SERODIAGNOSIS OF STAPHYLOCOCCAL SEPSIS

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SERODIAGNOSIS OF STAPHYLOCOCCAL INFECTION

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SUMMARY

The coagulase-negative staphylococci are the most frequent cause of sepsis associated with indwelling intravascular catheters. Current microbiological investigations to support the diagnosis of catheter-related sepsis (CRS) include the culture of blood and catheter tips, however positive results may reflect specimen contamination, or colonisation of the catheter rather than true sepsis. Previous serological approaches to assist in the diagnosis of CRS based on cellular staphylococcal antigens have been of limited value. In this current study, the serodiagnostic potential of an exocellular antigen produced by 7 strains of coagulase-negative staphylococci cultured in brain heart infusion broth was investigated. Antigenic material isolated by gel permeation from liquid culture was characterised by immunological techniques and chemical analysis. Characterisation of the exocellular antigen revealed a novel glycerophosphoglycolipid, termed lipid S, which shared antigenic determinants with lipoteichoic acid, but differed by comprising a glycerophosphate chain length of only 6 units. In addition, lipid S was immunologically distinct from diphosphatidyl glycerol, a constituent cell membrane phospholipid. An indirect enzyme linked immunosorbent assay (ELISA) based on lipid S was subsequently developed and used to determine serum antibody levels (IgM and IgG) in 67 patients with CRS due to staphylococci, and 67 patients with a central venous catheter (CVC) *in situ* who exhibited no evidence of sepsis. The sensitivity and specificity of the lipid S IgG ELISA was 75% and 90% respectively whilst the IgM assay had sensitivity and specificity of 52% and 85%. The addition of GullSORB™ reagent to the ELISA procedure to remove competing serum IgG and rheumatoid factor did not significantly improve the performance of the IgM assay. The serological response in serial serum samples of 13 patients with CRS due to staphylococci was investigated. Elevated levels of antibody were detected at an early stage of infection, prior to the isolation of microorganisms by standard culture methods, and before the clinical presentation of sepsis in 3 patients.

The lipid S ELISA was later optimised and a rapid 4-hour assay developed for the serodiagnosis of CRS. Serum IgG levels were determined in 40 patients with CRS due to staphylococci and 40 patients with a CVC *in situ* who exhibited no evidence of sepsis. The sensitivity and specificity of the rapid IgG assay was 70% and 100% respectively. Elevated serum antibody levels in patients with endocarditis, prosthetic joint infection and pyogenic spondylodiscitis due to Gram-positive cocci were also detected with the lipid S ELISA suggesting that the assay may facilitate the diagnosis of these infections. Unexpected increased levels of anti-lipid S IgG in 31% of control patients with sciatica suggested a possible microbial aetiology of this condition. Further investigation of some of these patients by culture of microdiscectomy tissue removed at operation, revealed the presence of low-virulent microorganisms in 37% of patients of which *Propionibacterium acnes* accounted for 85% of the positive culture isolates. The results suggested a previously unrecognised association between *P. acnes* and sciatica, which may have implications for the future management of the condition.

Key words: Lipid S, coagulase-negative staphylococci, enzyme linked immunosorbent assay, catheter-related sepsis, pulsed field gel electrophoresis.

DEDICATION

To my wife

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ABBREVIATIONS

ACTC	American Collection of Type Cultures
ADNB	anti-deoxyribonuclease B
AOLC	acridine orange leucocyte cytospin
ASO	anti-streptolysin O
BE	bacterial endocarditis
BHI	brain heart infusion
BZK	benzalkonium chloride
CABG	coronary artery bypass graft
CAPD	continuous ambulatory peritoneal dialysis
CDSC	Communicable Disease Surveillance Centre
CFU	colony forming unit
CHEF	contour-clamped homogenous electric field
CI	confidence interval
CL	confidence limit
CLED	cysteine lactose electrolyte deficient
CNS	coagulase-negative staphylococci
COV	coefficient of variation
CRP	C-reactive protein
CRS	catheter related sepsis
CSF	cerebrospinal fluid
CT	computerised topography
CVC	central venous catheter
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPG	diphosphatidyl glycerol
DTP	differential time to positivity
EDTA	ethylenediaminetetraacetic acid
EIU	enzyme immunoassay units

ELISA	enzyme linked immunosorbent assay
ES	EDTA-sarcosyl
ESP	EDTA-sarcosyl-proteinase K
ESR	erythrocyte sedimentation rate
ESS	extracellular slime substance
FAME	fatty acid methyl ester
FIGE	field inversion gel electrophoresis
FPLC	fast protein liquid chromatography
GC	gas-liquid chromatography
G+C	Guanine and Cytosine
h	hours
HHW	Hussain-Hastings-White
LTA	lipoteichoic acid
µg	microgram(s)
mg	milligram(s)
ml	millilitre(s)
M	molar
MRI	magnetic resonance imaging
min	minute(s)
NCIMB	National Collection of Marine and Industrial Bacteria
NCTC	National Collection of Type Cultures
NET-100	NaCl-EDTA-Tris
NHS	normal human serum
NMR	nuclear magnetic resonance
NPV	negative predictive value
NVE	native valve endocarditis
OD	optical density
OFAGE	orthogonal field agarose gel electrophoresis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PG	phosphatidyl glycerol

PIA	polysaccharide intercellular adhesin
PMSF	phenylmethylsulfonylfluoride
PPV	positive predictive value
PS	pyogenic spondylodiscitis
PS/A	polysaccharide adhesin
PVC	polyvinylchloride
PVE	prosthetic heart valve endocarditis
rpm	revolutions per minute
RAPD	random amplification of polymorphic DNA
ROC	receiver operator curve
S_D	Dice coefficient
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
SNC	standardised negative control
SOP	standard operating procedure
SPC	standardised positive control
TAFE	transverse alternating field electrophoresis
TBE	Tris-boric acid-EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
TFA	trifluoroacetic acid
TMB	tetra methyl benzidine
UPGMA	unweighted pair group method of arithmetic averages
UTI	urinary tract infection
w/v	weight per volume

Chapter 1 Introduction

1.1 The Diagnosis of central venous catheter (CVC) related sepsis and its associated problems

Intravascular central venous catheters (CVC) are essential for the successful treatment of patients with a chronic or critical illness. The indications for the use of a CVC include drug administration, monitoring central venous pressure and the infusion of hypertonic fluids and blood products. They are widely used throughout the world, and it estimated that over two million CVC are used annually in the United Kingdom (Elliott, 1993).

The CVC is placed within a vein with its distal end located within the superior or inferior vena cava (figure 1.1). They are usually 20-30cm in length (figure 1.0), predominantly manufactured from polyurethane and can be either single or multilumen (Elliott *et al.*, 1997). It has been well documented that sepsis associated with intravascular catheters and other medical devices is a significant cause of morbidity and mortality (Elliott, 1988; Elliott and Faroqui, 1992; Cree *et al.*,1995). The coagulase- negative staphylococci (CNS), in particular *Staphylococcus epidermidis* cause more device-related infections than any other microorganism. Indeed, this group of microorganisms is also the major cause of infections of not only intravascular catheters but also orthopaedic devices, continuous ambulatory peritoneal dialysis (CAPD) catheters, cerebrospinal fluid shunts and prosthetic valves (Christenson *et al.,* 1994).

The diagnosis of catheter related sepsis (CRS) is however difficult, both from the clinical and laboratory viewpoint (Elliott, 1988). At present, the most commonly used technique for laboratory diagnosis is that described by Maki *et al.* (1977), whereby a short length of the distal portion of an explanted catheter (approximately 2-3 cm) is rolled back and forth at least four times across the surface of a nutrient agar plate. After appropriate incubation, usually up

to 48 h, colonies are then counted and numbers of greater than 15 per agar plate are considered indicative of catheter related sepsis. In comparison, a clinical diagnosis is made by interpreting the isolates from the catheter in association with any from corresponding blood cultures and also taking into account the clinical presentation of the patient including the presence of erythema, oedema and exudate at the catheter insertion site, low grade pyrexia and no other obvious focus of infection.

The problems associated with the method of Maki *et al.* (1977) in the diagnosis of CRS are many. The catheter may become contaminated with the patient's own skin flora upon removal and subsequently any isolated organisms may not be representative of the pathogen, and the bacterial colony count on the agar plate may be falsely high. Also, the roll plate method only samples the external surface and not the internal lumen of the catheter. Microbial colonisation of plastic devices can occur both externally and internally (Cheesbrough *et al.*, 1985). Internal culture of a catheter lumen is however also associated with inaccuracies. The flushing of the lumen with a known volume of broth and subsequent culture onto solid agar does not allow for accurate quantitation of the internal colonising microflora, although in one study, internal culture by flushing the catheter proved superior to external culture giving a better predictive value of a positive isolate being associated with infection (Kristinsson *et al.*, 1989).

With regard to blood cultures, they should be obtained via the catheter and also from a separate peripheral venepuncture site (Elliott and Faroqui, 1992; Kloos and Bannerman, 1994). When identical organisms are isolated from both of these sources, it is strongly indicative of CRS, however, it is not definitive and the clinical interpretation still remains unclear as the isolates may indicate another septic focus not related to the catheter. Quantitative blood cultures prove useful in the diagnosis of CRS as a finding of a 5 to 10 fold increase in the concentration of microorganisms drawn through the catheter as compared to that via a peripheral venepuncture site is confirmation of a CVC- related bacteraemia (Elliott, 1997).

Figure 1.0

A triple lumen CVC (length-20-30 cm)

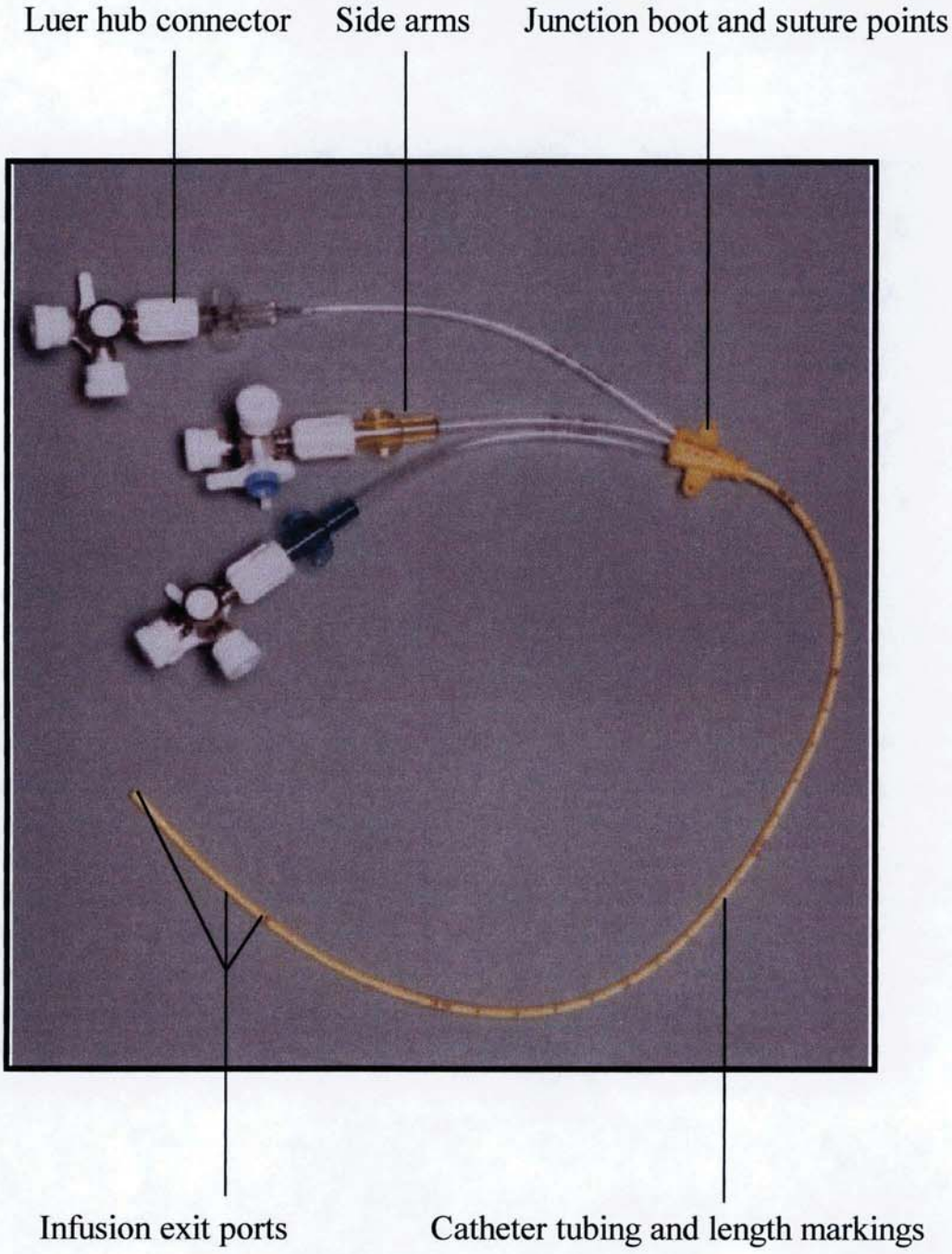
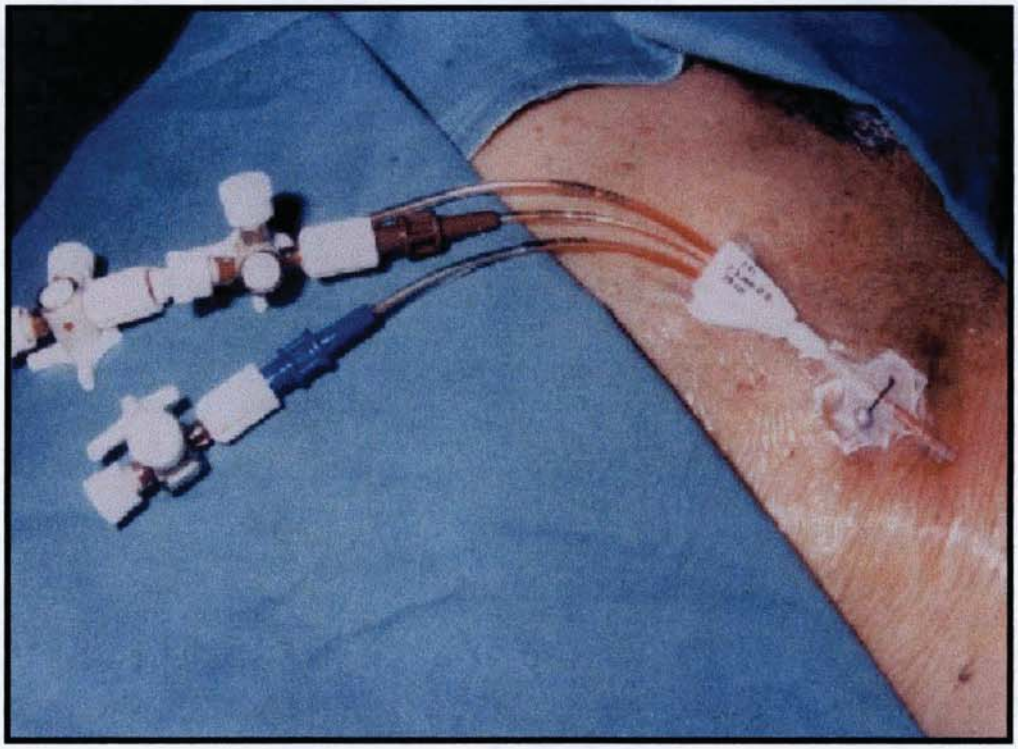


Figure 1.1

A triple lumen CVC inserted into the jugular vein of a patient prior to cardiac surgery



Unfortunately, routine microbiology laboratories at present do not generally identify their isolates of CNS further than to the group level based on their coagulase reaction. As the full identification of the microorganisms is the factor in determining whether isolates from catheters, blood cultures and insertion sites are the same organism, further typing of the CNS is necessary incorporating methods such as biotyping and where necessary, genotyping. However, such procedures are not routinely performed in medical microbiology laboratories.

It is likely that the use of medical devices will continue to increase, hence innovative laboratory techniques are required for rapid and accurate diagnosis of sepsis due to implanted devices. In this study, the serological response to staphylococcal antigens using a novel enzyme linked immunosorbent assay (ELISA) was investigated as a potential marker of coagulase negative staphylococcal CRS and deep seated infection. Clinical isolates were also characterised using conventional methods and by a system of pulsed field gel electrophoresis (PFGE) whereby an analysis of the chromosomal DNA restriction patterns was made.

1.2 Staphylococci – past and present

The name Staphylococcus was first introduced in 1882 by Sir William Ogston, a Scottish surgeon who described "cluster forming cocci" which he observed to be causing pyogenic abscesses (Rupp and Archer, 1994). The first attempt at classification of staphylococci was made shortly after by Rosenbach in 1884 when pigment production was used to classify staphylococci into two groups; virulent, golden *Staphylococcus aureus* and avirulent, white *Staphylococcus albus.*

A century later 20 species had been identified to belong within the genus Staphylococcus, 12 of which were associated with humans and the remaining species with animals (Koneman *et al.*, 1988). However, advances in the identification of new staphylococcus species including methods such as colonial morphology, enzyme activity and sugar fermentation tests, and also commercial rapid identification systems, for example, API STAPH IDENT, ID 32 STAPH (bioMérieux, Marcy-l'Etoile, France) has resulted in an increase in the number of recognised species within the genus.

There are now 31 recognised species of CNS, about half of which are indigenous to humans (Kloos and Bannerman , 1994). The species related to humans include *S. aureus, S. epidermidis, S. haemolyticus, S. saprophyticus, S. cohnii, S. xylosus, S. capitis, S. warneri, S. hominis, S. simulans, S. saccharolyticus, S. auricularis, S. caprae, S. lugdunensis* and *S. schleiferi.* As the identification of CNS down to species level becomes more standardised, the number of reports due to these species will no doubt increase.

1.2.1 The distribution of the staphylococci

The skin serves as a diverse environment and several species of staphylococci have adapted to colonise the many microenvironments present in this location (Kleeman *et al.,* 1993). The CNS in particular constitute a major component of the normal skin flora and mucus membranes and their presence is saprophytic. If however the cutaneous barrier is broken, for example, on insertion of a synthetic device such as a catheter or prosthesis, then the microorganisms may gain entry into the host and act as pathogens. The numbers of staphylococci colonising different areas of the skin range from 10 to 10^6 CFU/cm^2 of surface (Kloos and Bannerman, 1994). Moist areas, for example, the anterior nares, axillae and the toe webs harbour larger concentrations of staphylococci than drier habitats. It appears that certain species of staphylococci demonstrate a preference for specific areas of the body.

S. epidermidis for example, is widely distributed over the body (Archer, 1990). Species such as *S. hominis* and *S. haemolyticus* are associated primarily in areas where apocrine glands are found such as the axillae. In comparison, *S. capitis* subsp. *capitis* is present in extremely large numbers on the scalps of adolescents and to a slightly lesser degree on the face, ears, eyebrows and forehead.

In the recent report by Kleeman *et al.* (1993), where the distribution of CNS was studied, it was found that *S. epidermidis* accounted for the largest number of clinical isolates over a wide range of sources (65%), followed by *S. haemolyticus* (13%) and *S. hominis* (7%).

S. warneri and *S. lugdunensis*, which are widely distributed over the body, accounted for 4% and 3% respectively. Other species isolated in the study but to a lesser degree included *S. auricularis, S. capitis, S. cohnii* and *S. simulans.* *S. saprophyticus* has an affinity for urogenital cells and is primarily isolated from the urine, particularly of sexually active women aged 18-30 years. The only coagulase - positive species within the group, *S. aureus*, is isolated frequently from the anterior nares in adults but is more widely distributed over the

whole body in preadolescent children. Not surprisingly, the staphylococci as a group are the most frequently isolated organisms in the hospital microbiology laboratory.

1.2.2 Incidence of disease due to the CNS

The CNS are recognised as causing a wide range of hospital acquired infections with increasing mortality and morbidity. Although once thought to be clinically insignificant contaminants in a range of clinical samples, they are now known to be increasingly important opportunistic pathogens associated with prosthetic materials ranging from intravenous catheters to hip prostheses (Elliott and Faroqui, 1992; Kleeman *et al.,* 1993; Kloos and Bannerman, 1994; Cree *et al.,* 1995; Lambert *et al.,* 1996).

The reported incidence of sepsis associated with intravascular catheters is variable, ranging from 0 - 15% (Elliott, 1997). This large range is to an extent reflective of certain problematic areas such as the lack of a clear definition for catheter related sepsis, difficulties in making the clinical diagnosis, and inconclusive laboratory results. From 1980 to 1989 the National Nosocomial Infections Study reported increases in nosocomial bacteraemia due to CNS, in particular *S. epidermidis,* ranging from 70% to 279% depending upon the type of hospital (Rupp and Archer, 1994).

Between 1989 and 1991 alone, a total of 3,647 cases of bacteraemias due to CNS in association with intravascular lines were reported to the PHLS Communicable Disease Surveillance Centre (CDSC) in England and Wales (Elliott, 1993). This represented a total of 36% of all cases of catheter related bacteraemia during the period. Other cases were caused by microorganisms such as *S. aureus, Candida sp.,* Gram-negative bacilli and diphtheroids.

Interestingly, the number of reported cases of line associated bacteraemias between 1989 and 1991 rose by 39%. Similarly, between 1991 and 1994, the number of cases of line associated bacteraemias rose from 3,984 to 5,290 (Elliott, 1997), indicating a continuing rise in the number of reported cases. These figures however are probably an underestimation of the true incidence due to the lack of clear definitions as mentioned previously and the difficulty in making a definitive diagnosis.

Apart from sepsis relating to indwelling devices, CNS are also documented as being the third most frequent cause of wound infections and also responsible for a small percentage of nosocomial urinary tract infections and pneumonia's (Rupp and Archer, 1994). In addition, infection of the heart valves (native valve endocarditis (NVE)) due to CNS has also become more common (Etienne and Eykyn, 1990). A total of 35 cases of NVE due to CNS in two hospitals were documented between the period of 1976 to 1989. This accounted for 14.8% of the total number of cases of NVE seen. *S. lugdunensis,* a species found on human skin and first described in 1988, is now being increasingly documented as causing a virulent form of infective endocarditis, particularly in patients with a preexisting cardiac abnormality (Vandenesch *et al.*, 1993).

1.3 The clinical spectrum of disease associated with the CNS

Biomaterials such as synthetic polymers, ceramics and metals are frequently used for therapeutic purposes in the form of prosthesis and catheter devices, and their application has undoubtedly led to advances in patient care (Jansen and Peters, 1993). However, their usage is also accompanied by various complications such as infection and, with more than one million prosthetic implants per year being used world wide, a figure that is exceeded by

catheters, the problem has both medical and economical implications. The clinical spectrum of disease associated with the CNS is illustrated in table 1.0

Table 1.0

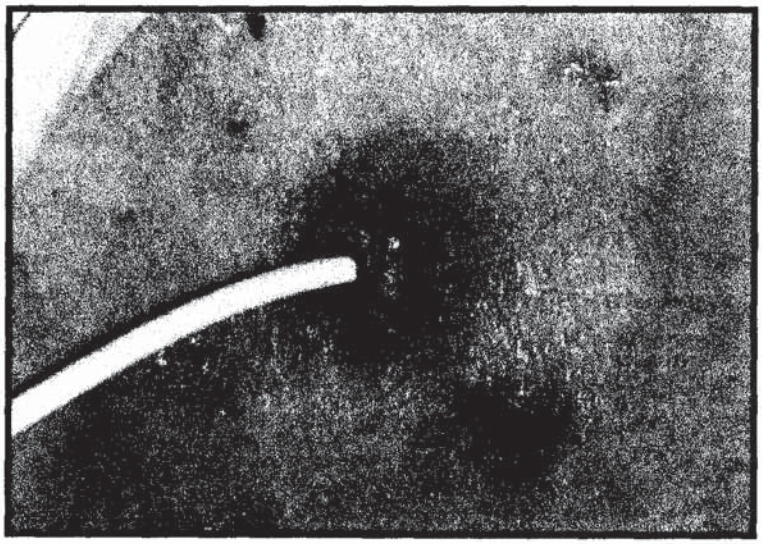
The clinical spectrum of disease associated with coagulase negative staphylococci

Infections of indwelling foreign devices	- Intravenous catheters - Prosthetic joints - Prosthetic cardiac valves - Haemodialysis shunts - Peritoneal dialysis catheters - Cerebrospinal fluid shunts - Pacemaker wires - Breast implants - Vascular grafts - Orthopaedic metal plates / pins - Intrauterine devices
Haematogenous	- Bacteraemia / Septicaemia - Endocarditis
Osteomyelitis	- Sternal wound - Haematogenous spread
Urinary tract infections	- Catheter related - Community acquired

1.3.1 Intravascular catheter related infections

Intravascular catheters are now imperative in the care and management of critically ill patients. In the United States alone over 150 million intravascular catheters, five million of which are central catheters, are used yearly (Elliott, 1997). In comparison, in the United Kingdom, approximately two million central line catheters are used per annum. The emergence of the CNS as the primary pathogen causing catheter related infections has been attributed to the increased use of prosthetic and indwelling devices, the higher demand for parenteral nutrition and the improved survival of immunocompromised patients (Nouwen *et al.*, 1998). Infections associated with catheters are divided into two major groups; localised and systemic. Localised infection is characterised by the presence of oedema, erythema, exudate and thrombophlebitis (figure 1.2). If a tunnelled device is used a subcutaneous infection may also occur. Systemic infection occurs usually as a consequence of microorganisms colonising the distal tip of the catheter and is characterised by low grade pyrexia. The commonest organisms associated with intravascular catheter related infections are the CNS, in particular *S. epidermidis* (Kloos and Bannerman, 1994).

Figure 1.2 **Localised infection characterised by oedema, erythema and exudate**



The microorganisms gain access to intravenous catheters via several routes (fig 1.3):

- extraluminal, through migration down the external surface of the catheter with the microorganisms originating from the skin of the patient or medical personnel (Elliott, 1997).
- intraluminal, through contaminated catheter hubs (Linares *et al.*, 1985), contaminated infusates (Elliott, 1997), and luer connectors (Tebbs *et al.*, 1996) and subsequent migration to the catheter tip through the lumen.
- haematogenous seeding of the catheter tip from a distant focus (Rupp and Archer, 1994).
- impaction of microorganisms onto the catheter tip at the time of insertion (Elliott, 1995).

Figure 1.3

Sources of microbial access

(Elliott, 1993, reproduced with permission)

1.3.2 Bacteraemia and septicaemia

Over the past few years, Gram- positive cocci have emerged as the most prevalent cause of nosocomial infections (Verhoef and Mattsson, 1995). This is due mainly to the increased incidence of bacteraemia caused by CNS, which is a serious medical condition associated with significant morbidity and mortality. Approximately 12 -22% of these patients are reported to suffer from septic shock (Rupp and Archer, 1994). CNS bacteraemia is often associated with indwelling foreign devices and about 75% of patients with bacteraemia are reported to have a device *in situ*, predominantly intravascular catheters. *S. epidermidis* is also recognised as an important nosocomial pathogen in immunocompromised patients and chronic bacteraemia due to this organism in patients following bone marrow transplantation has been reported (Lina *et al.*, 1995).

Due to the ubiquitous nature of *S. epidermidis* and its pathogenic potential, differentiation between significant blood culture isolates and those representing contamination must be made, hence strain characterisation has become increasingly important. Pulsed field gel electrophoresis (PFGE), a recently developed molecular fingerprinting technique provides useful information for typing by production of stable and discriminating chromosomal restriction patterns (Lina *et al.*, 1992; Kaufmann and Pitt, 1994; Lina *et al.*, 1995; Tenover *et al.*, 1995). The previous use of genotyping studies has indicated particular virulent, persistent strains of CNS isolated from patients with septicaemia as well as highlighting potential sources of infection. For example, one study showed that 30% of CNS from blood cultures taken from a heart-lung machine during cardiac surgery belonged to a single genotype (Van Belkum *et al.*, 1995). Similarly, Nouwen *et al.* (1998) demonstrated that two clones of *S. epidermidis* were involved in the colonisation and subsequent infection in neutropenic haemato-oncology patients from an environment with a high incidence of catheter associated infection. These 2 clones were responsible for more than 70% of the infections and appeared

to be confined to the haematology department, as their PFGE macrorestriction patterns were totally different from those strains of CNS outside of the department.

1.3.3 Endocarditis

The CNS are responsible for only 1-3% of cases of native valve endocarditis (NVE) (Rupp and Archer, 1994). Native valve infections are generally community acquired and many of the patients will have a predisposing abnormality, which leads to infection, such as previously damaged valves or abnormal cardiac anatomy. Unlike infections associated with medical devices, more than half of NVE cases are due to species other than *S. epidermidis,* including species such as *S. warneri* (Wood *et al.,* 1989). Also, several cases of a virulent form of endocarditis due to *S. lugdunensis,* a recently described CNS, have been documented (Vandenesch *et al.,* 1993).

Specific species of the CNS have been reported to be associated with prosthetic heart valves (Baddour *et al.,* 1990). One report documents a case of NVE due to *S. epidermidis* in which two colonial phenotypes were detected on primary isolation (Deighton *et al.,* 1992). It was proposed in this study that multiple phenotypes of CNS may function synergistically in infections to cause disease. The application of restriction endonuclease analysis of plasmid DNA and PFGE of whole cell DNA proved that the two variants of *S. epidermidis* were identical or closely related and may have been derived from the same parent.

The CNS, in particular *S. epidermidis* are the most common infecting microorganisms of prosthetic heart valve endocarditis (PVE) (Takeda *et al.,* 1991; Rupp and Archer, 1994) accounting for approximately 50% of cases. The majority of patients with PVE due to CNS present with complications including prosthetic heart valve dysfunction and fever despite appropriate therapy.

The diagnosis of PVE is also difficult. False positive blood cultures due to contaminating strains of CNS and also false negative results due to intermittent shedding of organisms from the infected heart valve, and prior use of antibiotics are common diagnostic problems. Clonal typing of multiple isolates from several blood cultures would assist in determining their significance. Common methods employed such as the use of antibiograms and species determination often results in poor discrimination between infecting and contaminating strains. However, the analysis of chromosomal DNA macrorestriction patterns through highly discriminative molecular typing methods, such as PFGE should be conclusive in determining the clonal origin of the isolates.

1.3.4 Peritonitis

Infection remains the most common complication of peritoneal dialysis. The CNS account for 17 to 53% episodes of continuous ambulatory peritoneal dialysis (CAPD) associated peritonitis (Rupp and Archer, 1994). *S. epidermidis* has been the most frequently isolated species of the CNS accounting for 69-84% of all isolates (Freeman and Falkiner, 1991).

Clinically, the diagnosis of peritonitis is based on a raised dialysate white cell count (>100 / mm^3) and symptoms including abdominal pain, pyrexia, nausea and vomiting.
The pathogenesis of CAPD infections is similar to that of infections associated with intravenous catheters, with microorganisms originating from the patients endogenous flora around the pericatheter skin gaining access to the peritoneum mainly through intraluminal or periluminal routes (Eisenberg *et al.,* 1987).

Epidemiological studies through typing of strains of CNS provide useful information by distinguishing strains associated with relapse from reinfection in CAPD patients and also by identifying sources of infection and areas of potential hazard (Freeman and Falkiner, 1991).

Due to the poor levels of discrimination achieved through conventional methods of typing alone, the use of combined typing systems, including molecular biological methods, would provide improved strain discrimination. The typing of strains also provides a useful means of evaluating clinical therapy. Hospital isolates of CNS are often resistant to methicillin, hence intraperitoneal vancomycin forms part of the recommended treatment of patients on CAPD. However, decreased susceptibility of *S. epidermidis* to glycopeptides has been documented in a case of CAPD peritonitis and also in neutropaenic patients with bacteraemia (Sanyal *et al.*, 1993), emphasising the need for a discriminative typing system.

1.3.5 Prosthetic joint infection

S. aureus and the CNS are the most common cause of prosthetic joint infection (Jansen *et al.*, 1989). It is estimated that approximately 185,000 cases of prosthetic joint implantations are performed in the United States each year (Jansen *et al.*, 1989). The infection rate associated with the prostheses is about 1-2% with the CNS accounting for up to 40% of the cases (Rupp and Archer, 1994). *S. epidermidis* is the major infecting organism of the group accounting for 50-80% of the isolates.

Early, accurate diagnosis is a major clinical problem in prosthetic infection. It is important to distinguish between septic and aseptic loosening of a prosthesis as the treatment of the two conditions differs. One recent report (Lambert *et al.*, 1996) suggests that an ELISA system based on non protein exocellular antigens of *S. epidermidis* may be used to detect elevated serum levels of IgG in patients with prosthetic joint infection and act as a marker of infection. Although limitations of the assay have been documented, the system potentially provides a rapid, non invasive method of detecting infection hence allowing prompt antimicrobial therapy. The system also provides a means of differentiating mechanical loosening from

infection. This thesis will investigate the potential of the ELISA for the diagnosis of deep seated Gram-positive sepsis.

1.3.6 Spondylodiscitis

Pyogenic spondylodiscitis is a rare, but severe complication of lumbar discectomy with the incidence varying between 0.1-3.0% (Tronnier *et al.*, 1992). The clinical symptoms include muscle cramps, severe spinal pain combined with mild symptoms of sciatica, and a temperature ranging from 37.3° C to above 39° C (Lindholm and Pylkkänen, 1982; El-Gindi *et al.*, 1976). The most common organisms associated with pyogenic spondylodiscitis are *S. aureus* and *S. epidermidis,* however organisms including *S. saprophyticus, S. capitis, S. warneri* and several species of streptococcus have been isolated from the intervertebral disc space (Tronnier *et al.*, 1992). In addition to post-operative infection, microorganisms may gain access to the intervertebral disc space through haematogenous spread in patients with predisposing infection at distant sites including intravenous cannulae, respiratory tract and genito-urinary tract.

1.3.7 Other infections

Aside from the major groups of infection involving CVC and prostheses, the CNS are also well documented aetiological agents of other types of disease. Infections of CSF shunts occur in approximately 5-12% of patients with the CNS accounting for about 40-60% of the cases. *S. saprophyticus* and *S. epidermidis* are common causes of disease of the urinary tract with *S. epidermidis* being responsible for cases of multiresistant nosocomial UTI occurring in the elderly catheterised patients. The CNS also account for some cases of post operative surgical wound infections, for example, following cardiac and ophthalmologic surgery. Finally, CNS have been reported to cause disease in the absence of prosthetic devices

including toxic shock syndrome, otitis media and septic arthritis (Kloos and Bannerman, 1994), whilst more recently, the microorganisms have been associated with cerebral palsy in neonates (Mittendorf *et al.*, 1999).

1.4 Pathogenesis

The CNS are a major cause of foreign body infections and it is well documented that the colonisation of these devices both *in vitro* and *in vivo* occurs through a series of sequential steps. An understanding of the mechanisms of microbial adherence and pathogenesis may allow for preventative measures to be initiated. There are three stages in the colonisation process:

- exposure of the device to bacteria and attachment
- biofilm formation
- slime production
- phase variation / phenotypic modulation (Christensen *et al.,* 1994).

1.4.1 Exposure and attachment

When a medical device is exposed to bacteria, the actual number of organisms that adhere is dependent upon a number of biological factors and physiochemical interactive forces between the device and the microbe. These include passive forces such as the movement of fluids over the surface of the device and gravity, and also non specific forces including van der Waals forces, surface tension, electrostatic forces and hydrophobic bonding. Conditioning of the prosthetic device also occurs *in vivo* whereby its surface is coated with host derived serum proteins and platelets forming a film which can then act as a target for microbial binding. The ability of bacteria to adhere to a device is also influenced by its surface morphology (Franson *et al.*, 1985; Cheesbrough *et al.*,1985; Tebbs *et al.*, 1994).

Surface defects such as scratches, cracks and fissures clearly aid in the ability of bacteria to adhere and multiply. In the early stages of microbial colonisation, hydrophobic bonds are recognised as the most significant forces, with adhesion of micoorganisms to hydrophobic polymers such as silicone being enhanced (Tebbs *et al.,* 1994).

1.4.2 Biofilm formation

The next stage in colonisation is the further formation of the biofilm (Costerton *et al.*, 1999; Costerton *et al.*, 1994). The bacterial biofilm theory describes bacterial populations in natural and pathogenic ecological systems in terms of a 'planktonic' population of bacteria interacting with a more important matrix enclosed 'sessile' population associated with or adherent to a surface. If the microorganism and the opposing surface have opposite charges, the bacterial cell may be attracted by electrostatic and van der Waals' forces and be reversibly held (Allison, 1993). The following stage of the biofilm development is the irreversible adhesion of the organisms through targeted binding. Targeted binding of microorganisms such as *S. aureus* is well recognised using surface adsorbed fibronectin, fibrinogen and fibrin to adhere to the surface of catheters. The CNS however appear to bind less readily to fibronectin but studies have shown that *S. epidermidis* does bind to fibrin-platelet clots through cell wall lipoteichoic acid and also platelets deposited on the surface of hydrophobic plastic (Christensen *et al.,* 1994). It has also been suggested that proteinaceous fimbrial or pili like structures are used by *S. epidermidis* and *S. saprophyticus* in the attachment process (Christensen *et al.,* 1994). Specific adhesion of *S. epidermidis* to silastic catheter surfaces by a capsular polysaccharide adhesin (PS/A) has been documented and provides a description of an adhesin for the CNS (Kloos and Bannerman, 1994; Christensen *et al.,* 1994). PS/A is a polymer of galactose and arabinose with a molecular weight of >50,000 and appears to be important in the initial stages of colonisation.

1.4.3 Slime production

The production of exocellular slime substance (ESS) or glycocalyx by the staphylococci is involved in the later stages of colonisation. The production of ESS is dependent upon the environment in which the organism is grown and is influenced by fluctuations in levels of oxygen (Barker *et al.,* 1990) and carbon dioxide (Hussain *et al.,* 1992). There is considerable debate about the nature of slime (Christensen *et al.*, 1994). A number of different polymers have been isolated from slime producing strains, including capsular polysaccharide/adhesin (Tojo *et al.*, 1988), slime associated antigen (Christensen *et al.*, 1990), a polysaccharide intercellular adhesin (Mack *et al.*, 1994) and teichoic acid (Hussain *et al.*, 1992). The function of ESS is to promote the colonisation of medical devices by binding bacterial cells to each other with the subsequent formation of a biofilm. ESS is also documented to interfere with host defence mechanisms (Kloos and Bannerman, 1994; Gray *et al.,* 1984). The inhibition of T-lymphocyte proliferation and interference of immunoglobulin production are two such mechanisms. ESS also appears to impede phagocytosis of the microorganisms and may contribute to antimicrobial resistance by a direct blocking of the penetration of antibiotics.

1.4.4 Phenotypic modulation

The final stage of surface colonisation involves the mechanism of phenotypic modulation whereby the production of ESS is switched on and off by phase variation and changes in environmental conditions. Non adherent progeny within the biofilm layer are released through the depletion of the environmental conditions necessary for slime production or by switching off slime production through phase variation. The colonisation process at other sites then begins (Christensen *et al.,* 1994).

1.5 Prevention and treatment of catheter associated infections

CRS results in a significant cost (Moss and Elliott, 1997). In the UK alone, the costs associated with CRS are estimated to be £2.5 million per annum for long term catheters and between £5-7 million for short term. A clear understanding of the risk factors associated with infections of catheters and other synthetic devices will help in determining strategies to prevent such infections. Efforts made in the prevention of sepsis will be repaid by savings of resources in the diagnosis and management of such infections. Some of the factors influencing microbial colonisation and strategies for prevention are summarised in table 1.1.

Table 1.1

Risk factors and preventative measures associated with catheter-related sepsis

RISK FACTORS	PREVENTATIVE MEASURES
1. DEVICE -Catheter material and topography	-Antibiotic / heparin coated catheter -Electrical charge
2. OPERATIONAL -Difficulty in catheter insertion -Duration of catheterisation -Site / route of catheter insertion	-Disinfection of catheter insertion site -Strict barrier precautions
3. PATIENT RELATED -Immunosuppresion / AIDS -Malignancy -Concurrent infection -Total parenteral nutrition	-Antibiotic prophylaxsis
4. MEDICAL PERSONNEL -Cross infection	-Increased Experience -Careful Hand washing -Disinfection of connectors

(adapted from Micalopoulos and Geroulanos, 1996 and Elliott, 1993).

1.5.1 The device

Intravascular catheterisation may be either short or long term. Short term CVC are usually single, double or triple lumen and made from polyurethane, polytetrafluoroethylene (Teflon), polythene, polypropylene or polyvinylchloride (PVC). Some catheters may be coated with heparin or hydrophilic agents to prevent fibrin deposition and subsequent colonisation (Roberts, 1993).

The use of impregnated antimicrobial agents on short term catheters, such as teicoplanin and irgasan into catheter materials has also been used to reduce the rate of CRS, however, they have yet to make a convincing impact on clinical practice. Compounds such as benzalkonium chloride (BZK), silver sulphadiazine and chlorhexidine have also been used (Elliott, 1997). BZK-impregnated catheters have been shown to reduce microbial colonisation for up to 3 weeks (Elliott and Tebbs, 1993). The results of one study however demonstrated that BZK catheters are unlikely to prevent infection over a longer period (Sampath *et al.*, 1995). Catheters coated with minocycline and rifampicin have also been used in clinical trials and shown to be effective in reducing colonisation of the catheter and also sepsis associated with its use (Darouriche *et al.*, 1999).

It is widely believed that most infections arise from the catheter hub which has been reported to be colonised in 70% of patients with CRS (Linares *et al.*, 1985). However, the use of 70% ethanol to disinfect the catheter hubs has been shown to be extremely effective in the eradication of microorganisms (Salzman *et al.*, 1993). The design of catheters is also related to the incidence of microbial colonisation. Catheters with a smooth topography are less likely to become colonised (Tebbs *et al.*, 1994). There is also some evidence that triple lumen catheters are associated with higher rates of infection, for example, patients receiving total parenteral nutrition through triple lumen catheters are three times more likely to become infected than those in whom single lumen catheters are used (Elliott *et al.*, 1994). Multi-lumen ports including luer connectors can also be a major source of sepsis resulting in

intraluminal microbial migration. It has been shown that 23% of luer locks may become contaminated with microorganisms within 3 days of use (Tebbs *et al.*, 1996). An alternative to the conventional luer system is the needleless connector, which also if cleaned appropriately before use, offers a safer system (Brown *et al.*, 1997).

The effect of direct electric current (10 μA) on the ability of microorganisms to attach to catheters has also been investigated (Lui *et al.*, 1993; Crocker *et al.*, 1992). Crocker *et al.* (1992) confirmed that bacteria are repelled by negatively charged current carrying cannulae *in vitro*, whilst Lui *et al.* (1993) demonstrated that the application of an electric current has bactericidal activity. Although further *in vivo* studies are needed in this area, the work suggests an alternative means in the prevention of sepsis.

1.5.2 The patient

Because the patients' own skin flora provides a source of infection, disinfection of the catheter insertion site is critically important. The use of 70% isopropanol or 1% iodine in 70% ethanol applied to patients skin two minutes before insertion of the catheter has been recommended (Elliott and Faroqui, 1992). Alcoholic chlorhexidine has also been found to be a useful skin disinfectant (Elliott, 1997). Antiseptic ointments such as bacitracin or neomycin offer some protection against cannulae contamination but their use should be restricted due to associated risks of fungal infection (Elliott, 1997). After catheter implantation, the insertion site should be covered with a dressing and monitored regularly for signs of sepsis. The dressing should be changed regularly or when they become soiled with exudate or blood. Although the transparent dressings allow for the monitoring of the insertion site, in a meta analysis, the risk of CRS was shown to be significantly increased by their use (Hoffman *et al.*, 1992). However, in a later contrasting report, the use of such dressings reduced the sepsis rate (Reynolds *et al.*, 1997).

1.5.3 Medical personnel

Careful aseptic technique and subsequent care of the catheter are the most important factors in the prevention of catheter sepsis (Elliott and Faroqui, 1992). Prior to disinfection of the insertion site, hand washing with soap or 70% ethanol will result in a significant reduction of microorganisms on the hands of the medical team. The use of sterile gloves and adherence to protocols concerning aseptic procedures and disinfection of catheter ports are also important measures. Regular inspection of the catheter *in situ* for signs of infection and changes of dressings, provides the necessary after care.

1.5.4 Treatment

Sepsis due to the CNS may be successfully treated with the catheter still *in situ.* The best antibiotic treatment of infections associated with intravascular catheters involves the use of vancomycin or teicoplanin (Elliott and Faroqui, 1992). However, the approach to treatment and management will depend upon the nature of infection, the aetiological agent and the patient's underlying condition (Elliott, 1993).

1.6 Laboratory diagnosis of catheter related sepsis

The accurate diagnosis of CRS continues to offer a diagnostic challenge since clinical presentation such is often silent or non specific (Elliott and Tebbs, 1998). Indeed, indicative symptoms such as local inflammation including erythema, odema and purulent exudate is evident in only 35-50% of cases (Michalopoulos and Geroulanos, 1996). The laboratory diagnosis of CRS is therefore imperative.

1.6.1 Culture of distal catheter tips

The semiquantitative culture of central venous catheter tips described by Maki *et al.* (1977), is probably the most common method used in hospital laboratories as it is simple and economical (Michalopoulos and Geroulanos, 1996; Widmer *et al.*, 1992). The method involves rolling the catheter tip over an agar plate and after appropriate culture, the numbers of colony forming units (CFU) are determined. It has been suggested that >15 CFU is indicative of CRS.

Although widely used, the technique has associated problems. The cut off figure of 15 CFU is disputed by some workers and levels of 5 CFU (Haslett *et al.*, 1988) and 25 CFU (Rello *et al.*, 1991) have been documented indicating that the interpretation of catheter tip culture results is both subjective and difficult. Adherent microorganisms and slime producing CNS make the diagnosis by this method complex. The microorganisms may be particularly difficult to remove from the external surface of the catheter by rolling and hence the number of CFU acheived on the surface of an agar plate may not reflect the number of organisms on the catheter. Slime production also interferes with counting procedures that rely upon determining the number of CFU. A single CFU may represent a single organism or a cluster of many microorganisms released during the rolling procedure.

Also, the roll plate technique does not always allow for discrimination to be made between microbial contamination of the catheter and true sepsis (Elliott, 1997). Another limitation is that it only detects microorganisms on the outside surface of the catheter and not the internal lumen, which needs be considered when interpreting cultures. In one study which evaluated three methods for culturing catheters, it was reported that the culture of the internal lumen was a better predictor of infection compared with external culture or ultrasonication (Kristinsson *et al.*, 1989).

Other workers have conflicting views on the usefulness of semiquantitative culture of tips in the diagnosis of sepsis. In the report by Widmer *et al.* (1992), only 4% of the semiquantitative tip culture results using the Maki roll plate technique had any clinical impact, and the negative culture results did not influence the clinical practice. This suggests that although widely used, the roll plate technique is not a valid "gold standard" for the diagnosis of CRS. In contrast, Collignon *et al.* (1986), concluded that semiquantitative culture using the roll plate method was a useful indicator for the diagnosis of sepsis particularly if a negative result is used to eliminate a suspected clinical bacteraemia. However, the main problem of catheter culture is that the diagnosis is made after the catheter has been removed, hence many catheters are removed unnecessarily (Elliott, 1993).

1.6.2 Surveillance cultures

The two main sources of microorganisms related to CRS are the skin insertion site and the catheter hub. It has been reported that the hub is the most important source and is one that will go undetected by routine semiquantitative culture techniques (Linares *et al.*, 1985). The clinical value of surveillance cultures such as the hubs however may only reflect contamination rather than sepsis and the laboratory results may be difficult to interpret (Elliott, 1997). Also, insertion site swabs should only be taken when there is clear evidence of local infection as again culture results may only reflect the presence of commensals. A highly discriminative typing technique would therefore be needed to correlate isolates of CNS from surveillance cultures with those from clinical samples to define the source of sepsis.

1.6.3 Blood cultures

Blood cultures are a major component in the diagnosis of CVC related sepsis (Elliott, 1997). The diagnosis can be confirmed by isolating the same organism from blood cultures taken from different sites, for example via a peripheral venepuncture and the catheter (Elliott, 1993), and also from sequential blood cultures (Kloos and Bannerman, 1994). The problem arises however when different microorganisms are assumed to be the same on the basis of limited antibiograms (Zaidi *et al.*, 1996). Isolates of CNS can be readily identified to species level in the diagnostic laboratory using commercial biotyping kits, yet it is rarely considered necessary to confirm the relatedness of less commonly isolated species. However, due to the ubiquitous nature of *S. epidermidis*, the most prevalent species of CNS on the skin surface, discriminate typing is necessary to confirm the relatedness of isolates and consequently identify true infection represented by repeated isolates of the same strain (Geary *et al.*, 1997). Quantitative blood cultures are also helpful for the diagnosis of CVC related sepsis (Elliott,1997; Kloos and Bannerman, 1994). The finding of a 5-10 fold increase in the concentration of microorganisms drawn via the CVC in comparison with that taken through the peripheral line has been suggested to indicate disease (Elliott, 1993)

1.6.4 *In situ* diagnostic techniques

Conventional methods to diagnose CRS generally require that the device is removed for culture, however, in many patients the culture proves negative despite the clinical suspicion. Rapid methods to diagnose CRS without catheter removal using the Gram stain and acridine orange leucocyte cytospin, to demonstrate intracellular microorganisms have been documented (Rushforth *et al.*, 1993; Kite *et al.*, 1999). In the study by Kite *et al.* (1999) the method was shown to have a sensitivity of 96% and specificity of 92%, which compares favourably with culture methods and the results are available in 30 minutes. However, a disadvantage of the method is that the blood is drawn through the catheter hub, which is a

common source of microbial contamination, therefore stringent cleaning of the hub prior to sampling and care in interpretation of the smears is necessary. The *in situ* diagnosis using the differential time to positivity (DTP) between catheter blood and peripheral blood has also been investigated (Blot *et al.*, 1999). In this study, a sensitivity of 94% and specificity of 92% was achieved if a DTP of 2 hours or more between paired samples was used as a cut off. However, these parameters varied considerably the method was investigated by another group and it was also shown to be influenced by the duration of patient catheterisation (Farr, 1999). Both methods potentially could prevent catheters being removed unnecessarily and hence reduce costs, but both methods require further work.

1.7 The Gram positive cell wall

Peptidoglycan is the major component of Gram-positive cell walls, accounting for 50% of the weight and it is responsible for the shape and mechanical integrity of the cell wall. It is composed of *N*-acetylglucosamine and *N*-acetylmuramic acid molecules linked alternately to form the glycan chain. The N-acetylmuramic acid molecules each carry a peptide side chain containing D- and L- alanine, D-glutamic acid and either L-lysine or diaminopimelic acid. The glycan chains are cross-linked by additional peptide chains to form the peptidoglycan network. The peptidoglycan is important in the pathogenesis of infection; it elicits the production of interleukin-1 and opsonic antibodies; it chemically attracts polymorphonucleocytes and activates complement (Jawetz *et al.*, 1987a).

In Gram-positive microorganisms, there are also two classes of teichoic acid. Wall teichoic acid is a linear anionic polymer of ribitol phosphate or glycerol phosphate, which is linked to the peptidoglycan and makes up about 20 % of the weight of the wall. Lipoteichoic acid (LTA) is located in the cytoplasmic membrane but protrudes through the cell wall and is exposed on the outer surface of the cell (figure 1.4). LTA is generally composed of a glycolipid anchor containing unsaturated fatty acids, and a 1,3-linked

poly(glycerophosphate)chain with substitution by oligoglucose or D-alanine at the 2-positions of the glycerols (Fischer, 1994). The poly(glycerophosphate) is assumed to have between 9 and 40 repeating units. The biological activities of LTA include cytokine induction (IL-1, IL-6, and TNF-α) (Verhoef and Mattsson, 1995). Protein-A is a cell wall component of many strains of *S. aureus* and binds to the Fc portion of IgG, with the exception of IgG3 and some strains also have capsules surrounding the cell wall which aid in pathogenesis by inhibiting phagocytosis. Clumping factor (coagulase) is also present on the cell wall surface of most strains of *S. aureus*.

Figure 1.4 **The Gram positive cell wall**

1.7.1 Serological approaches in diagnosing Gram-positive infections

The serological diagnosis of sepsis related to CVC may offer a rapid and non-invasive method of identifying patients with sepsis allowing prompt intervention with appropriate therapy. Serological approaches to diagnosing other infections related to staphylococci have been investigated. In the mid eighties, an ELISA for the detection of IgM and IgG antibodies against cell wall teichoic acid was developed for the diagnosis of intravascular staphylococcal disease (West *et al.*, 1985). The assay failed to discriminate between healthy patients and those with intravascular disease due to CNS but did detect a significant difference in IgG titres between those patients with endocarditis due to *S. aureus* and the controls. The detection of IgM antibody as a marker of infection proved of no value in this study. In the study by Verbrugh *et al.* (1983), a panel of staphylococcal antigens including peptidoglycan, teichoic acid, extracellular alpha toxin and nuclease was used in an ELISA for the diagnosis of staphylococcal disease. The peptidoglycan component was shown to be the most useful antigen to differentiate between complicated and uncomplicated staphylococcal bacteraemias, particularly when serial serum samples are tested. The alpha toxin and teichoic acid component respectively discriminated between the groups to a lesser degree, whilst the use of nuclease as an antigen proved of no value. Julander *et al.* (1983), compared the serological analysis of antibodies against staphylococcal alpha-haemolysin to those produced against teichoic acid using ELISA in the diagnosis of staphylococcal disease. Their work suggested that the antibody response elicited against alpha-haemolysin is more common than to teichoic acid. However, the use of the two tests run in parallel was the preferred approach. The study also demonstrated that the IgM antibody response against both antigens was found less frequently than IgG. The IgM titres were found to be very low and the specificity questionable due to the problem of rheumatoid factor of the IgM class, giving rise to false positive IgM reactions. The conclusion was that IgM antibody determination was of little diagnostic value.

The antibody response to staphylococcal cell wall teichoic acid has also been investigated using a method of double diffusion in agar gel plates (Crowder and White, 1972) and by an ELISA (Julander *et al.*, 1983). Crowder and White described how the serological analysis using staphylococcal teichoic acid as a test antigen allowed for the discrimination between patients with staphylococcal and non-staphylococcal endocarditis to be made. However, cross reactions were noted from patients with endocarditis due to *S. aureus* and *S. epidermidis* and the potential for cross reaction between antibodies against the cell wall *N*-acetylglucosamine of *S. aureus* and some strains of streptococci was described.

A solid phase radioimmunoassay for detecting IgG antibodies to *S. epidermidis* in patients with endocarditis has also been described (Epersen *et al.*, 1987). This assay differentiated between patients with endocarditis and uncomplicated bacteraemia due to CNS. The antibody response to *S. aureus* protein-A has also been investigated as a marker of infection (Greenberg *et al.*, 1990), and significant differences between patients with endocarditis and uninfected controls were observed. However, no serological difference was demonstrated between patients with endocarditis and uncomplicated bacteraemia.

Later, an ELISA system was developed for the detection of antibodies to exocellular proteins of *S. aureus* in bone infections (Krikler and Lambert, 1992), which differentiated those patients with bone infections due to *S. aureus* from healthy patients and also those with infections due to other Gram positive organisms including *S. epidermidis* and *S. sanguis*. The assay therefore offered a potential tool in differentiating between infections and mechanical loosening of prosthetic joints. More recently, an ELISA for serodiagnosis of prosthetic infection due to *S. epidermidis* has been developed (Lambert *et al.*, 1996). This test utilised exocellular carbohydrate antigens of *S. epidermidis* purified from brain heart infusion (BHI) culture medium to detect serum levels of IgG in patients with bone infection due to the organism and in those with uninfected joints. Although the assay proved not to be specific for *S. epidermidis* and detected raised antibody levels in infections due to other Gram-positive organisms, it did discriminate between test and control patients. These initial studies proved

encouraging in potentially offering an ELISA system for the detection of raised antibody levels in serious Gram positive infections.

Alpha-haemolysin toxin is a highly potent exotoxin produced by most clinical isolates of *S. aureus* and the detection of raised antibodies to the toxin has been used for many years as a diagnostic tool in human staphylococcal disease (Julander *et al.*, 1983). It has been widely used in the diagnosis of staphylococcal osteomyelitis (Taylor and Plommet, 1973) and is also recommended as a diagnostic tool for other staphylococcal diseases such as pneumonia and joint infections (Behring Diagnostics Inc., 1995). Healthy persons were found to have antibody levels against alpha-haemolysin of up to 2 international units (IU) /ml and it is recommended that levels of 2 IU/ml or above be considered significant.

Although widely used, the test suffers several disadvantages. Firstly, false positive values have been noted in non-staphylococcal disease, for example, tuberculosis and secondly, the anti alpha-haemolysin levels are not raised in all cases of staphylococcal disease (Taylor and Plommet, 1973). The anti alpha-haemolysin test has also been found to have a low sensitivity (Julander *et al.*, 1983). Approximately 86-95% of strains of *S. aureus* from human origin form alpha-haemolysin, however, it is rarely formed by the CNS (Easman and Goodfellow, 1991) and specific exoproteins and toxins have not yet been convincingly associated with the pathogenesis of CNS infections.

Laboratory tests widely used for the diagnosis of streptococcal disease include the anti-streptolysin (ASO) and anti-deoxyribonuclease B (ADNB). These tests are used in the detection of streptococcal infection and their sequelae, rheumatic fever and glomerulonephritis. Streptolysin-O is an extracellular product of group A, C, and G streptococci and is more widely used as a diagnostic test as it is elevated in 80-85% of cases

(Behring Diagnostics Inc., 1995). However, many sera tested demonstrate a certain level of antibody against streptolysin-O, dependent upon patient age and geographical locality. It is therefore recommended that each laboratory should determine its own "normal range" but as a guideline an orientation value of 200 IU/ml is accepted internationally as the upper limit of the "normal range".

1.8 Characterisation of the staphylococci

The goal of strain typing is to provide laboratory evidence that epidemiologically related isolates collected for example, during an outbreak, are related. The characterisation of the staphylococci through strain identification has become important since the recognition of their clinical significance. The repeated isolation of the same strain is indicative of true infection rather than contamination (Geary *et al.*, 1997).

1.8.1 Conventional methods

Conventional methods used in the typing of staphylococci have been largely based on colonial morphology, antibiogram and biotyping, and bacteriophage typing (Kloos and Bannerman, 1994). Other methods evaluated have included the use of a lectin binding assay (Jarlor *et al.*, 1992) and also slime production (Davenport *et al.*, 1986). The ease of obtaining an antibiogram and a biotype make these systems attractive for routine diagnostic laboratories, and when used in combination they provide a moderate degree of discrimination (Kloos and Bannerman, 1994). However, as single techniques, both methods have associated disadvantages. The epidemiological value of antibiograms is low for nosocomial strains of CNS that have, by definition been acquired within an environment that acts as a reservoir of antibiotic resistance genes. Antibiogram typing for example, would fail to type a strain whose susceptibility pattern varies due to loss or gain of resistance markers encoded by plasmid

DNA influenced by patient treatment (Geary *et al.*, 1997). Antibiograms however, can be useful markers for "local" strains of CNS within hospitals whose resistance patterns are unique. Biotyping as a single system lacks discriminatory power (Geary *et al.*, 1997; Dryden *et al.*, 1992) and strains of *S. epidermidis* exhibit too few biotypes for the system to provide useful epidemiological information. Also, certain biochemical characters demonstrate clonal variation, which may accumulate upon repeated subculture. Epidemiological studies of staphylococci are notoriously difficult to evaluate and these traditional methods of biotyping and antibiogram typing have low discrimination for the CNS. Previous work has shown the use of phenotypic markers such as biotype and antibiogram to have little use in determining the pathogenesis and epidemiology of CNS infection in oncology patients when compared to molecular methods such as random amplification of polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE) (Nouwen *et al.*, 1998).

A suggested method which is simple, rapid and cost effective, of distinguishing true pathogens from contamination is the detection of slime production (Christensen *et al.*, 1983) which could be used along with other phenotypic markers such as antibiogram and biotyping to characterise CNS. Davenport *et al.* (1986) used slime positivity as a marker for infection and found it to be significantly associated with clinical infection and recommended its use as a tool for early diagnosis of infections due to CNS. A simple, rapid and sensitive method of detecting slime production is by the use of congo red agar (Freeman *et al.*, 1989). Congo red incorporated into the agar indicates the presence of exopolysaccharide substance (slime) through blackening of the staphylococcal colonies, however the exact mechanism is unknown.

1.8.2 Molecular approaches to characterisation

As conventional methods have been regarded as generally unsatisfactory, many have been replaced or supplemented with newer molecular methods such as plasmid fingerprinting, random amplified polymorphic DNA analysis (RAPD), ribotyping, PCR-based methods, multilocus enzyme electrophoresis and analysis of chromosomal DNA restriction patterns by pulsed field gel electrophoresis (Tenover *et al.*, 1995; Geary *et al.*, 1997; Kloos and Bannerman, 1994).

1.8.2.1 Pulsed field gel electrophoresis (PFGE)

Analysis of bacterial DNA by PFGE has several significant uses. Firstly, a physical map of the bacterial chromosome can be developed by using known genes as probes. Secondly, transposon insertions into previously uncloned loci can be mapped. Finally, PFGE is highly effective in molecular epidemiologic studies of bacterial isolates and is superior to both phenotypic and Southern blotting techniques in discriminating bacterial isolates (Maslow *et al.*, 1993).

By the application of a pulse or the change of direction to an electric field as in PFGE, the upper limit of fragment size of DNA separation is greater compared with conventional electrophoresis methods (Kaufmann and Pitt, 1994). The movement of fragments of DNA in an agarose gel is influenced by changes in orientation of an electric field, therefore by switching the field at known intervals many points along the DNA molecule will move in the direction of the new field until one end eventually leads the molecule in that direction. Upon reapplication of the first field a reorientation process occurs within the DNA molecule. The reorientation is dependent upon DNA fragment size and provides the basis by which the fragments are separated. PFGE of the complete staphylococcal chromosome following

cleavage with a selective rare cutting restriction endonucleases generates a profile of up to 18 bands and allows a comparison to be made through analysis of the macrorestriction patterns (Tenover *et al.*, 1995). Although PFGE is expensive and technically demanding, it is currently the most sensitive technique available for epidemiological investigation of clonal relatedness between staphylococcal isolates (Lina *et al.*, 1992; Snopková *et al.*, 1994).

1.8.2.2 PFGE methodology

PFGE involves embedding microorganisms in agarose, lysing them *in situ* and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently, for example, *Sma*I (Tenover *et al.*, 1995). Molecular weight standards are also included with the test isolates, for example, a "lambda ladder" which consists of concatamers of lambda bacteriophage and thus provides a means of calculating the fragment sizes.

Segments of the agarose containing the DNA fragments are inserted into the wells of an agarose gel and the restriction fragments are resolved into a series of bands in the gel by apparatus that alters the direction of the electric current. The choice of restriction enzyme(s) is influenced by several factors (Maslow *et al.*, 1993). Firstly, the guanine and cytosine (G+C) content of the bacterial species is important. DNA with low G + C content, for example, *S. aureus*, will cut infrequently when treated with restriction enzymes with a G + C-rich recognition site e.g. *Sma*I, which recognises a CCCGGG sequence. Secondly, enzymes with 8 base-pair recognition sequences will cut less frequently than comparable 6 base-pair cutters. After staining with ethidium bromide, the resulting DNA restriction patterns of the organisms are compared with one another to determine their relatedness.

1.8.2.3 PFGE systems

All PFGE systems rely on DNA reorientation for fragment separation by subjecting the molecules to at least two alternating electric fields. Early models such as orthogonal field agarose gel electrophoresis (OFAGE) and transverse alternating field electrophoresis (TAFE) suffered from problems in electrode configuration resulting in difficulties in DNA mobility (Kaufmann and Pitt, 1994). Field inversion gel electrophoresis (FIGE) and contour-clamped homogenous electric field (CHEF) are upgraded systems of PFGE, which are versatile, and realtively easy to operate. FIGE involves a system of electric fields which move forward and backward through the gel whilst CHEF uses a system of multiple electrodes to generate homogenous electric fields. The CHEF system uses an angle of reorientation of 120° with gradations of electropotential radiating from the positive to negative poles allowing for molecules up to 7000 kb to be separated. Irrespective of which system of PFGE is used, the parameters affecting the separation of DNA remain the same including agarose concentration, DNA quality and concentration, electric field switching times and strength, and also the temperature and ionic strength of the system buffer (Kaufmann and Pitt, 1994).

1.8.2.4 Interpretation of chromosomal DNA restriction patterns produced by PFGE

Due to lack of standardised criteria for the analysis of restriction patterns, a set of guidelines for interpreting the patterns have been proposed by Tenover *et al.* (1995). The criteria take into account the random genetic events, which occur amongst microorganisms such as point mutations and insertions or deletions of DNA, which would generate small changes in PFGE restriction patterns during the course of an outbreak. The guidelines presented by Tenover *et al.* (1995) are intended for use in analysing discrete sets of isolates obtained during epidemiological studies and are summarised in table 1.2. In potential outbreak situations

however, the guidelines should only be used for outbreaks occurring over a short period of time, for example, 1-3 months.

