Development of Improved Diagnostic Testing Methods for Invasive Pneumococcal Disease

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Abstract

Streptococcus pneumoniae (the pneumococcus) is one of the most important bacterial pathogens and contributes extensively to global infectious disease morbidity and mortality, especially in children. To unequivocally identify *S. pneumoniae* as the causative pathogen of diseases like pneumonia, septicaemia or meningitis, has been a challenge ever since its first description in the late 19th century, reflecting, in part, short-comings of available diagnostic tests in terms of sensitivity and specificity. In recent history pneumococcal diagnostic advances have been slow and the urine antigen test and several molecular-based techniques appear to be the sole novel diagnostic approaches over the last three decades.

The focus of this study was to explore new diagnostic avenues and evaluate their usefulness in the clinical setting. The thesis starts with a landscape analysis looking at the diagnostic methods and clinician's ordering patterns over the last 11 years, followed by the evaluation of diagnostic assays. Three methods were included in the study, namely antigen detection, polymerase chain reaction (PCR) and mass spectrometry (MS). The targets chosen for the antigen detection assays, pneumolysin (Ply) and pneumococcal surface adhesin A (PsaA) were known to be immunogenic and present in most, if not all pneumococcal serotypes. The PCR target, autolysin (*lytA*), though not a novel PCR target was investigated in a quantitative assay as a potential marker for disease severity. Finally, matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS, a technology that has been used in other areas, such as biochemistry for a few decades, has only in the last decade made advances into routine microbiological diagnostics, was explored as a tool to differentiate *S. pneumoniae* from related non-pneumococcal viridians streptococci.

Both antigen detection assays were developed successfully. However, the Ply-assay revealed a problem with the non-specificity of the polyclonal antibody in serum specimens and was therefore not evaluated on clinical specimens. The PsaA-assay was applied to a selection of well characterised patient sera, and was showed to be specific, but needs to be of greater sensitivity to be clinically useful. The quantitative *lytA* PCR assay did demonstrate correlation of bacterial load and severity of disease and might be a useful adjunct in the clinical management of patients. MALDI-TOF MS is a novel tool applied to microbiological

bacterial and fungal identification which needs further development in the area of typing and species level identification of certain bacteria, such as the viridians streptococci. This study demonstrated that species differentiation of *S. pneumoniae* from non-pneumococcal streptococci of the *S. mitis* group might be possible by this method.

In summary, this study describes the investigation of novel diagnostic approaches which aim to provide solutions to three main ongoing challenges in the diagnosis of pneumococcal disease: (i) to increase the sensitivity of assays to detect *S. pneumoniae*, (ii) to differentiate between colonisation and invasive disease and (iii) to ensure more reliable identification of pneumococcal and non-pneumococcal viridians streptococci at the species level.

The findings of this thesis have allowed an in depth assessment of the current state of pneumococcal diagnostics and have allowed the realistic assessment of the implementation of technologies such as antigen detection assays, quantitative PCR and mass spectrometry in this area; the latter two almost certainly will become more firmly established in the diagnosis of pneumococcal disease in future.

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Publications arising from this thesis

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5. Kelly DF, Thorson S, Maskey M, Mahat S, Shrestha U, Hamaluba M, Williams E, Dongol S, **Werno AM**, Portess H, Yadav BK, Adhikari N, Guiver M, Thomas K, Murdoch DR, Pollard AJ. **2011.** The burden of vaccine-preventable invasive bacterial infections and pneumonia in children admitted to hospital in urban Nepal. Int J Infect Dis. Jan; 15 (1): e17-23. Epub 2010 Nov 30.

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List of abbreviations

AAT	adenine/aminopterin/thymidine
amp	ampicillin
AOM	acute otitis media
APRT	adenine phosphoribosyl transferase
APS	ammonium persulfate
ATCC	American type culture collection
BC	blood culture
BLAST	Basic Local Alignment Search Tool
BLD	biological limit of detection
bp	base pairs
BSA	bovine serum albumin
CAP	community-acquired pneumonia
CDC	Centers of Disease Control
CDCs	cholesterol-dependent cytolysins
CDHB	Canterbury District Health Board
cDNA	complimentary deoxyribonucleic acid
CFU	colony-forming unit
CHL	Canterbury Health Laboratories
CI	confidence interval
cm	centimetre
^{o}C	degrees centigrade
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
CSF	cerebrospinal fluid
C_T	crossing threshold
CV	coefficient of variation
Da	Dalton
dd H_2O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

dNTP	deoxy nucleotide triphosphate
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ERMA	Environmental Risk Management Authority
ESI MS	electrospray ionisation mass spectrometry
ESR	Environmental Science & Research Limited
E.T.T.	endotracheal tube
g	gram
GBD	Global Burden of Disease Study
gHCl	guanidine HCl
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
HPLC	High Performance Liquid Chromatography
HRP	horseradish peroxidase
IANZ	International Accreditation New Zealand
ICT	immunochromatographic test
ICU	intensive care unit
Ig	immunoglobulin
ILI	influenza-like illness
IMAC	Immobilised Metal Affinity Chromatography
IPD	invasive pneumococcal disease
IPTG	isopropylthio-β-D-galactopyranoside
IQR	interquartile range
kb	kilobase
LB	Luria Bertani
LLD	lowest limit of detection
Luminol	5-amino-2,3-dihydro-1,4-phthalazine dione
LytA	pneumococcal autolysin
μ	(prefix) micro- (10^{-6})
m	(prefix) micro- (10^{-3})
M	mol/litre

MAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption ionisation time-of-flight
MC	monoclonal antibody
mg	milligram
mL	millilitre
mm	millimetre
MS	mass spectrometry
MSP	main spectral projection
n	(prefix) nano-
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometres
OD	optical density
OR	odds ratio
PAGE	polyacrylamide gel elctrophoresis
PBS	phosphate-buffered saline
PC	polyclonal antibody
PCR	polymerase chain reaction
Pd-B	recombinant pneumolysin
PEG	polyethylene glycol
Ply	pneumolysin
PLY-7	monoclonal antibody to Ply
PMN	polymorphonuclear leukocyte
PsaA	pneumococcal surface adhesin A
PSSA	designation of monoclonal antibodies raised against PsaA
psi	pound-force per square inch
PSI	pneumonia severity index
RCPA	Royal College of Pathologists of Australasia
rpm	revolutions per minute
RT	room temperature
SBA	sheep blood agar
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SEC	squamous epithelial cells
SOC	super optimal broth with catabolite repression
TBE	Tris-Borate-EDTA
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tm	primer melting temperature
TMB	tetramethylbenzidine
Tris	tris[hydroxymethyl]aminomethane
Tween	polyoxyethylene sorbitan monolaurate
U	units
UAT	urinary antigen detection test
V	volts
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. General introduction

Streptococcus pneumoniae (the pneumococcus), is a Gram-positive organism requiring CO_2 for its growth. It is one of the most important human pathogens. It is a major cause of pneumonia, bacteraemia, meningitis, sinusitis and acute otitis media (AOM), but is also known to occasionally infect tissues at other body sites. The collective term invasive pneumococcal disease (IPD) refers to bacteraemia, meningitis, pneumonia in the presence of bacteraemia and infections of other normally sterile sites by *S. pneumoniae*.

IPD is a leading cause of illness and death globally. The World Health Organisation (WHO) estimates that 1.6 million deaths annually can be attributed to IPD, of which more than 800,000 are children under the age of 5 years [1]. IPD is likely to be the most common cause of childhood death worldwide due to infectious disease and, not surprisingly, the developing world bears the greatest burden [2]. Deaths due to IPD in HIV-seronegative children under 5 years of age contribute 11% to the overall childhood mortality of that age group [3]. Amongst vaccine-preventable diseases, IPD is the leading cause of death [4]. One of the millenium development goals (United Nations) is the reduction of mortality in young children under 5 years by two thirds, between 1990 and 2015 [5, 6].

Nasopharyngeal colonisation precedes IPD [7] and generally becomes established during the first year of life. The peak prevalence of pneumococcal colonisation occurs around age 3 years and is estimated to be between 20-60% in this age group. A steady decline of colonisation prevalence follows thereafter [8]. The frequency of pneumococcal carriage varies due to ethnic, environmental and socioeconomic factors. Once colonisation has occurred in an individual it readily spreads to other individuals within the community. More than 90 different capsular serotypes of *S. pneumoniae* have been described to date [9] and certain serotypes appear to be linked to distinct epidemiological properties [10]. Some serotypes are more associated with colonisation, others with invasive disease; some are linked to particular disease syndromes, such as complicated pneumonia in children.

With the development of the pneumococcal conjugate vaccine, a cost-effective tool in the prevention strategy of IPD has been implemented [11]. The vaccine is efficacious and effective in disease prevention in children as demonstrated in several large-scale trials around

the world [12-14], as well as having an indirect impact on pneumococcal disease in the elderly [15]. Continued annual reductions of vaccine-strain IPD in the vaccine target as well as unvaccinated populations have been observed, and the effect is thought to be due to herd-protection [16].

Despite efforts to develop novel vaccines with better coverage of serotypes and efficacy especially in children under the age of 2, and concerted efforts to keep abreast of developing antimicrobial resistance, the development of diagnostic tools has lagged behind. Diagnostic methods that have been used for decades are still used, and newer technology, such as antigen detection tests and nucleic acid amplification methods, have proven challenging to develop and are limited to date in their implementation.

In the battle against IPD it is invaluable to have ongoing surveillance to monitor global epidemiological changes [17] as well as antimicrobial resistance [18], but it is also essential to develop reliable and cost-effective diagnostic tests, which can be implemented in particular in resource-constrained countries.

1.2. Historical perspective

"Pneumococcus is an altogether amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death." [19]

It is likely that the pneumococcus was first seen in pulmonary tissues in 1875 [20] and was termed *Bacillus malariae* at that time. Subsequently Koch and Ebert [21, 22] visualised similar organisms in stained histological sections of human tissues.

S. pneumoniae, in the past referred to as *Micrococcus lanceolatus*, was discovered in 1880 independently by two researchers. In the United States, September 1880, Sternberg made his discovery by inoculating rabbits subcutaneously with his own saliva [23]. The animals died within 48 hours of inoculation. In France Pasteur performed a similar experiment in December of the same year inoculating rabbits with the saliva of a child who had died from rabies. Pasteur's findings were presented at a scientific meeting of the Académie de Médicine in Paris in January 1881 [24]. Sternberg published his findings 3 months later. This sequence of presentation of the findings gave Pasteur the credit of

discovery of the organism, despite Sternberg's discovery pre-dating Pasteur's. Both scientists had grown the organism *in vitro* and had described its morphological features as an encapsulated diplococcus.

The role of the pneumococcus in causing pneumonia was established in 1886. The first report on the bacterial aetiology of pneumonia appeared in 1882, when Friedländer described an organism he found in lung sections of eight patients who died of pneumonia [25]. The histology material was stained with aniline dyes by the Weigert-Koch stain. The organism identified may have been the pneumococcus. In 1883 he isolated a bacterium, which was named the Friedländer's bacillus, from the right upper lobe of a patient with pneumonia and liver cirrhosis [26]. It is now known that the isolate was *Klebsiella pneumoniae*. However, he also recovered organism from two additional cases; these differed from the Friedländer's bacillus. At the time, Gram was working in Friedländer's laboratory and had developed a new stain for the identification of bacteria in mammalian tissues, the Gram stain. In his 1883 publication Friedländer made a brief reference to this new stain and in 1884 Gram published his own findings [27]. The stain was only intended to enhance the visualisation of microorganisms in tissues; its great value in distinguishing two bacterial groups from one another remained unappreciated for some time. A reference to the Gram stain in 1885 indicates that Hueppe had picked up on the decolorisation of some bacteria and not others, and its usefulness for a differentiating test [28], Friedländer clearly recognised the apparent value of the Gram stain in 1886 [29]. If the Gram stain had been fully appreciated in 1883, the heated debate between Fraenkel and Friedländer regarding the causative organism of pneumonia would not have taken place [30]. At the time of Friedländer's publication, the scientific community believed that only one microrganism was involved, and not two as later confirmed.

At the Third Congress for Internal Medicine in Berlin in 1884, Fraenkel presented the isolation of an organism from a 30 year-old man who had died of pneumonia [31]. This bacterial isolate behaved different from Friedländer's in terms of culture characteristics and virulence in laboratory animals.

In 1886 Weichselbaum reported his findings on 129 fatal cases of pneumonia. He made the necessary differentiation of two organisms on the basis of culture characteristics and pathogenicity. He described the pneumococcus and named it *Diplococcus pneumoniae*, and the Friedländer's bacillus, which he named *Bacillus pneumoniae*. He described both organisms as encapsulated and the *Bacillus pneumoniae* as morphologically variable with occasional coccoid forms [32].

In the decade following the initial isolation of the pneumococcus, the extent of the range of pneumococcal disease became apparent [33-40]. It was Sternberg, who suggested a name change for the *Diplococcus pneumoniae* to streptococcus in 1897 [41].

Progress in recognising the protective effect of antisera was made in 1891 by the Klemperers [42] through pneumococcal challenge experiments in the offspring of immunised rabbits and subsequent injection of immune sera into patients.

For many years after 1900 Neufeld contributed to pneumococcal research, including the discovery of the lysis of the pneumococcus after exposure to bile salts [43], the description of the Quellung reaction [44] and the immune sera's recognition of part of the pneumococcus, namely the capsule [45, 46].

Insights regarding the pneumococcal capsule culminated in Avery and Morgan's publication in 1925, which described the individual antigenic properties of both constituents of the capsule, carbohydrate and nucleoprotein [47] and led to Heidelberger and Kendall founding the basis of quantitative immunology in 1929 [48].

Several studies on various animal species followed, in the pursuit of developing a potent vaccine against the pneumococcus. Finally in 1945 MacLeod demonstrated that type-specific pneumococcal disease can be prevented in humans by a vaccine composed of four different capsular polysaccharides [49].

In 1928 Griffith made a significant observation: the capsular transformation reaction. Intraperitoneal injection into mice of live non-encapsulated pneumococcal variants together with heat-killed encapsulated pneumococci resulted in the recovery of viable encapsulated pneumococci from the animals [50]. Avery continued to work on the phenomenon of capsular transformation and described an induction of the transformation by polymerised deoxyribonucleic acid isolated from pneumococcus as early as 1944, a first step towards modern molecular genetics [51].

In his overview of the first one hundred years of pneumococcal research, Robert Austrian underscored the great achievements that had been made as well as the remaining lack of understanding of this very complex microorganism [52].

1.3. Taxonomy

An excellent summary of the taxonomy of *Streptococcus* involved in human disease is given in the Manual of Clinical Microbiology [53].

The genus *Streptococcus* has been subject to several classification changes over recent decades. Traditionally, haemolytic reactions and detection of Lancefield antigens have been the phenotypic criteria by which streptococci were grouped. The same Lancefield antigens, however, are expressed by unrelated species within the genus and related strains may express heterogenous Lancefield antigens [54]. Still, characteristic haemolysis and Lancefield serological tests can be used to divide streptococci into broad groups.

Lancefield group A, C or G antigen positive β-haemolytic streptococcal isolates can be subdivided into the large-colony (>0.5 mm in diameter) and the small-colony (<0.5mm in diameter) formers. The large-colony formers are also known as the "pyogenic" streptococci, of which group A streptococcus *(S. pyogenes)* is one example. The small-colony formers with group A, C or G Lancefield antigens are genetically distinct from the large-colony formers and belong to a species group called the "*Streptococcus milleri*" group, including *S. anginosus, S. constellatus*, and *S. intermedius*. To complicate matters further the members of the "*S. milleri*" group can produce β-haemolysis, but the majority of strains display α-haemolysis or no haemolytic reaction at all. In their ability to cause clinical disease, the small-colony formers differ from the "pyogenic" streptococci in terms of pathogenic ability. Although they are known to cause clinical disease, notably abscesses, they can also be part of the commensal gastro-intestinal flora [55].

Another large-colony forming β-haemolytic streptococcus, not groupable within Lancefield A, C or G, is *S. agalactiae*. This organism consistently produces Lancefield group B antigen. The group of α-haemolytic streptococci can be divided into the complex group of viridans streptococci and the species *S. pneumoniae*.

Recent advances in molecular technology have led to a closer understanding of the relatedness of streptococcal species and as a result, a re-grouping based on their genetic sequence similarities. Genome-based techniques group the viridans streptococci into the following species groups: mitis group, anginosus group (previously referred to as the "*S. milleri*" group), mutans group, salivarius group, and bovis group including *S. bovis*, a Lancefield group D antigen producing non-haemolytic species.

16S rRNA sequence comparison revealed a close phylogentic relationship (99% identical nucleotide sequence) between *S. pneumoniae* and the viridans streptococci, in particular two species in the mitis species group, *S. mitis* and *S. oralis* [56]. Single locus analysis of the superoxide dismutase gene (*sodA*) [57] or the *groESL* genes [58] also position *S. pneumoniae* closest to *S. mitis* and *S. oralis*. **Figure 1.1** illustrates the genetic relatedness of

strains belonging to the *S. mitis* group by using a phylogenetic tree based on concatenated sequences of *sodA* and *rnpB* (a) and *psaA* (b) [59].

Both, *S. mitis* and *S. oralis* have been implicated as donors of chromosomal DNA to pneumococci with regards to the development of penicillin resistance in *S. pneumoniae* [60] [61]. In return, pneumococci are known to be able to transfer altered penicillin-binding protein genes to viridans streptococci [62].

Allelic replacement via horizontal gene transfer is not restricted to the genes conferring antimicrobial resistance; it extends to a range of pneumococcal genes involved in virulence and competence either through recombination events or with the aid of bacteriophages [63].

The variety, and ready exchange of genetic material within this group of streptococci not only makes the task of defining phylogenetic relationships difficult, it also impacts on the selection of diagnostic targets and the development of new diagnostic tests and vaccines.

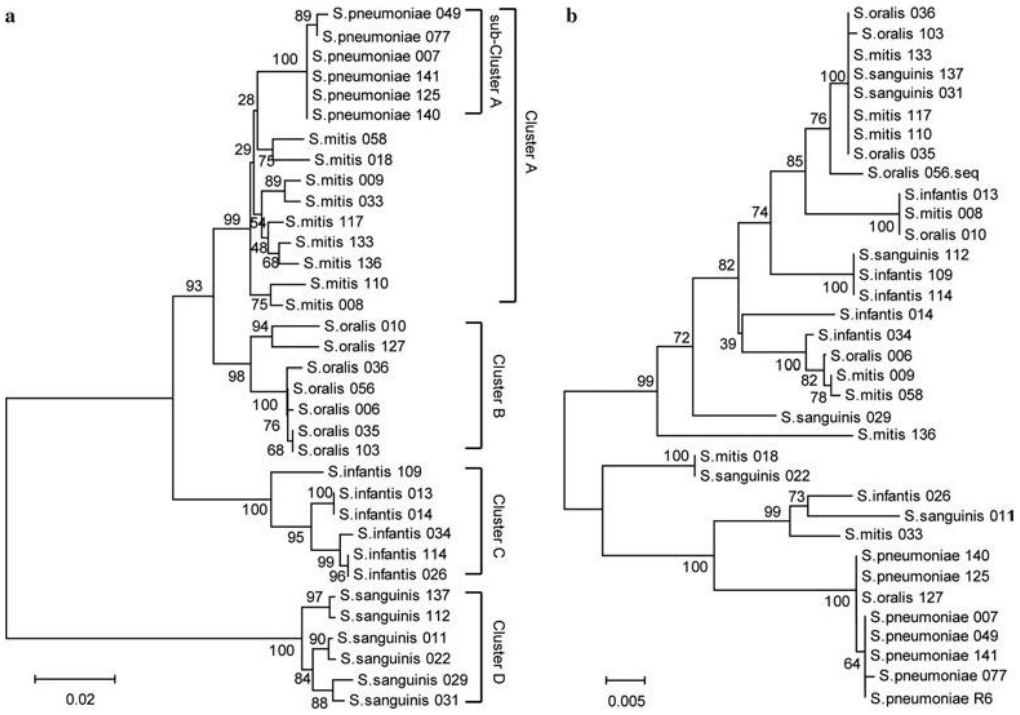


Figure 1.1: Phylogenetic trees demonstrating the genetic similarity of species belonging to the *S. mitis* group

The cluster analysis is based on the concatenated sequences of a) *sodA* and *rnpB* (housekeeping genes) and b) *psaA* (Reproduced from Zhang *et al*. 2010 [59]).

1.4. Virulence and clinical significance

IPD is the leading cause of illness and death globally, by the high mortality, especially in children. The following paragraphs will summarise the factors which make the pneumococcus such a potent and widespread pathogen.

1.4.1. Virulence factors

The pathogenicity of *S. pneumoniae* is no different from that of many other microorganisms in that it depends on a complex array of factors from the organism itself, and its interaction with the host's immune system, as well as predisposing environmental circumstances in order to produce disease.

The pneumococcal polysaccharide capsule, producing strains of the smooth phenotype, is considered the primary virulence factor, since non-encapsulated strains (rough phenotype) do not appear to have the same potential for invasion of host tissues. An understanding of an organism's virulence factors is vital in the process of developing effective vaccines. Currently, licensed vaccines are based on varying combinations of capsular antigens, but ever increasing knowledge regarding other pneumococcal proteins contributing to virulence has led to their consideration as potential vaccine targets [64-68].

1.4.2. Capsule

Pneumococci have three distinguishable surface layers: the capsule, the cell wall and the plasma membrane. The thickest layer is the capsule, consisting of high-molecular-weight polymers. The polymers are made up of repeating oligosaccharide units composed of 2 to 8 monosaccharides each. The cell wall anchors the capsule. It consists of a triple-layered peptidoglycan backbone, called the cell wall polysaccharide (CWPS), and proteins [69]. The CWPS is common to all pneumococci whereas the capsular polysaccharides are serotype-specific.

The main role of the capsule is to ensure that the organisms escape phagocytosis, and from the host immune defence by blocking access to the cell-wall-localised C3b. The capsule can also affect the amount of complement deposited [70].

Very early experiments in mice demonstrated that encapsulated pneumococcal strains were at least 105 times more virulent than non-encapsulated strains [71]. Through the production of pneumococcal mutants expressing different types of capsular polysaccharide from the parent strain it has been demonstrated that components outside the capsule are required for full virulence [72].

1.4.3. Cell wall or CWPS

The Gram-positive cell wall consists of glycan chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues, cross-linked via peptide side chains which have alanine as their first residue [73]. The CWPS is a complex teichoic acid that contains phosphorylcholine. The phosphorylcholine residues are the recognition site for N-acetylmuramic acid-L-alanine-amidase, also called autolysin, the enzyme that cleaves peptidoglycan. Autolysin is involved in the process of cell division as well as the lysis of pneumococci during the stationary growth phase [74]. CWPS activates the alternative pathway of complement. During that process C3a and C5a enhance vascular permeability, induce mast cell degranulation and the recruitment and activation of polymorphonuclear cells (PMN) [75].

1.4.4. Pneumolysin

This 53-kDa cytoplasmic pneumococcal protein belongs to the family of thiol-activated toxins [76]. It appears conserved among all the pneumococcal strains and is released during autolysis of the organism. It is not surface exposed. The cytotoxin can stimulate the production of inflammatory cytokines [77] and inhibits the cilial movement on human respiratory epithelial cells as well as disrupting the integrity of the bronchial epithelial cell monolayer. Low concentrations of the toxin have an effect on neutrophils by decreasing their bactericidal activity and migration [78] and the toxin inhibits lymphocyte proliferation and antibody synthesis [79]. Pneumolysin (Ply) activates the classical complement pathway in the absence of anti-toxin antibodies. Interestingly, Ply has sequence homology with C-reactive protein (CRP) [80-82]. As both can bind to C1q, competition between them might cause an abrogation of the protective effects of CRP [76]. Ply-negative mutants are less pathogenic

than their parent strains [83] and immunisation with the protein can prolong survival post pneumococcal challenge in mice [84].

Ply is part of a group of proteins present in pathogenic Gram-positive organisms, called the cholesterol-dependent cytolysins. They bind to cholesterol in the host cell cytoplasmic membrane and by insertion into the membrane form large pores. Lysis of the host cell follows. These cytolysins can exist in various forms. The water-soluble forms exist in aqueous solution as monomers and dimers [85], the cholesterol-bound form as dimers, and the membrane-bound form as large oligomers embedded in the lipid bilayer of a cell membrane.

1.4.5. Pneumococcal surface protein A

As an extracellular organism, *S. pneumoniae* has mechanisms to adhere to mammalian cells. Pneumococcal surface protein (PspA) is one of the surface-exposed choline binding proteins (CBPs) that are necessary for the attachment of bacteria to host cells through a specific choline binding motif. PspA is found in most, but not all clinically relevant pneumococcal isolates [86]. PspA proteins have been grouped into two major families consisting of five clades [87, 88]. The protein is required for full virulence in mouse models of IPD [89]. It is still unclear in what way exactly PspA contributes to virulence, but it seems likely that it inhibits complement activation [90].

1.4.6. Choline binding protein A

Choline binding protein A (CbpA) is a major CBP. It is surface exposed and adheres to host tissues. The host cell interaction is restricted to cytokine-activated human cells and could be a significant factor in the progress from colonisation to IPD [91].

1.4.7. Autolysin

This enzyme degrades the peptidoglycan backbone of the cell wall, which ultimately leads to cell lysis and cell death [92]. In *S. pneumoniae* autolysin is also termed LytA amidase or N-acetylmuromoyl-L-alanine amidase. The action of autolysin has a direct impact on virulence [93], which is actioned by releasing cell wall components that are highly

inflammatory in some animals [94] and indirect impact by releasing cytoplasmic proteins, including Ply.

1.4.8. Pneumococcal surface adhesin A

Pneumococcal surface adhesin A (PsaA) is a 37-kDa protein common to all pneumococcal serotypes [95]. The protein is known to be immunoreactive and has been considered a potential vaccine candidate and a diagnostic target [96]. PsaA has been shown to contribute significantly to adherence to nasopharyngeal epithelia [97]. The human cellular receptor that PsaA interacts with has been identified as E-cadherin [98]. E-cadherin also serves as a receptor for *Listeria monocytogenes* [99].

1.4.9. Other virulence factors

Neuraminidase, peptide permeases, hydrogen peroxide and IgA1 protease have all been implicated as potential contributors to virulence, either by mechanisms which enhance pneumococcal adhesion to host cells, or by direct tissue injury [100].

The expression of some of the virulence genes of *S. pneumoniae* appears to be modulated by heat shock and proteases [101]. As in other bacterial species the induction of heat shock proteins follows as a stress response mechanism to various environmental stressors. A heat shock induced serine protease HtrA (high-temperature requirement A) is involved in the pneumococci's ability to grow at elevated temperatures, to resist oxidative stress and to be genetically competent [102]. Another heat shock protein, the ATP-dependent caseinolytic protease ClpCP is required for the release of LytA and hence the cytotoxin Ply. A schematic overview of the location of pneumococcal virulence factors is given in **Figure 1.2.**

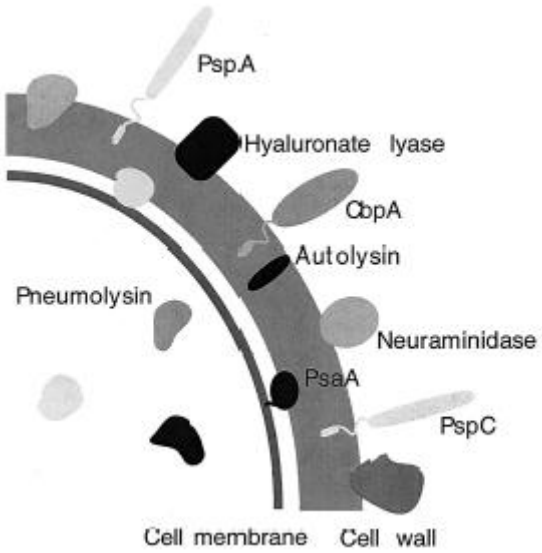


Figure 1.2: Pneumococcal virulence factors

Localisation of virulence factors in relationship to the bacterial anatomy (Adapted from Jedrzejas [103]).

1.4.10. Host defences

In order to protect the host from overwhelming infection with *S. pneumoniae*, the following parameters need to be ensured: the production of specific antibodies, the activation of the complement pathways leading to opsonisation and phagocytosis by tissue-fixed macrophages.

T cell immunity is important in the production of serotype-specific antibody, but does not appear to be a major factor in the prevention of overwhelming pneumococcal disease. Outside the obvious host cell defences, the complement system and the spleen, non-immunologic mechanisms like an intact respiratory integument, normal air flow and vascular perfusion within the respiratory tract, and undisturbed commensal microbiological flora, play an important role [104].

1.4.11. Complement system

It has been recognised for decades that the complement system plays an important role for both natural and acquired immunity to *S. pneumoniae*. In the early 1930s Ward and Enders demonstrated a role of complement as a pneumococcal opsonin [105]. In general, the

complement system functions as a mediator of inflammation and host defence [106], especially the terminal components C3 to C9. Microorganisms can activate C3–C9 either by the classical or by the alternative complement pathway.

Pneumococci can activate the classical pathway through its C polysaccharide complexed with C-reactive protein [107] or when sensitised with anticapsular antibody which binds to C1. However, this ability of different anticapsular antibodies varies greatly. In the classical pathway, antibodies of the IgG or IgM class bind to the antigen to form an immune complex. A conformational change in the Fc fraction of the antibody subsequently leads to binding to and activation of C1, the start of the pathway. C3 is cleaved into C3a and C3b, the latter is bound to the bacterial surface and acts as an opsonin or allows further activation of the rest of the complement cascade.

Pneumococci are known to be potent activators of the alternative pathway [108, 109]. Here the activation of C3-C9 occurs differently. C3 is cleaved by a bimolecular complex composed of C3b and factor B (Bb). It is designated C3b, Bb.

The initial generation of C3b can occur through a converting enzyme directly from C3, or by a labile enzyme outside the complement pathways. The stability of the C3b, Bb complex is influenced by control proteins working to destabilise the complex. It is thought that bacteria can bind to C3b and protect the protein and the complex from the action of those destabilisers [110, 111]. In principle, antibody is not required for the activation of the alternative pathway; however, aggregated immune complexes can activate the alternative pathway directly [112] or by mediation of an antibody's Fab portion [113].

In the immunocompetent host the alternative pathway activation in the defence against pneumococcal disease would be especially important in the non-immune host, early in the infection before the formation of specific antibodies. The classical pathway would become relevant in the later course of an infection once antibody production has begun or in an immune host when being challenged for a second time with the same pneumococcal serotype. Several *in vivo* studies with experimental animals have demonstrated the protective effect of C3 against IPD mainly through its action as an opsonin. A number of complement deficiency conditions have been researched in depth as to their susceptibility to IPD, including C3 deficiency, C3b inactivator deficiency, C2 deficiency and sickle cell disease (due to a defective function of the alternative pathway) [114].

1.4.12. Spleen

The increased risk that splenectomised patients will succumb to bacterial sepsis has been well described [115, 116]. The pathogens most frequently implicated in such infections are *S. pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Staphylococcus aureus*, with *S. pneumoniae* being responsible for about 50% of the cases [117]. Reasons for this include the capsule requiring efficient opsonisation for clearance of the pathogen from the bloodstream, the impaired formation of specific antibodies and rapid pneumococcal replication in the host.

In order to understand the role of the spleen in defence against these overwhelming infections it is important to reflect on its anatomy. The white pulp is the location where lymphocytes, plasma cells and macrophages are concentrated around a central artery, the red pulp is the reticular spongiforme tissue which makes up most of the organ and contains the Billroth endothelial cords and the macrophage-lined splenic sinusoids. The blood flow through the spleen is estimated to be 150mL/min. Due to its anatomical structure large volumes of blood are concentrated down so that particulate matter comes in close contact with a great number of splenic macrophages [118, 119].

Although in humans there are many lymphoreticular organs that are responsible for the production of antibodies it is likely that the large splenic blood flow favours the rapid production of specific antibody in the presence of particulate antigen.

Some interesting clinical observations have been made with regards to the differing risk of overwhelming sepsis post splenectomy. Children splenectomised before age 2 are at greater risk than children aged 5 and over at the time of splenectomy. Also, patients splenectomised due to trauma are at greatest risk during the first two years post-splenectomy. The latter observation might be due to the regeneration of some splenic tissue, called splenosis. It is also known that partial splenectomies can preserve a functioning host defense [120].

1.4.13. Predisposing factors for IPD

The single obligatory factor preceding IPD is nasopharyngeal colonisation with the homologous strain. Two host defense mechanisms play an important role in preventing infection: antibody production and complement, as well as the ready availability of functional phagocytic cells. Patients with a number of congenital and acquired defects in antibody production, namely Bruton’s agammaglobulinaemia, common variable immunodeficiency and

IgG subclass deficiency, are known to be prone to serious pneumococcal infection [121, 122]. Other immune system disorders, such as multiple myeloma, lymphoma, and chronic lymphocytic leukaemia make patients more susceptible to IPD [123]. The incidence of pneumococcal bacteraemia in patients with HIV is near 10 per 1000 per year [124]. Only those defects in complement leading to a deficiency in C3b are linked with IPD [125].

The reasons why the elderly are more prone to IPD are diverse. For one, the overall functioning of immune response appears diminished, reflecting a decreased number of immunoglobulins or the production of poorly functioning ones, as well as an impaired response to cytokines and the presence of other contributing illnesses.

A strong association of prior viral respiratory infection followed by serious pneumococcal disease has been described, foremost in the case of prior influenza infection. A preceding viral infection may up-regulate surface receptors on mammalian cells and therefore increase pneumococcal adherence leading to invasion of the pathogen. This was demonstrated by Håkansson in a study looking at the effect of adenovirus on bacterial adherence to human respiratory tract cells [126]. **Table 1.1** lists the conditions that are considered predisposing factors for patients to develop IPD.

Table 1.1: Conditions predisposing to IPD (Adapted from Musher [125])

Defective antibody formation	Congenital agammaglobulinaemia
	Common variable hypogammaglobulinaemia
	Selective IgG subclass deficiency
	Multiple myeloma
	Chronic Lymphocytic leukaemia
	Lymphoma
	HIV infection
Defective complement	Decreased or absent C1, C2, C3, C4
Insufficient numbers of PMNs	Cyclic neutropenia
	Drug induced neutropenia
	Aplastic anaemia
Poorly functioning PMNs	Alcoholism, liver cirrhosis
	Diabetes mellitus
	Glucocorticosteroid treatment
	Renal insufficiency
Defective clearance of	Congenital asplenia or hyposplenia
pneumococcal bacteraemia	Splenectomy, sickle cell disease
Multifactorial causes	Infancy and aging
	Chronic disease, hospitalisation
	Stress and fatigue, cold exposure
Excess likelihood of exposure	Day-care centres
	Prisons
	Military training camps
	Homeless shelters, household crowding
Prior respiratory infection	Influenza and other viruses
Inflammatory condition	Cigarette smoking
	Asthma, chronic obstructive pulmonary disease

1.4.14. Colonisation versus IPD

S. pneumoniae, amongst other microorganisms, has its ecological niche in the human nasopharynx. The nasopharyngeal flora in humans is established during the first months of life [127]. Every person is likely to be colonised with microorganisms including *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. aureus* during at least one period in their life [7]. Whether the colonisation results in asymptomatic carriage or develops into invasive disease depends on a complex array of factors involving the bacteria, the host's immune system and their interaction. One important concept is that IPD will not occur without prior colonisation with the homologous strain in the nasopharynx [127].

Colonisation plays an important role in horizontal disease spread. First the pathogen is disseminated to individuals in the immediate environment, followed by spread within a wider community [128]. It is intuitive, and has been reported, that crowded conditions such as day-care centres, hospitals, prisons, and over-crowded households encourage pneumococcal spread [129, 130]. With the highest incidence of nasopharyngeal colonisation in young children, and the modern reliance on day-care centres it seems logical to focus prevention strategies of IPD on pneumococcal colonisation, especially in very young children.

The dynamics of nasopharyngeal colonisation with potential pathogens is greatly influenced by the balance between the resident flora, which includes α-haemolytic streptococci, and the opportunity of transient microorganisms to establish themselves in their new ecological niche. Under ideal circumstances for the host the resident flora plays an inhibitory role preventing potential pathogens to find a niche. This can however be altered during upper respiratory tract infections and under the influence of antimicrobial therapy [131-133]. To further complicate issues, the different pathogenic bacterial species occupying the nasopharynx demonstrate a competitive relationship, with interference in each other's growth most likely linked to the effect of bacterial enzymes [134].

The local mucosal immune response of the host has an important regulatory role. In case of a successful response a transient pathogen is eliminated and re-colonisation is prevented. However, a poor host response can lead to persistent colonisation and ultimately to invasive infection. Local mucosal immunity matures earlier than systemic immunity in the infant and should be present from the age of 6 months [132, 133]. In that context there is an age-related decline in pneumococcal colonisation whereas *S. aureus* carriage rates increase from 10% in the first year of life up to 50% by age 10 [7].

While colonisation is the first step to developing invasive disease it is usually self-limiting and lasts from 2 to 18 weeks depending on the serotype. Clearance of the organism is $CD4^+$ T cell dependent and B cells do not appear to be essential [135, 136]. In fact, colonisation induces specific anti-capsular polysaccharide antibodies and studies have demonstrated significant levels of these antibodies in adults prior to vaccination [137, 138].

Pneumococcus is able to directly invade the respiratory epithelial cell *in vitro* [139], which could be an important factor in the transition process from colonisation to IPD. To understand the transition process it is important to consider the known virulence factors as having dual roles; one in the establishment and maintenance of colonisation, and the other in some of the stages leading to invasive disease. In order to be able to function in such dual capacity the expression of these virulence genes needs to be under the control of transcriptional regulators. These regulators control metabolic pathways by either direct or indirect activation or repression of virulence gene expression [140]. Another mechanism of gene regulation is reflected in the organism's ability to exist in two different phenotypic phase variants. The different variants are associated with different virulence properties [141, 142]. The more adhesive transparent colony variants predominately present during colonisation have less polysaccharide capsule, more cell wall teichoic acid, more CbpA, and less PspA than the opaque variants associated with invasive isolates [142, 143]. Environmental oxygen influences the production of capsular polysaccharide in these phase variants. Under reduced oxygen conditions the opaque variant produces more, whereas that of the transparent variant remains almost unchanged [144].

Interestingly, microarray analysis of pneumococcal gene expression during invasive disease has demonstrated that the majority of genes do not change expression. In blood, cell wall and cell membrane synthesis and competence were unchanged, but ribosomal transcription was decreased as was glycolysis, but increased production of some CBPs occurred. In cerebrospinal fluid (CSF) the bacteria demonstrated decreased gene expression for competence, autolysis and toxin production, but increased synthesis of amino acids and choline on the cell surface [145]. A study by Oggioni proposed that pneumococci during bacteraemic sepsis resemble planktonic growth, and those found in tissue infections, such as pneumonia and meningitis, closely resemble a biofilm-like state [146]. Further complex analysis of genetic expression patterns during different stages of the disease and in multiple differing bacterial strains is necessary to gain a better understanding of the processes involved in the change from a coloniser to invasive pathogen.

1.4.15. The clinical spectrum

Direct spread of *S. pneumoniae* from the nasopharyngeal colonisation site to adjoining anatomical sites can cause infection of the middle ear, sinuses, the bronchi and the lung tissues. Other sites like the central nervous system (CNS), heart valves, bones, joints and peritoneal cavity are usually reached via haematogenous spread of the organism. However, the CNS, pleural space and peritoneal cavity can be infected through direct spread from the middle ear and lungs, respectively. In clinical cases it is often impossible to determine the route of infection.

In principle, the process that leads to clinical disease starts with *S. pneumoniae* becoming established in the nasopharynx, followed by adherence to host cells and extracellular replication, which then can lead to local invasion into the host tissues. If the patient has no pre-existing immunity and other conditions favourable to IPD are present, local invasion can proceed to invasion of the blood stream and lymphatic vessels or other body sites where the organism resists phagocytosis, replicates and causes inflammation and subsequent tissue damage [104].

The following paragraphs discuss the clinical syndromes individually.

1.4.16. Otitis media

Among children, acute otitis media (AOM) is the most common bacterial infectious disease with *S. pneumoniae* contributing to approximately 40% of the cases [147]. Studies investigating the causative organisms of AOM in children have implicated *S. pneumoniae* in about 40-50% of the cases in children aged 6 months to 4 years in which an aetiological agent had been identified. In adults, the pneumococcus also prevails as the major pathogen [148]. Prior viral infection of the upper respiratory tract is thought to cause congestion of the Eustachian tube due to local inflammation. In turn, any drainage from inflammation and infection of the middle ear is prevented.

Generally AOM follows on from a nasopharyngeal colonisation with a different serotype. The serotypes most often implicated are 6, 14, 19F and 23F, perhaps due to their ability to adhere more avidly to mammalian cells [149].

1.4.17. Sinusitis

Similar to AOM, sinusitis is caused predominantly by either *S. pneumoniae* or *H. influenzae*. An allergic pre-condition or a preceding viral respiratory infection again leads to local inflammation and congestion of mucosal membranes, which prevents clearance from the sinus cavity. The accumulation of fluid during the inflammatory process provides an ideal growth medium for bacterial proliferation and subsequent acute infection.

1.4.18. Meningitis

S. pneumoniae is, excepting epidemics caused by *N. meningitidis*, the most common cause of bacterial meningitis in adults [150]. In countries where routine vaccination against *H. influenzae* type b has been introduced, *S. pneumoniae* is the leading cause of sporadic meningitis in children over the age of 6 months.

Pneumococcal meningitis can be the result of direct organism spread from the middle ear and the sinuses, or can result from haematogenous dissemination of bacteria [151]. There is supportive evidence for both routes of infection of the CNS, but whether one route is favoured over the other remains inconclusive [152, 153]. It is proposed that pneumococci enter the meninges by an upregulation of platelet activating factor on the cells of the vascular epithelium to which the bacteria adhere [154]. As in other body compartments the pathogen avoids phagocytosis and induces inflammation.

Meningitis caused by *S. pneumoniae* is clinically indistinguishable from that caused by other infectious agents.

1.4.19. Pneumonia

Once pneumococci reach the alveolar spaces and replicate, pneumonia can follow. Bacterial proliferation leads to activation of complement, cytokine production, up-regulation of receptors on vascular endothelial cells and as a result, an increased opportunity for the pathogen to adhere to cellular surfaces. Exudate, white blood cells and pneumococci accumulate within the alveoli. The filling of alveolar cells is observed on a chest radiograph as an area of consolidation.

In most cases of pneumococcal pneumonia, patients have two or more predisposing factors [155]. A significant proportion of patients have concomitant cardiac infarction and some have been discharged from hospital within the past 6 months.

Clinically, cough, fever, chills, fatigue and shortness of breath prevail. These symptoms are more common in younger age groups [156, 157]. Elderly patient may have only mild fevers or remain afebrile, yet present with increased respiratory rate [156]. The absence of fever or the presence of hypothermia in younger patient groups is associated with higher morbidity and mortality.

On physical examination, diminished respiratory excursion due to pain (splinting) may be a feature, as is dullness to percussion in almost 50% of cases. Crackling sounds can be heard on auscultation almost universally. Still, in patients who have chronic obstructive pulmonary disease (COPD), this finding can be difficult to attribute with certainty to pneumonia. Bronchial breathing may be present as well as flatness to percussion at the lung base and limited diaphragmatic motion on the affected side. In any case, unless all vital signs are normal and the patient is unlikely to suffer from pneumonia, the definitive diagnosis of pneumonia is made through a chest radiograph [158].

Radiographically, most cases of pneumococcal pneumonia show an area of infiltration of one or more segments within the affected lobe. In rare cases a lung abscess can be demonstrated [159]. Since abscesses are unusual in the context of *S. pneumoniae* infection, it is imperative to rule out a concurrent anaerobic infection or an anatomical abnormality encouraging abscess formation such as bronchial obstruction, cancer, or pulmonary infarction.

A pleural effusion can be present in cases of pneumococcal pneumonia, but they generally have insufficient fluid for aspiration. Empyema is a known complication of pneumococcal pneumonia, but has been a rare occurrence since the introduction of antimicrobials [160].

General laboratory investigations can assist diagnosis. Haemoglobin is $\leq$ 10mg/dL in 25% of cases. Leukocytosis is common (WBC >12000/mm^3), but it can remain normal or even be decreased (< 6000/mm^3) in about 5-10% of hospitalised patients and is indicative of a poor prognosis [161-164]. Other biochemical markers can be affected, such as a decreased serum albumin, and increased serum bilirubin and lactate dehydrogenase levels. However, these biochemical abnormalities have to be evaluated with great care; they may be attributed to the patient's underlying disease.

1.5. Prevention and treatment strategies

With the introduction of effective treatment and preventative vaccines the pneumococcus has been placed under increasing biological selection pressure. Bacteria, including *S. pneumoniae*, develop mechanisms to help them adapt to novel environmental changes and ensure their survival.

1.5.1. Pneumococcal vaccines

The history of the first attempt at pneumococcal vaccination dates back to Sir Almoth Wright in 1911 [165]. The discovery and isolation of the pneumococcal capsular polysaccharides was followed by the first, univalent, polysaccharide vaccine being trialled in the late 1930s [166]. The first successful clinical trial with a multivalent vaccine took place in 1945 [49] and led to the licensure in the USA by the US Food and Drug Administration in 1946. The advent of introduction of the antimicrobial penicillin intervened with progress in pneumococcal vaccine development due to a lack of demand. The licensure was withdrawn. Only the emergence of penicillin and multi-drug resistance in pneumococci revived the interest in vaccine prevention strategies. A 14-valent polysaccharide vaccine [167] was licensed in 1977. This vaccine was superseded by the current 23-valent polysaccharide vaccine which was licensed internationally in 1981.

The currently used 23-valent pneumococcal polysaccharide vaccine is used in adults and children over the age of 2 years, as well as to some degree in high risk patients, including those with sickle cell disease or asplenia. Responses and particularly longer-term maintenance of antibody titres in HIV-infected individuals are dependent on CD4 counts and suppression of HIV replication is associated with better serological responses. A recent study shows an improved serological response with the 7-valent conjugate vaccine vaccine compared to the 23-valent polysaccharide vaccine [168, 169]. The 23-valent polysaccharide vaccine has several well documented limitations. Firstly, it is not effective in children under 2 years of age which is the patient age group mainly affected by pneumococcal colonisation and disease. When given the 23-valent vaccine these young infants show a grossly impaired immune response [170]. Nasopharyngeal colonisation itself is not affected by the vaccine [171]. Other limitations are a lack of effectiveness against acute pneumococcal otitis media and non-bacteraemic pneumococcal pneumonia [172, 173]. A recent publication from the United Kingdom was looking at the impact and effectiveness of the 23-valent vaccine against IPD in

the elderly and concluded that there was modest overall effectiveness and lack of discernible impact on the incidence of IPD and PCV-induced serotype replacement. However, the vaccine was considered a viable cost-effective intervention for older low risk individuals [174].

By its inherent composition the vaccine does not elicit a T cell-dependent immune response. As a consequence, the duration of protection through the vaccine is limited and it fails to produce immunological memory. Antibody levels to the 23-valent polysaccharide vaccine usually wane about 5 years post immunisation, but can decline more rapidly in immunocompromised patients [175]. However, the coverage of serotypes known to cause IPD in the UK is excellent at about 96% [176]. In the recommended age groups the overall efficacy of preventing IPD ranges from 50-70% [175, 177-179].

Despite all its shortcomings, a role for the 23-valent polysaccharide vaccine will remain in the near future. It can be used as a booster vaccine following primary immunisation with the newer conjugate vaccines in infancy [180]. In that setting it may extend the duration of antibody response against the serotypes included in the conjugate vaccines as well as eliciting antibody response to those serotypes not included in the conjugate, but included in the polysaccharide vaccine.

The pneumococcal conjugate vaccine is based on linking the capsular polysaccharide to a protein carrier, mutant diphtheria protein (CRM197), to increase its immungenicity. The conjugation leads to a switch from a T cell- independent to a T cell-dependent response, in turn leading to the induction of a B cell memory response and better immune responses in the younger age groups. The necessary proportional balance between carrier protein and polysaccharide antigens limits the number of serotypes that can be included in the conjugate vaccine [181].

To date, several different conjugate pneumococcal vaccines have been developed containing the serotypes most frequently causing IPD and most commonly associated with antimicrobial resistance. The number of included serotypes ranges from 4 to 13. Currently, 8-, 10-, 11-, and 13-valent vaccines have been in phase II or III trials. PCV-7, licensed in the United States since 2000, includes a composition of serotypes mainly based on epidemiological data concerning the USA. These serotypes were responsible for 86% of bacteraemia cases, 83% of meningitis cases, and 65% of AOM cases in children under 6 years in the USA during the years 1978-1994 [182]. The geographical coverage of the vaccine depends on the geographic variation of prevalent serotypes, varying disease patterns and differing epidemiological distribution of serotypes nationally and globally. This explains why

the rate of coverage averages 85% in the USA, but only 60-70% in Europe and 55% in Asia [183].

To date, over 50 countries worldwide have incorporated the 7-valent vaccine into their national immunisation programs, including Latin American and Caribbean countries [184]. More than 100 million doses of vaccine have been distributed. Within the first year after routine introduction of the vaccine, rates of IPD covered by the vaccine serotypes decreased significantly in the < 2 years age group. Recent reports claim a 94% decline of IPD caused by the vaccine serotypes in the age groups recommended for vaccination, and indirect beneficial effects in unvaccinated groups have been documented [15]. Although the anticipated increase in disease caused by serotypes not included in the PCV-7 ("replacement disease") has been described it appears small in comparison to the overall reduction of disease burden by IPD [185].

Research in the development of new pneumococcal vaccines has led to protein-based vaccines, of which some have undergone pre-clinical or phase I and II clinical trials. The most popular targets for these protein vaccines include Ply, PspA, PspC, and the histidine triad proteins. Reverse vaccinology has contributed significantly to the identification of suitable proteins as vaccine candidates [186]. It has even been suggested that a combination of several pneumococcal virulence proteins may offer superior protection than a single antigen [187].

Despite huge progress in the last decade, including the introduction of PCV-10 and PCV-13 in some countries, the challenge still remains to find the most appropriate vaccine globally, one that covers all strains known to cause invasive disease, one that is easy to administer and cheap to produce. With the discrepancy between serotypes prevalent in the developed versus the developing world, a single vaccine may not be the answer. Several vaccine compositions targeting individual geographic areas could solve this challenge. However, in light of the cost of vaccine production, this option seems the least likely.

1.5.2. Treatment strategies

For years penicillin was the drug of choice in the treatment of IPD. Until the late 1970s penicillin resistance did not pose a problem in clinical isolates and *S. pneumoniae* was susceptible to antimicrobial concentrations easily achievable by the oral or parenteral route. However, since then emergence of antimicrobial resistance has complicated treatment approaches [104]. Resistant strains are more often found in children than adults, most likely

due to children harbouring *S. pneumoniae* in the nasopharynx as a coloniser, and their more frequent exposure to antimicrobials, which promotes the selection of antimicrobial-resistant pneumococci.

An important pharmacokinetic and pharmacodynamic consideration for antimicrobials targeting *S. pneumoniae* is the organism's extracellular existence. A sufficient concentration of unbound antimicrobial needs to be present extracellularly at the site of pneumococcal infection or colonisation in order to be effective.

Kathryn Edwards gives an excellent overview over the recommended treatment regimens of various diseases caused by pneumococci in children and adults in "The pneumococcus" [188].

Recent reports state that the global prevalence of macrolide resistance among pneumococci is now > 35%, and >40% for penicillin [189, 190]. What can be done to minimise the clinical and epidemiological impact of resistance? In his recent (2007) report, Klugman summarises some strategies that warrant implementation, such as the reduction of inappropriate use of antimicrobials, especially in the primary care setting, the use of appropriate drug dosages, and the drug selection according to its half-life and bactericidal versus bacteriostatic properties. In summary, the drug selection should focus on limiting selective pressures on the pathogen [191].

In 2008, the Clinical and Laboratory Standards Institute (CLSI) revised and published new breakpoints for penicillin minimal inhibitory concentrations (MICs) for the treatment of non-meningitis cases. It supported the prescription of penicillin for pneumococcal infections outside the CNS [192] and to this day beta-lactam antimicrobials continue to be the drug of choice for the treatment of pneumococcal pneumonia, with fluoroquinolones providing an alternative therapy. Combination therapies of beta-lactam therapy with macrolides are an option in the treatment of severe cases [193].

1.6. Epidemiology

Prospective surveillance to determine the incidence of pneumococcal disease has become even more important since the introduction of the 7-valent conjugate vaccine (PCV-7) into several routine immunisation programmes around the world. It is vital to obtain accurate data to assess vaccine efficacy and follow the change of serotype distribution at a global level. The accuracy of data is dependent on the case definition of pneumococcal

disease, on the sensitivity and specificity of the diagnostic methods used to obtain the data, and their availability in clinical settings, especially in under-resourced countries. These important factors contribute to the difficulties faced by epidemiological studies. The lack of a sensitive and specific "gold standard" in the laboratory makes it difficult to extract useful data from a comparison of newer versus older diagnostic tests. It is important to bear this in mind when studying epidemiological assessments of pneumococcal disease, and it is safe to assume that the reported global disease burden is underestimated.

1.6.1. Global epidemiology

The WHO estimated that in 2002 there were 716,000 deaths globally attributable to IPD in children less than 5 years. In this age group, IPD is the most common cause of vaccine-preventable death [194]. A recent analysis of the Global Burden of Disease Study 2010 (GBD 2010) published in the Lancet produced estimates looking at specific disease causes. The GBD 2010 estimated annual deaths globally for 21 regions between 1980 and 2010. Here, 168,000 deaths were reported for pneumococcal lower respiratory infection in children younger than 5 years compared to 381,000 deaths in 1990. For all ages, 827,300 deaths were attributable to pneumococcal pneumonia and 118,400 deaths to pneumococcal meningitis in 2010, a 3.6% and 5.2% decrease in numbers compared to 1990, respectively [195].

The reported incidence of IPD in the United States before 2000 in children less than 2 years of age was 188 per 100,000 [196]. The serotypes responsible for these cases were largely included in the preparation of PCV-7.

In the United States, surveillance for IPD is conducted via the Active Bacterial Core Surveillance system (ABCs), a branch of the Centers for Disease Control and Prevention (CDC). They found a 94% decline in IPD caused by the vaccine serotypes in children younger than 5 years, but also in older persons, presumably through herd protection effect [197].

In Canada PCV-7 was licensed in 2001. Surveillance is conducted via the Calgary Area *S. pneumoniae* Epidemiological Research (CASPER) in the Calgary region. They recently reported a 92% decrease in IPD caused by vaccine serotypes, whereas the rate of IPD caused by non-vaccine serotypes remained unchanged in children less than 2 years of age [198].

Several countries in Europe have introduced PCV-7. In Spain, for example, it was introduced in 2001 and in France in 2003. The vaccine uptake in Spain was patchy and no national laboratory-based surveillance system was in place to supervise IPD reporting and to

ensure comprehensive data collection. This may be reflected in the less dramatic reduction of cases of IPD than in the US and Canada.

In France, laboratory-based surveillance is conducted by the Institut de Veille Sanitaire (EPIBAC). France has reported significant reductions in pneumococcal bacteraemia and meningitis since 2003 [185].

Australia recommended the use of the PCV-7 in 2001 for all Aboriginal infants. The baseline incidence for cases of IPD was 219.2 per 100,000 in 2001 and dropped to 91.5 per 100,000 in 2004 in children less than 2 years old [199]. Universal vaccination for all Australian infants was started in 2005 and further reduction in IPD cases is to be expected.

In New Zealand, PCV-7 became part of the childhood immunisation schedule in June 2008 and was replaced by PCV-10 in July 2011 [200] and a decline of IPD in children younger than 2 years has been observed (section 1.6.2.).

1.6.2. Serotype epidemiology

The majority of cases of IPD are caused by a relatively small number of serotypes. Serotypes 1 and 2 are particularly associated with invasive disease. In the UK, serotypes 1, 4, 14 and 18C were found mostly in IPD, whereas serotype 23F was generally found to be present in colonising strains. After the first decade of life, the decline in certain serotypes, namely 6, 14, 19, and 23, is rapid. For serotype 1, however, the decline occurred gradually through adulthood. Serotypes 3 and 8 appear to have a predilection to infect adults. Serotype distribution differs in various regions of the world. Serotype 1 is common in children and adults in Asia, Africa, Latin-America, and parts of Europe, but uncommon in the USA, Canada, Scandinavia, and Oceania. It is important to remember that serotype 1 is not included in PCV-7, which renders the vaccine far less suitable for use in countries of the developing world. Still, serotypes 6, 14, and 19 are vaccine serotypes and are among the leading serotypes in IPD around the world [201].

Molecular typing methods like Multi Locus Sequence Typing (MLST) have provided a vast quantity of new information regarding pathogenic pneumococcal serotypes. Under stress pneumococci have been known to replace serotypes [202, 203]. In that situation sequence based data will be invaluable to gain new insights into the frequency of these events and the specific predisposing stressors.

1.6.3. Host-related influences on epidemiology

Age is a well documented risk factor for development of IPD. The very young and very old are most frequently and severely affected. Gender also appears to play a role as demonstrated by overrepresentation of males in infants, children and immunocompetent adults [204]. In the United States the rate of IPD is higher for black and Native Americans than any other racial group, and in Australia IPD is highest among Aboriginal communities.

Socioeconomically the rate of IPD is positively associated with poverty and crowded living conditions. Other host factors for increased risk of developing IPD were discussed in section 1.4.3 [182].

1.6.4. Local epidemiology

In a paper published in 2007, Heffernan *et al.* gives an overview over the epidemiology of IPD within New Zealand [205]. The data were collected through the national laboratory-based surveillance system, whereby all diagnostic laboratories in New Zealand refer their pneumococcal isolates from blood, CSF and other normally sterile sites to the national reference laboratory at the Institute of Environmental Science and Research (ESR). Only the first isolate from a case was included in the surveillance data analysis. Clinical details, ethnicity and demographic data were inconsistently supplied. Isolates from 3903 cases of IPD were collected over an 8 year period from 1998 until 2005. During that time IPD was not notifiable to the Medical Officers of Health. The lack of a formalised notification system for cases of IPD is likely to have contributed to a higher level of inaccuracy in incidence data than would be expected within a notification framework. Nearly one-third of these cases were in children aged less than 5 years, and another third were cases of adults age 65 and over. The highest incidence was in infants less than 2 years of age and adults of 75 years or older. There was no significant change in the overall incidence of IPD during the study period. The incidence averaged 12.4 per 100,000 per annum, which is consistent with reports from other developed countries [206]. It had its lowest annual incidence in 1998 (9.9 per 100,000) and the highest in 2001 (13.8 per 100,000). The fact that children under the age of 2 are most frequently affected was illustrated in the annual incidence of 100.8 per 100,000 confirming the findings of a previous study in the Auckland region of New Zealand [207]. Incidence in males was greater than that of females, except in the 55-64 age groups, and age and gender distribution remained constant throughout the 8-year period.

Serotype prevalence amongst these isolates from cases with IPD showed a consistent predominance of serotypes 14, 6B, 4, 19F, 9V, 23F and 18C, with serotype 14 being the most common. However, within the study period, serotypes 14, 6B and 4 were increasing and 9V, 7F, 1 and 12F were found to be decreasing. Looking at the vaccine coverage with PCV-7 and keeping the above distribution of common serotypes in mind, the coverage rate for infants younger than 2 years ranged from 69.5% in 1998 to 91.5% in 2002.

The four serotypes, 19F, 9V, 23F, and 14 accounted for 93.2% of penicillin-resistant pneumococci over the whole 8-year period. All cases of penicillin-resistant IPD in children less than 5 years were serotypes included in PCV-7.

The susceptibility testing results were interpreted according to the CLSI standards from 2006 [208]. In New Zealand, resistance to penicillin was uncommon among invasive pneumococcal isolates prior to 1995, but increased thereafter [209, 210]. Compared with other countries the rate of penicillin resistance in New Zealand is high. In 2004 the rate in New Zealand was 10.1% penicillin-resistant IPD isolates compared to <1% in the United Kingdom and 4.4% in Australia in the same year [199, 211].

The study susceptibility data from the time between 1998 and 2005 demonstrated no overall significant change in the prevalence of penicillin resistance, however, resistance decreased between 1998 and 2002, but then increased thereafter. In contrast, the resistance to other antimicrobials, namely cefotaxime, erythromycin and tetracycline increased significantly over the 8 year time frame.

In the age groups of less than 5 years and over 65 years, the prevalence of antimicrobial resistance was the highest, with multi-resistance being most common among isolates from children less than 5 years old.

In New Zealand data obtained through the national laboratory-based surveillance system led by ESR contributes to national policy development regarding treatment and prevention strategies, including the national immunisation policy and national guidelines for empirical antimicrobial therapy. PCV-7 (Prevnar 7, Pfizer NZ Ltd.) and PCV-10 (Synflorix, Pfizer NZ Ltd.) have been part of the New Zealand childhood immunisation program from June 2008 and July 2010, respectively. PCV-13 (Prevnar 13, Pfizer NZ Ltd.) is available for some high-risk children. The other licensed vaccine is the 23-valant pneumococcal polysaccharide vaccine, PPV-23 (Pneumovax®, MSD), which is recommended and funded 5 years after the first vaccination post splenectomy and for all those over age 65 years [200]. **Table 1.2** lists the serotypes covered by the polysaccharide protein conjugates vaccines.

A decline in cases of IPD in children younger than 2 years has been noted since the introduction of PCV-7, but herd protection has not been observed yet. In the United States the herd effect was noticeable 3 years post introduction of PCV-7 [200] and it is expected to see an effect on herd protection in New Zealand soon.

IPD has recently been included as a notifiable disease to the Medical Officers of Health in New Zealand. Relevant demographic and ethnicity data are submitted with referred pneumococcal isolates. The incidence of IPD among Maori and Pacific Island children in an Auckland-based study was almost twice and four times above the rate in other ethnic groups, respectively [207]. It will be interesting to analyse the data available post PCV introduction and post national notification of IPD to assess the effect the national immunisation program has had on the prevalence of disease in ethnic groups living in New Zealand. Such data would improve the understanding of the national epidemiology of pneumococcal disease and would assist in measuring the effects the changes in New Zealand's childhood immunisation policy have over time.

Table 1.2: Vaccine serotype coverage

Coverage of 3 different pneumococcal conjugate vaccines and one polysaccharide vaccine (Adapted from the NZ Immunisation Handbook 2011 [200]).

Vaccine	***Covered serotypes***
PCV-7	Serotypes 4, 6B, 9V, 14, 18C, 19F, 23F
PCV-10	Serotypes in PCV-7 plus 1, 5, 7F
PCV-13	Serotypes in PCV-10 plus 3, 6A, 19A
PPV-23	Serotypes in PCV-13 plus 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, 33F

1.7. Current diagnostic methods

In an editorial John Bartlett pointed out the shortfalls of microbiological diagnostic methods when it comes to diagnosing pulmonary infections [212]. Bartlett claims that instead of having more rapid and conclusive diagnostic tests available, our capabilities have, if

anything declined. He concludes that *"either the pneumococcus is disappearing or microbiology is disappearing"*. Aside from the microbiological difficulties in diagnosis of IPD, another set of questions could be raised: Do clinicians have a higher threshold for performing diagnostic tests in the climate of funding constraints? Has the clinician's attention to obtaining good quality diagnostic samples diminished?

Isolation of *S. pneumoniae* in bacterial culture from a normally sterile body site provides conclusive evidence of infection, however this evidence is surprisingly difficult to obtain and it is achieved only in the minority of cases [213-215]. In the case of pneumococcal pneumonia the clinician can be faced with problems obtaining a good quality lower respiratory tract specimen for testing, and as a result, differentiation between true invasive infection and nasopharyngeal colonisation without infection can be impossible to make. Prior administration of antimicrobials also significantly reduces the isolation rate of viable *S. pneumoniae* from clinical samples.

The consequences deriving from a lack of rapid and conclusive diagnostic tests for IPD extend beyond those for the individual patient. Without suitable and reliable diagnostic tests it will be impossible to assess the effectiveness of preventative measures, such as pneumococcal vaccination, and to gain accurate data regarding IPD disease burden locally and globally. On the other hand, the increasing use of molecular tests in favour of culture-based methods poses a threat to pneumococcal strain surveillance in terms of high cost associated with or inability to perform strain-typing and resistance gene testing.

The laboratory diagnosis of IPD today relies still on methods or variations of methodologies which have been around for many decades.

1.7.1. Microscopy and culture

The laboratory identification of *S. pneumoniae* isolates is based on their morphology and phenotypic tests. As a member of the genus S*treptococcus*, the organism is Gram-positive, catalase-negative, facultatively anaerobic, with a DNA G+C content ranging from 34 to 46 mol%. Although they can grow in the presence of oxygen, they cannot synthesise haem compounds and are therefore incapable of respiratory metabolism [216]. Microscopically, *S. pneumoniae* appear as lancet-shaped Gram-positive diplococci or short chains of cocci (**Figure 1.3**).

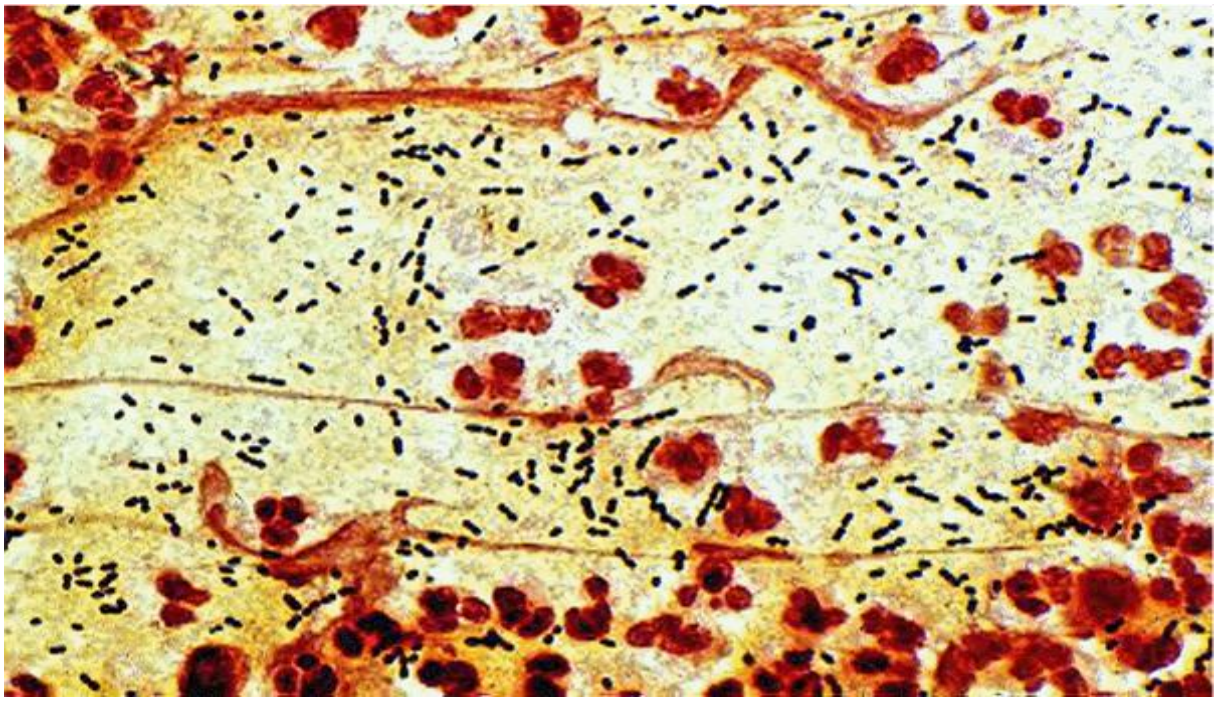


Figure 1.3: Gram stain of the pneumococcus in sputum

For an experienced microscopist, these features are highly specific to *S. pneumoniae*. However, for the less experienced scientist, misidentifications can occur due to alterations of the typical appearance of the organism by antimicrobial therapy, which can change the shape as well as the staining pattern. Over-decolourisation of the Gram stain can produce the false impression of Gram-negative diplococci, potentially resulting in an initial misdiagnosis of a clinical case.

The Quellung test is infrequently used, but it is a specific method for pneumococcal identification. The principle of this test is that the pneumococcus reacts with streptococcal anticapsular antisera, which in turn leads to visual enhancement of the pneumococcal capsule. The bacterial cell appears to be surrounded by a halo (**Figure 1.4**).

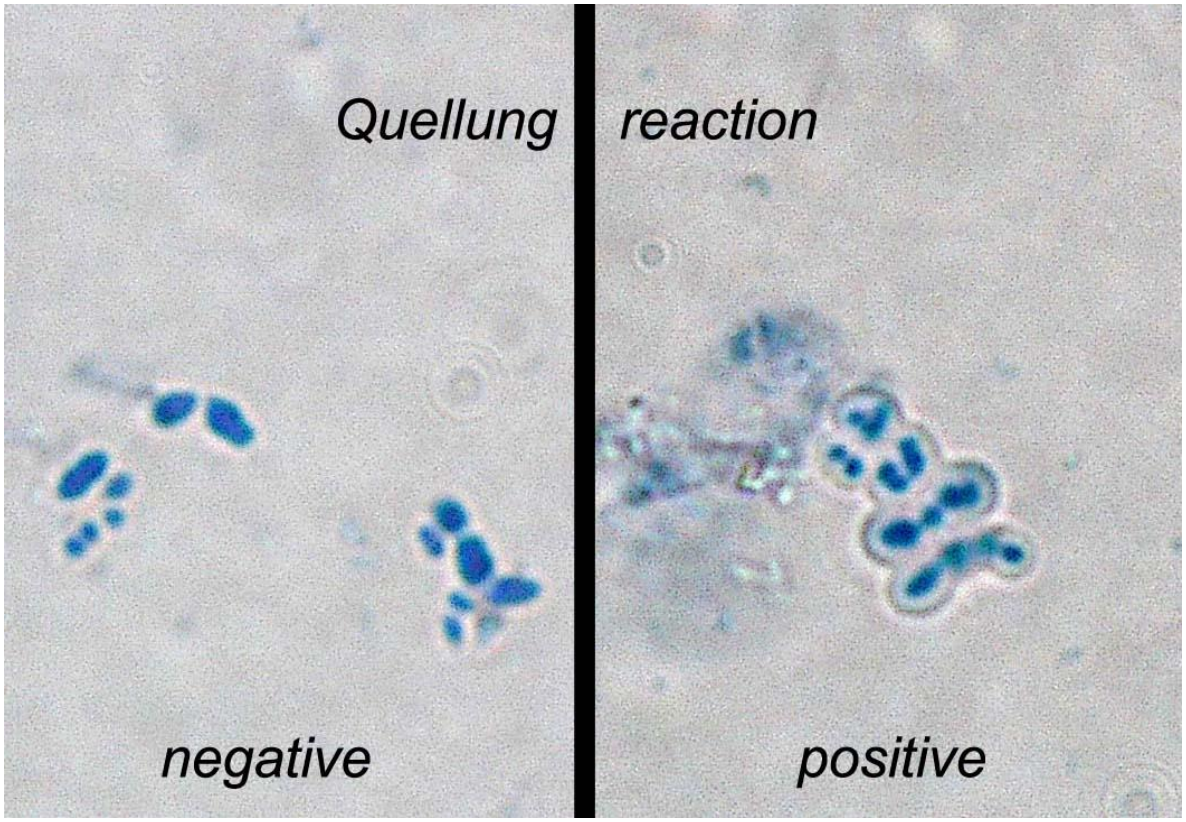


Figure 1.4: The Quellung reaction [217]

After overnight incubation at 35°C with 5% CO_2 on 5% sheep blood agar (SBA) or chocolate agar, pneumococcal colonies appear small, greyish and mucoid. The colonies are surrounded by a greenish zone of α-haemolysis. After 24-48 hours of incubation the colonies display a central depression; they are called "draughtsman" colonies (**Figure 1.5**). Further phenotypic tests are necessary to confirm identification. Two key reactions presumptively differentiate between *S. pneumoniae* and other viridans streptococci: optochin susceptibility (**Figure 1.5**) and bile solubility. Optochin (ethylhydrocupreine) has antibacterial properties. It is however not used therapeutically but only in the context of laboratory identification of streptococci. To obtain the most reliable results, optochin susceptibility should be performed in 5% CO_2 and on SBA [218]. In the presence of the surfactant sodium deoxycholate *S. pneumoniae* autolyses, this forms the basis of the bile solubility test. *S. pneumoniae* isolates are typically optochin-susceptible and bile-soluble, while other viridans streptococci are the opposite; they are optochin-resistant and bile-insoluble. It has been reported however that up to 10% of *S. pneumoniae* isolates can be optochin-resistant, which renders the test less reliable than bile solubility [219].

A further complication of pneumococcal diagnosis was introduced by the recent description of *Streptococcus pseudopneumoniae* [220]. *S. pseudopneumoniae* is distinct from *S. pneumoniae* and other viridans streptococci in its phenotypic and genotypic features. Its key characteristics are the absence of a capsule, bile insolubility, and resistance or intermediate susceptibility to optochin when incubated in 5% CO_2, yet remaining optochin-susceptible when incubated in ambient air. Positive reactions of *S. pseudopneumoniae* in antigen detection tests and DNA probe hybridisation assays further complicate differentiation from *S. pneumoniae* [220, 221]. The clinical significance of *S. pseudopneumoniae* is yet to be determined.

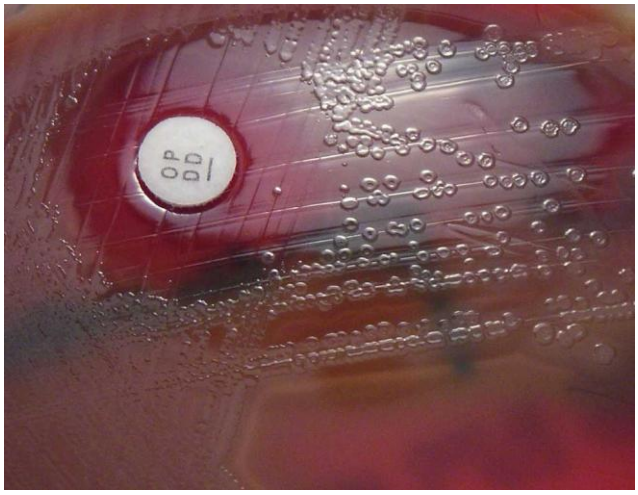


Figure 1.5: "Draughtsman" colonies and optochin susceptibility [217]

Commercial slide agglutination, coagulation and DNA probe hybridisation tests are other available rapid diagnostic tests for pneumococcal isolates and are used in some laboratory facilities [219, 222].

1.7.2. Blood cultures

The isolation of *S. pneumoniae* from blood provides a conclusive diagnosis of IPD. Unfortunately the isolation rate of pneumococci from blood is only achieved in a minority of cases in patients with IPD. Blood culture positivity rates for *S. pneumoniae* in cases of community-acquired pneumonia (CAP) in all age groups are low. They range from 3-8% in adult patients hospitalised with pneumonia [213, 214, 223, 224] and are even lower in children [215, 225]. Documented bacteraemia occurs more frequently in patients with

pneumococcal meningitis as opposed to pneumonia. Positivity rates of up to 50% have been reported [226, 227]. Positivity rates for pneumococcal bacteraemia in the absence of pneumonia or meningitis are thought to be low. Accurate published data could not be obtained.

Several factors are responsible for the low recovery rates from blood cultures. Prior administration of antimicrobials and inadequate volume of blood sample, resulting in a suboptimal blood to blood culture media ratio, represent external factors. Like other bacterial infections aside from bacterial endocarditis, blood stream invasion occurs intermittently. Additionally, *S. pneumoniae* releases a cell wall enzyme, autolysin, during its stationary growth phase which leads to bacterial cell death and a significant reduction in the number of viable cells. Hence, pneumococcal autolysis makes traditional culture of the organism difficult [228] and sets the pneumococcus apart from other pathogenic bacteria isolated in blood cultures.

1.7.3. Cerebrospinal fluid

The combination of Gram stain and bacterial culture of CSF samples will diagnose most cases of pneumococcal meningitis. However, as in culturing other specimens the rate of recovery is influenced by the prior administration of antimicrobial therapy [229]. The prompt transport of the CSF to the laboratory and its timely processing will ensure a sufficient quantity of viable organism and helps to optimise culture recovery rates.

1.7.4. Sputum

In the absence of documented bacteraemia, the diagnosis of pneumococcal pneumonia may rely on the isolation of the pneumococcus from sputum. Some cases of pneumonia (most frequently in children, but also in adults) do not produce sputum which poses a diagnostic challenge.

If a sputum specimen can be obtained, its quality is of vital importance. On microscopy, a sputum sample demonstrating large numbers of Gram-positive diplococci in the presence of <10 squamous epithelial cells (SEC) and >25 PMN cells per low-power field (magnification x100), or ≥10 PMN for each SEC, is strongly suggestive of pneumococcal pneumonia, especially when *S. pneumoniae* is cultured from sputum as the predominant bacterial isolate.

Poor quality sputum should not be processed as it is most likely to represent commensal oropharyngeal flora [230].

A recent study by Musher supported the usefulness of sputum culture in adults in the diagnosis of pneumococcal pneumonia providing the specimen obtained was of high quality and ideally taken prior to antimicrobial administration, or taken no later than 24 hours after the initiation of therapy with appropriate antimicrobials [231].

The sensitivity of sputum culture varies greatly in different studies. This is not necessarily due to the inadequacy of culture itself, but rather due to a variety of factors such as processing of an inadequate sample, delayed processing of samples, the patient's inability to produce sputum and a decrease in viable organisms present in sputum from prompt administration of antimicrobials prior to obtaining the sputum sample.

Under ideal circumstances, when an adequate specimen is obtained prior to therapy, the Gram stain and bacterial culture have sensitivities of 80% and 83%, respectively, in patients with bacteraemic pneumococcal pneumonia [231].

1.7.5. Transthoracic lung aspirates

In patients, especially children, who fail to produce a sputum specimen, transthoracic needle aspiration has the potential to increase the diagnostic yield in cases of pneumococcal pneumonia with large radiographic peripheral lesions present [232-235]. Unfortunately this procedure is not widely adopted; it is invasive, but safe when performed by experienced staff.

1.7.6. Antibody detection assays

The use of assays detecting pneumococcal antibodies or immune complexes for the diagnosis of IPD is limited to research settings [236-239] or to retrospective diagnosis of IPD [236]. The assays suffer from problems inherent in most serological tests, namely the time delay that occurs until seroconversion following acute infection, and suboptimal sensitivity and specificity [237]. In addition, the patients most likely to suffer from IPD are also those who may have difficulties in mounting a detectable immune response.

In one recent study it was observed that pneumococcal bacteraemia not only failed to elicit an antibody response but could also lead to antibody depletion in children with a previous antibody response to pneumococcal surface proteins [240].

1.7.7. Antigen detection assays

The detection of pneumococcal antigen in clinical samples, foremost urine, dates back to the beginning of the last century [241]. Over the past few decades, commercially available latex agglutination assays have been widely used. These tests target the capsular polysaccharide antigens of *S. pneumoniae*. Their usefulness compared with the value of Gram stain and bacterial culture methods alone have been debated [242]. Still, they have retained a role for the diagnosis of pneumococcal pneumonia and meningitis in laboratory facilities with limited resources [243, 244].

A revival and renewed interest in antigen detection assays arrived with the development of a rapid immunochromatographic test (ICT) which detects the C polysaccharide cell wall antigen common to all pneumococcal strains (NOW *S. pneumoniae* urinary antigen test by Binax, Inc., Scarborough, ME, USA). Arguably, this test has been the only recent major advance in pneumococcal diagnostic methods. Its sensitivity on urine samples is 70-80% with a specificity of > 90% compared to conventional diagnostic methods for the detection of pneumococcal pneumonia in adults [245-250]. In all studies, a proportion of patients with positive cultures of blood or sputum specimens had negative results in the NOW antigen detection test. This emphasises that its greatest usefulness is in conjunction with other, molecular or traditional diagnostic methods. Limitations of the test are that it can remain positive for several weeks post acute infection, that it lacks the ability to provide information regarding serotype and antimicrobial resistance, and that it may produce false-positive results after pneumococcal vaccination [251]. In children its utility remains unclear because of a high false-positive rate in this age group due to high rates of nasopharyngeal colonisation with *S. pneumoniae* [252, 253].

Originally designed for testing urine samples the NOW test has been successfully applied to other clinical specimens. It produces excellent results when used on CSF for the diagnosis of pneumococcal meningitis [254, 255]. Its application to pleural fluid has also been evaluated on children and adult cases with pneumonia [256, 257]. Additionally it can be used as a rapid provisional diagnostic tool in positive blood culture bottles [228], which has the potential to increase the generally poor sensitivity of blood cultures and their diagnostic usefulness in IPD.

Recently, a study demonstrated the usefulness of the urinary antigen detection test to discriminate between cases of CAP and control samples, which would make it not only a tool in directing clinical management, but also strengthen its usefulness as a test to measure vaccine efficacy [258]. But in another study, the usefulness of the test as a diagnostic tool in CAP cases was questioned, as its implementation showed no substantial benefit to the hospitalised patient with regards to outcome and it was not economically beneficial to the institution [259].

Other potential diagnostic targets for antigen detection assays are currently under investigation, but their usefulness has yet to be demonstrated to be superior to the C polysaccharide cell wall antigen [260].

1.7.8. Nucleic acid amplification tests

The current microbiological Zeitgeist revolves around molecular methods as diagnostic and as typing tools. Since the late 1990s nucleic acid amplification tests, especially the polymerase chain reaction (PCR), have established a definite and important role in the modern routine diagnostic microbiology laboratory. They are attractive in the diagnostic setting as they can detect minute quantities of nucleic acids from potential pathogens, as well as not being reliant on the viability of the organism, and results become rapidly available to the treating clinician.

The performance of nucleic acid amplification tests in pneumococcal diagnostics is variable. In the setting of pneumonia the sensitivity of PCR for the detection of *S. pneumoniae* in blood samples ranges from 29-100% [261] with a tendency to perform better in children than in adults. Overall the performance of pneumococcal PCR on blood samples is poor and may be due to rapid clearance of the organism from the blood stream, the transient or intermittent nature of organisms invading the blood stream, and sampling errors resulting from the small volumes used in PCR reactions. Dagan *et al*. reported ≥10cfu/mL (100fg of DNA) of *S. pneumoniae* as the lowest limit of detection in a *ply* PCR assay, whereas another study found that down to 10fg of pneumococcal DNA (4.3 genome copies) could be detected [262, 263]. These bacterial concentrations were obtained from 100µl of extracted blood. Unfortunately, positive PCR results can also be obtained from asymptomatic control subjects complicating the interpretation of a positive result [262-264]. However, a study in children, using blood specimens, has found *lytA* PCR potentially useful to distinguish carriers from

patients with IPD. In that study no pneumococcal *lytA* DNA was detected in healthy carriers colonised with pneumococcus in their nasopharynx [265]. A cut-off value of *lytA* ≥8000 copies/mL was suggested recently for distinguishing pneumococcal CAP from asymptomatic colonization [266].

PCR positivity rates on sputum samples from patients with suspected pneumococcal pneumonia can range from 68% to 100% [261]. It is difficult in some cases to ascertain whether the PCR positive results reflect IPD or nasopharyngeal colonisation [267]. One of the commonly used gene targets in pneumococcal PCR is pneumolysin (*ply*). This pneumococcal protein is thought to be common to all pneumococcal strains, but is also known to be present in some non-pneumococcal viridans streptococci, commensal organisms of the human oropharyngeal flora [221]. Refinement of currently available PCR assays by inclusion of multiple targets rather than targeting a single pneumococcal gene can increase the assay's specificity [268].

Preliminary published data suggest that quantitative PCR may distinguish colonisation from IPD by the anticipated higher bacterial burden in IPD. However, quantitation in a diagnostic setting needs further exploration [269]. A study from Malawi demonstrated the association between high pneumococcal DNA loads in blood and CSF in children with IPD with fatal outcome [270], and another study concluded that high bacterial loads can be a marker of severity of disease in cases of CAP [271]. Furthermore, a recent publication demonstrated the association of a high pneumococcal load in the nasopharynx with radiologically confirmed pneumonia and pneumococcal virulence in children [272] strongly suggesting that quantitative diagnostic approaches hold clinical value.

Applied to CSF, pneumococcal DNA can be readily detected via PCR in the case of meningitis. The bacterial concentration in CSF is expected to be high in pneumococcal meningitis, and interpretative difficulties due to local colonisation with *S. pneumoniae* are not an issue. Several studies have demonstrated a high sensitivity and specificity of PCR applied to CSF, but this needs further evaluation [273-276].

Other samples from normally sterile sites, such as pleural fluid and lung aspirates have successfully been tried in PCR tests [277-280].

Overall, PCR assays have improved the ability to detect many pneumonia pathogens, although the application of PCR to diagnose pneumococcal pneumonia has to date been limited. However, the use of quantitative PCR methods may be more promising.

1.7.9. "Atypical" pneumococci and *S. pseudopneumoniae*

Bacterial isolates resembling *S. pneumoniae* that give negative results in one or more of the classical assays used for identification are referred to as "atypical pneumococci". Essentially this group encompasses all members of the mitis group within the genus *Streptococcus*.

In 2004 Arbique described an unusual group of viridans streptococci, resembling atypical isolates of *S. pneumoniae*. This novel species could be differentiated via DNA-DNA homology studies and was termed *S. pseudopneumoniae* [220]. The primary differentiating phenotypic characteristics of *S. pseudopneumoniae* were the lack of a pneumococcal capsule, resistance to optochin when incubated at 5% CO_2 (<14mm with a 6mm optochin disk), and retained susceptibility when incubated at ambient air, as well as bile insolubility. In genetic probe tests for the pneumolysin gene (*ply*), and the manganese-dependent superoxide dismutase gene (*sodA*) and in the commercial GenProbe, AccuProbe Pneumococccus, *S. pseudopneumoniae* produces a positive reaction and cannot be differentiated from *S. pneumoniae*.

In real-time PCR assays, pneumolysin seems the least useful, since it can easily produce false-positive reactions with genetically similar organisms like *S. pseudopneumoniae* [281].

A New Zealand study looked at strains of *S. pseudopneumoniae* from sputum samples and revealed that *S. pseudopneumoniae*, like *S. mitis*, gives a positive reaction in the NOW antigen detection assay [282], indicating shared antigenic properties of all three species. None of these isolates were resistant to penicillin, but 60% were resistant to erythromycin and 77% to tetracycline. Clinically, all patients whose sputum grew *S. pseudopneumoniae* had lower respiratory tract symptoms, and the majority were known to suffer from COPD [221].

A recent study using a mouse peritonitis/sepsis model suggested a potential pathogenic effect of *S. pseudopneumoniae* [283].

Molecular assays to identify *S. pseudopneumoniae* and to accurately differentiate *S. pneumoniae* from other members of the *S. mitis* group have been published [284-287]. It is of clinical value to differentiate *S. pneumoniae* from atypical isolates with diagnostic methods that are easily accessible and of low cost to routine diagnostic facilities, because *S. pseudopneumoniae*, although a potential pathogen, appears to be less virulent and members of the *S. mitis* group cause a disease spectrum that differs from *S. pneumoniae* and have an antimicrobial susceptibility profile that cannot be inferred from that of *S. pneumoniae*.

1.7.10. Novel diagnostic approaches

Progress in pneumococcal diagnosis has been slow for various reasons, including the genetic variability of the capsular polysaccharide and the limitations of currently available methodologies. The promise of nucleic acid amplification tests has not yet been realised and the usefulness of quantitative amplification assays has yet to be fully evaluated.

The NOW antigen detection assay has been the most useful recent addition to pneumococcal diagnostics, but suffers from important limitations, especially when used in children.

A novel approach to the diagnosis of IPD is necessary, either by looking at technologies that have traditionally not been used in the diagnosis of pneumococcal disease, or by optimisation of current diagnostic tools.

There has been a tendency to downplay the role of microbiological diagnostics for pneumonia, partly because of the readily available empiric antimicrobial therapy, and partly because of disappointing results from traditional microscopy and culture based methods, possibly due to collection of suboptimal specimens, inappropriate timing of specimen collection and the limitations of commonly used diagnostic tests. The inherent ability of *S. pneumoniae* to autolyse and cause negative results in traditional culture methods has contributed to the speed and variety of development of molecular assays, which are not reliant on the presence of viable organisms.

Still, increased efforts need to be directed towards new diagnostic tools for IPD. The search should continue for other pneumococcal antigens which could be used as diagnostic targets or as potential vaccine candidates. These novel targets would have to have some features in common: presence in all pneumococcal strains, preferably surface exposure, suitable quantities present in patient specimens (unless amplification techniques are applied), and a clear association with virulence and invasive disease or with the carrier state. Some of the currently investigated virulence factors for vaccine development might reveal themselves as candidates for antigen detection assays either as single targets or in combination with other already characterised factors.

Some novel diagnostic tools, not yet routinely used in the diagnostic microbiology laboratory, hold hope for the future in pneumococcal diagnosis. These new avenues include microarray technology and deep sequencing, with a particular focus on capsular serotype

identification as well as detection of antimicrobial resistance markers by other than traditional means [284, 288].

The most recent and very promising introduction into more and more routine diagnostic laboratories has been matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). The method is rapid and user friendly and, apart from a high instrument purchase cost, individual test cost is low. It has been demonstrated that *S. pneumoniae* can successfully be identified, but misidentification of the other members of the *S. mitis* group are to be expected [287, 289, 290]. An earlier publication, however, alludes to the possibility that viridians streptococci can be differentiated by MALDI-TOF MS by direct comparison of their mass spectra [291]. This finding needs further investigation.

In future, techniques that have refined or combined amplification, mass spectrometry and array technology may provide the solution to the diagnosis of IPD. However, at present they are either still under development, in need of improved sensitivity or specificity or have restricted availability due to high cost.

Lastly, one of the challenges in the development of a useful diagnostic tool for IPD is to address the need where it is greatest, in the developing world. Therefore a test needs to not only be sensitive, specific and easily reproducible, but it also will have to be easy to use, rapid and economical in order to be successfully implemented into resource-limited settings.

1.8. Thesis objectives

This PhD project focuses on the development of improved diagnostic testing methods for invasive pneumococcal disease.

The study aim was to improve on and determine the limits of current methodologies in the diagnosis of pneumococcal disease:

(i) To increase the sensitivity of assays to detect *S. pneumoniae.*

(ii) To differentiate between colonisation and invasive disease.

(iii) To ensure more reliable identification of pneumococcal and non-pneumococcal viridians streptococci at the species level.

The specific objectives that formed the basis of this thesis were to:

1) Obtain a clear baseline of the current testing strategies and their limitations.
2) Decide upon suitable protein targets for an antigen detection assay.
3) Develop an assay for the chosen antigen and evaluate then in the clinical setting.
4) Develop a quantitative real-time PCR assay and then evaluate (i) its performance when compared to other available methods and (ii) its usefulness as a marker of disease severity.
5) Evaluate the role of MALDI-TOF MS in pneumococcal diagnostics, particularly focussing on species differentiation within the *S. mitis* group.

To address these objectives, this thesis comprises four parts.

Part 1 (Chapter 3) represents an analysis of the current diagnostic methods employed by Canterbury Health Laboratories (CHL). This part outlines the current state of pneumococcal diagnostics in a real medical microbiology laboratory setting. It relates the current state of diagnostic strategies, and their limitations, to the other parts of this thesis which aim to address some of these limitations and provide solutions.

Part 2 (Chapters 4-7) focused on antigen detection methods and their evaluation. Pneumococcal proteins were examined as potential diagnostic targets for the development of an antigen detection test, which could more specifically reflect the presence of invasive disease. As a pre-requisite these targets needed to be present in all pneumococcal strains and

have known antigenic properties. This held true for pneumolysin (Ply) and pneumococcal surface adhesin A (PsaA). Both proteins had previously been investigated as vaccine targets and had been used as gene targets in PCR and probe technology assays. In comparison to some of the other pneumococcal proteins Ply and PsaA are well characterised in their role in the pathogenesis of *S. pneumoniae*, they are present in all or virtually all *S. pneumoniae* strains and they are well conserved. In comparison, some of the surface exposed choline binding proteins (CBP), such as PspA, have a significant variation in amino acid sequence and *S. pneumoniae* strains can produce heterologous PspA types. PspA is also known to elicit antibodies that cross-react with human cardiac myosin which could pose a problem in the development of an antigen detection assay. Two iron transporter lipoproteins, PiuA and PiaA, would have been potential targets for a serological assay as they are also present in all pneumococcal strains, are highly conserved and are known to contribute to virulence. However, they are less well characterised than Ply and PsaA, and were not chosen for that reason. Sandwich ELISA assays to detect Ply and PsaA were developed and optimised. The clinical usefulness and limitations of both assays were tested on clinical samples of well characterised adult patients admitted to hospital with CAP. Of notice, this body of work does not cover serotyping or antimicrobial resistance of the clinical strains. This does not allow drawing conclusions in how far a novel diagnostic test would allow augmenting surveillance of IPD.

Part 3 (Chapters 8 and 9) looked at a quantitative PCR assay targeting the *lytA* gene. The assay is evaluated with regards to its clinical usefulness, its potential suitability as a marker of disease severity as well as its microbiological diagnostic value compared to other, previously and currently employed, methods.

Part 4 (Chapter 10) concluded this thesis by looking at a recently introduced microbiological diagnostic tool, the MALDI-TOF MS, and its application to improve pneumococcal diagnostics. MALDI-TOF MS was evaluated as a method to simply and rapidly differentiate *S. pneumoniae* from other closely related streptococci, the *S. mitis* group, in a routine diagnostic setting.

CHAPTER 2

GENERAL MATERIAL AND METHODS

2.1. Introduction

This chapter lists the materials and methods which have been used more than once in Chapters 4 to 7.

2.2. Materials

2.2.1. General materials

All chemicals listed in this thesis were obtained from Sigma-Aldrich, St. Louis, MO, USA, unless stated otherwise.

Double distilled water was used for making up solutions and media. Autoclave sterilisation was carried out on a liquid cycle for 20 min at 15 psi [1.05 kg/cm^2] and 121°C in a Mercer autoclave (Little Mercer J. Co., Rehoboth, MA, USA). Non-autoclavable reagents were made up in sterile water from a Milli-QTM water purification system (Millipore Inc., Bedford, MA, USA), and filter sterilisation was carried out using 10 and 20mL disposable syringe barrels (Becton Dickinson Ltd., Lincoln Park, NJ, USA).

2.2.2. Immunological materials

2.2.2.1. 10% SDS Minigel (x1)

Resolving gel buffer:

1.5M	Tris
0.4% (w/v)	SDS

Adjust to pH 8.8.

Resolving gel:

1.25mL	40% Acrylamide/Bis-acrylamide
1.25mL	resolving gel buffer
2.5mL	H_2O
30µL	10% APS
10µL	TEMED

Stacking gel buffer:

0.5M	Tris
0.4% (w/v)	SDS

Adjust to pH 6.5.

Stacking gel:

0.19mL	40% Acrylamide/Bis-acrylamide
0.625mL	stacking gel buffer
1.7mL	H_2O
30µL	10% APS
10µL	TEMED

Running buffer x5:

25mM	Tris
192mM	glycine
0.1% (w/v)	SDS

Sample buffer:

125mM	Tris
40% (v/v)	glycerol
4% (w/v)	SDS
0,002% (w/v)	bromophenol blue

For reducing buffer 10µL β-mercaptoethanol (10% v/v) was added.

Coomassie stain:

25% (v/v)	isopropanol
10% (v/v)	glacial acetic acid
0.1% (w/v)	Coomassie Brilliant Blue R

Destaining solution:

25% (v/v)	methanol
7.5% (v/v)	glacial acetic acid

2.2.2.2. 10x TBE (Tris Borate EDTA) buffer

180mM	Tris
90mM	Boric acid
2mM	EDTA

Adjust to pH 8.3.

2.2.2.3. TBS Tween buffer

57mM	Tris
0.15M	NaCl

pH 7.4, 0.1% Tween 20

2.2.2.4. 1% Agarose gel in 0.5xTBE buffer

1% (w/v)	agarose
1% (w/v)	ethidium bromide

2.2.2.5. Loading buffer

40% (w/v)	sucrose
0.25% (w/v)	bromophenol blue
0.25% (w/v)	xylene cyanol FF

2.2.2.6. Blocking buffer

5% (w/v)	low-fat milk powder (0.1% fat) (Fonterra Brands NZ Ltd, Manurewa, NZ) in TBS Tween buffer (plus sodium azide).

2.2.2.7. Antibody solutions

All antibodies were diluted in TBS Tween buffer containing 1% BSA (v/v) (bovine serum Cohn V fraction, Sigma-Aldrich, St. Louis, MO, USA).

2.2.2.8. Sample preparation

A bacterial suspension or lyophilised suspension was diluted with ddH_2O to obtain a concentration of 10-15µg per reaction. The concentration was of bacteria was determined by using the Bradford ELISA method, measuring against a BSA standard. The absorbance reading is performed at 620nm [292]. An equal volume of sample dye (3% mercaptoethanol, 10% SDS, 20% glycerol, 0.1% Bromophenol blue; sample dye can also be made up without mecaptoethanol) was added. In the case of recombinant PsaA (rPsaA) the final concentration was 5µg per reaction.

The samples were heated to at least 100°C for 5 min to fully denature the proteins.

2.2.2.9. TMB substrate (tetramethylbenzidine) staining

Citrate EDTA buffer: 10mM sodium citrate, 10mM sodium EDTA, pH 5.0

TMB substrate:

0.1M sodium acetate (anhydrous) and 18mM citric acid, 0.86mM TMB, 40% methanol.

Substrate was stored at room temperature (RT) in the dark. For extended use, 250µL 30% H_2O_2 was added to 500mL substrate and mixed.

When used in an ELISA reaction, TMB produces a soluble blue end-product that turns into a yellow chromogen upon addition of an equal volume of 0.5M/L HCl. This can be detected at 450nm. Stability of the yellow chromogen at RT is about 1 hour during which time the loss in absorbance is estimated to be less than 1%.

2.2.2.10. Bacterial suspensions

Bacterial suspensions were obtained by harvesting from overnight culture at 37°C, 5% CO_2, in 50mL Todd Hewitt broth (Difco Laboratories, Detroit, MI, USA) or from overnight growth on 5% sheep blood agar (SBA) (Fort Richard Laboratories, Auckland, New Zealand), harvested into sterile water or phosphate buffered saline (PBS), washed twice and centrifuged for 5 min at 7550 x g, then reconstituted in 0.5mL PBS or sterile water. Thiomersal was added for preservation. 100µL aliquots were stored at –80°C.

2.2.2.11. Bacterial lysates

Bacterial lysates were obtained by harvesting from overnight culture of bacteria grown on 5% Sheep blood agar (SBA) at 37°C, 5% CO_2. Bacteria were harvested into 1mL bacterial lysis buffer (1% Triton-X-100, 10mM Tris HCl pH 8.0, 5mM EDTA). The bacteria in lysis buffer were incubated at RT for 10 min, vigorously mixed, vortexed and centrifuged at 7550 x g for 2 min. Cellular debris was removed and the supernatant was stored at –80°C in 500µL aliquots.

Protein concentration for each bacterial strain was measured by using the Bradford ELISA method [292]. All lysates and suspensions were diluted with PBS to a final protein-concentration of 15µg/200µL for use in the ELISA and 1µg/µL for both, immunodot blots and Western blots (unless specified otherwise).

2.2.2.12. Patient samples

From July 1999 to July 2000, 304 adult patients aged ≥ 18 years were admitted to Christchurch Hospital (Christchurch, New Zealand) and enrolled in an adult community-acquired pneumonia (CAP) study if they met the inclusion criteria [293]. The inclusion and exclusion criteria were based on the ones used in the original British Thoracic Society study [294]. Patients were only included if pneumonia was the main reason for admission, was not a terminal event, and when the affected lung tissue was not due to pneumonia distal to bronchial obstruction, bronchiectasis or previous tuberculosis. Patients with severe immunosuppression (neutropenia, AIDS, or receiving cancer chemotherapy) were excluded. Patients with less profound immunosuppression on account of co-existing illnesss (renal failure, diabetes, liver impairment, alcoholism) or prolonged treatment with prednisone (≥ 7.5mg/day), methotrexate, or cyclosporine were included. On enrolment into the study patients had bloods taken for haematology, biochemistry and microbiology. Pneumonia was defined as an acute illness as well as radiographic shadowing in at least one lung segment or in one lobe, which was neither pre-existing, nor attributable to a known cause. The mean age of the enrolled patients was 63.7 years, and ranged from 18 to 99 years. Fourty seven % were female patients. “A microbial diagnosis was established in 197 cases (42%), of which 76 met the criteria for a definite diagnosis. More than one organism was diagnosed in 44 cases (9%). Blood cultures were positive in 32 cases (7%)” [293]. The criteria for a microbiological diagnosis were: isolation of a bacterial pathogen from sterile sites, including blood, pleural fluid or lung aspirate; detection of pathogens that do not colonise the upper respiratory

airways, such as *Legionella* spp, Influenza A and B by antigen detection or a four-fold or greater rise in antibody titre in paired sera. A presumptive diagnosis was made when a bacterial pathogen was isolated from a good quality sputum specimen and was seen in the Gram stain or when a defined high single titre of a pathogen was found in a serological assay [293]. Out of the 304 CAP patients 41 patients were diagnosed with pneumococcal disease. The patient samples were stored at -80°C. The samples that were used in this thesis were serum and plasma, if serum was not available.

2.3. Methods

2.3.1. Immunological methods

2.3.1.1. ELISA

The basic principle and reagents used in the various ELISAs performed during this project are the same and the underlying method is described in detail here. ELISAs described in this thesis have adhered to this method and if modifications occurred they are specified.

ELISA plates (Falcon 3912 microtest III; Beckton Dickinson, Oxnard, CA, USA) were coated overnight with capture antibody, a monoclonal or a polyclonal, or capture antigen, in the form of recombinant protein, cell lysate or cell suspension. The solution made to coat to the bottom of the well was diluted to 1:500 or 1:1,000 (or specified concentrations) in PBS or 6M guanidine HCl, for antibodies or antigens respectively. The plates were left at 4°C overnight.

The plates were washed four times with PBS containing 0.1% Tween-20 (v/v) and afterwards blocked for 15 min with 150µL/well assay buffer (PBS containing 0.1% Tween-20 (v/v) and 0.1% gelatine (w/v)) at RT.

After the plates were emptied, 100µL of either antigen or second antibody were added, incubated at RT for 1 hour and subsequently washed as described above.

Anti-mouse (using the monoclonal antibody as the second antibody) (IgG1 rat anti-mouse HRP, BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) or anti-rabbit (using the polyclonal antibody as the second antibody) (peroxidise conjugated Affini Pure goat anti-rabbit IgG (H+L), Jackson ImmunoResearch Laboratories Inc., West Baltimore, PA, USA) was added at 1:1,000 (unless specified otherwise) in assay buffer (100µL/well). After 15 min of incubation the plates were again washed and substrate solution (40mg *o*-

phenylenediamine and 60µL of 30% H_2O_2 in 100mL of 0.05M Na_2PO_4 and 0.025M citric acid, pH 5.0) was added (100µL/well). Colour development was stopped after 10 min (unless specified otherwise) by adding 100µL/well of 1.25M H_2SO_4. