site. This may be the result of a point mutation in the chromosome creating an extra restriction site. The following month another closely related *S. aureus* strain was recovered from a blood culture. This strain had lost a restriction site and thus exhibited one band less. This strain was subsequently recovered from the haemodialysis catheter exit site and the tip of the catheter when it was explanted.

This patient became infected with an MRSA strain in June, which was repeatedly recovered from a number of different sites over the following months. In addition, other closely related MRSA strains were also repeatedly recovered during the same time. Interestingly, *S. aureus* isolates that had indistinguishable macrorestriction patterns to those reported as MRSA were recovered in two closely related strains. This may have resulted from anomalies in

antimicrobial sensitivity testing whereby the zone of inhibition to methicillin is measured incorrectly and the microorganism is reported as sensitive when it is intermediate or resistant. Without macrorestriction analysis, this would be overlooked, as no visual comparison of microorganisms would be made.

The persistence of *S. aureus* strains in infecting new sites and reinfecting other sites suggests that these strains are particularly virulent. The selective pressure placed on the patients micro flora by months of continuous antimicrobial therapy may have induced the expression of resistant genes over time. This is suggested by the change in predominance of *S. aureus* to MRSA.

The migration of *S. aureus* and MRSA strains to new sites are supported by figures 5.17 and 5.18. These data demonstrate the transfer of local catheter infection into the systemic circulation resulting in bacteraemia and catheter tip colonisation (figure 5.17).

In conclusion, patients with ESRD are a high-risk infection group as they rely on polyurethane catheters and are immunocompromised as a consequence of their disease state. These patients have frequent hospital admissions and this supports the acquisition of microorganisms and their horizontal transfer to other patients and the dialysis environment. This study did not identify any specific endemic strain but did highlight the transfer of related staphylococcal strains between anatomical sites, different patients and clinical environments. In addition, the need to review the antibiotic protocols was demonstrated. One amendment to the current protocol could be the calculation of the antibiotic dose on a mg per kg basis for each patient as opposed to a blanket regimen.

Standard microbiological identification of microorganisms is valuable, however in patients that are likely to get a high incidence of infection, it was demonstrated that diagnosis and treatment may be greatly aided by molecular methods such as PFGE. Identification of indistinguishable isolates from the clinical specimens of single patients over time by macrorestriction profiling, can predict the efficacy of antimicrobial treatment and enable both early intervention for catheter removal and microorganism specific treatment (Toldos, *et al.*, 1997); (Lina, *et al.*, 1995); (Breen and Karchmer, 1994). This reduces the need for the use of broad-spectrum agents and therefore delays the development of antimicrobial resistance.

CHAPTER 6: PHENOTYPIC CHARACTERISATION OF COAGULASE-NEGATIVE STAPHYLOCOCCI

6.1 INTRODUCTION

Coagulase negative staphylococci are reported to express few enzymes and toxins that have been clearly associated with pathogenicity. However, the majority of strains isolated from both peritoneal dialysate and blood culture reveal CNS to be the causative organism for peritonitis and bacteraemia in renal dialysis patients. The aim of this section of study was to investigate differences in the phenotypic characteristics of clinically significant CNS isolated from the blood and peritoneal dialysate cultures of renal patients who presented with infection and those obtained from the ante-cubital fossa of non-septic volunteers.

6.2 MATERIALS AND METHODS

One hundred and twenty five CNS isolates were assessed for their ability to produce *in vitro* virulence factors. Sixty isolates were recovered from blood or peritoneal dialysate cultures of renal patients and 65 were isolated from the anti-cubital fossa of non-septic volunteers. Each isolate was tested for the production of eight different phenotypic characteristics on at least two different days. The phenotypic characteristics tested were based on the work of Lang (2000).

For each assay, the bacterial isolate was cultivated in 20mL of tryptone soya broth (TSB) (except for the haemolysin assays for which the bacteria were cultivated in 20mL brain heart infusion broth (BHI) at 37°C in air on an orbital shaker (200 rpm) for 18 to 20h. Each culture was inoculated onto a blood agar (BA) plate as a purity control measure.

6.2.1 Non-specific proteinase activity

The large bore end of a sterile glass Pasteur pipette was used to cut 8 equidistant wells into a skim milk agar plate [1% (w/v) agar no. 1 (LabM, UK), 1% (w/v) skim milk]. For each bacterial strain, two wells were inoculated. Into each well 75μL of culture supernatant, harvested from 1mL of the bacterial culture centrifuged at 13,000 rpm for 5 minutes, was dispensed. The agar plates were incubated at 37°C in air for 18 to 20h. A clearing zone around the wells indicated proteinase activity. Supernatant from the proteinase-positive bone

fracture-associated *S. epidermidis* (NU1) and proteinase-negative endocarditis-associated *S. lugdunensis* (LON2) were used as controls.

6.2.2 Elastase activity.

6.2.2.1 Qualitative plate method.

Bacterial cultures, diluted 1:100 with fresh broth, were streaked onto an elastin-agar plate [BHI agar supplemented with 0.3% bovine neck ligament elastin (Sigma)] (Janda, 1986). The inoculated plates were incubated at 37°C in air for 2 days before transfer to room temperature for a further 19 days. The cultures were observed on days 3, 5, 7, 11, 14, and 21 for elastin degradation. *P. aeruginosa* (PAO1) was included as a positive control and elastase negative *S. lugdunensis* LON2 as a negative control.

6.2.2.2 Quantitative elastin-Congo-red method.

The reaction mixture consisted of 5mg of elastin-Congo-red (Sigma), 1mL of Tris-malate buffer [0.1M Tris-malate buffer (pH 7.0) supplemented with 1mM $CaCl_2$ (Aldrich Chemical Co. Ltd.)] and 0.5mL of culture supernatant, harvested at 13,000 rpm for 15 minutes, in a disposable 5mL bijou bottle. The reaction bijou bottles were continuously rolled for 20h at 37°C until the reaction was terminated by the addition of 1mL of sodium phosphate buffer [0.7M Na_2HP0_4/NaH_2P0_4 (pH 6.0)] (Bjorn *et al.*, 1979). Particulate elastin-Congo-red was removed by centrifugation at 13,000 rpm for 8 minutes. An Anthos 2001 (Anthos Labtec Instruments) microtitre plate reader, initially referenced with air, was used to measure the optical density (492nm) of 0.2mL of supernatant dispensed in duplicate into a flat-bottomed microtitre tray (Immulon 2, Dynatech, Germany). Elastase positive *S. epidermidis* NU1 was included as the positive control and *S. epidermidis* (NCTC 11047) as a negative control.

6.2.3 Lipase/esterase activity

6.2.3.1 Non-specific lipase/esterase activity

Using a sterile glass Pasteur pipette wells were cut into glycerol tributyrate agar plates [1.5% (w/v) agar no. 1 (Lab M) and 1% (v/v) glycerol tributyrate] as previously outlined (section 4.2.1) (Molnàr, *et al.*, 1994). Into each well 75μL of culture supernatant, harvested from 1mL

of the bacterial culture centrifuged at 13,000 rpm for 5 minutes, was dispensed into duplicate wells. The plates were incubated in air at room temperature for 18 to 20h. A clearing zone around the well indicated non-specific lipase/esterase activity. Supernatant from cultures of *S. epidermidis* NU1 and *S. lugdunensis* LON2 were included as positive and negative controls respectively.

6.2.3.2 Lipase activity

The agar constituents were mixed and autoclaved [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.3%g/L agar no. 1 (Lab M)]. Once the agar had cooled to 60°C, 2.5% (v/v) dry heat sterilised olive oil (J Sainsbury Plc., UK) and 0.001% (w/v) filter sterilised aqueous solution of rhodamine B were added to the autoclaved agar whilst stirring vigorously and the plates poured immediately (Kouker and Jaeger, 1987).

Bacterial cultures, diluted 1:100 using fresh TSB, were streaked onto an olive oil agar plate and incubated at 37°C in air for 18 to 20h. Strains that were found to be negative at 18 to 20h were incubated for a further 18 to 20h. Lipase activity was indicated by colonies that fluoresced pink when examined under UV light (254nm). Lipase producing *S. epidermidis* NU1 was included as a positive control and *S. lugdunensis* LON2 as a negative control.

6.2.3.3 Esterase assay

Bacterial cultures, diluted 1:100 with fresh TSB, was streaked onto a Tween-80 agar plate [tryptone soya agar (TSA) supplemented with 1% (v/v) Tween-80 (George T Gurr Ltd.), 0.5% NaCl, 10μg/mL $CaCl_2$] (Barrow and Feltham, 1991). The inoculated plates were incubated at 37°C in air for 2 days followed by room temperature incubation for a further 12 days. On days 1, 2, 3, 5, 7, 11 and 14 the area around the bacterial streak was observed for esterase activity indicated by a halo of precipitated calcium chloride. The positive control was *S. epidermidis* NU1 and the negative control *S. lugdunensis* LON2.

6.2.4 Extracellular polysaccharide slime layer.

6.2.4.1 Congo-red agar method

The bacterial culture, diluted 1:100 with sterile TSB, was spread onto a quarter of a Congo-red agar plate [BHI broth supplemented with 5% (w/v) sucrose, 1% (w/v) agar no.1, 0.8mg/mL Congo-red (Hopkin and Williams Ltd., UK)] (Freeman, *et al.*, 1989).
The basal medium was autoclaved and cooled to 55°C prior to the addition of the Congo-red (concentrated aqueous solution autoclaved separately). The plates were incubated at 37°C in air for 18 to 20h and then transferred to room temperature for a further 24h. Extracellular polysaccharide slime production was indicated by dry black discolouration of the agar. Slime producing *S. epidermidis* (9865) and nonslime-producing *S. epidermidis* (023) strain were included as control isolates.

6.2.4.2 Agglutination by concanavalin A

Bacterial cells were harvested from 1mL of an 18 to 20h TSB broth culture by centrifugation at 10,000 rpm for 4 minutes. The pellet was washed by re-suspending the cells in 1mL of phosphate buffered saline (PBS) (Oxoid) and gently vortex mixing to loosen the pellet without destroying the polysaccharide layer, if present. The cells were once again pelleted and re-suspended in 0.5mL of PBS. An equal volume of concanavalin A [1% (w/v) concanavalin A (Sigma) prepared in PBS] and bacterial suspension (20µl) were mixed on a glass slide. The slide was gently rotated for 1 minute or until agglutination occurred (Ludwicka, *et al.*, 1984). Concanavalin A-PBS solution was included as a negative control, whilst a bacterial cell-PBS suspension indicated the lack of auto-agglutination of the bacterial cells.

6.2.5 Adherence of coagulase-negative staphylococci to polystyrene

An 18 to 20h broth culture was diluted 1:100 with fresh TSB and dispensed into eight wells of a 96 well flat-bottomed polystyrene microtitre plate, 0.2mL in each well. Each microtitre plate contained 10 test strains and 1 control strain (*S. epidermidis* 023). One column of wells was inoculated with sterile TSB as a negative control. The plate was sealed using a microtitre plate sealing strip (Titertek plate sealers) and incubated at 37°C for 21h.

With the plate held at a slight angle and the pipette tip placed against the side of the well the culture was completely aspirated. This technique was maintained throughout the assay to minimise disruption of the biofilm. The plate was washed three times with 0.2mL of PBS to

remove non-adherent cells. To fix the biofilm, 0.2mL of Bouin's solution [0.9% (w/v) picric acid, 5% (v/v) acetic acid, 9% (v/v) formaldehyde] was dispensed into each well and the plate left at room temperature for 30 minutes in a fume hood (Wilcox, 1994). The Bouin's solution was aspirated from the wells and then completely removed by gentle washing with running tap water. The biofilm was stained for 10 minutes at room temperature with 0.2mL of filtered crystal violet; 20mL of crystal violet solution [10% (w/v) crystal violet in 95% (v/v) ethanol] combined with 80mL of 1% (w/v) aqueous solution of ammonium oxalate (Barrow and Feltham, 1991). Excess stain was removed by aspiration with a pipette followed by washing under running tap water until the water ran clear. The plate was tapped dry on an absorbent material. Finally, the crystal violet was leached from the stained cells, by the addition of 0.2mL of 50% (v/v) aqueous ethanol, to create a solution of even optical density and thus improve the accuracy of the spectrophotometric reading. The optical density of each well was read at 595nm, following a blank reading with air, using an Anthos 2001 microtitre plate reader (Christensen, *et al.,* 1985).

6.2.6 DNase activity

A diluted bacterial culture, 1:100 in TSB, was inoculated, by a nichrome bacteriological loop (Medical Wire and Equipment Ltd, LTK) onto a surface dried DNase agar plate [3.9% (w/v) DNase agar (Oxoid)]. The plates were incubated at 37°C in air for 18 to 20h. Subsequently the plate was flooded with 1N HCl and left to stand for 2 min. A clearing zone around the bacterial colonies indicated DNase activity. A *S. aureus* strain was included as a positive control and *S. epidermidis* NCTC 11047 as a negative control.

6.2.7 Detection of haemolytic extracellular toxins

6.2.7.1 Haemolysis of horse erythrocytes

Freshly washed defibrinated horse red blood cells (RBC) were prepared by the addition of 10mL of PBS to 0.4mL of horse RBC in a sterile centrifuge tube. The RBC suspension was gently centrifuged at 2,000 rpm for 4 min (Beckman J2 centrifuge, JA-25.50) to loosely pellet the cells without causing lysis. Carefully, 10mL of the supernatant was removed, the cells gently re-suspended in the remaining supernatant and 10mL of fresh PBS added. The suspension was homogenised by brief vortex mixing before the cells were harvested by centrifugation at 2,000 rpm for 4 min. The wash was repeated as before and then a 2% RBC

suspension was achieved by re-suspending the cells in 20mL of PBS. Culture supernatant was harvested from 1mL of an 18 to 20h bacterial culture in BHI broth by centrifugation at 13,000 rpm for 5 minutes. Using a flat-bottomed 96 well polystyrene microtitre tray 0.2mL of the supernatant was dispensed (in duplicate) into the first well of the first row. The second specimen was dispensed into the first well of the second row and so forth for the first column. Into the wells of column 2 to column 6 inclusive, 0.1mL of PBS was dispensed using a multi-channel pipette. A 2-fold serial dilution of the supernatant was made for all the wells across the plate to column 6. The microtitre tray plate was incubated at 37°C for 15 minutes. Finally, 0.1mL of an evenly suspended 2% RBC suspension was dispensed into each well and the plate incubated at 37°C in air for 1h followed by 1h at 4°C. Haemolysis was recorded after 1h at 37°C and 1h at 4°C on the Anthos 2001 microtitre plate reader. Optical density was measured at 570nm (OD_{570}). CAPD-associated peritonitis strains *S. sciuri* (CAPD 17) and *S. caprae* (ROH 7) were included as positive control strains and *S. simulans* (CAPD 24) as a negative control.

To determine the OD_{570} of haemolysed RBC, a 1:100 dilution of horse erythrocytes in PBS was dispensed (0.2mL into each well) into 16 wells of a flat-bottomed polystyrene microtitre tray. With distilled water as the diluent a 1:100 dilution of horse RBC was prepared, similarly dispensed and the OD_{570} of the two sets of RBC ascertained. The average OD_{570} of the haemolysed RBC (diluted in distilled water) was compared to the non-haemolysed cells (diluted in PBS) OD_{570}.

6.2.8 Urease activity

6.2.8.1 Polyacrylamide gel electrophoresis (PAGE).

Production of extracellular urease was determined by native polyacrylamide gel electrophoresis (PAGE) using a modified Gatermann, and Marre method * (1989). A 10% (w/v) separating gel (80x70x1mm) (Table 6.1) was cast using the PAGE gel casting apparatus (BioRad).

Table 6.1 Composition of the native PAGE separating and stacking gels, sample buffer and electrode buffer used to detect urease activity in culture supernatant.

	Separating gel 11% (w/v)	Stacking gel 5% (w/v)	Sample Buffer	Electrode Buffer (pH 8.0)
Acrylamide stock I	5mL	-	-	-
Acrylamide stock II	-	2.5mL	-	-
1.5M Tris-HCl (pH 8.8)	6mL	-	-	-
0.5M Tris-HCl (pH 6.8)	-	3.75mL	2.5mL	-
Distilled water	8mL	8mL	5mL	1L
TEMED (N,N,N'N'-tetramethyl-ethylenediamine)	50μL	40μL	-	-
10% (w/v) ammonium persulphate – freshly prepared	70μL	50μL	-	-
Glycerol	-	-	2.5mL	-
5% (w/v) bromophenol blue	-	-	0.2mL	-
Tris	-	-	-	3g
Glycine	-	-	-	14.4g

Acrylamide stock I – 44% (w/v) Acrylamide and 0.8% (w/v) Bis(N,N^1,-methylene-bis-acrylamide) (Severn Biotech Ltd, UK).

Acrylamide stock II – 30% (w/v) Acrylamide and 0.8% (w/v) Bis(N,N^1,-methylene-bis-acrylamide).

A layer of distilled water was poured onto the mobile gel layer to ensure a sharp interface between the separating and the stacking gels. Following polymerisation of the separating gel the water layer was poured off and residual water removed with a strip of blotting paper. Onto the separating gel the stacking gel was cast with a 15-lane comb inserted to form the wells. The gel, still within the glass casting plates, was transferred to vertical support apparatus and loaded into the electrophoresis tank together with a Tris-Glycine electrode buffer (pH 8.0). The comb was removed from the stacking gel. The sample consisted of equal volumes of sample buffer and culture supernatant harvested at 13,000 rpm for 8 minutes from an 18 to 20h bacterial culture in TSB.

A glass microtitre syringe (Hamilton, Switzerland), cleaned with distilled water between each specimen, was used to load 15μL of the prepared sample into the appropriate well. Native PAGE of culture supernatant was electrophoresed at 200V using a Mini-PROTEAN ® II Cell (BioRad).

A Jack Bean urease standard (Sigma), re-suspended in PBS (1mg/mL), which was a mixture of hexamer (molecular weight (M_w) 545KDa) and trimer (M_w 272 KDa) was run in one lane to indicate the approximate size of any CNS urease detected. Urease producing *S. epidermidis* NU1 and non-urease producing *S. epidermidis* NCTC 11047 were included with every gel as positive and negative controls respectively.

After electrophoresis, the gel was washed three times in distilled water over a 1h period with agitation to remove the electrode buffer. The gel was transferred to a 2% (w/v) urea solution (Fisons, UK), pre-warmed to 37°C, for 30 min with constant agitation. Subsequently the gel was placed in a 0.002% (w/v) aqueous solution of cresol red (BDH) until red bands indicating urease activity became visible (Mobley, *et al.,* 1987).

6.2.8.2 Urease agar

Urease agar slopes (Oxoid) were inoculated using a nichrome bacteriological loop (Christensen, 1946). The slopes were incubated at 37°C for 24h and read in accordance with the manufactures instructions. The hydrolysis of urea to form ammonia was indicated by pink colouration of the media.

6.2.9 Statistical analysis of potential virulence factors

The significance of the differences in the observed frequencies of each potential virulence factor for CNS associated infection and the control group was investigated by the application of Chi square test and two-sided p value using the InStat (GraphPad Software Inc.) computer programme (Kanji, 1993).

6.3 RESULTS

6.3.1 Proteinase activity

The presence of proteinases in the supernatant of CNS strains from renal patients with dialysis catheter associated sepsis and non-septic volunteers as investigated by the skimmed milk agar plate method. A clearing zone around the well indicated the activity of non-specific proteinases (figure 6.1).

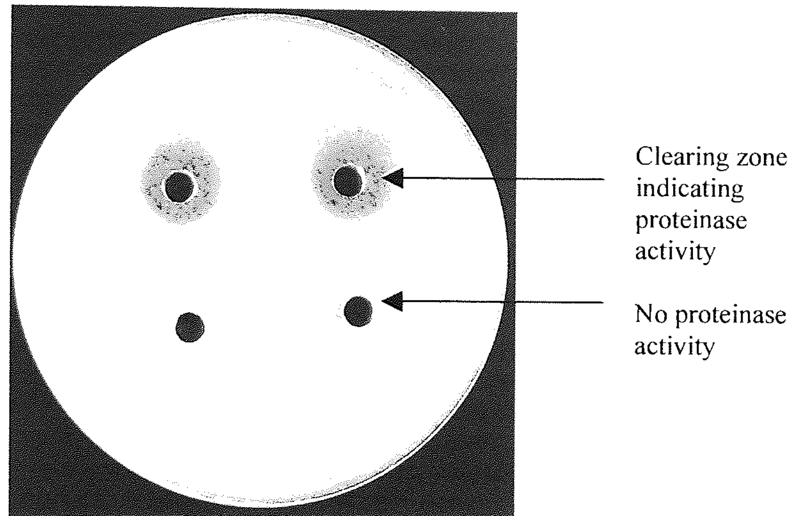


Figure 6.1 Skimmed milk agar method for detection of proteinase activity.

Table 6.2 The number of CNS strains expressing non-specific protease activity.

Source of isolates	Number of CNS strains expressing protease	
	Protease-positive	Protease-negative
CAPD-associated peritonitis (n=28)	15	13
CRI (n=32)	15	17
Non-septic volunteers (n=65)	15	50

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status $p = 0.0064$).

The presence of elastases was determined by their ability to degrade elastin suspended in agar. A clearing zone around the bacterial colony indicated elastase activity (figure 6.2).

6.3.2 Elastase activity

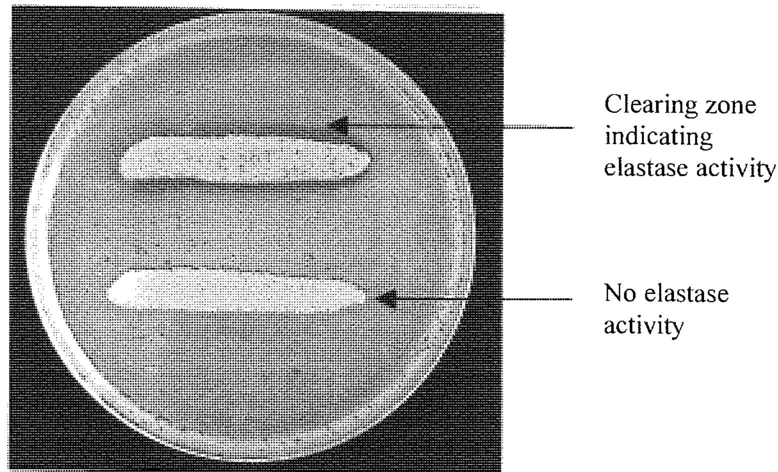


Figure 6.2 Elastin-agar plate method for detection of elastase activity.

Table 6.3 The number of CNS strains expressing elastase activity.

Source of isolates	Number of CNS strains expressing elastase	
	Elastase-positive	Elastase-negative
CAPD-associated peritonitis (n=28)	7	11
CRI (n=32)	10	22
Non-septic volunteers (n=65)	0	60

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status ($p<0.0001$). As one value entered into this test is <1 the significance of the result was not validated.

An elastase positive and negative value range was determined by assessment of the numerous results obtained for the positive and negative CNS control strains generating value ranges of 0.081 to 0.321 (n=13) and 0.042 to 0.083 (n=13) respectively. Thus an absorbance at 492nm of ≥0.084 was considered positive, ≤0.080 negative and 0.081 to 0.083 intermediate (+/-) for elastase activity.

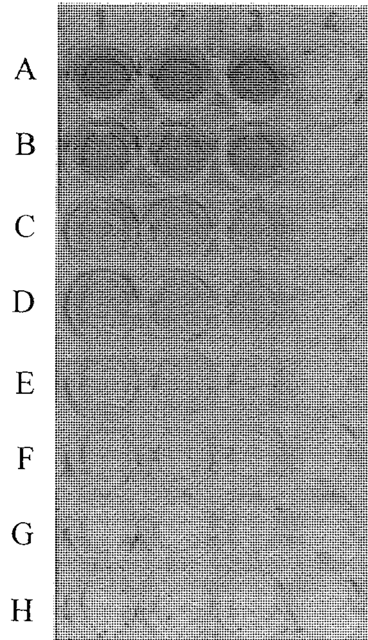


Figure 6.3 Elastin-Congo-red method for the detection of elastase activity.

Elastase activity was quantified by the release of soluble Congo-red from the insoluble elastin-Congo-red complex. Each row contains 0.2mL of reaction mixture recovered from the assay of a different bacterial strain. The strains in rows A-H exhibited decreasing levels of elastase activity.

Table 6.4 Number of CNS strains expressing elastase activity by the elastin Congo-red method.

Source of isolates	Number of CNS strains expressing elastase	
	Elastase-positive	Elastase-negative
CAPD-associated peritonitis (n=28)	11	7
CRI (n=32)	19	13
Non-septic volunteers (n=65)	7	58

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status ($p<0.0001$).
There was no significant association when comparing the three methods used for the detection of protease $p=0.0528$).

6.3.3 Lipase/esterase activity

6.3.3.1 Non-specific lipase/esterase activity by glycerol tributyrate agar plate method

Non-specific lipase/esterase activity was assessed by glycerol tributyrate agar plate. A clearing zone around the wells containing culture supernatant indicated production of lipase/esterase (figure 6.4).

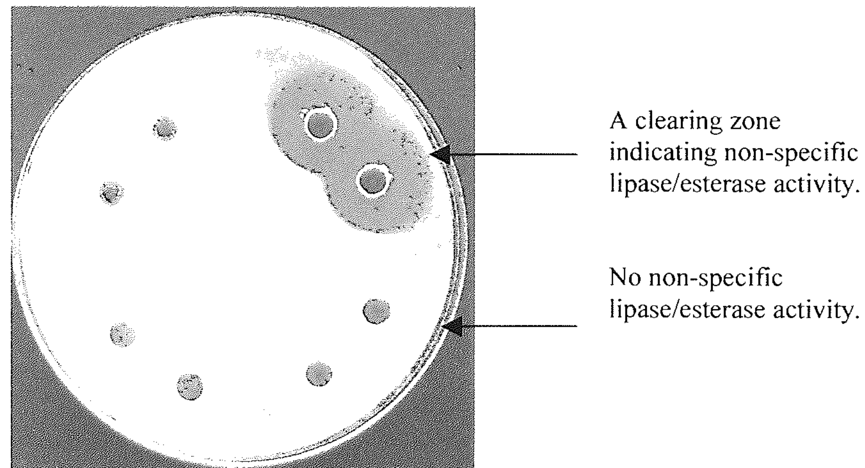


Figure 6.4 Glycerol tributyrate agar plate method.

Table 6.5 The number of CNS strains expressing non-specific lipase and esterase activity.

Source of isolates	Number of CNS strains expressing non-specific lipase/esterase.	
	Lipase/esterase-positive	Lipase/esterase-negative
CAPD-associated peritonitis (n=28)	24	4
CRI (n=32)	28	4
Non-septic volunteers (n=65)	35	30

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status p=0.0004).

6.3.3.2 Lipase activity

The hydrolysis of lipid (olive oil) by lipase activity in the presence of rhodamine B produced a fluorescent product (Kouker and Jaeger, 1987). On exposure to UV light lipase-producing staphylococci fluoresced indicated by bright pink colonies. Lipase-negative strains produced white/pink colonies under UV inspection (figure 6.5).

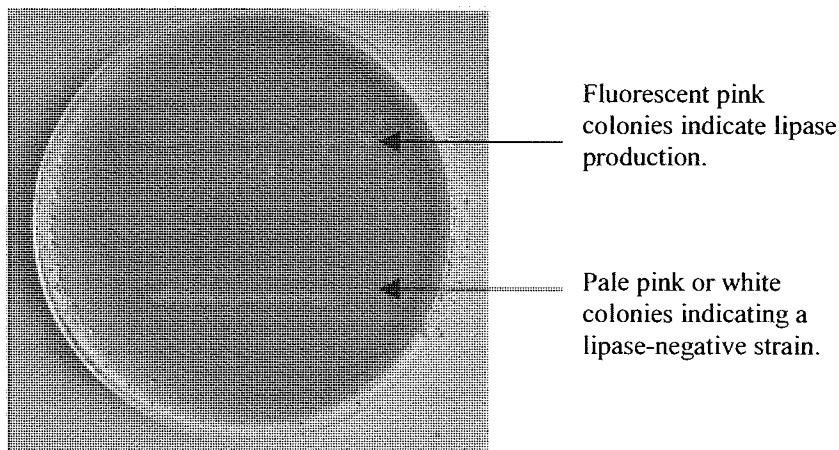


Figure 6.5 Lipase activity by olive oil agar plate method.

Although these isolates are not shown in the presence of UV light, the lipase-positive colonies show a much brighter pink colouration than the lipase-negative colonies.

Table 6.6 The number of CNS strains expressing lipase activity.

Source of isolates	Number of CNS strains expressing lipase	
	Lipase-positive	Lipase-negative
CAPD-associated peritonitis (n=28)	26	2
CRI (n=32)	32	0
Non-septic volunteers (n=65)	40	25

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status ($p<0.0001$). As one value entered into this test is <1 the significance of the result was not validated.

6.3.3.3 Tween agar

The hydrolysis of Tween-80 by staphylococcal esterase resulted in the precipitation of calcium chloride from the agar. This was indicated by a white halo of precipitation around the bacterial colonies of the esterase producing strains (figure 6.6).

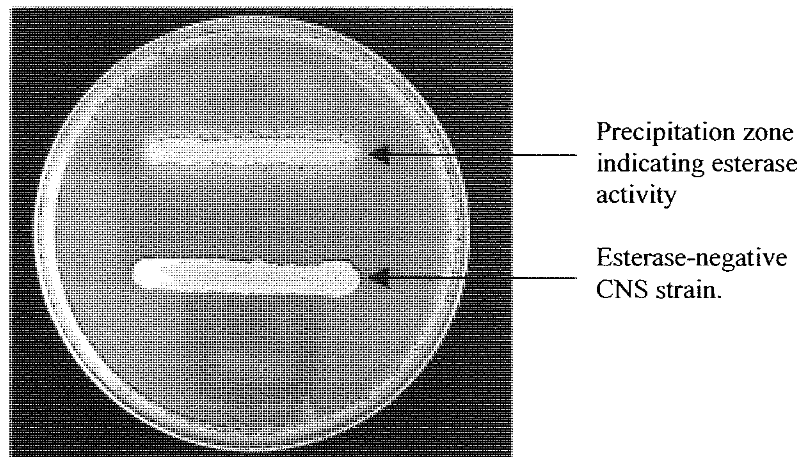


Figure 6.6 Esterase activity by Tween-80 agar plate method.
Calcium chloride precipitation from the Tween-80 agar is indicated by a white halo around the bacterial colonies.

Table 6.7 The number of CNS strains expressing esterase activity.

Source of isolates	Number of CNS strains expressing esterase	
	Esterase-positive	Esterase-negative
CAPD-associated peritonitis (n=28)	24	4
CRI (n=32)	32	0
Non-septic volunteers (n=65)	47	18

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status p=0.0030). As one value entered into this test is <1 the significance of the result was not validated.

There was no significant association when comparing the three methods used for the detection of lipase and esterase activity (p=0.9347).

6.3.4 Extracellular polysaccharide layer

Extracellular polysaccharide layer (slime) producing staphylococcal colonies cultured on congo-red agar plated developed a characteristic dry black appearance compared to non-slime producing CNS colonies which had a moist red/brown presentation (figure 6.7) (Freeman, *et al.*, 1989).

6.3.4.1 Congo-red agar

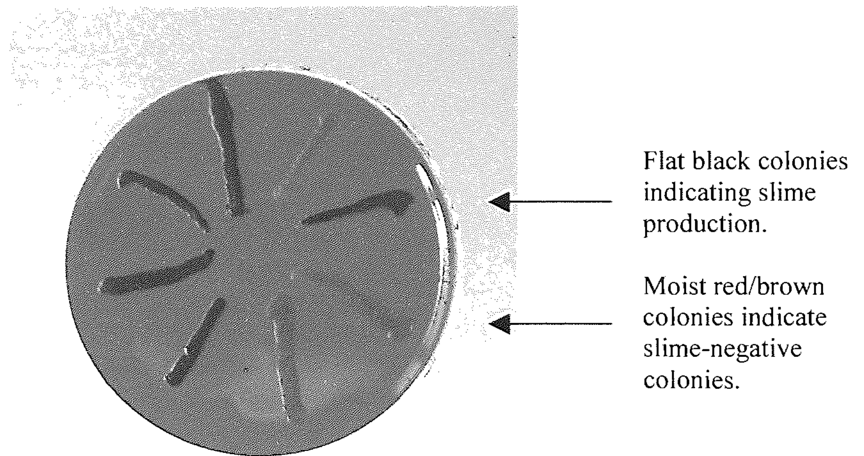


Figure 6.7 Comparison of slime-positive and slime negative CNS strains cultured on Congo-red agar.

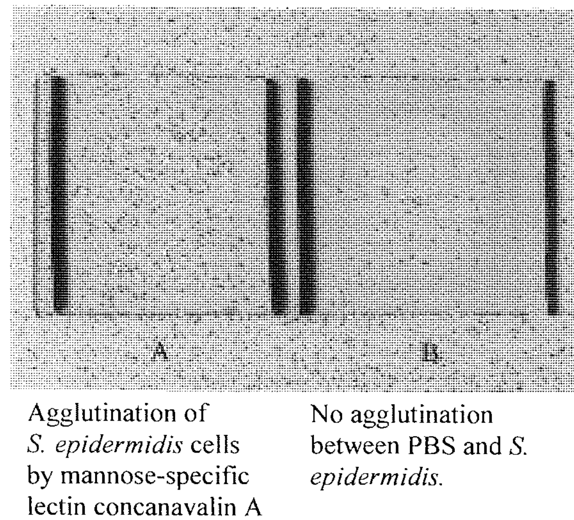
Table 6.8 The number of CNS strains expressing extracellular polysaccharide slime.

Source of isolates	Number of CNS strains expressing extracellular polysaccharide slime	
	Slime-positive	Slime-negative
CAPD-associated peritonitis (n=28)	14	14
CRI (n=32)	16	16
Non-septic volunteers (n=65)	51	14

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status (p=0.0039).

6.3.4.2 Concanavalin A

Agglutination of the lectin concanavalin A with a CNS suspension was undertaken to further evaluate production of extracellular slime (Ludwicka, *et al.*, 1984). Agglutination was indicated by the formation of aggregates within the suspension mixture. CNS strains that did not show signs of agglutination after 1 min were deemed negative.



Agglutination of *S. epidermidis* cells by mannose-specific lectin concanavalin A	No agglutination between PBS and *S. epidermidis*.

Figure 6.8 Extracellular polysaccharide slime detection by agglutination with concanavalin A

Table 6.9 The number of CNS strains that expressed aggregates.

Source of isolates	Number of CNS strains expressing aggregates	
	Aggregate-positive	Aggregate-negative
CAPD-associated peritonitis (n=28)	6	22
CRI (n=32)	10	22
Non-septic volunteers (n=65)	33	32

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status (p=0.0165).

There was no significant association between the methods used to detect extracellular polysaccharide slime (p=0.7403).

6.3.5 Adherence to polystyrene

The ability of the CNS strains to adhere to polystyrene and form an adherent biofilm was investigated by microtitre plate method.

The results of each test batch were only accepted if the absorbance of the control strain fell within a defined range (absorbance at 595nm of 0.085 to 0.125). This range was derived from the majority of twenty-nine repeated assay results obtained for the control strain. For each isolate eight absorbance values were obtained during the assay. The highest and/or lowest values were not included if another value did not fall within 15% range to exclude anomalous

results. The mean value of the accepted absorbance results was calculated and the final recorded absorbance (mean values of the repeated assay values) for each strain determined.

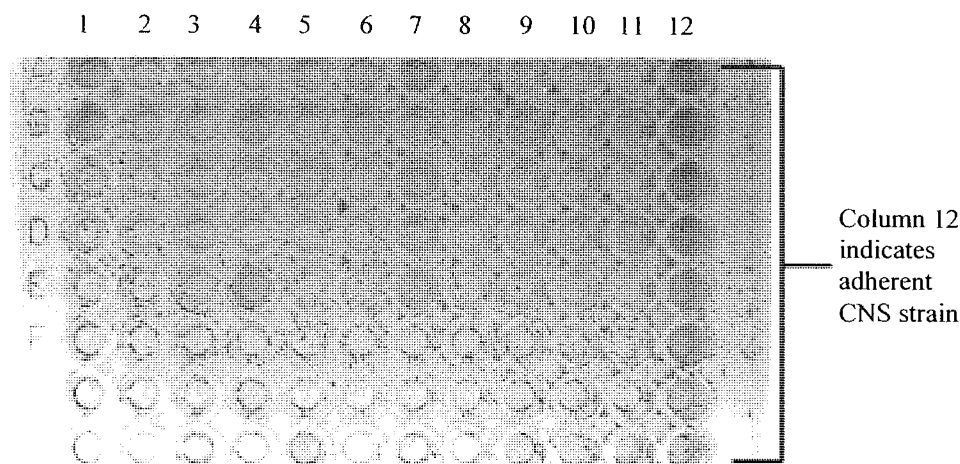


Figure 6.9 Adherence to polystyrene determined by microtitre plate method. Column 12 indicates adherent strain of CNS.

Table 6.10 The number of CNS strains expressing adhesins.

Source of isolates	Number of CNS strains expressing adhesins	
	Adhesin-positive	Adhesin-negative
CAPD-associated peritonitis (n=28)	27	1
CRI (n=32)	32	0
Non-septic volunteers (n=65)	54	11

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status (p=0.0137). As one value entered into this test is <1 the significance of the result was not validated.

6.3.6 DNase activity

DNase activity was assessed by the DNase agar method. The addition of 1M HCl to the surface of a DNase plate precipitated polymerised DNA, resulting in the plate becoming opaque. However, in areas of DNase activity the hydrolysed DNA was not precipitated resulting in a clearing zone around the bacterial colonies (figure 6.11).

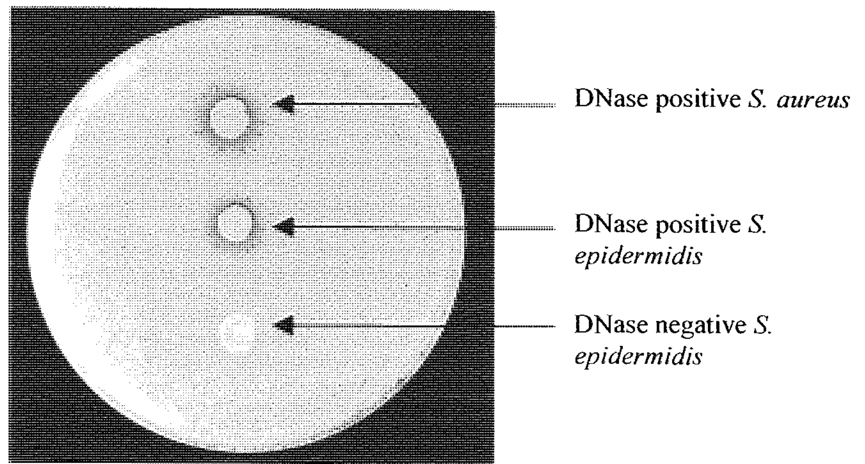


Figure 6.10 DNase activity indicated by a clearing zone around the bacterial colony.

Table 6.11 The number of CNS strains expressing DNase activity.

Source of isolates	Number of CNS strains expressing DNase	
	DNase-positive	DNase-negative
CAPD-associated peritonitis (n=28)	8	20
CRI (n=32)	5	27
Non-septic volunteers (n=65)	3	62

Analysis by means of a two-tailed Chi square test for independence indicated significant association between observed frequencies and the infection status p=0.0056).

6.3.7 Cytolytic toxins

The average absorbance at 570nm of haemolysed RBC (diluted in distilled water) was 0.062 in contrast to a mean of 0.732 for the non-haemolysed cells (diluted in PBS). Therefore, a sample with an absorbance of <0.1 was recorded as haemolysed whilst a value of ≥0.100 indicated no detectable haemolytic activity (figure 6.12). The reciprocal of the highest supernatant dilution with an absorbance of <0.100 was recorded as the haemolytic titre.

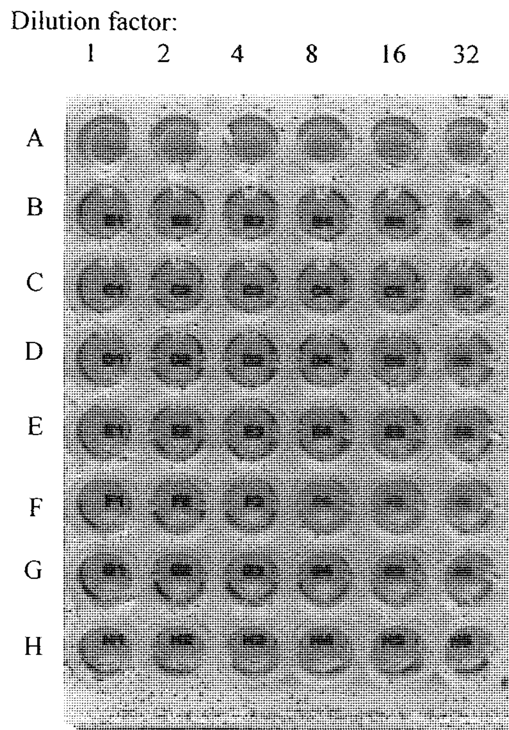


Figure 6.11 Haemolysis of horse erythrocytes by culture supernatant assessed by a microtitre plate method.

Rows A-G each contain the culture supernatant, serially diluted across the plate from left to right, of seven CNS. Row A has expressed no observable haemolysis. Row H contains haemolysed RBC, diluted 1:100 with distilled water. A numbered card has been placed beneath the microtitre plate to aid the detection of haemolysis in the illustrated figure (visible letters indicate haemolysis).

Table 6.12 The number of CNS strains expressing cytolytic toxins.

Source of isolates	Number of CNS strains expressing haemolysin	
	Haemolysin-positive	Haemolysin-negative
CAPD-associated peritonitis (n=28)	3	25
CRI (n=32)	2	30
Non-septic volunteers (n=65)	13	52

Analysis by means of a two-tailed Chi square test for independence indicated no significant association between observed frequencies and the infection status p=0.1583).

6.3.8 Urease activity

6.3.8.1 Native polyacrylamide gel electrophoresis (PAGE).

Initially a PAGE method was used to determine urease activity in the CNS strains recovered from patients with CAPD-associated peritonitis and those with CRI. This was later replaced by the urease agar slope method.

Native-PAGE separated the culture supernatant proteins whilst retaining enzymatic activity. Urea was hydrolysed to ammonia by the urease enzyme within the acrylamide, leading to a localised pH increase. After 10 min in cresol red (a pH indicator solution) the gel developed red bands on a pale yellow background. These bands indicated areas of increased pH and therefore the location of urease activity (figure 6.13). In addition to the urease negative and positive staphylococcal controls, a Jack Bean urease was incorporated into three random gels to confirm the specificity of the test and to indicate the general size of the staphylococcal enzyme.

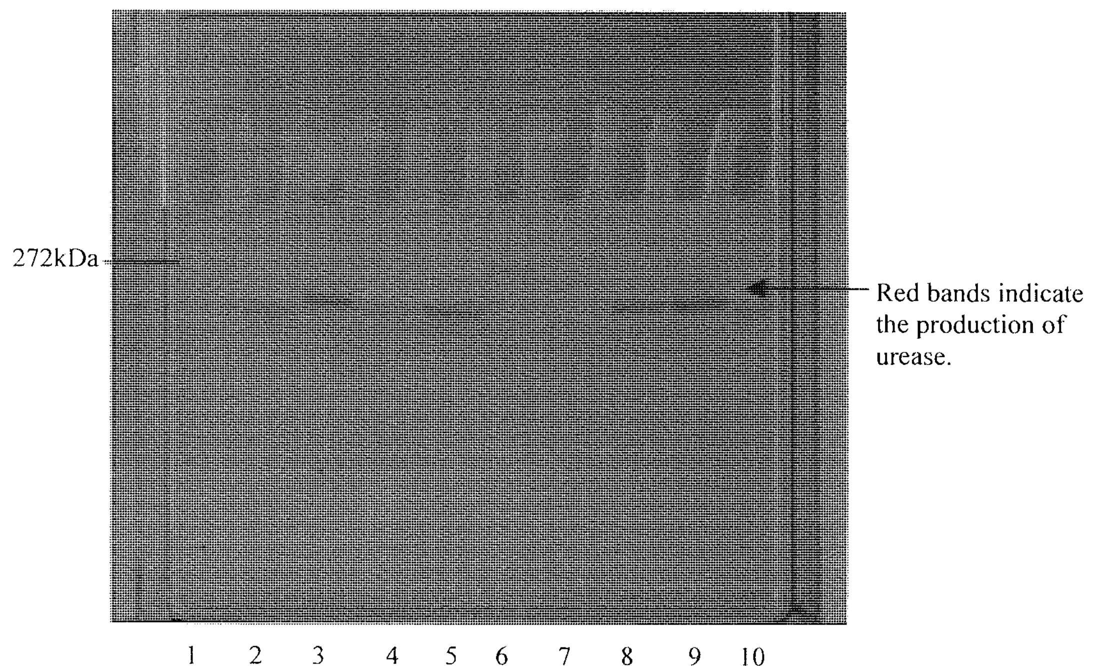


Figure 6.12 The formation of ammonia from urea indicated by red bands within the PAGE gel.

Lane 1 contains supernatant from a urease-negative CNS, lanes 2, 3, 4, 7, 8, 9, 10 contain supernatant from urease-positive CNS and lane 6 contains Jack Bean urease. The dominant band of lane 6 is 272-kDa.

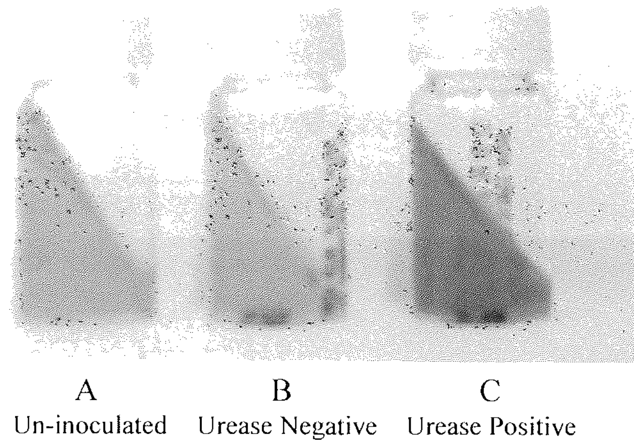
Table 6.13 The number of CNS strains expressing urease activity by PAGE method.

Source of isolates	Number of CNS strains expressing urease	
	Urease-positive	Urease-negative
CAPD-associated peritonitis (n=28)	15	13
CRI (n=32)	23	9

Analysis by means of a two-tailed Chi square test for independence indicated no significant association between observed frequencies and the infection status (p=0.2340).

6.3.8.2 Urease agar

Urease activity was also investigated using the urease agar slope method (Christensen, 1946).



A Un-inoculated B Urease Negative C Urease Positive

Figure 6.13 Agar slope method for the detection of urease activity.

Table 6.14 The number of CNS strains expressing urease activity by the agar slope method.

Source of isolates	Number of CNS strains expressing urease	
	Urease-positive	Urease-negative
CAPD-associated peritonitis (n=28)	21	7
CRI (n=32)	25	7
Non-septic volunteers (n=65)	47	18

Analysis by means of a two-tailed Chi square test for independence indicated no significant association between observed frequencies and the infection status (p=0.8237).

There was no significant association between the methods for urease detection when comparing the CNS strains recovered from patients with CAPD-associated peritonitis and CRI (p=0.5690).

The production of certain phenotypic characteristics by CNS appeared more likely to be associated with infection (table 6.15). This may suggest a way of predicting infection in renal patients.

Table 6.15 Probability of developing infection based on phenotypic characteristic produced by CNS strains.

Category*	**Phenotypic characteristic produced.**	**Significance (p value)****
High	Elastase; Non-specific lipase and esterase; Lipase	<0.0001
High-Moderate	Extracellular polysaccharide slime; Esterase; DNase; Non-specific protease.	<0.0065
Moderate-Low	Adhesin; Extracellular polysaccharide slime.	<0.0166
Low	Urease; Cytolytic toxins	>0.1000

* Category refers to the propensity to cause infection. ** Significance values were calculated comparing patients with infection versus healthy non-septic volunteers.

6.4 DISCUSSION

6.4.1 Proteinase activity

The expression of proteinases by CNS strains in this study was significantly associated with renal patients who had CAPD-associated peritonitis and those with CRI. CNS isolates recovered from the ante-cubital fossa of healthy volunteers were significantly less likely to express specific and non-specific proteinases ($p<0.0001$ and $p=0.0064$ respectively). Proteinase is implicated in host cell death leading to destruction of cellular complexes and the degradation of both serum proteins and immunoglobulins. Expression of these characteristics may assist the development of infection by reducing opsonization and permitting evasion of the immune response (Travis, *et al.,* 1995); (Projan and Novick, 1996).

Elastase expression was also significantly associated with strains of CNS isolated from renal patients with CAPD-associated peritonitis and those with CRI. Elastase produced by CNS species, particularly *S. epidermidis*, is known to cleave immunoglobulins (Sloot, *et al.,* 1992) thereby depressing host defence mechanisms through inactivation of immunoglobulins. Elastase can also modify the fibinogen-fibronectin layer covering the surface of plastic prosthetic devices, which promotes microbial colonisation of plastic (Sloot, *et al.,* 1992). In this study, a significant number of *S. epidermidis* species were shown to express elastase by both the agar plate and elastin-Congo-red methods in comparison with non-septic volunteers ($p<0.0001$). This suggests that the production of elastase is a prerequisite for the development of infection in renal patients with indwelling medical devises.

6.4.2 Lipase and esterase activity

The role of lipase/esterase in staphylococcal pathogenicity has been identified in the formation of staphylococcal abscess. During the development of an abscess, the host imports bactericidal lipids in particular fatty acids. In response to this, the staphylococci secrete a fatty acid modifying enzyme (FAME), which converts the bactericidal fatty acids into non-bactericidal fatty acid esters (Mortensen, *et al.,* 1992). The host also imports FAME-inhibiting glycerides. A staphylococcal lipase removes these inhibitory glycerides, allowing FAME to resume activity and the microorganism to evade the host's immune response (Long, *et al.,* 1992). Further to facilitating virulence within an abscess, staphylococcal lipase has been implicated in the damage of human granulocytes structure and function (Rollof, *et al.,* 1988).

Lipase production by *Staphylococci* has been associated with deep-seated infections such as septicaemia as opposed to superficial infections including impetigo; this suggests that this enzyme plays a role in tissue invasion (Rollof, *et al.,* 1987). The CNS strains in this study were recovered from patients with invasive infections and as illustrated in the work by Rollof *et al.* (1987) a significant number were found to express lipase compared to the non-septic volunteers ($p<0.0001$). Irrespective of the method of lipase/esterase detection, 92% of patients with CAPD-associated peritonitis and CRI expressed lipase/esterase, compared to 62% of the non-septic volunteers. This suggests that the expression of both specific and non-specific lipase/esterase is important for tissue invasion and biofilm formation and is implicated in the pathogenicity of device-related infections (Rupp, *et al.,* 1999a); (Rupp, *et al.,* 1999b).

6.4.3 Extracellular polysaccharide slime layer

CNS are recognised as significant pathogens causing infection in patients with indwelling medical devices, including peritoneal and haemodialysis catheters (Elliott, 1988); (Christensen, *et al.,* 1985). Bacterial adherence to biomaterials is mainly attributed to CNS strains that express extracellular highly adhesive material known as slime. Slime is described as a macroscopic adherent biofilm (Peters, *et al.,* 1982). The expression of slime promotes interbacterial shielding and adherence between bacterial cells and synthetic devices or biological tissues (Ishak, *et al.,* 1985); (Christensen, *et al.,* 1982). The mechanisms of biofilm formation are not fully understood, however, it is postulated that two distinct pathogenic mechanisms contribute to colonisation and biofilm formation (Christensen, *et al.,* 1990). Rapid initial adhesion of bacterial cells to a polymer surface is mediated by protein and/or carbohydrate bacterial adhesins accumulated in multiplayer cell clusters. These are embedded within an exopolymer substance and formation occurs over a period of hours with the presence of an *S. epidermidis* polysaccharide antigen (Mack, *et al.,* 1992). The composition of slime has not yet been fully characterised. It is suggested that the lectin may be interacting with terminal glucose molecules in wall teichoic acid (Hussain, *et al.,* 1991b).

In this study, expression of a polysaccharide slime layer was assessed by two methods: agglutination by concanavalin A and colony phenotype on congo-red agar. Ziebuhr *et al.* (1997) described the mechanism of accumulation of biofilm was mediated by the products of a gene locus comprising three intercellular adhesins genes (*icaABC*) which are organised into an operon structure. It was reported that the *ica*-encoded genes lead to the biosynthesis of the polysaccharide intercellular adhesin (PIA), which contains the *N*-acetylglucosamine as a

major component and is involved in the accumulative stage of biofilm formation. This *ica* cluster provides the capacity for phase variation, polymer adherence, autoaggregation and colony morphology on Congo-red agar (Ziebuhr, *et al.*, 1997).

Concanavalin A was used as a method of agglutinating mannose residues on the bacterial surface with the mannose/glucose/galactose-specific lectin. This formed visible aggregates in the bacterial suspension in those CNS strains that produced slime. Only 26% of CNS strains recovered from renal patients with CAPD-associated peritonitis and CRI were shown to produce slime by this method, compared to 50% in the non-septic volunteers (p=0.165). In contrast, when assessment was made by the congo-red method, 50% of CNS strains recovered from renal patients with CAPD-associated peritonitis and CRI were shown to produce slime compared to 79% in the non-septic volunteers (p=0.0039). This suggests that the congo-red agar plate method was superior and this is supported by previous work (Ziebuhr, *et al*., 1997). As shown in this study the validity of the lectin-based method is questionable, this is supported by previous work (Hussain, *et al.*, 1991b); (Deighton and Ballkau, 1990).

As discussed, it is widely reported that the production of extracellular polysaccharide slime is an important factor in the adherence of CNS to prosthetic devices and the evasion of the host immune response. In contrast, studies have also debated the association between slime production and the development of infection (Kotilainen, 1990); (Davenport, *et al.*, 1986); (Ishak, *et al.*, 1985); (Christensen, *et al.*, 1982). In this study the production of slime did not appear to be an important determinant of infection in renal patients with CAPD-associated peritonitis and CRI.

6.4.4 Adherence to polymer surface

Bacterial adherence to and growth on biomaterials have been shown to be important contributing factors to CNS infection (Karamanos, *et al.*, 1997). Attachment of staphylococci onto a polymer surface is the first step of prosthetic device related infection (figure 1.3) (Hogt, *et al.*, 1987). The blockade of this first step is reported to significantly reduce the ability of a microorganism to cause infection (Takeda, *et al.*, 1991).

In this study, 98% of CNS strains associated with renal patients with clinically significant infection expressed the ability to adhere to polystyrene. Indeed, 83% of CNS strains isolated from the ante-cubital fossa of non-septic volunteers expressed adhesins. This suggests that

while adherence to polymers may be essential to facilitate infection in indwelling medical devices, it is also essential for adherence in the local environment (i.e. skin cells). Therefore, the ability of a CNS strain to express adhesins is not specifically a measure of its pathogenicity. These data support the work of Lang (2000).

6.4.5 Urease activity

Mobley, Island and Hausinger (1995) demonstrated the production of urease in strains of *S. aureus, S. epidermidis, S. xylosus* and *S. saprophyticus*. In this study, urease activity was also observed in strain of *S. capitis* and *S. hominis*. Strains of *S. lugdunensis, S. haemolyticus, S. cohnii* and *S. warneri* recovered form renal patients with CAPD-associated peritonitis and those with CRI did not exhibit urease production. However, in the non-septic volunteers urease production was associated with *S. warneri, S. cohnii* and *S. haemolyticus*. Only *S. lugdunensis* did not express urease in the non-septic volunteer group. This may be attributed to under representation of strains of *S. lugdunensis* in the study.

Two different methods were used to assess the expression of urease, a native-PAGE and urease agar slope. Initially a native-PAGE method was employed and demonstrated no significant association in the urease production in the renal patients with CAPD-associated peritonitis and those with CRI (p=0.2304). Seventy one percent of patients with CRI expressed urease compared to 53% of patients with CAPD-associated peritonitis. None of the enzymically active protein bands detected by native-PAGE were larger than the Jack Bean sub-unit marker (272-kDa). The number of bands detected varied from 1 to 3 per strain. This banding pattern was thought to represent the holoenzyme and catalytic active degradation products (Schäfer and Kaltwasser, 1994). The urease agar slope method demonstrated no significant association between CNS strains recovered from patients with clinically diagnosed infection and those recovered from non-septic volunteers (p=0.8237). Seventy six percent and 72% respectively demonstrated CNS strains that expressed urease.

Urease activity has not been shown to act as a virulence factor during episodes of CRI but it is suggested that at localised points where biofilm formation is developing, the high pH generated by diffusion limiting processes such as slime expression, physical damage to the biomaterial surface may occur (Jansen, *et al.*, 1991). No significant association was demonstrated when comparing the two methods.

6.4.6 DNase activity

Thirteen out of 60 CNS strains recovered from patients with clinically diagnosed infection and 3 out of 65 CNS strains recovered from non-septic volunteers expressed DNase. This suggests that although the production of DNase is historically associated with *S. aureus*, the ability to hydrolyse DNA is within the capabilities of some CNS strains (Duguid, 1989). In this study the ability of CNS strains to hydrolyse DNA was demonstrated by a range of strains including *S. haemolyticus*, *S. capitis*, *S. hominis*, *S. lugdunensis*, *S warneri* and *S. caprae*. A significant association between the two groups of individuals was determined ($p=0.0056$) suggesting that although DNase production is only expressed in a minority of CNS strains, it is more likely to be expressed in CNS strains involved in infection than those isolated from the skin of non-septic volunteers.

6.4.7 Cytolytic toxins

Horse RBC were used to determine production of δ-toxin in CNS strains from renal patients with CAPD-associated peritonitis, CRI and from the ante-cubital fossa of non-septic volunteers. This toxin is reported to be active against both horse and sheep RBC (Hébert and Hancock, 1985). A microtitre plate method was employed to demonstrate haemolysis after 1h incubation at 37°C. A further 1h incubation at 4°C was included to stimulate "cold-shock" β-toxins to further haemolyse the RBC suspension. This was determined by reading the optical density at 570nm (Jordens, *et al.*, 1989).

In this study, no significant association was observed between the CNS strains from different patient categories ($p=0.1583$). Only 8% of CNS strains isolated from renal patients with clinically diagnosed sepsis expressed δ-toxin, however 20% of the CNS strains recovered from the ante-cubital fossa of healthy volunteers produced this toxin. Gemmell (1987) reported that CNS strains recovered from patients with CAPD-associated peritonitis were more likely to produce cytolytic toxins compared to patients with CRI. These findings were not upheld by this study. Incubation at 4°C did not further haemolysis in either patient group. This suggests that β-toxin is not active against horse RBC and is supported by the findings of Hébert and Hancock, (1985).

6.4.8 Are phenotypic characteristics a predictor for infection?

This study has shown that certain phenotypic characteristics are more likely to be seen in CNS strains recovered from patients with infection than in those recovered from the skin of non-septic volunteers. Table 6.15 suggests that based on probability, CNS strains producing a certain combination of phenotypic characteristics are more likely to cause infection in renal patients with indwelling medical devices. Ziebhur *et al.* (1997) reported that one function of the *ica* gene cluster is the initiation of phase variation. This suggests that CNS strains have the ability to modify the expression of phenotypic characteristics to suit their environment. This study has illustrated that the environment from which the CNS strains was recovered influenced both the proportion and combination of phenotypic characteristics expressed. This suggests that CNS strains recovered from non-septic patients cannot be used as a model to predict the likelihood of future infection as all CNS strains retain the ability to undergo phase variation.

CHAPTER 7: THE EFFECT OF ANTIMICROBIAL THERAPY ON THE FAECAL CARRIAGE OF VANCOMYCIN RESISTANT ENTEROCOCCI AND *CLOSTRIDIUM DIFFICILE*-ASSOCIATED DIARRHOEA.

7.1 INTRODUCTION

It is commonly acknowledged that the administration of antimicrobial therapy is associated with gastrointestinal disturbance, namely diarrhoea. This therefore provides clinicians with a complex challenge when treating patients with infection. The time disparity between the onset of clinical symptoms of infection and the availability of culture results, dictates that clinicians rely on standard treatment protocols that incorporate broad-spectrum antimicrobial therapy covering both Gram-positive and Gram-negative microorganisms. At UHB, empiric therapy protocols used within the renal service have previously included ceftazidime plus vancomycin and levofloxacin plus vancomycin. Third generation cephalosporins such as ceftazidime have been specifically linked with the development of *Clostridium difficile*-associated diarrhoea and thus alternative regimes were sought.

This section of study took place in two phases, phase I aimed to evaluate rates of faecal colonisation with VRE and carriage rates of *C. difficile* toxins in patients treated with intraperitoneal vancomycin plus ceftazidime. Phase II aimed to evaluate rates of faecal colonisation with VRE and carriage rates of *C. difficile* toxins in patients treated with intraperitoneal vancomycin plus piperacillin/tazobactam. In addition, phase II considered safety and efficacy of using intraperitoneal piperacillin/tazobactam.

7.2 MATERIALS AND METHODS

7.2.1 Phase I of study – Patients, antibiotic administration and sample collection

Fifty patients presenting at the CAPD unit with clinical signs of CAPD-associated peritonitis were treated with 1-2g vancomycin (dependent on the patients weight) and 1g ceftazidime administered in to a dialysate bag that was drained into the peritoneal cavity and allowed to dwell for 6-8 hours. Then each night, the patient administered 1g ceftazidime into their overnight dialysate bag, until peritoneal fluid culture results were available and they were asked to stop by the CAPD unit. They were asked to provide a faecal sample on day 6 or 7 of treatment. The protocol for antimicrobial treatment is shown in figures 7.1 and 7.1.1.

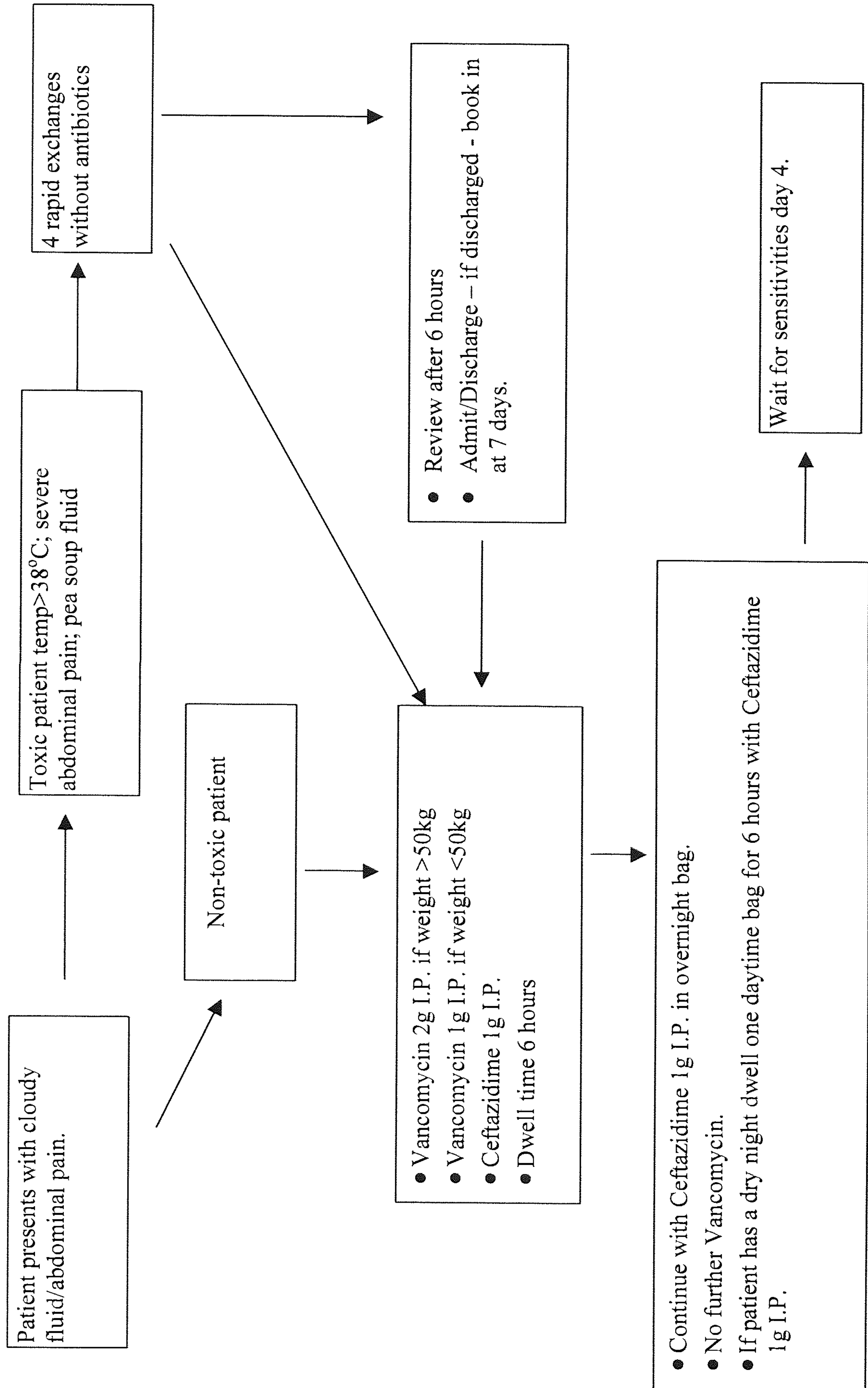
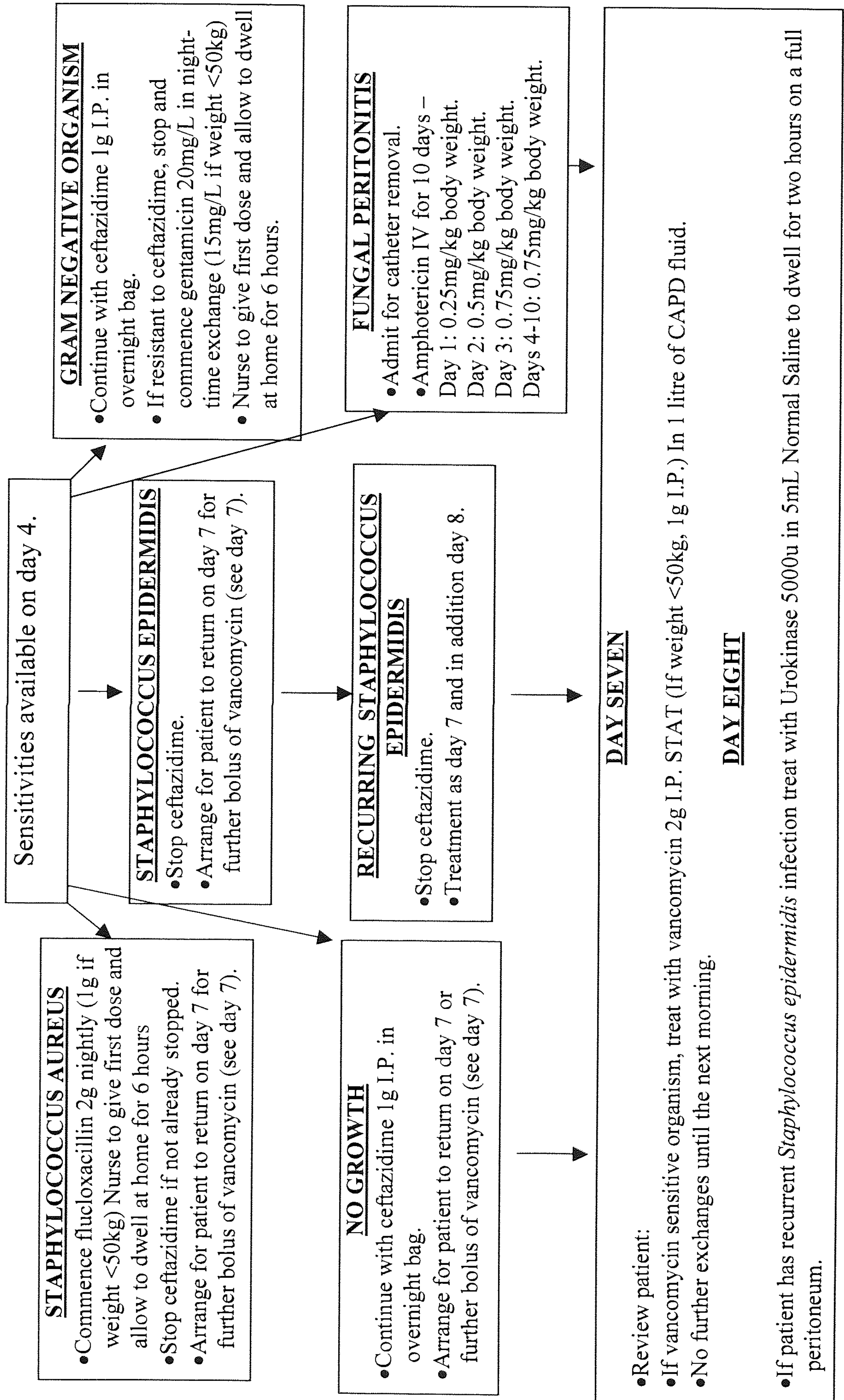


Figure 7.1 Antimicrobial treatment protocol for administration of ceftazidime plus vancomycin (Day one)

Figure 7.1.1 Antimicrobial treatment protocol for administration of ceftazidime plus vancomycin (Days 4-7)



In addition, surfaces and articles with the CAPD outpatient area were swabbed and cultured for the presence of VRE. Swabs were taken from sinks, tables used for CAPD fluid exchanges, trolleys and automated peritoneal dialysis machines.

7.2.2 Sample processing

7.2.2.1 Determination of vancomycin resistant enterococci from faecal samples

One gram of faeces was inoculated into 5mL saline. One drop of faecal suspension was inoculated onto Slanetz and Bartley medium, a selective media for enterococci. A 5μg vancomycin disc (Oxoid, UK) was then placed on the agar plate after the primary inoculum. The plate was incubated at 42°C for 48h in air and then examined for the presence of typical colonies.

7.2.2.2 Determination of vancomycin resistant enterococci from environmental swabs

A sterile cotton wool swab saturated in sterile saline was used to sample each item. The swabs were then aseptically transported to the laboratory and were inoculated onto a Slanetz and Bartley agar plate. A 5μg vancomycin disc was placed on the area following the primary inoculum. The plate was then incubated at 42°C for 48h and examined for the presence of typical colonies.

Typical colonies isolated from faeces culture and environmental swabs were subjected to further testing to confirm enterococcal speciation.

7.2.2.3 Gram stain

One colony was emulsified in one drop of saline on a microscope slide. The sample was heat-fixed and flooded with crystal violet for 2 minutes. After washing with water, Lugol's iodine was applied and left for 1 minute. The slide was then decolourised with acetone for 2 to 3 seconds and finally counter-stained with carbol fuchsin, which was removed by washing with water after 1 minute. The slide was allowed to dry and viewed by bright field microscopy using oil immersion x 100 objective.

7.2.2.4 Catalase test

A small volume of 3% hydrogen peroxide (H_2O_2) was aspirated using a capillary tube. The capillary tube was inverted and the end used to touch one colony. The hydrogen peroxide was then run down the tube to the colony and the resulting reaction observed. The tube was examined for the presence of hydrogen gas bubbles, indicating a positive catalase reaction.

7.2.2.5 Bile aesculin stab

Suspect colonies were stabbed into bile aesculin agar (Oxoid, UK) using a sterile straight wire. The plate was then incubated at 37°C for 24h in air. Plates were examined for blackening around the stab indicating a positive aesculin reaction.

7.2.2.6 Determination of *Clostridium difficile* toxin

Faecal specimens were stored up to 72h at 2 to 8°C before testing or frozen at –20°C upon receipt if testing could not be performed within 72h. The test was carried out using an enzyme immunoassay, ImmunoCard® Toxin A (Meridian Diagnostics, Inc.) in accordance with the manufacturer's instructions (appendix 6) and results recorded as *C. difficile* toxin A positive or negative.

7.2.3 Characterisation of vancomycin resistant enterococci

7.2.3.1 Antibiogram determination

Each isolate was tested for sensitivity against a panel of antibiotics using the disc diffusion method.

7.2.3.1.1 Disc diffusion sensitivity testing method

1 to 2 colonies were inoculated into 3mL saline so that the turbidity was equal to the 0.5 McFarland standard. A sterile cotton wool swab was then used to inoculate the suspension onto 1% lysed blood Isosensitest agar with a rotary plater.

Discs impregnated with the required antibiotic concentration were applied with the aid of a disc dispenser. The following antibiotic discs were used: gentamicin 200µg, ampicillin 10µg, vancomycin 5µg, teicoplanin 30µg, vancomycin 30µg, piperacillin/tazobactam 85µg, ciprofloxacin 1µg, erythromycin 5µg, and trimethoprim 1.25µg.

To prevent overlapping of zones a maximum of 6 discs were applied to a 90mm agar plate, so for each isolate 2 agar plates were used. The plates were then incubated for 24h at 37°C. The sensitivity pattern of each strain was established by measuring the zones of inhibition. These zone sizes were then compared to British Society for Antimicrobial Chemotherapy (BSAC) and Swedish Reference Group on Antibiotics (SRGA) guidelines for enterococci and were recorded as either sensitive or resistant. Where no guidelines were available zone sizes were compared to known sensitive laboratory isolates (table 7.1).

Table 7.1 Zone breakpoints for enterococci

Antibiotic	Disc content (µg)	Zone diameter (mm)	
		Resistant	Sensitive
Gentamicin	200	≤9	≥10
Ampicillin	10	≤19	≥20
Vancomycin	5	≤12	≥13
Teicoplanin	30	≤18	≥19
Vancomycin	30	≤19**	≥20**
Piperacillin/tazobactam	85	≤28**	≥29**
Ciprofloxacin	1	≤12*	≥32*
Erythromycin	5	≤21**	≥22**
Trimethoprim	1.25	***	

* Swedish Reference Group on Antibiotics

** Zone sizes established using sensitive laboratory isolate, NCTC 795

*** SRGA guidelines only for 5µg disc: 5≥17, ≤13. If isolates sensitive to trimethoprim 1.25µg had zone sizes larger than 17mm they were considered sensitive.

7.2.3.2 Biotyping

The biotype of each isolate was established using BBL CRYSTAL™ Gram-Positive Identification system (Becton Dickinson, France).

One colony was suspended in a BBL CRYSTAL inoculum tube and vortexed for 10 seconds. The inoculum fluid was then transferred to a crystal base and rolled so that each well in the base was filled. Using a sterile loop a sample of the inoculum was inoculated onto Columbia agar containing 7% defibrinated horse blood supplemented with 20mg/L NAD to check the

purity of the inoculum. A lid containing dehydrated biochemical substrates was attached to the base and the panel was incubated for 24h in humid air at 37°C. After the incubation period, the reactions were read using a BBL CRYSTAL™ panel viewer.

7.2.3.3 Pulsed field gel electrophoresis (Lina *et al.*, 1992)

Bacterial cells were harvested from an overnight culture in brain-heart infusion broth (Oxoid). Twenty milligrams (wet weight) of cells were re-suspended in 1mL NET-100 (0.1M Na_2 EDTA (pH 8.0), 0.1M NaCl, 0.01M Tris-HCl (pH 8.0)) and mixed with an equal volume of molten low melting point chromosomal grade agarose (0.9% (w/v) in NET-100; BioRad). Digestion of the bacterial wall and cellular components was achieved essentially by the method of Lina *et al* (1996) Slivers of the agarose blocks (1x1x9mm) containing chromosomal DNA were digested with 50 units of *Sma*I (Boehringer Mannheim, Germany) RE in 200µL buffer overnight at 25°C. The RE digested DNA slivers were subjected to PFGE (CHEF-DR III system BioRad) under the following conditions:

Voltage gradient of 6 V/cm at an angle of 120° at the following switch times for a period of 23 hours:

Block 1	Initial time:	1 second	Block 2	Initial time:	10 seconds
	Final time:	10 seconds		Final time:	25 seconds
	Run time:	10 hours		Run time:	13 hours

7.2.3.3.1 Analysis of PFGE profiles

Interpretation of the macrorestriction fragment profiles was performed using the Phoretix ID Advanced gel analysis computer programme (Phoretix International UK) together with supporting visual assessment. The size of the profile bands were evaluated by comparing against the bands of the lambda phage DNA standard. Only bands >30 kb were included in the analysis. Completed gel analysis data were transferred to the Phoretix ID Database programme to allow strain comparison by calculation of the Dice correlation coefficient. Isolates were clustered by the unweighted pair group method of arithmetic averages (UPGMA) to permit the construction of a dendrogram. In addition, multiple isolates from individual patients were subjected separately to PFGE and visual analysis of gels to identify any similarity between strains (Tenover, *et al.*, 1995). A 2 to 3 band difference within the

profile indicated that the isolates are closely related, 4 to 6 band difference suggested that the two isolates may be related whilst >7 band deviation indicated that the two strains are not related.

7.2.4 Phase II safety study - Patient recruitment

Patients presenting at the CAPD unit with clinical signs of CAPD-associated peritonitis were assessed according to the criteria shown in table 7.2.

Table 7.2 Inclusion and exclusion criteria for recruitment into the study

Patient Inclusion Criteria	Patient Exclusion Criteria
Greater than 18 yrs of age	Known hypersensitivity to any component of study treatment regime
Not acutely unwell	Pregnant or lactating mothers
Discharge post antibiotic administration	Known colonisation with organisms resistant to study treatment regime
	Known to have received any of the study antibiotics in the previous 24 hours
	Known HIV positive status
	Acute renal failure associated with multi organ failure and/or septicaemia

Those patients who matched the study criteria were given the opportunity to take part in the study. Informed consent was obtained following detailed explanation of the study.

7.2.5 Antibiotic administration

1-2g vancomycin (dependent on the patients' weight) and 1.125g piperacillin/tazobactam (pip/taz) were administered in to a 2L dialysate bag that was drained into the peritoneal cavity and allowed to dwell for 6-8 hours. Then each night, the patient administered 1.125g piperacillin/tazobactam into their overnight dialysate bag (2L), until peritoneal fluid culture results were available and they were asked to stop by the CAPD unit (figures 7.2 and 7.2.1).

Figure 7.2 Antimicrobial treatment protocol for administration of ceftazidime plus vancomycin (Day one)

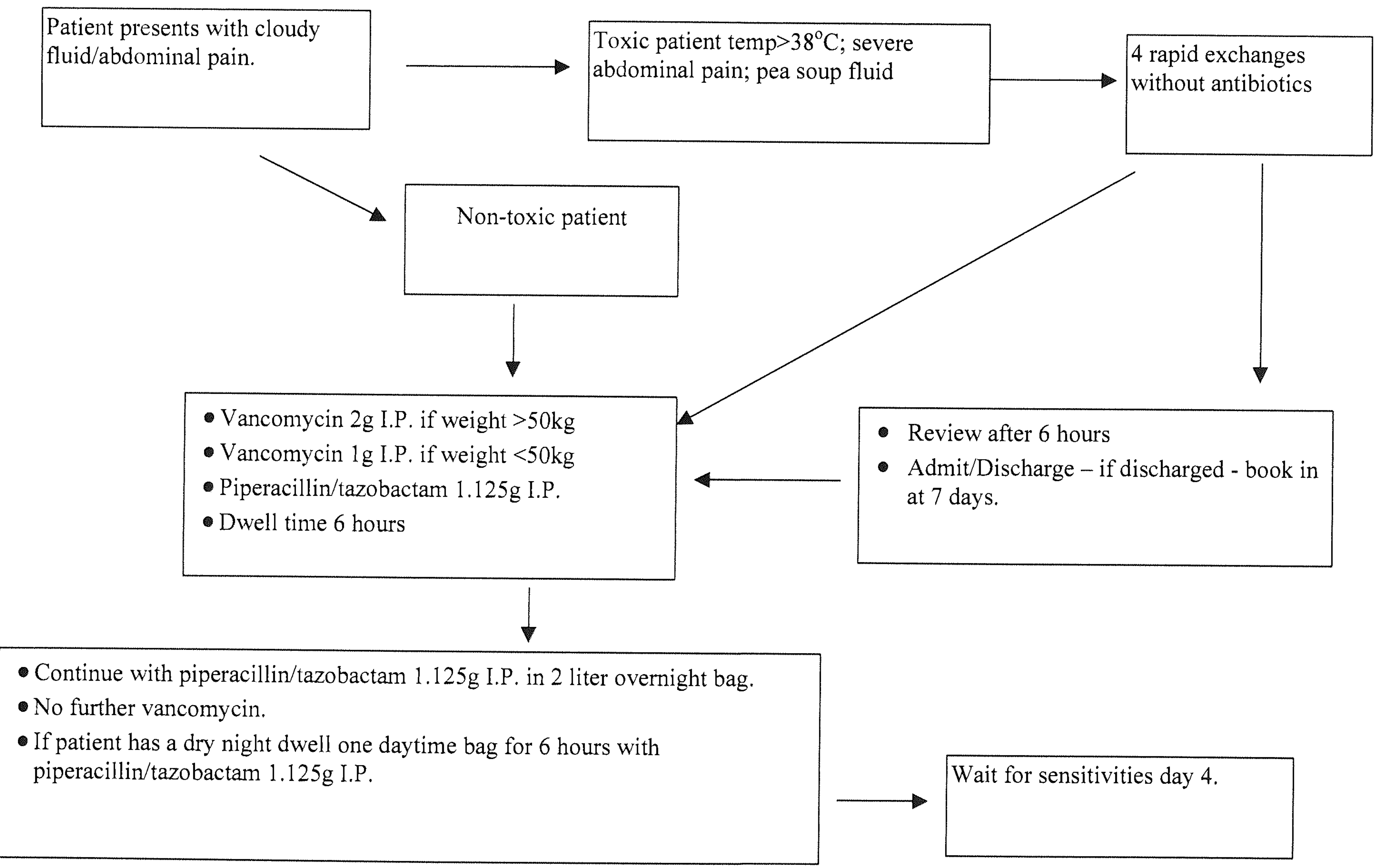
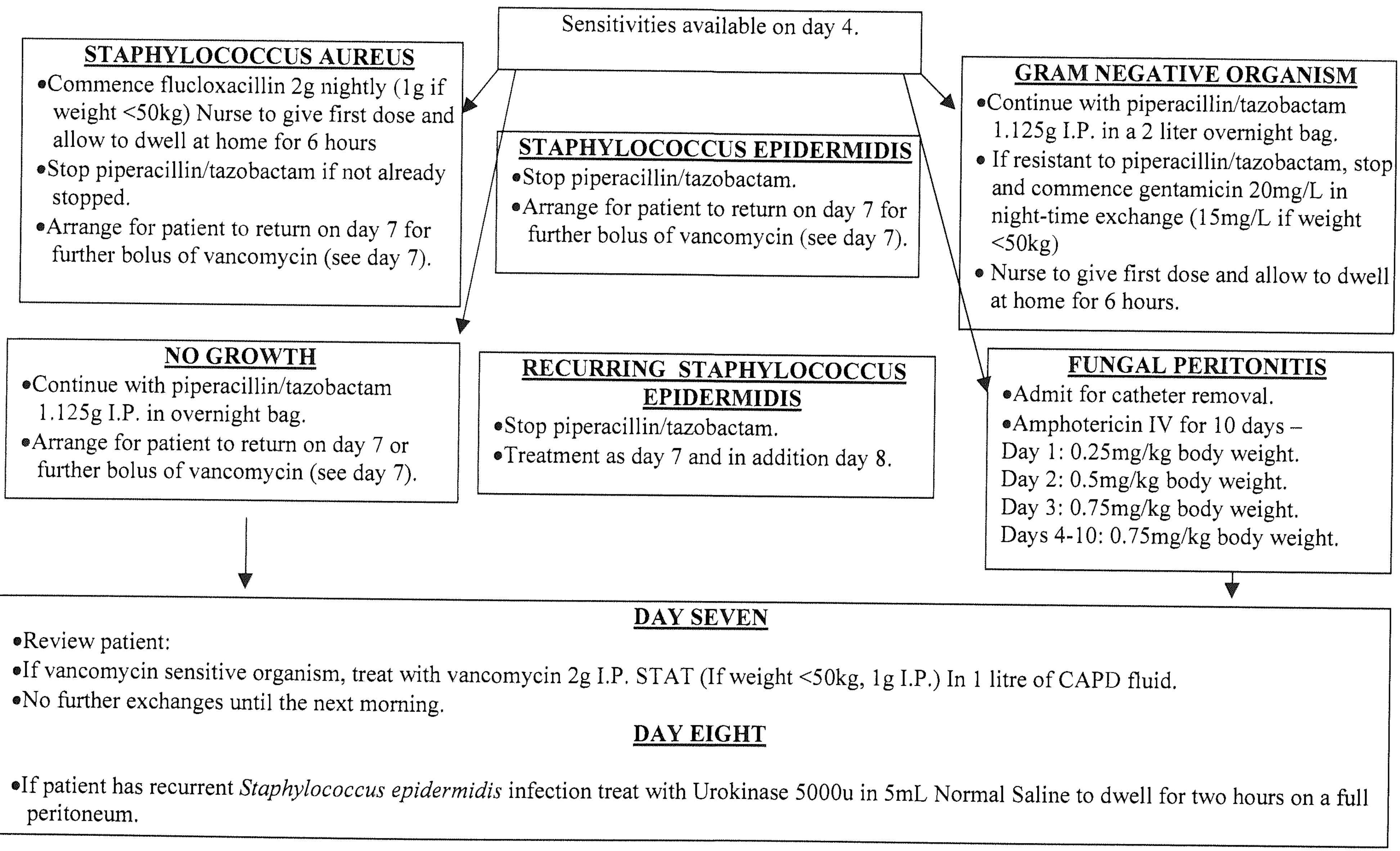


Figure 7.2.1 Antimicrobial treatment protocol for administration of ceftazidime plus vancomycin (Days 4-7)



Given that dialysate culture, microorganism identification and result availability would take three to four days, it was assumed that each patient would receive at least 4 doses of pip/taz.

7.2.6 Sample collection

To establish a baseline with which to compare the amount of pip/taz removed from the peritoneum during antimicrobial treatment, the patients were asked to save two bags of peritoneal dialysis effluent (PDE); the first bag to be collected was the last daytime bag on day 3 of treatment. This bag formed the baseline of antibiotic concentration prior to treatment. The second bag to be collected was the one containing the overnight dwell of day 3 that had contained the antibiotic treatment regimen (table 7.3). In addition, the patients were asked to provide a faecal specimen on day 6 or 7 of treatment.

Table 7.3 Typical peritoneal fluid exchange regime and exchange regime when experiencing peritonitis

Typical Day	Peritonitis Day 1	Peritonitis Day 2	Peritonitis Day 3	Peritonitis Day 4
08.00 Overnight fluid drained	08.00 Overnight fluid drained - Appears cloudy	08.00 Overnight fluid drained	08.00 Overnight fluid drained	08.00 Overnight fluid drained
10.00 Exchange performed	08.45 Exchange performed - Remains cloudy	10.00 Exchange performed	10.00 Exchange performed	10.00 Exchange performed
14.00 Exchange performed	10.00 CAPD unit contacted	14.00 Exchange performed	14.00 Exchange performed	14.00 Exchange performed
18.00 Exchange performed	12.00 Assessed in unit - Exchanges performed	18.00 Exchange performed	18.00 Exchange performed	18.00 Exchange performed
22.00 Overnight fluid inserted	14.00 Antibiotics administered	22.00 Overnight fluid inserted containing 1.125g pip/taz	22.00 Overnight fluid inserted containing 1.125g pip/taz	22.00 Overnight fluid inserted containing 1.125g pip/taz
	15.00 Discharged home with antibiotics for 5 days – Fluid remains in for 6-8 hours			
	22.00 Overnight fluid inserted - No further antibiotics today			

7.2.7 Sample processing

7.2.7.1 Determination of vancomycin resistant enterococci from faecal samples

As described in sections 7.2.2.1 to 7.2.2.5

7.2.7.2 Biotyping

The biotype of each VRE isolate was established using the Analytical Profile Index (API®20-Strep) (BioMérieux, France). This was carried out in accordance with the manufacturer's instructions.

7.2.7.3 Determination of *Clostridium difficile* toxin

Faecal specimens were stored up to 72h at 2 to 8°C before testing or frozen at –20°C upon receipt if testing could not be performed within 72h. The test was carried out using an enzyme immunoassay, for the detection of toxins A and B (Meridian Bioscience, Inc.) in accordance with the manufacturer's instructions (appendix 6) and results recorded as *C. difficile* toxin A & B positive or negative.

7.2.7.4 Determination of tazobactam and piperacillin in dialysis fluid by high performance liquid chromatography (HPLC) and ultra violet (UV) detection.

7.2.7.4.1 Test compounds and internal standard

Piperacillin and tazobactam were compared to an internal standard of cefotaxime sodium salt. The structures of these three compounds can be seen in figures 7.3 to 7.5.

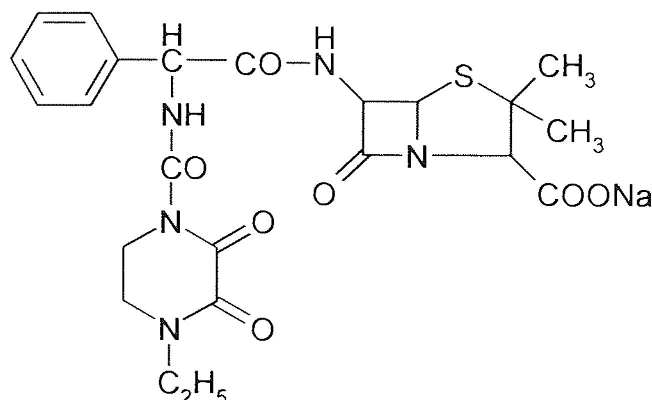


Figure 7.3 Chemical structure of piperacillin ($C_{23}H_{27}N_5O_7S$) Mol Wt =517.56

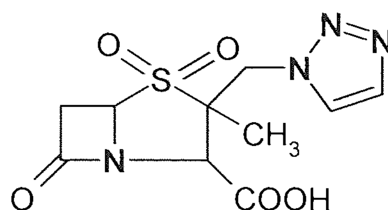


Figure 7.4 Chemical structure of tazobactam ($C_{10}H_{12}N_4O_5S$) Mol Wt = 300.30

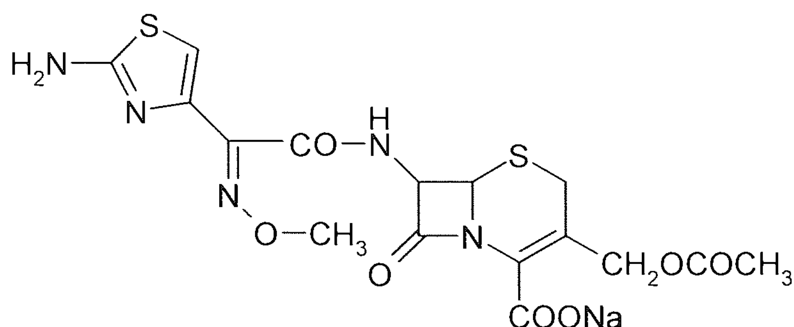


Figure 7.5 Chemical structure of cefotaxime sodium salt ($C_{15}H_{16}N_5NaO_7S_2$) Mol Wt =477.47

7.2.7.4.2 HPLC apparatus

HPLC Pump:	Thermoseparations model P200.
HPLC Autosampler:	Thermoseparations model AS3000 with sample tray cooler option.
HPLC Detector:	Thermoseparations model UV2000

PE Nelson 900 series interface unit and a personal computer loaded with Perkin-Elmer Turbochrom 4 software (Version 4.1) for data collection and a printer.

7.2.7.4.3 HPLC conditions

HPLC column:	Spherisorb ODSC-18-5 (Water Ltd) 250 X 4.6mm, 5micron with in line guard column containing the same packing material maintained at temperature of 26°C in a column oven.
Flow rate:	1.2mL/minute (Gradient run).

Table 7.4 Thermoseparations System's Mobile Phase Gradient Program.

Time (minutes)	Flow (mL/min)	%A	%B	Gradient
0.0	1.2	100	0.0	
2.0	1.2	100	0.0	
9.0	1.2	50	50	Linear
10.0	1.2	5	95	Linear
12.5	1.2	5	95	
13.5	1.2	70	30	
15.5	1.2	80	20	
16.5	1.2	100	0	
18.5	1.2	100	0	Linear

Injection Volume: 65μl from a polypropylene 250μl autosampler vial contained in a 1.8mL glass step vial with a PTFE snap cap (Capital Analytical, Leeds, UK)

Autosampler Temperature: Room Temperature

Mobile phase: HPLC buffer A - 97mL of stock sodium phosphate buffer was diluted to 970mL using water. 30mL of Acetonitrile was added and the solution and mixed. This solution was then filtered through a fibreglass filter under vacuum.

HPLC buffer B - 12mL of Stock Sodium Phosphate Buffer was diluted to 120mL with water in a 1-litre beaker. To this solution, 900mL of Acetonitrile was added in small volumes while constantly stirring the contents of the beaker. This solution was then filtered through a fibreglass filter under vacuum.

Detector: UV was set at 210nm from time = 0 to time = 18.5.

7.2.7.4.4 Chemicals and reagents

Table 7.5 Chemicals and reagents

REAGENT	GRADE	SUPPLIER
Cefotaxime sodium salt	99.8%	Sigma
Piperacillin (Sodium salt)	99%	WyethAyerst Research
Tazobactam	99%	WyethAyerst Research
Sodium dihydrogen Orthophosphate monohydrate	Analytical grade	Sigma
Sodium hydroxide	Analytical grade	BDH
Orthophosphoric acid	Analytical grade	BDH
Dialysis fluid		Baxter Healthcare, UK
Acetonitrile	HPLC	Hichrom Ltd.
Water	HPLC	Hichrom Ltd.
Dichloromethane	HPLC	Hichrom Ltd.

7.2.7.4.5 Preparation of stock standard solutions

7.2.7.4.5.1 Piperacillin/tazobactam 4mg/mL

Combined piperacillin/tazobactam solution was prepared by adding 41.39mg of piperacillin monohydrate to 40mg of tazobactam and quantitatively transferring to a 10mL volumetric flask, using methanol. The solution was sonicated for 5 minutes to ensure dissolution. Equilibration to ambient temperature was allowed prior to adjusting to final volume of 10 mL with methanol. The flask was labelled and stored in a freezer at -20°C. The solution was used within 3 months of preparation.

7.2.7.4.5.2 Cefotaxime sodium 1mg/mL (Internal standard)

10mg of solid cefotaxime sodium standard was quantitatively transferred to a 10mL grade A volumetric flask and dissolved in methanol. The flask was labelled and stored in a freezer at -20°C. The solution was used within 3 months of preparation.

7.2.7.4.5.3 0.1M Phosphate buffer for HPLC Mobile Phase

27.6g of NaH_2PO_4 were dissolved in 800 mL of HPLC grade water. The pH adjusted 2.7 with 85% (w/w) phosphoric acid. The solution was diluted to 1 litre with HPLC grade water, labelled and store at room temperature. The solution was used within 6 months of preparation.

7.2.7.4.5.4 0.05M Phosphate Buffer for internal standard

1.725g of NaH_2PO_4 were dissolved in 200 mL of HPLC grade water. The solution was adjusted to pH 6.0 with 4.0M NaOH.

7.2.7.4.6 Preparation of Working Internal Standard Solution

1mL of the stock internal standard solution was diluted to 20mL with 0.05M Phosphate Buffer (pH=6). This was prepared fresh on the day of the assay and then discarded after use.

7.2.7.4.6.1 Preparation of spiking working solutions.

The combined piperacillin/tazobactam stock solution was used to prepare working solutions (C1 to C7) as shown in table 7.6. These solutions were used to prepare calibration standards in the concentration range 1 to 200µg/mL in dialysis fluid.

Table 7.6 Working solutions of piperacillin and tazobactam

Working Std No.	Volume of stock or working std µl	Volume of methanol µl	Concentration µg/mL
C7	Stock Solution	0.0	4000
C6	750 Stock Solution	250	3000
C5	500 Stock Solution	500	2000
C4	250 Stock Solution	750	1000
C3	50 Stock Solution	950	200
C2	50 Stock Solution	1900	100
C1	50 Stock Solution	10,000	20

7.2.7.4.6.2 Preparation of spiked calibration standards in dialysis fluid.

Working standard solutions C1 to C7 were used as in table 7.7 below to prepare a calibration standards DFC1 to DFC7.

Table 7.7 Calibration standards

Calibration Standard No.	Volume of Dialysis Fluid mL	Working Methanolic Std mL	Final Concentration µg/mL
DFC7	9.5	0.5 of C7	200
DFC6	9.5	0.5 of C6	150
DFC5	9.5	0.5 of C5	100
DFC4	9.5	0.5 of C4	50
DFC3	9.5	0.5 of C3	10
DFC2	9.5	0.5 of C2	5
DFC1	9.5	0.5 of C1	1
BLANK	9.5	0.5 Methanol	0.0

7.2.7.4.6.3 Preparation of spiked Quality Control Samples in dialysis fluid

Quality control working standards (low, medium and high) were prepared as outlined in table 7.8.

Table 7.8 Spiking solutions for preparation of working Quality Control Samples

Working Std No.	Volume of stock µl	Volume of methanol µl	Concentration µg/mL
WS-QCL	925	75	3700
WS-QCM	300	700	1200
WS-QCH	225	775	90

Quality control spiking solutions of working standards (WS-QCL, WS-QCM and WS-QCH) were used as in table 7.9 to prepare Quality Control Samples in dialysis fluid, to represent the lower, middle and upper regions of the calibration range.

Table 7.9 Quality Control Samples

Working Std No.	Volume of stock µl	Volume of Dialysis fluid mL	Concentration µg/mL
QCL-45	250 of WS-QCL	4.75	45
QCM-60	250 of WS-QCM	4.75	60
QCH-185	250 of WS-QCH	4.75	185

Each level was divided into 0.7 mL aliquots and frozen at -20°C until required for the assay. Note: During the method validation LLOQ samples, spiked at 1µg/mL were prepared fresh on the day of the assay in the same manner as for preparation of the C1 calibration standard.

7.2.7.4.6.4 Preparation of samples for HPLC analysis

0.2 mL of dialysis fluid was added to 10mL glass screw capped tubes and labelled as standard, quality control or blank. 0.2mL of the working internal standard solution was added to each tube. To the blanks without internal standard, 0.05M, Phosphate buffer (pH 6.0) was added instead of internal standard. The tubes were mixed and 0.8mL of acetonitrile added to all the samples, vortex mixed for 2 x 30 seconds and then centrifuged for 10 minutes. The resulting supernatant was transferred to an appropriately labelled glass screw capped tube. To all the tubes 2mL of dichloromethane was added, vortex mixed for 2 x 30 seconds and then centrifuge for 10 minutes at 3000 rpm to separate the aqueous phase. The aqueous phase (200μL) was transferred to a clean glass tube and purged with nitrogen to evaporate any residual organic phase. 65μl of the aqueous phase was transferred to the auto-injector vial and injected onto the HPLC.

Table 7.10 Summary of sample preparation procedure

Sample	**Blank (DF)**	**Blank + IS (DF)**	**C1-C7 (DF)**	**QC**	**Test (DF)**
Calibration standard (μL)	-	-	200	-	-
DF (μL) Test and QC	200	200	-	200	200
IS (μL)	-	200	200	200	200
0.05M phosphate buffer (μL)	200	-	-	-	-
Acetonitrile (μL)	800	800	800	800	800
Vortex (sec)	30	30	30	30	30
Centrifuge 3000 rpm (min)	5	5	5	5	5
Transfer the resulting supernatant to a glass screw top tube					
Dichloromethane (mL)	2	2	2	2	2
Vortex (sec)	30	30	30	30	30
Centrifuge 3000 rpm (min)	5	5	5	5	5
Transfer aqueous layer to glass tube (μL)	200	200	200	200	200
Purge tubes with nitrogen (sec)	30	30	30	30	30
Transfer to auto-sample vial and inject on to HPLC (μL)	65	65	65	65	65

7.2.7.4.6.5 Data processing

The raw chromatographic data was recorded using the Perkin Elmer Turbochrom 4 software (Version 4.1). The calibration curve data from the calibration standards in each analytical run was used to construct a calibration line. The equation of the line was used to calculate the concentration of piperacillin and tazobactam in each sample automatically. The calibration was an expression of the ratio of the peak area response of piperacillin or tazobactam to the internal standard (IS), versus the nominal concentration. A weighing of $1/X^2$ was used throughout the method validation. The statistical analysis of this data was carried out using Excel spreadsheet. Under the conditions described in section 7.2.7.4.3, baseline separation of tazobactam from the cefotaxime (IS) was achieved with retention times of approximately 6.0 minutes for tazobactam, 11.5 minutes for piperacillin and 8.0 minutes for cefotaxime.

7.2.7.4.6.6 Analysis

Prior to processing, all frozen samples (control and test samples) were thawed for approximately 30 minutes at room temperature, vortexed and transferred to appropriately labelled tubes.

The analytical run consisted of two control blank matrix samples, calibrators C1-C7, quality control samples (minimum two sets of Quality Control Low -QCL, Quality Control Medium - QCM and Quality Control High – QCH per run), one set run after blanks and calibrators and then after approximately every 15 test samples or an appropriate number of subject period batches. Each analytical run was prepared and assayed within a 28-hour period.

The precision and accuracy data were generated from analytical runs consisting of the following: two blank dialysis control blanks – one labelled blank and the other blank + internal standard, Calibration standards in dialysis fluid C1 to C7, 6 x LLOQ samples, 6 x QCL, 6 x QCM, 6 x QCH (where LLOQ=Lower Limit Of Quantification, QCL=Quality control low – representing the lower part of the calibration curve, QCM= Quality control Medium - representing the middle of the calibration curve, and QCH= Quality control High – representing the upper part of the calibration curve).
Analyte concentrations were calculated on a personal computer with data collection software (Turbochrom, version 4.1) and a printer.

The raw data (calculated concentrations, chromatograms, parameters of the HPLC method) were obtained and stored as hard copy printouts with electronic backup. Statistical calculations were performed with Microsoft Excel version 7.0 and manually checked using a scientific calculator. Precision and accuracy were calculated according to the formulae:

Precision = (Standard deviation of found concentration / mean of found concentration) x 100
Accuracy = (Mean of found concentration / spiked concentration) x 100.

Results obtained may be omitted only if there is adequate justification, i.e. temporary instrument fault, evidence of operator error etc. Any values omitted from the statistical analysis were listed and the reason for their omission documented. For the sample analysis, an analytical run was accepted if no more than six out of nine quality controls are out of range with at least one complete set within range.

7.2.8 Characterisation of vancomycin resistant enterococci

As described in sections 7.2.3.1 to 7.2.3.3.1

7.2.9 Monitoring adverse events

Patients were required to attend the CAPD unit on day seven of treatment in accordance with the standard treatment protocol. At this time, the patient was asked about their health and well being while receiving pip/taz. This process was repeated 14 days after the end of pip/taz treatment.

7.3 RESULTS

7.3.1 Phase I - Patient demographics

Fifty patients were recruited into the study and the patient demographics can be seen in table 7.11

Table 7.11 Patient demographics

Patient demographics		
Age (years)	Mean	58
	Range	18-76
Male: Female		33:17
Out patients		90%
In patients		10%

7.3.2 Microorganisms causing peritonitis

The range of microorganisms isolated from clinically significant peritoneal dialysis cultures are listed in table 7.12.

Table 7.12 Range of microorganisms causing peritonitis in patients treated with ceftazidime and vancomycin for CAPD-associated peritonitis.

Total Gram positive orgs	37
Staphylococcus aureus	8
Coagulase-negative staphylococci	22
Streptococcus	1
Other Gram-positive microorganisms	6
Total Gram negative microorganisms	6
Escherichia coli	1
Serratia	2
Coliform	2
Bacteroidies	1
Polymicrobial infections	5
CULTURE NEGATIVE/NO GROWTH	8

7.3.3 VRE faecal colonisation rates

Of the 50 patients with CAPD associated peritonitis recruited to the study 46% were colonised with VRE. The colonisation rate did not correlate with the number of previous episodes of CAPD associated peritonitis treated with vancomycin and ceftazidime over the previous year; this is shown in table 7.13. However, the colonisation rate of VRE did correlate to the number of days spent in hospital in the 6 months prior to recruitment to the study ($p<0.05$). This is shown in table 7.14.

Table 7.13 Number of peritonitis episodes treated with vancomycin and ceftazidime in the previous year compared with VRE colonisation rate (n=46‡).

N° of peritonitis episodes in the last year	% Patients	VRE colonisation rate in faecal samples of patients (%)*
0	28 (n=14)	57 (n=8)
1	30 (n=15)	46.6 (n=7)
2	18 (n=9)	55.5 (n=5)
3	4 (n=2)	50 (n=1)
4	6 (n=3)	50 (n=1)
5	6 (n=3)	0

* Unable to confirm previous episodes of peritonitis for one patient colonised with VRE.

‡ Unable to confirm no previous episodes of peritonitis for four patients.

Table 7.14 Gastrointestinal carriage rate of VRE and the number of days attendance at renal outpatients clinics (n=30)

Total number of days attendance at renal outpatients in the 6 months prior to current episode of peritonitis.	Percentage of patients colonised with VRE
0	36 (n=12)
1-15	30 (n=9)
16-75	100 (n=9)

7.3.4 Treatment outcomes in patients treated with vancomycin plus ceftazidime

Nine out of 42 patients treated with vancomycin plus ceftazidime had a relapse in peritonitis within 21 days of finishing treatment.

7.3.5 Phenotypic characteristics of VRE isolated from patients with CAPD associated peritonitis treated with vancomycin and ceftazidime

E. faecium and *E. durans* accounted for 90% of isolates obtained. All isolates except 2 had different identification profiles as determined by BBL CRYSTAL™. Strains identified as *E. durans* shared similar profiles with all but 1 isolate differing by 4 digits out of 10. *E. faecium* BBL CRYSTAL™ profiles showed greater diversity.

7.3.5.1 Preliminary identification of VRE isolated from faecal samples

Suspect colonies, which were resistant to vancomycin on Slanetz and Bartley media, were further investigated. VRE appeared as Gram-positive cocci that were catalase-negative and aesculin-positive.

7.3.5.2 Antibiogram patterns of VRE isolates.

Seven different sensitivity patterns were obtained from 27 isolates with the majority of isolates resistant to all antibiotics tested. Other than vancomycin all isolates were resistant to ciprofloxacin and 95% of isolates were resistant to teicoplanin, piperacillin/tazobactam and erythromycin.

7.3.6 Genotypic characterisation of VRE isolates from patients with CAPD-associated peritonitis treated with vancomycin and ceftazidime.

*Sma*I digestion macrorestriction patterns of VRE yielded 17-20 fragments ranging from 48.5-436.5 kb. VRE identified as *E. durans* were genotypically different from other isolates, however a cluster of *E. durans* with a common genotypic pattern appeared within this group (figure 7.6). Three isolates were genotypically identical, 2 were possibly related and 2 were unrelated when macrorestriction patterns were analysed according to the criteria of Tenover *et al.* (1995). The cluster of *E. durans* was isolated within a 4-month period. Four fragments in the range 97-194 kb, 3 fragments between 242.5-291 kb and 1 at 339.5 kb were common to all isolates in the *E. durans* cluster.

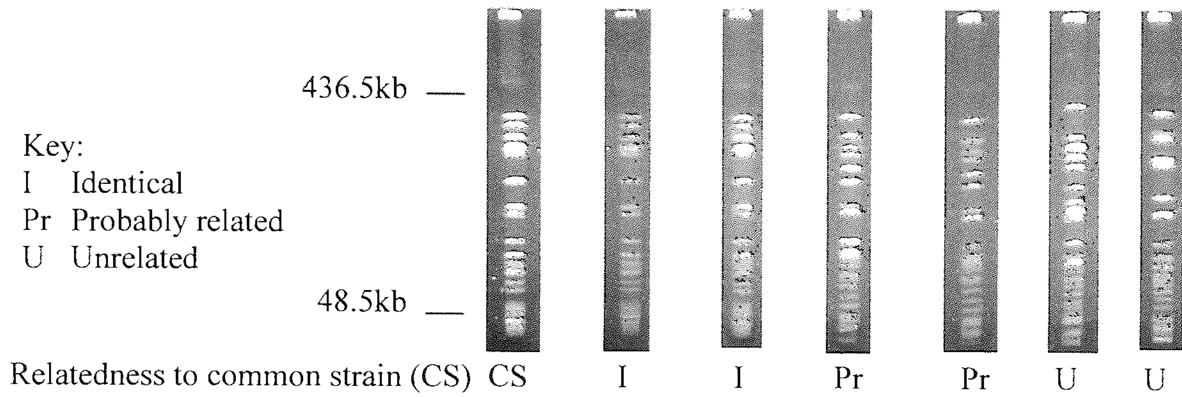


Figure 7.6 PFGE profiles of *E. durans* isolates from patients with CAPD-associated peritonitis.
Devised using the criteria of Tenover *et al.*, (1995).

The macrorestriction patterns of *E. avium* and *E. faecalis* isolates are shown in figure 7.7

These isolates were genetically different from each other and unique from other strains of VRE.

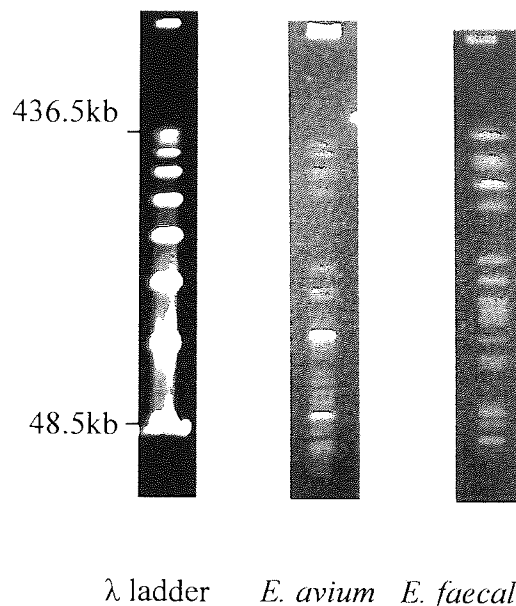


Figure 7.7 PFGE profiles of *E. avium* and *E. faecalis* isolated from patients with CAPD-associated peritonitis.

7.3.6.1 PFGE of environmental swabs

Macrorestriction profile analysis of the environmental isolates (n=30) taken from equipment in the CAPD outpatient unit revealed 10% of VRE strains isolated were genetically related to clinical strains, all of which were recovered within a 4-week period.

7.3.7 *C. difficile* toxin detection

6.3 % of patients with CAPD-associated peritonitis yielded positive *C. difficile* toxin A results following treatment with ceftazidime and vancomycin. None of the control faecal samples tested positive for *C. difficile* toxin A.

7.3.8 Phase II – Intraperitoneal piperacillin/tazobactam safety study

Seventeen patients were recruited into the safety study and the patient demographics are shown in table 7.15

Table 7.15 Patient demographics – Safety study

Patient demographics		
Age (years)	Mean	57
	Range	27-88
Male: Female		11:6
Out patients		100%
In patients		0%

7.3.9 Microorganisms causing peritonitis

The range of microorganism isolated from clinically significant peritoneal dialysis cultures are shown in table 7.16.

Table 7.16 Range of microorganisms causing peritonitis in patients treated with ceftazidime and vancomycin for CAPD-associated peritonitis.

Total Gram positive orgs	**10**
Staphylococcus aureus	5
Coagulase-negative staphylococci	2
Streptococcus	2
Corynebacterium	1
Total Gram negative orgs	5
Escherichia coli	2
Klebsiella	1
Pseudomonas aeruginosa	1
Coliform	1
Culture negative/no growth	2

7.3.10 Treatment and outcomes in patients treated with vancomycin plus pip/taz

The range of the number of doses of IP pip/taz received by patients was 2 to 14 with a mean of 6 days treatment. Six out of 15 patients treated with vancomycin plus pip/taz had a relapse of peritonitis within 21 days of finishing treatment.

7.3.11 VRE faecal colonisation rates

Of the 17 patients with CAPD associated peritonitis recruited to the study 15 provided a faecal sample on day 6 or 7 of treatment. Three of these samples (20 %) were colonised with VRE.

7.3.12 Preliminary identification of VRE isolated from faecal samples

Suspect colonies, which were resistant to vancomycin on Slanetz and Bartley media, were further investigated. VRE appeared as Gram-positive cocci that were catalase negative and aesculin positive.

7.3.12.1 Phenotypic characteristics of VRE isolated from patients with CAPD associated peritonitis treated with vancomycin and piperacillin/tazobactam

The numerical profiles obtained by the Analytical Profile Index (API®20-Strep) identified the three VRE isolates recovered from faecal samples of patients with CAPD-associated peritonitis treated with vancomycin plus pip/taz as *E. faecium* and *E. avium*. All isolates had different identification profiles as determined by API®20-Strep. Strains identified as *E. faecium* shared similar numerical profiles differing by 3 digits out of 7.

7.3.13 Genotypic characterisation of VRE isolates from patients with CAPD-associated peritonitis treated with vancomycin and piperacillin/tazobactam

*Sma*I digestion macrorestriction patterns of VRE yielded 11-16 fragments ranging from 48.5 to 436.5kb. The three VRE isolated recovered from faecal samples were all genotypically different. When macrorestriction patterns were analysed according to the criteria of Tenover *et al.* (1995) the three isolates were unrelated when. The macrorestriction patterns of these isolates are shown in figure 7.8.

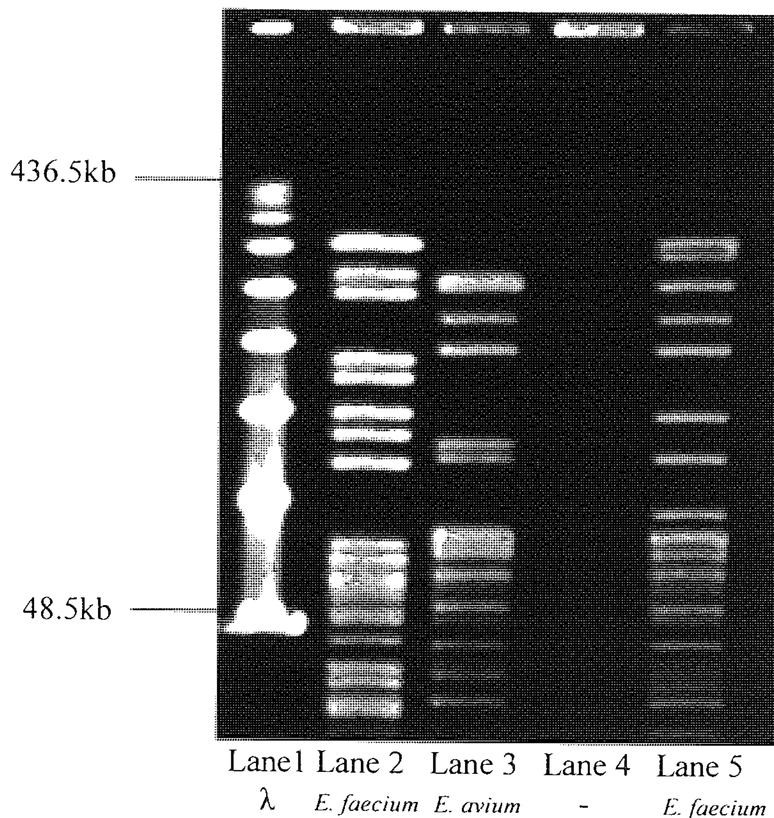


Figure 7.8 PFGE profiles of vancomycin resistant enterococci recovered from faecal samples of patients with CAPD-associated peritonitis treated with vancomycin and piperacillin/tazobactam.

Devised using the criteria of Tenover *et al.* (1995).

7.3.14 *C. difficile* toxin detection

One patient out of 15 (6.6%) with CAPD-associated peritonitis was positive for *C. difficile* toxin following treatment with vancomycin and piperacillin/tazobactam.

7.3.15 Recovery of piperacillin/tazobactam from peritoneal dialysis fluid by HPLC

This component of the work was carried out in conjunction with and in accordance to the methodology developed by Diagnostic Healthcare Ltd, London, UK.

7.3.15.1 Intra and Inter assay Accuracy and Precision

The accuracy and precision of the method was determined by assaying 0.2 mL aliquots of dialysis fluid fortified with 1.0, 4.5, 60 and 185μg/mL of pip/taz (representing quality controls: LLOQ, QCL, QCM and QCH respectively).

7.3.15.2 Intra-Assay Accuracy and Precision

The Intra-assay accuracy and precision for pip and taz were calculated from results obtained from 6 replicate analyses of quality controls at each of 4 concentrations (1.0, 4.5, 60 and 185μg/mL of pip/taz in dialysis fluid, representing LLOQ, QCL, QCM and QCH respectively) in a single batch. They are summarised below in tables 7.17 and 7.18 (Run-1).

Table 7.17 Intra-assay accuracy and precision for piperacillin

Piperacillin		1.0μg/mL	4.5μg/mL	60μg/mL	185μg/mL
Intra-Assay (Validation Run-1)	Mean Concentration	1.00	4.26	62.78	183.02
	Precision, C.V. (%)	2.95	1.29	2.22	0.39
	Accuracy (%)	99.67	94.63	104.64	98.93
	N =	6	6	6	6

Table 7.18 Intra-assay accuracy and precision for tazobactam

Tazobactam		1.0μg/mL	4.5μg/mL	60μg/mL	185μg/mL
Intra-Assay (Validation Run-1)	Mean Concentration	1.03	3.99	55.76	167.65
	Precision, C.V. (%)	2.28	2.51	2.56	1.38
	Accuracy (%)	102.67	88.59	92.94	90.62
	N =	6	6	6	6

Precision data for pip/taz was less than 3% and the accuracy figure was in the range 88.59% to 104.64% for LLOQ, QCL, QCM and QCH. These values were within the assay criteria for accuracy of nominal concentration ±15%.

7.3.15.3 Inter-Assay Accuracy and Precision

The Inter-assay accuracy and precision were calculated from results obtained from quality control samples analysed (N=18) at 4 concentrations (1.0, 4.5, 60 and 185μg/mL of pip/taz in dialysis fluid, representing LLOQ, QCL, QCM and QCH respectively) on three separate occasions. The results are summarised below in table 7.19 and 7.20.

Table 7.19 Inter-assay accuracy and precision for piperacillin

Piperacillin		1.0μg/mL	4.5μg/mL	60μg/mL	185μg/mL
Inter-Assay (Validation Runs-1-3)	Mean Concentration	0.98	4.24	64.88	189.88
	Precision, C.V. (%)	6.57	3.80	4.23	3.20
	Accuracy (%)	97.94	94.20	108.14	102.64
	N =	18	18	18	18

Table 7.20 Inter-assay accuracy and precision for tazobactam

Tazobactam		1.0µg/mL	4.5µg/mL	60µg/mL	185µg/mL
Inter-Assay (Validation Runs-1-3)	Mean Concentration	0.99	3.95	55.58	168.29
	Precision, C.V. (%)	4.57	2.07	3.20	2.03
	Accuracy (%)	98.67	87.74	92.63	90.97
	N =	18	18	18	18

Precision data for pip/taz was less than 6.6% and the accuracy figure was in the range 87.74% to 108.14% for LLOQ, QCL, QCM and QCH. These values are within the assay criteria for accuracy of nominal concentration $\pm 15\%$.

For inter and intra batch accuracy and precision data please see appendix 6.

7.3.16 Linearity

The ratio of UV area response for the peak corresponding to pip and taz in the HPLC chromatogram to the UV area response of the internal standard against the nominal concentrations of pip/taz represent the linearity. Applying linear regression to the calibration data obtained from seven calibration standards gives the equation of the linear line. The plots were linear over the concentration range 1 to 200µg/mL and the fit statistically tested by slope, intercept and correlation coefficient (r). Linear regression with $1/X^2$ was appropriate fit and a plot of calibration graph is presented in figures 7.9 and 7.10.

A calibration point could be rejected as an outlier if the back-calculated concentration for a calibrator (on the basis of the corresponding calibration curve) deviated more than 15% at all concentrations covered by the calibration range. A calibration curve was accepted with a minimum of 4 acceptable calibration levels.

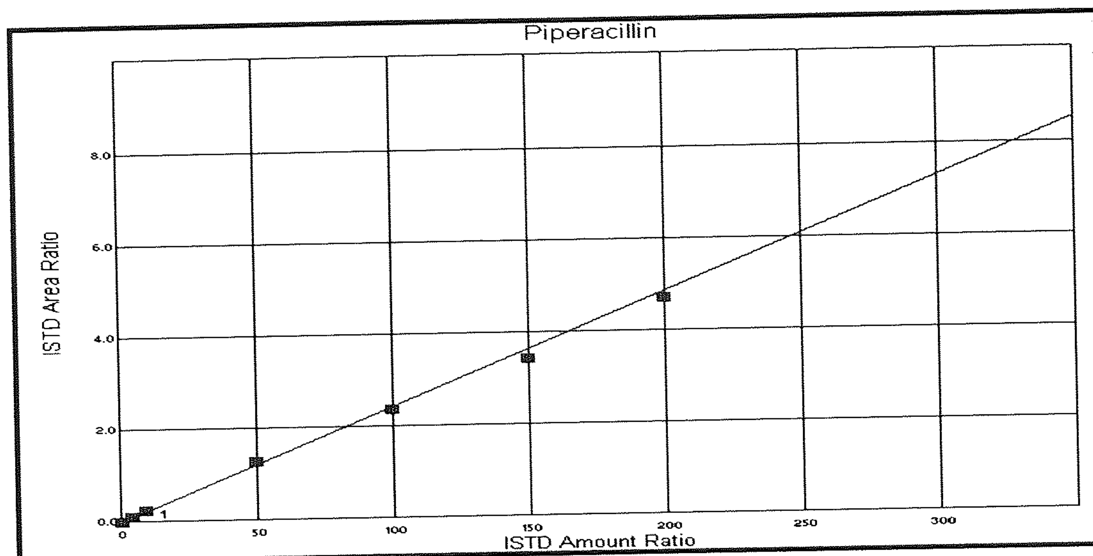


Figure 7.9 Calibration curve for piperacillin (1μg - 200μg/mL)

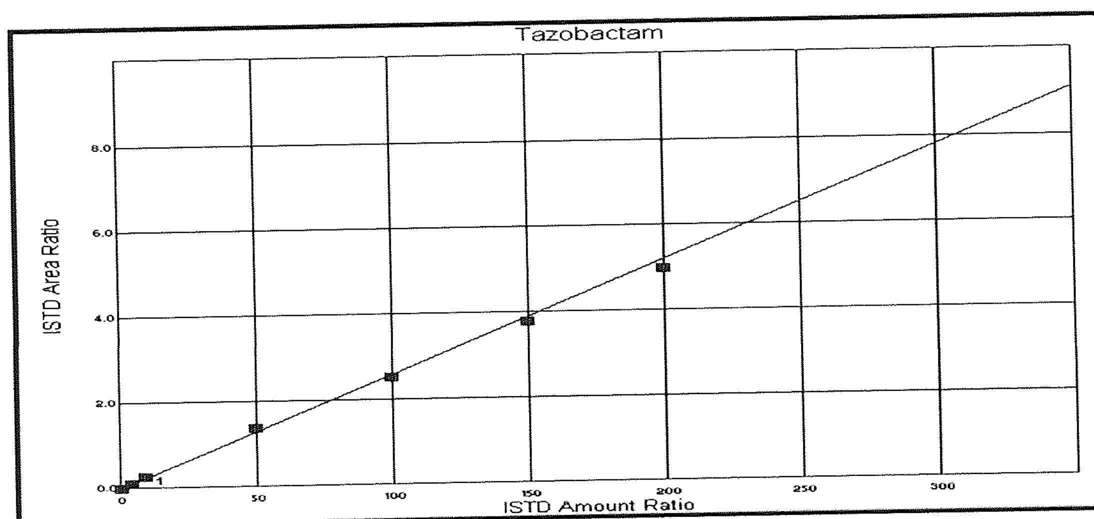


Figure 7.10 Calibration curve for tazobactam (1μg - 200μg/mL)

7.3.17 Recovery and Lower Limit of Quantitation

The recovery from the dialysis fluid was determined by first processing a series of un-spiked samples of dialysis fluid from a single batch (2 X n=5). The same batch of dialysis fluid was spiked with pip and taz at two concentrations (60μg/mL QCM and 185μg/mL QCH). The extracted blank dialysis fluid samples were spiked with pip and taz and the samples processed for HPLC.

These represent samples with:

(I). Dialysis fluid samples containing added known amount of pip/taz (Extracted sample)

(II). Dialysis fluid samples containing added known amount of pip/taz (Unextracted sample).

From this data, the percent recovery (R%) from Dialysis fluid samples was calculated using the following equations:

$$R\% = \frac{\text{(mean Area ratio of pip or taz to internal standard in I}}{\text{(mean Area ratio of pip or taz and to internal standard in II)}} \times 100$$

The recovery of piperacillin at concentration of 60 and 185μg/mL was found to be 81.73% and 79.78% respectively. The recovery of Tazobactam at concentration of 60 and 185μg/mL was found to be 89.16% and 91.42% respectively. The internal standard was quantitatively recovered from dialysis; range was 98.45 to 103.11% at working concentration of 10μg/mL.

Lower Limit of Quantitation (LLOQ) is defined as the lowest concentration that can be determined by a given procedure with good precision and accuracy (FDA method validation guidelines states the limits for precision and accuracy as nominal spiked level ±15% with a C.V. (%) within 20%). The analytical method has an LLOQ of 1μg/mL in dialysis fluid for piperacillin and tazobactam.

7.3.18 Study sample analysis

A single analytical run was required to analyse the study samples. In total 24 samples were analysed from 12 patients. There were two samples for each subject one obtained prior to the administration of pip/taz treatment and one recovered from the dialysis effluent that had contained the pip/taz treatment. The calibration curve and the Quality controls gave values similar to those obtained during the validation. A chromatogram of a study sample, superimposed on a calibration standard is shown in figure 7.11. The results of the study samples are presented in table 7.21.

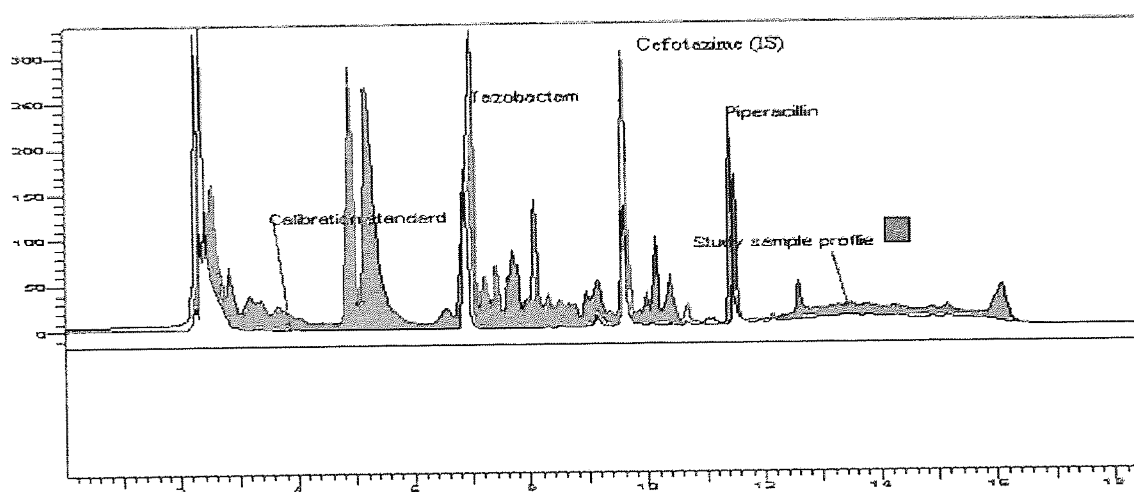


Figure 7.11 A superimposed HPLC chromatogram of a study sample profile, and a calibration standard containing tazobactam, piperacillin and the internal standard cefotaxime.

Table 7.21 Recovery of piperacillin and tazobactam from patient peritoneal dialysis samples

Subject ID	Piperacillin (500µg/mL)			Tazobactam (62.5µg/mL)		
	Pre treatment concentration (µg/mL)	Post treatment concentration (µg/mL)	Percentage absorption	Pre treatment concentration (µg/mL)	Post treatment concentration (µg/mL)	Percentage absorption
1	BLQ	31.56	93.69	2.04	4.56	55.20
2	20.07	19.55	96.09	17.35	15.64	37.48
3	BLQ	10.75	97.85	BLQ	BLQ	
4	BLQ	25.85	94.83	29.41	67.52	
5	25.49	33.39	93.32	1.97	2.25	58.9
6	BLQ	3.67	99.27	19.37	3.86	56.32
7	BLQ	6.09	98.78	26.69	1.67	59.83
8	BLQ	BLQ		14.55	12.01	43.28
9	BLQ	12.43	97.51	5.08	5.03	54.45
10	BLQ	40.52	91.90	16.17	4.79	54.84
11	35.97	25.8	94.84	5.83	4.75	54.9
12	4.63	4.33	99.13	17.06	12.78	42.05

The absorption of piperacillin from dialysis fluid in the peritoneum ranged from 91-99% however absorption of tazobactam was found to be much lower (37-59%). In addition, piperacillin was generally less likely to be retained in the peritoneal fluid between treatments.

7.3.19 Adverse events

None of the patients recruited into the study experienced serious adverse events during or after treatment with piperacillin/tazobactam.

7.3.20 Faecal VRE colonisation rates and *C. difficile* toxin positivity rates in patients treated for CAPD-associated peritonitis with vancomycin plus ceftazidime compared to patients treated with vancomycin plus pip/taz

There was a decrease in the rate of faecal VRE colonisation when patients were treated with vancomycin plus pip/taz, compared to patients treated with vancomycin plus ceftazidime. *C. difficile* toxin detection was similar in both groups. These data are shown in figure 7.12

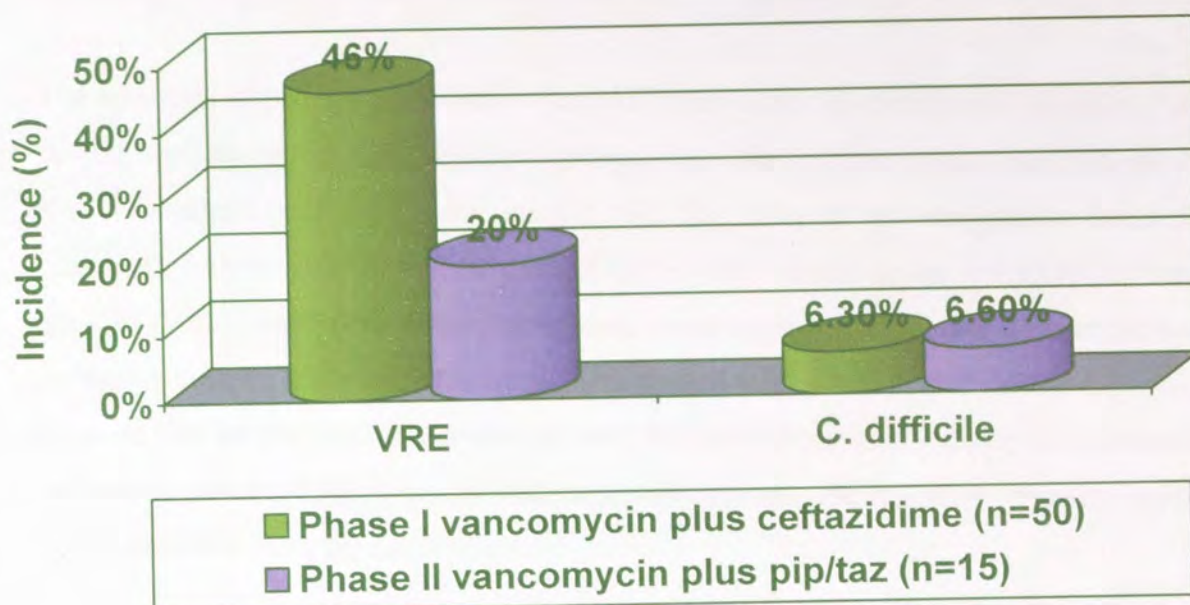


Figure 7.12 Faecal VRE colonisation rates and *C. difficile* toxin positivity rates in patients presenting with CAPD-associated peritonitis treated with vancomycin plus ceftazidime, compared to patients treated with vancomycin plus pip/taz.

7.4 DISCUSSION

The mean age of patients presenting with CAPD-associated peritonitis and the ratio of male to female were similar in both treatment groups (vancomycin plus ceftazidime 58 years; 33:17 and vancomycin plus pip/taz 57 years; 11:6). The types of microorganisms isolated from peritoneal dialysis effluent culture did not differ with the change in antimicrobial treatment for CAPD-associated peritonitis. In addition, there were no significant differences between peritonitis relapse and cure rates in patients treated with either antimicrobial regimen. This suggests that as the common routes of entry for microorganisms causing CAPD-associated peritonitis remained the same in both treatment groups, the selective pressure applied to commensal skin flora appeared unaltered.

In phase I of the study, the incidence of colonisation with VRE was shown to increase with extended hospital attendance (table 7.14). This was further supported by the recovery of VRE from dialysis equipment in the CAPD unit that was genotypically identical to those isolated from dialysis effluent. This illustrates that the patient's environment is contaminated with VRE and consequently, supports the acquisition of VRE from the hospital environment and the horizontal transfer of microorganisms between patients.

Faecal VRE colonisation rates showed a decrease in those patients treated with vancomycin plus pip/taz. However, this was probably due to the small number of patents recruited in phase II. *E. faecium* was the most common enterococcal species recovered from faecal samples in both treatment groups, however in those patients treated with vancomycin plus pip/taz no *E. durans* isolates were recovered. This may suggest that *E. durans* were more susceptible to pip/taz and as such, its effect on the gut flora selected for *E. faecium* proliferation. However it may also have been due to the disproportionate numbers in the two treatment groups.

The analysis of macrorestriction profiles showed heterogeneity amongst the enterococcal isolates recovered from dialysis effluent; however, the fragment profiles of *E. durans* isolates showed relatedness between strains. This supports the assumption that the selective pressure of vancomycin plus ceftazidime on gut flora promotes the proliferation of *E. durans*, whereas treatment with pip/taz suggests greater biocidal activity. When strains isolated from each treatment group were compared according to the Tenover criteria (Tenover, *et al.,* 1995) no relatedness was established.

The rate of *C. difficile* toxin detection in faecal samples remained the same in both treatment groups. This was an unexpected result due to the superior activity of pip/taz against *Clostridium* species as compared to cephalosporins (Bradley, *et al.,* 1999) (Spangler, *et al.,* 1994). A report to the Department of Health by the National *Clostridium difficile* Standards Group (2003) (C Difficile Standards Group, 2004) reported evidence suggesting that the incidence of faecal carriage of *C. difficile* is more prevalent in those over 65 years of age. There are a large number of elderly patients within the renal population furthermore, in phase I of the study 16 out of 50 patients were over 65 as compared to 3 out of 15 patients in phase II. This therefore did not support the evidence put forward by the National *Clostridium difficile* Standards Group, nonetheless it may reflect treatment modality. Patients undergoing CAPD are treated in their own homes, only go to the hospital in cases of peritonitis or to attend clinic appointments, and so are less likely to acquire *C. difficile* from the hospital environment. Furthermore, *C. difficile* toxin detection assays may not necessarily reflect the presence of the organism as some strains are not toxin producers and it is thought that toxin is not present in formed faecal samples. All of these patients were asymptomatic at the time of specimen collection and therefore maybe isolation of *Clostridia* by culture would be a better form of identification. This would lend itself to genotypic investigation and detection of endemic strains, thus enabling specific treatment based on sensitivity testing as opposed to a blanket regimen of metronidazole.

Recovery of piperacillin and tazobactam from dialysis effluent by HPLC showed good reproducibility in terms of accuracy and precision for both compounds at a range of concentrations. It is estimated that approximately 100mL of fluid is naturally contained within the peritoneal cavity and therefore this volume will remain after a dialysis exchange allowing the possible retention of antibiotics within the peritoneum between treatments. The recovery rates of piperacillin showed all patients had a greater than 90% absorption of piperacillin during the 6-8 hour dwell time. In addition, most patients (8 out of 12) had concentrations of piperacillin that were below the limit of quantitation prior to treatment, this suggests that residual piperacillin was eluted from the peritoneal cavity between treatments and therefore even with the extended half-life in patients with renal impairment there is little or no antimicrobial protection between treatments with piperacillin.

Tazobactam was rarely eluted from the peritoneal cavity between treatments and absorption ranged from 37% to 59%. The metabolism of tazobactam is via glomerular filtration where 80% of the dose is excreted as unchanged drug (Wyeth Information Sheet, 2000). This

suggests that although tazobactam is widely distributed in tissues such as the intestinal mucosa, it is not easily metabolised and the absorption rates shown in table 7.21 are typical of the drug's pharmacokinetic profile. The persistence of tazobactam concentrations in the peritoneal cavity may represent diffusion from tissue back to peritoneal fluid due to a decreasing concentration gradient within the peritoneal fluid following elution of the initial treatment. In addition, the half-life of tazobactam is increased 4-fold in patients with a GFR <20mL/min and has been reported to accumulate in patients with impaired renal function (Valtonen, *et al.,* 2001). Since the recovery of 41%-63% of tazobactam from dialysate effluent was as unchanged drug, it could be postulated that tazobactam may give some enduring piperacillin-protection between treatments, when patients are infected with β lactamase producing microorganisms.

The primary aim of conducting a safety study was to determine the safety and efficacy of the intraperitoneal administration of pip/taz and results showed that no serious adverse events were experienced during or after completion of IP treatment with pip/taz. However, one patient experienced a rash and another had joint aches. In both cases, stopping therapy alleviated symptoms and neither patient required additional treatment.

In the case of patients treated with pip/taz who presented with Gram-negative peritonitis (n=5) three patients were readmitted to hospital within 48h with un-clearing peritonitis and in 2 of these patients this resulted in peritoneal catheter removal. The prescribed dose of pip/taz administered into a 2L dialysate bag was 1.125g each night. The recommended MIC_{90} values for pip/taz against common Gram-negative microorganisms recovered from dialysis effluent such as *K. pneumoniae, E. coli* and *P. aeruginosa* are reported as 4-128mg/L, 1-32mg/L and 8-256mg/L respectively (Adis Drug Evaluation, 1999). While these concentrations were achieved during treatment, the HLPC results show that once the dialysate containing pip/taz was eluted from the peritoneum, the required MIC for bactericidal activity was not met and microorganisms might proliferate between treatments.

There is a paucity of studies investigating the intraperitoneal (IP) administration of piperacillin/tazobactam however; (Zaidenstein, *et al.,* 2000) investigated the pharmacokinetics of IP pip/taz in patients on CAPD with and without *Pseudomonas* peritonitis. All patients (n=6) received a 4g IP loading dose then 24 h later a maintenance dose of 0.5g was administered with each dialysate bag for 1 week. This achieved a peak plasma concentration of piperacillin, which was 51.6mg/mL at 45 min. During the

maintenance period, plasma piperacillin concentration was 5.2mg/mL. Tazobactam was only detected in the plasma of 1 patient. They concluded that a 4g loading dose resulted in plasma concentrations comparable to IV administration. This was not extended to tazobactam. During the maintenance period they reported that 0.5g of pip/taz resulted in piperacillin concentrations below the MIC for *Pseudomonas* species.

In contrast, the recommended IV dose for mild/moderate infection is 2.25g every 6 – 12h and for severe infection 4.5g every 6-8h by slow intravenous infusion. Patients should receive treatment until 48h after symptoms have subsided (Bryson and Brogden, 1994); (Perry and Markham, 1999). In healthy volunteers and patients with intra abdominal infections, pip and taz each have plasma elimination half-lives of 0.8-1h. Piperacillin and tazobactam have been shown to achieve concentrations of >60mg/kg and >8mg/kg respectively in the GI tract (with the exception of the omentum) (Kinzig, *et al.,* 1992). Halstenson *et. al.* (1994) demonstrated that 3.375g pip/taz achieves a predicted maximum steady state plasma concentration of the inactive tazobactam M1 metabolite of 48.8 μg/mL in patients on CAPD when dosed every 12 hours. Bacterial eradication is reported to range from 76-100%.

Kim *et. al.* (2001) suggests that based on pharmacodynamic principals the dosing interval for pip/taz can be extended if larger doses are given (9 and 6.75g per 12h). They demonstrated that pip/taz was 50% T > MIC for most pathogens including *Pseudomonas aeruginosa*. Conventional dosing of 3.375-4.5g only achieve 20-30% T >MIC for pathogens with an MIC of 64mg/L.

In renal patients, the increase in dose (9g and 6.75g) results in an increase in $t_{½}$ 5.6h and 4.6h respectively which will be further increased due to renal impairment. Therefore, administration of 9g pip/taz would increase the half-life to 11.2h for piperacillin and 22.4h for tazobactam. Kim and colleagues (2001) also demonstrated that with the higher dose (9g) the mean trough concentration was significantly higher than in the 6.75g dosing regime (4.2mg/L and 3.0 mg/L respectively). This suggests that the 9g/12h regime may have a beneficial impact on piperacillin efficacy against β-lactamase producing pathogens.

Pedeboscq (2001) showed that when pip/taz (12g/day) is given on a continuous regime the %T > MIC was 100% for enterobacteria and 99% for *Pseudomonas* species. When given on an intermittent regime (4g TDS) the %T > MIC decreased to 74% and 62% respectively. They also demonstrated that tazobactam concentration was low and often undetectable in between

each injection in the intermittent regime. They concluded that intermittent administration allowed periods prior to each injection where the concentration of piperacillin was below the MIC for most organisms and taz concentrations were below the efficacy threshold.

In summary, it is recognised that the small number of patients recruited in phase II may have had an effect on the diversity of VRE species recovered from faecal samples and a larger sample may have enabled significant conclusions to be drawn. However, previous studies indicate that the results obtained in this study may have been impeded by the prescribed dose of pip/taz administered. It is suggested that the dose and the administration regimen may be insufficient to achieve clinically therapeutic concentrations of pip/taz, for effective bactericidal activity between treatments. In addition, the need for *Clostridia* culture and genotypic investigation to support valid epidemiological study is recommended. In preventing the spread of enteric microorganisms in the hospital, environment strict cleaning regimens must be employed. The similarity in VRE colonisation rates and presence of *C. difficile* toxins in faecal samples from both treatment groups and the low incidence of adverse events demonstrates that treatment of patients with CAPD-associated peritonitis with vancomycin plus pip/taz is equal to treatment with vancomycin plus ceftazidime.

CHAPTER 8; GENERAL DISCUSSION

8.1 RENAL DISEASE

Chronic renal failure (CRF) is a consequence of progressive disease that includes immunoglobulin A nephropathy, hypertension, diabetes and systemic lupus erythematosus. In the USA diabetic nephropathy, hypertension and glomerulonephritis cause approximately 75% of all adult cases of ESRD (Krause, *et al.,* 2002). Patients with ESRD often have a complex array of comorbid processes that reduce the survival rate and impede the management of their renal disease; such processes include ischaemic heart disease, diabetes and immunodeficiency.

Management of patients with ESRD is therefore a complicated process. The long-term goal, where possible, is transplantation, however this method of treatment presents with its own complications due firstly to a lack of organ donors and secondly to organ rejection. In 2001 only 46.6% of ESRD patients in the UK benefited from transplantation (Renal Registry 2002), of the remaining patients 37.1% were managed by means of haemodialysis and 16.3% by peritoneal dialysis. It is estimated that there are approximately 15,000 people in the UK being treated with dialysis (Smith, 2002). The National Renal Register (2002) reported that the number of patients being treated with dialysis is increasing annually by 566 per million of the population (7%). This is attributed primarily to the ageing population, increasing life expectancy of patients with a high risk of ESRD and the growth of population subsets with a greater risk of ESRD. Without dialysis, the patient is unable to regulate electrolyte, fluid and acid-base balance. This results in an accumulation of toxic products from amino acid metabolism in the serum. Presenting clinically with a range of symptoms that include anorexia, nausea, peripheral neuropathy, anaemia and abnormalities in white cell and platelet function, leading to increased susceptibility to infection. The 2002 annual report from the Renal Registry announced a 33% increase in infection related deaths in renal patients (Renal Registry, 2002).

Patients with ESRD are a high-risk group for the acquisition of infection. The most frequent microorganism isolated from clinical samples in these patients is Gram-positive cocci, specifically staphylococci. Much work has been published in the scientific press suggesting the nature of opportunistic infection in patients with ESRD treated with RRT and strategies for its control.

8.2 AIMS OF THE STUDY

The aims of this study were to:

1a. Determine the rate of infection associated with renal dialysis catheters by means of a novel clinical audit tool and to identify common causative microorganisms.

1b. Establish genotypic macrorestriction profiles of CNS strains associated with infection in dialysis catheters.

1c. Identify specific CNS genotypes that may predict the likelihood of infection in renal dialysis catheters.

1d. Identify the phenotypic characteristics associated with staphylococcal strains associated with infection in dialysis catheters.

2a. Investigate the value of haematological and serological diagnosis of infection in renal dialysis patients.

2b. Assess any identified phenotypic characteristics as markers of infection in renal patients with dialysis catheters *in situ*.

3a. Determine the rate of faecal carriage of vancomycin-resistant enterococci and *Clostridium difficile* toxin in renal patients treated with intraperitoneal ceftazidime and vancomycin for CAPD-associated peritonitis.

3b. Determine the rate of faecal carriage of vancomycin-resistant enterococci and *Clostridium difficile* toxin in renal patients treated with piperacillin/tazobactam and vancomycin for CAPD-associated peritonitis.

8.3 SURVEILLANCE OF INFECTION

Surveillance of infection in renal patients at UHB Trust is a complex task. There are over 800 patients being treated in nine dialysis units, six of which are located in different geographical locations around the West Midlands. Each dialysis unit records their own infection data however there is no central database to collect all of the data generated, therefore clinical audit of infection rates across the whole renal service is difficult.

With the reliance on polyurethane catheters for vascular access the risk of infection is greatly increased. The Meditrend audit tool was developed to monitor infection rates in central venous catheters and improve catheter management by suggesting best practice. The results from this section of study indicated that the questionnaire was labour intensive and required

modification for the specific use in the renal population. It did however highlight the need for practice revision and after implementation of an evidence based catheter management protocol, clinical audit of catheter-related infection and catheter management demonstrated the positive benefits of clinical improvement by a reduction in the incidence of CRI within the renal service.

8.4 DIAGNOSIS OF INFECTION

The diagnosis of infection in renal patients is a complex problem due to the effect of the disease process upon many facets of the patients health. Techniques for the diagnosis of catheter-related infection have historically relied upon culture of the distal tip following removal of the catheter, however negative culture results are common despite clinical suspicion. In the absence of visible signs of infection or positive microbiology, clinicians often rely upon changes in biochemical and haematological blood results to predict the activity of the immune system and thus likelihood of infection. The tests most commonly observed include C-reactive protein (CRP), white cell count (WCC), serum albumin and erythrocyte sedimentation rate.

In renal patients, the continuing performance of the dialysis catheter is of optimum importance and therefore methods of diagnosing CRI without catheter removal are invaluable. Assays that detect exocellular antigens have been developed that distinguish serious staphylococcal infection from uncomplicated septicaemia (Christensson, *et al.,* 1985), (Espersen, *et al.,* 1986). Antibody to a number of exocellular *S. aureus* antigens has been demonstrated (Lambert, *et al.,* 1996), (Lambert, *et al.,* 1992), (Krikler and Lambert, 1992). An enzyme-linked immunosorbent assay (ELISA) based on the detection of anti-lipid S (a secreted short chain form of lipoteichoic acid) developed at Aston University and the University Hospital Birmingham NHS Trust (Lambert, *et al.,* 2000), (Elliott, *et al.,* 2000) has proved valuable in the detection and diagnosis of infection within the renal service. Patients who had positive microbiology culture results were found to produce high concentrations of anti-lipid S antibody in response to antigenic stimulation with lipid S. Interestingly patients who did not have positive microbiology culture results were also found to be producing anti-lipid S antibodies, albeit at a lower concentration. This suggests that renal patients may have an increased exposure to a wide variety of Gram-positive antigens.

Renal replacement therapy, especially chronic haemodialysis, supports the acquisition and spread of infection within the renal patient population. This can be attributed to several factors that include the prolonged need for vascular access and treatment within an environment where multiple patients receive dialysis concurrently. This presents continual opportunities for horizontal transfer of infectious agents between patients and clinical personnel, either by direct person-to-person contact or indirect contact with contaminated devices, equipment and supplies or environmental surfaces (CDC, 2001b). In addition, haemodialysis patients are immunosuppressed (Hörl, 1999) due to both their disease state and the frequency with which they require the insertion of dialysis needles and catheters. The clinical management of this patient group requires frequent hospital attendance/admission and this increases the risk of exposure to nosocomial pathogens and contraction infection.

These observations were supported by the results of the anti-lipid S antibody concentrations recovered from culture negative patients. In addition, the effect of ESRD on the immune response in this patient group was evident when comparing other routine immune markers with the anti-lipid S antibody concentrations. The ESR was higher than the upper normal limit in virtually all of the patients tested, suggesting that dialysis associated anaemia impacts on the function of the immune system. This makes diagnosis of infection based on immune markers alone unreliable.

Much work has discussed the immune deficiency in this patient group however, this study found that the majority of renal patients are able to secrete antibody to lipid S. In contrast, the antibody response to hepatitis B vaccine was shown to be low (25%) in this group of patients. This suggests that antibodies to hepatitis B have specific epitopes for the recognition of that antigen, whereas anti-lipid S antibodies have epitopes that recognise a variety of Gram-positive antigen. In those patients who did not produce antibody to lipid S the AOLC test was able to detect microorganisms in blood samples thus complimenting the anti-lipid ELISA.

8.5 MOLECULAR IDENTIFICATION OF MICROORGANISMS

The most frequently isolated microorganism from renal patients is staphylococci. PFGE of macrorestricted chromosomal DNA is ideally suited to the genotyping of staphylococci. The choice of restriction enzyme (RE) affects the number of bands generated in the profile; *Sma*I (cutting at CCC↓GGG) has been reported to be the most suitable RE for use in PFGE as it provides an adequate number of clearly separated fragments (Snopkovà, *et al.*, 1994). PFGE

supports the identification of the source of infection (Lang, *et al.,* 1999); (Lina, *et al.,* 1992) by allowing the relatedness of strains from the patient, clinical staff and the environment to be examined. In addition, it is an invaluable tool for the differentiation of relapse, recurrence and reinfection in patients with indwelling medical devices such as dialysis catheters (Chang, *et al.,* 2000).

The most common of the staphylococci to be recovered from the clinical specimens of this patient group are CNS. They are ever-present within the environment and on the skin of both patients and attending clinical staff and therefore frequently contaminate specimens. Assessing the clinical significance of CNS isolates recovered from single patient samples can be difficult (Hedin, 1996); (Zaidi, *et al.,* 1996) therefore the availability of multiple specimens from a single patient improves the interpretation of results, as repeated recovery of the same microorganism aids the accurate diagnosis of infection.

The relatedness of staphylococcal strains recovered from different patients was examined. Comparison of macrorestriction patterns of CNS, *S. aureus* and MRSA identified strains recovered from different types of clinical specimens and different patients that were from a common ancestor. This suggests that horizontal transfer of staphylococcal strains between patients is occurring. In addition, the horizontal transfer of staphylococcal strains may also be taking place from staff to patients. Houang and coworkers (1986) reported the transfer of gentamicin-resistant CNS from hospital personnel to patients (Houang *et al.,* 1986). The investigation of such transfer could be undertaken using an anonymised screening programme.

Macrorestriction profile analysis of one patients staphylococcal isolates demonstrated repeated skin infection with an indistinguishable *S. aureus* strain, at the exit site of their haemodialysis catheter. This suggests that the causative strain may have been part of the natural micro flora of the patient and thus was able to reinfect the exit site on completion of antimicrobial treatment. Conversely, the *S. aureus* strain may have been transferred from a specific dialysis nurse at dressing change. This could be confirmed by comparing the macrorestriction profiles of staphylococci recovered from the anterior nares of attending clinical staff and those recovered from the patient.

The migration of microorganisms from one site to another was illustrated in one patient who had 49 positive microbiology specimens over a ten-month period. The patient presented with

a *S. aureus* infection in a suture line after a graft insertion. Two weeks later, an indistinguishable *S. aureus* strain was recovered from a blood culture. This suggests the transfer of the strain from the skin into the systemic circulation. A closely related *S. aureus* strain with the addition of one band was then recovered from the infected graft site. This may be the result of a point mutation in the chromosome creating an extra restriction site. The following month another closely related *S. aureus* strain was recovered from a blood culture. This strain had lost a restriction site and thus exhibited one band less. This strain was subsequently recovered from the haemodialysis catheter exit site and the tip of the catheter when it was explanted.

This section of study demonstrated the extensive migration of common commensal flora to different anatomical patient sites, transfer between patients and the wider dialysis environment.

8.6 PHENTOTYPIC CHARACTERISTICS OF STAPHYLOCOCCI

The selective pressure placed on microorganisms to evolve in order to proliferate in an ever-changing environment is driven by the developing practice of modern medicine. The ability of staphylococci to express a variety of phenotypic characteristics that enable them to adapt to these changes was investigated.

Bacterial adherence to and growth on biomaterials have been shown to be important contributing factors to CNS infection (Karamanos, *et al.*, 1997). Attachment of staphylococci onto a polymer surface is the first step of prosthetic device related infection (figure 1.3) (Hogt, *et al.*, 1987). The blockade of this first step is reported to significantly reduce the ability of a microorganism to cause infection (Takeda, *et al.*, 1991). CNS are recognised as significant pathogens causing infection in patients with indwelling medical devices, including peritoneal and haemodialysis catheters (Elliott, 1988); (Christensen, *et al.*, 1985). Bacterial adherence to biomaterials is mainly attributed to CNS strains that express extracellular highly adhesive material known as slime. Slime is described as a macroscopic adherent biofilm (Peters, *et al.*, 1982). The expression of slime promotes interbacterial shielding and adherence between bacterial cells and synthetic devices or biological tissues (Ishak, *et al.*, 1985); (Christensen, *et al.*, 1982). The mechanisms of biofilm formation are not fully understood, however, it is postulated that two distinct pathogenic mechanisms contribute to colonisation and biofilm formation (Christensen, *et al.*, 1990). Rapid initial adhesion of bacterial cells to a

polymer surface is mediated by protein and/or carbohydrate bacterial adhesins accumulated in multiplayer cell clusters. These are embedded within an exopolymer substance and formation occurs over a period of hours with the presence of an *S. epidermidis* polysaccharide antigen (Mack, *et al.*, 1992).

In this section of study, 98% of CNS strains associated with renal patients with clinically significant infection expressed the ability to adhere to polystyrene. Indeed, 83% of CNS strains isolated from the ante-cubital fossa of non-septic volunteers expressed adhesins. This suggests that while adherence to polymers may be essential to facilitate infection in indwelling medical devices, it is also essential for adherence in the local environment (i.e. skin cells). Therefore, the ability of a CNS strain to express adhesins is not specifically a measure of its pathogenicity. These data support the work of Lang (2000).

Lipase production by staphylococci has been associated with deep-seated infections such as septicaemia as opposed to superficial infections including impetigo; this suggests that this enzyme plays a role in tissue invasion (Rollof, *et al.*, 1987). The CNS strains in this study were recovered from patients with invasive infections and as illustrated in the work by Rollof *et al.* (1987) a significant number were found to express lipase compared to the non-septic volunteers ($p<0.0001$). Irrespective of the method of lipase/esterase detection, 92% of patients with CAPD-associated peritonitis and CRI expressed lipase/esterase, compared to 62% of the non-septic volunteers. This suggests that the expression of both specific and non-specific lipase/esterase is important for tissue invasion and biofilm formation and is implicated in the pathogenicity of device-related infections (Rupp, *et al.*, 1999a); (Rupp, *et al.*, 1999b).

The ability of staphylococci to express lytic enzymes was investigated using horse RBC. The production of δ-toxin in CNS strains from renal patients with CAPD-associated peritonitis, CRI and from the ante-cubital fossa of non-septic volunteers was compared. This toxin is reported to be active against both horse and sheep RBC (Hébert and Hancock, 1985). A microtitre plate method was employed to demonstrate haemolysis after 1h incubation at 37°C. A further 1h incubation at 4°C was included to stimulate "cold-shock" β-toxins to further haemolyse the RBC suspension. This was determined by reading the optical density at 570nm (Jordens, *et al.*, 1989).

This section of study demonstrated that certain phenotypic characteristics are more likely to be seen in CNS strains recovered from patients with infection than in those recovered from

the skin of non-septic volunteers. Based on probability, CNS strains producing a certain combination of phenotypic characteristics are more likely to cause infection in renal patients with indwelling medical devices. Ziebhur *et al.* (1997) reported that one function of the *ica* gene cluster is the initiation of phase variation. This suggests that CNS strains have the ability to modify the expression of phenotypic characteristics to suit their environment. This study has illustrated that the environment from which the CNS strains was recovered influenced both the proportion and combination of phenotypic characteristics expressed. This suggests that CNS strains recovered from non-septic patients cannot be used as a model to predict the likelihood of future infection as all CNS strains retain the ability to undergo phase variation.

8.7 ANTIMICROBIAL TREATMENT OF INFECTION

Antimicrobial treatment of infection is a contentious issue. The overuse of antimicrobial therapy places selective pressure on microorganisms and supports the development of antimicrobial resistant strains. In addition, the disequilibria of commensal flora by antimicrobial agents create a complex situation for clinicians when assessing the treatment options.

In this section of study, patients were assessed for the faecal carriage of vancomycin resistant enterococci and *Clostridium difficile* toxin. Patients treated with intraperitoneal ceftazidime for CAPD-associated peritonitis were found to have a high incidence of faecal VRE carriage (46%). Patients treated with piperacillin/tazobactam had >50% reduction in the faecal carriage of VRE. However, it must be acknowledged that the numbers in the group treated with piperacillin/tazobactam were small. Interestingly, recovery of piperacillin and tazobactam from peritoneal dialysis effluent by HPLC demonstrated that the concentration of piperacillin in the peritoneum between dialysis treatments was below the MIC for Gram-positive microorganisms. This may allow the proliferation of microorganisms within the peritoneum between treatments and contribute to treatment failure.

VRE and *C. difficile* have been shown to survive in the hospital environment and in phase I of the study, the incidence of colonisation with VRE was shown to increase with extended hospital attendance. This was further supported by the recovery of VRE from dialysis equipment in the CAPD unit that was genotypically identical to those isolated from dialysis effluent. This suggests the ward environment is therefore contaminated with VRE and

consequently, supports the acquisition of VRE from the hospital environment and the horizontal transfer of microorganisms between patients.

Previous studies indicate that the results obtained in this section of study may have been impeded by the prescribed dose of pip/taz administered. It is suggested that the dose and the administration regimen may be insufficient to achieve clinically therapeutic concentrations of pip/taz, for effective bactericidal activity between treatments. In addition, the need for *Clostridia* culture and genotypic investigation to support valid epidemiological study is recommended. In preventing the spread of enteric microorganisms in the hospital, environment strict cleaning regimens must be employed. The similarity in VRE colonisation rates and presence of *C. difficile* toxins in faecal samples from both treatment groups and the low incidence of adverse events demonstrates that treatment of patients with CAPD-associated peritonitis with vancomycin plus pip/taz is equal to treatment with vancomycin plus ceftazidime.

In summary, patients with renal failure are a rapidly expanding group at a rate of 7-8% per year. It is well established that they are a high-risk infection group and this will not change over time. This body of work has verified the widespread migration of microorganisms within and between patients and the environment in which they are treated. Macrorestriction analysis of local staphylococcal strains identified indistinguishable isolates recovered from different patients treated with different modalities. Furthermore, the ability of microorganisms to evolve and exploit their host by the production of phenotypic characteristics has been highlighted. Treatment protocols employed in the management of renal dialysis patients have been shown to promote the acquisition and proliferation of local staphylococcal strains and encourage antimicrobial resistance. The detection and diagnosis of infection in renal patients in the absence of positive microbiology is difficult. Comorbid processes have been shown to modulate the immune response and the exposure to Gram-positive antigenic stimulus within the clinical environment, creates a steady state of immune activation, reducing the diagnostic value of routine immune markers.

Improvement of infection rates in this patient group will only be achieved by the continual development of the service we provide. This would include reducing the number of polyurethane dialysis catheters, surveillance of local microbial strains by macrorestriction analysis and exploring alternative treatment options such as probiotic therapy. In addition,

reduction in the microbial load within the treatment areas, by improved infection control practices and the use of air filters in the dialysis units would reduce the risk of infection.

8.8 FUTURE WORK

A natural progression of the work presented in this thesis would include the further development of the Meditrend audit tool to encompass long-term tunnelled dialysis catheters. In addition, a computer programme that enables data collection on the incidence of catheter-related infection and catheter survival rates over time would be of clinical value.

In conjunction with clinical practice improvements a surveillance programme of mupirocin resistance could be developed. The collection of these data within the UHB renal service has recently been introduced however, at UHB there is no data on high level mupirocin resistance prior to the introduction of prophylactic use at the exit site of newly implanted dialysis catheters. Ongoing collection of these data would provide an informed view of the impact clinical practice is having on the local microbial population.

Further investigation of the anti-lipid S IgG antibody over time may enable recommendations to be developed concerning the point at which antimicrobial intervention in renal patients is appropriate. Moreover, serological testing from the onset of infection followed by collection of serum samples at specific time points for anti-lipid S and WCC, CRP and ESR may provide data to support the development of treatment protocols for catheter-related infection.

Horizontal transfer of staphylococci between patients and their environment has been established by macrorestriction profile analysis. The role clinical personnel play in the transfer of these microorganisms at UHB has yet to be established. The next step would be to investigate the extent of colinisation of clinical personnel by the introduction of an anonymised screening programme that would serve to compare the macrorestriction profiles of clinical personnel to those obtained from patient routine screening swabs. This would provide an evidence base for the continuing improvement of infection control practices within the dialysis setting.

The ability to produce intercellular adhesins is the first step in the pathogenesis of device-related infections. The production of this adhesin has been shown to be regulated by the ica gene cluster therefore investigation of the prescence of this gene by southern hybridisation and northern blot analysis. This may help to distinguish pathogenic from non-pathogenic strains and therefore highlight clinically significant CNS infection from contaminated samples.

A detailed epidemiological study of *Clostridium* species within the renal service would be of great clinical value. Investigation of the genotypic and phenotypic characteristics of local strains and antibiotic sensitivity testing would be of great clinical interest. In addition, a study of the impact of probiotic therapy on the incidents of *C. difficile* associated diarrhoea in hospitalised patients, could significantly improve patient experience and save lives.

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APPENDICES

APPENDIX 1: MEDITREND QUESTIONNAIRE

Patient Hospital Registration Number

Please ask the patient the following questions.

1. **Have you had any infections in the last 6 months?** YES/NO
   If **YES** please fill in the following:

Site	**Date**	**Duration**	**Treatment given**	**Outcome**

2. **Do you suffer with any skin problems?** YES/NO
   If **YES** please fill in the following:

Skin Complaint	**Onset**	**Site**	**Treatment given**	**Current status**

3. How many catheters has the patient previously had inserted?

a. ☐ None **b.** ☐ 1-3 **c.** ☐ 4-7 **d.** ☐ 8-10 **e.** ☐ >10

1.0 Patient Details

1.1 Audit Number | Ward Number | Date of catheter insertion

1.2 Date of Birth | Hospital Registration Number | What date was the patient admitted?

1.3 Ethnicity | Gender

1.4 Clinical details/comorbid processes

1.5 Was the patient?
- a. ☐ Surgical
- b. ☐ Medical

1.6 What is the location of the patient **after** catheter insertion?
- a. ☐ Ward (please state) ....
- b. ☐ ITU
- c. ☐ High Dependency Unit
- d. ☐ Discharged Home
- e. ☐ Other

2.0 Catheter Insertion Details

2.1 What month was the catheter inserted?
- a. ☐ January
- b. ☐ February
- c. ☐ March
- d. ☐ April
- e. ☐ May
- f. ☐ June
- g. ☐ July
- h. ☐ August
- i. ☐ September
- j. ☐ October
- k. ☐ November
- l. ☐ December

2.2 What year was the catheter inserted?
- a. ☐ 1999
- b. ☐ 2000
- c. ☐ 2001
- d. ☐ 2002
- e. ☐ 2003
- f. ☐ 2004

2.3 Who inserted the catheter?

a. ☐ Senior Anaesthetist/Intensivist
b. ☐ Intensivist
c. ☐ Senior Surgeon
d. ☐ Junior Surgeon
e. ☐ Radiographer
f. ☐ Consultant Physician
g. ☐ Registrar
h. ☐ SHO

2.4 Where did the insertion take place?

a. ☐ Ward (please state) ....
b. ☐ Theatre (please state) ....
c. ☐ Intensive Care Unit
d. ☐ High Dependency Unit
e. ☐ Accident and Emergency
f. ☐ Radiology
g ☐ Procedure Room (E4A)

2.5 Was it tunneled?

a. ☐ Yes
b. ☐ No

2.6 What was the biomaterial used to make the catheter?

a. ☐ Polyurethane
b. ☐ Polyurethane + Hydrophilic coating
c. ☐ Polyurethane + Antiseptic
d. ☐ Polyurethane + Antibiotic
e. ☐ Silicone
f. ☐ PTFE
g. ☐ Other (please state)

2.7 What type of catheter was used?

a. ☐ Single lumen central line (Go to section 4)
b. ☐ Double lumen central line (Go to section 4)
c. ☐ Triple lumen central line (Go to section 4)
d. ☐ Quad lumen central line (Go to section 4)
e. ☐ Quin lumen central line (Go to section 4)
f. ☐ PICC (Go to section 3)
g. ☐ Sheath Introducer (Go to section 4)
h. ☐ Midline (Go to section 3)
i. ☐ Vascular Port (Go to section4)
j. ☐ CVC&Sheath introducer (Go to section 4)
k. ☐ PA& Sheath introducer (Go to section 4)
l. ☐ Tessio (Go to section 4)
m. ☐ Other (please state)

3.0 Parenteral Nutrition

3.1 What was the patients Body Mass Index?

a. ☐ Low (<19)
b. ☐ Normal (20-30)
c. ☐ High (=31)

3.2 How long was long the catheter inserted before PN commenced?

a. ☐ One day
b. ☐ Two days
c. ☐ Three days
d. ☐ Four days
e. ☐ Five days
f. ☐ > five days

3.3 Was lipid infused?

a. ☐ Yes
b. ☐ No

4.0 Catheter and Insertion Details

4.1 Which side is the catheter entry site?

a. ☐ Left — **b.** ☐ Right

4.2 Is the catheter entry site:

a. ☐ Subclavian
b. ☐ Internal Jugular
c. ☐ External Jugular
d. ☐ Femoral
e. ☐ Other

4.3 Was a gloved finger used to enlarge the insertion site?

a. ☐ Yes (Go to 7) — **b.** ☐ No (Go to 6)

4.4 What was the insertion site enlarged with?

		Yes	No
6.1	Dilator	☐	☐
6.2	Scalpel	☐	☐
6.3	Surgical procedure	☐	☐
6.4	Other	☐	☐

4.5 Were any of the following connectors fitted to the hubs?

		Yes	No
5.1	Stopcock	☐	☐
5.2	Needleless IV access valve	☐	☐
5.3	Injection port	☐	☐
5.4	IV line	☐	☐
5.6	Luer lock plug	☐	☐

4.6 What catheter insertion technique was used?

a. ☐ Seldinger
b. ☐ Cut down
c. ☐ Introducer
d. ☐ Guidewire exchange
e. ☐ Tunneled
f. ☐ Surgical
g. ☐ Other

4.7 Was the same vessel used for any other cannulation?

a. ☐ Yes — **b.** ☐ No

4.8 What were the patient risk factors at the time of catheter insertion?

		Yes	No
9.1	Assisted ventilation	☐	☐
9.2	Trachiostomy	☐	☐
9.3	Concurrent central line	☐	☐
9.4	PN infusion	☐	☐
9.5	Anticoagulant therapy	☐	☐
9.6	Colonization by resistant microorganisms	☐	☐
9.7	Neutropaenic	☐	☐
9.8	Immunocompromised	☐	☐
9.9	Other (please state)	☐	☐

4.9 Prior to insertion were hands washed?

a. ☐ Yes — **b.** ☐ No

4.10 Was hand disinfectant used?
a. ☐ Yes **b.** ☐ No

4.11 Which of the following were used in the preparation procedure?

		Yes	No
11.1	Gloves	☐	☐
11.2	Drapes	☐	☐
11.3	Gowns	☐	☐
11.4	Mask	☐	☐
11.5	Cap	☐	☐

5.0 Insertion Site Preparation and Dressing.

5.1 What type of skin disinfectant was used on the insertion site?
a. ☐ Isopropyl alcohol
b. ☐ Chlorhexidine/Aqueous %( )
c. ☐ Chlorhexidine/Alcohol %( )
d. ☐ Iodine
e. ☐ Other (please state)
f. ☐ None

5.2 How long prior to cannulation was disinfectant used?
a. ☐ Less than two minutes **b.** ☐ More than two minutes

5.3 What type of dressing was used immediately following insertion?
a. ☐ Non-occlusive semi-permeable dressing
b. ☐ Sterile gauze/dry dressing
c. ☐ Occlusive
d. ☐ Non-sterile gauze/dry
e. ☐ IV3000
f. ☐ Tielle
g. ☐ None

5.4 Did the dressing contain an antimicrobial?
a. ☐ Yes **b.** ☐ No

FOR CATHETER INSERTION COMPLETE TO THIS POINT

6.0 Insertion Site Management

6.1 What type of dressing was used in the ward?
a. ☐ Non-occlusive semi-permeable dressing
b. ☐ Sterile gauze/dry dressing
c. ☐ Occlusive
d. ☐ Non-sterile gauze/dry
e. ☐ IV3000
f. ☐ Tielle
g. ☐ None

6.2 Did the dressing contain an antimicrobial?
a. ☐ Yes **b.** ☐ No

6.3 If the dressing appeared soiled was it changed?
a. ☐ Yes **b.** ☐ No

6.4 How often were dressings changed?
a. ☐ Every day
b. ☐ 2-3 day intervals (at dialysis)
c. ☐ 4-7 day intervals
d. ☐ Less than once a week

6.5 What was used to clean the insertion/exit site at dressing change?

- **a.** ☐ Normal saline
- **b.** ☐ Iodine/Povidone
- **c.** ☐ Isopropyl Alcohol
- **d.** ☐ Iodine/ Alcohol
- **e.** ☐ Chlorhexadine/ Alcohol
- **f.** ☐ Chlorhexadine/Aqueous
- **g.** ☐ Soap and Water
- **h.** ☐ Not cleaned

6.6 How frequently is the catheter insertion/exit site cleaned?

- **a.** ☐ Every day
- **b.** ☐ 2 - 3 days (at dialysis)
- **c.** ☐ > 3 days
- **d.** ☐ Not cleaned

7.0 IV line management

7.1 Was PN being infused?

- **a.** ☐ Yes
- **b.** ☐ No

7.2 Was a dedicated lumen used to infuse PN?

- **a.** ☐ Yes
- **b.** ☐ No

7.3 Was the catheter flushed after line usage?

- **a.** ☐ Yes
- **b.** ☐ No

7.4 Did any of the lumens become blocked?

- **a.** ☐ Yes
- **b.** ☐ No

7.5 Which lumen became blocked?

- **a.** ☐ Arterial
- **b.** ☐ Venous

7.6 Post insertion did the blockage occur after:

- **a.** ☐ 0-3 days
- **b.** ☐ 4-7 days
- **c.** ☐ 1-2 weeks
- **d.** ☐ 2-4 weeks
- **e.** ☐ 1-6 months
- **f.** ☐ More than 6 months

7.7 Which flush was used to unblock the catheter?

- **a.** ☐ Urokinase
- **b.** ☐ Heparinised saline
- **c.** ☐ Saline
- **d.** ☐ Heparin
- **e.** ☐ Other
- **f.** ☐ None

7.8 Was an endoluminal brush used to unblock the catheter?

- **a.** ☐ Yes
- **b.** ☐ No

8.0 Catheter Removal

8.1 How long was the catheter in situ?

- **a.** ☐ Up to 24 hours
- **b.** ☐ 1-3 days
- **c.** ☐ 3-4 days
- **d.** ☐ 5-7 days
- **e.** ☐ 8-30 days
- **f.** ☐ More than 30 days

8.2 Why was the catheter removed?

- a. ☐ No longer required
- b. ☐ Blocked
- c. ☐ Suspected infection
- d. ☐ Extended duration of catheterisation
- e. ☐ Misplacement
- f. ☐ Accidental removal
- g. ☐ Other

8.3 Was the catheter replaced by guidewire exchange?

- a. ☐ Yes
- b. ☐ No

9.0 Localised Infection at the Insertion Site

9.1 Did the patient have an infection at the insertion site?

- a. ☐ Yes (Go to 9.2)
- b. ☐ No (Go to 10)

9.2 How many days post insertion did the infection develop?

- a. ☐ 0-3 days
- b. ☐ 4-7 days
- c. ☐ 1-2 weeks
- d. ☐ 2-4 weeks
- e. ☐ Greater than 1 month

10.0 Catheter-Related Sepsis

10.1 Did the patient have a systemic infection associated with the catheter?

- a. ☐ Yes (Go to 10.2)
- b. ☐ No (Go to 11)

10.2 How many days was the catheter was in situ before systemic infection was diagnosed?

- a. ☐ 0-3 days
- b. ☐ 4-7 days
- c. ☐ 1-2 weeks
- d. ☐ 2-4 weeks
- e. ☐ 1-6 months
- f. ☐ More than 6 months

10.3 If the patient did have a systemic infection, what was the infection profile?

	Yes	No
18.1 Pyrexia (low grade < 38.5°C)	☐	☐
18.2 Pyrexia (high grade > 38.5°C)	☐	☐
18.3 Pyrexial and unresponsive to broad spectrum antibiotics	☐	☐
18.4 Septic rigors on catheter flushing	☐	☐
18.5 Hypotension	☐	☐
18.6 CRP	(please state)	

11.0 How many days did the patient stay in hospital?

☐

12.0 Microbiology

12.1 If catheter related sepsis was suspected, was the distal tip of the catheter cultured?

Yes (Go to 12.2)	No (Go to 13)
☐	☐

12.2 Which of the following microorganisms were isolated from the distal tip?

		Yes	No
20.1	Coagulase negative staphylococcus	☐	☐
20.2	Staphylococcus aureus	☐	☐
20.3	Staphylococcus aureus (MRSA)	☐	☐
20.4	Gram-negative aerobic bacilli	☐	☐
20.5	Gram-positive aerobic bacilli	☐	☐
20.6	Yeast	☐	☐
20.7	Other	☐	☐

13.0 If catheter related sepsis was suspected, was a peripheral blood cultures analysed?

Yes	No
(Go to 13.1)	(Go to 14)
☐	☐

13.1 Which of the following microorganisms were isolated from the peripheral blood cultures?

		Yes	No
22.1	Coagulase negative staphylococcus	☐	☐
22.2	Staphylococcus aureus	☐	☐
22.3	Staphylococcus aureus (MRSA)	☐	☐
22.4	Gram-negative aerobic bacilli	☐	☐
22.5	Gram-positive aerobic bacilli	☐	☐
22.6	Yeast	☐	☐
22.7	Other	☐	☐

14.0 If catheter related sepsis was suspected was a blood culture taken via the catheter?

Yes	No
(Go to 14.1)	(Go to 15)
☐	☐

14.1 Which of the following microorganisms were isolated from the blood cultures taken via the catheter?

		Yes	No
24.1	Coagulase negative staphylococcus	☐	☐
24.2	Staphylococcus aureus	☐	☐
24.3	Staphylococcus aureus (MRSA)	☐	☐
24.4	Gram-negative aerobic bacilli	☐	☐
24.5	Gram-positive aerobic bacilli	☐	☐
24.6	Yeast	☐	☐
24.7	Other	☐	☐

15.0 If local infection was suspected, was a swab from the exit site analysed?

Yes	No
(Go to 15.1)	(Go to 16)
☐	☐

15.1 Which of the following microorganisms were isolated from the exit site swab?

	Yes	No
26.1 Coagulase negative staphylococcus	☐	☐
26.2 Staphylococcus aureus	☐	☐
26.3 Staphylococcus aureus (MRSA)	☐	☐
26.4 Gram-negative aerobic bacilli	☐	☐
26.5 Gram-positive aerobic bacilli	☐	☐
26.6 Yeast	☐	☐
26.7 Other	☐	☐

16.0 Patient Outcome

16.1 Were antibiotics given to treat the infection?
a. ☐ Yes **b.** ☐ No

16.2 If yes, which antibiotics were given for related sepsis?

	Yes	No
27.3 Flucloxacillin	☐	☐
27.4 Vancomycin	☐	☐
27.5 Teicoplanin	☐	☐
27.6 Other (please state)	☐	☐

16.3 Did the patient respond to the antibiotics?
a. ☐ Yes **b.** ☐ No

16.4 Was the catheter removed as a consequence of the infection?
a. ☐ Yes **b.** ☐ No

16.5 Did the patient die as a result of sepsis?
a. ☐ Yes **b.** ☐ No

Comments
If you have any further comments, please list them in the space provided below.

APPENDIX 2: HPLC INTRA-BATCH PRECISION AND ACCURACY

The inter- and intra-batch precision and accuracy for pip and taz in dialysis fluid at three different concentrations of 4.5μg/mL (Quality Control Low, QCL), 60μg/mL (Quality Control Medium, QCM) and 185μg/mL (Quality Control High, QCH) are summarised in tables below and described in more detail in the main body of the report.

Inter- batch precision and accuracy for tazobactam and piperacillin

Tazobactam		4.5μg/mL	60μg/mL	185μg/mL
Inter-Assay (Validation Runs-1-3)	Precision, C.V. (%)	2.51	2.56	1.38
	Accuracy %	88.59	92.94	90.62
Piperacillin		4.5μg/mL	60μg/mL	185μg/mL
Inter-Assay (Validation Runs-1-3)	Precision, C.V. (%)	1.29	2.22	0.39
	Accuracy %	94.63	104.64	98.93

Intra-batch precision and accuracy for tazobactam and piperacillin

Tazobactam		4.5μg/mL	60μg/mL	185μg/mL
Intra-Assay (Validation Runs-1-3)	Precision, C.V. (%)	2.07	3.20	2.03
	Accuracy %	87.74	92.63	90.97
Piperacillin		4.5μg/mL	60μg/mL	185μg/mL
Intra-Assay (Validation Runs-1-3)	Precision, C.V. (%)	3.80	4.23	3.20
	Accuracy %	94.20	108.14	102.64

The recovery values of pip and taz obtained from fortified dialysis fluid at two concentrations were 89.2% and 81.7% at concentration of 60μg/mL and 91.4% and 79.8% at concentration of 185μg/mL respectively.

Table 1: Intra-assay accuracy and precision data for tazobactam and piperacillin in Dialysis Fluid
Data from Validation Run 1
Intra-Assay data for tazobactam

	Back calculated values for Tazobactam			
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
1	164.99	53.66	3.96	1.00
2	167.41	56.97	3.89	1.03
3	169.66	55.25	4.08	1.04
4	170.86	54.77	3.93	1.06
5	165.32	57.30	3.92	1.03
6	167.64	56.62	4.14	1.00
Mean	167.65	55.76	3.99	1.03
S.D.	2.32	1.43	0.10	0.02
C.V. (%)	1.38	2.56	2.51	2.28
%Accuracy	90.62	92.94	88.59	102.67

Intra-Assay data for piperacillin

	Back calculated values for piperacillin			
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
1	182.82	60.16	4.30	0.98
2	182.83	63.46	4.17	1.01
3	183.14	63.48	4.28	0.99
4	184.32	62.43	4.29	1.03
5	182.90	64.08	4.21	1.02
6	182.12	63.09	4.30	0.95
Mean	183.02	62.78	4.26	1.00
S.D.	0.72	1.39	0.05	0.03
C.V. (%)	0.39	2.22	1.29	2.95
%Accuracy	98.93	104.64	94.63	99.67

Table 2: Validation data from analytical run 2

Back calculated values for Tazobactam				
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
1	175.94	51.42	3.96	0.93
2	174.56	53.72	3.89	0.93
3	164.05	53.03	3.85	0.94
4	165.36	55.87	3.81	0.95
5	171.97	56.33	4.01	0.94
6	169.55	54.20	4.01	0.91
Mean	170.24	54.10	3.92	0.93
S.D.	4.83	1.82	0.08	0.01
C.V. (%)	2.84	3.37	2.15	1.46
%Accuracy	92.02	90.16	87.15	93.33

Back calculated values for piperacillin				
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
1	194.36	62.32	4.57	0.89
2	191.75	64.07	4.28	0.87
3	187.06	63.11	4.34	0.89
4	185.02	62.57	4.27	0.97
5	193.64	65.40	4.52	0.95
6	191.50	63.53	4.33	0.88
Mean	190.56	63.50	4.39	0.91
S.D.	3.72	1.13	0.13	0.04
C.V. (%)	1.95	1.77	2.92	4.53
%Accuracy	103.00	105.83	97.44	90.83

Table 2A: Validation data from analytical run 3

Back calculated values for Tazobactam				
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
1	166.45	56.48	3.89	1.02
2	166.12	57.63	3.97	1.00
3	166.89	55.92	4.00	1.02
4	165.58	57.94	3.86	0.96
5	170.87	56.13	3.97	0.98
6	165.98	57.13	3.93	1.02
Mean	166.98	56.87	3.94	1.00
S.D.	1.96	0.82	0.05	0.03
C.V. (%)	1.17	1.45	1.36	2.53
%Accuracy	90.26	94.79	87.48	100.00

Back calculated values for piperacillin				
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
1	193.23	68.87	3.94	1.09
2	197.19	68.17	4.04	1.07
3	194.18	67.46	4.24	1.02
4	193.84	68.85	4.06	0.98
5	200.59	68.41	4.12	1.03
6	197.36	68.46	4.04	1.01
Mean	196.07	68.37	4.07	1.03
S.D.	2.83	0.52	0.10	0.04
C.V. (%)	1.44	0.76	2.46	3.90
%Accuracy	105.98	113.95	90.52	103.33

Table 3: Inter-assay accuracy and precision data for tazobactam and piperacillin in dialysis fluid
Data from Validation Runs 1 to 3
Inter-Assay data for tazobactam

	Back calculated values for tazobactam			
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
Run 1-1	164.99	53.66	3.96	1.00
2	167.41	56.97	3.89	1.03
3	169.66	55.25	4.08	1.04
4	170.86	54.77	3.93	1.06
5	165.32	57.30	3.92	1.03
6	167.64	56.62	4.14	1.00
Run 2-1	175.94	51.42	3.96	0.93
2	174.56	53.72	3.89	0.93
3	164.05	53.03	3.85	0.94
4	165.36	55.87	3.81	0.95
5	171.97	56.33	4.01	0.94
6	169.55	54.20	4.01	0.91
Run 3-1	166.45	56.48	3.89	1.02
2	166.12	57.63	3.97	1.00
3	166.89	55.92	4.00	1.02
4	165.58	57.94	3.86	0.96
5	170.87	56.13	3.97	0.98
6	165.98	57.13	3.93	1.02
Mean	168.29	55.58	3.95	0.99
S.D.	3.42	1.78	0.08	0.05
C.V. (%)	2.03	3.20	2.07	4.57
N=	18.00	18.00	18.00	18.00
%Accuracy	90.97	92.63	87.74	98.67

Table 3: Continued
Inter-Assay data for piperacillin

	Back calculated values for piperacillin			
Sample No	QCH-185μg/mL	QCM-60μg/mL	QCL-4.5μg/mL	QCLOD-1μg/mL
Run 1-1	182.82	60.16	4.30	0.98
2	182.83	63.46	4.17	1.01
3	183.14	63.48	4.28	0.99
4	184.32	62.43	4.29	1.03
5	182.90	64.08	4.21	1.02
6	182.12	63.09	4.30	0.95
Run 2-1	194.36	62.32	4.57	0.89
2	191.75	64.07	4.28	0.87
3	187.06	63.11	4.34	0.89
4	185.02	62.57	4.27	0.97
5	193.64	65.40	4.52	0.95
6	191.50	63.53	4.33	0.88
Run 3-1	193.23	68.87	3.94	1.09
2	197.19	68.17	4.04	1.07
3	194.18	67.46	4.24	1.02
4	193.84	68.85	4.06	0.98
5	200.59	68.41	4.12	1.03
6	197.36	68.46	4.04	1.01
Mean	189.88	64.88	4.24	0.98
S.D.	6.07	2.75	0.16	0.06
C.V. (%)	3.20	4.23	3.80	6.57
N=	18.00	18.00	18.00	18.00
%Accuracy	102.64	108.14	94.20	97.94

Table 4: Recovery of tazobactam, piperacillin and cefotaxime from dialysis fluid

	Tazobactam at 60μg/mL level		Tazobactam at 185μg/mL level	
	Extracted Area	Unextracted Area	Extracted Area	Unextracted Area
1	1799872.00	1895975.00	5824573.00	6439565.00
2	1831929.00	1976276.00	5533875.50	6083857.50
3	1727589.00	1976068.50	5591941.00	5946065.00
4	1768279.00	2091326.00	5322549.00	6049185.00
5	1780204.00	2034917.00	5609498.00	5885492.00
6	1764218.00	*	5470687.50	*
Mean	1778681.83	1994912.50	5558854.00	6080832.90
S.D.	35254.79	73121.42	166435.25	215650.13
C.V. (%)	1.98	3.67	2.99	3.55
Recovery (%)	89.16	-	91.42	-

	Piperacillin at 60μg/mL level		Piperacillin at 185μg/mL level	
	Extracted Area	Unextracted Area	Extracted Area	Unextracted Area
1	2008868.00	2386625.00	6050965.00	7836562.50
2	2025471.00	2362646.00	5710905.50	7324310.00
3	1934314.00	2401961.00	5893734.50	7120032.50
4	1975602.00	2598660.00	5573415.50	7329398.50
5	1974735.00	2397903.00	5897801.00	6826408.00
6	1995362.00	*	5754439.50	*
Mean	1985725.33	2429559.00	5813543.50	7287342.30
S.D.	31857.69	95759.57	168278.45	369198.85
C.V. (%)	1.60	3.94	2.89	5.07
Recovery (%)	81.73	-	79.78	-

	Cefotaxime (IS)		Cefotaxime (IS)	
	Extracted Area	Unextracted Area	Extracted Area	Unextracted Area
1	5755458.00	5509080.00	5998426.00	6124261.00
2	5804057.00	5611522.00	5552751.00	5385425.50
3	5597391.00	5644724.00	5755523.00	5277737.50
4	5720780.00	6129088.00	5641142.50	5322609.00
5	5885391.00	6304013.00	6056973.00	5992122.00
6	5730647.00	*	5767757.00	*
Mean	5748954.00	5839685.40	5795428.75	5620431.00
S.D.	95701.58	353101.40	197316.10	404154.44
C.V. (%)	1.66	6.05	3.40	7.19
Recovery (%)	98.45	-	103.11	-

CONFERENCES AND WORKSHOPS ATTENDED

Presentations and tutorials

2000 Presentation of research findings (The Piperacillin/tazobactam Study - Phase I). UHB, NHS Trust, CAPD Unit.

2001 Presentation of Ph.D. research outline. UHB, NHS Trust, Clinical Microbiology.

2001 The Streptococci. UHB, NHS Trust, Microbiology research group seminar.

2001 Presentation of research outline (The Piperacillin/tazobactam study – Phase II). UHB, NHS Trust, CAPD Unit.

2001 Presentation of research outline (The Piperacillin/tazobactam study – Phase II). UHB, NHS Trust, CAPD Unit.

2002 The Classification of Microorganisms. UHB, NHS Trust, CAPD staff training day.

2002 Antibiotics and How They Work. UHB, NHS Trust, CAPD staff training day.

2002 Faecal *Clostridium difficile* carriage rates within the renal unit. UHB, NHS Trust, Renal Seminars.

2002 The use if intra-peritoneal Piperacillin/tazobactam in CAPD patients. UHB, Clinical Microbiology Seminar.

2002 Presentation of research findings (The Piperacillin/tazobactam study - results to date). UHB, NHS Trust, Renal Seminars.

Conferences and courses attended

2000 Seventh Conference of the Federation of Infection Societies, Manchester.

2001 Speechcraft, Selly Oak Hospital, UHB, NHS Trust.

2001 Risk management and Communication, Selly Oak Hospital, UHB, NHS Trust.

2002 Research In Progress, Selly Oak Hospital, UHB, NHS Trust.

2002 Working for your Ph.D. Aston University, Birmingham.

2002 Writing up your research for publication, Aston University, Birmingham.

2002 Fifth International Conference of the Hospital Infection Societies, Edinburgh, Scotland.

2002 The American Society of Nephrology 35th Annual Meeting and Scientific Exposition, Philadelphia, USA.

2002 The Myths and Challenges of Clinical Research, UHB, NHS Trust.

2003 A Practical Guide to Managing and Controlling Hospital Acquired Infection under Clinical Governance. Royal College of Surgeons, London.

2003 The American Society of Nephrology 36th Annual Meeting and Scientific Exposition, San Diego, USA.

2004 A Practical Guide to Managing and Controlling Hospital Acquired Infection under Clinical Governance. Royal College of Surgeons, London.

2004 Tackling hospital infection. Health Service Journal, London

2004 First International *Clostridium difficile* Simposia. Slovenia

Membership of professional societies

Hospital Infection Society

The Renal Association

PUBLICATIONS

LIPID S - A NOVEL MARKER FOR THE SERODIAGNOSIS OF DEEP-SEATED GRAM POSITIVE SEPSIS

T. Worthington,[1] M.K. Spare,[1] P.A. Lambert,[2] S. Lang,[2] M. Connaughton,[1] W.A. Littler,[1] M. Rafiq,[3] A. Stirling,[3] T.S.J. Elliott.[1]

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Journal of Infection (2000) 40, 262–266
doi:10.1053/jinf.2000.0656, available online at http://www.idealibrary.com on IDEAL

A Novel Serological Test for the Diagnosis of Central Venous Catheter-associated Sepsis

T. S. J. Elliott*[1], S. E. Tebbs[1], H. A. Moss[1], T. Worthington[1], M. K. Spare[1], M. H. Faroqui[3] and P. A. Lambert[2]

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The Treatment of CAPD Associated Peritonitis With Intraperitoneal Piperacillin/Tazobactam

Spare, M K[1]; Adu, D[2]; Lipkin, G[2]; Ball, S[2]; Worthington, T[3]; Lambert, P A[3]; Elliott, T S[1].

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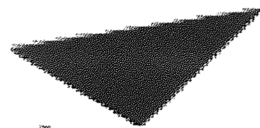

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Journal of Hospital Infection (2003) 54, 272-278

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Genotypic and phenotypic properties of coagulase-negative staphylococci causing dialysis catheter-related sepsis

M.K. Spare[a], S.E. Tebbs[a], S. Lang[b], P.A. Lambert[b], T. Worthington[a], G.W. Lipkin[c], T.S.J. Elliott[a,*]

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Control of *Clostridium difficile* on a renal unit

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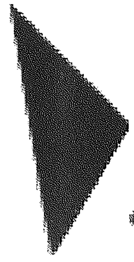

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