Table 1.2

Guidelines proposed for interpreting restriction patterns

Category of isolate	Typical no. of fragment differences compared with "outbreak" pattern
Indistinguishable	0
Closely related	2-3
Possibly related	4-6
Different	> 7

Adapted from Tenover *et al.* (1995)

Another method of determining the levels of relatedness between strains is by conducting comprehensive pairwise comparisons of macrorestriction fragment sizes by using the Dice coefficient (S_D), which is defined as the ratio of twice the number of bands common to each pair of restriction profiles to the total number of macrorestriction profiles in each restriction profile (Grothues and Tümmler, 1991; Rainey *et al.*, 1994). The Dice coefficient (S_D) is expressed as:

$$S_D = \frac{2ni}{(n_A + m_B)}$$

where n_A and m_B are the numbers of fragments in patterns A and B and ni is the number of common bands. The statistical significance of pairwise comparisons can be evaluated and

indications of a 95% confidence interval for the statistical similarity of restriction fragment patterns made (Grothues and Tümmler, 1991). Interpretation of the macrorestriction fragment profiles can also be performed using software, for example, Phoretix 1D Advanced gel analysis computer programme (Phoretix international, UK). Completed gel analysis data can be transferred to the database programme to allow strain comparison by calculation of the Dice correlation coefficient. Isolates are clustered together by the unweighted pair group method of arithmetic averages (UPGMA) to permit the construction of a dendrogram.

1.8.2.5 PFGE in the microbiology laboratory

As a molecular typing system, the validity of PFGE is well established (Tenover *et al.,* 1995). Zaidi *et al.* (1996) used PFGE to assess the similarity of CNS isolated from sequential blood cultures of neonates, and although they found the technique time and labour consuming, it provided them with an excellent system for studying differences amongst strains of CNS especially in an environment where the strains isolated were highly resistant to the commonly used antibiotics. The system has also been used to study the molecular epidemiology of the CNS involved in catheter related infections within haemato-oncology patients (Nouwen *et al.*, 1998). PFGE has been successfully used to examine the persistence of virulent clones of CNS in neonatal intensive care units responsible for catheter related sepsis (Vermont *et al.*, 1998).

Lina *et al.* (1992) observed that epidemiologically unrelated strains of *S. epidermidis* isolated from patients with community acquired NVE showed identical profiles using PFGE. This finding suggests that PFGE may be a useful tool for not only discriminating between strains of microorganisms but also for identifying strains that are potentially more pathogenic or that can disseminate more readily than others. Also, Lina *et al.* (1995) used PFGE to identify the

aetiological agent and its source in a case of chronic bacteraemia due to *S. epidermidis* in a patient following bone marrow transplantation, and recommended its use as a tool for understanding the pathogenicity and transmission of CNS. Finally, PFGE has more recently been used to analyse the macrorestriction fragment profiles and investigate the relatedness, of strains of CNS recovered from patients with CAPD, endocarditis, non-union bone fractures, prosthetic hip replacement and CVC-related sepsis (Lang *et al.*, 1999). PFGE, in this study, highlighted the genomic diversity of the CNS and demonstrated that there was no apparent segregation of CNS by associated infection type, medical unit or medical centre.

1.9 Aims of the current study

- To develop a serological test to assist in the diagnosis of deep-seated infections caused by staphylococci and to investigate the epidemiology of these infections by conventional and molecular methods.

- To obtain, prepare and characterise antigenic material derived from coagulase negative staphylococci (CNS) isolated from patients with a clinical diagnosis of catheter related sepsis.

- To develop a prototype ELISA system incorporating the exocellular antigen derived from CNS and investigate the parameters influencing the assay including selection of suitable internal standards, the evaluation of the best method of determining end points within the assay and a study of intratest variation and intertest variation.

- To determine the levels of both IgM and IgG to the novel antigen in the normal population and in patients with CRS, and to evaluate the parameters of the prototype ELISA including sensitivity, specificity, positive predictive value, negative predictive value and accuracy.

- To investigate the potential cross reactivity of the antibodies with cardiolipin, a structurally similar phospholipid to the ELISA antigen and present in mammalian cell membranes.

- To determine the similarity of strains of CNS isolated from patients with CRS using PFGE genotyping and also conventional phenotypic typing methods including antibiograms, biotypes and slime production.

- To develop the prototype ELISA into a format which could be incorporated and used in routine clinical Microbiology laboratories.

- To assess the potential of the ELISA in the serodiagnosis of other deep-seated Gram-positive infections including prosthetic joint infection, endocarditis and pyogenic spondylodiscitis.

Chapter 2 Isolation and characterisation of a novel exocellular glycolipid antigen produced by coagulase-negative staphylococci

2.1 Introduction

Many attempts have been made to identify the exocellular and surface components of *S. epidermidis* which are responsible for the colonisation of prosthetic devices and the formation of biofilm. Although the slime material produced by many strains of *S. epidermidis* has been difficult to characterise (Christensen *et al.*, 1994), a number of distinct exocellular polymers have been identified. Tojo *et al.* (1988) isolated a purified capsular polysaccharide adhesin (PS/A) which is highly immunogenic in its purified form and appears to enhance the very early stages of colonisation of biomaterials. PS/A was isolated from strain RP62A (ATCC 35984) and was predominantly carbohydrate with low to non-detectable levels of protein, nucleic acids, and phosphate. This adhesin contained no detectable lipids, and glucosamine, galactose and galactosamine were the principal monosaccharides. In addition, Timmerman *et al.* (1991), characterised a 220-kDa proteinaceous surface antigen of *S. epidermidis* 354 which also mediates attachment to polymer surfaces.

Mack *et al.* (1994) chemically characterised one component of the biofilm as a hexosamine containing polysaccharide adhesin. This adhesin, referred to as the polysaccharide intercellular adhesin (PIA), is important in staphylococcal cell clustering and in the accumulation phase of the biofilm formation, and has been shown by Baldassarri *et al.* (1996) to be the same as the slime-associated antigen first characterised by Christensen *et al.* (1990). The chemical composition of extracellular slime substance (ESS), another polymer important in biofilm formation, is at present undefined although work by Hussain *et al.* (1992) showed that ESS had a similar composition to glycerol teichoic acid when grown in a chemically defined medium. More recently, Karamanos *et al.* (1997) isolated a highly immunogenic, 20-kDa acidic polysaccharide which constitutes a major component of the extracellular slime of *S. epidermidis*. Serum samples from patients with bacteraemia due to

slime producing strains of *S. epidermidis* were shown to have significantly higher levels of antibody to the 20-kDa polysaccharide than sera from healthy individuals. Despite the wide range of components that have been identified to date, no single component appears to be associated with adhesion and biofilm formation in strains causing infection.

Krikler and Lambert (1992) demonstrated the immunological importance and potential of exocellular protein antigens of *S. aureus* as markers of bone infection by immunoblotting, whilst Lambert *et al.* (1996) used exocellular carbohydrate antigens from a strain of *S . epidermidis* in an enzyme linked immunosorbent assay (ELISA) to facilitate the diagnosis of orthopaedic prosthetic infection. In the study of Lambert *et al.* (1996) the exocellular antigenic material was prepared from the culture supernatant of a strain of *S. epidermidis* isolated from an orthopaedic infection, and grown in brain heart infusion (BHI) broth (Oxoid, Basingstoke, U.K.). The antigen was partially purified by gel permeation liquid chromatography. High molecular weight fractions were selected for binding to the solid phase in an ELISA. The ELISA was shown to differentiate between IgG antibody levels of healthy controls and patients with deep-seated staphylococcal infection.

The aim of this part of the study was to isolate exocellular antigenic material derived from 7 strains of coagulase negative staphylococci (CNS). The microorganisms from which the antigen was prepared were isolated from the blood cultures of patients with defined catheter-related sepsis (CRS) with a view to incorporating the antigen in an ELISA for the serodiagnosis of the infection. In contrast to the ELISA of Lambert *et al.* (1996) which incorporated antigen prepared from a single strain, a number of strains of CNS were chosen to prepare the antigen in order to represent a range of colonial morphologies, biotypes and slime / non-slime producing strains of CNS associated with CRS. The exocellular antigenic material was then characterised by competitive ELISA, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), silver stain and western blotting. Further characterisation by chemical analysis was performed on a single strain of CNS selected from the 7 isolates, which had been cultured in a chemically defined medium (Hussain *et al.*, 1992).

2.2 Materials and Methods

2.2.1 Bacterial strains

Seven phenotypically different strains of CNS, which were isolated from the blood cultures and central venous catheters (CVC) of patients with defined CRS, were selected for antigen preparation. Seven isolates were chosen to include a wide range of colonial variants. The strains were grown on blood agar plates containing 7% defribinated horse blood incubated at 37°C in CO_2 for 24 h and identified by API STAPH (bioMérieux, Marcy-l'Etoile, France). The ability of each strain to produce exocellular slime was determined by colonial appearance on congo red agar (figure 9.0). The strains were stored on polystyrene beads at -20°C and nutrient agar plates (Oxoid, Basingstoke, U.K.). The strains have also been deposited at the National Collection of Marine and Industrial Bacteria as NCIMB 40896, NCIMB 40945, NCIMB 40946, NCIMB 40947, NCIMB 40948, NCIMB 40949, and NCIMB 40950.

2.2.2 Preparation and recovery of exocellular antigenic material from culture supernatants

The BHI-7 antigen was prepared from 7 strains of CNS (5 *S. epidermidis,* 1 *S. haemolyticus*, 1 *Micrococcus kristinae*) which were identified by API STAPH (bioMérieux, Marcy-l'Etoile, France). Starter cultures for each strain were prepared by inoculating 2 to 3 colonies from nutrient agar plates into 20 ml of BHI in 100 ml conical flasks. The broths were incubated for 18 h at 37°C on a rotary shaker (200 rpm). One ml of each starter culture of each strain was inoculated separately into conical flasks containing 200 ml of BHI and incubated for 18 h at 37°C on a rotary shaker (200 rpm). The purity of each culture was checked by inoculation onto nutrient agar plates, which were incubated for 18 h at 37°C. The cells were harvested by centrifugation at 10,000 g for 10 min and the culture supernatants collected. Aliquots of each supernatant (100 ml) were dispensed into 500 ml flasks and the water was removed by freeze drying. The freeze dried material was

resuspended in 10 ml of distilled water resulting in a 10-fold concentration of the original culture supernatent. After centrifugation at 15,000 g for 5 min to remove any residual insoluble material, 1 ml aliquots of each supernatant fluid were applied to a Superose 12 HR 10/30 gel filtration column equilibrated with water (dimensions: 30cm length x 1 cm diameter), (Amersham Pharmacia Biotech, St. Albans, Hertfordshire, U.K.). The column was eluted with water at 0.4 ml/min, monitoring the eluate at 280 nm and 50 fractions of 0.8 ml each were collected (Pharmacia FPLC). The void volume of the column (determined by elution of blue dextran) was 7.2 ml. In addition, to identify any components unrelated to the material in the culture supernatants, uninoculated BHI broth was also applied to the FPLC column.

2.2.3 Determination of hexose and protein content in fractions 1 to 50

2.2.3.1 Hexose assay (Dubois *et al.*, 1956)

Phenol solution (0.1ml of 80% w/v) was added to 0.1ml of each fast protein liquid chromatography (FPLC) fraction, followed by addition of 2ml concentrated H_2SO_4 S.G. 1.84 (98%). The fractions were left at 20°C for 10 min. A brown colour developed if hexose was present in the solution. One hundred microlitres of each fraction was transferred to a microtitre plate and the absorbance read at 492nm. The assay was calibrated using glucose as a standard (concentration range:10 to 50µg) and the hexose content of the fractions expressed in terms of equivalent glucose content.

2.2.3.2 Protein assay (Folin-Lowry) (Lowry *et al.*, 1951)

Reagents A and B were prepared as follows:

Reagent A: 1% w/v $CuSO_4$, 2% w/v potassium tartrate in 0.1N NaOH
Reagent B: 2% w/v Na_2CO_3

Equal volumes of reagent A and B were mixed and 0.4ml of the solution added to 0.1ml aliquots of the FPLC fractions. Forty microlitres of Folin Ciocalteu reagent (Sigma, U.K.) was added, mixed and left at 37°C for 30 min. Fifty microlitres of each fraction was transferred to a microtitre tray and the absorbance read at 692nm. The assay was calibrated using bovine serum albumin as a standard (concentration range: 10 to 300μg).

2.2.4 Detection of antigenic material in FPLC fractions by enzyme linked immunosorbent assay (ELISA)

An indirect ELISA was used to assay FPLC fractions (1 to 50) of each culture supernatant for antigenic content. The ELISA measured binding of IgG in serum from a patient with CRS and *S. epidermidis* isolated from the blood culture. Individual fractions were diluted in 100 volumes of sodium carbonate/bicarbonate buffer (0.05M, pH 9.6) and 100μl of the diluted antigen was dispensed into wells of a 96-well flat bottomed microtitre plate (Immulon 2, Dynex Technologies, Chantilly, VA). Plates incorporating *S. epidermidis* and *S. aureus* LTA (section 2.2.5) and control material (fractions of BHI alone) were also prepared (section 2.2.3). The plates were stored at 4°C for 18h to allow for binding of the fractions to the polystyrene surface of the wells (Lambert *et al.*, 1996). The residual material was then removed, and the plates washed 3 times in TBS-Tween buffer (0.01M Tris-HCl pH 7.4, NaCl 0.9%, 0.3% v/v Tween-20) using an ELP-40 automated plate washer (Launch Diagnostics, U.K.). Unbound antigen sites in the wells of the plates were blocked by completely filling the wells with TBS-Tween buffer and allowing the plates to stand at 4°C for 1 h, after which, the buffer was removed and the coated plates stored at -20°C until required.

The patient's serum was diluted 1:800 (5μl of serum in 4ml of TBS-Tween buffer) and 100μl added to the wells of the microtitre plate coated with fractions 1 to 50, which was then covered with an acetate plate sealing strip and incubated for 2 h at 4°C. After removal of the serum and washing with TBS-Tween, bound IgG was detected by the addition of 100μl of

protein A-horseradish peroxidase conjugate (0.5μg/ml in TBS-Tween; Sigma, Poole, Dorset, U.K.) for 2 h at 4°C. The conjugate was then removed by washing with TBS-Tween, and the ELISA developed by the addition of 100μl of the chromogenic substrate to all of the wells. The substrate contained 10 mg of 3,3',5,5'-tetramethylbenzidine (Sigma, U.K.) dissolved in 1 ml of dimethyl sulphoxide and diluted into 100 ml of sodium acetate/citrate buffer (0.1M, pH 6.0) containing 20μl of 6% v/v H_2O_2. The plate was incubated at room temperature for 10 minutes and the reaction was stopped by addition of 100μl of 1M sulphuric acid (BDH chemicals, U.K). The yellow coloured product in each well was measured immediately at 450nm with an Anthos 2001 plate reader (Labtech, Ringmer, East Sussex, U.K.).

2.2.5 Preparation of control lipoteichoic acid (LTA)

Lipoteichoic acid (LTA) from *S. aureus* and *S. epidermidis* was also used as control antigen to coat the wells of microtitre trays. LTA from *S. aureus* was obtained from Sigma, whilst LTA from *S. epidermidis* was prepared from strain NCIMB 40896. *S. epidermidis* LTA was prepared by culturing the strain in 4 litres of BHI for 18 h with shaking at 37°C and harvesting the cells by centrifugation at 10,000g for 10 min. The cells were then washed in saline, resuspended in water and freeze dried. Phospholipids were removed by extraction with chloroform:methanol (2:1). The de-fatted cells were dried *in vacuo* and the LTA was then extracted by stirring with 80% w/w phenol for 30 min. After centrifugation (10,000g, 10 min), the upper (aqueous) phase was collected and analysed against water to remove the phenol. The crude LTA was incubated with RNA-ase/DNA-ase (1 mg/ml each, Sigma U.K.) for 2 h at 37°C then re-extracted with phenol. Finally, the LTA was purified by gel filtration on a Superose 12 10/30 FPLC column, eluted with water at 0.4ml/min, collecting 0.8ml fractions.

2.2.6 Competitive ELISA of LTA absorbed serum against a panel of antigens (BHI-7, HHW6 and LTA).

The preliminary results of the analysis of FPLC fractions by ELISA (section 2.2.4) indicated that antigenic material was present in fractions 10 to 15 and was either LTA or a substance that was chemically related to LTA. To support these initial findings, ELISA were performed on the FPLC fractions as described in 2.2.4 on serum samples from patients with a defined CRS and controls that had been pre-absorbed with LTA prepared from *S. epidermidis*. In addition, the future characterisation of antigenic material prepared in BHI broth, which is chemically undefined would be complicated. Therefore, the use of a chemically defined medium (Hussain-Hastings-White, HHW) designed for the growth of *S. epidermidis* (Hussain *et al.*, 1991) was investigated. However, upon preparation of the starter cultures (2.2.2), only 6 of the 7 strains grew in this medium, therefore the antigen was prepared using only 6 strains and was designated HHW6.

Microtitre plates were coated with pooled fractions 10 to 15 (BHI-7) and HHW6 as described previously (2.2.4). In addition, LTA prepared from a single strain of *S. epidermidis* and LTA from *S. aureus* (Sigma, U.K.) (2.2.5) at a concentration of 0.05mg/l was also coated onto microtitre trays. The performance of each antigen was assessed by ELISA on a panel of sera from 27 patients with CRS and 14 controls (chapter 4). The sera were tested at a single dilution of 1:800 on each plate so that direct comparisons of the responses could be made. Each serum sample was applied to the plates before and after absorption with *S. epidermidis* LTA. Absorption was performed by the addition of 50µl of LTA (10mg/l) to 2 ml of each serum sample diluted 1:800 in TBS-Tween, incubating for 18 h at 4°C, and centrifuging at 10,000 g for 10 min to remove anti-LTA antibody / LTA immune complexes. The remainder of the ELISA was performed by the method described in 2.2.4. A statistical comparison (unpaired t-test) of the means of the absorbencies of the 2 panels of sera (absorbed and unabsorbed) against each antigen was made.

2.2.7 Separation of the individual components of BHI-7 antigen by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The composition of the individual components of the BHI-7 antigen and their contribution to the ELISA reaction was investigated by separation on SDS-PAGE. Immunoreactive fractions of culture supernatants (fractions 10 to 15) as determined by the crude ELISA (2.2.4), were firstly pooled and freeze dried. Aqueous solutions of pooled fractions from each strain (1mg/ml dry weight) were denatured for 5 min at 100°C in an equal volume of sample buffer containing SDS and mercaptoethanol. Following denaturation of the proteins, 15µl of each sample and molecular weight standards were loaded into lanes of 12% w/v acrylamide gels (Lugtenberg *et al.*, 1975) and electrophoresed at 200 V for 45 min in Mini-Protean II apparatus (BioRad, Hemel Hempstead, Hertfordshire, U.K.). Gels were then investigated for protein and polysaccharide bands, and also used for immunoblotting studies

2.2.8 Detection of protein and polysaccharide constituents by silver staining

The polyacrylamide gel was fixed in a solution containing 40% v/v ethanol and 5% v/v acetic acid for 18 h. A solution containing 0.7% periodic acid in 40% v/v ethanol / 5% v/v acetic acid was added for 45 min to oxidise any polysaccharide. After 3 washes in double distilled water, staining reagent was added and the gel was agitated for 45 min at 37°C.
Staining reagent was prepared by the addition of 2ml of concentrated ammonium hydroxide to 28ml of 0.1M NaOH followed by 5ml of 20% w/v silver nitrate. The reagent was made up to 150ml with double distilled water. The gel was washed 3 times in double distilled water, followed by the addition of developing solution which contained 10mg citric acid, 0.1ml 37% v/v formaldehyde and 200ml of double distilled water. When the stain had developed, the gel was washed with water and photographed immediately.

2.2.9 Detection of antigenic components by immunoblotting

The components separated by SDS-PAGE were transferred onto a nitro-cellulose membrane sheet (0.45μm pore size, BioRad, U.K.). Transfer was performed in an ice-cooled transblot buffer containing 25mM Tris, 192mM glycine and 20% v/v methanol pH 8.3. The polyacrylamide gel and nitrocellulose membrane were rinsed briefly in transblot buffer and placed between pre-soaked chromatography paper, scotch brite pads and support grids. The immunoblot "sandwich" was placed in a transblot cell (BioRad Transblot apparatus) containing transblot buffer at 4°C and a constant potential of 100V applied for 45 min. Unbound sites on the nitrocellulose membrane were blocked with TBS-Tween for 2 h at 4°C. The TBS-Tween was removed and replaced with immune antisera diluted 1:400 in TBS-Tween in which the blot was immersed for 18 h. The antisera was removed and the blot washed 3 times in TBS-Tween buffer, after which bound IgG was detected by the addition of protein-A horseradish peroxidase conjugate (50μg/ml) (Sigma, Poole, Dorset, U.K.) for 2 h at 4°C. After washing, bands were visualised by the addition of chromogenic substrate (10mg 4-chloro-1-naphthol (Sigma)), dissolved in 1ml methanol and diluted in 100ml of 0.01M Tris HCl (pH 7.4) containing 20μl of 6% v/v H_2O_2. After the appearance of any bands, the reaction was stopped by the addition of distilled water.

2.2.10 Chemical characterisation of the antigen

Indirect evidence from the results of the previous experiments indicated that the BHI-7 antigen contained a small amount of protein which was non-immunoreactive; contained hexose sugars; was of low molecular weight and negatively charged and was predominantly LTA or an antigenically related substance. No structural determination of the LTA of CNS has been reported, however the LTA of *S. aureus* consists of approximately 25 glycerol phosphate units linked to a diglucosyldiacylglycerol glycolipid (Fischer, 1994). Confirmation by direct chemical analysis of the BHI-7 antigen could not be performed due to the presence of contaminating components derived from the BHI culture medium.

Therefore, exocellular antigen was prepared for chemical analysis from a single strain (strain 1 CAN 6KIII, NCIMB 40896) after growth in the chemically defined medium, HHW. The chemical nature of this material was investigated by negative electrospray mass spectrometry, ^{31}P and ^{1}H NMR and chemical analysis of acid hydrolysed material.

2.2.10.1 Preparation of the antigen for chemical analysis

The method for antigen preparation was as described for the BHI-7 antigen (2.2.2).
Strain CAN 6KIII, NCIMB 40896 was cultured in 2 L of HHW at 37°C for 18 h on a rotary shaker. Culture medium was recovered by centrifugation, freeze dried and reconstituted in distilled water to 10-times the original concentration. Aliquots of 1ml were applied to the Superose 12 column and eluted in water at 0.4 ml/min collecting 0.8ml fractions. Fractions 10 to15 were pooled and freeze-dried. Of the pooled fractions 10 to 15, 45ml yielded 5.5mg dry weight of material as white powder, which was subjected to analysis.

2.2.10.2 Negative electrospray mass spectrometry

The position of elution of the antigen from the superose 12 FPLC column (fractions 10 to15) indicated that the antigenic material was of high molecular weight (≥ 100 KDal from the elution of standard protein markers). Conventional mass spectrometry only permits analysis of molecules with mass / charge ratio up to 1600. However, the presence of a number of negative charges (as on LTA) enables higher molecular weight substances to be analysed. The electrospray technique does not break the molecule into fragments but detects the relative abundance of molecules with different mass / charge ratios. The antigenic material (0.1 mg) was dissolved in 1ml of methanol/water (95/5 v/v) containing 50µl ammonia (0.88 s.g.) and examined by negative ion electrospray mass spectrometry using a Hewlett Packard MS 5989B mass spectrometer with a 59987A electrospray unit (Hewlett-Packard, Palo Alto,

CA) operating at 10µl of sample per min. LTA from strain NCIMB 40896 and *S. aureus* LTA (section 2.2.5) were also subjected to analysis.

2.2.10.3 ^{31}P nuclear magnetic resonance (NMR) spectra to detect the presence of phosphorus

^{31}P NMR analysis was performed using a Bruker AC-250 spectrometer (Bruker, Coventry, U.K.) operating at 101.3 MHz. The ^{31}P spectrum was obtained using 2mg of antigenic material dissolved in D_2O. The phosphorus chemical shift was measured at 20°C as parts per million (ppm) relative to an internal standard of 85% phosphoric acid.

2.2.10.4 ^{1}H NMR spectra to detect the presence of fatty acids

The ^{1}H NMR spectrum was obtained using 2mg of antigenic material dissolved in dimethyl sulphoxide (DMSO), (Sigma, Poole, Dorset, U.K.). The chemical shift of the signals in ppm was measured relative to the DMSO signal at 2.5 ppm.

The mass spectrum and NMR data of the antigen from NCIMB 40896 suggested an LTA structure analogous to that of *S. aureus* but with a much shorter glycerol phosphate chain length. Chemical analysis of the material was undertaken to confirm the presence of the structural components of LTA (fatty acids, glucose, glycerol and phosphate) and to determine the ratio of each component.

2.2.10.5 Analysis of fatty acids

Fatty acid content was determined by alkaline methanolysis and gas-liquid chromatography (GC). One mg of antigenic material was suspended in 1 ml of 3.8 M NaOH in 50% aqueous methanol in a glass hydrolysis tube. The tube was sealed and incubated at 100°C for 30 min and then cooled to room temperature. Any fatty acids liberated were converted to the corresponding fatty acid methyl esters (FAMEs) by the addition of 6 ml of 6 M HCl / methanol (1:1) and heating at 80°C for 10 minutes. FAMEs were extracted with 1 ml hexane/diethyl ether (1:1). The upper solvent layer was removed and placed in a glass tube containing 3 ml of 0.3 M NaOH. After mixing by repeated inversion the organic phase was recovered and transferred to a 2 ml glass sample tube. The solvent was evaporated to dryness by the passage of nitrogen gas into the tube at room temperature. For quantitative analysis by GC the sample was redissolved in 100 µl of hexane and 1 µl was loaded onto a Hewlett Packard HP-1 capillary column (crosslinked methyl silicone gum, ID 0.32 mm, film thickness 0.17 µm, length 25 m) on a Unicam 610 series GC operating with 1:50 sample splitting and flame ionisation detector (Unicam, Cambridge, United Kingdom). The column temperature was programmed to maintain 150°C for 4 min then increased at 4°C per min to 250°C and held at this temperature for 2 min. The gas phase comprised helium at 6.5 psi with nitrogen as the makeup gas. The flame ionisation detector used hydrogen and air, the injector temperature was 200°C and the detector temperature was 280°C. Fatty acids were identified and estimated quantitatively by comparison with the profile obtained for 1 µl of a standard bacterial acid methyl esters CP™ mix containing 26 bacterial FAMEs (Matreya, Pleasant Gap, PA) diluted in hexane to a concentration of 5 µg/µl. Integration readings were calculated for each peak and the total amount of fatty acid in the original sample calculated.

2.2.10.6 Determination of hexose, phosphate and glycerol content

Hexose, phosphate and glycerol content were determined after acid hydrolysis of the antigen. A 2.4 mg sample of the purified antigen was dissolved in 0.3 ml of 2M

trifluoroacetic acid (TFA), the tube was sealed and heated at 100°C for 18 h. A white precipitate formed when the TFA was initially added. After hydrolysis the TFA was removed by repeated drying under vacuum with addition of water. The hydrolysis products were then dissolved in 0.24 ml of water to give a concentration equivalent to 10 mg/ml of original antigen. Samples of this TFA hydrolysate were subjected to different analyses to determine the nature of hexoses present (as alditol acetates by gas chromatography (GC)) and the relative amounts of hexose, phosphate and glycerol by quantitative colorimetric assays. An empty hydrolysis tube was treated with TFA in the same way as the sample, this was used in all assays as a control (hydrolysis blank).

2.2.10.7 Analysis of the nature of the hexoses by GC

The conversion of hexoses in the TFA hydrolysate to the alditol acetates and their identification by GC was carried out to determine which sugars were present. The method gave a qualitative estimation of the total amount of the alditol acetates. 0.1 ml of the TFA hydrolysate (equivalent to 1mg of antigen) was placed in a glass tube with 50 µl of 3M NH_4OH. Three milligrams of sodium borohydride was added and the solution left in the dark at 22°C for 18 h. One drop of glacial acetic acid was added to destroy excess borohydride. The borate was converted to the methyl derivative by addition of methanol (0.5 ml) and rotary evaporated to dryness at 50°C. Another aliquot of methanol (0.5 ml) was added and the sample again evaporated to dryness. Acetic anhydride (0.1 ml) was then added to the dried alditol sample, the tube sealed and heated in an autoclave at 121°C for 3 h. Excess acetic anhydride was destroyed by adding water (0.4 ml) and the alditol acetates were purified by passage through a Sep-Pak C18 cartridge (Waters, Milford, MA). The cartridge was prepared by washing with acetonitrile (2 ml) followed by water (1 ml). The sample was loaded onto the cartridge, washed with 10% acetonitrile (2 ml) and eluted in 40% acetonitrile (2 ml). The eluted alditol acetates were rotary evaporated to dryness, redissolved in chloroform (0.10 ml) and analysed by GC. One microlitre was loaded onto a Supelco SP-2380 capillary column (ID 0.25 mm, film thickness 0.2 µm, length 30 m) on a Unicam 610 series GC operating with 1:100 sample splitting and flame ionisation detector.

The column temperature was maintained at 250°C. The gas phase comprised helium at 6.5 psi with nitrogen as the makeup gas (flow rate 25.05 cm/sec). The flame ionisation detector used hydrogen and air, the injector temperature was set at 200°C and the detector temperature was set at 280°C. A mixture (1 µl) of the alditol acetates of mannitol, galactitol, glucitol and inositol (5 mg/ml total in chloroform, Supelco, Bellefonte, PA) was run as a standard giving retention times of 7.91, 8.66, 9.38 and 10.37 min respectively.

2.2.10.8 Estimation of the hexose content by phenol sulphuric acid assay

Assay of the total hexose in the TFA hydrolysate was carried out using the phenol sulphuric acid reagent (Dubois *et al.*, 1956). Measured volumes of a standard glucose solution (1 µg/µl) were placed in glass tubes to give a range of 0 to 50 µg of glucose per tube, the volumes were then adjusted to 0.2 ml with water. Both 10 µl and 20 µl samples of the TFA hydrolysate and blank hydrolysate were placed in separate tubes and the volumes also made up to 0.2 ml with water. In addition, 50 µl of 8% w/v aqueous phenol solution was added to each tube followed by 0.5 ml of concentrated sulphuric acid. The tubes were allowed to stand for 10 min then transferred to a water bath at 30°C for 20 min. The absorbance was then measured at 492 nm and the amount of hexose (as glucose) present in the sample was calculated from the standard glucose calibration curve.

2.2.10.9 Estimation of the phosphorus content

Phosphorus present in the sample TFA hydrolysate was measured as phosphate after treatment with alkaline phosphatase to release phosphate from any remaining glycerol phosphate residues. 0.1ml samples of the TFA hydrolysate and the hydrolysis blank were treated with 10 µl of an aqueous solution (1 mg/ml) of alkaline phosphatase (calf intestinal phosphomonoesterase, Sigma, Poole, Dorset, U.K.) for 2 h at room temperature. Both 5 µl and 10 µl samples were then placed in glass tubes. Separate tubes were prepared which contained 1 to 5 µg of phosphorus made from a standard solution containing 10 µg/ml

phosphorus (prepared by making a stock solution of 87.8 mg of KH_2PO_4 in 100 ml water and diluting 5 ml of this solution to 100 ml with water). Colour reagent was prepared by mixing 3 M sulphuric acid (10 ml) with 2.5% w/v ammonium molybdate (10 ml) and adding to 1 g of ascorbic acid. One ml of the colour reagent was added to each tube and incubated at 37°C for 1.5 h. The absorbance was read at 750 nm and the total phosphorus content of the sample calculated from the standard curve.

2.2.10.10 Estimation of the glycerol content

The glycerol content of the TFA hydrolysate was determined by periodate oxidation and measurement of the formaldehyde released with chromotropic acid. 100 µl of glycerol standard containing 2 to 20 µg glycerol, 10 µl of TFA hydrolysate and blank hydrolysate were placed in separate glass tubes. In addition, 20 µl of concentrated sulphuric acid was added followed by 20 µl of 0.1 M aqueous sodium periodate. The tubes were allowed to stand at room temperature for 5 min. Twenty microlitres of 10% w/v aqueous sodium bisulphite was added followed by 0.5 ml of chromotropic acid solution (containing 0.1 g of chromotropic acid in 10 ml of water and 45 ml of concentrated sulphuric acid). The tubes were heated at 100°C for 30 min, cooled and 50µl of saturated aqueous thiourea added. The absorbance was then measured at 570 nm. The amount of glycerol in the TFA hydrolysate was calculated from the standard curve.

2.3 Results

2.3.1 Bacterial isolates

The phenotypic characteristics of the 7 strains of CNS selected for inclusion in the BHI-7 antigen, along with the corresponding NCIMB details are shown in table 2.0.

Table 2.0 Phenotypic characteristics and NCIMB details of the 7 strains of CNS included in the preparation of the BHI-7 antigen

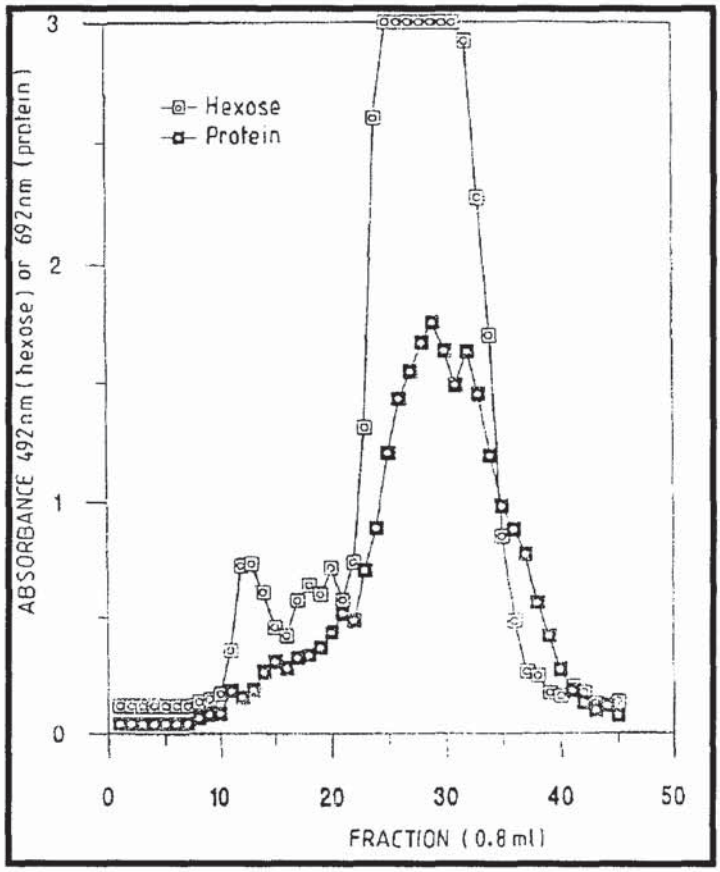
Strain	Designation	Biotpye	API profile	Slime production*	NCIMB Accession No
1	CAN 6KIII	*S. epidermidis*	6706113	+	40896
2	HAR 6KIV	*S. epidermidis*	6306152	-	40945
3	COS 6KV	*S. epidermidis*	6706112	+	40946
4	MIL 6LI	*S. epidermidis*	6706113	-	40947
5	HED 6LIII	*S. epidermidis*	6706113	+	40948
6	ONE 6KVI	*S. haemolyticus*	6632151	-	40949
7	MAT 6LII	*Micrococcus kristinae*	6310114	+	40950

* Slime production determined by the colonial morphology of the strains on congo red agar (section 9.2.4)

2.3.2 Protein and hexose content in fractions 1 to 50 of CAN 6KIII grown in BHI

The protein and hexose content in the 50 fractions of CAN 6KIII eluting from the FPLC column are shown in figure 2.0. The large peaks of material in fractions 20 to 40 represent hydrolysed protein components of the BHI and were also present in the elution profile when fresh uninoculated BHI was applied to the column (section 2.2.3). Fractions 10 to 15, eluting immediately after the void volume of the column, contained hexose and a limited amount of protein.

Figure 2.0 **Purification of exocellular antigen from CAN 6KIII brain heart infusion culture medium. Elution profile of culture supernatant from a Superose 12 gel permeation column. Fractions 1 to 50 were assayed for hexose and protein.**



2.3.3 Detection of antigenic material in fractions 1 to 50 eluting from the gel permeation column. Analysis was performed on elution fractions of the 7 strains of CNS, commercial and laboratory prepared LTA and uninoculated BHI.

The gel permeation elution profiles of the exocellular antigen in the 50 fractions obtained for the 7 strains of CNS, *S. aureus* LTA, *S. epidermidis* LTA and BHI are shown in figures 2.1 to 2.10. In each case the profiles comprised of a major peak of antigen in fractions 10 to15. In addition, the strains HED 6LIII, HAR 6KIV, CAN 6KIII, MIL 6LI and MAT 6LIII had a secondary peak of antigenic material between fractions 20 to 25. Both of the LTA samples (*S. aureus* and *S. epidermidis*) resulted in single broad antigen peaks in fractions 10 to 15, whilst the BHI control did not comprise of any immunoreactive material between the fractions.

Figure 2.1 Superose 12 gel permeation profiles of exocellular antigen of strain HED 6LIII grown in brain heart infusion broth

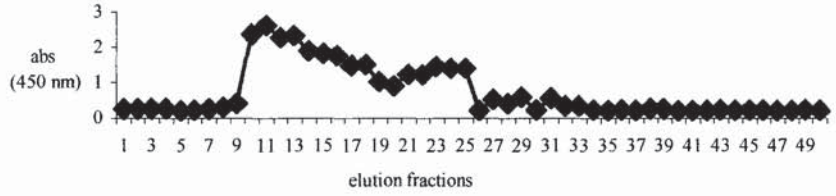


Figure 2.2 Superose 12 gel permeation profiles of exocellular antigen of strain COS 6KV grown in brain heart infusion broth

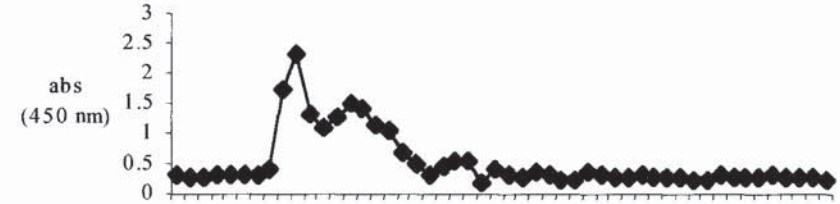


Figure 2.3 Superose 12 gel permeation profiles of exocellular antigen of strain CAN 6KIII grown in brain heart infusion broth

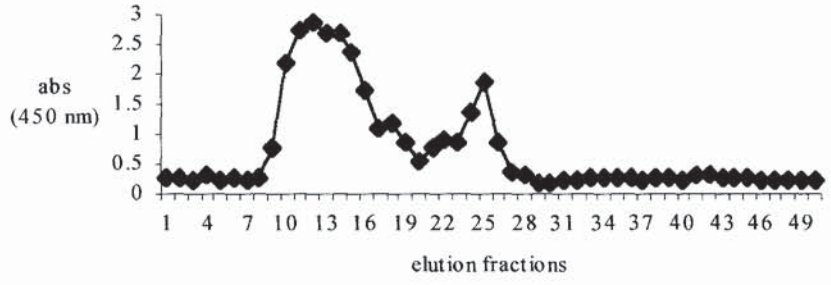


Figure 2.4 Superose 12 gel permeation profiles of exocellular antigen of strain HAR 6KIV grown in brain heart infusion broth

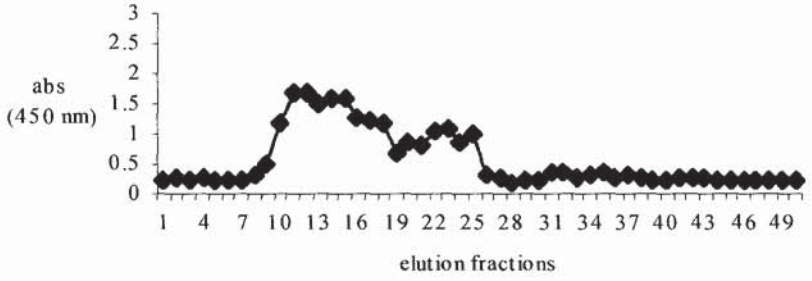


Figure 2.5 Superose 12 gel permeation profiles of exocellular antigen of MIL 6LI grown in brain heart infusion broth

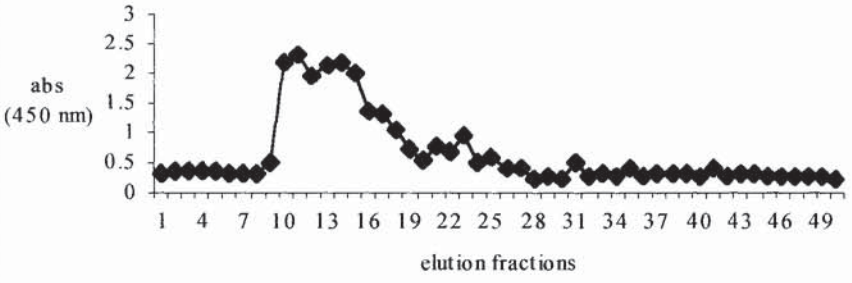


Figure 2.6 Superose 12 gel permeation profiles of exocellular antigen of ONE 6KVI grown in brain heart infusion broth

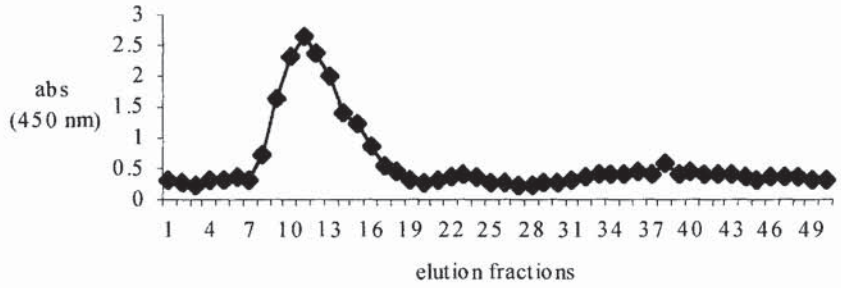


Figure 2.7 Superose 12 gel permeation profiles of exocellular antigen of strain MAT 6LII grown in brain heart infusion broth

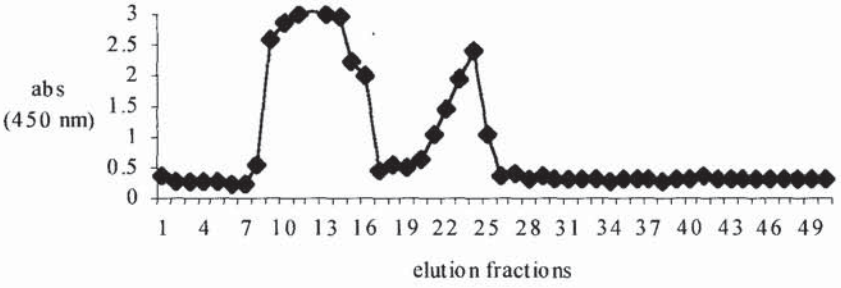


Figure 2.8 Superose 12 gel permeation profiles of brain heart infusion broth

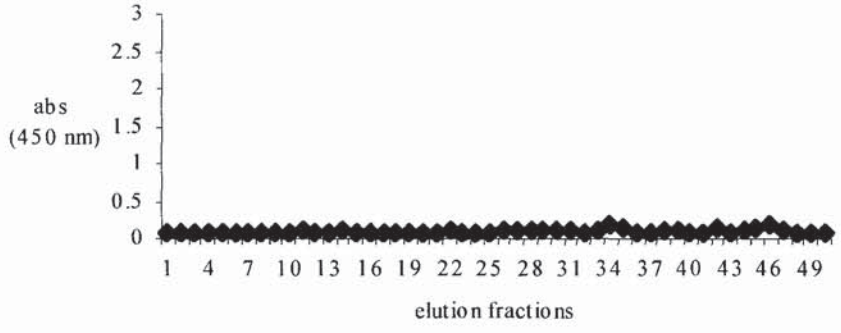


Figure 2.9 Superose 12 gel permeation profiles of *S. aureus* LTA obtained from Sigma, Poole, Dorset U.K.

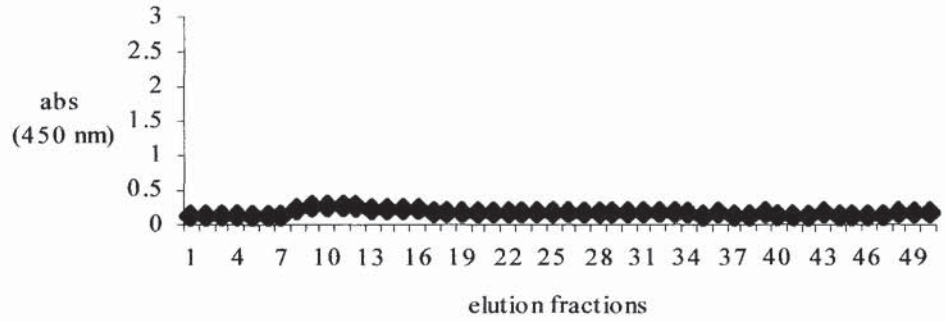
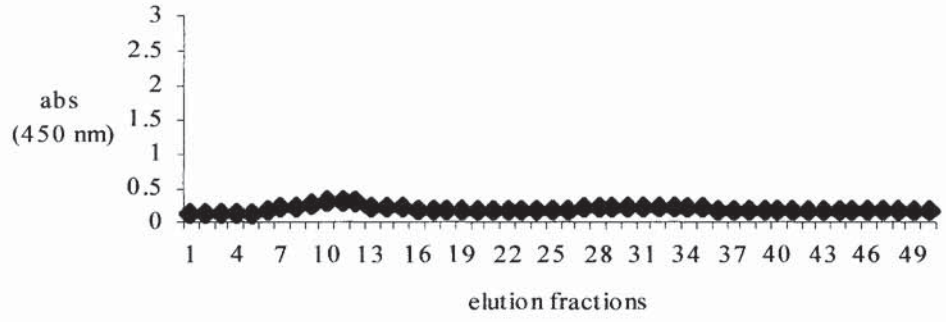


Figure 2.10 Superose 12 gel permeation profiles of *S. epidermidis* LTA (phenol extracted)



2.3.4 Competitive ELISA of LTA absorbed serum against a panel of antigens (BHI-7, HHW6, LTA)

A comparison of the means of serum absorbancies from patients with CRS and the control group in a competitive ELISA is shown in tables 2.1 and 2.2.

Table 2.1 Comparison of IgG ELISA mean serum absorbancies from patients with CRS (n=27) and controls (n=14) before and after absorption with LTA. Serum tested against BHI-7, HHW6 and LTA antigens.

Antigen	CRS patients mean absorbance (450nm) before and after absorption with LTA		control patients mean absorbance (450nm) before and after absorption with LTA	
	Before	*After*	*Before*	*After*
BHI-7	0.4040	0.1766	0.2859	0.1357
HHW6	0.2760	0.1303	0.1541	0.1012
LTA	0.5631	0.1272	0.0987	0.0848

Table 2.2 Statistical analysis (unpaired t-test) of IgG ELISA mean serum absorbancies from patients with CRS and controls before and after absorption with LTA. Serum tested against BHI-7, HHW6 and LTA antigens.

Antigen	Difference in means CRS patients (n=27); controls (n=14)	Difference in means CRS patients (n=27); controls (n=14) After absorption with LTA
BHI-7	0.2275, $p<0.0001$	0.1502, $p<0.0001$
HHW6	0.1367, $p<0.0001$	0.0529, $p<0.0001$
LTA	0.4359, $p<0.0001$	0.0139, $p<0.0259$

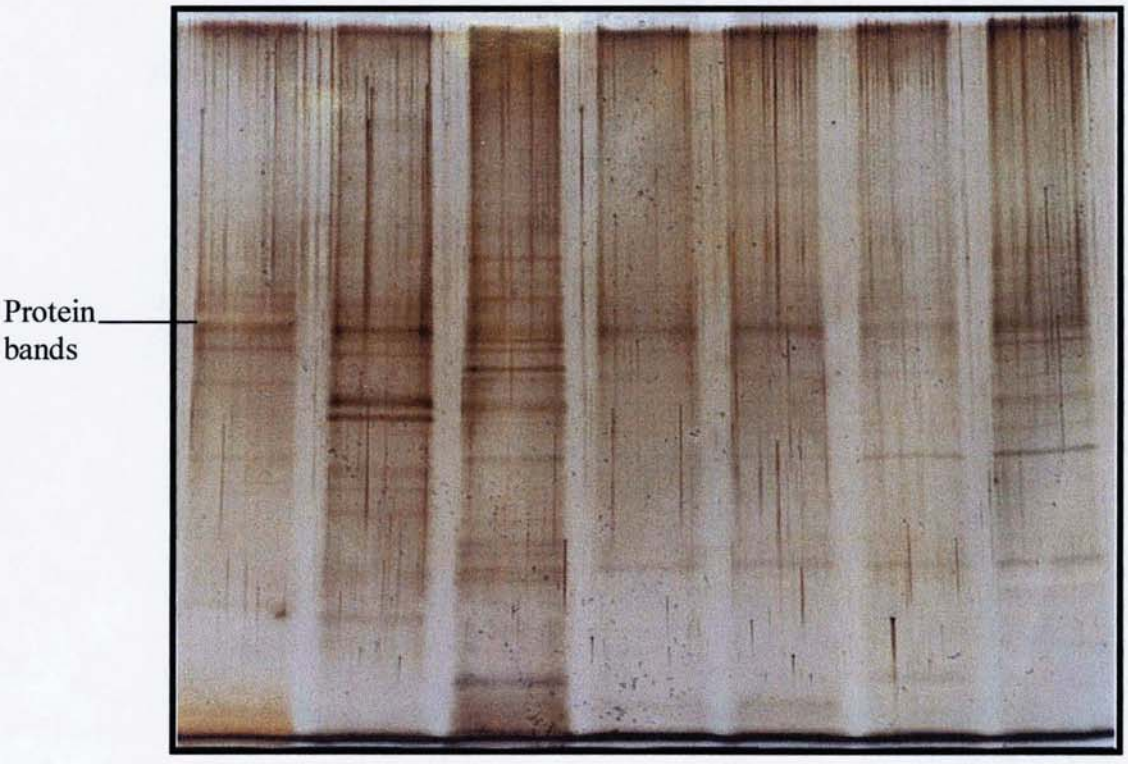
A significant difference in IgG ELISA absorbancies was achieved between patients with CRS and controls with BHI-7, HHW6 and LTA antigen preparations. In addition, the serum absorbancies were considerably reduced in the ELISA following a single absorption with LTA prior to assay. The reduction in absorbancies in the assays were as follows:

BHI-7 = 70% reduction
HHW6 = 44% reduction
LTA = 83% reduction

2.3.5 Analysis of the individual components of BHI-7 by SDS-PAGE and silver staining

The silver stained polyacrylamide gel containing lanes of separated antigen from the 7 individual strains of CNS is shown in figure 2.11. Antigen preparation from each strain contained some protein as shown by sharp bands on the stained gel.

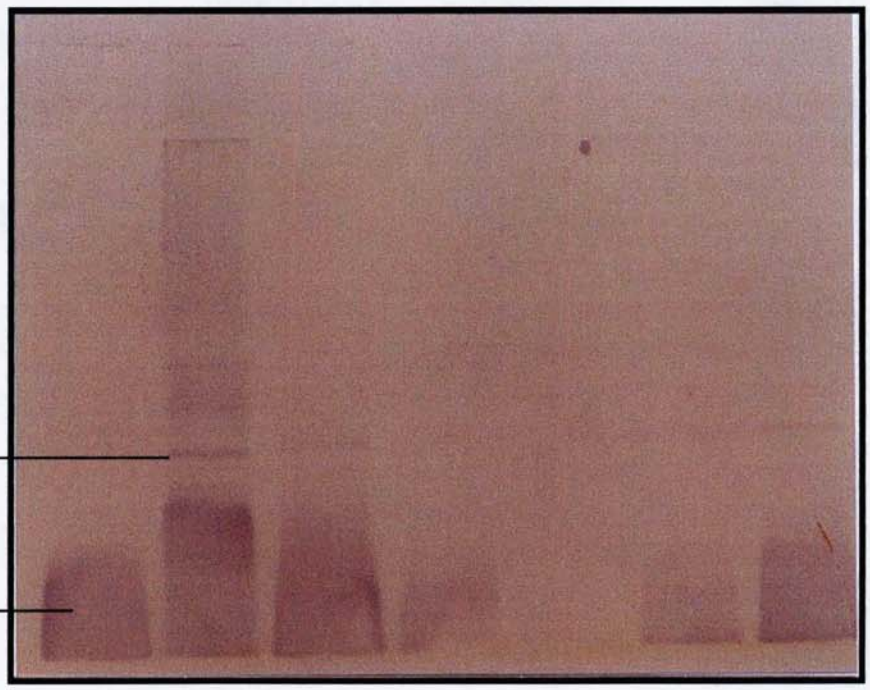
Figure 2.11 **Individual components of BHI-7 by SDS-PAGE on 12% polyacrylamide silver stained gel. Lanes 1-7 contain 10μl of purified antigen (pooled fractions 10 to 15 as in figures 2.1 to 2.7) from the 7 strains of coagulase negative staphylococci.**



2.3.6 Analysis of the individual components of BHI-7 by SDS-PAGE and immunoblotting

The western blot containing lanes of separated antigen from the 7 individual strains of CNS after immunoblotting is shown in figure 2.12. The dominant antigenic material migrated as fast-moving, low molecular weight and negatively charged, and is shown as diffuse bands of immunoreactive material at the bottom of the gel in figure 2.12. This indicates that the antigen is neither protein, which is present in small amounts but not immunoreactive (compare figures 2.11 and 2.12), nor polysaccharide which should be detected by silver staining and would migrate more slowly on the gel.

Figure 2.12 **Individual components of BHI-7 by SDS-PAGE on 12% polyacrylamide and probed with serum from a patient with defined catheter-related sepsis. Lanes 1-7 contain 10μl of purified antigen (pooled fractions 10 to 15 as in figures 2.1 to 2.7) from the 7 strains of coagulase negative staphylococci.**



2.3.7 Chemical analysis of the antigenic material prepared from CAN 6KIII cultured in HHW

2.3.7.1 Negative electrospray mass spectrophotometry

The position of elution of the antigen from the Superose 12 gel permeation column in fractions 10 to15 suggested that the material has a high molecular weight (estimated as >100,000 Daltons from the elution of standard protein markers). The negative ion electrospray mass spectra of FPLC fractions 10 to 15 for CAN 6KIII, is shown in figure 2.13. Major fragments of mass/charge (m/z) peaks at 804 and 1206 were obtained in fractions 10, 11 and 12. The other fractions (9, 13, 14, 15) contained a limited amount of detectable material. The m/z peaks of 804 and 1206 (difference of 402) were deconvoluted by the Hewlett Packard electrospray software to give an actual mass for the material present in the fractions of 2415.16 with a negative charge of 6 (Fenn *et al.*, 1990). Pooled fractions 10-15 from the control of HHW medium alone gave no peaks of abundance greater than 300. In contrast, the phenol extracted LTA from strain CAN 6KIII did not give m/z peaks at 804 and 1206. Instead, the mass spectrum contained a single major peak at 450 together with other minor peaks which were deconvoluted to give a total mass of 18,474.59 and a total negative charge of 40 to 42. The LTA from *S. aureus* gave numerous peaks of low abundance which were deconvoluted to three separate masses of 19,492.60, 7395.83 and 6,273.05 and estimated negative charges of 58 to 59, 21 to 22 and 19 to 20 respectively.

2.3.7.1.1 Manual calculation of the charge state of the individual ions and molecular mass of CAN 6KIII (Hewlett-Packard, 1994)

n = charge status of ion $n1>n2$

m = the m/z ratio of the ion $m1<m2$

x = molecular weight of the adduct ion, typically H^+ (1.008)

M = molecular weight of the compound

Therefore:

$m_1 = 804$

$m_2 = 1206$

$X = 1.008$

$M = n_2(m_2 - x)$

$M = 802.992n_1$

$M = 1204.992n_2$

Determination of the number of charges on the ion (charge status)

$802.992n_1 = 1204.992n_2$

but $n_1 = n_2 + 1$

$802.992 (n_2 + 1) = 1024.992n_2$

$802.992 = 402n_2$

$n_2 = 2$ (applied to 1206 peak)

$n_1 = 3$ (applied to 804 peak)

Determination of molecular mass of CAN 6KIII

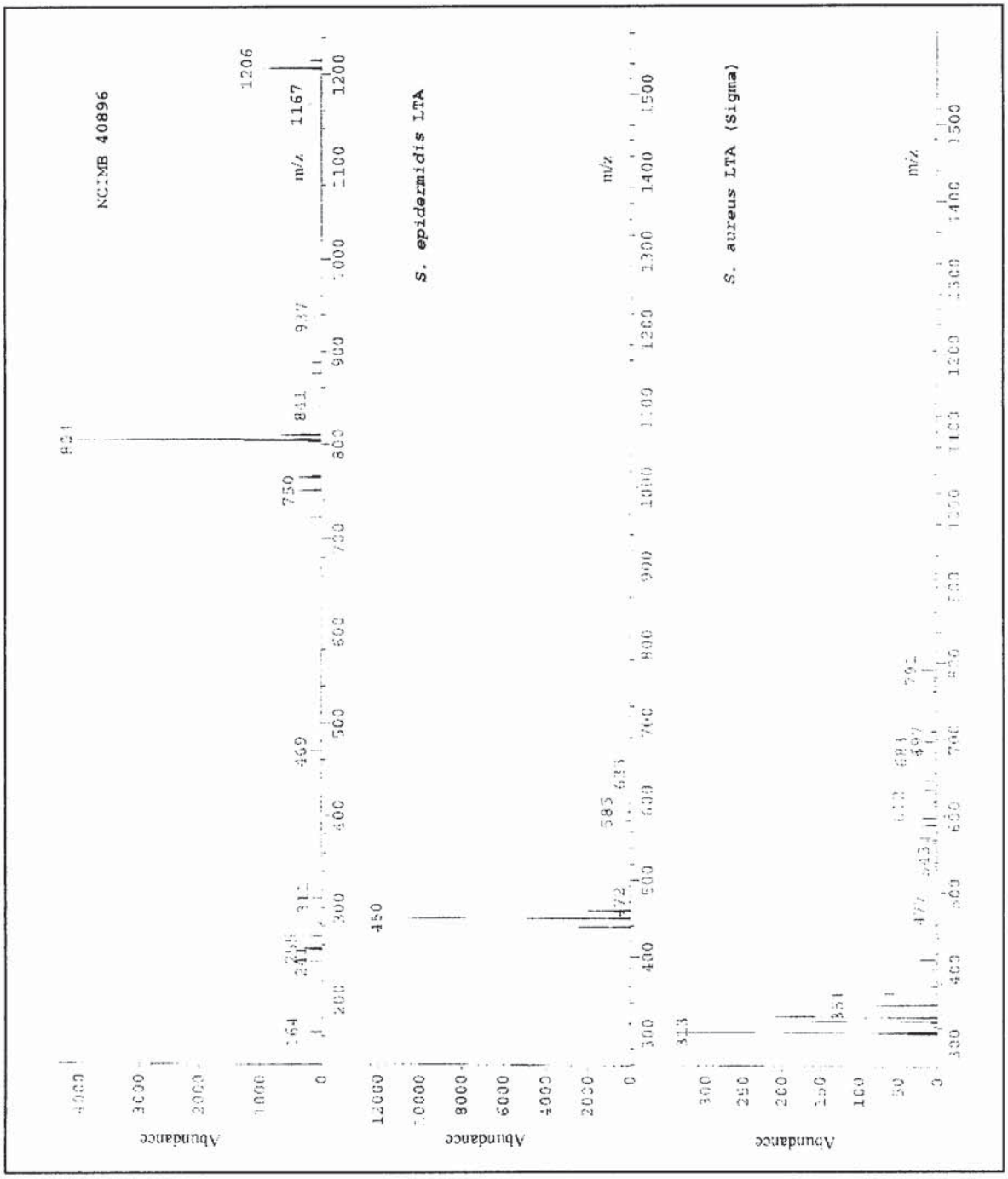
$M = n_2(m_2 - x)$

$M = 2 (1206 - 1)$

$M = \mathbf{2410}$

overall charge= **6**

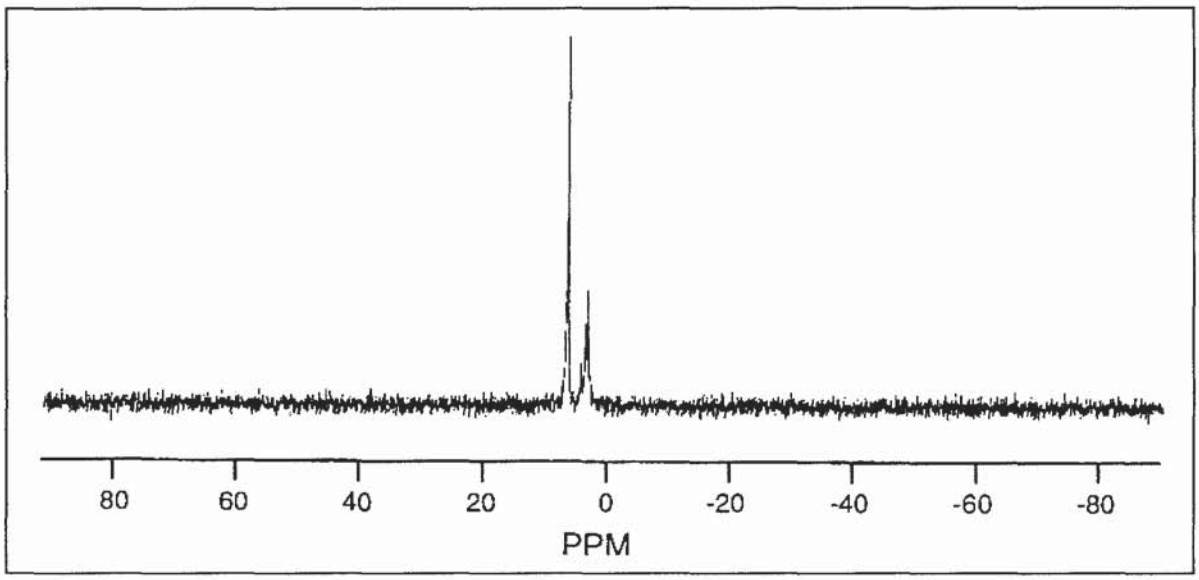
Figure 2.13 **Negative electrospray mass spectrum of CAN 6KIII (NCIMB 40896) grown in HHW, *S. epidermidis* LTA, and *S. aureus* LTA.**



2.3.7.2 ^{31}P NMR analysis.

The ^{31}P spectrum of the pooled FPLC fractions 10 to 15 for strain CAN 6KIII (freeze dried and dissolved in D_2O to 2 mg/ml) is shown in figure 2.14. The spectrum contained two major signals at 6.33 and 3.01 ppm together with a number of smaller peaks, confirming the presence of phosphorus in the material. The chemical shifts of the peaks suggest the presence of phosphate esters in which the phosphorus atoms occur in a number of different magnetic environments.

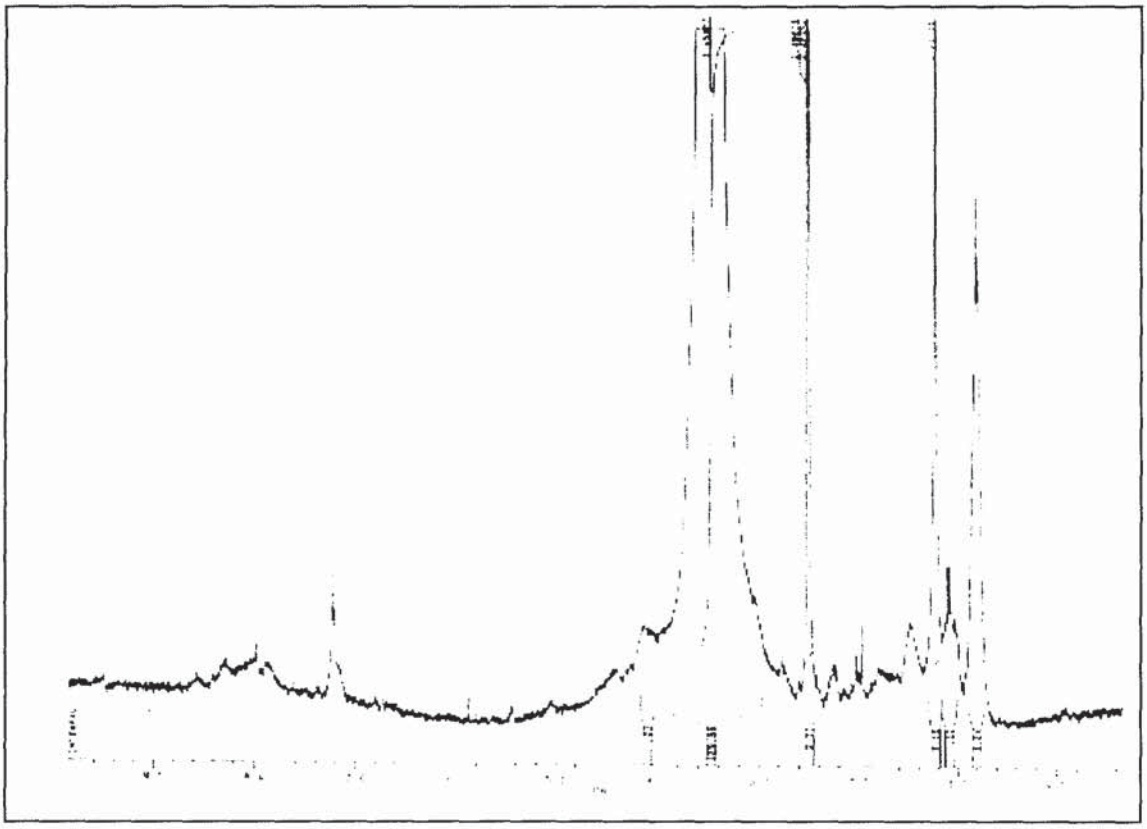
Figure 2.14 **^{31}P spectrum of CAN 6KIII grown in HHW**



2.3.7.3 ^1H NMR analysis.

The ^1H NMR spectrum is shown in figure 2.15. The signals at 0.8 and 1.21 ppm are typical of aliphatic protons CH_3 and $(CH_2)_n$ present in methylene chains and are similar to those reported for the fatty acid moiety of LTA (Batley *et al.*, 1987). Peaks at 3.459 ppm and 2.489 ppm are from contaminating water and dimethylsulphoxide respectively.

Figure 2.15 **^1H spectrum of CAN 6KIII grown in HHW**



2.3.7.4 Fatty acid analysis

The fatty acid composition of the antigenic material from CAN 6KIII grown in HHW is shown in table 2.3. The total amount of fatty acid present in the antigen was 36 μg/mg (0.31μmol/mg, assuming an average carbon chain length of 16 from table 2.3). Fatty acid analysis of the whole cell pellet of CAN 6KIII was similar to those reported for *S. epidermidis* (Behme *et al.*, 1996).

Table 2.3 The fatty acid composition of the antigenic material from CAN 6KIII grown in HHW

Fatty acid	**Retention time (min)**	**Relative amount (%)**
13-methyltetradecanoate (iso-15:0)	11.0	2.5
12-methyltetradecanoate (anteiso-15:0)	11.2	37.8
15-methylhexadecanoate (iso-17:0)	16.0	8.8
cis-9-octadecanoate (18:1)	18.3	0.8
Octadecanoate (18:0)	19.0	4.5
Unidentified	20.4	2.5
cis-9,10-methyleneoctadecanoate (19:0 cycloprop)	20.6	13.1
Eicosanoate (20:0)	23.4	26.6
Unidentified	25.0	3.4

2.3.7.5 Analysis of the nature of the hexoses (alditol acetates) by GC

The antigenic material contained a major peak of retention time 9.38 min, which ran close to that of the glucitol standard. In addition, there were 9 minor peaks detected on the chromatogram, which were also present in the hydrolysis blank. The antigen therefore was presumed to contain glucose as the major hexose component.

2.3.7.6 Determination of hexose content by phenol sulphuric assay

The amount of hexose (as glucose) was calculated from the standard glucose curve. In 20μl of the TFA hydrosylate, 16.7μg of glucose was present. This equated to 84μg of glucose per mg of antigenic material.

2.3.7.7 Determination of the phosphorus content

The amount of phosphorus present in the antigenic material was calculated from the standard curve. In 10μl of the TFA hydrosylate, 3μg of phosphorus was detected. This equated to 30μg of phosphorus per mg of antigen.

2.3.7.8 Determination of the glycerol content

The amount of glycerol present in the antigenic material was calculated from the standard curve. In 10μl of the TFA hydrosylate, 6.7 μg of glycerol was detected. This equated to 67 μg of glycerol per mg of antigen.

2.3.7.9 Ratio of constituents of the antigen

The combination of results from the chemical analyses gave the following ratios:

Glycerol 0.73 μmol/mg (67 μg/mg)

Phosphorus 1 μmol/mg (30 μg/mg)

Glucose 0.46 μmol/mg (0.84 μg/mg)

Fatty acid 0.31 μmol/mg* (36 μg/mg)

*assuming an average carbon chain length of 16 (table 2.3)

The following molar ratio of the constituents was therefore indicated:
5 glycerol units: 6 phosphorus (as phosphate): 2 fatty acids: 3 glucose

The molecular weight of each component analysed and the total molecular weight of the antigen based on the chemical analyses is shown in table 2.4

Table 2.4 Molecular weights of the constituents of the antigen and relative ratios as determined by chemical analysis

Component	No. of mols	Molecular wt. of unit	Weight in antigen
Glycerol	5**	74	444
Phosphate	6	80	480
Glucose	3	162	486
Fatty acid	2	241	482
Total molecular weight of antigenic material			**1892**

**assuming 1 glycerol also present as diglyceride

2.4 Discussion

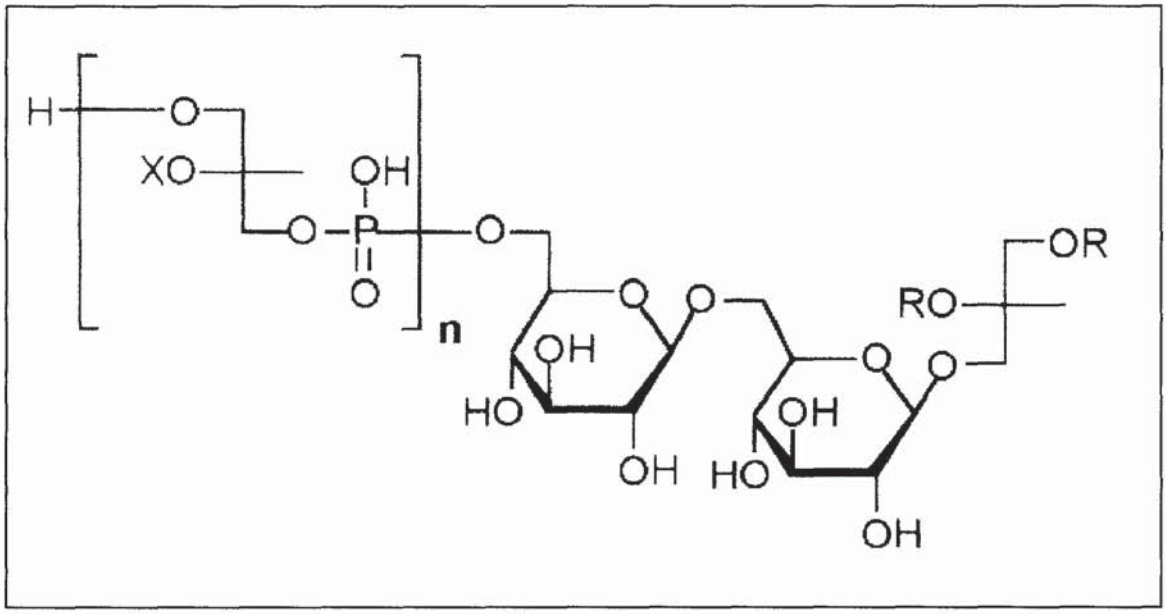
Each of the 7 strains of CNS, as were identified by API STAPH (bioMérieux, Marcy-l'Etoile, France), produced exocellular antigen in BHI which eluted from the Superose 12 gel permeation column as high molecular weight material immediately after the void volume in fraction 9. Analysis of fractions 1 to 50 for hexose and protein content indicated that fractions 10 to 15 contained hexose and a limited amount of protein. Probing of the fractions that eluted from the column with serum from a patient with CRS indicated that antigenic material was present in fractions 10 to 15 of all 7 strains (figures 2.1 to 2.10). This was reflected by a major peak of absorbance between the fractions in a crude ELISA. In addition, secondary peaks of absorbance were also observed between fractions 20 to 25 from the exocellular material from 5 of the strains, indicating a second antigen of lower molecular weight. Results obtained from the competitive ELISA studies suggest that the high molecular weight antigen in fractions 10 to 15 is LTA or a material sharing similar characteristics. Elution as high molecular weight material on gel permeation could be explained by the formation of micelles, through the aggregation of the fatty acid components in solution. In addition, lower molecular weight long chain "free" LTA or deacylated LTA that has lost some or all of its fatty acid component, might account for the secondary peaks of antigen eluting from the column after the micelles.

The reaction of the pooled BHI-7 antigen with immune serum in a competitive ELISA incorporating a panel of antigens, was inhibited by pre-incubation of the serum with LTA prepared from *S. epidermidis* NCIMB 40896. This suggests that the antigen present in fractions 10 to 15 either contained LTA or shared antigenic determinants with LTA. Failure to decrease the absorbance on plates coated with BHI-7 (and HHW6) to the level observed on LTA coated plates, indicated additional antigenic determinants in these preparations compared with purified LTA. Alternatively, antigenic sites on the LTA component of BHI-7 (and HHW6) preparations may be blocked by binding to other components, such as the small amount of protein as indicated by the Folin-Lowry assay and silver stained gels

following SDS-PAGE (figure 2.11). Further evidence supporting the hypothesis of the antigen being LTA (or similar) in nature, was gained by probing the SDS-PAGE gels containing the separated fractions of strains 1 to 7 with immune sera (figure 2.12). The results suggested that the antigenic material was fast migrating, of low molecular weight, and negatively charged. However, the small amount of antibody binding to the 24 kDal protein might reflect genuine antigenicity of this protein or the co-migration of a portion of the fast migrating antigenic material.

Negative ion electrospray mass spectrometry on the column fractions 10 to 15 gave common m/z peaks at 804 and 1206. These peaks (differing by 402 m/z units and multiples of 402) indicate the presence of material of total mass 2414 and 6 negative charges. Similar peaks were not detected in either of the LTA samples. Indeed, the mass spectra show that the *S. aureus* LTA obtained from Sigma is heterogeneous with respect to size. The *S. epidermidis* LTA (prepared by phenol extraction of whole cells) was homogeneous in size but was significantly larger than the antigen recovered from the culture supernatant of CAN 6KIII. To distinguish the exocellular antigen from the cellular LTA, it has been designated as "lipid S" (figure 2.16).

Figure 2.16 **Schematic representation of the potential structure of lipid S. R=fatty acid esters, X=alanyl esters or N-acetylglucosamine, n=6**



The positions at which the lipid S eluted from the Superose 12 column indicate a high molecular weight, which is not consistent with the values determined by mass spectrometry. LTA molecules behave as high molecular weight species on gel permeation chromatography due to micelle formation in solution. The critical micelle concentration reported for LTA of 5×10^{-6} M (Fischer, 1994) would be exceeded at the concentrations used during gel permeation chromatography. The lipid S preparation eluted from the column just before the higher molecular weight LTA, suggesting that lipid S forms larger micelle clusters than LTA. An alternative explanation is that lipid S is bound to other high molecular weight material produced by the organisms in the culture medium such as excreted proteins.

LTA of *S. aureus* comprises a polyglycerophosphate teichoic acid chain of approximately 28 units linked via a diglucosyl unit to diacylglycerol (Fischer, 1994). Although a detailed structure of *S. epidermidis* LTA has not been reported, the mass spectra results indicated that the *S. epidermidis* LTA has a longer chain length than that of *S. aureus*. By contrast, the lipid S antigen recovered from the culture medium of CAN 6KIII had a much shorter chain length of just 6 glycerophosphate units. Evidence supporting this short chain length structure was provided by chemical analysis of acid hydrolysates of lipid S. Despite the variation likely in the analyses (estimated as to be +/- 10% based on volume and weight measurements) and the possible loss of material during hydrolysis, the results suggested that lipid S comprised a glycolipid (for example, diglucosyldiacylglycerol) linked to a glycerol phosphate chain of 6 units. However, the chemical analysis did not account for all of the material present in the antigen on a weight basis. This may be partly due to the losses during hydrolysis, but also to the presence of other components of the LTA which were not analysed (D-alanyl esters or N-acetylglucosamine substituents on the glycerol phosphate chain). The negative electrospray mass spectrum indicated a total mass of 2415 with 6 negative charges. The calculated molecular mass for the structure containing 6 glycerol phosphate units would be 1892. Therefore additional mass units of 522 need to be identified to account for the molecular weight of 2415. The two possible candidate components not analysed (N-acetylglucosamine and D-alanine) have masses of 203 and 61 respectively. With 6 potential glycerol units available for substitution, the presence of a combination of

these additional components on the glycerol units could account for the extra molecular weight (e.g. 2 x N-acetylglucosamine, molecular weight 406, and 2 x D-alanine, molecular weight 122). The uncertainty in assigning a precise molecular mass could be attributed to the range of fatty acids present (table 2.3).

The ^{31}P NMR spectrum of lipid S supports the presence of phosphate esters and is consistent with the polyglycerol phosphate structure of the teichoic acid chain. Similar ^{31}P spectra have been reported for LTA from a range of organisms (Batley *et al.*, 1987). The splitting of the peaks is thought to indicate high flexibility in the glycerophosphate chains rather than different chemical linkages of the phosphates. One explanation is the variation in substituents on the 2-position of the glycerol units in the poly(glycerophosphate) chain. Phosphate esters on unsubstituted glycerols give a signal at higher ppm than phosphates on glycerol units bearing alanine substituents.

The possible origin of lipid S and its relation to LTA based upon the biosynthetic pathway for LTA in *S. aureus* (Fischer, 1994), needs to be considered. The glycerophosphate units that make up the teichoic acid chain of LTA are added sequentially from the phospholipid phosphatidyl glycerol, to the glycolipid diglucosyldiacylglycerol in the cytoplasmic membrane. A range of shorter chain length LTA analogues would therefore be synthesised within the cells during the assembly of the LTA. The isolation of a short chain length form of LTA from the culture medium is a novel finding suggesting that this material is specifically released from the cells. Long chain length LTA was recovered from whole cells using conventional phenol extraction methods. If this long chain length LTA had also been present in the culture medium, it would have been detected as full chain length LTA during the characterisation.

Owing to their structure, LTAs display peculiar physiochemical properties. The large hydrophilic moiety renders them less hydrophobic than membrane lipids and this is reflected by the critical micellar concentration, which is higher for LTA. This comparatively low hydrophobicity of LTA is one possible reason for its spontaneous loss from the membrane of dividing bacteria into the surroundings (Fischer, 1994), and hence the presence of lipid S

in culture supernatants. In addition, the loss of LTA may be greatly stimulated by the action of beta-lactam antibiotics, which account for half of the antimicrobials used to treat infection (Wilks, 1997). In infections, released LTA may interact with humoral and cellular components of the mammalian host (Fischer, 1994). When released from bacterial cells, LTA stimulates a range of responses in the host. LTA elicits a humoral response, it also activates the complement system in an antibody independent manner and stimulates blood monocytes and macrophages (Fischer, 1994). In addition LTA stimulates the induction of cytokines, chemokines and inducible nitric oxide synthase which are presumed to mediate Gram-positive shock (Kengatharan *et al.*, 1998). Indeed, because of the structural similarity between lipid S and LTA and the quantity of lipid S released from cells, it may be that lipid S is responsible for many of the physiological functions ascribed to LTA.

In summary, a novel exocellular glycolipid antigen has been isolated from the culture supernatants of 7 strains of CNS. Characterisation studies suggest that the antigen is a short chain LTA with a structure analogous to that of *S. aureus* LTA. However, further analyses are required to determine the nature of the components which may account for the molecular weight differences as determined by electrospray mass spectrophotometry and chemical analysis. The serodiagnostic potential of the antigen for deep-seated Gram-positive infection including CRS, prosthetic hip infection, pyogenic spondylodiscitis and endocarditis is investigated in the following chapters. In the forthcoming chapters of this thesis where other experimental work was concurrent with the characterisation of the antigen, the antigen is referred to as BHI-7. However, in the concluding chapters following its characterisation, the antigen is referred to as lipid S.

Chapter 3 Development of an indirect enzyme linked immunosorbent assay for the serological diagnosis of catheter-related sepsis due to staphylococci.

3.1 Introduction

The enzyme linked immunosorbent assay (ELISA) developed by Lambert *et al.* (1996), incorporated non-protein antigenic material prepared from 1 strain of *S. epidermidis* which was isolated from a patient with an orthopaedic infection. The ELISA was subsequently used to investigate the IgG antibody levels in different patient groups including those with prosthetic joints infected with *S. epidermidis* and *S. aureus*, those with uninfected prosthetic joints, and healthy adults. Increased levels of IgG antibody were detected by the ELISA in patients with infected prosthetic joints as compared to those with uninfected prosthesis and healthy patients. However, no investigations had been undertaken to examine the quality control of the ELISA, the reproducibility of the results, or methods of end-point determination (Lambert, personal communication). In addition, the IgM response in the patients had not been investigated.

When developing a new assay several parameters need to be assessed. The microtitre tray should be evaluated for reproducibility of results across the plate (intratest), as well as between plates (intertest) and the coefficient of variation (COV) determined. The COV, which is also referred to as the coefficient of error, expresses the standard deviation as a proportion of the mean and is applicable to the quality control of ELISA (Balfour and Harford, 1990). The COV can be used to compare assay results and acts as a measure of confidence in any individual assay by reflecting both its accuracy and reproducibility. A COV of <10% is acceptable in ELISA, but a level of <5% should be aimed for (Wood and Wreghitt, 1990). However, a level of variation of <5 % in an assay may be difficult to achieve, especially if a non-protein antigen is bound to the solid phase (Wood and Wreghitt, 1990).

The coefficient of variation is expressed as:

$$\mathbf{COV} = \frac{\boldsymbol{S}}{\boldsymbol{X}} \mathbf{x\ 100\%}$$

where **S** = standard deviation

$\boldsymbol{X}$ = mean value of a set of measurements

Other parameters that can influence ELISA performance include the choice of solid phase to which the antibody or antigen is bound. The solid phase is available in a variety of materials including polystyrene, nylon, polyvinyl and polycarbonate, which may be gamma irradiated. Results can also vary between wells on the same plate and between plates of the same batch (Wood and Wreghitt, 1990). Due to these variations, a solid phase should be used which is specifically manufactured for ELISA and the variation of results across the plate and between plates assessed. The choice of buffer used to coat the antibody or antigen to the solid phase may also influence how the molecules adsorb. The buffers used include carbonate/bicarbonate pH 9.6, phosphate buffered saline pH 7.2 and tris-saline pH 8.5. Antigen prepared from different sources or batches can also vary considerably and influence ELISA results. The antigen should remain stable throughout the assay, retain its antigenicity, and not dissociate from the solid phase on storage. This however will depend on the storage temperature, the nature of the antigen bound to the solid phase and the coating buffer used. These parameters highlight the need for quality control systems within the ELISA

The aim of this section of the study was to firstly modify and further develop the original ELISA (Lambert *et al.*, 1996) by incorporating antigenic material prepared from culture supernatants of 7 strains of CNS grown in brain heart infusion broth (BHI) (chapter 2). The strains used in the preparation of the antigen (BHI-7) were isolated from the blood cultures of patients with clinically defined catheter-related sepsis, and were chosen with a view to

utilising the assay to investigate the serological response in patients with the infection. The number of isolates of CNS used to prepare the antigenic material was increased from the original work of Lambert *et al.* (1996) to include isolates with different phenotypic characteristics including biotype and slime production. An investigation of the IgM and IgG response in selected positive and negative control sera was undertaken to assess the suitability of the samples as internal control sera. In addition, the ELISA intratest / intertest variation, and method of end-point determination was investigated to assess the reproducibility and robustness of the modified ELISA.

3.2 Methods and Materials

3.2.1 Preparation of stock solutions and reagents

All chemicals and reagents used in the ELISA system were obtained from Sigma, Poole, Dorset, U.K. unless otherwise stated.

3.2.1.1 TBS-Tween buffer (0.01M Tris-HCl pH 7.4, 0.9% NaCl w/v, 0.3% Tween 20 v/v)

A 10 times concentrated solution was prepared and diluted 10 fold prior to use with distilled water. Trishydroxymethylamino methane (Tris) (12.1g) was dissolved in 800ml of distilled water and 90g of NaCl added. Thirty millilitres of Tween 20 was then added and dissolved by stirring. The pH of the buffer was adjusted to 7.4 with concentrated hydrochloric acid (BDH Ltd.) and the volume made up to 1 litre with distilled water. The buffer was autoclaved at 121°C for 15 minutes and stored at 4°C.

3.2.1.2 Citrate-acetate buffer

Sodium acetate trihydrate (13.6g) was dissolved in 800ml of distilled water (0.1 M sodium acetate). A separate solution of 0.1 M citric acid was prepared by dissolving 10.5g of citric acid in 500ml of distilled water. The pH of the sodium acetate solution was then adjusted to 6.0 by addition of 0.1 M citric acid and the final volume made up to 1 litre with distilled water. The buffer was autoclaved at 121°C for 15 minutes and stored at 4°C.

3.2.1.3 Carbonate buffer

0.1 M sodium carbonate was prepared by dissolving 5.3g of sodium carbonate in 1litre of distilled water. Sodium bicarbonate (4.2g) was dissolved in 1 litre of distilled water. The sodium carbonate solution was added to the sodium bicarbonate solution until a pH of 9.6 was achieved. The buffer was then autoclaved at 121°C for 15 minutes and stored at 4°C.

3.2.1.4 Protein-A horseradish peroxidase conjugate

One milligram of the freeze-dried protein-A peroxidase conjugate was dissolved in 4ml of TBS-Tween buffer (0.25mg/ml), dispensed into 50µl aliquots and stored at -20°C. Prior to use, the conjugate was thawed and diluted in 50 ml of TBS-Tween buffer (in-use concentration 0.25µg/ml).

3.2.1.5 Goat anti-human IgM peroxidase conjugate

One millilitre of anti-human IgM peroxidase conjugate was diluted into 4ml of TBS-Tween buffer and stored at -20°C in 50µl aliquots. The conjugate was diluted to 1:5,000 for use by the addition of one 50µl aliquot to 50 ml of TBS-Tween.

3.2.1.6 Chromogenic substrate

Ten milligrams of 3,3',5,5'-tetramethylbenzidine was dissolved in 1ml of dimethyl sulphoxide. Fifty microlitres of 3% hydrogen peroxide (Thornton and Ross, U.K) was added and the substrate was then made up to 100ml with the citrate-acetate buffer giving a final concentration of 0.1mg/ml. The chromogenic substrate was prepared fresh when required.

3.2.2 Preparation of the BHI-7 antigen by fast protein liquid chromatography (FPLC)

Antigenic material used to coat microtitre plates was prepared as described in section 2.2.2. However, to prepare the BHI-7 antigen, the culture supernatants from each strain were pooled together prior to FPLC.

3.2.3 Preparation of the BHI-7 antigen coated microtitre plates

A 1ml aliquot of frozen FPLC-purified BHI-7 antigen was thawed at room temperature and diluted 100 fold in carbonate buffer. The antigenic concentration when diluted in 100 volumes of buffer was 5μg/ml (Lambert, personal communication). One hundred microlitres of the diluted antigen was dispensed into each well of a 96-well flat bottomed microtitre plate (Immulon 2, Dynex Technologies, Chantilly, VA) to provide 0.5μg of antigen per well. One millilitre of BHI-7 antigen diluted in 100ml of carbonate buffer was sufficient to coat

the wells of 10 microtitre plates. The plates were stored at 4°C for 18 h for antigen binding to the polystyrene surface of the wells to occur (Lambert *et al.*, 1996). The residual antigen was then removed, and the plates washed 3 times in TBS-Tween buffer with an ELP-40 automated plate washer (Launch Diagnostics, U.K.). Unbound antigen sites in the wells of the plates were blocked by filling the wells with TBS-Tween buffer and allowing the plates to stand at 4°C for 1 h, after which, the buffer was removed and the coated plates stored at -20°C until required.

3.2.4 Selection of the BHI-7 ELISA internal quality control standards

The positive control serum sample was obtained from a patient with a clinical diagnosis of CRS including localised signs of infection and pyrexia. Laboratory diagnosis confirmed the clinical suspicion with repeated isolation of *S. epidermidis* from blood culture samples and isolation of the same organism from the distal tip of the CVC. The negative control sample obtained was "normal human serum" (NHS) (Bradsure Biologicals, U.K.), prepared from a pool of serum samples from healthy blood donors and is used routinely in antibiotic assay serum binding studies.

3.2.5 The ELISA method for the detection of antibodies to the BHI-7 antigen

All solutions used throughout the ELISA attained room temperature before use. An initial 1:400 serum dilution was performed on all test samples and controls (5µl of serum diluted into 2 ml of TBS-Tween). One hundred microlitres of TBS-Tween was added to all wells of a BHI-7 coated microtitre plate except for the first column. Two hundred microlitres of the diluted test samples and controls were then added to the first column of the plate and then titrated from column 2 to 11 by serial transfer of 100 µl. Column 12 contained 100µl of TBS-Tween only and was used to blank the assay. The plates were covered with an acetate plate sealing strip and stored at 4°C for 18h, after which, they were washed 3 times in TBS-Tween using the ELP-40 plate washer. The wash cycle also included a 30s hold time in between washes (Wood and Wreghitt, 1990).

For the detection of IgG antibodies, 100µl of protein A-horseradish peroxidase conjugate was added to all of the wells. For the detection of IgM, 100µl of goat anti-human IgM peroxidase conjugate was added to all the wells. The plates were stored at 4°C for 2 h to allow the conjugate to bind, after which, they were washed 4 times in TBS-Tween to remove the unbound conjugate. The ELISA was developed by the addition of 100µl of the chromogenic substrate to all of the wells. This stage was incubated at room temperature for 10 minutes until the colour had developed sufficiently. The reaction was stopped by addition of 100µl of 1M sulphuric acid (BDH chemicals, U.K). The product, which was yellow, was measured immediately at 450nm using a Titertek plate reader (Life Sciences, U.K.). The optical density readings were plotted against the corresponding serum dilution using computer software (Microsoft ™ Excel). The antibody titre was determined as the serum dilution required to reduce the absorbance to 0.1 (Lambert *et al.*, 1996).

3.2.6 Determination of the IgM and IgG titres of the internal quality standards to BHI-7 antigen and an investigation of ELISA intratest variation

As a pilot study, the assay was performed using only the positive and negative standards (3.2.4) to investigate the BHI-7 ELISA intratest variation and also the suitability of the chosen internal standards. Four microtitre plates coated with BHI-7 antigen (3.2.3) were removed from –20°C and allowed to reach room temperature. The positive and negative controls were titrated a total of 8 times each on separate plates and the COV determined. The titres for both IgG and IgM classes of antibody were measured.

3.2.7 An assessment of the BHI-7 ELISA intertest variation with plates incorporating different preparations of antigen, freshly bound to the solid phase and stored coated to the solid phase for 2 weeks at -20°C.

Ten samples of serum were chosen at random from the patients described in chapter 4. Microtitre trays were coated with BHI-7 antigen prepared from 2 different stored aliquots of the same batch and termed antigen 1 and antigen 2. The plates were used to assess the intertest variation of antibody levels in the 10 serum samples by the BHI-7 ELISA method, and to investigate any variation in titres when different stored aliquots of the antigen were used to prepare the ELISA plates. Also, plates that had been freshly coated with the antigen and those that had been stored for 2 weeks at -20°C were used to evaluate the titres and determine whether the BHI-7 antigen lost antigenicity up on freezing. The serum samples were tested in duplicate and the mean titre was calculated. The COV was determined and a

statistical correlation of the results was made using the Spearman rank test. The ELISA experiments were designated as follows:

(a) Assay 1: antigen 1 coated onto plates, stored for 2 days

(b) Assay 2: antigen 1 coated onto plates, stored for 2 weeks

(c) Assay 3: antigen 2 coated onto plates, stored for 2 days

(d) Assay 4: antigen 2 coated onto plates, stored for 2 weeks

The positive and negative internal standards were included with each assay.

3.3 Results

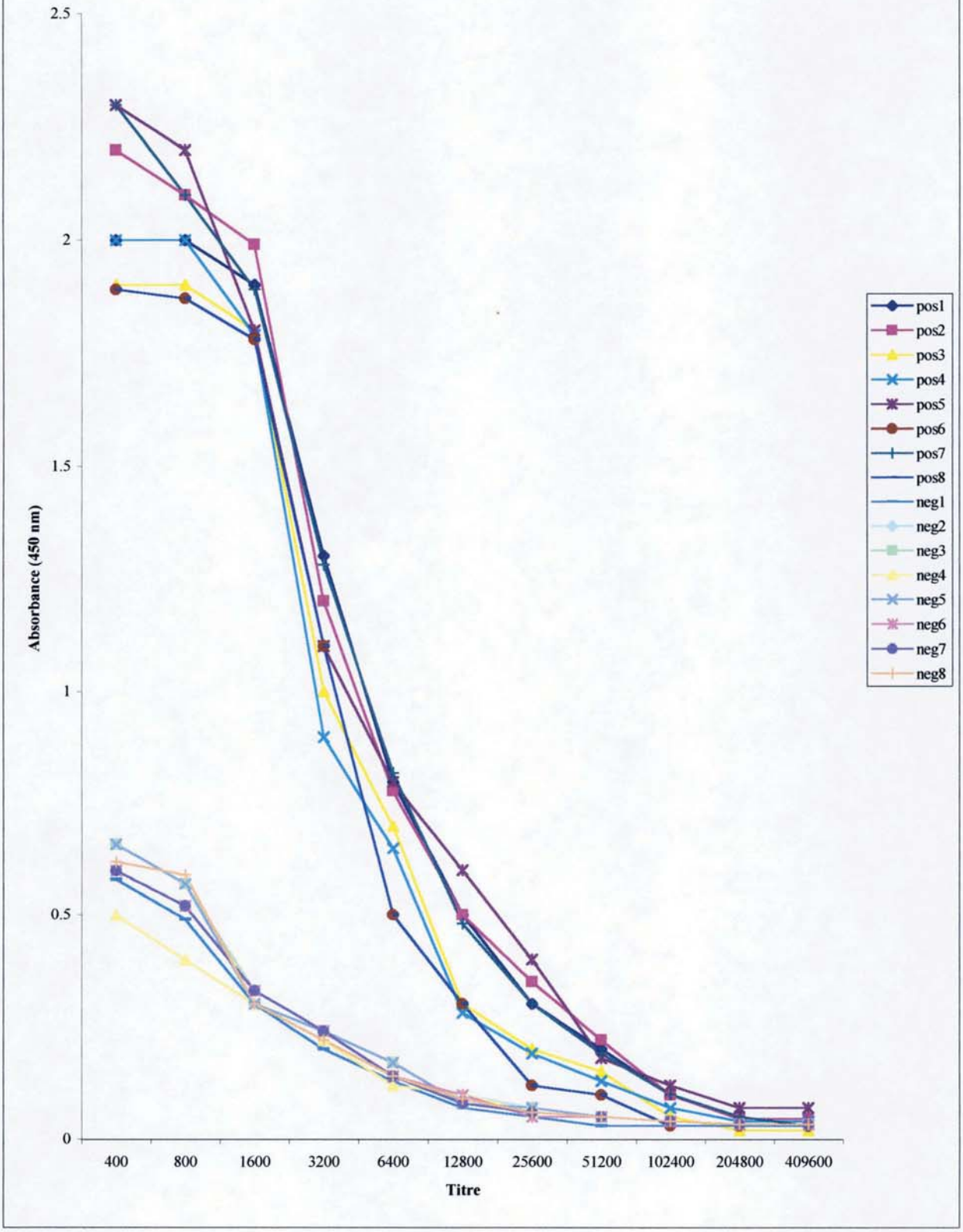
3.3.1 The positive and negative internal standard titres and intratest variation

The IgG and IgM titres obtained for both positive and negative controls repeated 8 times, together with the intratest variation, are shown in table 3.0. The corresponding titration curves are shown in figure 3.0. Reproducibility of antibody titres was demonstrated in the negative control (NHS), where a constant level of 1:10,000 was repeatedly achieved for both classes of antibody. However, the IgG titres of the positive ranged from 1: 50,000 to 1:100,000 (mean 82,500) with a COV of 25% whilst the corresponding IgM titres were reproducible with levels of 1: 25,000 being consistently achieved.

Table 3.0 BHI-7 ELISA control titres and intratest variation

POSITIVE IgM	*POSITIVE IgG*	*NEGATIVE IgM*	*NEGATIVE IgG*
1:25,000	1:100,000	1:10,000	1:10,000
1:25,000	1:100,000	1:10,000	1:10,000
1:25,000	1:80,000	1:10,000	1:10,000
1:25,000	1:100,000	1:10,000	1:10,000
1:25,000	1:100,000	1:10,000	1:10,000
1:25,000	1:50,000	1:10,000	1:10,000
1:25,000	1:80,000	1:10,000	1:10,000
1:25,000	1:50,000	1:10,000	1:10,000
COV 0%	**COV 25%**	**COV 0%**	**COV 0%**

Figure 3.0 Titration curves of the BHI-7 ELISA positive and negative controls. Each control was titrated 8 times. The IgG titres were estimated at an absorbance of 0.1.



3.3.2 The intertest variation of a panel of serum samples observed in the BHI-7 ELISA using an absorbance of 0.1 as assay end point.

The IgG levels and the COV of the 10 serum samples are shown in table 3.1.

Table 3.1 Mean IgG titres of serum obtained from 10 patients determined by the BHI-7 ELISA and a cut off absorbance of 0.1.

Patient	Assay 1	Assay 2	Assay 3	Assay 4	%COV
1	30000	85000	79000	29000	47.2
2	14500	34500	33000	15000	39.2
3	12500	31000	41500	12000	51.8
4	21000	45500	43000	14000	44.1
5	14000	23000	48500	12000	59.6
6	21000	22500	35000	15000	31.1
7	25500	40500	33000	21500	24.1
8	32500	71000	46500	33500	33.8
9	12500	18500	20500	11000	25.4
10	36500	51500	73500	52000	24.6
mean	**22000**	**42300**	**45350**	**21500**	**37.9**
+ CONTROL	80000	100000	109000	70000	17
- CONTROL	15000	21000	25000	14000	24

The mean IgG titre per assay and the intertest COV between the 4 assays is shown. The mean intertest COV with an assay cut off of 0.1 absorbance (Lambert *et al.*, 1996) was 37.9%. Assays 1 to 4 were as follows:

Assay 1: antigen 1 coated onto plates, stored for 2 days

Assay 2: antigen 1 coated onto plates, stored for 2 weeks

Assay 3: antigen 2 coated onto plates, stored for 2 days

Assay 4: antigen 2 coated onto plates, stored for 2 weeks

3.3.3 The ELISA intertest variation when comparing test serum results to standardised control values to determine antibody levels

The statistical analysis of the results achieved for the 10 test serum samples in 3.3.2 demonstrated a high assay intertest variation (range 24.6% to 59.6%, mean 37.9%). A high intertest variation of 17% and 24% was also obtained for the positive and negative internal standards respectively. Similarly, the intratest variation of the positive control IgG titre was also 25% (3.3.1). As these results indicated that the assay had low reproducibility, the next stage in the evaluation and development of the ELISA was to investigate comparison of test results to reference sera as a method of end point determination (Balfour and Harford, 1990). Standardised IgG control values of 1:100,000 and 1:10,000 were chosen and set to the positive and negative internal standards respectively based on the results achieved in 3.3.1. The titres of the panel of 10 serum samples (table 3.1) were adjusted accordingly by the factor required to set the internal controls to the standardised IgG titres. The resulting antibody levels and COV of the serum panel are shown in tables 3.2 and 3.3. Variation or trends in the antibody titre results did not correlate with aliquot of antigen used to prepare the BHI-7 ELISA plates or length of storage of the microtitre plates at -20°C (tables 3.4 and 3.5).

Table 3.2 Mean IgG titres of 10 patients using ELISA plates prepared from 2 aliquots of BHI-7 antigen stored at -20°C for 2 days and 2 weeks (section 3.2.7). Results were compared to a standardised positive control IgG titre of 1:100,000

PATIENT	TITRE				%COV
	Assay 1	Assay 2	Assay 3	Assay 4	
1	39000	85000	72000	41000	33.5
2	18000	34500	30000	21000	25.1
3	16000	31000	38000	17000	26.0
4	26000	45500	39500	20000	30.6
5	17500	23000	44500	17500	43.2
6	26500	22500	32000	21500	16.2
7	32000	40500	30000	30500	12.6
8	41000	71000	42500	48000	23.2
9	15500	18500	18500	16000	8.8
10	46000	51000	67500	74500	19.6
mean	**27700**	**42250**	**41450**	**30700**	**23.9**
+ control IgG	80000	100000	109000	70000	
Titre adjustment *	1.25	0	0.9	1.43	

The mean IgG titre per assay and the intertest COV between the 4 assays is shown. The mean intertest COV with reference to a standarised positive titre of 1:100,000 was 23.9%. Assays 1 to 4 were as follows:

Assay 1: antigen 1 coated onto plates, stored for 2 days

Assay 2: antigen 1 coated onto plates, stored for 2 weeks

Assay 3: antigen 2 coated onto plates, stored for 2 days

Assay 4: antigen 2 coated onto plates, stored for 2 weeks

*Titre adjustment: the multiplication factor required to achieve a positive control titre of 1:100,000.

Table 3.3 Mean IgG titres of 10 patients using ELISA plates prepared from 2 aliquots of BHI-7 antigen stored at -20°C for 2 days and 2 weeks (section 3.2.7). Results were compared to a standardised negative control IgG of 1:10,000

PATIENT	TITRE				%COV
	Assay 1	Assay 2	Assay 3	Assay 4	
1	21000	40500	31500	20000	29.7
2	9500	16500	13000	10500	21.1
3	8500	14500	16500	9000	30.6
4	14000	21500	17000	10000	27.3
5	9500	11000	19000	8500	35.7
6	14000	11000	14000	10500	13.7
7	17000	19000	18500	15000	9.4
8	22000	33500	18500	23500	23.2
9	8000	9000	8000	8000	4.9
10	24500	24500	29500	36500	17.4
mean	**14800**	**20100**	**18550**	**15150**	**21.3**
- control IgG	15000	21000	25000	14000	
Titre adjustment *	0.66	0.47	0.4	0.7	

The mean IgG titre per assay and the intertest COV between the 4 assays is shown. The mean intertest COV with reference to a standarised negative titre of 1:10,000 was 21.3%. Assays 1 to 4 were as follows:

Assay 1: antigen 1 coated onto plates, stored for 2 days

Assay 2: antigen 1 coated onto plates, stored for 2 weeks

Assay 3: antigen 2 coated onto plates, stored for 2 days

Assay 4: antigen 2 coated onto plates, stored for 2 weeks

*Titre adjustment: the multiplication factor required to achieve a negative control titre of 1:10,000.

Table 3.4 Correlation of assays using plates coated with different aliquots of antigen and storage times at -20°C. Results compared to a positive reference serum

Statistical test	**Spearman rank correlation**		
Assay comparison	R value	P value	correlation
1 with 2	0.79	0.0088	Yes
1 with 3	0.67	0.0400	Yes
1 with 4	0.96	<0.0001	Yes
2 with 3	0.67	0.019	Yes
2 with 4	0.81	0.0072	Yes
3 with 4	0.51	0.1334	No

Correlation between assay results from ELISA incorporating different antigen preparation and storage conditions was achieved with the exception of assay 3 and 4. Assays 1 to 4 were as follows:

Assay 1: antigen 1 coated onto plates, stored for 2 days

Assay 2: antigen 1 coated onto plates, stored for 2 weeks

Assay 3: antigen 2 coated onto plates, stored for 2 days

Assay 4: antigen 2 coated onto plates, stored for 2 weeks

Table 3.5 Correlation of assays using plates coated with different aliquots of antigen and storage times at -20°C. Results compared to a negative reference serum

Statistical test	**Spearman rank correlation**		
Assay comparison	R value	P value	correlation
1 with 2	0.79	0.0088	Yes
1 with 3	0.66	0.0438	Yes
1 with 4	0.96	<0.0001	Yes
2 with 3	0.69	0.015	Yes
2 with 4	0.79	0.008	Yes
3 with 4	0.62	0.060	No

Correlation between assay results from ELISA incorporating different antigen preparation and storage conditions was achieved with the exception of assay 3 and 4. Assays 1 to 4 were as follows:

Assay 1: antigen 1 coated onto plates, stored for 2 days

Assay 2: antigen 1 coated onto plates, stored for 2 weeks

Assay 3: antigen 2 coated onto plates, stored for 2 days

Assay 4: antigen 2 coated onto plates, stored for 2 weeks

3.4 Discussion

Lambert *et al.* (1996) developed an ELISA that had the potential to detect specific serum IgG levels in patients with an infected prosthesis due to staphylococci. However, the antigenic material incorporated in the assay was prepared from a single strain of *S. epidermidis*, and therefore potentially lacked antigenic components that may be expressed by other strains of *S. epidermidis* or CNS associated with infection. Also, the quality control aspects of the ELISA including intratest / intertest variation and result reproducibility had not been investigated. In the clinical environment therefore, the application of the assay in this format was limited.

The aim of this part of the study was to further develop the ELISA described by Lambert *et al.* (1996) by incorporating antigenic material prepared from 7 strains of CNS, with a view to broadening the application of the assay. The initial studies with the modified ELISA determined the levels of IgM and IgG in selected positive and negative control sera, and assessed the assay intratest variation. In agreement with Lambert *et al.* (1996), the results demonstrated the potential of the ELISA to differentiate between the levels of serum IgG in patients with Gram-positive coccal infection, in this case CRS, and healthy subjects. Indeed, an 8-fold difference in mean antibody titre was achieved between the IgG titres of the positive and negative control sera. However, an intratest COV of 25% was also obtained between the IgG levels of the positive control, which may indicate low assay reproducibility. Conversely, the IgM titres achieved for the positive and negative control sera were reproducible, but the assay failed to clearly discriminate between them with both sera having similar detectable levels of IgM. The apparent low level of IgM in the positive control serum may have been due to the high levels of IgG (1:100,000), blocking the antibody binding sites on the solid phase. Further work is therefore necessary in the development of the IgM assay by incorporating a method to remove competing IgG in serum samples. This is investigated in chapter 7. Alternatively, the positive control serum may have low levels of IgM, therefore further studies are required to investigate the IgM levels in more patients with CRS, with possible re-selection of a positive control for the IgM ELISA (chapter 4).

The intertest variation of results in the ELISA was high (table 3.1). Lambert *et al.* (1996), determined the IgG levels of patients as the serum dilution required to give an absorbance of 0.1 in the assay. By adapting this method of titre determination, the intertest variation of results ranged from 17% to 59.6%, mean 37.9% (table 3.1). This method was technically difficult to reproduce and may of accounted for some of the variation, as it required antibody titres to be interpolated from the lower part of the sigmoidal plot and then estimated from the x-axis (figure 3.0). Small variations in repeat titration curves of serum samples therefore equated to large differences in antibody level estimates. In addition, this method of end point determination did not compensate for any fluctuations in assay conditions such as reagent and room temperature at the time of assay, and incubation conditions, all of which may affect the assay absorbancies (Balfour and Harford, 1990). An alternative method of estimating antibody levels in ELISA is the comparison and correction of test results by reference to standard serum with assigned titres or absorbancies (Wood and Wreghitt, 1990; Balfour and Harford, 1990; Balfour, 1990). By assigning titre values of 1:100,000 and 1:10,000 to the positive and negative reference sera respectively and comparing serum antibody levels to these figures, the intertest variation was reduced (tables 3.2 and 3.3).

The variation between the results however did not correlate with microtitre plates prepared from different aliquots of antigen, or storage of the coated plates at -20°C for 2 weeks (tables 3.4 and 3.5). A large number of technical factors will influence assay variation including pipetting, dilution preparation, assay incubation times and temperatures, type of conjugate and substrate, concentration of antigen and the method of interpreting the results. These technical parameters and steps to optimise the ELISA are investigated in chapter 8. In addition, biological factors including the nature of the antigen may influence the level of result variation in an assay. Indeed, a level of variation of <10% may be difficult to achieve if a non-protein antigen is bound to the solid phase (Wood and Wreghitt, 1990). The antigen incorporated in this ELISA was a short chain lipoteichoic acid (chapter 2).

In conclusion, the ELISA incorporating antigenic material prepared from 7 strains of CNS differentiated between IgG levels in serum from a patient with CRS due to *S. epidermidis* and the negative control serum, thus supporting the original work by Lambert *et al.* (1996). However, to enhance the reproducibility of the ELISA and reduce the variation between results, the comparison and correction of test titres with reference to a standardised positive serum of assigned IgG titre (1:100,000), has been incorporated as the method of determining antibody levels. A positive control titre of 1:25,000 was also assigned as the cut off figure for estimating IgM levels. The current ELISA provides a potential method for the serodiagnosis of CRS due to staphylococci, and its value in the diagnosis of the infection is assessed in chapter 4. At present there is no simple serological test available for the diagnosis of CRS.

Chapter 4 Serological response to BHI-7 antigen in patients with intravascular catheter-related sepsis due to staphylococci

4.1 Introduction

One of the main problems associated with catheter-related sepsis (CRS) is establishing the clinical diagnosis due to the non-specific clinical presentation. To add to this problem there is also a poor correlation between the clinical diagnosis and laboratory findings (Smith *et al.*, 1994). Also, many of the microbiological methods available to assist the diagnosis of CRS may give misleading results (Maki and Mermel, 1988). Due to these difficulties in making a diagnosis, many hospital units, as a pre-emptive prophylactic measure, routinely remove CVC (Elliott and Faroqui, 1992).

Lambert *et al.* (1996) used a serological approach to examine the IgG response in patients which included a healthy control group, those with prosthetic device infection due to *S. epidermidis* and *S. aureus,* 1 patient with endocarditis due to a beta-haemolytic streptococcus, 1 patient with an enterococcal osteomyelitis and patients with a CVC-related sepsis. The ELISA system used non-protein antigenic material prepared from the culture medium of a strain of *S. epidermidis* isolated from a patient with prosthetic device infection. The study concluded that the ELISA provided a means of not only diagnosing prosthetic infection due to *S. epidermidis*, but also a potential method for the diagnosis of other serious Gram-positive infections, including CRS. In order to study the serological response in patients with a clinical diagnosis of CRS, antigenic material was prepared from bacterial strains isolated from the blood cultures of patients with intravascular CRS (chapter 2). The initial studies on the development of the ELISA demonstrated that the modified antigen differentiated between positive and negative control sera (chapter 3).

In validating a diagnostic test, there are several important features of the test which need to be considered (Greenhalgh, 1997). A new test should be validated against an established gold standard, however, the diagnosis of CRS is mainly clinical and there is not a "gold standard" diagnostic test to which a new assay could be compared. The sensitivity (detection of true positives) and specificity (exclusion of true negatives) need to be evaluated. Also, the positive predictive value (probability of those patients testing positive having the condition) and negative predictive value (probability of those patients testing negative not having the condition) require determination. Finally, the accuracy of the test (proportion of the tests giving the correct result) is an equally important test parameter, which needs to be measured. The aim of this study was to determine the levels of IgM and IgG to the BHI-7 antigen in patients with confirmed CRS and in a control group. In addition, the sensitivity, specificity, positive and negative predictive values and accuracy of the BHI-7 ELISA were to be evaluated.

4.2 Materials and Methods

4.2.1 Patient groups and sample collection

4.2.1.1 Patients with catheter-related sepsis

Sixty-seven patients over 18 years of age who were diagnosed as having CRS were entered into the study between September 1996 and March 1999. All of the patients had a CVC inserted into the internal jugular vein and no patient received total parenteral nutrition. The clinical criteria included the presence of two or more of the following: 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 exudate at the site of catheter insertion; the pyrexia being unresponsive to broad spectrum antibiotics but settling on CVC removal (Elliott and Tebbs, 1998). The diagnosis of a CRS was supported by the isolation of CNS or *S. aureus* from one or more blood culture samples (either via the CVC, a separate peripheral venepuncture or both) and when available, from the corresponding distal catheter tip.
Patients were excluded if any of the following were identified: skin infections including boils and cysts or dermatological conditions such as eczema or dermatitis, recent infections including urinary tract infection, upper respiratory tract infection, wound infection.

Ethical committee approval and patient consent was obtained prior to entry into the study. The patient details collected included demographic information, clinical information incorporating antibiotic therapy and signs of post flushing pyrexia, evidence of localised and systemic signs of CRS, other foci of sepsis, peripheral white cell count, serum C-reactive protein and erythrocyte sedimentation rates, and microbiology culture results. Blood samples from the patients were also collected in vacutainer® tubes, free of anticoagulant and allowed to clot for 24h at 4° C. The samples were centrifuged at 3000 rpm for 5 minutes to separate

the serum which was then stored in micocentrifuge tubes at – 20° C. Microorganisms isolated from the patients' blood cultures were stored on polystyrene beads at –20° C.

4.2.1.2 Control group

Sixty seven patients over 18 years of age who were admitted to hospital between September 1996 and March 1999 for cardiac surgery (coronary arterial bypass grafting), hip surgery (some of whom subsequently required intensive care treatment), and haematology patients were entered into the study. The patients recruited had no clinical evidence of catheter associated infection and no other signs or indication of another septic focus. Blood samples from the patients were collected in vacutainer® tubes, free of anticoagulant and allowed to clot for 24h at 4°C. The samples were centrifuged at 3000 rpm for 5 minutes to separate the serum which was then stored in microcentrifuge tubes at – 20° C.

4.2.2 Estimation of antibody titres to the BHI-7 antigen

The samples collected from test and control patients were removed from –20°C and thawed at room temperature. The IgG and IgM titres were then determined using the BHI-7 ELISA methodology as described in chapter 3 (section 3.2.5).

4.2.3 Statistical analysis

The non-parametric Mann-Whitney test was used to analyse the serological data.

4.2.4 Blood cultures and distal catheter tips

Blood culture samples received from the patients were processed according to the laboratory standard operating procedure (SOP). This involved incubation of the blood sample for 7 days in Signal liquid blood culture medium (Oxoid, Basingstoke, U.K.). Positive blood cultures were subcultured onto solid agar, which included; blood agar containing 7% defibrinated horse blood, incubated in CO_2 and anaerobically at 37°C for up to 48 hours, cysteine lactose electrolyte deficient agar (CLED) and chocolate agar incubated in CO_2 for up to 48h. Corresponding catheter tips, when received from the patients were cultured according to the method of Maki *et al.* (1977) using blood agar supplemented with 7% horse blood (Oxoid, Basingstoke, U.K.).

4.2.5 Estimation of the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the BHI-7 ELISA

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy for the IgM and IgG were estimated using a range of cut-off titres for each test. A cut-off range of 8,000-28,000 for IgG estimation and a range of 5,000-20,000 for IgM were used. To determine the sensitivity and specificity of the assay, the antibody titres obtained using the BHI-7 ELISA were compared with the clinical evidence of CRS based on the strict criteria

noted in section 4.2.1.1. The following formulae were used to calculate each of the ELISA parameters (Greenhalgh, 1997):

$$\text{Sensitivity} = a / (a+c)$$

$$\text{Specificity} = d / (b+d)$$

$$\text{PPV} = a / (a+b)$$

$$\text{NPV} = d / (c+d)$$

$$\text{Accuracy} = (a+d) / (a+b+c+d)$$

Where a = true positives, b = false positives, c = false negatives, d = true negatives.

The Youden index (sensitivity + specificity -1) was also estimated as a statistical criterion of test performance and a method of evaluating diagnostic accuracy whereby sensitivity and specificity are assumed equally important (Siegman-Igra *et al.*, 1997).

4.2.6 Receiver operating characteristic (ROC) analysis

A ROC analysis (Siegman-Igra *et al.*, 1997) was performed to assess how the accuracy of the IgM and IgG ELISA systems varied with sensitivity and specificity of titre cut-off points. The results of the true positive rates (sensitivity) determined in 4.2.5 were plotted against false positive rates (1- specificity) in a ROC space.

4.3 Results

4.3.1 Patients with catheter-related sepsis and controls

One hundred and thirty four patients were recruited into the study, 67 with CRS and 67 control patients. The characteristics of the two patient groups are presented in table 4.0. None of the patients had any recent infection or signs of skin infections / dermatological conditions such as eczema or dermatitis. All of the patients within the group were receiving intra-venous antibiotic infusions, mainly the glycopeptides, vancomycin or teicoplanin. The CNS were the most frequently isolated organisms being cultured from 64 (96 %) of the patients with 11 (17 %) having repeated isolates. *S. aureus* accounted for the remaining 4% of isolates.

Table 4.0 **Patient demographic details**

	Patients with CRS	**Control patients**
Mean age **range**	49 years (18-82)	60 years (24-81)
Male:Female ratio	39:28	44:23
Patient group: **Haematology** **General** **Medical/Surgical**	 35 32	 20 47

4.3.2 Serological response in patients with CRS and controls

The IgG and IgM titres estimated in the 67 control patients and the 67 patients with a CRS are shown in tables 4.1 and 4.2 respectively. The means, ranges and standard deviations for the IgG and IgM titres within the two groups were determined and are shown in tables 4.3 and 4.4. There was a significant difference between the mean IgG titres of the patients with CRS and non-septic control subjects ($p<0.001$). A significant difference was also observed with the IgM titres ($p<0.001$). The IgM and IgG titre scatter plots are shown in figures 4.0 and 4.1. The BHI-7 ELISA microtitre plate is shown in figure 4.2 and demonstrates the IgG assay after the addition of the substrate and prior to stopping the colour generation by the addition of sulphuric acid.

Table 4.1 Serum IgG and IgM titres in the control group of patients

pt	IgG titre	IgM titre	pt	IgG titre	IgM titre	pt	IgG titre	IgM titre
1	8000	2000	**26**	2800	3900	**51**	20000	2300
2	1200	3200	**27**	11900	32900	**52**	3000	9600
3	10000	4000	**28**	11400	1600	**53**	13000	2700
4	6400	1600	**29**	10900	1400	**54**	5000	800
5	6600	2000	**30**	19200	43900	**55**	6000	2300
6	3200	2000	**31**	5700	2400	**56**	13000	1000
7	5000	1600	**32**	12800	25000	**57**	1000	4800
8	6000	2000	**33**	4100	8700	**58**	3000	400
9	10000	2500	**34**	11900	10000	**59**	6000	10000
10	6400	3000	**35**	6400	5400	**60**	17000	2800
11	10800	1600	**36**	1900	< 400	**61**	1000	1600
12	6400	2000	**37**	12800	3000	**62**	23000	2300
13	10000	5000	**38**	6400	15500	**63**	5000	400
14	7600	3200	**39**	19200	31000	**64**	6000	1300
15	5500	1600	**40**	3900	10900	**65**	10000	1200
16	3800	1000	**41**	10500	21900	**66**	12000	1300
17	7000	7000	**42**	21900	3000	**67**	22000	400
18	1000	5000	**43**	27400	4800			
19	6000	3200	**44**	11300	4300			
20	12000	7500	**45**	10000	5700			
21	20000	7000	**46**	6000	1600			
22	24000	5000	**47**	8000	4800			
23	17000	5000	**48**	5000	3000			
24	5500	1300	**49**	9000	1600			
25	5000	10500	**50**	6000	700			

Table 4.2 Serum IgG and IgM titres patients with catheter-related sepsis due to staphylococci

pt	IgG titre	IgM titre	pt	IgG titre	IgM titre	pt	IgG titre	IgM titre
1	15000	6000	**26**	50000	25600	**51**	11600	1600
2	25000	9000	**27**	50000	23800	**52**	58200	1200
3	100000	25000	**28**	20000	10200	**53**	34000	400
4	46000	6400	**29**	15000	8200	**54**	400000	3100
5	6000	9600	**30**	10000	3200	**55**	20000	400
6	51000	9100	**31**	50000	2400	**56**	63000	1500
7	6000	1900	**32**	40000	6400	**57**	43000	1200
8	26000	12800	**33**	50000	25600	**58**	51000	400
9	51000	25600	**34**	50000	11400	**59**	39000	5000
10	16000	38400	**35**	30000	25600	**60**	120000	2000
11	38000	3900	**36**	50000	47500	**61**	38000	400
12	46000	18300	**37**	20000	51200	**62**	11000	650
13	13000	14600	**38**	40000	45700	**63**	23000	400
14	20000	69500	**39**	15000	10100	**64**	23000	400
15	37000	6400	**40**	30000	14600	**65**	65000	4600
16	29000	12800	**41**	50000	20100	**66**	18000	1400
17	85000	20000	**42**	50000	50000	**67**	22000	3600
18	50000	102000	**43**	10000	40200			
19	50000	12300	**44**	40000	7300			
20	40000	12800	**45**	25000	3200			
21	15000	6400	**46**	50000	9600			
22	15000	2900	**47**	50000	19900			
23	200000	11000	**48**	20000	25600			
24	20000	9600	**49**	40000	1300			
25	10000	3200	**50**	409600	322000			

Table 4.3

Statistical analysis of the titres achieved in the control group

	IgG TITRES	IgM TITRES
number (n)	67	67
range	1200-27400	< 400-43900
mean	9216	6744
standard deviation (SD)	5815	8921

Table 4.4

Statistical analysis of the titres achieved in patients with catheter-related sepsis due to staphylococci

	IgG TITRES	IgM TITRES
number (n)	67	67
range	6000-409600	1300-322000
mean	45492	24744
standard deviation (SD)	60042	46597

Figure 4.0 Scatter plot of the IgM levels from patients with catheter related sepsis and controls. The 5,000 cut off for positivity is indicated

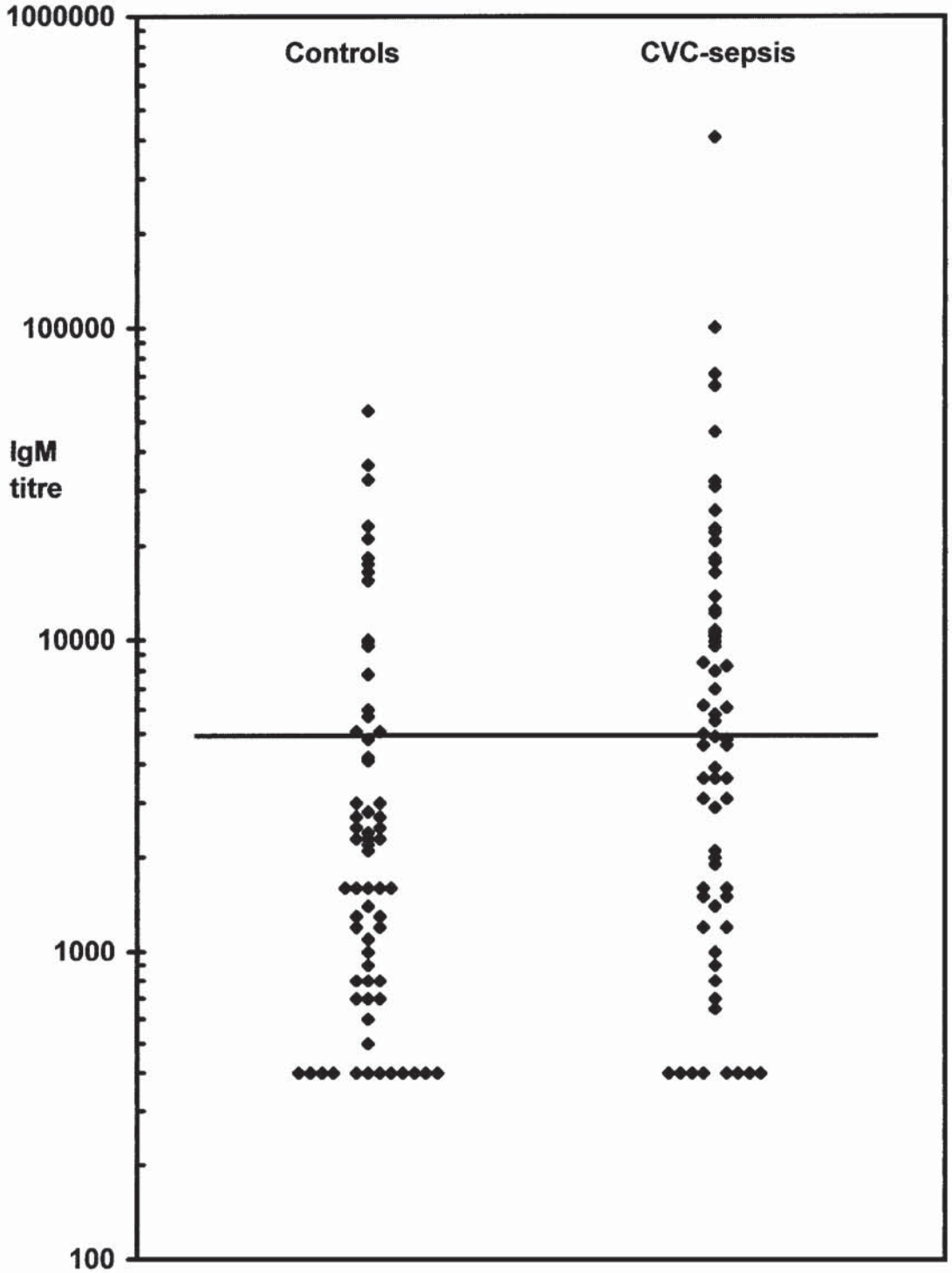


Figure 4.1 Scatter plot of the IgG levels from patients with catheter related sepsis and controls. The 20,000 cut off point for positivity is indicated

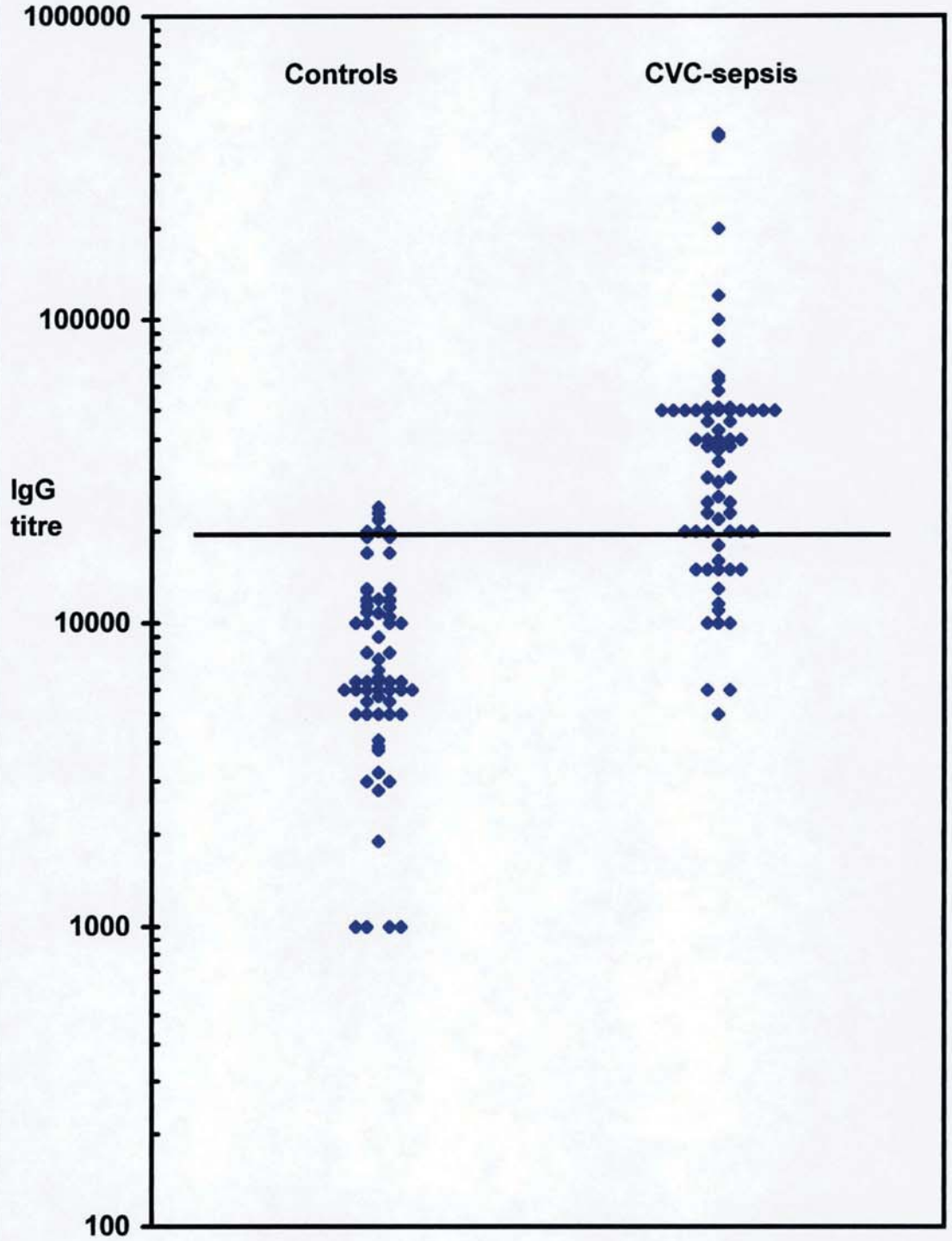
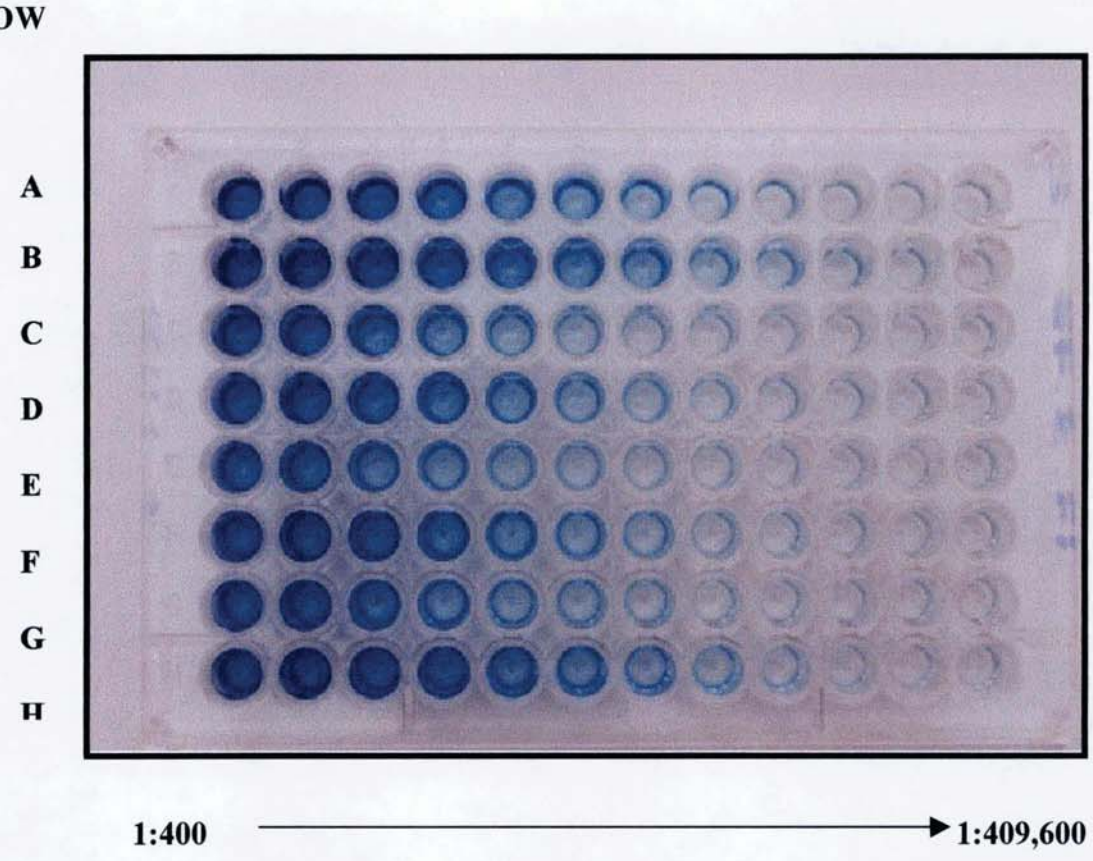


Figure 4.2 **Detection of IgG antibodies in patients with CRS and controls with the BHI-7 ELISA**



Eight serum samples were tested (rows A-H). Each sample was double diluted horizontally across the microtitre tray from 1:400-1:409,600. Patient serum IgG levels were determined by reference to the positive control titre of 1:100,000 (chapter 3).

ROW A= positive serum: IgG titre 1:120,000

ROW B= positive serum: IgG titre 1:400,000

ROW C= positive serum: IgG titre 1:50,000

ROW D= positive serum: IgG titre 1:85,000

ROW E= negative serum: IgG titre 1:5000

ROW F= positive serum: IgG titre1:85,000

ROW G= Normal Human Serum: IgG titre1:10,000

ROW H= Positive Control Serum: titre 1:100,000

4.3.3 The sensitivity, specificity, PPV, NPV and accuracy of the BHI-7 ELISA

The sensitivity, specificity, PPV, NPV and accuracy of both IgG and IgM ELISA using different cut-off values are shown in tables 4.5 and 4.6. The most discriminatory cut off titres within the IgG system were 1:14,000 and 1:20,000 as indicated by Youden indexes of 0.74 and 0.68 respectively. In agreement, the method of accuracy testing by Greenhalgh (1997), yielded results of 87% and 84% for cut-off titres of 1:14,000 and 1:20,000 respectively. The most discriminatory cut off titre of the IgM system was 1:6,000, with a Youden index of 0.4 and an accuracy result of 70%. The mean Youden index for the IgG ELISA was 0.64 for and 0.23 for the IgM ELISA, indicating a greater accuracy in the IgG system.

Table 4.5 Sensitivity, specificity, PPV, NPV and accuracy of BHI-7 IgG estimation using cut-off values of 1:8,000-1:28,000

TITRE CUT-OFF	TRUE POS a	FALSE NEG c	TRUE NEG d	FALSE POS b	**SENSITIVITY %**	**SPECIFICITY %**	**PPV %**	**NPV %**	**ACCURACY %**	**YOUDEN INDEX**
8,000	65/67	2	35/67	32	**97**	**52**	**67**	**95**	**75**	**0.49**
10,000	65/67	2	40/67	27	**97**	**60**	**70**	**95**	**78**	**0.57**
12,000	60/67	7	51/67	16	**90**	**76**	**79**	**88**	**83**	**0.66**
14,000	60/67	7	56/67	11	**90**	**84**	**84**	**89**	**87**	**0.74**
16,000	55/67	12	56/67	11	**82**	**84**	**83**	**82**	**83**	**0.66**
18,000	53/67	14	58/67	9	**79**	**87**	**85**	**81**	**83**	**0.66**
20,000	52/67	15	61/67	6	**75**	**90**	**90**	**80**	**84**	**0.68**
22,000	48/67	19	64/67	3	**72**	**95**	**94**	**77**	**84**	**0.67**
24,000	44/67	23	65/67	2	**66**	**97**	**96**	**74**	**81**	**0.63**
26,000	42/67	25	66/67	1	**63**	**99**	**98**	**73**	**80**	**0.62**
28,000	41/67	26	67/67	0	**61**	**100**	**100**	**72**	**80**	**0.61**

Table 4.6 Sensitivity, specificity, PPV, NPV and accuracy of BHI-7 IgM estimation using cut-off values of 1:5,000-1:20,000

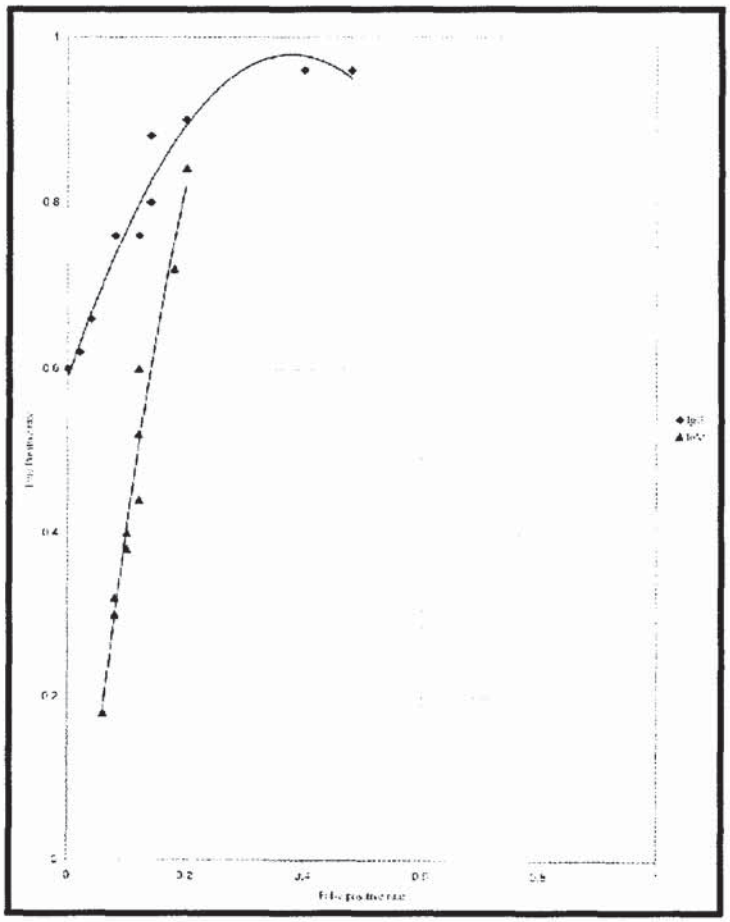
TITRE CUT-OFF	TRUE POS a	FALSE NEG c	TRUE NEG d	FALSE POS b	**SENSITIVITY %**	**SPECIFICITY %**	**PPV %**	**NPV %**	**ACCURACY %**	**YOUDEN INDEX**
5,000	35/67	32	50/67	17	**52**	**85**	**67**	**61**	**63**	**0.37**
6,000	42/67	25	52/67	17	**63**	**77**	**71**	**68**	**70**	**0.40**
8,000	36/67	31	55/67	12	**54**	**82**	**75**	**64**	**68**	**0.36**
10,000	30/67	37	58/67	9	**45**	**87**	**77**	**61**	**66**	**0.32**
12,000	26/67	41	61/67	6	**39**	**91**	**76**	**59**	**65**	**0.30**
14,000	22/67	45	61/67	6	**33**	**91**	**78**	**58**	**62**	**0.30**
16,000	20/67	47	62/67	5	**30**	**93**	**80**	**57**	**61**	**0.23**
18,000	20/67	47	62/67	5	**30**	**93**	**80**	**57**	**61**	**0.23**
20,000	19/67	48	62/67	5	**28**	**93**	**79**	**56**	**60**	**0.21**

4.3.4 Receiver operator curve analysis

A graph of receiver operator curves (ROC) plotting true positive rates against false positive rates for the IgG and IgM ELISA in patients with CRS is shown in figure 4.3. The ROC curve for IgG estimation positioned closer to the upper left hand corner of the graph as compared with the curve for IgM estimation and had a larger area under the curve, indicating higher test accuracy.

Figure 4.3 **ROC graph plotting true positive rates against false positive rates for the IgM and IgG ELISA in patients with CRS**

TRUE POSITIVE RATE



FALSE POSITIVE RATE

4.4 Discussion

For successful treatment of catheter-related infections it is important to make the diagnosis at an early stage, which requires a rapid and reliable laboratory method. Many methods have been described but their accuracy and cost-effectiveness remains unclear (Siegman-Igra *et al.*, 1997). The serological method based on measurement of antibody to the novel BHI-7 antigen however appears to offer a clinically useful test. Although there was a significant difference between the IgM titres of the two patient groups, there was a considerable overlap in measured titres, which did not allow a clear differentiation for between septic, and control patients. The lack of sensitivity and specificity of the IgM assay may have reflected the relationship between the date of onset of the CRS, which is difficult to determine with any accuracy and the actual sampling time, which followed the clinical diagnosis of sepsis. It is evident that further studies are necessary to investigate the history of CVC-related sepsis and colonisation related to the IgM response. In comparison, the serological test showed a significant difference in serum IgG levels to the short chain length lipoteichoic acid antigen in the patients with catheter-related infection associated with CNS as compared to control subjects with an acceptable sensitivity and specificity. The test had a sensitivity of 75% and a specificity of 90% which compares favourably to those of semi-quantitative catheter segment culture (sensitivity 85%, specificity 85%) and quantitative blood cultures (sensitivity 79%, specificity 94%) reported in a meta-analysis of diagnostic methods used for vascular catheter sepsis. Indeed, the ROC analysis of the IgG results was very similar to that of quantitative blood cultures (Siegman-Igra *et al.*, 1997).

As the diagnosis of CRS is difficult to make and there is no "gold standard" definition to compare new methods to, a sensitivity of 75% may in fact be an underestimation and may reflect the true rate of infection. Conversely, it may reflect the difficulties experienced in the patient selection criteria. Indeed, some of the patients with an antibody response of <20,000 and considered clinically to have a CRS may not have been infected. Within the IgG antibody titre range of 15,000-25,000 an overlap between the control group and

patients with a clinical sepsis was also observed. This may reflect catheter colonisation in the septic group of patients, or alternatively be an indication of an infection at the early stages of development. Patients undergoing immunosuppressive treatment may also elicit a poor antibody response, which might account for some of the titres that fell within this range. The kinetics of the antibody response in patients with a colonised CVC and those with device-related sepsis has not yet been studied and needs further investigation. In addition, high antibody levels in some control patients might be explained by prior exposure the antigen produced by commensal skin CNS through minor skin conditions or unreported earlier infection.

The current results however suggest that this ELISA test based on a novel lipoteichoic acid antigen to detect corresponding IgG levels may offer a rapid diagnostic method for CRS. This new test allows, in the majority of cases, for the diagnosis of CRS to be made without having to remove the device or to wait for blood cultures to become positive and for the microorganisms to be identified. Similarly the problems associated with microbial contamination from other sources, for example from hubs which are colonised in 22% of cases (Tebbs *et al.*, 1996) and may contaminate blood cultures or blood for Gram or acridine orange stain leucocyte cytospin (AOLC), is eliminated. Thighe *et al.* (1996) used an endoluminal brush technique for the diagnosis of CRS and compared it to the AOLC. The endoluminal brush sampled the internal lumen of the CVC for microorganisms *in situ* whilst in the AOLC test, blood taken from the CVC was examined for the presence of microorganisms. In combination, the endoluminal brush significantly improved the yield of the AOLC test. However, both of these methods require specialist techniques and may not distinguish colonisation of the internal lumen of catheters and luer connectors from infection. The potential of the BHI-7 ELISA and its possible role in assisting diagnosis of these infections due to Gram-positive cocci is outlined in table 4.7.

Table 4.7 Potential of the IgG ELISA in aiding the diagnosis of a CRS

Presence of staphylococci in blood cultures	IgG ELISA result	Interpretation
+	+	CRS
+	-	Possible contamination. Repeat blood cultures and serology
-	+	Treated / previous sepsis
-	-	CRS unlikely

The test offers serological evidence of sepsis and may also allow appropriate treatment to be commenced earlier, which in turn could result in an improved outcome. The test may also support the relatively indeterminate clinical suspicion of CVC infection and thereby allow clinicians to more confidently keep catheters *in situ* without having to resort to removal based on non-specific clinical indications. Similarly it could reduce the likelihood of inappropriate treatment resulting in increased selective pressure for antimicrobial resistance. Catheter related sepsis results in a significant cost (Moss and Elliott, 1997). In the U.K. alone, the costs associated with catheter related sepsis are estimated to be £2.5 million per annum for long term catheters and between £5-7 million for short term catheters and the use of this test may therefore also result in significant savings.

Chapter 5 A preliminary investigation into the antibody response to the BHI-7 antigen in serial blood samples from patients with catheter-related sepsis.

5.1 Introduction

The humoral immune response including IgM and IgG serum levels is of major diagnostic importance as it is exploited in the serodiagnosis of many viral, bacterial and fungal diseases including syphilis, legionnaires disease, cryptococcosis and candidiasis. Within several days following primary antigenic stimulation, B lymphocytes derived from haemopoietic cells of the foetal liver, and the foetal and adult bone marrow, produce immunoglobulin resulting in a rise in detectable antibody in serum. This is termed the primary humoral response. IgM antibodies are usually detectable in serum earlier than IgG, however the levels of IgM subsequently decline more rapidly than IgG. In addition, IgA is produced and is the principal immunoglobulin in external secretions of the respiratory, intestinal, and genitourinary tracts and in tears, saliva and breast milk. When using antibody titre results to support the diagnosis of infection, several factors concerning the humoral response need to be considered. Following the primary antigenic challenge there is an initial lag phase of several days dependant on the infection, when no antibody can be detected. This is followed by phases when the antibody titre rises logarithmically to reach a plateau usually within 1to 10 weeks. The levels of antibody decline as they are catabolised or bind to the antigen and are cleared from the circulation (Roitt *et al.*, 1985). On second and subsequent encounters with the antigen, the type of response is largely determined by the outcome of the first antigenic challenge, however the quantity and quality of the response differ in 4 major respects;

- Time course. The secondary response has less of a lag phase but an extended plateau and decline.
- Antibody titre. During the secondary response, IgM production may be similar to that of the primary whereas the IgG level is usually greater.

- Antibody class. IgM class antibodies constitute a major proportion of the primary response and are detectable in serum before IgG antibodies. However, the levels of IgM antibodies decline after several weeks, whilst the IgG antibodies may persist for years. In the secondary humoral response to the same antigen, the antibody class is predominantly IgG.
- Antibody affinity. The affinity of the antibodies in the secondary response is usually much greater.

In addition to the quality and quantity of antibody observed in primary and secondary humoral responses, host factors which may influence immunoglobulin production also need to be considered when interpreting the results of antibody assays. Immunodeficiency for example due to extremes of age, malnutrition, infection due to human immunodeficiency virus and neoplastic disorders such as lymphoma and leukaemia are associated with a decline in the antibody response (Woolf, 1986).

Serodiagnosis of infection may be accomplished from the antibody assay results achieved on a single sample of serum. However, low titres due to immunodeficiency, the time the blood was taken after antigen presentation and cross-reacting antibodies (chapter 6) can make the interpretation of assay results difficult. Therefore, serial samples of serum should be assayed if antibody titres are to be fully evaluated. Verbrugh *et al.* (1983), for example, incorporated peptidoglycan and other antigenic constituents of *S. aureus* in an ELISA to investigate the serological response in patients with bacteraemia, and to determine the value of testing serial samples. A significant increase in the antibody titre was detected in the serial serum samples of 64% of the patients. Ideally, when monitoring the course of an infection, serum samples should be taken early in the course of illness, 2 to 3 weeks after onset, during convalescence and several weeks after recovery. A fourfold or greater rise in antibody titre in a second serum sample taken 2 weeks after the first is usually diagnostic of infection.

The aim of this part of study was to investigate the serological response of immunocompetent and immunodeficient patients with catheter-related sepsis (CRS) due to staphylococci (chapter 4), by determining specific antibody titres in serial serum samples against the BHI-7 antigen. In addition, baseline levels of antibody in those patients with and without previous episodes of CRS were estimated prior to the diagnosis of CRS. The value of determining antibody in serial serum samples as compared to single sera in supporting the diagnosis was assessed in a preliminary investigation.

5.2 Materials and Methods

5.2.1 Patients

Serial samples of blood were collected from 13 patients with CRS studied in chapter 4. The serum was separated and stored at -20°C. In addition, sera from 11 out of 13 (85%) patients were collected prior to the diagnosis of CRS. The sera had been previously sent to the laboratory for routine viral serology screening and required storage at -20°C post testing. The samples were included in the study to investigate baseline levels of antibody prior to CRS and designated pre CRS samples. All sera were removed from -20°C and thawed at room temperature prior to assay.

5.2.2 BHI-7 ELISA

The BHI-7 ELISA was performed on all serum samples by the method described in section 3.2.5. However, IgM and IgG antibody titres were estimated by reference to the standard positive control serum titres of 1:25,000 and 1:100,000 respectively (chapter 3).

5.3 Results

5.3.1 Sample collection

Of the 13 patients recruited into this part of the study, multiple serum samples post CRS were obtained from 9 (69%). These patients are designated 1 to 9 in tables 5.0 to 5.12. The numbers of serum samples received ranged from 2 to 11 (mean, 5) per patient and were obtained over time ranging from 1 week to 5 months post CRS, depending upon the length of patient hospitalisation. Of the 9 patients with multiple post CRS sera, pre CRS sera were collected from 7 (78%) patients (range 2 days to 8 months prior to CRS).

One sample of post CRS serum was received from the remaining 4 out of 13 (31%) patients. Pre CRS sera were collected from all of these patients (range 10 days to 6 months prior to CRS) and they were therefore included in the study. These patients are designated 10 to 13 in tables 5.0 to 5.12.

5.3.2 Serology

The IgM and IgG titres, and clinical details of the 13 individual patients with CRS are shown in tables 5.0 to 5.12. The serological response over time in immunodeficient and immunocompetent patients with and without previous episodes of CRS is shown in figures 5.0 to 5.7. The time of serum sampling is standardised for the figures and reference to tables 5.0 to 5.12 should be made for exact sample dates. The cut-off titres indicative of positive serology were ≥1:20,000 for IgG antibody and ≥1:5,000 for IgM (chapter 4). A summary of patient and serological data is shown in table 5.13.

Table 5.13 Summary of the clinical data and serological response in 13 patients with CRS due to staphylococci

Pt	Pre-CRS IgM	Pre-CRS IgG	Post-CRS IgM	Post-CRS IgG	Immuno-deficient patient	Previous Clinical history of CRS
1	NT	NT	+, rising titre	+, rising titre	yes	no
2	+	+	+, rising titre	+, rising titre	no	no
3	+	+	+, rising titre	+	no	yes
4	-	-	-	-	yes	yes
5	+	+	+, rising titre	+, rising titre	no	no
6	NT	NT	-	-	yes	no
7	+	+	+, rising titre	+, rising titre	no	yes
8	-	+	+	+	yes	yes
9	+	+	+, rising titre	+, rising titre	no	yes
10	+	-	+	-	yes	no
11	+	+	+	+	no	yes
12	+	+	+	+	yes	yes
13	+	+	+	+	yes	yes

NT = not tested

Pts 1-9 had multiple serum samples post CRS, + indicates a constant positive titre, - indicates a constant negative titre.

Pts 10-13 had a single sample of serum collected post CRS

Of the 11 pre CRS samples of serum, 10 (91%) had elevated levels of antibody detected with the BHI-7 ELISA. In the 9 patients where multiple serum samples were obtained post CRS, 6 (67%) had rising levels of IgM and IgG during the course of sample collection. Seven out of 13 (54%) patients had underlying immunodeficiency, 5 (71%) of which had elevated levels of antibody detected in post CRS serum samples. Five out of 13 (38%) patients had no clinical history of CRS of which 4 (80%) had pre CRS serum samples collected. Interestingly, antibody levels were detectable in the serum of 3 (75%) of these patients before staphylococci were isolated from the blood cultures. Patients 2 and 5 had elevated antibody levels 5 and 4 days respectively prior to positive blood cultures respectively and patient 10 had a positive IgM titre 10 days prior to positive culture.

Tables 5.0 to 5.12 Clinical details of 13 patients with catheter-related sepsis and antibody levels in serial samples of serum

Table 5.0 PATIENT 1

Underlying disease: Acute myeloid leukaemia

Clinical evidence of CRS: Pyrexia, positive blood culture, inflammed insertion site

Previous clinical history of CRS: No

Antibiotic therapy: Vancomycin

SERUM DATE	IgG TITRE	IgM TITRE
CNS isolated from blood culture 1.4.96		
4.4.96	15000	6000
7.4.96	15000	13000
11.4.96	25000	13000
14.5.96	30000	13000
17.5.96	40000	13000
20.5.96	40000	5000

Table 5.1 PATIENT 2

Underlying disease: Aortic valve replacement

Clinical evidence of CRS: Pyrexia, positive blood culture

Previous clinical history of CRS: No

Antibiotic therapy: Vancomycin

SERUM DATE	IgG TITRE	IgM TITRE
15.3.96 (pre)	25000	9000
CNS isolated from blood culture 20.3.96		
21.3.96	30000	20000
22.3.96	30000	25000
23.3.96	30000	25000
26.3.96	40000	25000

Table 5.2 PATIENT 3

Underlying disease: Short bowel syndrome

Clinical evidence of CRS: Pyrexia, positive blood culture

Previous clinical history of CRS: Yes

Antibiotic therapy: Vancomycin

SERUM DATE	IgG TITRE	IgM TITRE
17.7.95 (pre)	100000	13000
CNS isolated from blood culture 17.3.96		
18.3.96	100000	13000
22.3.96	100000	25000
28.3.96	100000	25000
17.5.96	100000	25000
19.5.96	100000	25000
20.5.96	100000	25000
23.5.96	100000	25000
4.6.96	100000	13000

Table 5.3 PATIENT 4

Underlying disease: Acute myeloid leukaemia

Clinical evidence of CRS: Pyrexia post CVC flushing, positive blood culture

Previous clinical history of CRS: Yes

Antibiotic therapy: Vancomycin, Teicoplanin, Ciprofloxacin

SERUM DATE	IgG TITRE	IgM TITRE
CNS isolated from blood culture 6.8.96		
4.9.96 (pre)	6000	4000
CNS isolated from blood culture 7.1.97		
14.1.97	5000	3200
29.4.97	3000	1500
29.6.97	2000	900

Table 5.4 PATIENT 5

Underlying disease: Renal failure and dialysis

Clinical evidence of CRS: Pyrexia, positive blood culture

Previous clinical history of CRS: No

Antibiotic therapy: Unknown

SERUM DATE	IgG TITRE	IgM TITRE
21.1.97 (pre)	11000	6400
20.2.97 (pre)	51000	9000
CNS isolated from blood culture 24.2.97		
28.2.97	70000	12800
3.3.97	90000	12800
10.3.97	90000	10000

Table 5.5 PATIENT 6

Underlying disease: Acute myeloid leukaemia

Clinical evidence of CRS: Septicaemia, pyrexia, positive blood culture, erythema

Previous clinical history of CRS: No

Antibiotic therapy: Teicoplanin

SERUM DATE	IgG TITRE	IgM TITRE
CNS isolated from blood culture 24.2.97		
27.2.97	6000	1900
13.4.97	8000	800

Table 5.6 PATIENT 7

Underlying disease: Renal dialysis,

Clinical evidence of CRS: pyrexia, positive blood culture

Previous clinical history of CRS: Yes

Antibiotic therapy: Vancomycin

SERUM DATE	IgG TITRE	IgM TITRE
20.5.96 (pre)	18000	9500
21.1.97 (pre)	26000	12500
CNS isolated from blood culture 11.2.97		
28.2.97	42000	9500
5.3.97	70000	11000

Table 5.7 PATIENT 8

Underlying disease: Acute myeloid leukaemia

Cinical evidence of CRS: Rigor post CVC flushing, positive blood culture

Previous clinical history of CRS: Yes

Antibiotic therapy: Teicoplanin

SERUM DATE	IgG TITRE	IgM TITRE
CNS isolated from blood culture 15.5.97		
17.5.97 (pre)	33000	3900
CNS isolated from blood culture 11.7.97		
11.7.97	33000	18000
17.7.97	33000	21000
18.7.97	33000	18000

Table 5.8 PATIENT 9

Underlying disease: Budd Chiarhi Syndrome

Clinical evidence of CRS: Pyrexia, positive blood culture, erythema

Previous clinical history of CRS: Yes

Antibiotic therapy: Vancomycin

SERUM DATE	IgG TITRE	IgM TITRE
25.6.97 (pre)	13000	14500
CNS isolated from blood culture 30/6/97		
30.6.97	20000	10000
1.7.97	30000	16500
2.7.97	30000	21000
3.7.97	26000	36000
4.7.97	26000	14600

Table 5.9 PATIENT 10

Underlying disease: Organ transplantation

Clinical evidence of CRS: Pyrexia, positive blood culture, erythema

Previous clinical history of CRS: No

Antibiotic therapy: Vancomycin

SERUM DATE	IgG TITRE	IgM TITRE
10.1.97 (pre)	3000	10000
***S. aureus* isolated from blood culture 20.1.97**		
23.1.97	6000	9600

Table 5.10 PATIENT 11

Underlying disease: Renal failure and dialysis

Clinical evidence of CRS: Pyrexia, positive blood culture, erythema, oedema

Previous clinical history of CRS: Yes

Antibiotic therapy: Vancomycin, Gentamicin

SERUM DATE	IgG TITRE	IgM TITRE
19.12.96 (pre)	33000	31000
***S. aureus* isolated from blood culture 21.3.97**		
8.4.97	51000	25500

Table 5.11 PATIENT 12

Underlying disease: Acute myeloid leukaemia

Clinical evidence of CRS: Pyrexia, positive blood culture

Previous clinical history of CRS: Yes

Antibiotic therapy: Teicoplanin

SERUM DATE	IgG TITRE	IgM TITRE
5.2.97 (pre)	16000	38500
21.3.97 (pre)	33000	25600
CNS isolated from blood culture 26.3.97		
29.3.97	26000	47500

Table 5.12 PATIENT 13

Underlying disease: Acute myeloid leukaemia

Clinical evidence of CRS: Pyrexia, positive blood culture

Previous clinical history of CRS: Yes

Antibiotic therapy: Vancomycin, Ciprofloxacin

SERUM DATE	IgG TITRE	IgM TITRE
10.9.96 (pre)	33000	18000
CNS isolated from blood culture 27/3/97		
1.4.97	46000	18000

Figure 5.0 IgM levels in serial serum samples from immunodeficient patients. Patients with no previous clinical history of catheter related sepsis

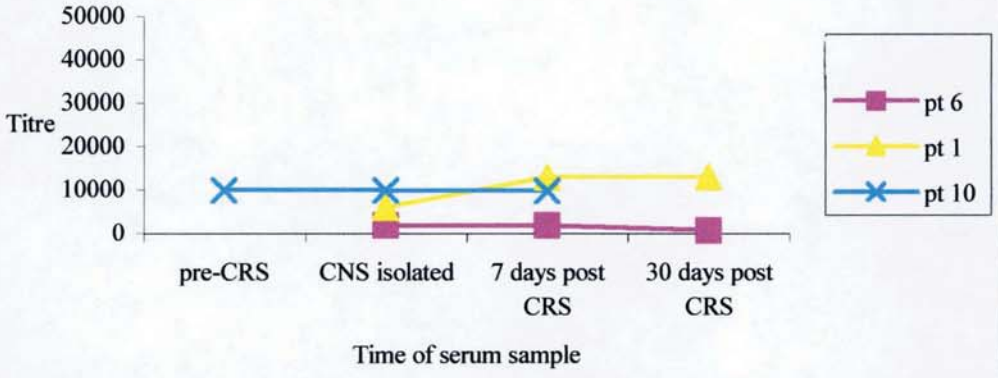
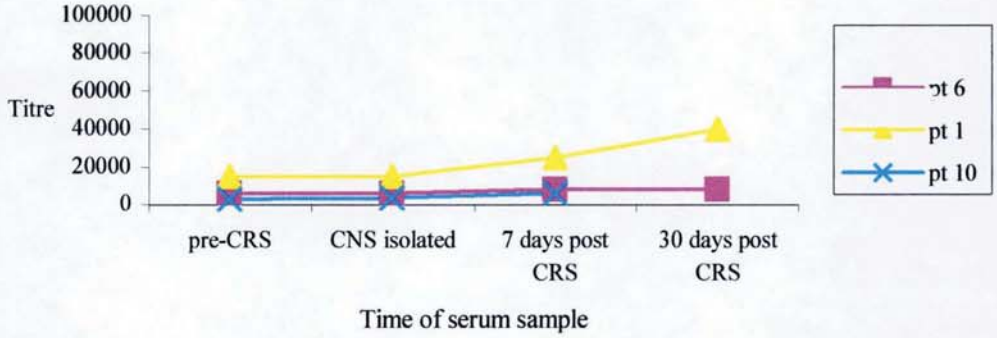


Figure 5.1 IgG levels in serial serum samples from immunodeficient patients. Patients with no previous clinical history of catheter related sepsis



Cut off titres for positivity are: IgM 1:5,000 and IgG 1:20,000.

Refer to tables 5.0 to 5.12 for individual patient serum sample dates

Figure 5.2 IgM levels in serial serum samples from immunodeficient patients. Patients with a previous clinical history of catheter related sepsis

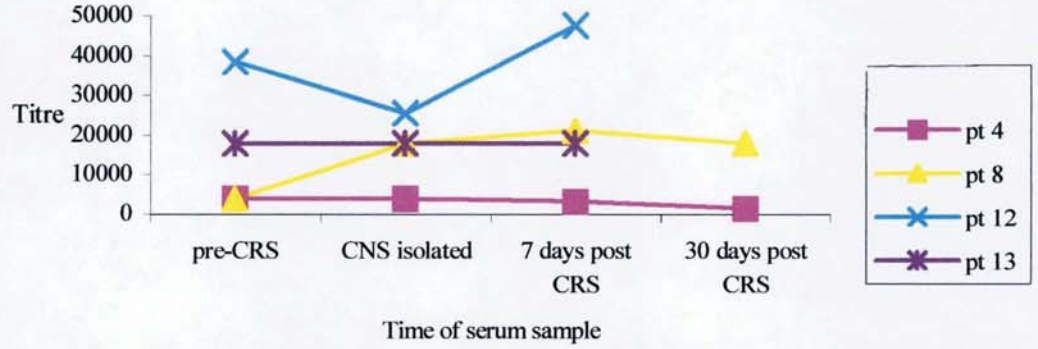
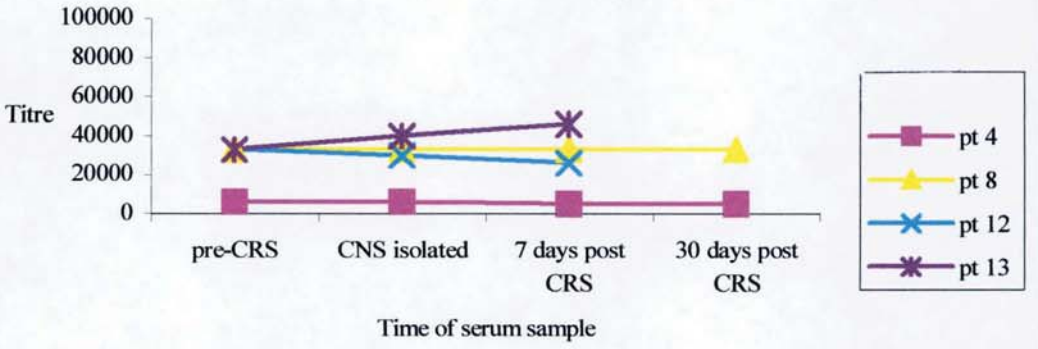


Figure 5.3 IgG in serial serum samples from immunodeficient patients. Patients with a previous clinical history of catheter related sepsis



Cut off titres for positivity are: IgM 1:5,000 and IgG 1:20,000

Refer to tables 5.0 to 5.12 for individual patient serum sample dates

Figure 5.4 IgM levels in serial serum samples from immunocompetent patients. Patients with no previous clinical history of catheter related sepsis

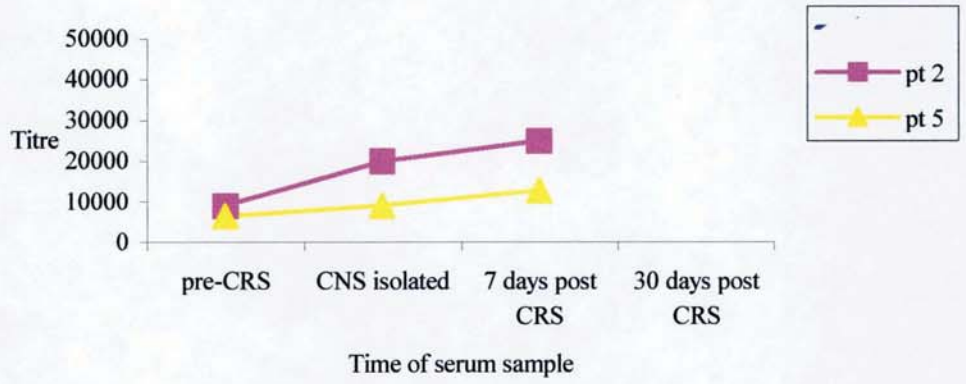
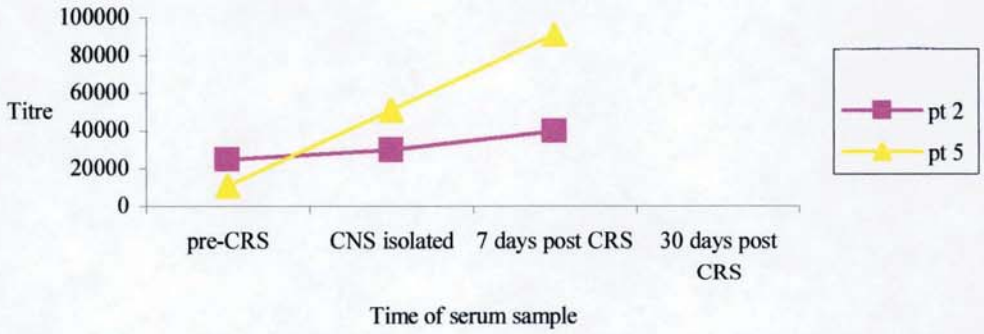


Figure 5.5 IgG levels in serial serum samples from immunocompetent patients. Patients with no previous clinical history of catheter related sepsis



Cut off titres for positivity are: IgM 1:5,000 and IgG 1:20,000

Refer to tables 5.0 to 5.12 for individual patient serum sample dates

Figure 5.6 IgM levels in serial samples of serum from immunocompetent patients. Patients with previous a clinical history of catheter related sepsis

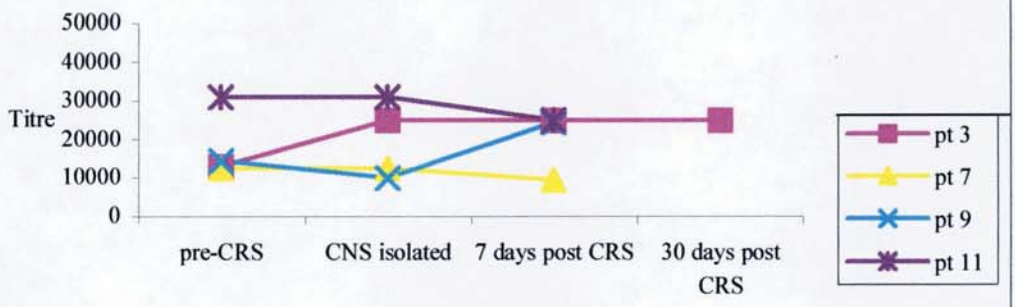
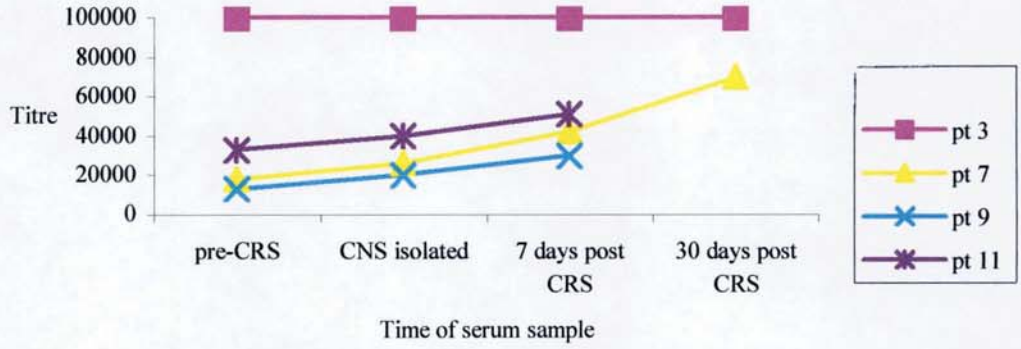


Figure 5.7 IgG levels in serial serum samples from immunocompetent patients. Patients with a previous clinical history of catheter related sepsis



Cut off titres for positivity are: IgM 1:5,000 and IgG 1:20,000

Refer to tables 5.0 to 5.12 for individual patient serum sample dates

5.4 Discussion

The study on the serological response in patients with CRS due to staphylococci (chapter 4) highlighted the serodiagnostic potential of the BHI-7 ELISA, particularly in the differentiation between IgG levels of infected patients and healthy controls. However, the study was based on the analysis of single samples of serum taken from patients after the isolation of staphylococci from blood cultures and therefore did not investigate the serological status of the patients prior to the diagnosis of CRS or following treatment.
When developing an assay incorporating novel antigenic material several factors that may influence the interpretation of antibody titres need to be investigated, especially if single serum samples are tested. These factors include the primary and subsequent humoral responses of the patient to the antigen, the persistence of antibodies following infection and treatment, and the influence of immune disorders. This can only be achieved by the determination of antibody titres of serial samples of serum.

The results of this current preliminary study highlighted several important issues that need to be considered when interpreting antibody levels by the BHI-7 ELISA. The value of determining the antibody titres to the BHI-7 antigen in the 11 pre CRS serum samples was clearly demonstrated. Elevated levels of IgM and IgG were detected in 82% of pre CRS sera prior to the isolation of staphylococci. However, 8 out of 11 (73%) of these patients had a clinical history of CRS due to staphylococci and therefore raised levels of antibody may be expected. Interestingly, 3 out of 11 (27%) patients had no previous history of CRS and positive antibody titres were detected in all these serum samples. Indeed, 2 patients (2 and 5) had elevated levels of IgM and IgG in pre CRS serum samples which were obtained 5 and 4 days before corresponding blood cultures were positive. The third patient (10), who was immunodeficient, had elevated levels of IgM in sera 10 days prior to positive blood cultures. These elevated antibody levels may be in response to a Gram-positive infection elsewhere in the patient, however this was not recorded in the clinical notes. Alternatively,

the positive titres may have reflected early signs of infection related to the indwelling intravascular device.

The monitoring of antibody levels in serial serum samples by ELISA may therefore provide an alternative diagnostic approach to conventional methods including blood cultures, by detecting infection at an early stage of sepsis. Indeed, the early signs of seroconversion in patients with CRS as indicated by the BHI-7 ELISA in this current study, may be exploited to influence the clinical management of patients with a CVC *in situ*. For example, blood withdrawn through the catheter on insertion would provide a sample from which the baseline levels of IgM and IgG could be determined, and subsequent samples during the period of catheter placement could be assessed for changes in the humoral response. An increasing antibody in patients with no other septic focus, may provide clinicians with the information needed to remove the catheter at an early stage of sepsis, and administer treatment when necessary. Conversely, serological monitoring of the patient from the day of catheter insertion may aid in unnecessary removal of catheters from those presenting with non-specific clinical symptoms.

The associated costs of CRS are high (Moss and Elliott, 1997). The implementation of a system of routine serological monitoring of catheterised patients by a rapid ELISA (chapter 8) may assist in the reduction of associated costs in terms of unnecessary catheter removal, treatment and extended hospital stay. However, studies are required to serologically monitor antibody levels in further immunocompetent and immunodeficient patients to fully assess the value of serial serum samples. Careful interpretation of antibody titre results from patients with immunosuppression is warranted as some may fail to elicit an antibody response (Woolf, 1986). In this current study, 5 out of 11 (45%) pre CRS serum samples were from patients with immunosuppression, and 4 (80%) had raised antibody levels. The level of serological response elicited by immunodeficient patients is likely to reflect the severity of the underlying immunodeficiency at the time of serum sampling.

Multiple post CRS serum samples were obtained from 9 patients of which 6 (67%) had increasing levels of antibody. However, 5 out of 6 patients (83%) were immunocompetent. This was similar to the findings of Verbrugh *et al.* (1983), who demonstrated a rising titre of antibody to staphylococcal call wall components in 64% of patients. In addition, the level of antibody in the post CRS serum samples was higher in patients with a clinical history of CRS due to staphylococci as compared to those eliciting a primary response. Indeed, high levels of antibody to the glycolipid antigen were detectable in the post CRS serum samples of patient 3 for 12 months following multiple previous episodes of CRS due to staphylococci. Three out of 9 (33%) patients did not have rising levels of antibody post CRS, all of whom had underlying immunodeficiency. Two of these patients (4 and 6) had negative serological results with the BHI-7 ELISA, which may be due to failure of these patients in eliciting a humoral response, or it may reflect a clinical misdiagnosis of CRS.

In summary, antibody levels in serial serum samples of patients with CRS should be estimated where possible to support the clinical diagnosis. Although a four-fold rise in antibody level was not observed in any of the serial samples, a rising titre was detected in the sera of all immunocompetent patients which was higher in those patients with a clinical history of CRS. Antibody titres from patients with underlying immune disorders should be interpreted with care as negative results with the BHI-7 ELISA may not exclude CRS in this patient group. However, the real value of testing multiple sera by the BHI-7 ELISA was in the detection of antibody at an early stage of infection, prior to the isolation of microorganisms by standard culture methods, and before the clinical presentation of sepsis. Serological monitoring of patients from the day of catheter insertion may be a future approach to the management of patients with intravascular devices, however, further studies are required to support this hypothesis.

Chapter 6 Potential cross-reactions in the BHI-7 ELISA due to antiphospholipid antibody

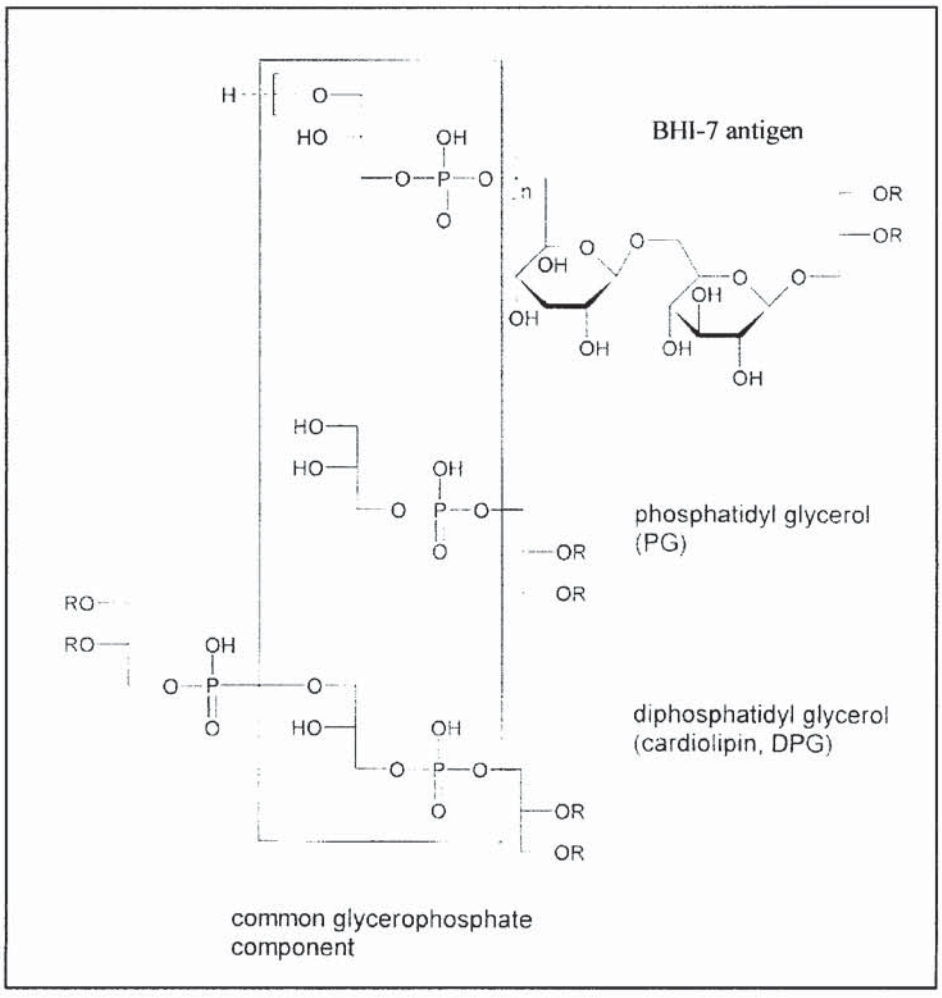
6.1 Introduction

Antibody-antigen reactions have a high level of specificity when the binding sites of antibodies are directed against determinants of a single antigen. However, if the determinants are shared between antigens, then a proportion of the antibodies directed towards one antigen will bind with others. This is termed cross-reactivity. In this chapter, the possibility of cross-reaction between the BHI-7 antigen (a short chain lipoteichoic acid, chapter 2) and the phospholipid, diphosphatidyl glycerol (cardiolipin, DPG) is investigated. Phosphatidyl glycerol (PG) and diphosphatidyl glycerol are constituent, negatively charged cell membrane lipids with similar molecular structures. PG is involved in the biosynthsis of lipoteichoic acid (LTA) which occurs through consecutive donation of glycerophosphate units from PG to diglucosyl diacylglycerol to form the glycerophosphate chain of LTA (Fischer, 1994). LTA (and presumably BHI-7 antigen), DPG and PG therefore share the common glycerophosphate moiety (figure 6.0) and thus have the potential for cross-reaction in immunological assays.

Cardiolipin is also found in the lipid membranes of many mammalian cell organelles, notably mitochondria and is widely distributed within tissues (Woolf, 1986). Anti-phospholipid antibodies are autoantibodies that react with most negatively charged phospholipids, including cardiolipin (Harris *et al.*, 1985). These antibodies may be found transiently in some infections such as leprosy, malaria, glandular fever, trypanosomiasis, mycoplasmal pneumonia and treponemal disorders (Woolf, 1986), or more persistently in autoimmune diseases such as systemic lupus erythematous (SLE). In addition, anticardiolipin antibodies have also been associated with a range of clinical conditions including endocarditis, thrombophlebitis, stroke, heart attack and autoimmune haemolytic anaemia (Buchanan *et al.*, 1989). Associated with cardiolipin is a phospholipid binding protein (β(2)glycoprotein I) that has been identified as a cofactor required for anticardiolipin antibody binding (Tincani *et al.*, 1998).

The aim of this part of the study was to determine the potential for cross- reactivity between the BHI-7 antigen incorporated in the novel ELISA and anticardiolipin antibody. The serum levels of anticardiolipin antibody in patients studied throughout this thesis were determined. Patient groups included those with catheter-related sepsis (CRS) (chapter 4), sciatica (chapter 10), endocarditis (chapter 8) and the control patients with a clinical history of heart disease who underwent coronary artery bypass grafting (CABG) (chapter 4). Serum samples with high and low levels of IgG to BHI-7 antigen previously determined in the above chapters, were selected for this investigation.

Figure 6.0 **The structures of BHI-7 antigen (n=6), phosphatidyl glycerol and diphosphadityl glycerol showing the common glycerophosphate in each structure. R=fatty acyl group**



6.2 Materials and methods

6.2.1 Estimation of anti-phospholipid antibodies in serum samples with the BINDAZYME™ anticardiolipin enzyme immunoassay

The assay was performed in accordance with the manufacturer's recommendations (The Binding Site Ltd, Birmingham, U.K.). One hundred microlitres of each assay calibrator, control and patient sample (diluted 1:100 in sample diluent) were dispensed into the assay wells coated with cardiolipin and its cofactor. The assay was incubated at room temperature for 30 min and then washed 3 times in assay wash buffer (diluted 1:20) with the ELP-40 plate washer. One hundred microlitres of antihuman IgG conjugate was added to all wells and the assay incubated at room temperature for 30 min, after which, the wash procedure was repeated. One hundred microlitres of TMB substrate was dispensed into each well and the assay incubated at room temperature for a further 10 min. The assay reaction was stopped by dispensing 100µl of stop solution (1 M sulphuric acid) into the wells and the optical density read at 450nm. Quantitative estimation of antibody level was made on samples having an optical density equal to or greater than the cut-off control. This was performed by reference to a calibration curve prepared with the assay calibrators.

6.2.2 Patient groups

Serum samples from 80 patients including those with CRS, sciatica, endocarditis and those who underwent CABG, were assayed for anticardiolipin IgG antibody. This incorporated patients with high and low levels of IgG as previously determined by the BHI-7 ELISA. The numbers of patients from each group were: 42 patients with a clinical diagnosis of sciatica (chapter 10), 20 patients with a diagnosis of endocarditis in accordance with the Duke criteria (chapter 8), 10 patients with CRS (chapter 4) and 7 patients who underwent CABG (chapter 4). The Spearman rank correlation test was used to correlate the levels of serum IgG determined with the BHI-7 ELISA and the bindazyme™ anticardiolipin ELISA.

6.3 Results

6.3.1 Cross-reactivity of IgG antibodies to the BHI-7 antigen (lipid S) and diphosphatidyl glycerol (cardiolipin)

The levels of serum IgG antibody to the BHI-7 antigen and cardiolipin in 42 patients with sciatica, 20 with endocarditis, 10 with CRS and 8 who underwent CABG with no evidence of sepsis, are shown in table 6.0. Elevated levels of anticardiolipin antibody were detected in 10 out of 80 (12.5%) patients, however there was no correlation between the levels of serum IgG antibody determined by the BHI-7 ELISA and levels of serum anticardiolipin antibody (Spearman rank; $r = 0.21$, $p = 0.14$). No correlation between anticardiolipin IgG levels and BHI-7 IgG titres was found, suggesting that BHI-7 IgG did not cross-react with cardiolipin.

Table 6.0 **Serum IgG levels to BHI-7 antigen and cardiolipin (CL) in patients with sciatica, endocarditis, catheter-related sepsis (CRS) and those who underwent coronary artery bypass graft (CABG)**

Patient No. & condition	BHI-7 IgG titre	CL GPL/Uml	Patient No. & condition	BHI-7 IgG titre	CL GPL/Uml
1 sciatica	47000	<6.25	**21 sciatica**	64300	<6.25
2 sciatica	42700	<6.25	**22 sciatica**	29500	<6.25
3 sciatica	63000	<6.25	**23 sciatica**	25300	<6.25
4 sciatica	40000	<6.25	**24 sciatica**	26900	<6.25
5 sciatica	38100	<6.25	**25 sciatica**	8000	8.45
6 sciatica	36300	<6.25	**26 sciatica**	12000	<6.25
7 sciatica	60400	12.5	**27 sciatica**	11200	<6.25
8 sciatica	46600	<6.25	**28 sciatica**	11500	<6.25
9 sciatica	70000	<6.25	**29 sciatica**	7200	<6.25
10 sciatica	34600	<6.25	**30 sciatica**	6100	<6.25
11 sciatica	51200	<6.25	**31 sciatica**	15700	<6.25
12 sciatica	48600	<6.25	**32 sciatica**	11000	<6.25
13 sciatica	123400	6.25	**33 sciatica**	11000	<6.25
14 sciatica	35000	<6.25	**34 sciatica**	3000	<6.25
15 sciatica	73500	<6.25	**35 sciatica**	16500	<6.25
16 sciatica	25000	<6.25	**36 sciatica**	12800	<6.25
17 sciatica	23000	<6.25	**37 sciatica**	12800	<6.25
18 sciatica	29500	<6.25	**38 sciatica**	12800	<6.25
19 sciatica	31500	<6.25	**39 sciatica**	15400	<6.25
20 sciatica	51200	<6.25	**40 sciatica**	4300	<6.25

Key to table 6.0:

Cut-off titre for BHI-7 IgG positivity: ≥ 20000 (chapter 4)

Normal serum level of anticardiolipin IgG: <11 GPL U/ml

Table 6.0 continued **Serum IgG levels to BHI-7 antigen and cardiolipin (CL) in patients with sciatica, endocarditis, catheter-related sepsis (CRS) and those who underwent coronary artery bypass graft (CABG)**

Patient No. & condition	BHI-7 IgG titre	CL GPL/Uml	Patient No. & condition	BHI-7 IgG titre	CL GPL/Uml
41 sciatica	23700	<6.25	**61 endocarditis**	38000	<6.25
42 sciatica	25600	<6.25	**62 endocarditis**	205000	<6.25
43 endocarditis	22000	57	**63 CRS**	409000	25
44 endocarditis	6000	<6.25	**64 CRS**	100000	<6.25
45 endocarditis	30000	<6.25	**65 CRS**	51000	<6.25
46 endocarditis	19000	<6.25	**66 CRS**	15000	<6.25
47 endocarditis	10000	<6.25	**67 CRS**	46000	<6.25
48 endocarditis	25000	<6.25	**68 CRS**	6000	<6.25
49 endocarditis	25000	<6.25	**69 CRS**	15000	21
50 endocarditis	26000	<6.25	**70 CRS**	51000	<6.25
51 endocarditis	22000	<6.25	**71 CRS**	10000	<6.25
52 endocarditis	26000	<6.25	**72 CRS**	15000	<6.25
53 endocarditis	270000	<6.25	**73 CABG**	9000	<6.25
54 endocarditis	149000	12.5	**74 CABG**	44000	<6.25
55 endocarditis	157000	<6.25	**75 CABG**	37000	14
56 endocarditis	80000	<6.25	**76 CABG**	9600	<6.25
57 endocarditis	77000	<6.25	**77 CABG**	12000	<6.25
58 endocarditis	51000	<6.25	**78 CABG**	50000	6.6
59 endocarditis	410000	<6.25	**79 CABG**	1000	<6.25
60 endocarditis	256000	8.45	**80 CABG**	35000	<6.25

Key to table 6.0:

Cut-off titre for BHI-7 IgG positivity: ≥ 20000 (chapter 4)

Normal serum level of anticardiolipin IgG: <11 GPL U/ml

6.4 Discussion

The possibility of cross-reactions between IgG antibodies produced against the BHI-7 antigen and diphosphatidyl glycerol (cardiolipin) was investigated for several reasons. The antigenic material on which the novel BHI-7 ELISA is based, is prepared from the culture supernatants of 7 strains of coagulase negative staphylococci which are grown in brain heart infusion (BHI) broth (Oxoid, Basingstoke U.K.) (chapter 2). BHI broth is a chemically undefined culture medium which is rich in cardiolipin, containing 1.25 %w/v calf brain infusion and 0.5% w/v beef heart infusion, and whilst cardiolipin is widely distributed throughout mammalian tissue, a common source is extract of beef heart (Woolf, 1986). Some contamination of the BHI-7 antigen preparation due to components of the BHI broth cannot be entirely excluded, therefore the possibility of cross-reactivity between serum antibodies to BHI-7 antigen and cardiolipin had to be determined. In addition, anticardiolipin antibodies are frequently present in the serum of patients with SLE (Harris *et al.*, 1985), malaria, glandular fever, mycoplasmal pneumonia, treponemal infections (Woolf, 1986), heart disease (Buchanan *et al.*, 1989) and also healthy individuals (Fields *et al.*, 1989). The potential for cross-reaction of anticardiolipin antibodies with BHI-7 antigen, a structurally similar molecule, in patients without Gram-positive sepsis was therefore possible. Moreover, the potential of the BHI-7 ELISA for the serodiagnosis of deep-seated Gram-positive sepsis was evaluated on patients with heart disease including those with endocarditis (chapter 8) and those who underwent CABG (chapter 4).

Previous work with animal models has demonstrated that immunisation with LTA induced anticardiolipin antibody production, which suggests that human infection with Gram-positive microorganisms may stimulate the synthesis of these antibodies, resulting in elevated serum levels (Gotoh and Matsuda, 1996). However, the results of this current study demonstrated elevated levels of anticardiolipin antibody in only 10 out of 80 (12.5%) patients studied. Moreover, there was no correlation between elevated levels of anti-BHI-7 IgG and increased levels of anticardiolipin. This suggests that anti-BHI-7 IgG did not cross-

react with cardiolipin, and that the short chain LTA antigen incorporated in the BHI-7 ELISA is immunologically distinct from cardiolipin.

Chapter 7 Effect of GullSORB™ on serum IgM levels in patients with catheter-related sepsis due to staphylococci

7.1 Introduction

The detection of serum IgM is a standard serological procedure for the early diagnosis of many infectious diseases (Schaefer *et al.*, 1988). However, testing for IgM in patients' sera can be compromised by the presence of IgG antibody. If IgG is present in the serum, it may result in either decreased or increased IgM titres being detected and should therefore be removed prior to testing (Doerr *et al.*, 1987). There are two ways in which immune IgG may interfere with IgM assays; it may compete with specific IgM for antigen binding sites resulting in falsely low IgM assay results, or it may form immune complexes with rheumatoid factor which may be of IgG or IgM class. The Fab portion of the rheumatoid factor binds to the Fc portion of IgG (Woolf, 1986) and forms the complex which becomes a reactive site for the μ-chain specific conjugate, resulting in falsely high IgM results (Smith *et al.*, 1987). Rheumatoid factor forms the complexes within the synovium and synovial fluid in patients with rheumatoid arthritis and it is also frequently produced in the acute phase of many viral infections and will therefore interfere with serum IgM estimations in many assays (Chandler *et al.*, 1990).

Several methods have been developed for the separation of IgG and IgM antibodies in serum. The most commonly used methods have depended on the fractionation of serum, usually by sucrose density gradient centrifugation or gel filtration, however these methods are labour intensive (Morgan-Capner *et al.*, 1990). More recent methods have incorporated the use of anti-human IgG preparations and recombinant protein G for the removal of IgG when estimating serum IgM levels (Martins *et al.*, 1995). In addition, the problems associated with rheumatoid factor in serum samples can be minimised by pre-treatment of sera with aggregated IgG (Morgan-Capner *et al.*, 1990). The preparation of conjugated antibodies with the Fc portion removed prior to conjugation has also been described, thus removing the binding site for rheumatoid factor (Chandler *et al.*, 1990).

Interference in IgM assays due serum IgG can also be eliminated by the incorporation of GullSORB™ reagent into the test procedure. GullSORB™ reagent is a commercially available product, which removes serum IgG through an immunoprecipitation reaction with antihuman IgG antibodies (GullSORB™ product information, 1993).

Earlier work in this thesis (chapter 4) indicated that the BHI-7 IgM ELISA had low sensitivity and specificity for the serodiagnosis of CRS. Similarly, previous studies of the serological response to staphylococcal α-haemolysin and cell wall teichoic acid have also shown the estimation of serum IgM to be of limited diagnostic value, and have concentrated on the IgG titre estimations (Julander *et al.*, 1983). The aim of this part of the study was to attempt to improve the sensitivity and specificity of the BHI-7 IgM ELISA for the serodiagnosis of catheter–related sepsis (CRS) due to Gram-positive microorganisms, by the incorporation of GullSORB™ reagent into the procedure.

7.2 Materials and Methods

The IgM titres of 50 patients with CRS and 50 controls (chapter 4) were re-estimated with the BHI-7 ELISA (section 3.2.5), and the levels determined by reference to the standard positive control IgM titre of 1:25,000 (chapter 3). All samples were tested in duplicate. An initial dilution of the serum samples was also made by the addition of 5µl of each sample to 50µl of GullSORB™ reagent (Gull Laboratories, Salt Lake City, Utah, USA). The sera were then further diluted to 1:400 by the addition of 1.95 ml of TBS-Tween buffer. The IgM titres in the serum samples after reaction with GullSORB™ reagent were estimated with the BHI-7 ELISA. All samples were tested in duplicate. A statistical analysis of the data was made using the Wilcoxon signed rank test. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the BHI-7 IgM ELISA with and without the incorporation of GullSORB™ reagent were determined.

7.3 Results

The serum IgM levels of patients with CRS and controls, determined with and without the addition of GullSORB™ reagent, are shown in tables 7.0 and 7.1 respectively. The statistical analysis of the data is shown in table 7.2. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the BHI-7 IgM ELISA, with and without the incorporation of GullSORB™ reagent are shown in tables 7.2 and 7.3.

Table 7.0 Serum IgM levels to BHI-7 antigen in control patients before and after the addition of GullSORB™ reagent

Pt	IgM titre - GullSORB™	IgM titre + GullSORB™	Pt	IgM titre - GullSORB™	IgM titre + GullSORB™
1	13900	18300	26	3400	200
2	3700	1100	27	12600	16500
3	5100	2700	28	1900	400
4	1400	400	29	4500	1600
5	1900	500	30	40300	54200
6	3100	800	31	900	400
7	2100	400	32	30400	32500
8	3200	1200	33	6700	4100
9	1700	600	34	13000	9900
10	1400	700	35	5500	3000
11	1800	800	36	3600	900
12	3000	1600	37	3800	2500
13	5300	2200	38	19700	23100
14	2600	2400	39	23600	36200
15	1500	700	40	12000	15500
16	1200	400	41	14400	21100
17	11000	5700	42	3000	1600
18	4500	2500	43	3000	400
19	55000	3000	44	6700	4200
20	9000	6000	45	12000	7800
21	12100	17500	46	3500	1400
22	5400	5100	47	5100	2100
23	3600	1600	48	2300	700
24	700	400	49	1400	400
25	12300	5100	50	600	400

Table 7.1 Serum IgM levels to BHI-7 antigen in patients with CRS (n=50) before and after the addition of GullSORB™ reagent

Pt	IgM titre - GullSORB™	IgM titre + GullSORB™	Pt	IgM titre - GullSORB™	IgM titre + GullSORB™
1	2600	1900	26	17600	10600
2	2200	1500	27	15200	16400
3	25000	22200	28	15700	26000
4	4100	900	29	4800	4600
5	5500	6200	30	1000	400
6	8400	10400	31	2600	800
7	2100	1000	32	4200	3600
8	8200	8000	33	103800	71500
9	18800	18200	34	7800	6100
10	32700	32200	35	96200	100800
11	12500	8500	36	29200	20700
12	14300	10800	37	51900	30900
13	28700	22700	38	21000	17800
14	50000	46600	39	3000	2100
15	6700	5800	40	6400	4800
16	11700	9600	41	12000	12200
17	12400	9900	42	3700	3100
18	81200	65500	43	11900	13800
19	15200	10300	44	5400	3600
20	8100	5500	45	2700	1600
21	7400	2900	46	6400	4900
22	2000	700	47	17700	12500
23	6200	3900	48	11600	8300
24	10300	7000	49	1400	400
25	1900	800	50	385200	409600

Table 7.2 Statistical analysis of serum IgM levels in patients with CRS and controls. Serum IgM levels were estimated with and without the addition of GullSORB™ reagent

	IgM titre (controls)	IgM titre + GullSORB™ (controls)	IgM titre (CRS)	IgM titre + GullSORB™ (CRS)
number	50	50	50	50
Mean titre	8008	6424	24332	21994
Range of titres	600-55000	400-54200	1400-385200	400-409600
Standard deviation	10364	10842	56772	59248

Pre-treatment of the serum samples with GullSORB™ reagent significantly reduced the detectable levels of serum IgM in both groups of patients (Wilcoxon test: controls $p = 0.0056$; patients with CRS $p = <0.0001$). Decreased serum levels of IgM were observed in 82% of the control group and 86% of patients with CRS.

Table 7.3 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the BHI-7 IgM ELISA without GullSORB™ reagent

Titre cut-off	True Positive	False Negative	True Negative	False Positive	Sens %	Spec %	PPV %	NPV %	Accuracy %
5,000	36	14	28	12	72	56	75	67	64
6,000	34	16	33	17	68	66	67	67	67
7,000	30	20	35	15	60	70	67	64	65
8,000	28	22	35	15	56	70	65	61	63
9,000	25	25	35	15	50	70	63	61	60
10,000	25	25	36	17	50	72	64	59	61

Table 7.4 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the BHI-7 IgM ELISA with GullSORB™ reagent

Titre cut-off	True Positive	False Negative	True Negative	False Positive	Sens %	Spec %	PPV %	NPV %	Accuracy %
5,000	32	18	35	15	64	70	75	67	67
6,000	29	21	39	11	58	78	73	65	68
7,000	27	23	39	11	54	78	71	63	66
8,000	26	24	40	10	52	80	72	63	66
9,000	22	27	40	10	44	80	69	60	62
10,000	21	29	41	9	42	82	70	59	62

Key to tables 7.3 and 7.4:

True positive = number of patients with CRS with a titre ≥ cut-off titre

False negative = number of patients with CRS with a titre ≤ cut-off titre

True negative = number of control patients with a titre ≤ cut-off titre

False positive = number of control patients with a titre ≥ cut-off titre

Treatment of serum samples with GullSORB™ reagent decreased the sensitivity of the BHI-7 IgM ELISA at the given cut-off titres. However, an increased assay specificity, positive predictive value and accuracy was achieved.

7.4 Discussion

The detection of elevated levels of serum IgM is important in the early diagnosis of infectious disease. However, previous studies have shown that the detection of IgM antibody in the serum of patients with staphylococcal disease to be of limited diagnostic value (Julander *et al.*, 1983; West *et al.*, 1985). Similarly, earlier work in this thesis indicated that the BHI-7 IgM ELISA lacked sensitivity and specificity, and its application as a serodiagnostic assay was limited (chapter 4). However, the time of serum collection in relation to date of onset of the CRS, and the levels of competing immune IgG and rheumatoid factor in the serum samples, may have affected the levels of serum IgM detected in the BHI-7 ELISA.

The inclusion of GullSORB™ reagent into the BHI-7 ELISA methodology significantly reduced the detectable levels of serum IgM. Surprisingly, 82% of the controls and 86% of the patients with CRS had decreased IgM titres following addition of GullSORB™ reagent. Many of the patients with CRS due to staphylococci had high levels of serum IgG (chapter 4), and its removal with GullSORB™ reagent should have resulted in elevated levels of serum IgM detected with the BHI-7 ELISA. However, technical difficulties in the interpolation of antibody titres from the lower part of a sigmoidal titration curve (chapter 3) may have accounted for some of the observed decrease in IgM levels, and this again reflected the need for an optimised ELISA system and methodology. In addition, the presence of rheumatoid factors of the IgM class in the serum of some patients may have accounted for the increased levels of serum IgM prior to treatment with GullSORB™ reagent. Indeed, approximately one third of the control patients recruited into the study had a clinical history of joint disease, which may have involved autoimmune antibodies. Rheumatoid factors are antibodies directed against antigenic sites on heavy chain determinants, located in the Fc portion of immunoglobulin. Whilst some of these antigenic sites are detected only in certain groups of individuals, others are present in all sera but may be restricted to subclasses of IgG. Additionally, rheumatoid factors may occur in the serum of individuals without any demonstrable disease (Maini, 1977).

The inclusion of GullSORB™ reagent into the BHI-7 IgM ELISA methodology resulted in an assay with decreased sensitivity but increased specificity, positive predictive value and accuracy and should therefore be incorporated when estimating levels of serum IgM. In summary, it is evident from the lack of assay sensitivity with or without the incorporation of GullSORB™ reagent, that further detailed studies are required to investigate the clinical history of CRS and colonisation in relation to the IgM response. Also, the decreased assay sensitivity following the use of GullSORB™ reagent may have reflected false positivity in untreated serum samples due to rheumatoid factor, especially those patients with a history of joint disease. Moreover, the technical difficulties related to the estimation of antibody levels from sigmoidal titration curves again highlighted the need for an optimised assay system. Due to the discouraging results achieved with the BHI-7 IgM ELISA to date, future work in this thesis will concentrate on the development and optimisation of an ELISA system for the detection of IgG antibodies in deep-seated Gram-positive infection (chapter 8).

Chapter 8 A rapid single absorbance ELISA for the detection of serum IgG to lipid S in patients with deep-seated Gram-positive sepsis

8.1 Introduction

The serological studies on patients with catheter-related sepsis (CRS) presented in the previous chapters, were performed using a prototype BHI-7 ELISA method as described by Lambert *et al.* (1996). The results of the earlier work showed that patients with CRS had elevated levels of antibody to lipid S and that the assay had potential for the serodiagnosis of deep-seated Gram-positive coccal infection. The indirect ELISA was found to have a sensitivity of 70% and specificity of 90% in the serodiagnosis of CRS (chapter 4). The lipid S ELISA had also been used in preliminary studies to examine antibody levels in the serum samples of patients with infected orthopaedic prosthesis due to Gram-positive organisms (Rafiq *et al.*, 1999) and endocarditis (Connaughton *et al.*, 1997) with significant differences being found between control and septic groups. The ELISA used in these preliminary investigations was based on a method whereby patients' sera were titrated and compared to a reference positive serum sample. The assay procedure, in this prototype form, took 24 hours to perform and required software analysis, it therefore served mainly as a research tool.

Optimisation of an ELISA system includes the determination of the optimum concentrations of the reactants in the assay, which are related to the incubation temperature and time. By adjusting the concentration of reactants, incubation times and temperatures, assays may be designed to fit into the routine laboratory timetable. Increasing the reaction temperature or reagent concentration, for example, can result in a quicker assay equilibrium being reached (Wood and Wreghitt, 1990). The assay conditions are based either on the use of optimum conditions, suitability to the laboratory routine or a compromise between the two (Wood and Wreghitt, 1990). The need to test serial dilutions of patients' sera, as in the prototype

BHI-7 ELISA, renders an assay unsuitable for busy routine clinical laboratories and it is not cost effective. Therefore, a method in which an absorbance value for a single dilution of patient serum is converted into specific antibody units is preferable (Cozon *et al.*, 1998).

Quality control, end point determination and assay reproducibility / robustness issues concerning the prototype titration ELISA, were investigated in chapter 3. It was determined that the comparison of test results to a standardised reference serum was the most satisfactory method of controlling the assay. However, the assay was shown to have an intratest COV of 25% and intertest COV of 24%, which indicated that the assay was operating under sub-optimal conditions. Also, the prototype ELISA would not easily fit into routine laboratory practice, as the assay turnaround time was approximately 24 hours. A quality assurance system of a high standard is an essential part of good laboratory practice. With systems such as ELISA, there are many stages where inaccuracies may arise such as pipetting, variations in temperature, change of reagent batch and hence these assays require the use of defined quality control procedures including standardisation and optimisation of reagents (Balfour and Harford, 1990). Internal quality control provides information on the daily reproducibility and precision of the assay and whether the results achieved fall within acceptable limits. The aims of this part of the study were to optimise the conditions of the assay and to investigate its potential as a rapid serodiagnostic test for the diagnosis of deep seated Gram-positive infection.

8.2 Methods and Materials

In the following experiments, the ELISA is described in 3 stages:

Stage 1= Antigen : **Antibody**

Stage 2= Antigen : Antibody : **Conjugate**

Stage 3= Antigen : Antibody : Congugate : **Substrate**

The part of the ELISA under investigation in each stage is highlighted above.

8.2.1 The determination of the optimal time and temperature for IgG antibodies to bind to lipid S antigen (ELISA stage 1)

A panel of 8 serum samples was selected to include a broad range of IgG titres, which were determined in an earlier study (chapter 4). Six of the samples were selected from patients with CRS and controls, whilst the remaining 2 samples were the reference sera; the standardised positive control (SPC) and the standardised negative control (SNC) (normal human serum used in previous chapters of this thesis). The range of serum titres included is shown in table 8.0

Table 8.0 Optimisation study serum panel incorporating a range of IgG titres determined by the prototype ELISA method

Patient	IgG titre
1	400,000
2 (SPC)	100,000
3	50,0000
4	20,000
5	15,000
6 (SNC)	10,000
7	5,000
8	2,000

All serum samples were diluted 1:6,400. This was performed by initially diluting 5µl of thawed sample into 2ml of TBS-Tween buffer, of which 1.6ml was further diluted into 24ml of TBS-Tween buffer. A serum dilution of 1:6,400 was chosen at it resulted in the greatest differentiation between absorbancies obtained for positive and negative samples (P:N ratio) (Wood and Wreghitt, 1990).

One hundred microlitres of each of the 8 diluted samples were pipetted into microtitre trays containing antigen at a concentration of 0.5µg per well. This was repeated 10 times. The microtitre plates had been stored with the antigen bound to the solid phase at –20°C for 1 week prior to use. The plates were incubated at 4°C or 37°C for 1, 2, 3 and 4 hour periods. These time periods were chosen to reflect those achievable in a routine laboratory. The remainder of the assay was the same as described in section 3.2.5. The mean absorbance for each sample was calculated and plotted against time. The optimal time and temperature for stage 1 were determined.

8.2.2 The determination of the optimal time and temperature for protein-A peroxidase conjugation (ELISA stage 2)

The serum panel was diluted to 1:6,400 and 100µl was pipetted into wells of an ELISA plate containing lipid S antigen. This was repeated 10 times for each sample. The plates were incubated at 37°C for 3 hours to allow for antibody to bind to antigen. The plates were then washed and 100µl of the protein –A peroxidase conjugate diluted to 25µg/ml was added to all wells. The plates were incubated at 4°C or 37°C for 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 hours. The remainder of the ELISA was performed as described in section 3.2.5. The mean absorbancies were calculated and plotted against time. The optimal time and temperature for stage 2 were determined.

8.2.3 The determination of the optimal time and temperature for the substrate reaction (ELISA stage 3)

The panel of sera was diluted to 1:6,400. One hundred microlitres of each sample was pipetted into a lipid S ELISA plate and incubated for 3 hours at 37°C. This was repeated 10 times for each sample. The plate was washed and 100µl of conjugate was added to all wells and incubated at 37°C for 2 hours. The plate was washed, 100µl of substrate was added to each reaction well and incubated at room temperature and at 37°C for 5, 10, 15, 20, 25 and 30 minutes. The remainder of the ELISA was performed as described in 3.2.5. The mean absorbancies were determined and plotted against time. The optimal time and temperature for stage 3 was determined as the point where equilibrium was reached.

8.2.4 The determination of the optimal lipid S concentration

One ml of lipid S antigen was diluted in bicarbonate/carbonate buffer (pH 9.6) to 1:100 concentration. A series of dilutions of the antigen were then prepared at 1:200, 1:400, 1:600, 1:800 and 1:1000. Microtitre trays were then coated using the various concentrations of antigen by the method described in section 3.2.3. The trays were coated so that each row of the tray represented increasing dilutions of the antigen. A condensed panel of 4 sera was selected from that shown in table 8.0, these were: pt 2 (SPC), pt 4, pt 6 (SNC) and pt 8. The sera were diluted 1:6,400 and 100µl added to each antigen concentration. This was repeated 5 times. The last row of the microtitre tray was used as a blank and contained no serum. The microtitre trays were incubated in the conditions found to be most suitable to allow for equilibrium to be reached in all stages of the ELISA.

Stage 1- antibody + antigen	37°C / 3 hours, wash
Stage 2- antibody + conjugate	37°C / 2 hours, wash
Stage 3- conjugate + substrate	37°C / 25 minutes.

The ELISA reaction was stopped using 1M sulphuric acid and the absorbancies read at 450nm. The mean absorbance for each sample was determined and plotted against antigen concentration.

8.2.5 The optimal conditions for lipid S ELISA stage 1 (antigen 1:400)

The panel of sera described in 8.2.4 was then used to determine the optimal time for the equilibrium of antibody and antigen to be reached at 37°C. Each sample was diluted 1:6,400 and 100µl pipetted into microtitre trays containing freshly prepared lipid S antigen at a concentration of 1:400. This was repeated 10 times. The plates were incubated at 37°C for 1, 2, 3 and 4 hours. The remainder of the assay was as described in section 3.2.5. The mean absorbance for each serum sample was calculated and plotted against time.

8.2.6 The optimal conditions for lipid S ELISA stage 2 (antigen 1:400)

The panel was diluted 1:6,400 and pipetted into micotitre trays. The plates were incubated at 37°C for 2 hours and then washed. Protein-A peroxidase conjugate was prepared (section 3.2.1.4) and 100µl added to all reaction wells including the blank row. The plates were incubated at 37°C for 0.5, 1, 1.25, 1.5, 1.75 and 2 hours. The remainder of the experiment was as described in 3.2.5. The mean absorbancies of the serum panel were calculated and plotted against time.

8.2.7 The optimal conditions for lipid S ELISA stage 3 (antigen 1:400)

The serum panel was diluted 1:6,400 and 100µl pipetted into microtitre trays coated with lipid S. The plates were incubated at 37°C for 2 hours and then washed. Protein-A peroxidse conjugate was prepared (section 3.2.1.4) and 100µl added to all reaction wells

including the blank row. The plates were then incubated at 37°C for 30 minutes. The microtitre trays were washed and the substrate prepared as described in 3.2.1.5. One hundred microlitres of substrate was added to all reaction wells and incubated at 37°C for 5, 10, 15, 20, 25 and 30 minutes. The reactions were stopped with 1M sulphuric acid. The mean absorbance for each serum sample in the panel was determined and plotted against time and the optimal time for stage 3 of the ELISA was then assessed.

8.2.8 A comparison of conjugates: protein-A peroxidase and anti-human IgG peroxidase

An alternative conjugate for detecting bound IgG is specific anti-human IgG peroxidase. The performance of this conjugate was compared with protein-A peroxidase. One ml of anti-human IgG peroxidase conjugate was diluted in 4ml of TBS-Tween, which was then stored at -20°C in 50µl aliquots. When required, 50µl was thawed and diluted in 50ml of TBS-Tween giving a working conjugate dilution of 1:5,000 (manufacturer's recommendation). The optimal time necessary for binding of the anti-human IgG peroxidase conjugate to antibody at 37°C was investigated. The method used was the same as described in section 8.2.6.

8.2.9 Statistical analysis on a control panel of sera – a measure of test reproducibility

A panel of control sera was selected from the patient groups studied in previous chapters, with the aim of providing the lipid S ELISA with a complete set of standards for internal quality control purposes. The chosen panel included "low" and "moderate" absorbance positives as determined by the prototype BHI-7 ELISA, the SPC (high positive) and SNC (negative).

The controls were stored at - 20°C and thawed each morning prior to use. All reagents were allowed to stand at room temperature for a minimum of 3 hours before use. Each sample was diluted 1:6,400 by adding 5µl of sera to 2ml of TBS-Tween which was then further diluted by taking 160µl into 2.4ml of TBS-Tween. This provided a sufficient volume of diluted sample to allow for each control to be tested repeatedly on one microtitre plate.

Column 1 of the microtitre tray was used as a blank and 100µl of each control was dispensed 22 times across 2 rows of the tray. The optimal conditions for the lipid S ELISA, determined in the previous sections of this chapter, were used and are shown below:

Stage 1- antibody + antigen	37°C for 2 hours
Stage 2- antibody + antihuman IgG conjugate	37°C for 1 hour
Stage 3 – conjugate + in house TMB substrate	37°C for 25 minutes

The ELISA was stopped using 1M sulphuric acid and the absorbancies determined at 450 nm. This procedure was performed on 5 consecutive days over a working week. The means, standard deviations, ranges and upper and lower 95% confidence intervals were assessed for each of the controls.

8.2.10 Effect of sample volume in the lipid S ELISA

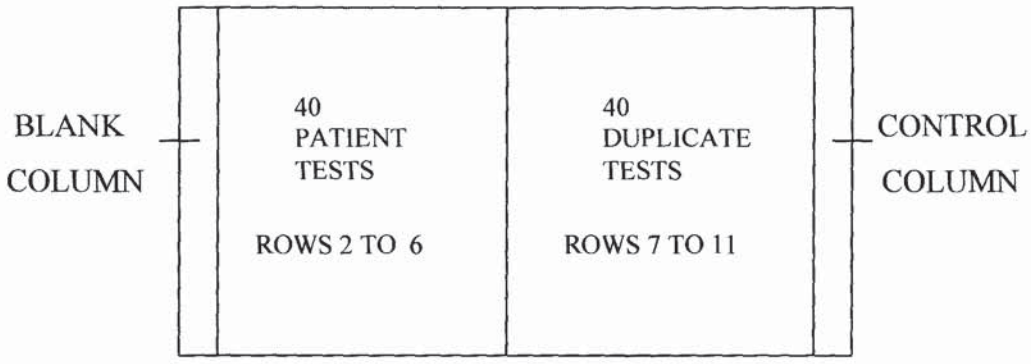
In the previous chapters, 5µl of serum had been used to prepare the initial dilution for sample titration. Many of the ELISA used in routine clinical laboratories use sample volumes of 10µl or above to prepare working dilutions, therefore a comparison of 5µl and 10µl volumes to prepare the dilution for the single absorbance method was made. The ELISA internal standards described in 8.2.9 were used throughout this study. Five microlitres of each control was added to 2ml of TBS-Tween from which 160µl was removed and added to a further 2.4ml of TBS-Tween to give a final dilution of 1:6,400. Ten microlitres of each control was added to 2ml of TBS-Tween from which 100µl was removed

and added to a further 3.1ml of TBS-Tween to give a final dilution of 1:6,400. Each sample was tested 22 times across 2 rows of the ELISA plate and the study was repeated on 3 consecutive working days. The lipid S ELISA was performed using the optimised parameters. The effectiveness of the pairing of the 2 sets of absorbancies was determined by the calculation of the Spearman linear correlation coefficient and the p value testing the null hypothesis.

8.2.11 Estimation of anti-lipid S IgG in patients with deep-seated Gram-positive sepsis

Forty patients with CRS and 40 control patients (chapter 4) were used to evaluate the optimised lipid S ELISA parameters, and variations of the conjugate and substrate. A number of 40 was chosen since each sample in the group could be tested in duplicate on the same plate whilst allowing 1 column for blank testing and 1 column for controls (fig 8.0). The 40 patients with CRS were selected to exclude patients with immunodefficiency. Following the evaluation of the assay using patients with CRS, 40 samples of sera were then tested from patients over the age of 18 years presenting with endocarditis. Patients were recruited into the study when identified as definite cases of endocarditis according to the Duke criteria (Hoen *et al.*, 1996). All of the patients had positive blood cultures for either staphylococci (68%) or streptococci, including enterococci (32%).

Fig 8.0 Diagram showing the layout of the lipid S ELISA plate



The samples and controls were diluted to 1:6,400 in TBS-Tween. All of the reagents were allowed to reach room temperature prior to use. One hundred microlitres of the diluted samples and controls were pipetted into the microtitre plate coated with lipid S antigen at a concentration of 1:400. The plates were then incubated under the conditions shown in table 8.1 using anti-human IgG peroxidase conjugate and protein-A peroxidase conjugate (prepared to the manufacturer's recommended concentration). The plates were washed between each incubation stage and absorbancies read at 450 nm. The mean absorbancies (A) of the samples from patients with sepsis, the control group and the SPC / SNC were determined. The COV of the SPC and SNC was also determined. The titre of each sample was then evaluated using the following formulae (Balfour and Harford, 1990).

$$\frac{A_{\text{sample}} - A_{\text{neg control}}}{A_{\text{pos control}} - A_{\text{neg control}}} \quad \text{X } 100{,}000 \text{ EIU}$$

The sensitivity and specificity of the optimised lipid S ELISA for the serodiagnosis of CRS and endocarditis was evaluated. A statistical comparison of the results using both the anti-human IgG peroxidase and protein-A peroxidase conjugates was made using the Chi-square test.

Table 8.1 The optimal working parameters for the lipid S ELISA using anti-human IgG and protein-A peroxidase conjugates

ELISA stage	Anti-human IgG peroxidase conjugate	Protein-A peroxidase conjugate
Stage 1 (antibody)	2 hours / 37°C	2 hours / 37°C
Stage 2 (conjugate)	1 hour / 37°C	30 minutes / 37°C
Stage 3 (substrate)	25 minutes / 37°C	25 minutes / 37°C

8.2.12 Comparison of laboratory-prepared substrate with commercial TMB substrate

TMB Microwell substrate containing 3,3', 5,5'-tetramethylbenzidine (TMB) and 0.01% H_20_2 in acidic buffer was obtained (Kirkegard & Perry Laboratories, Guildford). The optimal conditions for the commercial substrate were determined using the method described in 8.2.7. The IgG levels in the 40 patients with CRS and 40 controls were re-evaluated using commercial TMB substrate and the sensitivity, specificity, PPV, NPV and accuracy of the assay determined.

8.2.13 Correlation of IgG levels in patients with CRS and controls obtained using the prototype 24 hour ELISA and the rapid 4 hour single absorbance ELISA.

The antibody levels achieved from the 40 patients with CRS and 40 controls using the prototype 24-hour ELISA (chapter 4), and the optimised 4-hour rapid ELISA were compared in a scatter plot. The most accurate cut-off values of 1:14,000 and 1:20,000, determined in earlier work, were set to the prototype titration method and the Spearman correlation coefficient was calculated.

8.2.14 Stability of the lipid S antigen

The stability of the lipid S antigen when stored bound to microtitre plates at -20°C was monitored over a 11 month period from March 1999 to January 2000. The antigen stability was monitored by documenting the absorbancies of the standard high, low and negative controls. The moderate positive control used was a new sample as repeated use had depleted the volume of the original positive control sample. The same controls were used throughout the study. Microtitre plates bound with fresh lipid S antigen were prepared and

used in April and November to determine whether any variation in control absorbance was due to deterioration of the controls. The control absorbancies documented over the study period were also used to determine 95% confidence limits (2 x SD of the calculated mean) for each control which could then be used to produce a Shewart plot as part of the routine assay quality assurance process (Balfour and Harford, 1990). The determination of the assay control limits therefore included absorbancies obtained from ELISA plates of varying age.

8.3 Results

8.3.1 Optimal conditions for ELISA stage 1

The effects of time and temperature on stage 1 of the lipid S ELISA are shown in figures 8.1 and 8.2. The time and temperature chosen to allow for equilibrium to be reached in the selected panel was 3 hours at 37°C. Sera with titres ranging from 1:2,000 – 1:50,000 had reached complete equilibrium by 3 hours. The remaining 2 sera with higher titres of 1:100,000 and 1:400,000 had begun to reach equilibrium at 3 hours, however, small increases of absorbance in the 2 samples were observed between 3 and 4 hours when an antigen concentration of 1:100 was used to coat the microtitre trays.

At 4°C none of the selected panel had reached full equilibrium at 3 hours and small increases in all sample absorbancies were noted between 3 and 4 hours. The absorbance of sample 3 was increased by incubation at 4°C compared with that at 37°C.

Figure 8.1 Effect of serum incubation time upon absorbance at 37°C. Patient numbers refer to table 8.0. Antigen: 1:100 dilution; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB

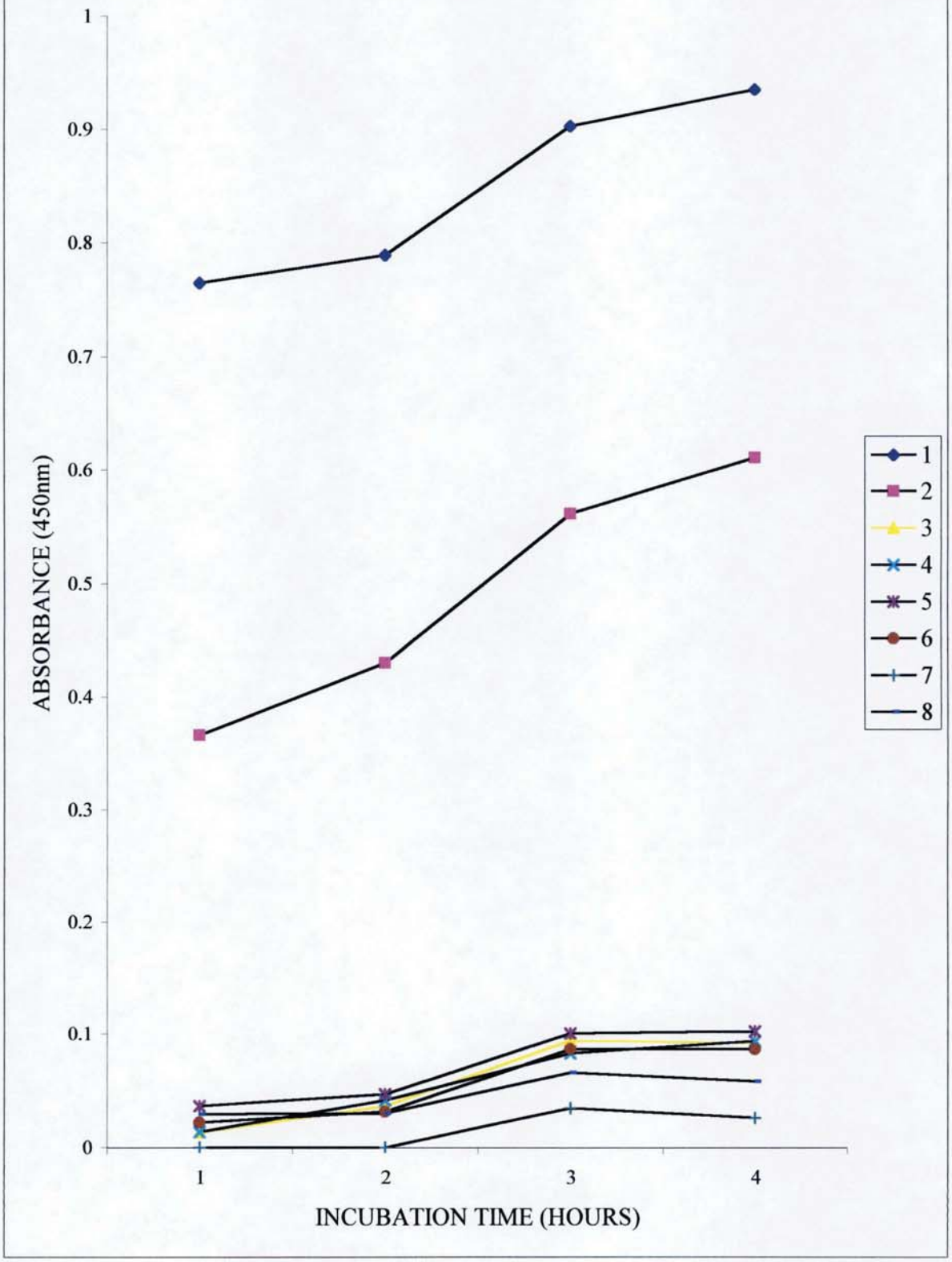
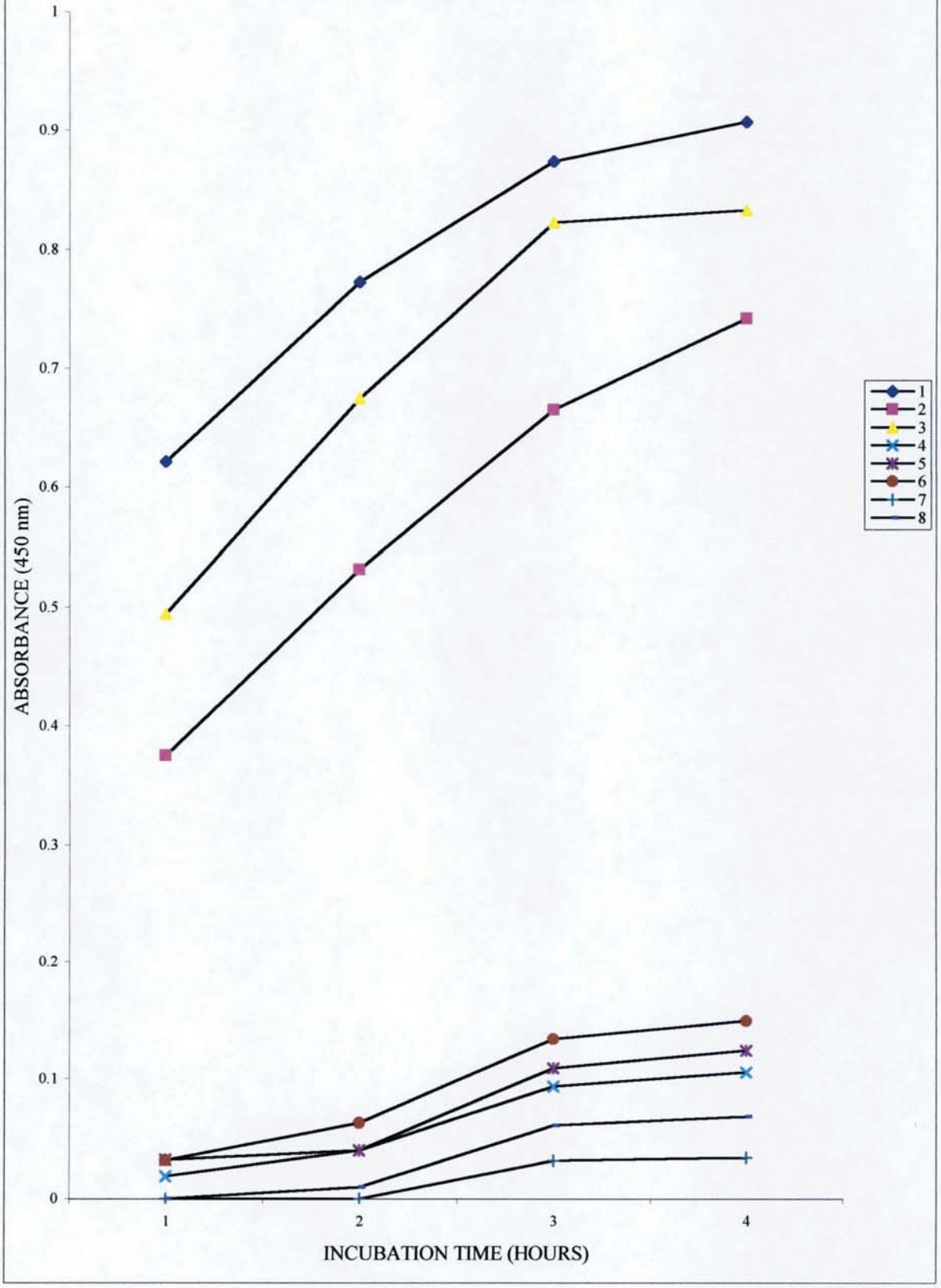


Figure 8.2 Effect of serum incubation time upon absorbance at 4°C. Patient numbers refer to table 8.0. Antigen: 1:100 dilution; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB



8.3.2 Optimal conditions for ELISA stage 2

The effects of time and temperature on the conjugate binding to antibody are shown in figures 8.3 and 8.4. The optimal time and temperature for conjugate binding was 2 hours at 37°C, at which positive and negative samples demonstrated the greatest difference in absorbance. At this temperature, samples with titres between 1:2,000 and 1:20,000 were completely bound by the conjugate within 15 minutes and showed no increase in absorbance over the time period. However, the sera with higher titres of 1:100,000 and 1:400,000 both demonstrated slight increases in absorbancies of over the 2 hour period, with the greatest increase occurring between 1.5 and 2 hours. Sample 3, with titre of 1:50,000 had a step-wise increase in absorbance over time.

At 4°C, samples with titres between 1:2,000 and 1:20,000 demonstrated a small increase in absorbance over the 2-hour period and results were comparable to those achieved at 37°C. The samples with high titres increased gradually over time but even after 2 hours had not produced the absorbancies achieved in 15 minutes at 37°C.

Figure 8.3 Effect of conjugate incubation time upon absorbance at 4°C. Patient numbers refer to table 8.0. Antigen: 1:100 dilution; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB.

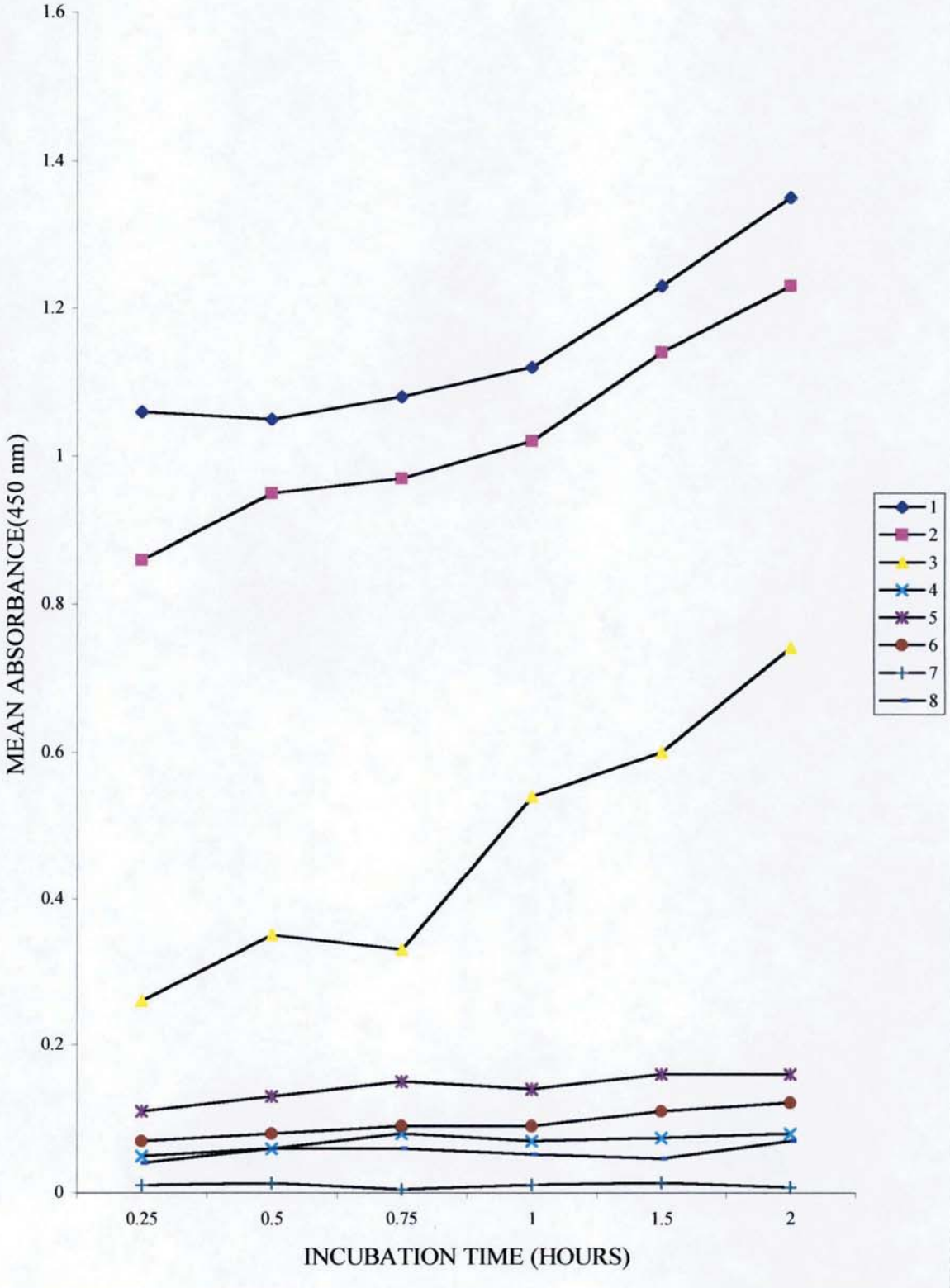
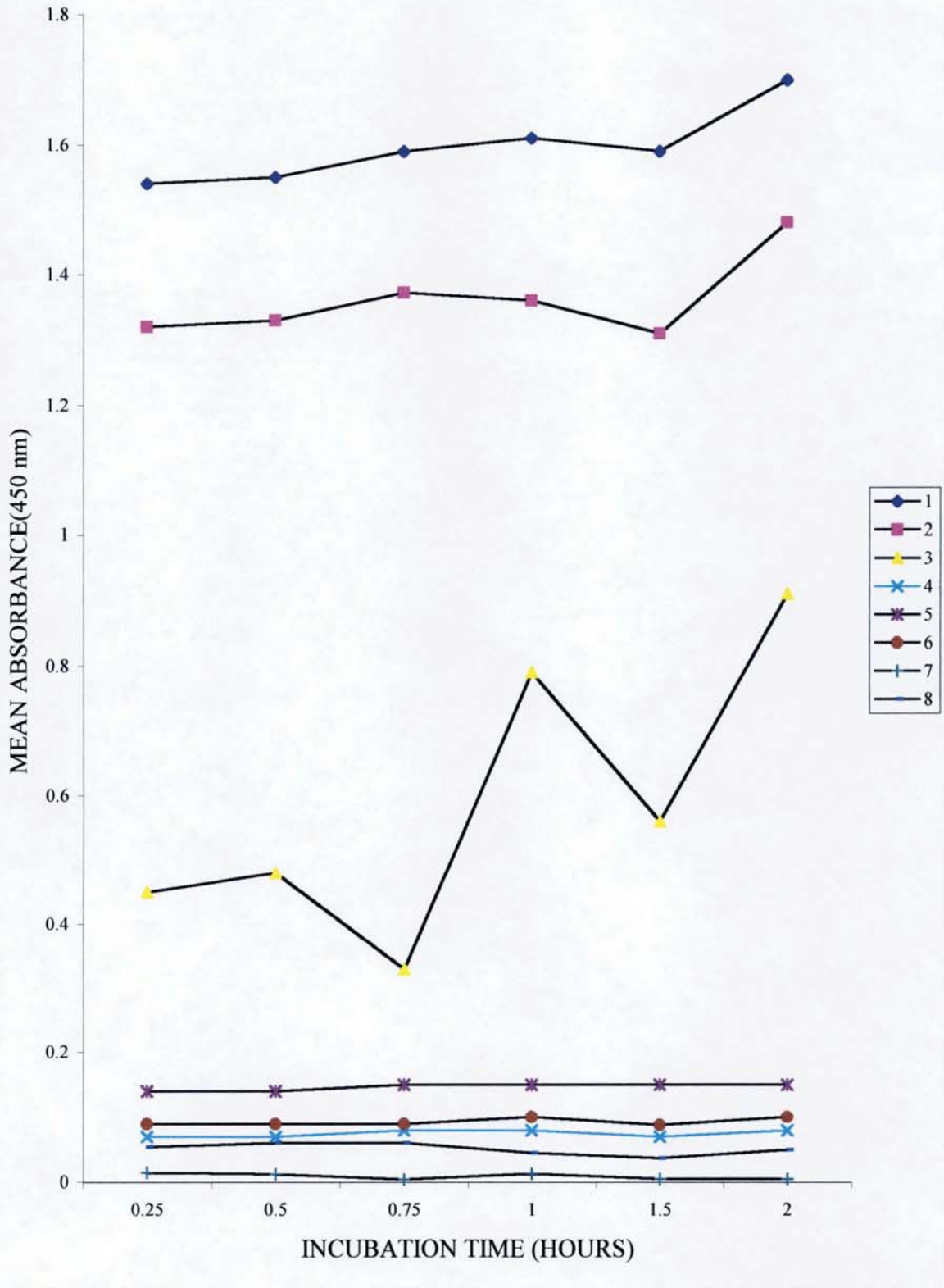


Figure 8.4 Effect of conjugate incubation time upon absorbance at 37°C. Patient numbers refer to table 8.0. Antigen: 1:100 dilution; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB



8.3.3 Optimal conditions for ELISA stage 3

The results obtained for substrate reaction with bound conjugate enzyme were comparable at both temperatures. However, a time and temperature of 30 minutes at 37°C was chosen as optimal as the sample with the highest titre of 1:400,000 had started to reach equilibrium between 25 and 30 minutes. Complete conversion had not occurred in this sample in the same time, at room temperature. The effects of time and temperature on substrate conversion are shown in figures 8.5 and 8.6.

Figure 8.5 Effect of substrate incubation time upon absorbance at 37°C. Patient numbers refer to table 8.0. Antigen: 1:100 dilution; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB

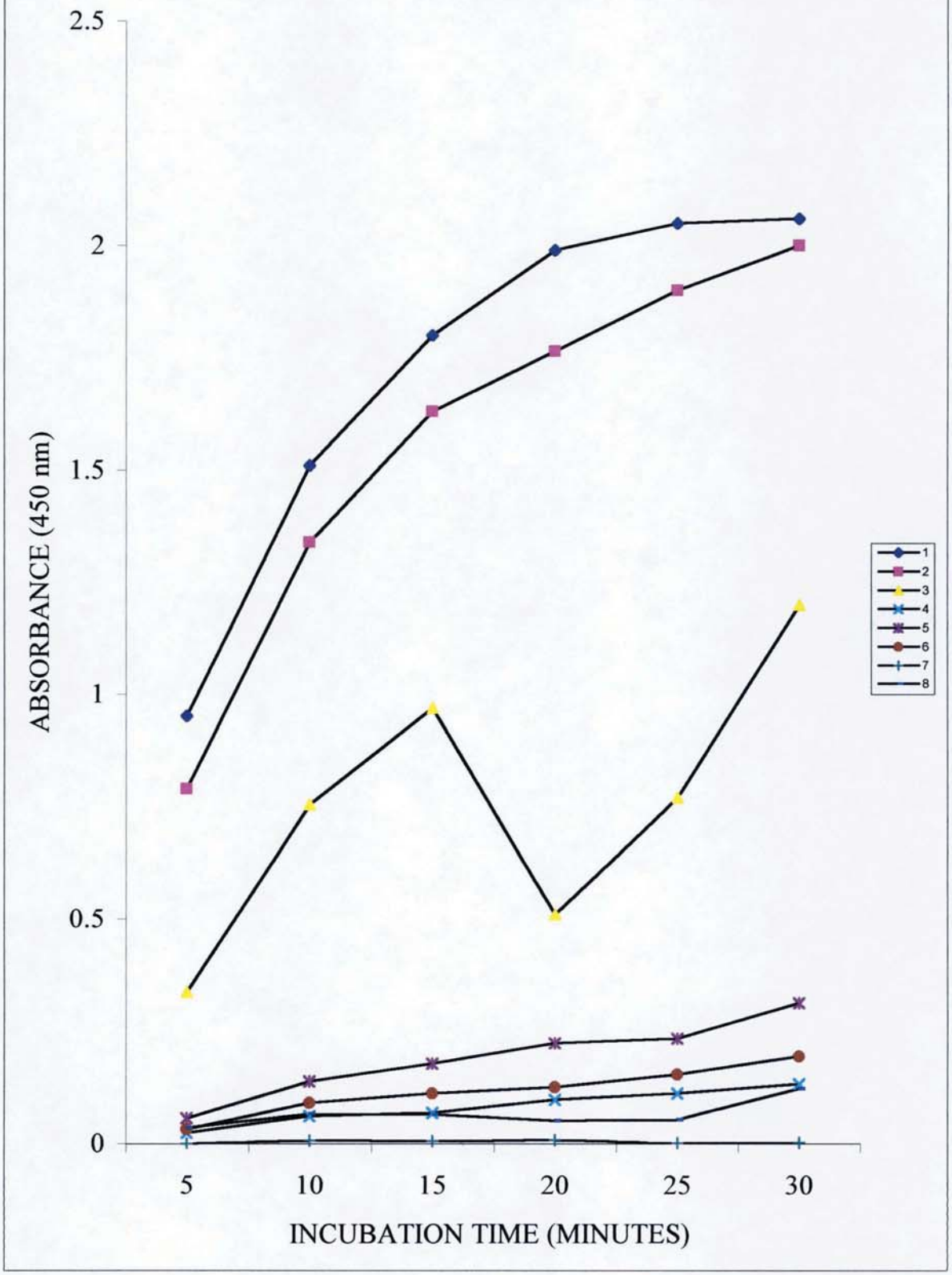
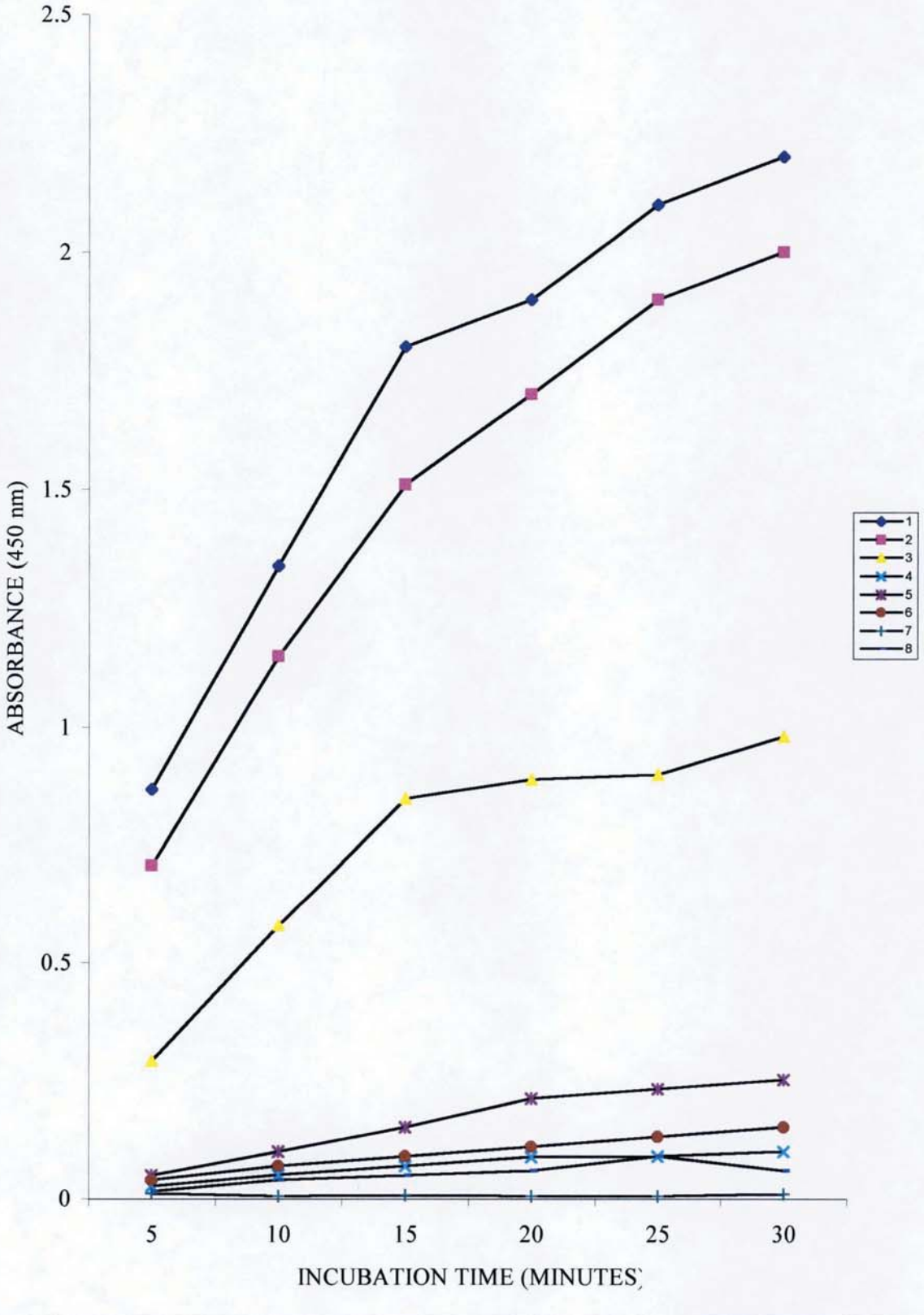


Figure 8.6 Effect of substrate incubation time upon absorbance at room temperature. Patient numbers refer to 8.0. Antigen: 1:100 dilution; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB



8.3.4 Optimal concentration of lipid S antigen

The chessboard titration of antibody against varying concentrations of antigen demonstrated that the optimal concentration of lipid S antigen was 1:400, which equates to 0.125μg of antigen per microtitre well. The observed increase in absorbance by all control sera in the panel at this antigen concentration is shown in figure 8.7.

Figure 8.7 Effect of antigen concentration upon absorbance at 37°C. Patient numbers refer to table 8.0. Serum: Antigen: 1:100-1:1000 dilution; conjugate:protein-A peroxidase; substrate: laboratory prepared TMB

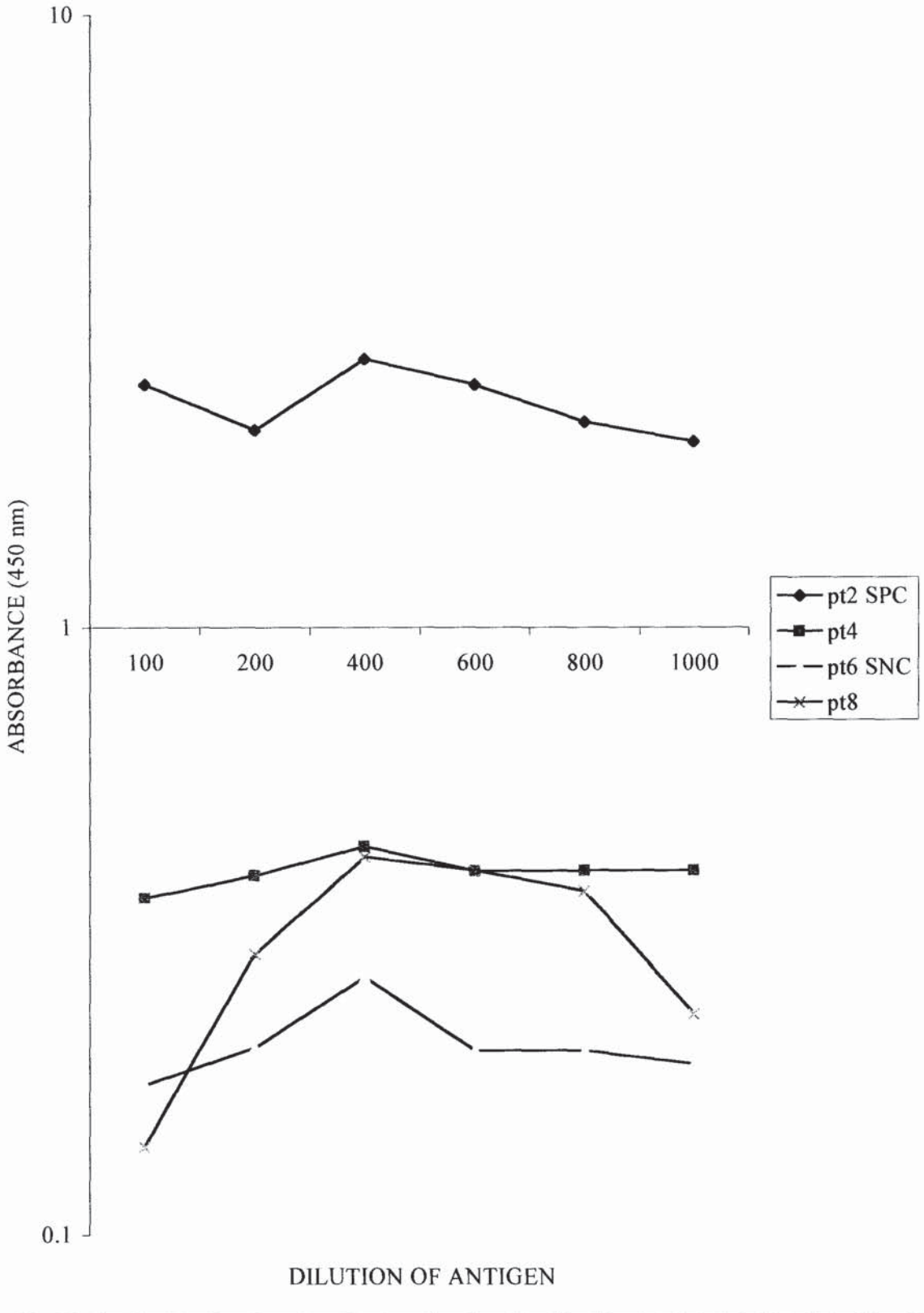
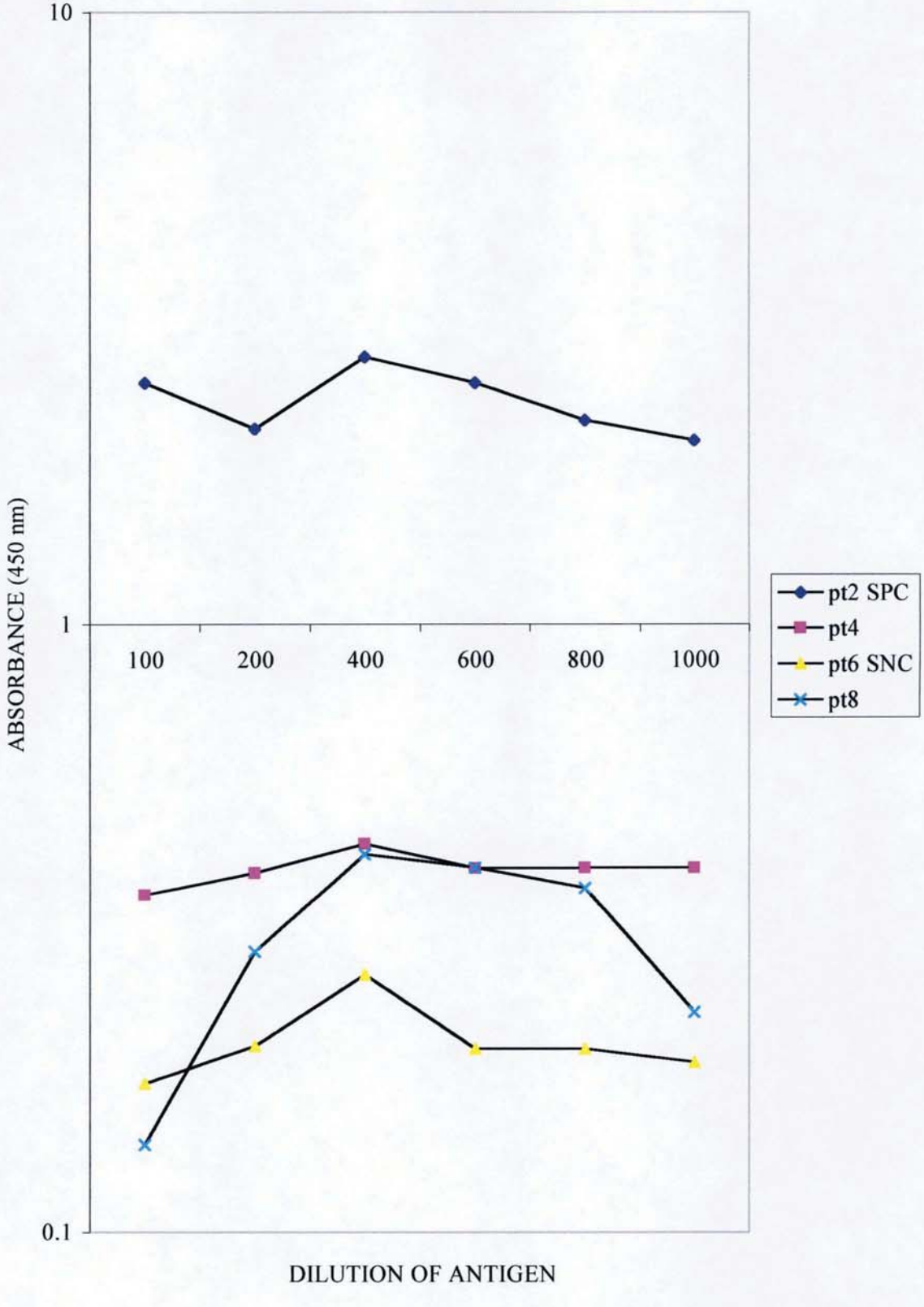


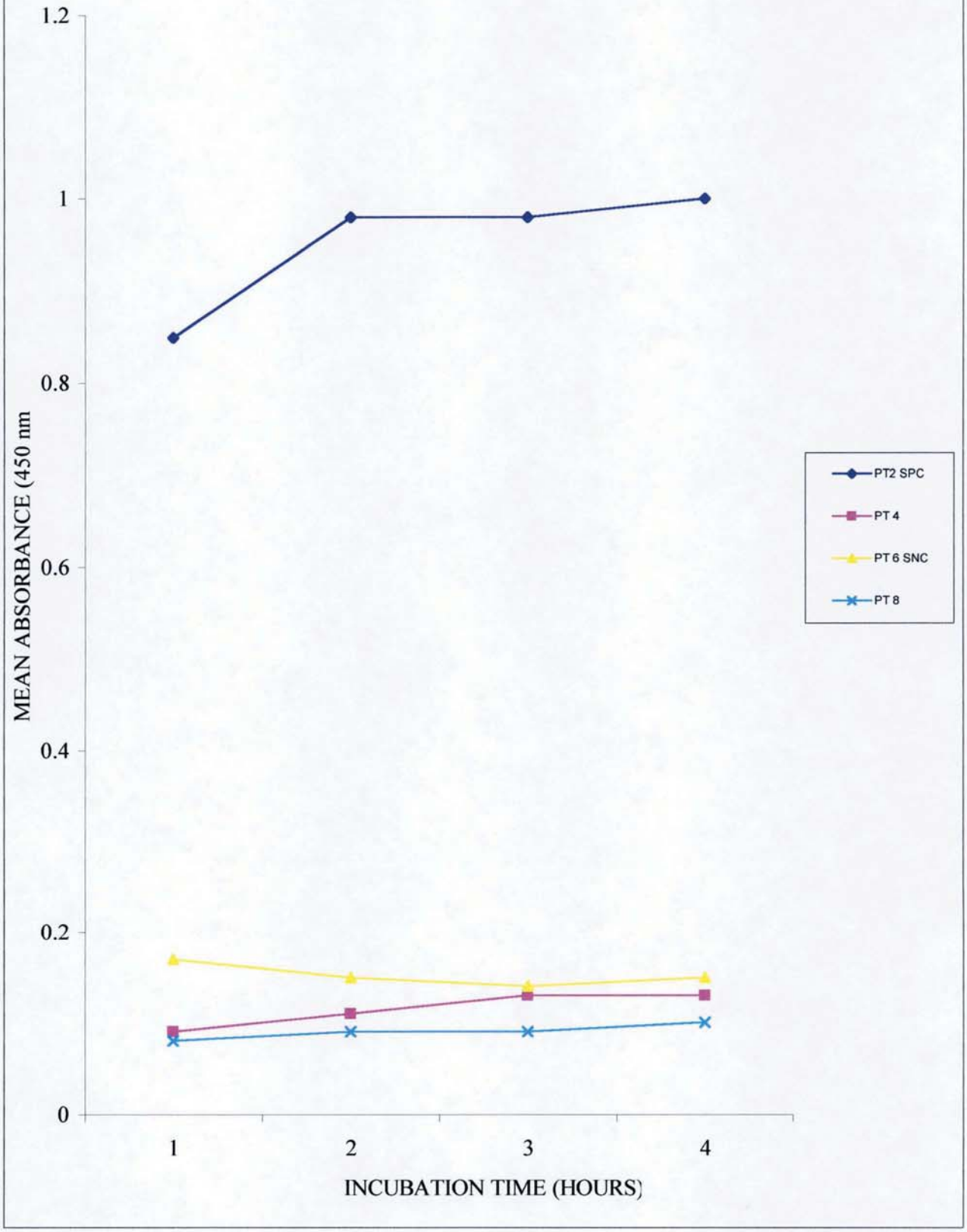
Figure 8.7 Effect of antigen concentration upon absorbance at 37°C. Patient numbers refer to table 8.0. Serum: Antigen: 1:100-1:1000 dilution; conjugate:protein-A peroxidase; substrate: laboratory prepared TMB



8.3.5 Optimal conditions for ELISA stage 1 (antigen 1:400)

The time curve for IgG antibody to bind to lipid S antigen at the reduced concentration of 1:400 is shown in figure 8.8. The optimal time for binding at 37°C was 2 hours
This allowed for the high titre serum sample to reach equilibrium. The remaining lower titre samples had reached equilibrium by the first time point of 1 hour and demonstrated minimal change thereafter.

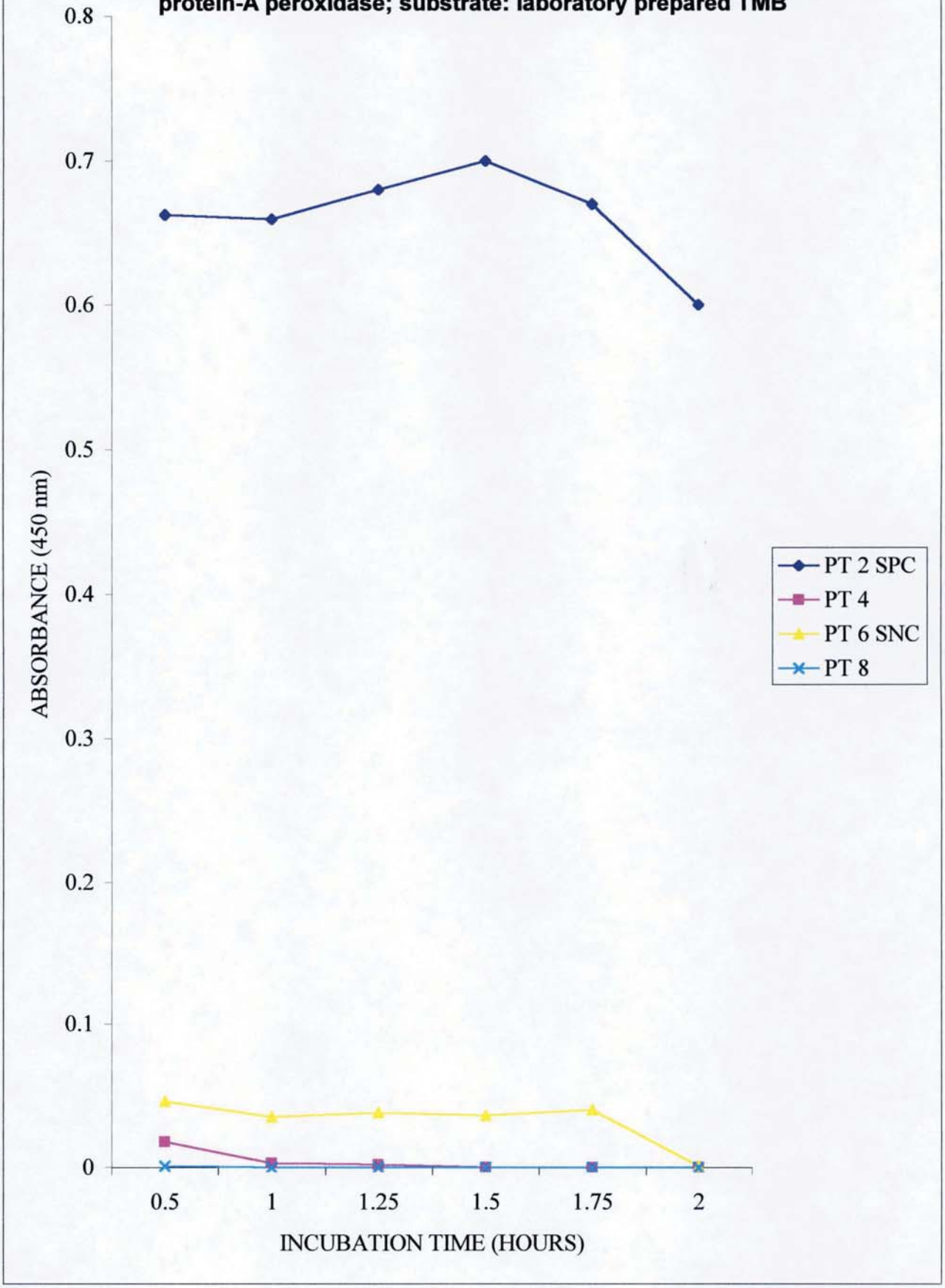
Figure 8.8 Effect of serum incubation time upon absorbance at 37°C. Patient numbers refer to table 8.0. Antigen: 1:400; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB



8.3.6 Optimal conditions for ELISA stage 2 (antigen 1:400)

The optimal time for protein-A peroxidase conjugate to bind to IgG antibody at 37°C was 30 minutes and the time curve is shown in figure 8.9. Only minimal changes in absorbance after this time were observed, however it was noted that a drop in the absorbance values of both PT 2 (SPC) and PT 6 (SNC) occurred as the incubation time exceeded 1.75 hours.

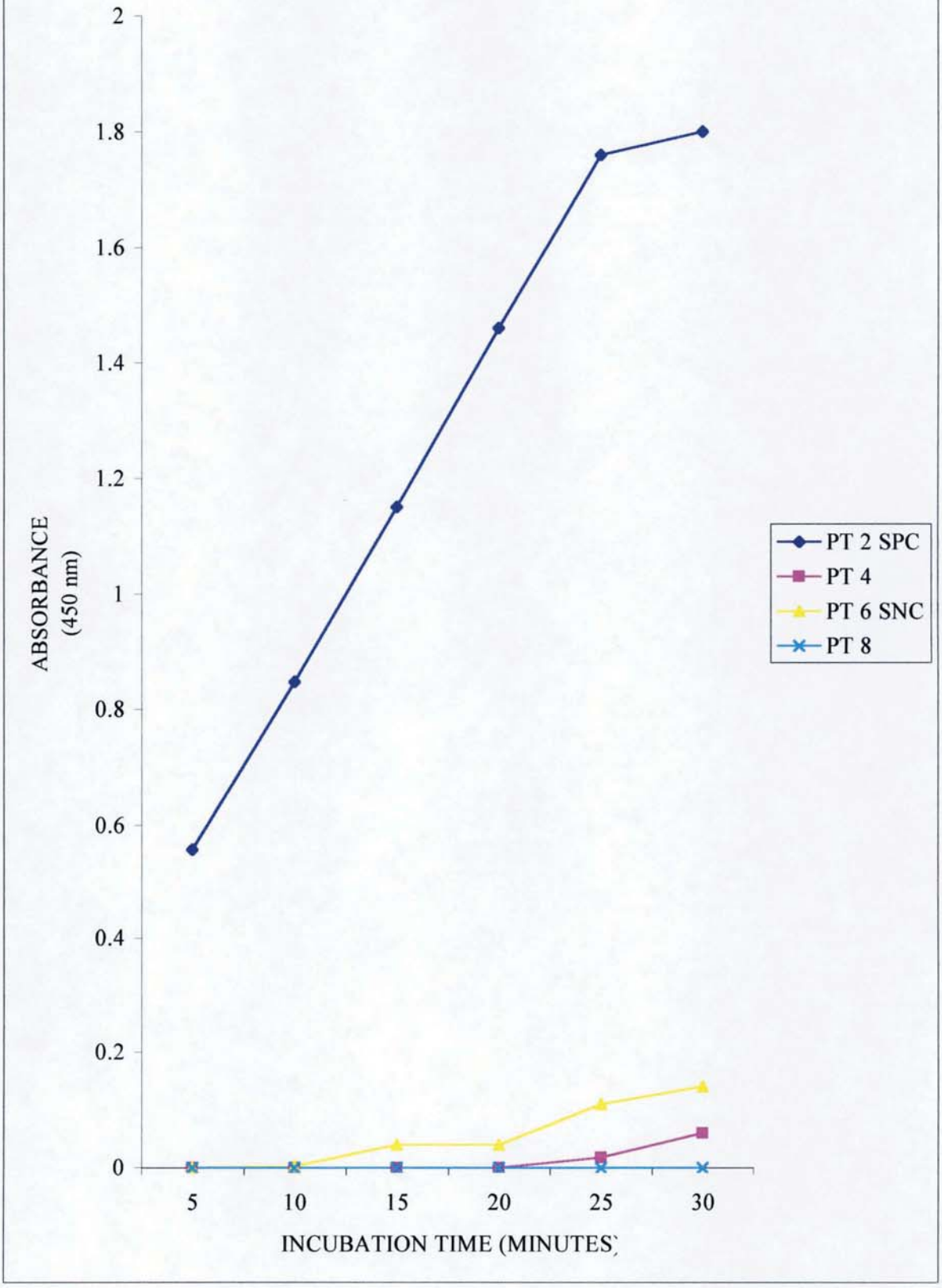
Figure 8.9 Effect of conjugate incubation time upon absorbance at 37°C. Patient numbers refer to table 8.0. Antigen: 1:400; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB



8.3.7 Optimal conditions for ELISA stage 3 (antigen 1:400)

The optimal time for maximum substrate conversion to product at 37°C was 25 minutes. The time curve for substrate conversion is shown in figure 8.10.

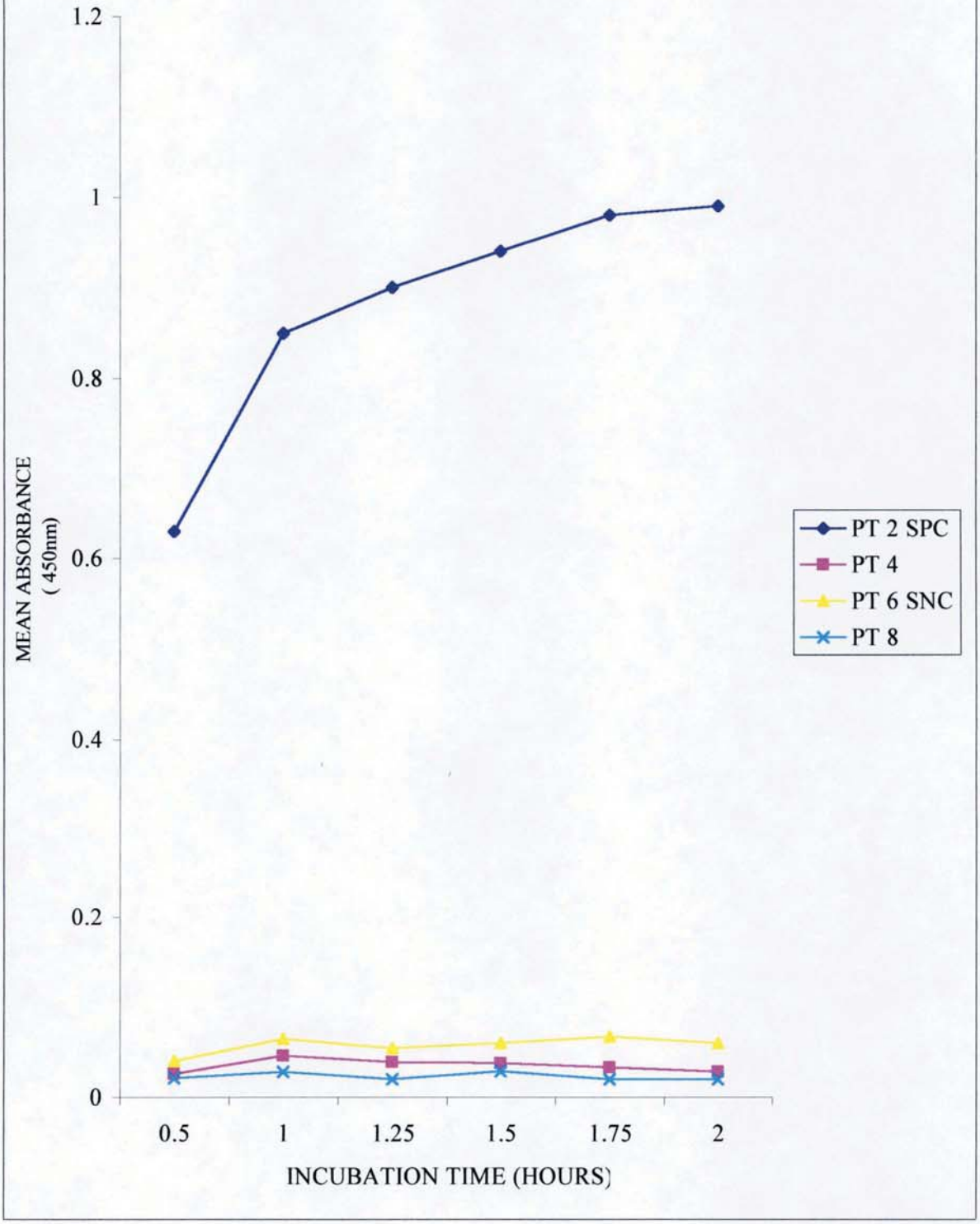
Figure 8.10 Effect of substrate incubation time upon absorbance. Patient numbers refer to table 8.0. Antigen: 1:400; conjugate: protein-A peroxidase; substrate: laboratory prepared TMB



8.3.8 Optimal conditions for anti-human IgG peroxidase conjugate – an alternative conjugate to protein-A peroxidase

The optimal time chosen for complete binding of anti-human IgG peroxidase conjugate to antibody at 37°C was 1 hour and is shown in figure 8.11. The binding of conjugate to the high titre control serum had almost reached completion after 1 hour, and a minimal increase in absorbance was observed between 1 and 2 hours. However for routine laboratory testing, 1 hour was considered sufficient. In the control samples with lower antibody titres, complete binding had occurred within 30 minutes.

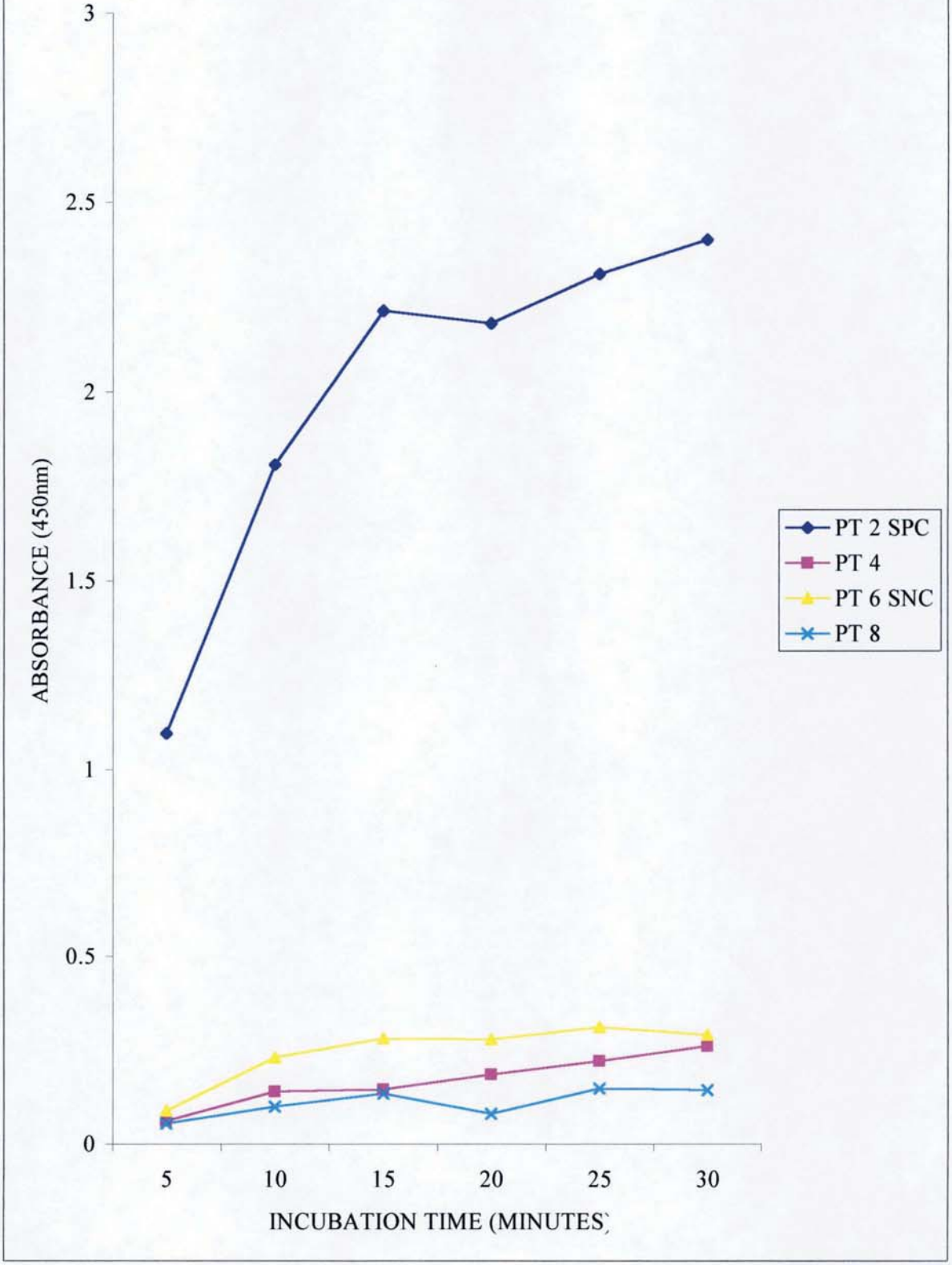
Figure 8.11 Effect of conjugate incubation time upon absorbance. Patient numbers refer to table 8.0. Antigen: 1:400; conjugate: anti-human IgG peroxidase; substrate: laboratory prepared TMB



8.3.9 Optimal conditions for Microwell TMB substrate

The optimal time to allow the commercial Microwell TMB substrate to be converted to product at 37°C was 15 minutes. The time curve for Microwell TMB substrate conversion is shown in figure 8.12.

Figure 8.12 Effect of substrate incubation time upon absorbance. Patient numbers refer to table 8.0. Antigen: 1:400; conjugate: anti-human IgG peroxidase; substrate: Commercial Microwell TMB substrate



8.3.10 Estimation of anti-lipid S IgG in septic and control patients using variations of conjugate and substrate

The mean absorbancies and IgG levels for the patients with CRS and the control group using variations of conjugate (Protein-A peroxidase and anti-human IgG peroxidase), and substrate (laboratory prepared and commercial TMB) are shown in tables 8.4 – 8.11. The sensitivity and specificity of the different ELISA systems are shown in table 8.2. The COV of the positive control (SPC) and negative control (SNC) within the systems are shown in table 8.3. There was no significant difference between test results using either the protein-A peroxidase conjugate or the anti-human IgG peroxidase conjugate (CRS $p=0.3$, controls $p=0.95$). However, the background absorbance observed in the blank column was clearly reduced in the blank column when anti-human IgG peroxidase conjugate was used. The sensitivity and specificity of the assay were maximised when the following combination of assay reactants was used:

1. Lipid S antigen concentration 1:400
2. Sample binding time 2 hours at 37°C
3. Conjugate: anti-human IgG peroxidase incubated for 1 hour at 37°C
4. Laboratory prepared TMB substrate incubated for 25 minutes at 37°C

The distribution of IgG titres in patients with CRS, endocarditis and the control group using anti-human IgG peroxidase conjugate and laboratory-prepared TMB substrate is shown as a logarithmic plot in figure 8.13. For all further work, the parameters listed above were used.

Figure 8.13 The IgG antibody levels using the single absorbance ELISA from patients with CRS, endocarditis and healthy controls (logarithmic scale) Antigen: 1:400; conjugate: anti-human IgG peroxidase; substrate: laboratory prepared TMB

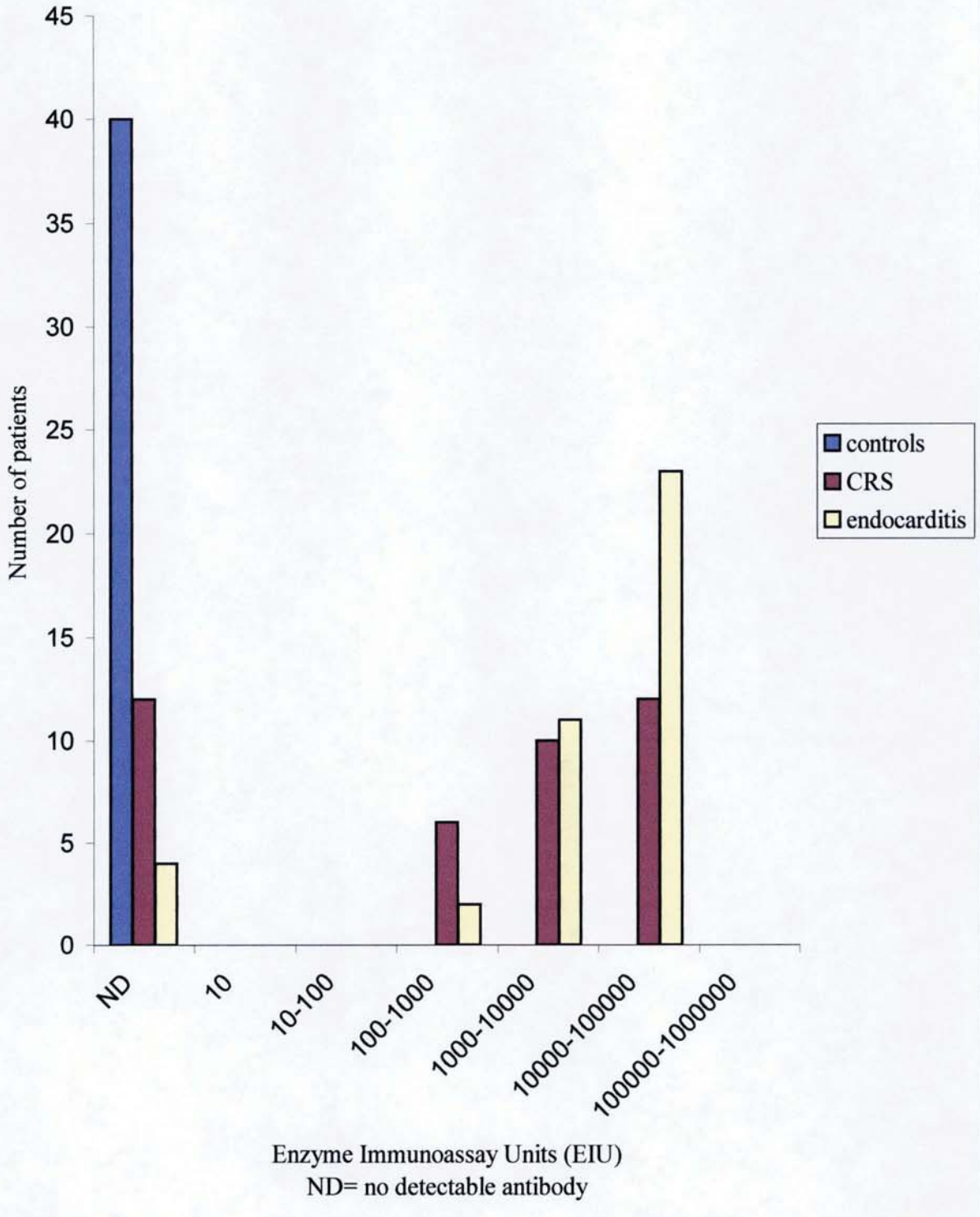


Table 8.2 The sensitivity and specificity of the lipid S ELISA for the serodiagnosis of CRS and endocarditis using different assay conjugate and substrate.

Conjugate used in assay	Substrate used in assay	Patient group	Sens %	Spec %	PPV %	NPV %	Acc %
Anti-human IgG	In-house	Endocarditis	90	100	100	91	95
Anti-human IgG	In-house	CRS	70	100	100	77	85
Protein-A	In house	CRS	53	95	91	67	74
Anti-human IgG	Microwell	CRS	53	93	84	66	73
Protein-A	Microwell	CRS	58	90	85	68	74

Key: sens = sensitivity, spec = specificity, PPV= positive predictive value, NPV= negative predictive value, Acc= accuracy

Table 8.3 The coefficient of variation (COV) of the internal controls in the lipid S ELISA using different assay conjugate and substrate.

Conjugate type	Anti-human IgG peroxidase		Protein-A peroxidase	
Assay control and substrate type	CRS plate	Control plate	CRS plate	Control plate
SPC +(laboratory)	0.7%	2.9%	3.8%	6.1%
SNC +(laboratory)	11%	22%	14.3%	16.9%
SPC + Microwell	4.7%	4.4%	2.6%	5.6%
SNC + Microwell	4.2%	2%	26%	10.8%

Key: SPC = standardised positive control, SNC = standardised negative control, laboratory = laboratory prepared TMB substrate, Microwell = commercial TMB substrate

Table 8.4 Mean absorbance and EIU of 40 patients with CRS.

Conjugate used: anti-human IgG peroxidase

PT	A	EIU	PT	A	EIU
1	0.808	39,159	**21**	0.145	ND
2	1.82	99,939	**22**	0.170	660
3	0.9	44,684	**23**	0.168	720
4	0.86	42,282	**24**	0.339	10,990
5	0.63	28,468	**25**	0.254	5,854
6	0.13	ND	**26**	0.168	720
7	0.088	ND	**27**	0.33	10,450
8	0.162	360	**28**	0.175	1,141
9	0.117	ND	**29**	0.173	1,621
10	0.33	10,450	**30**	0.174	1,081
11	0.159	180	**31**	0.246	5,405
12	0.332	10,570	**32**	0.63	28,468
13	0.073	ND	**33**	0.089	ND
14	0.0311	9,309	**34**	0.6	26,666
15	0.188	1,921	**35**	0.127	ND
16	0.996	50,450	**36**	0.039	ND
17	0.062	ND	**37**	0.14	ND
18	0.117	ND	**38**	0.202	2,762
19	0.172	577	**39**	0.46	18,252
20	0.063	ND	**40**	0.205	2,942

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 1.821

Mean SNC absorbance = 0.156

Table 8.5 Mean absorbance and EIU of 40 control patients

Conjugate used: anti-human IgG peroxidase

PT	A	EIU	PT	A	EIU
1	0.057	ND	21	0.172	ND
2	0.039	ND	22	0.096	ND
3	0.067	ND	23	0.065	ND
4	0.083	ND	24	0.084	ND
5	0.066	ND	25	0.078	ND
6	0.059	ND	26	0.009	ND
7	0.065	ND	27	0.096	ND
8	0.083	ND	28	0.008	ND
9	0.171	ND	29	0.094	ND
10	0.054	ND	30	0.060	ND
11	0.003	ND	31	0.087	ND
12	0.027	ND	32	0.1	ND
13	0.049	ND	33	0.064	ND
14	0.071	ND	34	0.071	ND
15	0.043	ND	35	0.092	ND
16	0.014	ND	36	0.054	ND
17	0.089	ND	37	0.083	ND
18	0.057	ND	38	0.048	ND
19	0.037	ND	39	0.1	ND
20	0.027	ND	40	0.067	ND

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 1.829

Mean SNC absorbance = 0.2

Table 8.6 Mean absorbance and EIU of 40 patients with CRS

Conjugate used: protein-A peroxidase

PT	A	EIU	PT	A	EIU
1	0.668	36,594	**21**	0.69	ND
2	1.589	94,109	**22**	0.13	3,247
3	0.618	33,440	**23**	0.055	ND
4	0.756	42,089	**24**	0.203	7,524
5	0.296	12,363	**25**	0.12	2,373
6	0.0525	ND	**26**	0.083	31
7	0.062	ND	**27**	0.98	1,000
8	0.104	1,378	**28**	0.1	1,124
9	0.0445	ND	**29**	0.142	3,746
10	0.136	3,372	**30**	0.042	ND
11	0.059	ND	**31**	0.079	ND
12	0.192	6,869	**32**	0.041	ND
13	0.05	ND	**33**	0.014	ND
14	0.197	7,181	**34**	0.571	30,532
15	0.103	1,311	**35**	0.034	ND
16	0.412	20,576	**36**	0.05	ND
17	0.05	ND	**37**	0.05	ND
18	0.065	ND	**38**	0.05	ND
19	0.058	ND	**39**	0.20	7,524
20	0.01	ND	**40**	0.1	1,124

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 1.683

Mean SNC absorbance = 0.082

Table 8.7 Mean absorbance and EIU of 40 control patients

Conjugate used: protein-A peroxidase

PT	A	EIU	PT	A	EIU
1	0	ND	**21**	0.08	ND
2	0	ND	**22**	0.133	ND
3	0	ND	**23**	0	ND
4	0.033	ND	**24**	0	ND
5	0	ND	**25**	0	ND
6	0	ND	**26**	0	ND
7	0.002	ND	**27**	0.033	ND
8	0.033	ND	**28**	0	ND
9	0.095	ND	**29**	0	ND
10	0.009	ND	**30**	0	ND
11	0	ND	**31**	0	ND
12	0	ND	**32**	0	ND
13	0	ND	**33**	0	ND
14	0.021	ND	**34**	0	ND
15	0.012	ND	**35**	0	ND
16	0	ND	**36**	0	ND
17	0.029	ND	**37**	0.019	ND
18	0	ND	**38**	0.018	ND
19	0	ND	**39**	0.088	ND
20	0	ND	**40**	0.019	ND

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 1.662

Mean SNC absorbance = 0.175

Table 8.8 Mean absorbance and EIU of 40 patients with CRS in the lipid S ELISA using anti-human IgG conjugate and Microwell substrate

PT	A	EIU	PT	A	EIU
1	0.914	36,132	**21**	0.097	ND
2	2.088	100,000	**22**	0.2	ND
3	1.159	49,668	**23**	0.29	2,154
4	1.132	48,176	**24**	0.525	14,640
5	0.75	27,071	**25**	0.477	11,988
6	0.235	ND	**26**	0.497	13,093
7	0.174	ND	**27**	0.227	ND
8	0.229	ND	**28**	0.284	1,325
9	0.151	ND	**29**	0.281	1,160
10	0.27	552	**30**	0.212	ND
11	0.4	7,734	**31**	0.26	ND
12	1.193	51,546	**32**	0.61	19,337
13	0.236	ND	**33**	0.16	ND
14	0.442	10,055	**34**	0.133	ND
15	0.345	4,696	**35**	1.69	ND
16	1.606	74,360	**36**	0.251	ND
17	0.147	ND	**37**	0.203	ND
18	0.2	ND	**38**	0.241	ND
19	0.196	ND	**39**	0.68	23,204
20	0.314	2,983	**40**	0.368	5,966

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 2.07

Mean SNC absorbance = 0.26

Table 8.9 Mean absorbance and EIU of 40 control patients in the lipid S ELISA using anti-human IgG conjugate and Microwell substrate

PT	A	EIU	PT	A	EIU
1	0.063	ND	**21**	0.239	ND
2	0.078	ND	**22**	0.239	ND
3	0.108	ND	**23**	0.101	ND
4	0.187	ND	**24**	0.116	ND
5	0.111	ND	**25**	0.132	ND
6	0.176	ND	**26**	0.043	ND
7	0.196	ND	**27**	0.146	ND
8	0.187	ND	**28**	0.098	ND
9	0.259	ND	**29**	0.069	ND
10	0.115	ND	**30**	0.096	ND
11	0.028	ND	**31**	0.125	ND
12	0.081	ND	**32**	0.190	ND
13	0.092	ND	**33**	0.090	ND
14	0.120	ND	**34**	0.259	ND
15	0.080	ND	**35**	0.111	ND
16	0.059	ND	**36**	0.087	ND
17	0.248	ND	**37**	0.063	ND
18	0.111	ND	**38**	0.057	ND
19	0.064	ND	**39**	0.178	ND
20	0.063	ND	**40**	0.120	ND

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 2.07

Mean SNC absorbance = 0.3

Table 8.10 Mean absorbance and EIU of 40 patients with CRS in the lipid S ELISA using protein-A peroxidase and Microwell substrate

PT	A	EIU	PT	A	EIU
1	0.909	49,957	**21**	0	ND
2	1.613	92,467	**22**	0.004	ND
3	0.528	26,951	**23**	0.08	201
4	0.66	35,435	**24**	0.26	10,980
5	0.197	6,964	**25**	0.45	22,301
6	0	ND	**26**	0.15	4,700
7	0	ND	**27**	0.02	ND
8	0.147	3,944	**28**	0.15	4,217
9	0.003	ND	**29**	0.088	382
10	0.03	ND	**30**	0.09	805
11	0.21	8,323	**31**	0	ND
12	0.98	54,335	**32**	0	ND
13	0.055	ND	**33**	0	ND
14	0.21	8,141	**34**	0	ND
15	0.98	3,643	**35**	0.19	6,600
16	0.055	68,072	**36**	0.01	ND
17	0.21	ND	**37**	0.022	ND
18	0.14	ND	**38**	0.04	ND
19	1.209	20	**39**	0.31	14,180
20	0.069	2,042	**40**	0.9	51,829

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 1.74

Mean SNC absorbance = 0.08

Table 8.11 **Mean absorbance and EIU of 40 control patients in the lipid S ELISA using protein-A peroxidase and Microwell substrate**

PT	A	EIU	PT	A	EIU
1	0	ND	**21**	0.047	ND
2	0	ND	**22**	0.011	ND
3	0	ND	**23**	0	ND
4	0.039	ND	**24**	0	ND
5	0	ND	**25**	0.066	ND
6	0.011	ND	**26**	0	ND
7	0	ND	**27**	0	ND
8	0.039	ND	**28**	0	ND
9	0	ND	**29**	0	ND
10	0	ND	**30**	0	ND
11	0	ND	**31**	0.005	ND
12	0	ND	**32**	0	ND
13	0	ND	**33**	0	ND
14	0	ND	**34**	0.004	ND
15	0	ND	**35**	0	ND
16	0	ND	**36**	0	ND
17	0.005	ND	**37**	0	ND
18	0.004	ND	**38**	0	ND
19	0	ND	**39**	0	ND
20	0	ND	**40**	0	ND

Key:

A = absorbance, EIU = enzyme immunoassay units, ND = no detectable antibody

Mean SPC absorbance = 1.6

Mean SNC absorbance = 0.076

8.3.11 Statistical analysis of the internal quality control panel

The statistical analysis of the negative, low positive, moderate positive and high positive controls over 5 consecutive working days is shown in tables 8.12 – 8.15.

Table 8.12 Statistical analysis of the high positive control (SPC)

PARAMETER	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Mean absorbance	1.75	2.00	2.16	1.94	1.84
SD	0.097	0.2	0.04	0.16	0.08
COV %	5.3	9	9	7.1	4.4
Absorbance range	1.62-1.96	1.77-1.68	2.10-2.21	1.76-2.20	1.75-2.03
Lower 95% CI	1.697	1.912	2.130	1.831	1.792
Upper 95% CI	1.818	2.190	2.189	2.047	2.026

Table 8.13 Statistical analysis of the moderate positive control

PARAMETER	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Mean absorbance	0.35	0.35	0.4	0.93	0.63
SD	0.05	0.06	0.05	0.09	0.07
COV %	13	17	9	17	12
Absorbance range	0.30-0.47	0.26-0.44	0.3-0.48	0.84-1.06	0.54-0.76
Lower 95% CI	0.3218	0.3081	0.3020	0.8664	0.5480
Upper 95% CI	0.3384	0.3964	0.4810	1.006	0.7660

Table 8.14 **Statistical analysis of the low positive control**

PARAMETER	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Mean absorbance	0.28	0.34	0.33	0.31	0.38
SD	0.01	0.04	0.02	0.009	0.02
COV %	3.6	12.6	6.3	3	5.7
Absorbance range	0.26-0.29	0.31-0.47	0.3-0.37	0.29-0.322	0.36-0.43
Lower 95% CI	0.2760	0.3125	0.3213	0.2890	0.3737
Upper 95% CI	0.2907	0.3737	0.3535	0.3220	0.4057

Table 8.15 **Statistical analysis of the negative control (SNC)**

PARAMETER	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Mean absorbance	0.13	0.15	0.15	0.19	0.19
SD	0.008	0.03	0.01	0.02	0.01
COV %	6.2	21	6.7	10	5.2
Absorbance range	0.12-0.15	0.11-0.2	0.13-0.17	0.16-0.26	0.16-0.21
Lower 95% CI	0.1273	0.1248	0.1393	0.1740	0.1839
Upper 95% CI	0.1390	0.1703	0.1670	0.2132	0.2120

8.3.12 Effect of sample volume

The Spearman correlation coefficients and one-tailed p values determined are shown in table 8.16. Effective pairing in a significant correlation between the sets of data utilising 5μl and 10μl of serum was achieved for all 4 control sera.

Table 8.16 Spearman correlation coefficients and one-tailed p values when using 5μl and 10μl of control serum in the lipid S ELISA

CONTROL	SPEARMAN COEFFICIENT	P VALUE
High (SPC)	0.8744	< 0.0001
Moderate	0.5075	0.0013
Low	0.4831	0.0022
Negative (SNC)	0.4201	0.0075

8.3.13 Correlation between the antibody levels achieved with the prototype 24 hour titration ELISA and the rapid 4 hour single absorbance ELISA.

The antibody levels in patients with CRS and controls achieved using the prototype ELISA and the 4 hour rapid assay are shown in tables 8.17 and 8.18. The scatter plots demonstrating the correlation of the serum IgG levels for patients with CRS and controls are shown in figures 8.14 and 8.15. Effective pairing of results using the 2 methods was observed for CRS (r= 0.5, p=0.0012) and control patients (infinite slope).

Table 8.17 **Comparison of IgG levels in patients with CRS using the prototype ELISA and rapid assay**

Patient	EIU (rapid assay)	Titre (prototype ELISA)	Patient	EIU (rapid assay)	Titre (prototype ELISA)
1	39,159	15,000	21	ND	15,000
2	99,939	100,000	22	670	10,000
3	44,684	51,000	23	720	50,000
4	42,282	26,000	24	10,990	40,000
5	28,468	38,000	25	5,885	50,000
6	ND	46,000	26	720	50,000
7	ND	20,000	27	10,450	30,000
8	360	37,000	28	1,141	50,000
9	ND	29,000	29	1,021	20,000
10	10,450	85,000	30	1,081	40,000
11	180	15,000	31	5,405	15,000
12	10,570	50,000	32	ND	30,000
13	ND	15,000	33	28,468	50,000
14	9,309	40,000	34	26,666	50,000
15	1,921	15,000	35	ND	40,000
16	50,450	200,000	36	ND	25,000
17	ND	20,000	37	ND	50,000
18	ND	50,000	38	37,673	50,000
19	600	50,000	39	18,252	20,000
20	ND	20,000	40	2,942	40,000

Key: ND = no detectable antibody, EIU = enzyme immunoassay units

Table 8.18 Comparison of IgG levels in the control group using the prototype ELISA and rapid assay

Patient	EIU (rapid assay)	Titre (prototype ELISA)	Patient	EIU (rapid assay)	Titre (prototype ELISA)
1	ND	8000	21	ND	20000
2	ND	1200	22	ND	24000
3	ND	10000	23	ND	17000
4	ND	6400	24	ND	5500
5	ND	6600	25	ND	5000
6	ND	3200	26	ND	2800
7	ND	5000	27	ND	11900
8	ND	6000	28	ND	11400
9	ND	10000	29	ND	10900
10	ND	6400	30	ND	19200
11	ND	10800	31	ND	5700
12	ND	6400	32	ND	12800
13	ND	10000	33	ND	4100
14	ND	7600	34	ND	11900
15	ND	5500	35	ND	6400
16	ND	3800	36	ND	1900
17	ND	7000	37	ND	12800
18	ND	1000	38	ND	6400
19	ND	6000	39	ND	19200
20	ND	12000	40	ND	3900

Key: ND = no detectable antibody, EIU = enzyme immunoassay units

Figure 8.14 The correlation of IgG levels evaluated in 40 patients with CRS utilising the prototype 24-hour ELISA and the 4-hour single absorbance ELISA

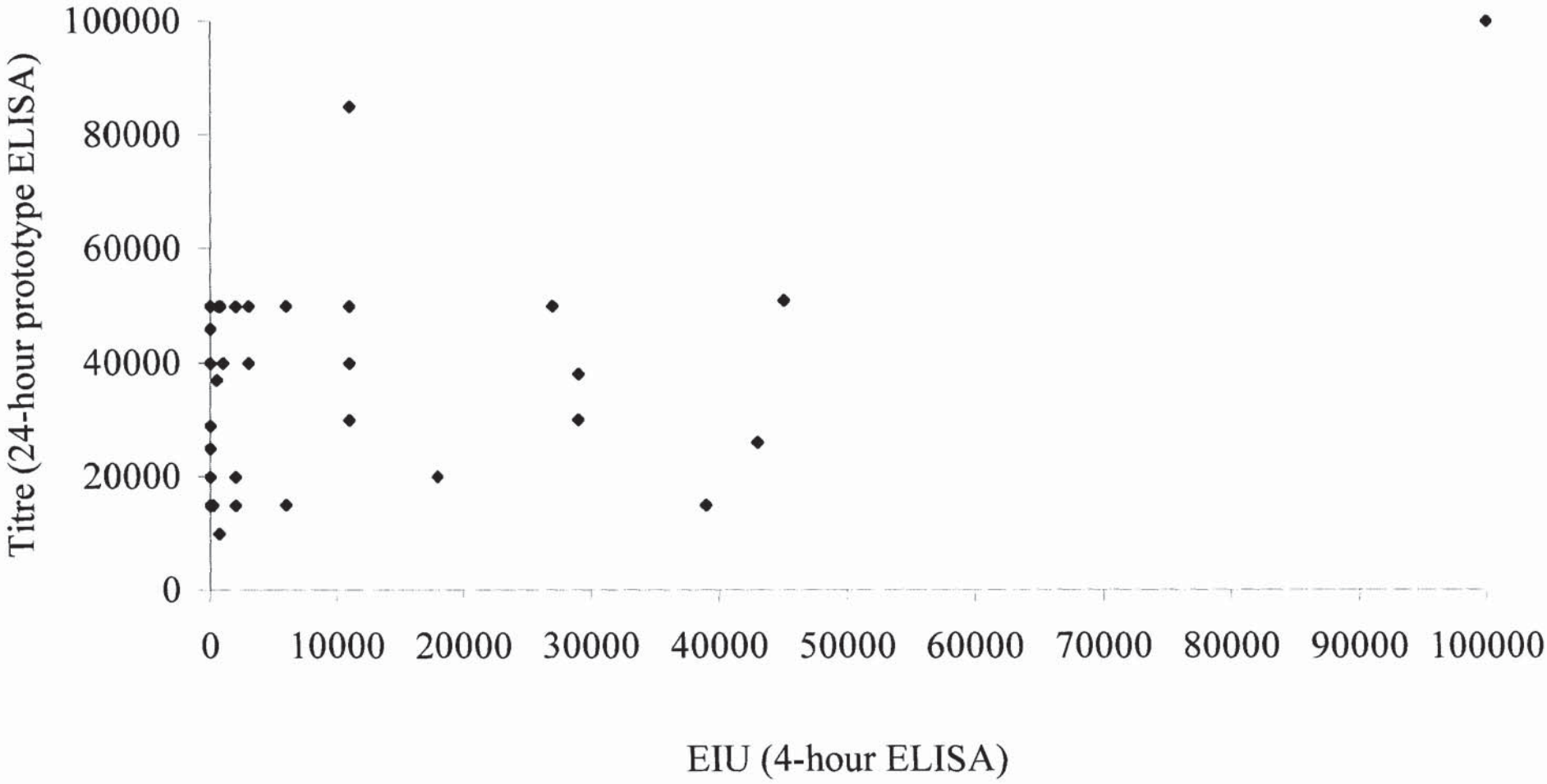
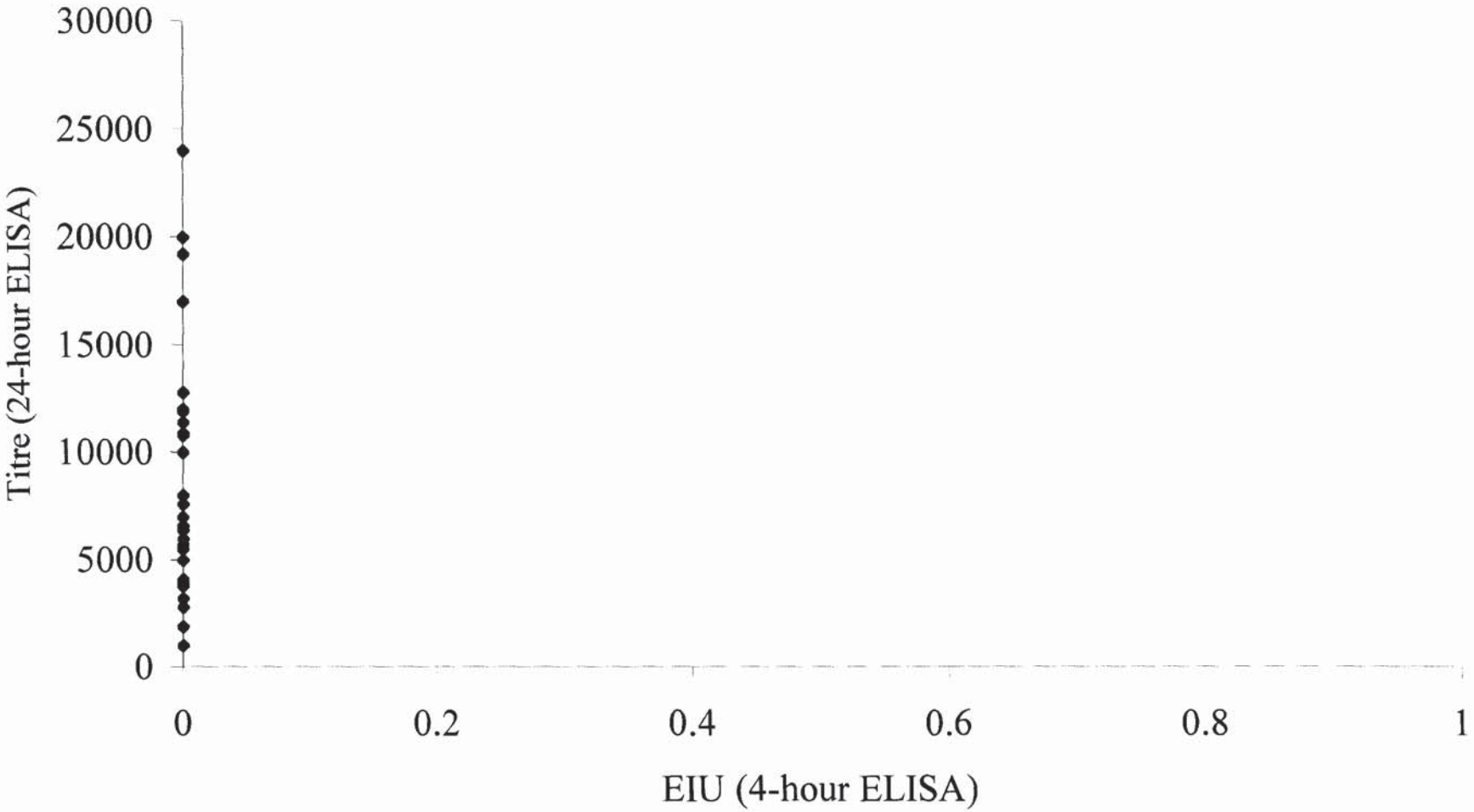


Figure 8.15 The correlation of IgG levels evaluated in control 40 control patients utilising the 24-hour prototype ELISA and the 4-hour single absorbance ELISA



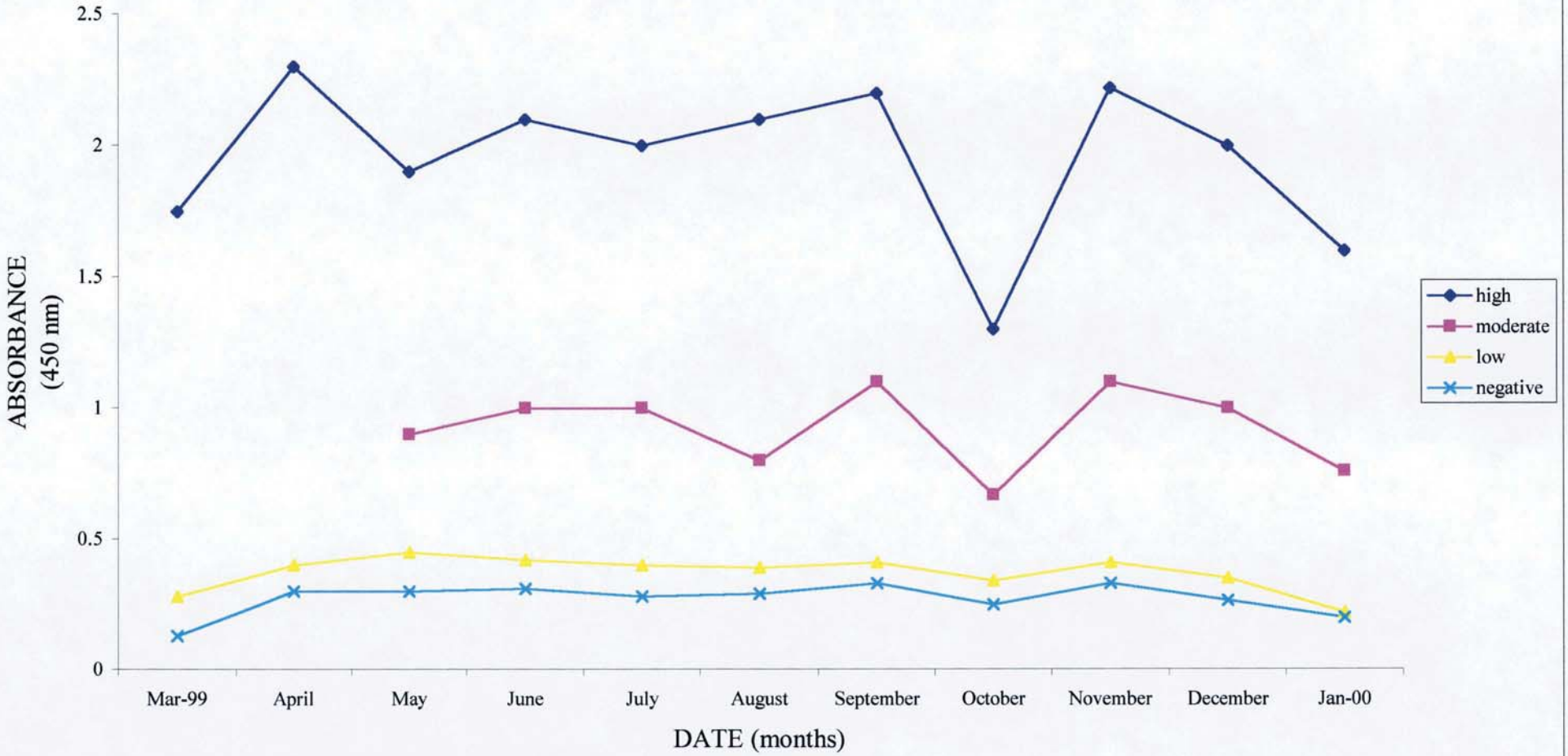
8.3.14 Stability of the lipid S antigen

The absorbancies of the high, moderate, low positive and negative controls recorded over 11 months are shown in figure 8.16. The stability of the bound antigen remained constant over the first 6 months when ELISA plates prepared in April 1999 were used, and any variation in absorbency was reflected across the panel of internal controls. After this, the antigen appeared to deteriorate gradually over the following 2 months (table 8.19). The decrease in absorbance reflected in the control panel, was greater in months 1-3 utilsing the ELISA plates prepared in November 1999 compared with the previous batch prepared in April 1999.

Table 8.19 The percentage decrease in absorbancies of the lipid S assay internal standards over time

plates prepared:	**April**		**November**
TIME / **CONTROLS**	MONTHS 1-6	MONTHS 6-8	MONTHS 1-3
HIGH	5 %	67 %	27 %
MODERATE	0 %	58%	36 %
LOW	0 %	63%	54 %
NEGATIVE	0 %	60%	44 %

Figure 8.16 Effect of storage at -20°C upon the absorbance of the lipid S ELISA internal standards over 11 months. Plates coated with fresh lipid S antigen were used in April and November 1999.



8.3.15 The 95% confidence limits of lipid S assay internal quality standards

The 95 % confidence limits for each control determined over the 11-month period is shown in table 8.20.

Table 8.20 The 95% confidence limits of the lipid S assay internal quality standards

Control (mean value)	Lower and upper limits
High (1.844)	1.634 – 2.055
Moderate (0.832)	0.684 – 0.983
Low (0.346)	0.300 – 0.392
Negative (0.22)	0.1802 – 0.290

8.4 Discussion

It was determined during the development of this assay, that the optimal antigen concentration to coat the wells of the microtitre was 0.125 μg per well (1:400 dilution of the stock) and not 0.5μg as was used in the prototype titration method. Antibody molecules bind to a particular part of the antigen called an antigenic determinant or epitope.
A particular antigen can have several different or several identical epitopes and the antibodies are specific for the epitopes rather than the whole antigen molecule. The binding of antibody to epitope takes place by the formation of multiple non-covalent bonds including hydrogen bonds, electrostatic, Van der Waals and hydrophobic, which are critically dependent on the distance between the interacting groups. Therefore, the strength with which multivalent antibodies bind to multivalent antigens (avidity) and single epitopes bind to single antibody (affinity) will depend upon the expression and availability of the epitopes on the antigen. One hypothesis for the increased test absorbance using a 1:400 dilution of the antigen stock (figure 8.7), is that epitopes on the lipid S antigen are more evenly distributed on the solid phase, which resulted in increased and stronger antibody binding.

The test COV was also reduced, on most occasions, to within acceptable limits of <10% (Balfour and Harford, 1990). This reduction in variation will in part be due to the optimisation of the antigen concentration. Also, a lower test COV was observed when anti-human IgG conjugate replaced protein-A peroxidase (table 8.3). Anti-human IgG peroxidase is a goat anti-human affinity purified antibody, which binds to specific sites on the Fc region of all classes of IgG. Protein-A peroxidase conjugate binds non-specifically to the Fc region in all classes except IgG3 (Jawetz *et al.*, 1987), which may indeed account for the increased variation.

The commercial negative control (NHS) used in the prototype ELISA had an IgG level to lipid S of 1:10,000 (chapter 3). Similarly, in chapter 4, it was shown that the mean IgG titre to lipid S of the 67 patients included in the control group was 1:9,216. Therefore, the inclusion of serum as a standardised negative control (SNC) in the rapid assay to which absorbancies of test sera could be directly compared proved of value. Indeed, patients with low levels of IgG to lipid S were effectively screened out, resulting in high assay specificity. The inclusion of the standard positive control that had a set titre of 1:100,000 allowed for quantitation of those sera giving absorbancies greater than the negative control (Balfour and Harford, 1990). Comparison of patient and control serum absorbancies as a method of estimating antibody levels generated problems when the test serum had readings marginally over that obtained for the negative control serum. These samples upon repeat testing sometimes gave discrepant results. It was also noted that small fluctuations in the absorbance of a sample upon repeat testing could result in large differences in observed EIU. This is governed by the absorbance readings of the negative and positive controls at the time of testing, which will be affected by experimental conditions.

To compensate for these inherent assay problems, the single absorbance ELISA procedure could be modified and improved by implementing two guidelines. Those samples having an EIU result of 1000 or less (equating to approximately a 10% increase in negative control optical density at 450 nm) should be regarded as equivocal / borderline and warrant repeat testing of both sample and patient. Also, using the assay as a semi-quantitative test rather than quantitative is recommended, and a scheme based on assay experience is shown in table 8.21.

Table 8.21 A scheme for using the lipid S ELISA as a semi-quantitative assay

EIU	*RESULT*
Not detectable	Negative
1- 1000	Equivocal. Repeat test. Request repeat sample in 7 days time
1000-5000	+ (weak positive)
5000-20000	++ (moderate positive)
〉20000	+++ (strong positive)

The rapid assay discriminated between patients with CRS and controls and was comparable to the prototype titration ELISA, with the test having a sensitivity of 70% and specificity of 100%. The rapid single absorbance assay failed to detect antibody in 10/40 (25%) patients who tested positive by the prototype method at a cut off of 1:20,000 (table 8.17). However, 6/10 (60%) of these patients generated titres of only 1:20,000 to 1:30,000. This highlights the difficulties experienced when interpreting the results of the prototype assay, whereby a sigmoidal titration curve was generated and used to estimate antibody levels on a logarithmic scale. Indeed, the rapid ELISA does not require the plotting of a titration curve and consequently offers a more accurate method of testing.

The stability of the lipid S antigen over time was variable. Fluctuations in absorbance on a daily basis, due to experimental conditions, were reflected consistently across the panel of internal standards and any small variations of ELISA conditions such as assay temperature or incubation times should not affect the test result. The antigen showed little deterioration in the first six months of storage at -20°C, however there was a marked reduction of about 60% in control absorbance observed in month 7 and 8. Indeed, this was again shown consistently across the entire panel of controls. There was no deterioration in control sera activity over the time period due to repeated thawing and freezing and this is highlighted in figure 8.16, when plates bound with fresh antigen were used (November 1999). The gradual

decrease in control absorbance in the first three months of using the November plates was unexpected and was not consistent with previous batches. The decrease in absorbance may be due to dissociation of the antigen from the solid phase on storage due to an error in the plate preparation, rather than antigen deterioration. The shelf life of the lipid S plate appeared to be six months, after which, fresh plates should be prepared. Moreover, fresh ELISA plates should be considered if control values repeatedly fall outside of the values shown in table 8.20. The stability of the plate may be increased for a longer period of time if different storage conditions are used, for example, -70°C (Balfour and Harford, 1990). However, further work is needed to investigate this parameter.

The accurate diagnosis of CRS continues to remain a problem since the clinical presentation is often silent or non-specific (Elliott and Tebbs, 1998; Siegman-Igra *et al.*, 1997). There are also difficulties in supporting microbiological investigations. The current standard method is the examination of blood cultures taken either via a peripheral venepuncture or the CVC. Positive blood cultures may however represent contamination rather than sepsis. Quantitative analysis of paired blood cultures is also available. However, many laboratories do not offer this investigation and the results can be complicated by microbial colonisation of the catheter rather than infection (Michalopoulos and Geroulanos, 1996).

The results of this study highlight the potential of the lipid S ELISA as a rapid diagnostic tool in the diagnosis of CRS. The assay had a sensitivity and specificity of 70% and 100% respectively, which compares favorably with other methods of diagnosis (Siegman-Igra *et al.*, 1997) and also its prototype (chapter 4). The prototype form of this optimised ELISA was used, and shown to be of value, in the serodiagnosis of culture positive, definite cases of endocarditis according to the Duke criteria (Connaughton *et al.*, 1997). It was reasoned therefore that a direct comparison of the results achieved from patients with CRS with those having endocarditis, would serve as a useful positive control. The clustering of results from both groups of patients was very similar and the ELISA clearly differentiated them from the

negative control group (figure 8.13). The lower test sensitivity observed in CRS compared to endocarditis, is likely be due the difficulties experienced in making the clinical diagnosis of the disease and hence the patient selection. Also, some patients were oncology patients receiving immunosuppressive therapy (chapter 4) which would influence their antibody response. The detectable IgG levels in patients with CRS was lower than those seen in endocarditis, but the antigenic challenge and persistence of sepsis is likely to be less aggressive in cases of CRS. The ELISA had a sensitivity and specificity of 90% and 100% in the serodiagnosis of endocarditis and proved to be superior compared with its prototype (Connaughton *et al.*, 1997). The ELISA therefore appeared a powerful tool not only in confirming a clinical diagnosis, but also in aiding the diagnosis of possible cases of endocarditis, where microbiological culture and endocardial imaging prove inconclusive. Patients with endocarditis demonstrated the highest detectable IgG levels to the lipid S antigen which is probably due to the severity of the disease and the persistence of antigen in damaged heart valves. In the control group of patients, antibody to lipid S was not detected, giving the test 100% specificity.

The lipid S ELISA has several advantages over currently available methods for the diagnosis of CRS. The assay is rapid, with results being available within 4 hours; there is no need for unnecessary catheter removal, whether it be a pre-emptive prophylactic measure or a suspected sepsis which in turn has added cost benefits; the problem of microbial contamination of catheter tips, blood cultures and blood drawn through potentially contaminated luers for staining is removed. In addition, the assay is relatively economical with a cost per test of £6. Previous attempts at developing serological tests based on staphylococcal cell surface antigens for the diagnoses of endocarditis have been unsuccessful (Bayer *et al.*, 1987). Others have developed ELISA using polysaccharide antigens that detect IgG antibodies allowing the differentiation between infections due to slime producing *S. epidermidis* and those strains that are slime negative (Karamanos *et al.*, 1997). In conclusion, the lipid S rapid ELISA was sensitive and specific in the serodiagnosis of endocarditis and CRS which may therefore aid in optimising patient care and management.

Chapter 9 The epidemiological typing of strains of coagulase negative staphylococci associated with catheter-related sepsis

9.1 Introduction

Coagulase negative staphylococci (CNS) are an important cause of infection of implanted medical devices such as intravascular catheters, prosthetic heart valves, pacemakers, continuous ambulatory peritoneal dialysis catheters and orthopaedic devices (Rupp and Archer, 1994). Infection associated with central venous catheters (CVC) is a significant cause of morbidity and mortality and is a primary cause of bacteraemia and septicaemia in hospitalised patients (Elliott, 1993). A major source of microbial contamination of CVC is the skin insertion site from where microorganisms may migrate down the intracutaneous tract on the external surface of the catheter leading to subsequent colonisation of the catheter or sepsis. Microorganisms may also gain access to catheters via the intraluminal route through catheter hubs, infusates and luer connectors (Sitges-Serra *et al.*, 1984), and they can be impacted onto the catheter distal tip at the time of insertion (Elliott *et al.*, 1997a).

Until recently, typing of CNS was based on conventional methods including biotyping, antibiograms and slime production. However, previous workers have found the majority of isolates of *S. epidermidis* to be contained within a few distinct biotypes (Pfaller and Herwaldt, 1988), and therefore such methods of limited value in determining the pathogenesis and epidemiology of CNS infection (Nouwen *et al.*, 1998). In addition, the existence of multi-resistant nosocomial strains of CNS limits the use of antibiogram typing to studies that investigate local endemic strains which are resistant to various combinations of antibiotics (Geary *et al.*, 1997). Typing methods that are both accurate and sensitive are essential for studies investigating the epidemiology and source of catheter-related sepsis (CRS). One such method is pulsed field gel electrophoresis (PFGE). The preparation of macrorestriction fragment profiles of chromosomal DNA by PFGE is the most sensitive method currently available for genotyping microorganisms since it analyses the entire genome

rather than specific sequences. Several studies have used PFGE to successfully type *S. epidermidis* from a range of clinical sources.

Distinct clones of *S. epidermidis* for example were shown to be endemic in neonatal intensive care units by PFGE of *Sma*I digested chromosomal DNA (Huebner *et al.*, 1994). Similar restriction digest conditions have been used to characterise *S. epidermidis* in patients undergoing bone marrow transplantation (Lina *et al.*, 1995) and also to demonstrate hospital acquired infection (Burnie *et al.*, 1997). In one recent study, genotyping methods were used to identify 2 virulent clones of *S. epidermidis* that were associated with 70% of cases of CRS in a haemato-oncology department (Nouwen *et al.*, 1998). Furthermore, identical macrorestriction patterns generated by PFGE have demonstrated that microorganisms impacted onto catheter distal tips or related equipment during insertion, have originated from the patient's skin flora (Livesley *et al.*, 1998).

To conclude the work in this thesis regarding CRS, the aim of this part of the study was to determine retrospectively the phenotypic and genotypic relatedness of the strains of CNS isolated from the blood cultures of patients with CRS (chapter 4). Conventional phenotypic typing strategies including biotyping, antibiogram typing and slime production, and the more recent molecular method of PFGE were used. In addition, the discriminatory power of each of the typing methods was assessed.

9.2 Methods and Materials

9.2.1 Patients and bacterial isolates

Sixty five strains of CNS were obtained from the blood cultures of 33 patients with CRS (chapter 4). Eleven of the patients had multiple sequential positive blood cultures. The patients were all in-patients and located on various wards of the University Hospital Birmingham NHS Trust, which included the haematology (60%), intensive care unit (17%) and renal unit (23%). All of the catheters were indwelling central venous catheters inserted into the patients' internal jugular vein. The microorganisms were preserved on polystyrene beads at –20°C. Strains were revived for characterisation by subculture onto blood agar plates containing 7% defribinated horse blood (Oxoid, U.K) and incubation for 18 hours at 37°C in 5% CO_2. A reference strain of *S. epidermidis* (NCTC 11047) was also included in the study. All sixty seven isolates were characterised by phenotypic methods and 56 of these (obtained from 30 of the patients, 23 of which acquired the infection nosocomially) were further characterised by PFGE. The relatedness of the isolates was determined on the 56 strains typed by conventional and molecular methods.

9.2.2 Biotyping

Sixty five strains were biotyped. The identification and biotypes of the staphylococcal isolates were determined by the API STAPH system (bioMérieux, France). The API strips were prepared and interpreted in accordance with the API STAPH kit insert.

9.2.3 Antibiogram typing

Antibiogram typing was performed on 65 isolates of CNS. With the exception of methicillin sensitivity testing, the comparative disc diffusion method for the determination of the antimicrobial susceptibility of all staphylococcal isolates was used (Working Party of the

British Society for Antimicrobial Chemotherapy, 1991). The sensitivity patterns of the strains were established against the following panel of 7 antibiotics: penicillin 1μg disc; erythromycin 5μg; ciprofloxacin 1μg; gentamicin 10μg; trimethoprim 1.25μg; vancomycin 5μg (Oxoid, U.K.); flucloxacillin 25μg strip of methicillin (Mast diagnostics, U.K).

9.2.3.1 Comparative disc diffusion method (Working party of the British Society for Antimicrobial Chemotherapy, 1991).

A suspension of test microorganism was prepared by inoculating 1 colony of staphylococci in 2ml of sterile distilled water. The suspension was then used to inoculate the inner circular zone of Isosensitest agar incorporating 1% lysed blood (Oxoid, U.K). A suspension of *S. aureus* Oxford strain (NCTC 6571) was also prepared in the same way and used to inoculate the outer circular1.5cm zone of the plate. The antimicrobial discs were placed onto the agar plate between the test and control organisms with a disc dispenser. The plates were incubated at 37°C for 18h in air and the sensitivity patterns determined.

9.2.3.2 Methicillin sensitivity testing

The test and sensitive control suspensions of microorganisms used for the comparative disc diffusion tests were inoculated as horizontal streaks across the surface of a nutrient agar plate (Oxoid, U.K) containing 5 % NaCl. A resistant control suspension (laboratory isolate of methicillin resistant *S. aureus* with a methicillin minimum inhibitory concentration of > 25mg/l) was prepared and inoculated in the same way. A 25μg strip of methicillin was placed vertically over the test and control organisms and the plate incubated at 37°C for 18 hours in air. The sensitivity to methicillin was determined as follows:

resistant: growth of the microorganism up to the methicillin strip

sensitive: zone of inhibition equal to or greater than *S. aureus* (NCTC 6571). A reduced zone of inhibition was considered resistant.

9.2.4 Slime production (Freeman *et al.*, 1989).

9.2.4.1 Preparation of congo red agar

Thirty seven grams of brain heart infusion broth (Oxoid, U.K.), 50g of sucrose (BDH Ltd, U.K.), and 10g of agar no.1 (Oxoid, U.K.) were dissolved in 900ml of distilled water. The media was dispensed into 90ml amounts and autoclaved at 121°C for 15 minutes. Separately, 0.08g of congo red (BDH Ltd, U.K) was dissolved in 100ml of distilled water and filter sterilised. To prepare the congo red agar, 10ml of congo red solution was added to 90ml of molten agar cooled to 50°C, gently mixed, and the plates poured. The congo red plates were dried prior to use. Congo red agar was quality controlled with a known slime positive strain of *S. epidermidis* RP2A(ACTC 35984), and a slime negative strain of *S. hominis* (ACTC 35982).

9.2.4.2 Detection of exocellular slime

One colony of the test strain was inoculated into 2 ml of sterile distilled water, and 2.5 µl then spread over the surface of a congo red plate. The plates were incubated at 37°C for 18h in air and the results determined as follows:

positive slime test: Black crystalline colonies, dry in appearance.

negative slime test: Pink colonies often darkening in the centre.

equivocal slime test: Dark colonies but not dry and crystalline.

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

9.2.5.1 Standard stock solutions

All reagents and chemicals were obtained from Sigma, U.K. unless otherwise stated.

9.2.5.1.1 1M Tris-HCl

Tris base (12.11g) was dissolved in 90ml of distilled water. The pH was adjusted to 7.6 with concentrated HCl (BDH Ltd, U.K.) and the volume was adjusted to 100ml with distilled water. The solution was autoclaved at 121°C for 15 minutes and stored at 4°C.

9.2.5.1.2 0.5 M EDTA pH 8.0

EDTA (18.61g) was added to 80ml of distilled water. The pH was adjusted to 8.0 with concentrated NaOH (BDH Ltd, U.K.). The volume was adjusted to 100ml with distilled water and autoclaved at 121°C for 15 minutes and stored at 4°C.

9.2.5.1.3 0.5 M EDTA pH 9.0

The solution was prepared as 9.2.5.1.2 and the pH adjusted to 9.0 with concentrated NaOH (BDH Ltd, U.K.).

9.2.5.1.4 TE solution (Tris-EDTA)

Five millilitres of Tris-HCl and 1 ml of 0.5M EDTA pH 8.0 were mixed and the volume adjusted to 500ml with distilled water. The solution was autoclaved at 121°C for 15 minutes and stored at 4°C.

9.2.5.1.5 10 times concentrated TBE solution (Tris-Borate-EDTA)

Tris base (60.5g), 27.5g of boric acid and 20 ml of 0.5 M EDTA pH 8.0 were mixed and the volume adjusted to 500ml with distilled water. The solution was autoclaved at 121°C for 15 minutes and stored at 4°C.

9.2.5.2 In-use solutions for PFGE

9.2.5.2.1 NET-100 solution (NaCl-EDTA-Tris)

Twenty millilitres of 0.5 M EDTA pH 8.0 and 1.0ml of Tris-HCl were mixed and 0.58g of NaCl added. The volume was adjusted to 100ml with distilled water and autoclaved at 121°C for 15 minutes and stored at 4°C.

9.2.5.2.2 0.9% chromosomal grade agarose

Chromosomal grade agarose (0.45g) (BioRad, U.K.) was dissolved in 50ml of sterile NET-100. The suspension was heated to dissolve the media and then cooled to solidify the agarose at room temperature.

9.2.5.2.3 Lysis solution

Tris-HCl (0.6ml) and 20ml of 0.5 M EDTA pH 8.0 were mixed and 5.8g of NaCl added. The volume was adjusted to 100ml with distilled water and autoclaved at at 121°C for 15 minutes and stored at 4°C. Prior to use, 0.5% sarcosyl (w/v), 1 mg/ml lysozyme and 10 units/ml of lysostaphin were added. For 25ml of lysis solution, 0.125g sarcosyl, 0.025g lysozyme and 240µl of reconstituted lysostaphin were added.

9.2.5.2.4 ES solution (EDTA-Sarcosyl)

ES solution consisted of 0.5 M EDTA pH 9.0 with 1% sarcosyl (w/v). For 25ml of ES solution, 0.25g of sarcosyl was added to 25 ml of 0.5 M EDTA pH 9.0.

9.2.5.2.5 ESP solution (EDTA-Sarcosyl-Proteinase K)

ESP solution consisted of ES solution with 1.5 mg/ml of proteinase K added. For 25ml of fresh ESP solution, 0.25g of sarcosyl and 0.0375g of proteinase K were added to 25ml of 0.5 M EDTA pH 9.0.

9.2.5.2.6 0.1M Phenylmethylsulfonylfluoride (PMSF) solution

PMSF crystals (17.5mg) were dissolved in 1ml of isopropanol (BDH Ltd, U.K.). The solution was prepared fresh when required.

9.2.5.2.7 Restriction enzyme buffer A (Boehringer Mannheim, Germany)

Twenty microlitres of 10 times concentrated buffer A was diluted in 180µl of sterile distilled water and stored at –20°C in microcentrifuge tubes.

9.2.5.2.8 Sma I restriction enzyme (Boehringer Mannheim, Germany)

The enzyme specific activity on receipt was 10 units/µl. Four microlitres of S*ma*I (40 units) was added to 176µl of sterile distilled water. Twenty microlitres of 10 times concentrated restriction enzyme buffer A was added and stored at –20°C in microcentrifuge tubes.

9.2.5.3 Preparation of the agarose blocks for PFGE

Strains stored on polystyrene beads at –20°C were revived by subculture onto blood agar plates containing 7% defribinated horse blood (Oxoid, U.K) followed by incubation for 18 h at 37°C in 5% CO_2. One colony was then inoculated into 5ml of brain heart infusion broth (Oxoid, U.K.) which was shaken for 24 hours at 37°C. One ml of the 24 hour suspension was dispensed into a pre-weighed microcentrifuge tube and centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and the remaining pellet weighed. The weight of the

pellet was adjusted with NET-100 to give a final wet weight of 40 mg/ml. A 20 mg/ml suspension was prepared by transfer of 0.5 ml to an equal volume of molten 0.9% chromosomal agarose (w/v) which was then dispensed into a PFGE mould (BioRad, U.K.) pre-cooled on ice. Agarose blocks were removed from the mould and placed into 3ml of lysis solution and shaken for 24 hours at 37°C. The lysis solution was replaced with fresh ESP solution and incubated at 50°C for 48 h.

9.2.5.4 Pre-digestion treatment of the agarose blocks

The ESP solution was replaced with 3ml of TE. Thirty microlitres of 0.1 M PMSF was also added and shaken for 2 hours. This stage was repeated. The agarose blocks were then washed in 5ml of TE solution for 30 minutes. This stage was repeated 3 times. The agarose blocks were stored in TE solution at 4°C until digestion.

9.2.5.5 Digestion of the agarose blocks

A 1cm x 1mm section was cut from the agarose block and placed into 200µl of restriction enzyme buffer. The agarose section was incubated in the buffer for 15 minutes on ice, after which the buffer was removed and replaced with 40 units of S*ma* I. The agarose section was incubated for 18 hours in a 37°C water bath. The enzyme was removed and replaced with 200µl of ES solution and incubated at 50°C for 15 minutes. Finally, the ES solution was replaced with 1 ml of TE solution prior to electrophoresis.

9.2.5.6 Electrophoresis of the *Sma* I digested sections

9.2.5.6.1 Casting the gel

One hundred millilitres of 1% agarose (w/v) (Molecular Biology grade, BioRad, U.K.) was prepared in 0.5 times concentrated TBE and heated to dissolve the agarose. The PFGE gel tray (BioRad, U.K.) was assembled according to manufacturer's instructions. The tray was then adjusted with the aid of a spirit level to ensure a level surface. On reaching 56°C, 95ml of the agarose was poured into the gel tray and left to set for 30 minutes at room temperature. The digested test sections were placed into the pre-formed wells of the gel. A bacteriophage lambda concatamer ladder (BioRad U.K.) was also incorporated into the gel as a DNA size standard. All embedded sections were sealed in the gel with 0.5% agarose (w/v) prepared in 0.5 times concentrated TBE solution.

9.2.5.6.2 The CHEF-DR III electrophoresis cell and parameters.

PFGE was carried out using CHEF DR III apparatus (BioRad, U.K.) operating under the following conditions:

Initial pulse time: 5 seconds
Final pulse time: 60 seconds
Run time: 22 hours
Voltage: 6V/cm
Angle: 120°

The temperature was maintained at 10° C by recirculation of buffer at a rate of 1 litre/minute.

9.2.5.6.3 Staining of the PFGE gel

The gel was stained with 0.5μg/ml ethidium bromide for 30 minutes followed by destaining in distilled water for 1 hour. The gel was visualised using a UV light scanner (UVP products, U.K.). The gel was photographed under UV transillumination and the image retained on a 3.5 inch floppy disc.

9.2.5.6.4 Analysis of the PFGE profiles

Macrorestriction fragment profiles were interpreted using the Phoretix 1D Advanced gel analysis computer programme (Phoretix International, U.K.) together with supporting visual assessment (Tenover *et al.*, 1995). The size of the profile bands was determined by reference to the lambda phage DNA standard. Strain comparison was performed by calculation of the Dice correlation coefficient and isolates were clustered together by the unweighted pair group method of arithmetic averages (UPGMA) to permit the construction of a dendrogram.

9.3 Results

9.3.1 Identification of the coagulase-negative staphylococci and biotypes

Sixty three out of 65 (97%) isolates of CNS were identified as *S. epidermidis* by API STAPH system. The 2 remaining isolates were identified as *S. lugdunensis* and *S. hominis.* Of the 63 isolates of *S. epidermidis* 10 distinct biotypes were identified. The 10 biotypes and the frequency at which they occurred are shown in table 9.0.

Table 9.0 Biochemical profile numbers of 63 isolates of *S. epidermidis* determined by API STAPH and the frequency of occurrence

API STAPH index number	Frequency (%)
6606113	26 (41)
6706113	18 (29)
6706112	9 (14)
6704112	4 (6)
6604153	2 (3)
6706152	2 (3)
6206113	1 (2)
6306152	1 (2)
6706753	1 (2)
6606111	1 (2)

9.3.2 Antibiogram typing

The antibiotic resistance patterns of the 63 isolates of *S. epidermidis* and the percentage occurrence are shown in table 9.1. Fourteen distinct antibiograms of *S. epidermidis* were identified through sensitivity testing. The resistance of the isolates to the antibiotics in the panel tested is shown in table 9.2.

Table 9.1 Antibiogram patterns of 63 isolates of *S. epidermidis* and the percentage occurrence of each antibiogram

Antibiogram pattern	P	F	E	C	G	V	T	%
1	R	R	R	R	R	S	R	**46**
2	R	R	S	S	S	S	S	**5**
3	S	S	S	S	S	S	S	**9**
4	R	R	R	R	S	S	R	**6**
5	R	R	R	S	S	S	R	**1**
6	R	R	R	R	R	S	S	**6**
7	R	R	S	R	S	S	R	**1**
8	R	S	R	S	S	S	S	**1**
9	R	R	R	S	S	S	S	**5**
10	R	R	S	S	S	S	R	**5**
11	R	R	R	S	R	S	R	**5**
12	R	S	R	R	S	S	R	**1**
13	S	S	R	S	S	S	S	**1**
14	R	R	S	S	R	S	R	**6**

Key to table 9.1: P = penicillin, F = flucloxacillin, E = erythromycin, C = ciprofloxacin, G = gentamicin, V = vancomycin, T = trimethoprim, R = resistant, S = sensitive.

Table 9.2 Percentage of strains of *S. epidermidis* resistant to the antibiotics included in the antibiogram panel

Antibiotic	% of isolates of CNS resistant
penicillin	90
flucloxacillin	88
erythromycin	72
ciprofloxacin	60
gentamicin	63
vancomycin	0
trimethoprim	71

9.3.3 Slime production

Thirty (48%) of the 63 isolates of *S. epidermidis* did not produce exocellular slime when grown on congo red agar. Thirty (48%) strains were slime positive and 3 of the isolates (4%) gave equivocal results. A slime producing strain of *S. epidermidis* grown on congo red agar is shown in figure 9.0.

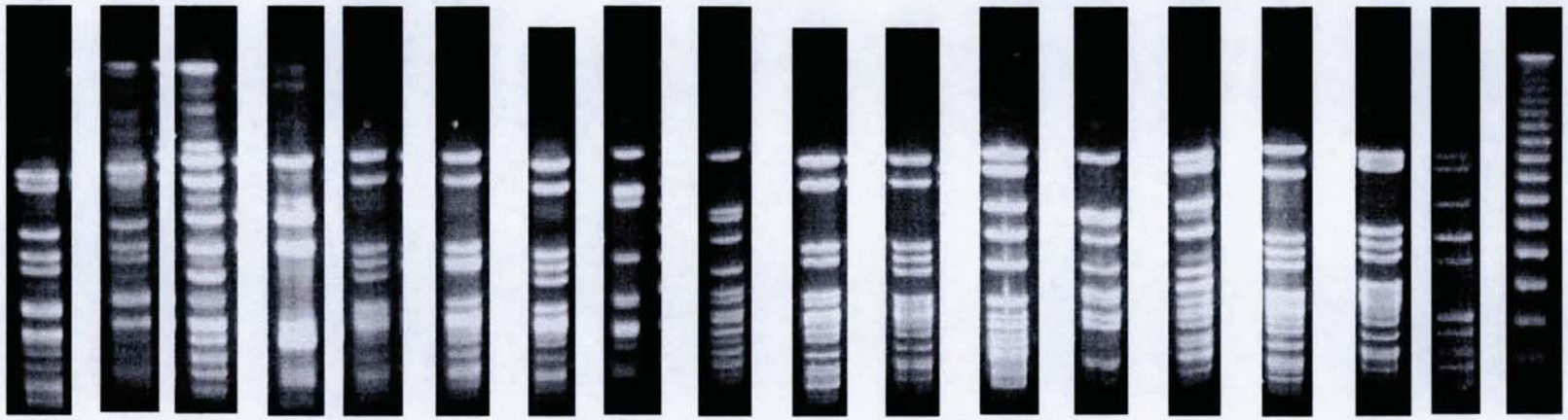
Figure 9.0 **Slime producing strain of *S. epidermidis* RP2A (ACTC 35984) cultured on congo red agar for 18 hours at 37° C in air**

Colonies of *S. epidermidis* RP2A (ACTC 35984) show distinctive blackening after growth on congo red agar for 18 hours at 37° C in air. Congo red was incorporated into the agar to indicate the presence of exopolysaccharide (Freeman *et al.*, 1989) however the exact mechanism of colonial blackening is unknown.

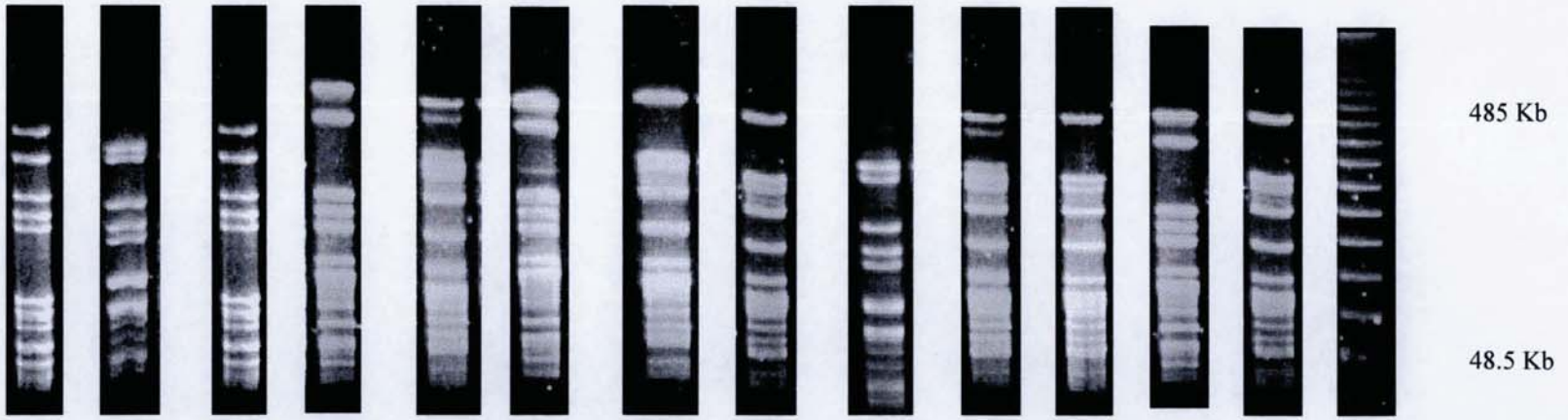
9.3.4 Pulsed field gel electrophoresis

The biotypes, antibiograms and slime production results for the 65 isolates of CNS isolated from 33 patients (A to G1) are shown in table 9.3. The corresponding PFGE lane numbers of the 56 isolates further characterised by PFGE are also shown in table 9.3. The macroestriction profiles of the 56 strains of CNS and the lambda ladder are shown in figure 9.1

Figure 9.1 **PFGE profiles of *Sma*I restricted coagulase negative staphylococci isolated from patients with catheter-related sepsis (lanes 1-30). λ =Lambda ladder.**

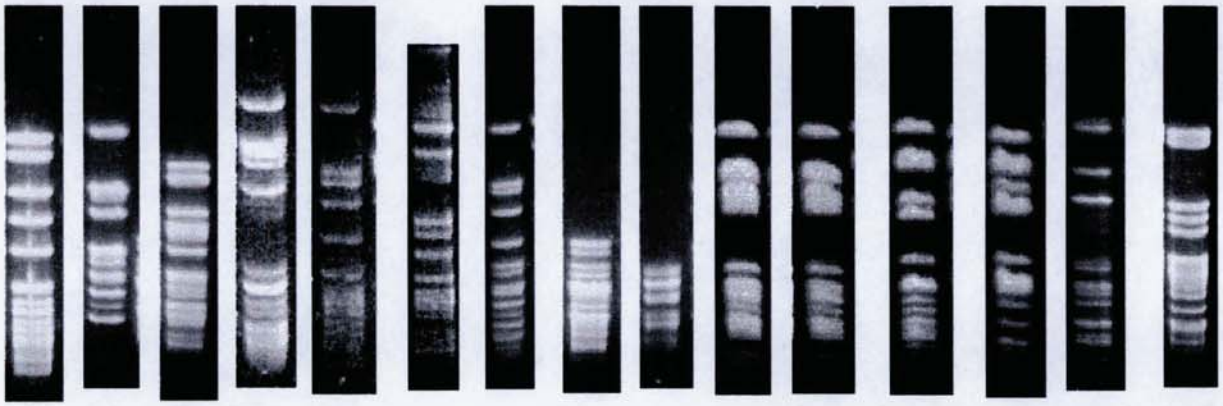


LANE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
PATIENT	A	A	B	C	D	D	D	E	F	G	H	I	I	J	K	L	M	λ

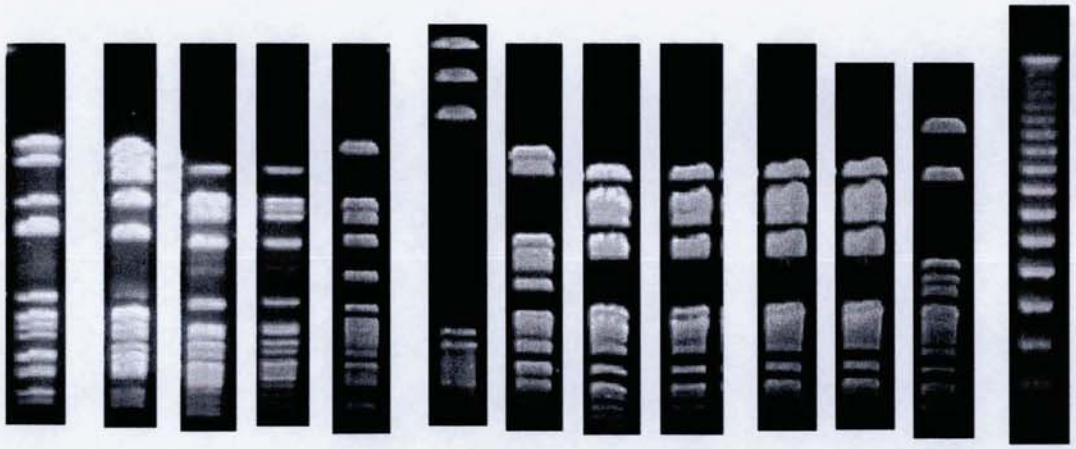


LANE	18	19	20	21	22	23	24	25	26	27	28	29	30	
PATIENT	M	M	N	O	O	O	P	P	P	P	P	P	P	λ

Figure 9.1 continued. **PFGE profiles of *Sma* I restricted coagulase negative staphylococci isolated from patients with catheter-related sepsis (lanes 1-30). λ =Lambda ladder.**



LANE	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
PATIENT	P	Q	R	S	T	U	V	W	W	X	X	X	X	Y	C1



LANE	46	47	48	49	50	51	52	53	54	55	56	57	
PATIENT	C1	C1	D1	D1	NCTC 11047	E1	F1	G1	G1	G1	G1	G1	λ

Table 9.3 Phenotypic characteristics (biotype, antibiogram, slime production) of 65 strains of coagulase negative staphylococci isolated from 33 patients with catheter related sepsis. Pulsed field gel electrophoresis lane numbers of 56 strains of the correlate to the macrorestriction patterns shown in figures 9.1

Patient ID	Site of CNS isolation	API profile number	P	F	E	C	G	V	T	Slime production	PFGE lane No.
A	cvc	6706113	R	R	R	R	R	S	R	-	1
A	bcc	6706113	R	R	R	R	R	S	R	-	2
B	bcc	6706113	R	R	S	S	S	S	S	+	3
C	bcc	6306152	S	S	S	S	S	S	S	-	4
D	bcc	6706113	R	R	R	R	S	S	R	+	5
D	bcc	6706113	R	R	R	R	S	S	R	+	6
D	bcc	6706113	R	R	R	R	R	S	R	+	7
E	bcc	6706152	R	R	R	S	S	S	R	-	8
F	bcc	6706112	R	R	R	R	R	S	S	+	9
G	bcc	6706112	R	R	S	R	S	S	R	+	10
H	bcc	6706112	R	R	R	R	R	S	R	+/-	11
I	bcc	6706112	R	S	R	S	S	S	S	+	12
I	bcc	6706113	R	R	R	R	R	S	S	+	13
J	bcc	6706112	R	R	R	R	R	S	S	-	14
K	bcc	6706113	R	R	R	R	R	S	S	+	15
L	bcc	6706113	R	R	R	R	R	S	S	+	16
M	bcc	6706113	R	R	R	S	S	S	S	-	17
M	bcc	6706112	S	S	S	S	S	S	S	-	18
M	bcc	6706112	R	R	R	R	R	S	R	-	19
N	bcc	6706112	R	R	R	S	R	S	R	-	20
O	bcc	6706753	R	R	R	R	R	S	R	+	21
O	bcc	6606113	R	R	R	R	R	S	R	+	22
O	bcc	6606113	R	R	R	R	R	S	R	+	nd
O	bcc	6206113	R	R	S	S	S	S	R	+	23
P	bcp	6706113	R	R	R	R	R	S	R	+	24
P	bcp	6606113	R	R	R	S	R	S	R	-	25
P	bcc	6606113	R	R	R	S	R	S	R	-	26
P	bcc	6606113	R	R	R	R	R	S	R	+	27
P	bcc	6606113	R	R	R	R	R	S	R	+	28
P	bcp	6606113	R	R	R	R	R	S	R	-	29
P	bcc	6606113	R	R	R	R	R	S	R	+	30
P	bcc	6606113	R	R	R	S	R	S	R	-	31

Key to table 9.3: P = penicillin, F = methicillin, E = erythromycin, C = ciprofloxacin, G = gentamicin, V = vancomycin, T = trimethoprim, nd = not done, bcp = peripheral line blood culture, bcc = central line blood culture, cvc = distal tip of catheter

Table 9.3 continued

Patient ID	Site of CNS isolation	API profile number	P	F	E	C	G	V	T	Slime production	PFGE lane No.
Q	bcc	6706113	R	R	R	R	R	S	R	+	32
R	bcc	6706113	R	R	R	R	R	S	R	+	33
S	bcc	6704112	R	R	R	R	R	S	R	+	34
T	bcc	6706113	R	R	R	R	R	S	R	+	35
U	bcc	6704112	R	R	R	R	R	S	R	+	36
V	bcc	6706113	R	R	R	S	S	S	S	-	37
W	bcc	6706112	R	R	R	R	R	S	R	+	38
W	bcc	6706152	R	R	R	R	R	S	R	+	39
X	bcc	6606113	R	S	R	R	S	S	R	+/-	40
Y	bcc	6606113	R	R	R	R	R	S	R	-	43
Z	bcc	6606113	R	R	R	R	R	S	R	+	nd
Z	bcc	6606113	R	R	R	R	R	S	R	+	nd
Z	bcc	6606113	R	R	R	R	R	S	R	+	nd
Z	bcc	6606113	R	R	R	R	R	S	R	+	nd
A1	bcc	6604153	R	R	S	S	S	S	R	-	nd
A1	bcc	6604153	R	R	S	S	S	S	R	-	nd
B1	bcc	6706113	R	R	R	R	S	S	R	-	nd
B1	bcc	6706113	R	R	R	R	S	S	R	-	nd
B1	bcp	6706113	R	R	S	S	S	S	S	-	nd
C1	bcp	6606113	S	S	S	S	S	S	S	-	45
C1	bcp	6606113	S	S	S	S	S	S	S	+	nd
C1	bcc	6606113	S	S	S	S	S	S	S	+	nd
C1	bcp	6606113	S	S	S	S	S	S	S	+	nd
C1	bcp	6606111	R	R	R	S	S	S	S	+	46
C1	bcp	6606113	R	R	S	S	S	S	S	-	47
D1	bcc	6704112	R	R	R	R	R	S	R	-	48
D1	bcc	6704112	R	R	R	R	R	S	R	+/-	49
S. epidermidis NCTC 11047											50
E1	bcc	6312150	R	R	R	R	R	S	R		51
F1	bcc	6606113	S	S	R	S	S	S	S	-	52
G1	bcc	6606113	R	R	S	S	R	S	R	-	53
G1	bcc	6606113	R	R	S	S	R	S	R	-	54
G1	bcc	6606113	R	R	S	S	R	S	R	-	55
G1	bcc	6606113	R	R	S	S	R	S	R	-	56
G1	bcc	6706113	R	R	R	R	R	S	R	-	57

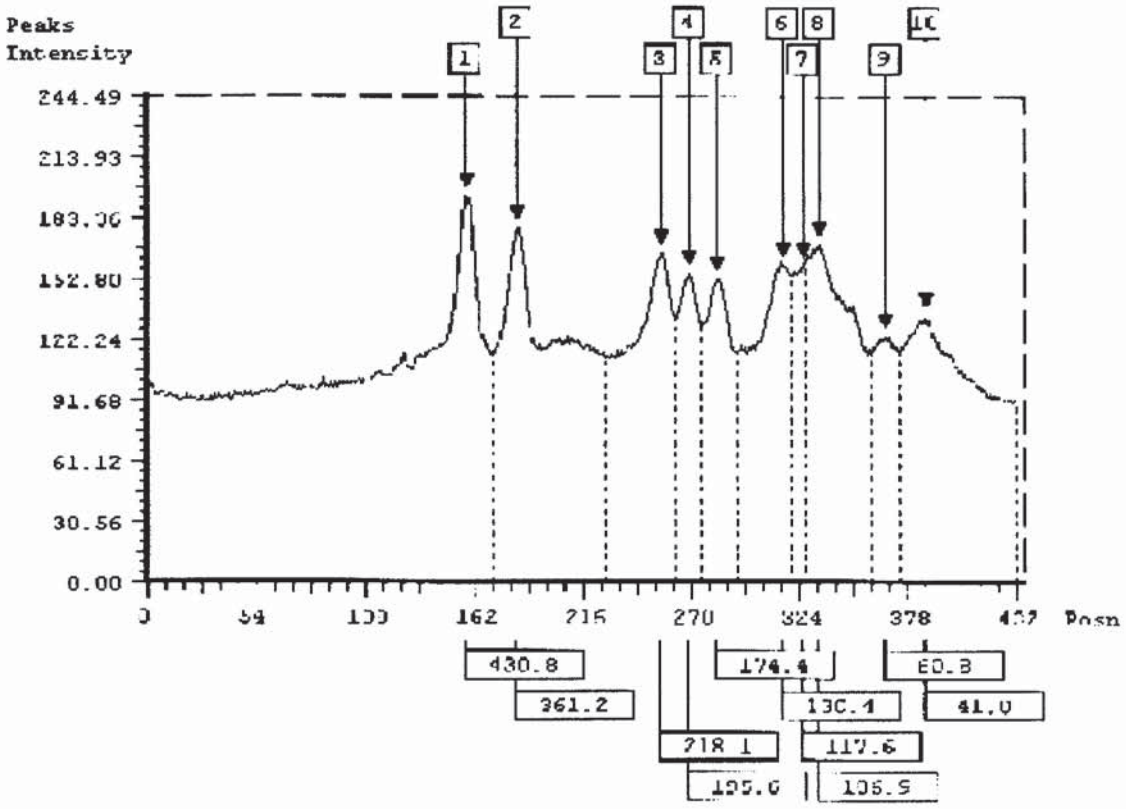
Key to table 9.3: P = penicillin, F = methicillin, E = erythromycin, C = ciprofloxacin, G = gentamicin, V = vancomycin, T = trimethoprim , nd = not done, bcp = peripheral line blood culture, bcc = central line blood culture, cvc = distal tip of catheter

9.3.4.1 Macrorestriction fragment analysis

Fifty-four of the strains of CNS were identified by API STAPH as *S. epidermidis*, 1 as *S. lugdunensis* and 1 as *S. hominis*. PFGE of the 54 isolates of *S. epidermidis*, however, produced a broad and diverse range of restriction patterns. All of the isolates yielded 13 to 15 fragments by PFGE and the weights of the fragments obtained from the strains of *S. epidermidis* were predominantly in the range of 30 to 450 kb. A cluster of between 6-10 restriction fragments was common to all isolates in the lower weight range of 30 to 150 kb followed by 5 to 7 fragments in a range of 150 to 450kb (figure 9.1). The restriction pattern of *S. lugdunensis* (lane 51), however, yielded only 7 fragments, which ranged from 49.4 to 825.5 kb in molecular weight and was uniquely different from the patterns of *S. epidermidis*. The macrorestriction pattern and Phoretix 1D analysis of *S. lugdunensis* is shown in figure 9.3. The isolate of *S. hominis* (lane 13) yielded 9 fragments, the molecular weights of which ranged from approximately 50 to 450kb.

A common restriction pattern was detected in *S. epidermidis* obtained from six patients. The macrorestriction pattern and Phoretix 1D analysis of the common strain of *S. epidermidis* is shown in figure 9.2. This consisted of 10 fragments in total and had the approximate following weights: five bands of 41.0, 61.0, 107.0, 118.0 and 130.0 kb; three bands of 174.0, 196.0 and 218.0 kb; and two bands of 361.0 and 430.0 kb. The common macrorestriction pattern is shown in figure 9.1 (lanes 5, 6 and 7, pt D; lane 10, pt G; lane 11, pt H; lane 15, pt K; lane 16, pt L; lane 45, pt C1). This particular genotype pattern was present in four (13%) of the patients studied. A further two (7%) isolates differed in no more than two bands (Tenover *et al.*, 1995). Strains of *S. epidermidis* with the common restriction pattern were isolated from three patients located on the haematology unit, two from the ITU and one patient from the renal unit. This genotype of *S. epidermidis* yielded three different biochemical profiles when identified by API and five different antibiograms, whilst four out of the six strains were slime positive. Clustering of genotypes was also observed in the multiple strains of *S. epidermidis* isolated from the sequential blood cultures of 8 out of 11 patients.

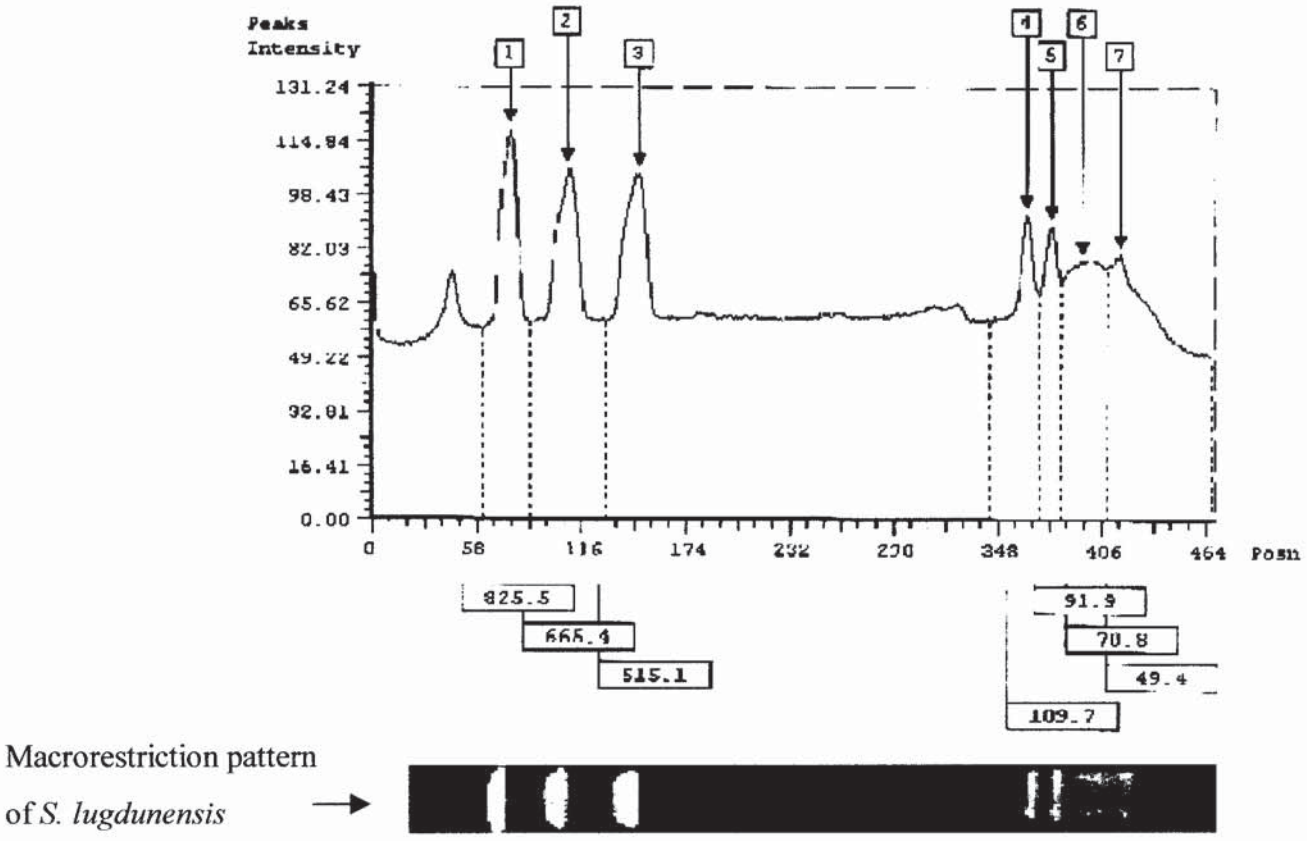
Figure 9.2 **Phoretix 1D advanced gel analysis demonstrating macrorestriction band peaks and sizes (Kb) of the common genotype of *S. epidermidis***



Macrorestriction pattern of the common genotype of *S. epidermidis*

Analysis of the macrorestriction pattern of the common genotype of *S. epidermidis* by Phoretix software identified 10 bands with weights ranging from 41 to 430 Kb

Figure 9.3 **Phoretix 1D advanced gel analysis demonstrating macrorestriction band peaks and sizes (Kb) of *S. lugdunensis***

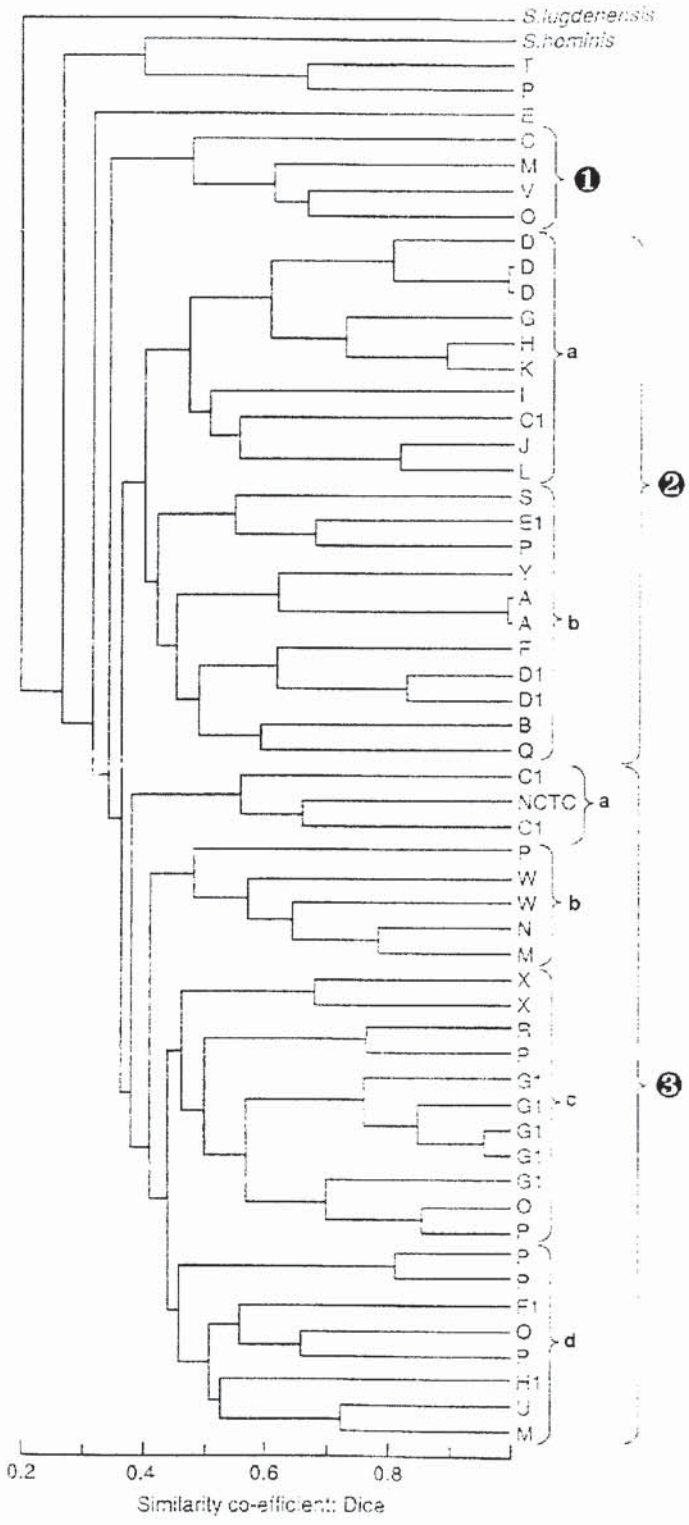


Analysis of the macrorestriction pattern of *S. lugdunensis* by Phoretix software identified 7 bands with weights ranging from 49 to 825 Kb

9.3.4.1.1 Phenotypic and genotypic correlation of strains of CNS associated with CRS

Analysis of the macrorestriction patterns of the 56 strains of CNS by Phoretix 1D software is shown in the dendrogram (figure 9.4). Three major clusters at a similarity Dice coefficient of 0.3 were generated. Two of the clusters could be subdivided into a further 6 clusters at a similarity Dice coefficient of 0.45 (figure 9.4, 2a, 2b, 3a-3d). The common macrorestriction pattern was located in cluster 2a. Software analysis showed no correlation between the genotypic and phenotypic characteristics (biotype, antibiogram and slime production) of strains of *S. epidermidis* isolated from patients with CRS (figures 9.5 to 9.7).

Figure 9.4 **UPGMA dendrogram using the Dice correlation coefficient based on *Sma* I macrorestriction fragment profiles of 56 strains of CNS**



*Sma*I macrorestriction profiles of the 56 strains of *S. epidermidis* isolated from the blood cultures of patients A-G1 are shown in figure 9.1

Figure 9.5 **Key and UPGMA dendrogram using the Dice correlation coefficient based on *Sma* I macrorestriction fragment profiles of 56 strains of CNS. Dendrogram demonstrates the correlation between genotype and biotype of the strains.**

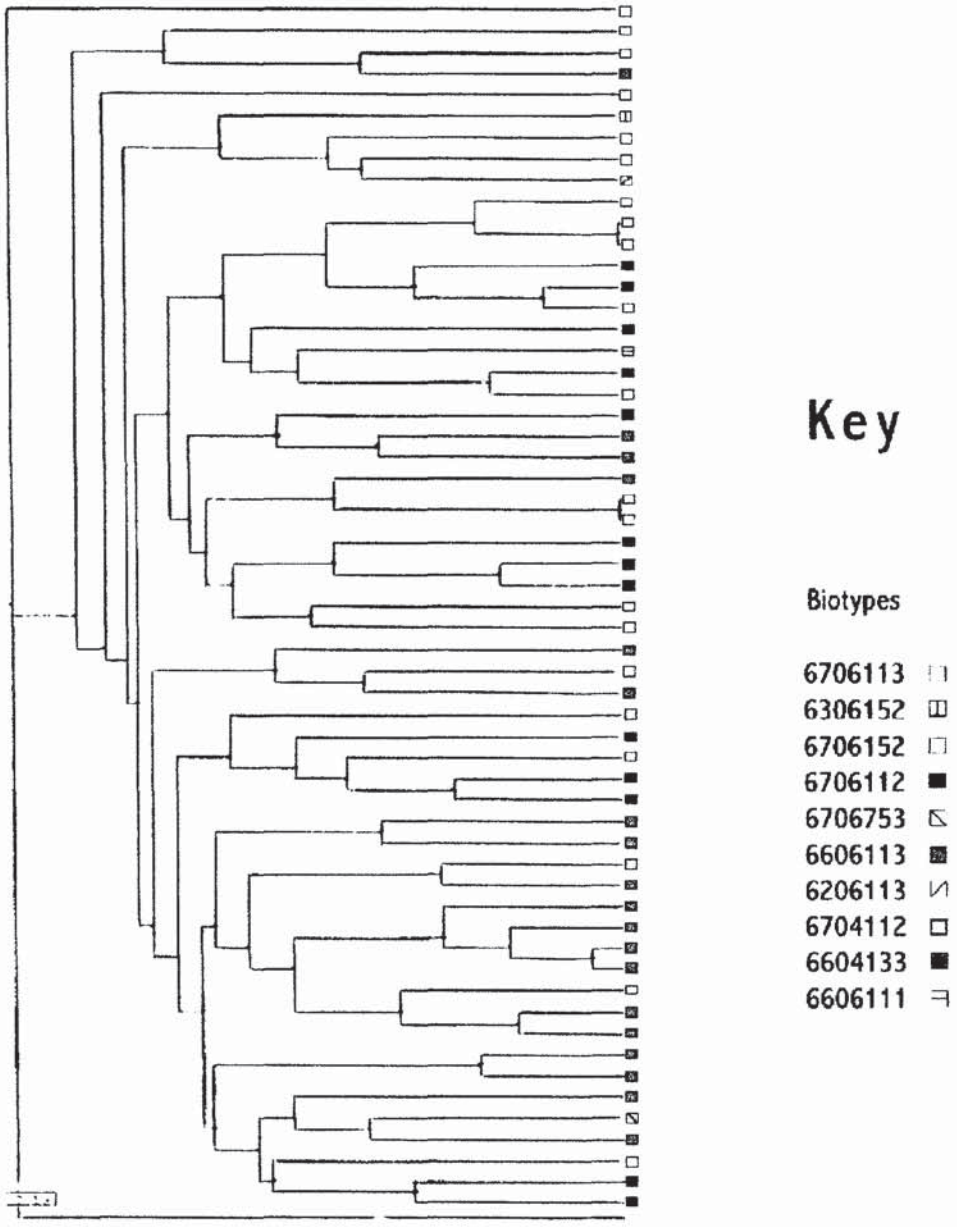


Figure 9.5 constructed to relate the genotype to biotype using colour code (see key)

Figure 9.6 **Key and UPGMA dendrogram using the Dice correlation coefficient based on *Sma* I macrorestriction fragment profiles of 56 strains of CNS. Dendrogram demonstrates the correlation between genotype and antibiogram pattern of the strains.**

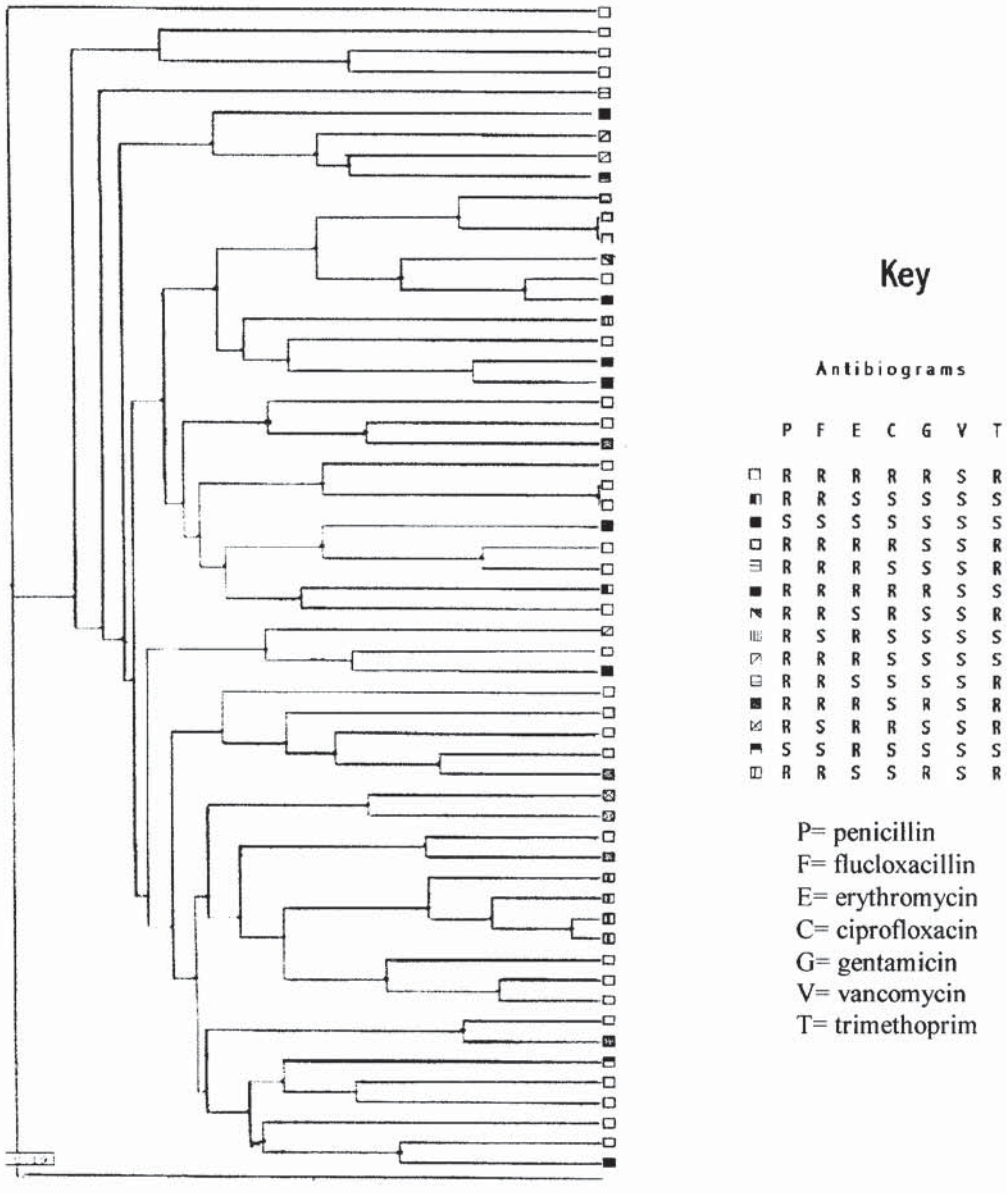


Figure 9.6 constructed to relate genotype to antibiogram pattern using colour code (see key)

Figure 9.7 **Key and UPGMA dendrogram using the Dice correlation coefficient based on *Sma* I macrorestriction fragment profiles of 56 strains of CNS. Dendrogram demonstrates the correlation between genotype and slime production within the strains.**

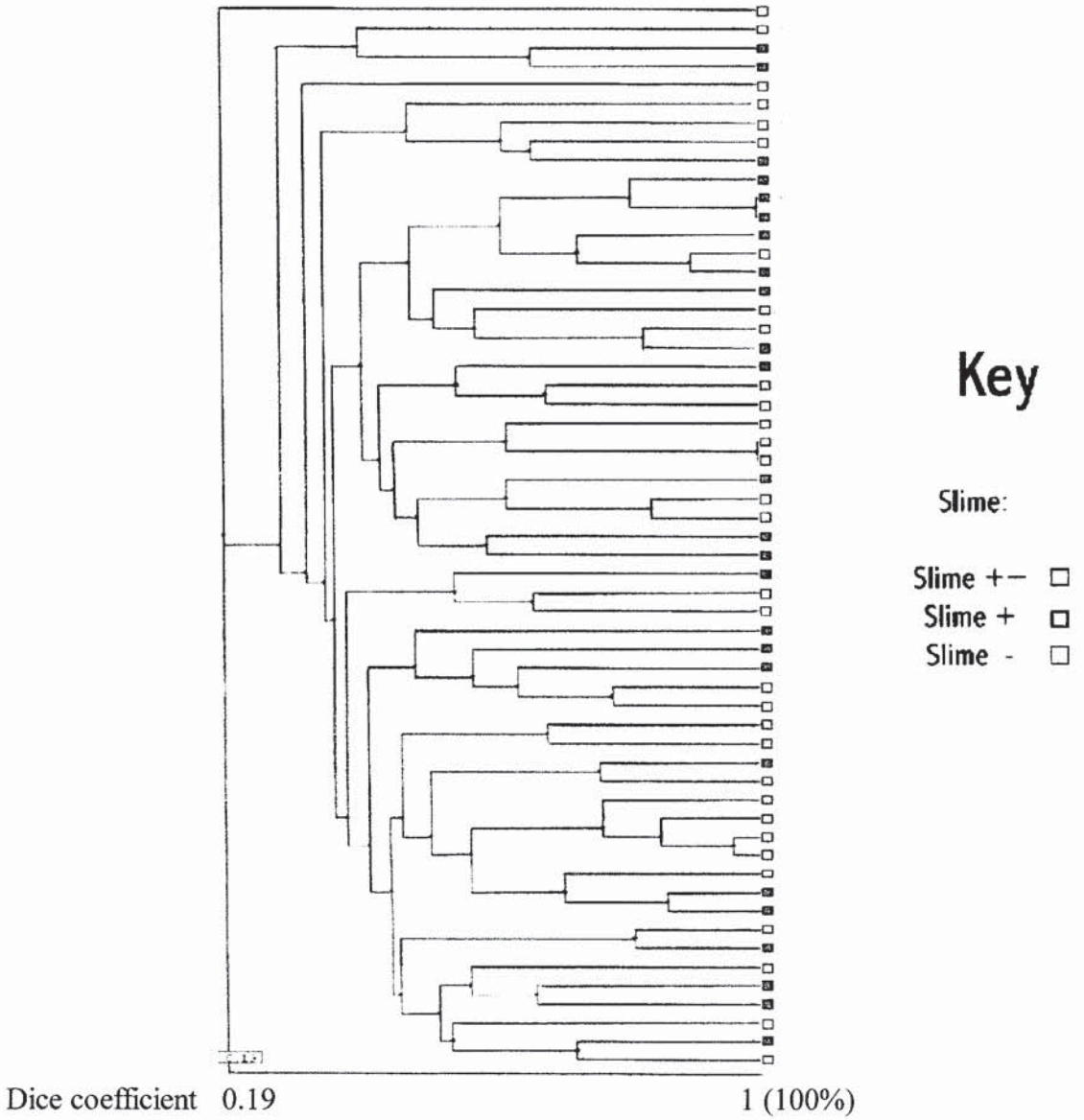


Figure 9.7 constructed to relate genotype to slime production using colour code (see key)

9.4 Discussion

The aim of this current study was to use phenotypic and genotypic typing methods to determine the relatedness of strains of CNS isolated from patients with CRS. *S. epidermidis* is the species of CNS most frequently isolated from the blood cultures of patients with intravascular related sepsis (Rupp and Archer, 1994) and methods of typing microorganisms must be able to identify individual strains within the species if they are to be of any value in epidemiological studies.

In this present study, 63 out of 65 (97%) of the CNS were identified as *S. epidermidis* by the API STAPH system (bioMérieux, France) and 10 different biotypes were obtained (table 9.0). However, 84% of the isolates clustered into 3 major biotypes that showed only minor differences in profile. Indeed, the 2 major biotypes that accounted for 70% of the total were differentiated on the basis of D-mannose fermentation, a reaction that is subjective in its interpretation. This is similar to the findings of Geary *et al.* (1997), who demonstrated that 72% of *S. epidermidis* isolated from nososcomial infections clustered into 3 major biotypes when identified by ATB 32 Staph™ (bioMérieux, France). The API STAPH system lacked power in discriminating between strains of *S. epidermidis* in this study, however it may be of value in identifying strains with unusual biotypes or in epidemiological investigations involving other species of staphylococci, for example *S. lugdunensis*.

Antimicrobial susceptibility testing against a panel of 7 antibiotics divided the 63 isolates of *S. epidermidis* into 14 antibiogram types. Discrimination amongst the strains was low and a single antibiogram reflecting multi-resistance predominated, which accounted for 46% of the types. Typing strains of *S. epidermidis* by antimicrobial susceptibility testing therefore proved to be of limited value in this current study. Antimicrobial resistance is increasing in many microbial pathogens reflecting *de novo* selection due to overuse of antibiotics and spread of resistant microorganisms in the hospital and community. Many of the strains of

S. epidermidis in this current study were resistant to all antibiotics included in the panel with the exception of vancomycin. However, strains of CNS and *S. aureus* with reduced susceptibility to vancomycin have now been reported (Sieradzki and Tomasz, 1997; Rahman, 1998; Sieradzki *et al.*, 1999).

Heterogeneity of strains of *S. epidermidis* isolated from 24 patients with a CRS was confirmed in this study by use of both molecular and conventional characterisation techniques. There was no correlation between the phenotype and genotype of the isolates as determined by PFGE, API, antibiograms and slime production, demonstrating that detailed information on potential sources of infection and nosocomial outbreaks caused by CNS cannot be obtained from phenotypic typing methods alone. Multiple blood culture isolates of CNS from the same patient in some instances also exhibited differing antimicrobial sensitivity patterns despite having the same biotype and genotype, supporting the observations of Geary *et al.* (1997). The isolation of CNS from multiple blood cultures therefore needs careful interpretation, as microorganisms shown to have similar phenotypic characters may in fact be distinct. This also raises the possibility that any one episode of CRS may be caused simultaneously by several strains of CNS.

Of the 23 patients with a nosocomially acquired catheter-related sepsis, 6 (26%) had a strain isolated from their blood cultures with a closely related genotype. This observation is similar to the 31% of genotypically related CNS isolated from sequential blood culture obtained from neonates using PFGE (Zaidi *et al.*, 1996). In a further study on neonatal bacteraemias using multilocus enzyme electrophoresis, 9% of the CNS strains were similar (Tan *et al.*, 1994), again suggesting a common source. Four isolates from 4 patients in our study had similar macrorestriction patterns when the PFGE gels were analysed using the criteria of Tenover *et al.* (1995). A further 2 patients had isolates which differed by no more than 2 bands. Isolates with identical macrorestriction patterns are considered to be clonal. Similarly, strains with only 1 or 2 band shifts are consistent with a single genetic event (point mutation resulting in

the loss or gain of a restriction site, an insertion, a deletion, or a chromosomal inversion) and are also considered to be clonally related (Tenover *et al.*, 1995). The common strain was isolated within a 2 week period from three patients located on a haematology unit and ITU, and again from a further two patients on the haematology unit one month later. The clinical data of these patients strongly suggested that they were nosocomially acquired and that there was a common source. Attempts to identify the source and carriage of this specific strain, for example by health care workers, were confounded by the numerous strains isolated from the various sources. Modes of transmission via medical personnel have however been investigated in a study of patients undergoing cardiac surgery in Rhode Island, USA, where a common isolate of CNS was found on the hands of a surgeon (Boyce *et al.*, 1990). The common genotype was isolated again from 1 more patient on the renal unit 4 months later which demonstrated the ability of this organism to persist in the hospital environment for extended periods of time.

In a recent study which investigated the genotypes of CNS isolated from central venous lines, continuous ambulatory peritoneal dialysis catheters and patients with endocarditis, the genetic heterogeneity of the strains was demonstrated using PFGE (Lang *et al.*, 1999). The majority of isolates were community-acquired and no evidence of cross-infection was demonstrated. In this current study however, 26% of the CNS isolated from the blood cultures of patients with nosocomially acquired CRS had identical or closely related genotypes suggesting possible cross-infection of a specific strain within the hospital. These preliminary studies suggest that whereas a diverse spectrum of CNS genotypes is associated with CRS, a significant percentage appear to be associated with cross-infection. This may reflect the propensity for certain strains of *S. epidermidis* to cause CRS and that outbreak strains may be occurring in the hospital environment previously undetected. Infection control teams need to consider this possibility when investigating central venous catheter-related sepsis.

In summary, conventional typing methods and PFGE were used to investigate the phenotypic and genotypic characteristics of isolates of *S. epidermidis* associated with CRS. The discriminatory power of phenotypic typing methods including biotyping, antibiogram typing and slime production was low, and although relatively inexpensive and simple to use, the methods were of limited value in this epidemiological study. Heterogeneity of the strains was demonstrated amongst the majority of strains isolated from patients with CRS by PFGE, which identified numerous genotypes. In addition, it was also shown that the phenotypic characteristics of closely related or identical genotypes may vary during the course of an infection. A common strain of *S. epidermidis* with a closely related genotype was isolated from 26% of patients with a nosocomially acquired CRS. This highlighted the discriminatory power of PFGE in identifying micoorganisms that may be associated with cross-infection and which would remain undetected if conventional typing methods alone were used.

Chapter 10 Application of the lipid S ELISA to prosthetic joint infection due to staphylococci

10.1 Introduction

Staphylococcus aureus and coagulase negative staphylococci (CNS) are the most common causes of prosthetic joint infection, with the CNS accounting for 20 to 40% of cases (Rupp and Archer, 1994). The incidence of infection following joint replacement has ranged from 0.6 to 11%, however, a 1 to 2% infection rate is often reported (Rupp and Archer, 1994). Early and accurate diagnosis of infection is a major problem and it is particularly important to distinguish between infection and mechanical (aseptic) loosening since the treatment of these conditions is different (Ling, 1984). False positive cultures arising from skin contaminants make the interpretation of microbiological culture from joint aspirates difficult. A major prospective evaluation of criteria for microbiological diagnosis of prosthetic joint infection recommended that the cut-off for a definite diagnosis of infection should be three or more operative specimens that yield an indistinguishable organism (Atkins *et al.*, 1998).

Current tests available to aid the preoperative diagnosis of prosthetic joint infection include erythrocyte sedimentation rate, C-reactive protein, plain radiography, isotope scans, aspiration, biopsy and histology (Garvin and Hanssen, 1995). A characteristic feature of infections of prosthetic devices, as with indwelling intravenous catheters, is the biofilm mode of growth in which the organisms adhering to the components of the device are embedded. It therefore follows that exocellular antigens produced by the microorganisms which are released from the biofilm layer on prosthetic joints, might stimulate a stronger antibody response than cellular antigens. Krikler and Lambert (1992) utilised exocellular proteins of *S. aureus* to determine the antibody levels in patients with osteomyelitis associated with artificial joints, and found the IgG response towards these antigens to be significantly higher than in patients with uninfected joints or in healthy normal controls. In

addition, Lambert *et al.* (1996) investigated the serodiagnostic potential of an unidentified partially purified exocellular antigen produced by a single CNS strain grown in brain heart infusion broth, and demonstrated elevated antibody levels to the antigen in patients with orthopaedic infections. Both investigations therefore clearly highlighted the potential of a serological approach to the diagnosis of osteomyelitis, by utilising exocellular antigenic material produced by staphylococci. The aim of this part of the study was to determine the levels of serum antibody in patients with prosthetic joint infection due to staphylococci utilising the lipid S ELISA, and assess the potential of the assay in aiding the diagnosis of osteomyelitis associated with prosthetic joints.

10.2 Materials and Methods

10.2.1. Patients with osteomyelitis associated with prosthetic joints

A prospective study was undertaken between July 1997 and July 1998. Strict inclusion criteria for the trial were used. Any patient with a recent history (within the last six months) of possible infection including the following were excluded: -

1 Upper respiratory infection or urinary tract infection
2 Abscess
3 Dental sepsis or procedures
4 Invasive urological procedures
5 Intra-abdominal sepsis
6 Osteomyelitis
7 Subacute bacterial endocarditis
8 Skin disorders (dermatitis, psoriasis, eczema)

Ethical committee approval and patient consent was obtained prior to entry into the study. Fifteen patients with prosthetic joint infection were included in the trial. The preoperative clinical evaluation assessed pain, function and range of motion following the Merle D`Aubingne and Postel scale (Merle *et al.*, 1954). Anteroposterior and true lateral radiographs of the pelvis were obtained prior to surgery. A complete blood cell count, erythrocyte sedimentation rate and C-reactive protein was obtained from 10 out of 15 patients. In addition, 10 ml of blood was obtained from each patient for anti-lipid S determination. All the operations were performed by senior surgeons who used the same operative technique. The revision prosthetic joint surgery was performed in an operating suite with horizontal laminar flow. Intraoperative antibiotics were withheld until appropriate cultures were taken from the joint capsule, acetabulum and femur using sterile instruments to biopsy each site. When phenotypically identical microorganisms were

isolated from all three sites and matched the clinical presentation, the patient was considered to meet the criteria for prosthetic joint infection.

10.2.2 Control patients

Thirty two patients were included in the control group of which 21 had closed recent fractures, and 11 had primary osteoarthrosis of the hip. Ten millilitres of blood was obtained from each patient for anti-lipid S determination.

10.2.3 Lipid S ELISA

Antibody titres of 15 patients with osteomyelitis associated with a prosthetic joint and 32 controls were determined with the lipid S ELISA (section 3.2.5). The IgM and IgG titres were estimated by reference to standard positive control titres of 1:25,000 and 100,000 respectively (chapter 3). In addition, IgM titres were determined in serum samples pre-treated with GullSORB™ reagent (chapter 7).

10.2.4 Statistical Analysis

The Mann U Whitney test was used to determine the significance of differences between the test and the control groups.

10.2.5 Culture of microbiological samples from patients with prosthetic joint infection

Blood culture and aspirates received from patients with prosthetic joint infection were processed according to the laboratory standard operating procedure. This involved incubation of the blood sample for 7 days in liquid blood culture medium (Oxoid,

Basingstoke, U.K.). Positive blood cultures were subcultured onto solid agar, which included: blood agar containing 7% defibrinated horse blood, incubated in 5% CO_2 and anaerobically at 37°C for 48 h; cysteine lactose electrolyte deficient agar (CLED) and chocolate agar incubated in 5% CO_2 for up to 48 h. Samples of soft tissue were homogenised prior to inoculation into liquid blood culture, which was incubated for up to 21 days at 37°C. Samples of spinal, acetabular, capsular and femoral tissue were placed into 15 ml of Robertson's cooked meat enrichment media and incubated for up to 21 days at 37°C.

10.3 Results

10.3.1 Patients demographics

The demographic details of patients with prosthetic joint infection and controls are shown in table 10.0

Table 10.0 Demographic details of patients with prosthetic joint infection and controls

	Prosthetic joint infection	**Controls**
Number (n)	15	32
Mean age (range)	69 (34-82)	52 (16-84)
Male:Female ratio	7:8	20:12

10.3.2 Patients with prosthetic joint infections

Positive serum IgG and IgM levels for patients with infected prosthetic joints were indicated by titres of $\geq$ 20,000 and $\geq$ 5,000 respectively (chapter 4) and are shown in figures 10.0 and 10.2. The results for the control group are shown in figures 10.1 and 10.3. The statistical analysis of the anti-lipid S titres from patients with infected prosthetic joints is shown in table 10.1. Coagulase negative staphylococci (CNS) were isolated from fifteen patients under going revision joint surgery. In all cases the same microorganism was cultured from 3 different tissue specimens. The CNS were identified in accordance with the laboratory standard operating procedure and their phenotypic relatedness was confirmed by identical antibiogram patterns. Fourteen patients (93%) with septic loosening of joint prostheses due

to Gram- positive bacteria had positive serology, 3 out of 13 (23%) patients in this group had elevated IgM titres. Serum IgG levels of patients with prosthetic hip infection due to CNS were significantly different from control IgG titres ($p = <0.0001$), however there was no significant difference in the IgM titres ($p = 0.7903$). A statistical analysis of the serum anti-lipid S levels obtained from patients with prosthetic joint infection due to staphylococci is shown in table 10.1.

10.3.2.1 ESR, CRP and WCC of patients with prosthetic joint infections

The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and white cell count was measured in 10 patients with septic loosening of joint prostheses due to Gram-positive bacteria. The ESR was elevated above 30-millimetres per hour in all patients. The CRP was > 6ng/l in 7 out of 10 (70%) patients, and normal in the remainder. The white cell count in all ten patients was within the normal range. The laboratory parameters were also measured in the control patients and none were abnormal.

10.3.3 Anti-lipid S levels in control patients

One patient had a raised IgG titre (22,000), representing a false positive rate of 3%. Serum IgM titres were determined in 21 patients from this group and 3 (14%) had elevated levels. Positive IgG and IgM levels for control patients were indicated by titres of ≥ 20,000 and ≥ 5,000 respectively (chapter 4) and are shown in figures 10.1 and 10.3. The statistical analysis of the anti-lipid S titres is shown in table 10.1.

Table 10.1 Statistical analysis of anti-lipid S serum titres from patients with prosthetic joint infections and controls

	Prosthetic joint infection	Controls
IgM (range)	500-12000	500-13000
IgM (mean)	3990	3500
IgM (SD)	4094	3136
IgG (range)	18000-200000	500-22000
IgG (mean)	65000	8500
IgG (SD)	47451	5136

Figure 10.0 Anti-lipid S serum IgG titres from patients with prosthetic hip infection due to staphylococci. Positive IgG cut off titre of 20,000 is indicated

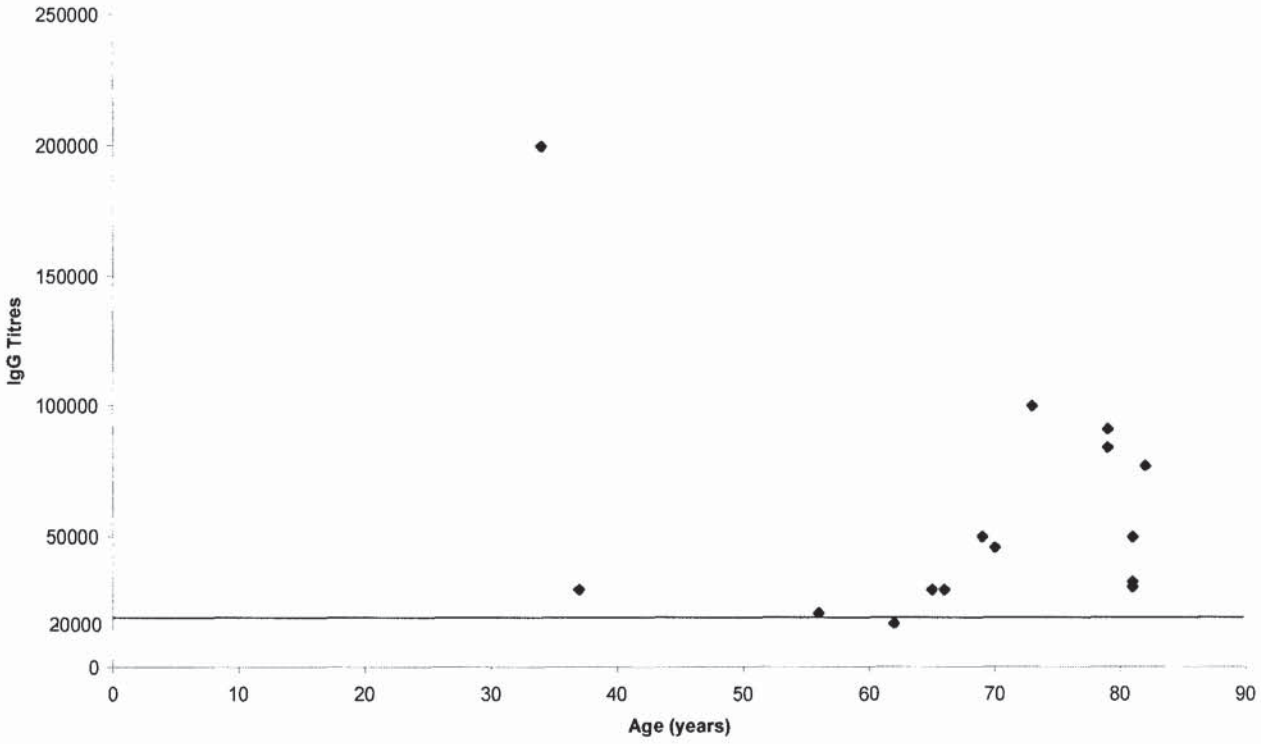


Figure 10.1 Anti-lipid S serum IgG titres from the control group of patients. Positive cut off titre of 20,000 is indicated

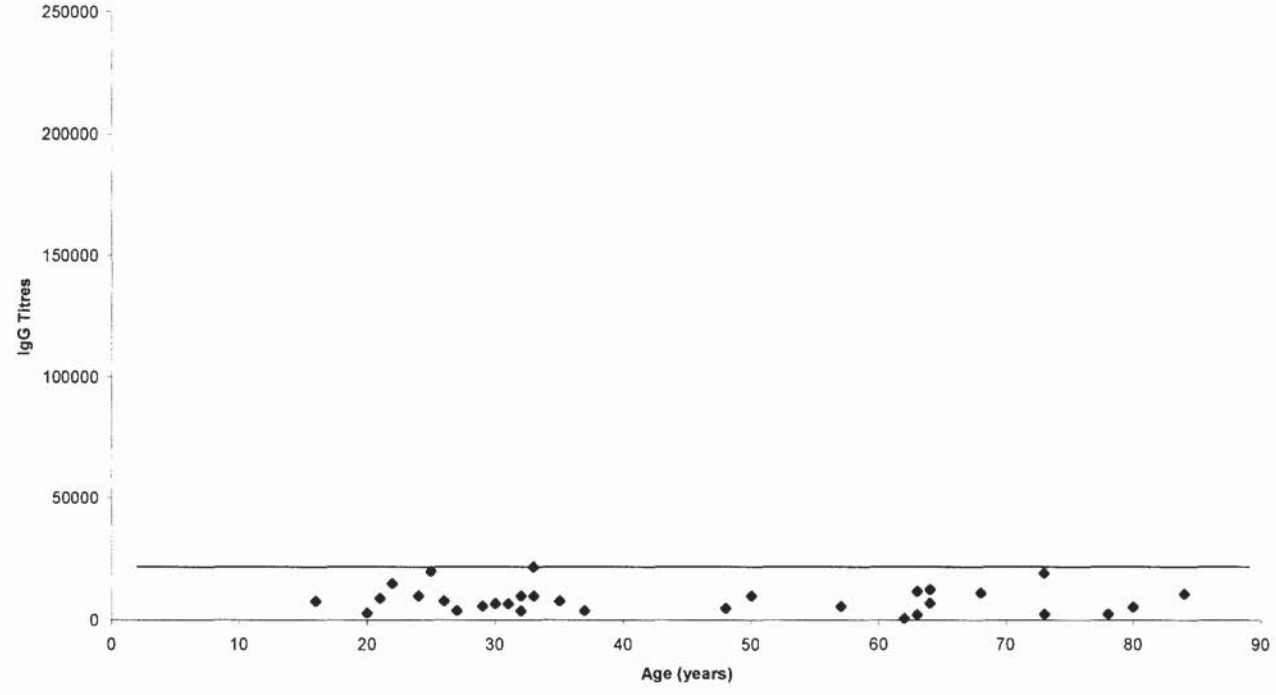


Figure 10.2 Anti-lipid S serum IgM titres from patients with prosthetic joint infection due to staphylococci. Positive cut off titre of 5,000 is indicated

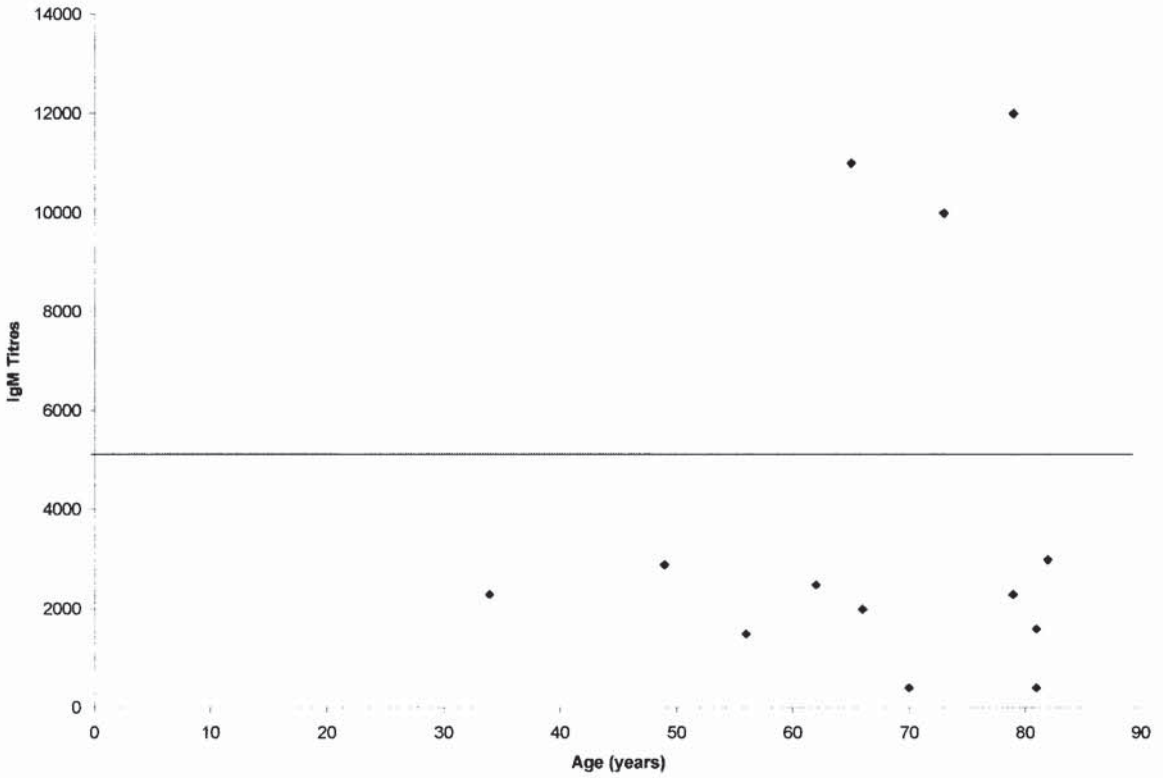
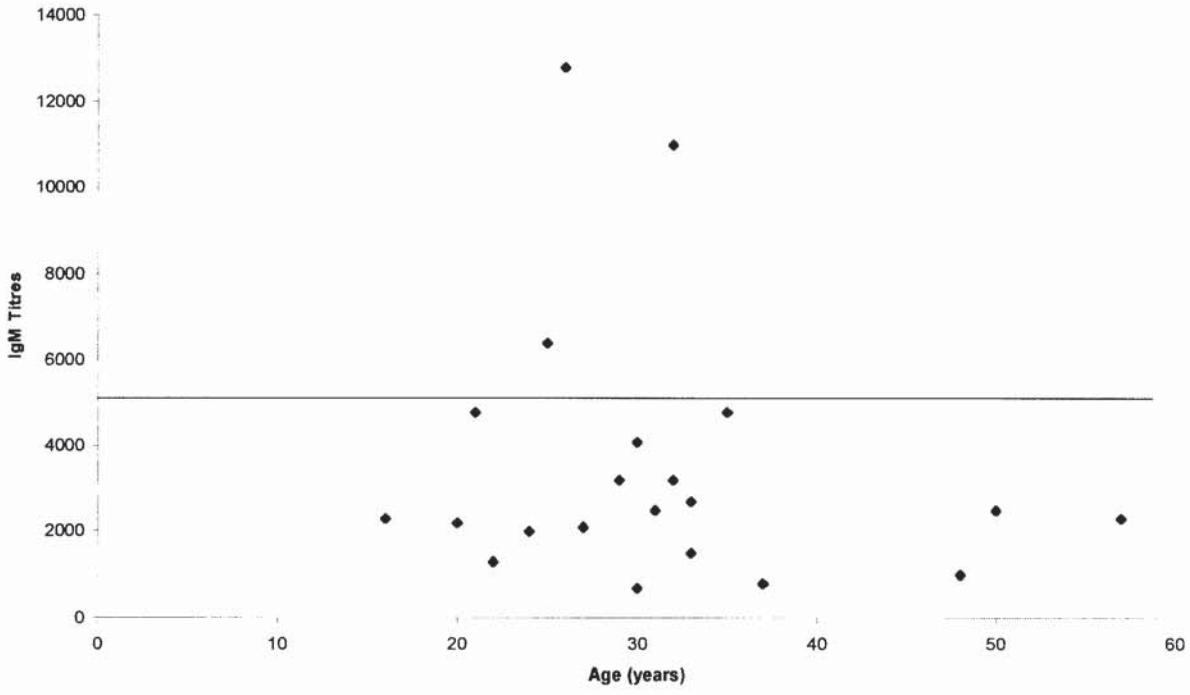


Figure 10.3 Anti-lipid S serum IgM titres from the control group of patients. Positive cut off of 5,000 is indicated



10.4 Discussion

It is important to distinguish loosening of a joint prosthesis that is due to low-grade sepsis from aseptic loosening, as the management of the revision procedure is different for the two conditions (Ling, 1984). Early identification of the infecting microorganism would also influence the antibiotic therapy in the perioperative period. However, at present, there is no single method to distinguish reliably between septic and aseptic loosening. Laboratory tests, including ESR and CRP measurements, have been determined in patients with total hip arthroplasty, and may aid in distinguishing mechanical loosening from sepsis if elevated (Shih *et al.*, 1987). However, an elevated CRP level, which is more indicative of infection than a rise in the sedimentation rate, may still be nonspecific (Shih *et al.*, 1987). In the study described in this thesis, the ESR was elevated in all patients whilst CRP was raised in 70%. Joint aspiration is used as a routine test prior to all revision procedures by some surgeons, whereas others use it more selectively in patients in whom they consider the presence of infection to be more likely. The accuracy of aspiration of the hip joint remains controversial. Some authors have reported a positive diagnosis of infection in up to 90% of hips that are infected (Phillips and Kattapuram, 1983), whereas others have found positive results in only 10% of cases (Barrack and Harris, 1993).

In this current study, the relatedness of the CNS isolated from the tissue samples was determined using antibiogram patterns, which have low discrimination (Geary *et al.*, 1997; chapter 9). To confirm the isolates as being identical, however, molecular genotyping by pulsed field gel electrophoresis would be necessary. Serum IgG and IgM levels to the novel lipid S antigen in patients with positive joint prosthetic infections due to the CNS were determined preoperatively. Serum IgM levels were not significantly elevated in comparison to the controls ($p = 0.7903$). This may be a reflection of the time of serum sampling during the course of the infection, and elevated IgM titres at the onset of sepsis may have been missed. Moreover, previous work in this thesis has shown the anti-lipid S IgM ELISA to lack sensitivity and specificity (chapters 4 and 7). The potential of the IgM assay in the diagnosis of prosthetic joint infection therefore remains unclear. Further studies are required to determine the baseline levels of IgM in patients prior to prosthetic implant with

subsequent monitoring of titres post operatively. However, serum IgG levels to this novel antigen were significantly elevated in comparison to the controls ($p<0.0001$). Using a threshold of 20,000 to indicate positivity (chapter 4), the sensitivity and specificity of the anti-lipid S IgG ELISA were 93% and 97% respectively. The results from the lipid S IgG ELISA may provide useful preoperative information in evaluating painful total joint prostheses.

In addition to aiding the serodiagnosis of catheter-related sepsis, the lipid S ELISA may provide a rapid, sensitive and inexpensive serodiagnostic method for the detection of Gram-positive bacterial infection of prosthetic joints, which could be easily adapted into the routine microbiology laboratory (chapter 8). The test may also assist in the management of patients, from whom microbiological results are either negative (for example due to antibiotic use), or inconclusive due to the isolation of a microorganism from a single tissue sample, which may be a contaminant or a possible pathogen. The ELISA needs further evaluation to confirm its value in the clinical setting.

In summary, the lipid S ELISA detected elevated serum levels of IgG in patients with prosthetic joint infection due to staphylococci. The assay may therefore serve as a useful adjunct in the diagnosis of prosthetic joint infection, and aid in the differentiation between infection and aseptic loosening. In addition, the ELISA may facilitate the interpretation of microbiological cultures from patients with prosthetic joint infection, where contamination due to skin microorganisms makes diagnosis difficult. However, further studies are required to determine the anti-lipid S levels in more patients with prosthetic joint infection.

Chapter 11 Application of the lipid S ELISA to spinal infection and the association between *Propionibacterium acnes* and sciatica

11.1 Introduction

11.1.1 Pyogenic spondylodiscitis

Although pyogenic spondylodiscitis is a relatively uncommon disease, the incidence is rising and has been associated with the increased use of indwelling intravenous devices and resultant bacteraemia (Torda *et al.*, 1995). The vertebral bodies are a typical site of haematogenous osteomyelitis and spondylodiscitis due to CNS associated with infected intravascular devices has been reported (Bucher *et al.*, 2000). In addition to iatrogenic infection there is also evidence for spontaneous development of discitis where a common cause is preceding infection of the genito-urinary tract. Microorganisms may also gain access to the intervertebral disc space due to intra-operative contamination (El-Gindi *et al.*, 1976; Tronnier *et al.*, 1992; Lindholm and Plykkänen, 1982). The most common microorganisms associated with infection of the intervertebral space are *S. aureus* and *S. epidermidis* (Torda *et al.*, 1995; Tronnier *et al.*, 1992).

The diagnosis of spondylodiscitis is often difficult and may be complicated non-specific results of many investigations. For example, infection, malignancy or degenerative joint disease may not be distinguished by radiological investigations (Torda *et al.*, 1995) and evident abnormalities may take 10 days or more to appear. Narrowing of the disc space is an early radiological finding, however this is non-specific, particularly in the elderly patient. Bone scanning procedures have a sensitivity of >90% and a specificity of approximately 78% but they do not reliably distinguish between infection, malignancy or trauma (Torda *et al.*, 1995). CT (computerised tomography) and MRI (magnetic resonance imaging) scans are the most sensitive and specific methods for detection of spondylodiscitis. Other investigations to aid in the diagnosis of spondylodiscitis include erythrocyte sedimentation rate, C-reactive protein and white blood cell count.

Microbiological investigation of the disease should include culture of both blood and if clinically appropriate bone biopsy, to support the diagnosis. Positive bone biopsy culture results along with supporting histological evidence provide the most definitive diagnostic evidence, however previous antimicrobial therapy and biopsy sampling error may result in misleading negative cultures. The serological response to lipid S in patients with CRS was investigated in the previous chapters of this thesis, and elevated levels of antibody were detected in many of the patients with sepsis due to staphylococci. The aim of the first part of this current study was to determine the serum levels of IgG and IgM to lipid S in patients with pyogenic spondylodiscitis, and assess the potential of the ELISA to support diagnosis of this infection.

11.1.2 An association between *Propionibacterium acnes* and sciatica

Patients with sciatica who were attending the spinal clinic at The Royal Othopaedic Hospital, Birmingham were included in the pyogenic spondylodiscitis study as the initial case-control group. Surprisingly, it was found that 31% of patients with sciatica had elevated serum IgG titres to the novel antigen in the ELISA and none of these patients had had infections in the previous 6 months to offer an explanation for the result. The elevated levels of serum IgG associated with sciatica raised the possibility of an unrecognised microbial cause, possibly with microorganisms of low virulence. Following these unexpected serological results, the possible association between microorganisms and sciatica was investigated and is described in the second part of this chapter.

The pathogenesis of low back pain and sciatica remains poorly understood despite being one of the commonest causes for consultation in primary care (OPCS, 1995; Biering-Sørensen and Bendix, 2000). Indeed, approximately 80% of all people will experience low back pain at some time in life and in most cases the cause is unknown (Biering-Sørensen and Bendix, 2000). The most common cause of true sciatica is a prolapsed intervertebral disc (herniated nucleous pulposus). Discogenic radiculitis is caused by pressure due to the herniated disc pressing on the sciatic nerve, which results in unilateral leg pain and may also involve the

knee and foot. Patients presenting with severe lumbosciatic pain may be treated with epidural corticosteroid injections, (e.g. methylprednisilone), which aid in pain relief and improved mobility, particularly if administered in the acute phase of the illness (Buchner *et al.*, 2000). In patients where sciatica does not resolve, CT or MRI scan are performed to examine for soft tissue inflammation and bone abnormalities which may subsequently require removal by microdiscectomy.

It is recognised that discogenic radiculitis is not caused by pressure alone and that the pathophysiology involves an inflammatory process. Cytokines including IL -1α have been associated with lumbar disc herniation (Takahashi *et al.*, 1996). Surgically induced disc lesions in animal models have similarly involved an inflammatory reaction in the nucleus pulposus with the production of IgG in surrounding tissues (Pennington *et al.*, 1988). The inflammation around the nerve root was considered to be an auto-immune reaction acting locally. Marshall *et al.* (1977) have also demonstrated raised serum immunoglobulin levels in patients with sciatica as compared to controls.

Over the last two decades the skin commensals, including coagulase-negative staphylococci (CNS) and propionibacteria, originally considered as primarily contaminants when isolated from patients, are being increasingly recognised as aetiological agents of infection. Tunney *et al.* (1998) for example, have demonstrated the presence of *Propionibacterium acnes* and CNS *in situ* on prosthetic joints studied at revision arthroplasty. Furthermore, *P. acnes* has also been associated with vertebral osteomyelitis in a patient who underwent microdiscectomy (Noble and Overman, 1987) whilst more recently, CNS have been implicated with cerebral palsy in neonates (Mittendorf *et al.*, 1999).

The role of propionibacterium species in the aetiology of human infection is well established, and the microorganism has been associated with skin infection (Brook and Frazier, 1991), endocarditis, endophthalmitis, central nervous system infections associated with shunts, arthritis, prosthetic joint infection and osteomyelitis (Funke *et al.*, 1997).
P. acnes produces many extracellular factors which may aid in its pathogenicity including proteinases, lipase, bacteriocins, phospholipase C, neuramidase, histamine and

hyaluronidase (Eady and Ingham, 1994). In addition, *Propionibacterium* species possess immunostimulatory mechanisms including the activation of complement, stimulation of lysosomal enzyme release from neutrophils, and the production of serum-independent neutrophil chemotactic factors (Brook and Frazier, 1991).

The aim of this part of the present study was to further investigate patients with sciatica based on the initial unexpected elevated serological results obtained with the lipid S ELISA. A prospective study was undertaken to determine the serum IgG levels by the rapid ELISA (chapter 8), in those patients with severe lumbosciatic pain requiring microdiscectomy. In addition, to attempt to fulfill Koch's postulates and support the hypothesis that the elevated levels of anti-lipid S IgG were associated with microbial infection, spinal material removed during surgery was cultured to investigate for the presence of microorganisms.

11.2 Materials and Methods

11.2.1 Patients with pyogenic spondylodiscitis

Ten patients with a clinical diagnosis of pyogenic spondylodiscitis were recruited into the study. Patients were diagnosed as having spondylodiscitis after presenting at the spinal clinic with severe back and leg pain, fever and night sweats and not responding to pain relief treatment for sciatica. Following MRI scan, all patients underwent spinal surgery where samples of spinal tissue, pus and aspirates were taken for microbiological culture. In addition, 10 ml of blood was obtained from each patient for anti-lipid S determination.
Blood cultures, aspirates and spinal tissue received from patients with pyogenic spondylodiscitis were cultured in accordance with the laboratory standard operating procedure (10.2.5).

11.2.2 Control patients

Thirty two patients were included in the control group of which 21 had closed recent fractures, and 11 had primary osteoarthrosis of the hip. Ten millilitres of blood was obtained from each patient for anti-lipid S determination.

11.2.3 Patients with sciatica

As described in the introduction to this chapter, this group of patients was included as controls for the study of anti-lipid S titres in patients with pyogenic spondylodiscitis. However, the preliminary results demonstrated that 31% of patients with sciatica had elevated anti-lipid S serum IgG levels. In response to these unexpected results a separate, prospective study was undertaken to determine anti-lipid S titres in patients with sciatica and to investigate a possible microbial cause for the elevated antibody levels.

11.2.3.1 Inclusion criteria

Patients were diagnosed as having sciatica based on the following criteria (Kellgren, 1941):

1. **Predominant complaint of root pain**: indicated by the presence of localised unilateral leg pain radiating to mid calf or below, which was more severe than back pain or parasthesia.

2. **Root irritation signs**: as indicated by the reproduction of root pain or parasthesia when an irritable nerve was stimulated.

3. **Root compression signs**: as indicated by muscle wasting or weakness, sensory alteration and reflex depression (but not decreased calf circumference or a depressed ankle jerk alone).

11.2.3.2 Exclusion criteria

Strict exclusion criteria were adopted for patients presenting with pyogenic spondylodiscitis and sciatica (10.2.1).

11.2.3.3 Estimation of anti-lipid S IgG serum levels in patients with sciatica

Serum IgG titres of 140 patients presenting with sciatica were determined by the rapid lipid S ELISA which incorporated anti-human IgG peroxidase conjugate (chapter 8, table 8.1).

11.2.3.4 Determination of cross reactivity of anti-lipid S serum IgG and vertebral disc material in patients with sciatica

Fresh intervertebral disc material was obtained from patients undergoing surgery for scoliosis. The disc material was snap frozen and cryostat tissue sections were cut, mounted on glass and fixed in acetone. Serum samples from 6 patients with high anti-lipid S titres and 6 patients with low levels were selected and incubated with the tissue sections for 1 h at room temperature. The slides were washed in TBS-Tween buffer and further incubated with anti-human IgG peroxidase (Sigma, U.K.) for 30 min at room temperature. After washing with TBS-Tween, chromogenic development was achieved by incubation of the tissue section in diaminobenzidine for 10 min at room temperature. Control slides of fixed tissue sections, which were not incubated with patient sera, were also included.

11.2.3.5 Culture of microdiscectomy tissue samples from patients with severe sciatica

Fifty-one samples of microdiscectomy tissue were received from 51 patients undergoing surgery for severe lumbosciatic pain. In addition, corresponding samples of clotted blood for anti-lipid S IgG estimation were received from 47 of the patients.

Using strict aseptic technique, microdiscectomy tissue was obtained for culture. Further samples obtained were stored frozen at -20°C. Due to the limited amount of tissue sample available for culture, material was placed into 15 ml of Robertson's cooked meat enrichment broth (Oxoid, U.K.) and incubated for up to 21 days (Funke *et al.*, 1997). Enrichment broths were subcultured onto blood agar containing 7% defribinated horse blood (Oxoid, U.K.) at 2, 14 and 21 days and incubated in 5% CO_2 and anaerobic conditions for 7 days, after which plates were examined for microbial growth.

11.2.3.6 Culture and microscopy of microdiscectomy tissue stored at -20°C

Sixteen samples of microdiscectomy tissue yielded *P. acnes* through enrichment culture (11.2.3.5). To determine whether strains of *P. acnes* isolated by enrichment culture could be recovered from the original sample, 10 stored samples of microdiscectomy tissue from patients who were culture positive for *P. acnes*, were removed from storage at -20°C and thawed at 4°C. Using stringent aseptic technique, a piece of each tissue was removed for thin section preparation and microscopy, and the remainder of each pressed 10 times over the surface of a blood agar plate containing 7% defribinated horse blood (Oxoid, U.K.). Each sample was finally submerged into the blood agar plate and incubated in anaerobic conditions for 72 h, after which the plates were examined for microbial growth. In addition, thin tissue section slides were prepared, Gram stained and examined for the presence of microorganisms.

11.2.3.7 Statistical analysis of culture and serology results

The Fischer exact test was used to compare patients with positive culture and serology with those having negative culture and negative serology.

11.2.3.8 Identification of microorganisms isolated from microdiscectomy tissue

11.2.3.8.1 Gas liquid chromatography for the detection of volatile fatty acids produced by Gram-positive bacilli

One colony of Gram-positive bacilli was inoculated into 15 ml Robertson's cooked meat enrichment broth (Oxoid, U.K.) and incubated at 37°C for 72 h. Two hundred microlitres of 50% sulphuric acid (BDH Ltd, U.K) and 1 ml of diethylether (BDH Ltd, U.K.) were added

to 1ml of culture broth and mixed by repeated inversion for 1 min. After centrifugation at 3000 rpm for 1 min, the upper solvent layer was removed and 1 µl loaded onto a Hewlett Packard HP-1 capillary column (crosslinked methyl silicone gum, ID 0.32 mm, film thickness 0.17 µm, length 25 m) on a Unicam 610 gas chromatograph. Volatile fatty acids were identified by comparison with the profile obtained for 1 µl of a Supelco volatile acid standard mix. The carrier gas phase was nitrogen 13 psi and the flame ionisation detector used hydrogen 15 psi and air 5 psi.

11.2.3.8.2 Biotyping of microorganisms

Isolates of anaerobic Gram-positive bacilli were identified with API 32 ATB (bioMérieux, Marcy-l'Etoile, France). Aerobic Gram-positive bacilli were identified with API Coryneform (bioMérieux, Marcy-l'Etoile, France). Isolates of coagulase negative staphylococci (CNS) were identified with API STAPH (bioMérieux, Marcy-l'Etoile, France). Identification procedures were performed in accordance with the manufacture's instructions.

11.2.3.9 Detection of exocellular lipid S production by P. acnes

To assess whether lipid S was produced by *P. acnes,* 1 strain which was isolated from the microdiscectomy tissue of a patient with severe sciatica and an elevated anti-lipid S IgG titre, was selected for investigation. The isolate was cultured in brain heart infusion (BHI) broth for 72 h under anaerobic conditions, after which the culture supernatant was concentrated 10-fold by freeze drying and applied to a gel permeation column and the eluting fractions assayed to determine the presence of lipid S (chapter 2, sections 2.2.2 and 2.2.4). In addition, to identify any components unrelated to the material in the culture supernatant, uninoculated BHI broth was applied to the column as a control.

11.3 Results

11.3.1 Patients demographics

The demographic details of patients with pyogenic spondylodiscitis and controls are shown in table 11.0

Table 11.0 Demographic details of patients with pyogenic spondylodicitis and controls

	Pyogenic spondylodiscitis	**Controls**
Number (n)	10	32
Mean age (range)	57 (21-80)	52 (16-84)
Male:Female ratio	5:5	20:12

11.3.2 Microbiological culture results and anti-lipid S titres from patients with pyogenic spondylodiscitis

The clinical samples received, culture results and anti-lipid S titres from 10 patients with pyogenic spondylodiscitis are shown in table 11.1. Spinal tissue was received from 9 out of 10 (90%) patients and corresponding blood cultures were received from 2 out of 9 (22%) patients. *S. aureus* (44%) and the CNS (44%) were the most frequently isolated microorganisms from spinal tissue of patients with pyogenic spondylodiscitis. A beta-haemolytic streptococcus (Lancefield group B) was isolated from 1 (22%) patient. *Mycobacterium tuberculosis* was also isolated in addition to CNS from 1 patient. Seven patients (70%) had elevated levels of anti-lipid S IgG and 8 (80%) had positive IgM titres.

The statistical analysis of the anti-lipid S titres is shown in table 11.2. There was a significant difference between IgM titres (p = <0.0001) and IgG titres (p = <0.0001) of patients with pyogenic spondylodiscitis and controls.

Table 11.1 Clinical samples received, culture results and anti-lipid S titres in sera from 10 patients with pyogenic spondylodiscitis

Patient	Clinical samples received and culture results	IgM titre	IgG titre
1	Blood culture: NG Spinal tissue: *S. aureus*	6400	204000
2	Spinal tissue: CNS	6400	409000
3	Spinal tissue: *S. aureus*	1500	15000
4	Spinal tissue: CNS	5500	15000
5	Spinal tissue: CNS, *Mycobacterium tuberculosis*	6500	22000
6	Blood culture: NG Spinal tissue: BHS group B	2500	25000
7	Spinal tissue: *S. aureus*	5000	40000
8	No samples received	10000	155000
9	Spinal tissue: *S. aureus*	9000	15000
10	Spinal tissue: CNS	8500	130000

Key to table 11.1: NG = no growth, BHS = beta-haemolytic streptococcus

Table 11.2 Statistical analysis of anti-lipid S serum titres from patients with pyogenic spondylodiscitis and controls

	Pyogenic spondylodiscitis	Controls
IgM (range)	2500-10000	500-13000
IgM (mean)	6130	3500
IgM (SD)	2557	3136
IgG (range)	15000-409000	500-22000
IgG (mean)	103000	8500
IgG (SD)	121129	5136

11.3.3 Patients with sciatica

11.3.3.1 Anti-lipid S IgG levels

Forty-three out of 140 (31%) patients with sciatica had elevated levels of serum IgG determined with the rapid lipid S ELISA. The IgG antibody levels ranged from 1,997 to 54,334 with a mean IgG titre of 10,100 (11,960, SD). Ninety-seven patients (69%) had no detectable levels of anti-lipid S IgG.

11.3.3.2 Cross reactivity of anti-lipid S IgG and vertebral tissue

Specific staining of vertebral tissue by diaminobenzidine after probing with anti-human IgG peroxidase was not observed, suggesting that cross-reaction between anti-lipid S IgG in the serum of patients with elevated titres and vertebral tissue did not occur.

11.3.3.3 Microbiology results and anti-lipid S serum IgG titres from patients with severe sciatica who underwent microdiscectomy

Microorganisms were cultured from 19 out of 51(37%) microdiscectomy samples within 7 days of enrichment culture incubation. Of the 19 positive cultures, 16 (85%) yielded *Propionibacterium acnes,* 2 of which also had associated *S. epidermidis*, 2 (10%) grew pure cultures of *S. epidermidis* and 1 (5%) yielded *Corynebacterium propinquum.* Of the 19 patients with positive microbiology cultures, 15 (79%) had corresponding blood samples taken for anti-lipid S serum IgG estimation and 7 (47%) had elevated titres.

Thirty-two out of 51(63%) microdiscectomy samples did not yield microorganisms and 31 (97%) of these patients had negative anti-lipid S serum IgG levels. There was a significant difference between patients with positive serology and culture as compared to those with both negative serology and culture ($p<0.0007$; Fischer exact test). Microorganisms isolated from microdiscectomy tissue of patients with severe sciatica and the corresponding anti-lipid S serum IgG titres are shown in table 11.3. In addition, the phenotypic characteristics of the 16 isolates of *P. acnes* are given. Nine biotypes of *P. acnes* were identified by API 32 ATB, of which the biochemical profile 0503 3706 accounted for 44%. Phenotypic variation of 2 isolates of *P. acnes* isolated from microdiscectomy tissue is shown in figure 11.0.

Table 11.3 Lipid S serology and microbiology culture results from 19 "culture positive" patients with severe sciatica. The phenotypic characteristics of 16 isolates of *P. acnes* are also shown

Pt ID	Microorganism isolated from tissue	Lipid S Serum IgG titre	Anaerobic growth	Growth in 5% CO_2	Beta haemolysis on blood agar	Volatile fatty acids	Biochemical Profile (API 32 ATB)
hs	*P. acnes*	1,600	+++	-	+	P, A	0503 3726
ma	*P. acnes*	5,000	+++	-	-	P, A	0503 1726
gw	*P. acnes*	1,000	+++	-	+	P, A	0103 3726
sm	*P. acnes* (+CNS)	11,000	+++	-	-	P, A	0503 3706
ac	*P. acnes* (+CNS)	4,400	+++	-	-	P, A	0503 3706
mt	*P. acnes*	ND	+++	+	+	P, A	0103 3726
bc	*P. acnes*	ND	+++	+	+	P, A	0503 3706
ag	*P. acnes*	ND	+++	++	+	P, A	0503 3706
gs	*P. acnes*	ND	+++	-	-	P, A	0503 1326
cl	*P. acnes*	ND	+++	-	-	P, A	0503 3306
jw	*P. acnes*	ND	+++	+	+	P, A	0103 3206
mh	*P. acnes*	ND	+++	-	-	P, A	0003 3326
sl	*P. acnes*	NSR	+++	+	-	P, A	0103 3706
ag	*P. acnes*	NSR	+++	-	+	P, A	0503 3706
tw	*P. acnes*	NSR	+++	-	+	P, A	0503 3706
jc	*P. acnes*	NSR	+++	-	+	P, A	0503 3706
hg	C. propinquum	2,000					
lw	CNS	1,800					
de	CNS	ND					

Key to table 11.3: P = propionic acid, A= acetic acid. CO_2 = carbon dioxide. CNS = coagulase negative staphylococci identified as *S. epidermidis.*
Growth conditions: +++ = heavy, ++ = moderate, + = light, - = no growth
Beta-Haemolysis: + = present, - = no haemolysis

Figure 11.0 **Colonial appearance of *P. acnes* isolated from the microdiscectomy tissue of 2 patients with severe sciatica and elevated lipid S IgG levels**

(A) Beta-haemoltyic strain of *P. acnes* isolated from patient **hs** (table 11.3)

(B) Non-haemolytic strain of *P. acnes* isolated from patient **ma** (table 11.3).

This strain of *P. acnes* was further investigated to determine if exocellular lipid S was produced after growth in brain heart infusion broth. Lipid S was produced by this strain of *P. acnes* (11.3.3.4).

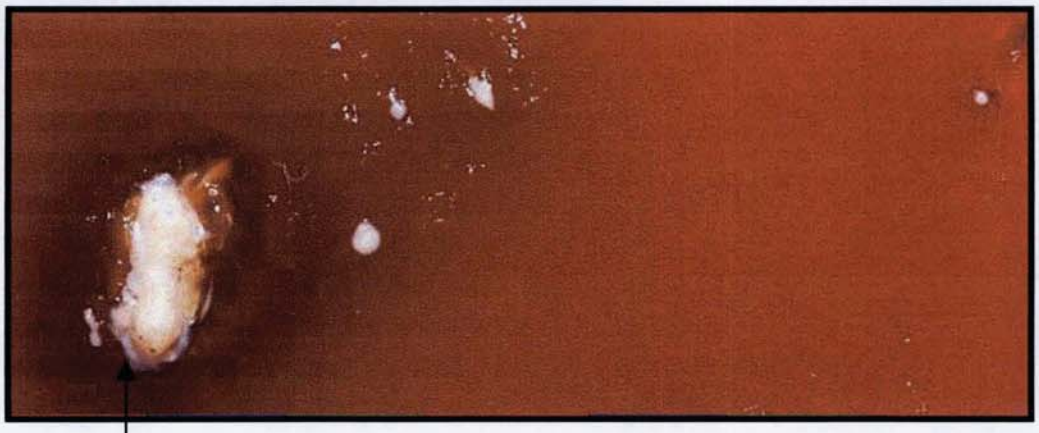
11.3.3.4 Detection of lipid S in the culture supernatant of P. acnes grown in BHI broth

Antigenic material was detected in fractions 10 to 15 eluting from the gel permeation column (data not shown). This indicated that the strain of *P. acnes* isolated from patient **ma** (table 11.3), produced exocellular lipid S when cultured in BHI broth. Fractions 1 to 50 of control BHI broth eluting from the gel permeation column did not contain immunogenic material.

11.3.3.5 Culture and microscopy of microdiscectomy samples stored at -20°C

Microorganisms were not observed in any of the 10 Gram stained thin sections of microdiscectomy tissue, however occasional polymorphonucleocytes were present in all samples. *P. acnes* was recovered from 6 out of 10 (60%) of the tissue samples following direct culture. Colonies of *P. acnes* were present on the surface impression marks on blood agar from 2 (20%) tissue samples (figure 11.1), whilst removal of the submerged tissue from the agar revealed microcolonies of *P. acnes* in all 6 samples, which were confirmed by Gram stain (figure 11.2).

Figure 11.1 **Colonies of *P. acnes* on 7% blood agar and nutrient agar following direct culture of microdiscectomy tissue. Colonies are present on surface impression marks and surrounding the submerged tissue**



Microdiscectomy tissue submerged in 7% blood agar. *P. acnes* is visible growing around the tissue after 72 hours incubation in anaerobic conditions

Microcolonies of *P. acnes* growing from a section of microdiscectomy tissue submerged in nutrient agar and incubated in anaerobic conditions for a minimum of 72 hours

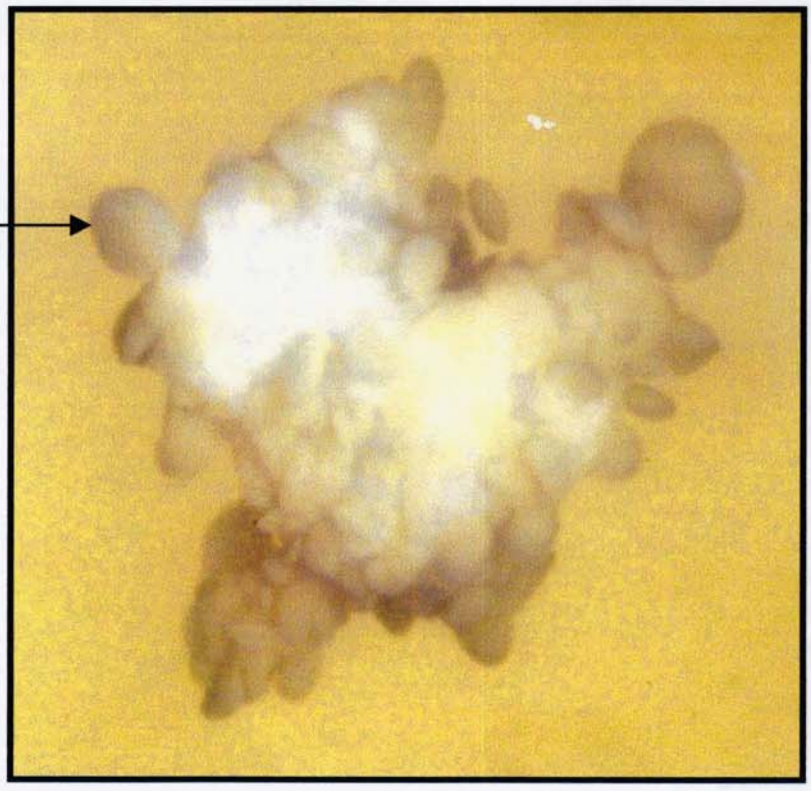
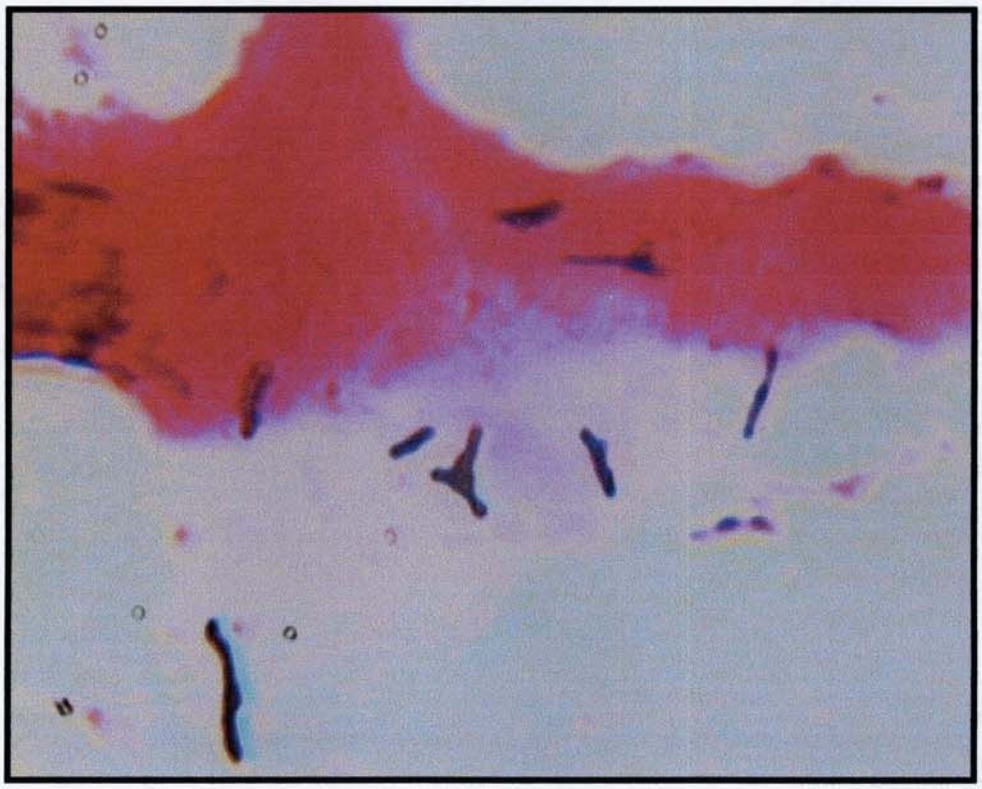


Figure 11.2 **Light photomicrograph of microdiscectomy tissue obtained from a patient with discogenic radiculitis showing the presence of *Propionibacterium acnes* which was isolated from the material** (see figure 11.1) (magnification: X 1000).



P. acnes was recovered from the tissue after submerging the sample in 7% blood agar for 72 h and incubation at 37°C in anaerobic conditions. Gram stain of the surface of the tissue revealed pleomorphic branching Gram-positive bacilli (average size 3 X 0.3 μm).

11.4 Discussion

Serum levels of IgM and IgG in patients with pyogenic spondylodiscitis were significantly different from the controls. In addition, the antibody titres observed in this group of patients were considerably higher than those achieved from patients with prosthetic joint infection or CRS (chapter 4). Indeed, the high titres observed in patients with pyogenic spondylodiscitis were similar to those demonstrated in patients with bacterial endocarditis (BE) (Connaughton *et al.*, 1997). The lower age groups of these patients (mean age 57 and 56 years respectively) compared to those patients with prosthetic hip infections (chapter 10) (mean age 69 years) may explain some of the higher titres achieved. The higher antibody levels observed in patients with pyogenic spondylodiscitis and BE may also be due to the nature of the infections. In BE for example, the antigenic challenge associated with haematogenous shedding of microorganisms from vegetations on heart valves, and persistence of sepsis may explain the high titres observed in this group of patients. Similarly, pyogenic spondylodiscitis is often associated with pre-existing infection, including that of intravenous cannulae, the urinary tract, genitourinary tract, and respiratory tract a with subsequent haematogenous spread to the intravertebral disc space (Torda *et al.*, 1995), offering one possible explanation for the high antibody levels achieved in some of these patients.

The potential of the lipid S ELISA for the serodiagnosis of pyogenic spondylodiscitis has been highlighted in this preliminary study. The IgG assay had a sensitivity and specificity of 70% and 97% respectively and may therefore provide a useful adjunct to the current microbiological approaches to diagnosis including the culture of bone biopsy specimens and blood. Indeed, Torda *et al.* (1995) reported positive blood cultures in only 50% of patients with pyogenic spondylodiscitis, which may have reflected prior antimicrobial therapy, whilst bone biopsy cultures were positive in 73%. Similarly, Frederickson *et al.* (1974) reported positive blood cultures and bone biopsies in 46% and 71% of patients respectively. In the current study, blood cultures were received from 2 out of 9 (22%) patients, both of which were negative whilst all biopsy samples grew Gram-positive microorganisms. In addition, the lipid S ELISA may aid clinicians in their choice of antimicrobial therapy when

microbiological cultures are negative. However, further studies are required to investigate the serological response in more patients with pyogenic spondyolodiscitis due to Gram-positive and Gram-negative microorganisms. In one study for example (Frederickson *et al.*, 1974), *Escherichia coli* was the predominant microorganism isolated from patients with pyogenic spondylodiscitis, the treatment of which would differ from infection due to *S. aureus* or CNS.

In utilising the lipid S ELISA to determine antibody levels in patients with pyogenic spondylodiscitis, unexpectedly high serum levels of anti-lipid S IgG were detected in 31% of patients with sciatica. One explanation for this may be that disc material contains antigenic determinants related to those in the lipid S antigen and as disc degeneration occurs, this constituent becomes recognised by an autoimmune mechanism. However, due to the lack of cross reactivity between anti-lipid S IgG and disc tissue from patients with scoliosis in this current study, this hypothesis seems unlikely. In addition, levels of antibody to cardiolipin (similar in structure to lipid S and found in many mammalian cells, chapter 6) from 42 sciatica patients with high and low titres of anti-lipid S IgG were estimated in chapter 6. However, only 2 (4%) patients had elevated levels of anti-cardiolipin IgG, and the study demonstrated no cross-reaction between cardiolipin and lipid S.

The elevated levels of serum IgG associated with sciatica also raised the question of a microbial aetiology. This hypothesis was investigated in the study by culturing microdiscectomy samples from patients with severe sciatica utilising an enrichment technique and extended incubation times. Interestingly, microdiscectomy samples from 37% of patients yielded microorganisms, and of these 47% had elevated anti-lipid S serum IgG titres, which was significantly different from patients with negative serology and culture. This result was similar to the 31% of patients with sciatica and elevated levels of anti-lipid S serum IgG in the preliminary investigations. The predominating microorganism recovered from microdiscectomy tissue was *P. acnes*, which accounted for 85% of the isolates and was grown in pure culture from 14 out of 16 (88%) samples.

P. acnes is a common saprophyte of human skin and predominates over other constituents of the normal flora in the pilosebaceous follicles where it reaches approximately 10^5 colony forming units per follicle (Funke *et al.*, 1997). The potential for contamination of microdiscectomy samples from the skin of the patient or the surgeon during surgery is therefore high. However, recognising the possibility of contamination in clean wound culture, stringent aseptic precautions were taken by the surgeon to minimise the risk. In addition, *P. acnes* was isolated in pure culture from 88% of the samples from which it was recovered. This indicates that contamination of the samples from the skin was unlikely, as one would expect polymicrobial cultures to be achieved, including microorganisms such as coagulase negative staphylococci (Twum-Danso *et al.*, 1992). The retrospective isolation of *P. acnes* from 60% of the original microdiscectomy samples stored at -20°C, also indicates that contamination of the enrichment broths during laboratory subculture did not occur.

Predisposing conditions for infections due to *P. acnes* include the presence of foreign bodies, immunosuppression, preceding surgery, trauma, diabetes and obstruction of sinuses or ducts (Funke *et al.*, 1997; Clarke and Shufflebarger, 1999). Patients with non-resolving severe sciatica may undergo microdiscectomy to remove bone and soft tissue abnormalities, and many will have had previous cordal or lumbar epidural steroid injections to aid in pain relief. One hypothesis for the high number of microdiscectomy samples yielding *P. acnes*, is that trauma due to injection of epidural steroid may have served as a route for microorganisms to gain access to the spinal disc and initiate a chronic inflammatory response. However, 50% of the patients from whom microorganisms were isolated did not have an epidural, in comparison 41% of the tissue culture negative patients underwent this procedure. This suggests that the epidural was not the source of the microorganisms.

A further hypothesis is that in patients with sciatica, minor trauma resulting in a breach in the mechanical integrity of the disc allows access by microorganisms of low virulence thereby initiating or stimulating an inflammatory response with accompanying symptoms. This would be consistent with the observed anatomical distribution of disc degeneration with greater stresses on the more distal levels of the spine. The observation that disc protrusions detected by MRI are frequently asymptomatic, perhaps awaiting infection before

becoming symptomatic, also supports this hypothesis. It is therefore conceivable that low virulent microorganisms including *P. acnes* are associated with the inflammation found with sciatica and may even be a primary cause of the condition. In addition, the long mean generation time of propionibacteria make them ideal candidates for chronic infection, which may explain why some patients experience lumbosciatic pain for many years.

Elevated serum levels of anti-lipid S IgG were detected in 42% of the patients where *P. acnes* was recovered from microdiscectomy tissue. Negative serological results in some of the remaining culture positive patients may be due to iatrogenic immune deficiency as a side effect from corticosteroid injection for pain relief (Woolf, 1986). In addition, the long incubation period of *P. acnes* which may range from a few days to several months, and occasionally for more than a year (Funke *et al.*, 1997), will influence the level of exocellular lipid S released by the microorganisms. The antigenic challenge of lipid S may therefore be less in infections due to *P. acnes* than in those due to staphylococci. Furthermore, *P. acnes* may also avoid recognition by the immune system as it can resist killing by phagocytes and is able to survive in macrophages (Funke *et al.*, 1997). In this current study, 9 different biotpyes of *P. acnes* were identified by API 32 ATB, which indicated that a common source was unlikely, for example the surgeon, and that the microorganism originated from the skin of the patient. However, there are no reliable phenotypic typing methods for *Propionibacterium* species (Funke *et al.*, 1997), and genotypic typing of the microorganisms may be necessary to discriminate between strains. More recently, pulsed field gel electrophoresis has been successfully used to compare *Not*I digested strains of *P. acnes* isolated from the vitreous humor of patients with chronic postoperative endophthalmitis following cataract surgery (Ting *et al.*, 1999).

Recent guidelines by Funke *et al.* (1997) have been published to facilitate establishing true disease associations of coryneform bacteria and include: positive microscopy and culture of clinical material; phenotypic characteristics including pigment and haemolysis of colonies; biochemical reactions; chemotaxanomic investigations and isolation of the microorganism in pure culture. This current study has attempted where possible to fulfil these published guidelines and the results indicate a strong association between *P. acnes* and patients with

severe sciatica. In addition to these guidelines and to support this association, the serological response to lipid S in patients with sciatica was investigated. Serum IgG levels to lipid S were elevated in 42% of patients where *P. acnes* was isolated from microdiscectomy tissue.

In summary, the lipid S ELISA detected elevated serum levels of IgG in patients with pyogenic spondylodiscitis. The assay may provide supporting serological evidence to aid in the diagnosis of pyogenic spondylodiscitis, a condition where diagnosis may be complicated by non-specific results of current procedures. However, further studies are required to determine the anti-lipid S levels in more patients with pyogenic spondylodiscitis. Incidental to these preliminary investigations, the lipid S ELISA unexpectedly detected elevated levels of serum IgG in patients with severe sciatica, which raised the question of a microbial aetiology. Interestingly, 37% of patients yielded positive cultures and *P. acnes* accounted for 85% of the isolates, which was significantly different to patients with both negative serology and culture. By reference to recently published guidelines, these current microbiological and serological results strongly suggest an association between microorganisms, in particular *P. acnes*, and severe sciatica. If this is the case, the future management of some patients with sciatica may include antimicrobial therapy as an adjunct to conventional treatment regimes, or in some patients the condition may be treated with antibiotics alone. *P. acnes* is susceptible to most antimicrobials including the β-lactam antibiotic and the macrolide (Brook and Frazier, 1991). However, further incorporating more patients and clinical trials are required to support this hypothesis.

Chapter 12 General Discussion

The accurate diagnosis of catheter-related sepsis (CRS) continues to pose a clinical problem. The clinical presentation of CRS is often non-specific which may result in the unnecessary removal of many catheters (Elliott and Tebbs, 1998). In addition, there are difficulties in many of the supporting microbiological investigations. Current standard methods include the examination of blood cultures taken either via a peripheral venepuncture or the central venous catheter (CVC) (Siegman-Igra *et al.*, 1997) and the culture of distal catheter tips removed from patients with suspected device related sepsis (Maki *et al.*, 1977). However, these methods do not always allow for clear discrimination to be made between microbial contamination of the samples and true CRS and interpretation of positive cultures may therefore be difficult (Elliott, 1997). More recent methods for the *in situ* diagnosis of the disease include the use of an endoluminal brush to sample the internal lumen of the catheter and staining of blood withdrawn through the catheter hub (Kite *et al.*, 1999). These techniques, however, need to be used with caution as it is important to differentiate between colonisation and contamination and further evaluation of the methods in a clinical setting is required.

Many attempts have been made to investigate the serological response to staphylococcal antigens including teichoic acid, peptidoglycan, and protein A (West *et al.*, 1985; Wheat *et al.*, 1984; Verbrugh *et al.*, 1983). However, these serological methods have been based on staphylococcal cell wall antigens, and are of limited use in aiding the diagnosis of sepsis related to intravascular devices. Indeed, staphylococci growing in a biofilm on intravascular catheters, may evade phagocytosis and recognition by the immune system due to the presence of slime, which encapsulates the microorganisms (Christensen *et al.*, 1994). More recently, Lambert *et al.* (1996) utilised an ELISA that incorporated an exocellular, non-protein antigen of *S. epidermidis* to detect elevated serum IgG levels in patients with bone infection due to Gram-positive cocci. The study suggested that the use of exocellular staphylococcal antigens that are readily detected by the immune system, may provide a convenient means of serodiagnosing deep-seated infections due to *S. epidermidis*. Work presented in this thesis showed that in agreement with Lambert *et al.* (1996), many patients

with CRS due to slime producing strains of *S. epidermidis* had elevated levels of antibody to the exocellular antigen (chapters 4 and 9), supporting the serodiagnostic application of the antigenic material.

The main objective of this current study was to isolate and characterise the exocellular antigen produced by strains of coagulase-negative staphylococci (CNS) associated with CRS (Lambert *et al.*, 1996) and to investigate its potential as a serodiagnostic marker of intravascular device related sepsis due to staphylococci. The previous study of Lambert *et al.* (1996) utilised exocellular antigen produced by a single strain of *S. epidermidis* isolated from a patient with bone infection. However, gel permeation and probing with immune serum (chapter 2), revealed that the exocellular antigen was also present in the culture supernatants of the 7 phenotypically distinct strains of CNS included in this current study. To improve the performance of the antigen in facilitating the serodiagnosis of CRS due to different strains of CNS, antigenic material pooled from 7 strains associated with sepsis was therefore adopted for this study. In addition, the diversity of genotypes of *S. epidermidis* associated with CRS and the possibility that a single episode of CRS may be caused simultaneously by several strains of CNS (chapter 9), further supported the need for antigenic material prepared from multiple isolates.

Initial characterisation of the exocellular antigen by competitive ELISA (chapter 2) indicated that the material was similar in structure to lipoteichoic acid (LTA), sharing common antigenic determinants, however subsequent probing with immune sera suggested that it was immunologically distinct from LTA (figures 2.1 to 2.10). In addition, further analysis of the antigen by negative electrospray mass spectrophotometry and nuclear magnetic resonance confirmed the distinction from LTA by revealing a previously undescribed structure subsequently termed lipid S (figure 2.16). The structure of lipid S differs from that of LTA by comprising a glycerophosphate chain length of 6 units compared to the average 25 units found in *S. aureus* (Fischer, 1994), and the 42 units found in the phenol extracted LTA of *S. epidermidis* in this thesis. Characterisation of the

exocellular antigenic material indicated the presence of glucose, fatty acid, glycerol and phosphate (table 2.4), which are present in LTA. In addition to LTA, the cell membrane of *S. aureus* (and presumably CNS) also contains phosphatidyl glycerol and diphosphatidyl glycerol (cardiolipin) (Fischer, 1994); both are lipids similar in structure to LTA (and lipid S) by comprising a common glycerophosphate component (figure 6.0). Cardiolipin is also present in mammalian cell membranes and is widely distributed within tissues (Woolf, 1986). Previous studies have shown that anti-cardiolipin IgG can be generated by immunisation of rabbits with LTA (Gotoh and Matsuda, 1996). Furthermore, serum antibodies to cardiolipin are detected in many diseases, including infection, and also in healthy individuals. The possibility that lipid S was related to cardiolipin was therefore investigated in this current study and no serological cross-reactivity between lipid S and cardiolipin was demonstrated, indicating the immunologically distinct nature of both lipids (chapter 6).

In the initial stages of this current study, a crude enzyme linked immunosorbent assay (ELISA) was developed to determine the potential of the pooled exocellular lipid S antigen as a serological marker of staphylococcal intravascular CRS (chapter 3). An ELISA format was selected as these tests are relatively inexpensive, simple to perform, avoid the necessity of hazardous reagents and are adopted easily by clinical laboratories (Wood and Wreghitt, 1990). However, immunoassays rely on complex immunochemical reactions with several stages for error to occur, which may be attributable to factors including pipetting, variations in temperature and change of reagent batch (Balfour and Harford, 1990). By adopting the method of Lambert *et al.* (1996) whereby serum titres were determined at a standard absorbance of 0.1, the intertest variation of the ELISA incorporating lipid S antigen was high (table 3.1). This method of titre determination however does not account for variations in temperature (environmental and incubation), therefore the poor assay reproducibility was not unexpected. By establishing internal quality control sera with standardised titres to which test sera could be referred (section 3.3.3) (Balfour and Harford, 1990), the intertest variation of the lipid S ELISA was reduced considerably (tables 3.2 and 3.3).

This present study has therefore demonstrated that by referring test sera to a standardised positive control titre, the lipid S ELISA detected significant differences in serum IgM and IgG levels of patients with CRS and controls (chapter 4). Indeed, elevated levels of antibody to lipid S were detected in the serum of patients with CRS caused by CNS, indicating that lipid S is produced by microorganisms growing in a biofilm on catheters and is released in a form which stimulates a strong immune response. The IgG assay had a sensitivity and specificity of 75% and 90% respectively, which compared favourably with current methods of diagnosis, many of which require catheter removal (Siegman-Igra *et al.*, 1997). The lower assay sensitivity of the ELISA may have reflected several factors:

- The patients recruited into the study included those with immunosuppression and subsequent negative serological results.
- The clinical presentation of CRS is often non-specific and some patients therefore may have been clinically misdiagnosed.
- The method of estimating antibody titres by a sigmoidal titration curve (figure 3.0) was technically difficult
- The assay may have been performed under sub-optimal conditions.

However, the potential of ELISA in aiding the diagnosis of CRS due to staphylococci was clearly demonstrated, and technical difficulties in titre estimation may be avoided if the rapid 4-hour IgG ELISA is utilised (chapter 8). Indeed, the assay may provide a novel means of indicating Gram-positive infection at a very early stage of sepsis through serological monitoring of patients with intravascular catheters *in situ* (chapter 5).

The clinical diagnosis of CRS often relies heavily on the results of single blood cultures, which may be misleading as false positive cultures can occur due to skin contamination during sample collection or from contaminated luers. The lipid S ELISA may also facilitate the full interpretation of such positive blood culture results with CNS and prove a useful adjunct for the diagnosis of CRS. The combination of negative serology and positive blood cultures may reflect catheter colonisation rather than CRS, whereas positive serology and blood cultures strongly suggest sepsis. The ELISA may also aid in a more accurate interpretation of the microbiological investigations of catheter tips, blood cultures and blood drawn through contaminated luers for staining.

In agreement with previous studies (Julander *et al.*, 1983; West *et al.*, 1985), the IgM ELISA was of limited serodiagnostic value. Although the assay detected significantly higher antibody levels in patients with CRS as compared to controls, it is evident from the assay sensitivity of 52% that the IgM ELISA would have limited use in the clinical setting. In addition, the incorporation of GullSORB™ reagent into the assay procedure did not significantly improve the IgM ELISA performance (chapter 7), and further detailed studies are required to investigate the kinetics of the IgM response to lipid S in patients with staphylococcal sepsis.

The high levels of serum antibody to lipid S detected in the sera of the control group of patients (tables 4.3 and 4.4) were not unexpected. The skin is colonised by several species of CNS which constitute a major component of the normal skin flora with numbers of microorganisms ranging from 10 to 10^6 CFU/cm^2 of surface (Kloos and Bannerman, 1994). In addition, 30 to 50 % of healthy adults are colonised with *S. aureus* (Lowy, 1998), whilst *P. acnes* is the predominant microorganism on sebaceous gland-rich areas of skin in adults (Eady and Ingham, 1994). High lipid S antibody levels in healthy adults may therefore be explained by prior exposure to lipid S produced by commensal skin CNS and *S. aureus* through minor skin conditions, psoriasis, eczema or unreported earlier infection. Moreover, careful interpretation of the serum IgG levels obtained with the lipid S ELISA is essential when investigating suspected Gram-positive sepsis related to intravascular cannulae or prosthetic devices, as patients with skin conditions, previous infection, or concurrent infection may give rise to misleading serological results. Reference to the patient's recent clinical history is therefore fundamental to the accurate interpretation of serological results achieved with the lipid S ELISA.

The elevated levels of lipid S antibody in the serum of patients with CRS (chapter 4) and prosthetic joint infection (chapter 10) may be a reflection of the large reservoir of microorganisms and diffusible lipid S present in biofilms associated with infections of intravascular devices and orthopaedic prostheses. In addition, a steady release of exocellular lipid S antigen over a prolonged period of time from cardiac vegetations (chapter 8),

prosthetic joints (chapter 10) and chronic spinal infection (chapter 11) may explain the generation of very high levels of anti-lipid S IgG observed in these patients.

Skin commensals, including CNS and propionibacteria are being increasingly recognised as aetiological agents of infection (Tunney *et al.*, 1998; Mittendorf *et al.*, 1999). The incidental but highly significant association between *Propionibacterium acnes* with sciatica (chapter 11), not only reflected the potential of lipid S as a novel marker of occult microbial infection, but has also laid the foundation for clinical trials to investigate treatment of this condition with antimicrobials. Indeed, the results presented in the concluding chapter of this thesis strongly suggest a microbial aetiology in 37% of the patients presenting with severe sciatica, approximately half of whom had elevated levels of anti-lipid S IgG. There is also considerable evidence that persistence of *P. acnes* in a variety of organs and tissues can lead to a chronic inflammatory response (Eady and Ingham, 1994). The presence of inflammation around the nerve root and in the nucleus pulposus, which is associated with sciatica, may therefore be due to the presence of low-virulent microorganisms including *P. acnes*. At present, one can only speculate as to why *P. acnes* was recovered from 85% of the positive microdiscectomy tissues. The microorganism is part of the normal gut and dental microbiota (Eady and Ingham, 1994), therefore previous surgery or invasive diagnostic procedures may have facilitated haematogenous spread to the vertebral bodies. Similarly, *P. acnes* is responsible for the polymorphic lesions of acne vulgaris which arise in sebaceous follicles and are distributed over the face and trunk of the body. Considering that these low-virulent microorganisms often cause delayed clinical infections with a long interval between inoculation and onset of symptoms (Tunney *et al.*, 1998), haematogenous spread from colonised lesions to the vertebrae in adolescents may result in symptoms of sciatica in later years.

Further work

Serological monitoring of patients with a CVC in situ

Estimation of the antibody levels to lipid S in serial serum samples from patients with a CVC *in situ* (chapter 5), indicated that the lipid S ELISA may provide a system for the early detection of Gram-positive sepsis related to intravascular devices. Indeed, elevated levels of antibody were detected in patients prior to positive blood cultures processed by standard techniques. Future work should concentrate on obtaining blood drawn through the CVC on insertion from which a baseline level of antibody to lipid S for individual patients can be established. Daily or weekly estimation of anti-lipid S IgG levels may provide an early indication of CRS through rising antibody titres in the absence of concurrent infection or pre-existing skin conditions. In addition, serological monitoring of patients from the day of CVC insertion would provide the necessary information and samples to further develop the lipid S IgM ELISA. Previous attempts to develop the lipid S IgM assay (chapters 4 and 7) were confounded by lack of clinical information regarding time of onset of sepsis in relation to blood collection.

Application of the lipid S ELISA to sternal osteomyelitis

The results from chapters 10 and 11 indicated that the lipid S ELISA may provide a useful adjunct to the diagnosis of osteomyelitis associated with prosthetic joints and vertebral discs. The diagnosis of sternal sepsis following coronary artery bypass grafting often relies on the clinical presentation including erythema and purulent exudate. The coagulase-negative staphylococci are a common cause of wound infection including sternal sepsis (Rupp and Archer, 1994) and laboratory culture results may therefore often be misleading, indicating the presence of skin commensals only. The lipid S ELISA may provide additional serological information to support the clinical suspicion whilst also aiding in the interpretation of sternal cultures yielding skin microorganisms.

Application of the lipid S ELISA to carpal tunnel syndrome and rheumatoid arthritis

The unexpected association between *Propionibacterium acnes* and sciatica (chapter 11), raises the question of a microbial aetiology due to low-virulent microorganisms in other conditions including carpal tunnel and rheumatoid arthritis. Indeed, antigenic polysaccharide material identical to that of the propionibacterium group has previously been recovered from the synovial fluid of patients with rheumatoid arthritis (Bartholomew and Bartholomew, 1979). Future application of the lipid S ELISA to patients with rheumatoid arthritis may reveal levels of anti-lipid S IgG suggestive of microbial infection. Similar to sciatica, where symptoms arise from pressure on the sciatic nerve, carpal tunnel syndrome is caused by compression of the median nerve at the wrist due to local inflammation. In severe cases of carpal tunnel syndrome, relief may be achieved by a relatively mild operative procedure to relieve pressure on the median nerve. A future approach may include extended culture of material removed at operation for the presence of low-virulent microorganisms and serological investigation by the lipid S ELISA.

Role of lipid S in pathogenesis

Lipoteichoic acid (LTA) stimulates a range of responses in the host, including induction of cytokines (Verhoef and Mattsson, 1995) and inducible nitric oxide synthase (Kengatharan *et al.*, 1998) which are presumed to mediate Gram-positive shock. Due to the structural similarity between LTA and lipid S, it may be that lipid S is responsible for many of the physiological ascribed to LTA. Future *in vitro* studies may therefore concentrate on establishing the role of lipid S in pathogenesis by investigating its ability to stimulate the release of proinflammatory cytokines including tumour necrosis factor α and interleukin 1, and nitric oxide from murine macrophage cell lines.

The protective role of anti-lipid S IgG in Gram-positive sepsis

The high serum titres of anti-lipid S IgG observed in many patients with Gram-positive sepsis throughout this thesis, raises the question concerning the protective role of the IgG antibody. One would expect the high serum levels of IgG to neutralise exocellular lipid S and cross-react with cellular LTA, thereby interfering with the cytokine and nitric oxide production associated with sepsis. Future studies may concentrate on patient outcome in relation to the levels of IgG in serial serum samples as determined by the lipid S ELISA. In addition, the efficacy of active and passive immunisation against lipid S in preventing Gram-positive sepsis in animal models warrants future investigation. Previous work by Takeda *et al.* (1991) demonstrated that immunoprophylaxis targeted at the capsular staphylococcal polysaccharide adhesin (PS/A), provided protection against bacteraemia and endocarditis in rabbit models challenged with *S. epidermidis* strain RP62A.

Clinical trials to determine the effect of antimicrobial treatment in patients with sciatica

It has been clearly demonstrated that low-virulent microorganisms, in particular *P. acnes*, are associated with some patients with severe sciatica (chapter 11). Extensive clinical trials are now required to determine the outcome of selected patients presenting with the condition following treatment with antimicrobials. Blood samples taken for anti-lipid S IgG levels at the time of clinic attendance, may determine those patients selected for trial.

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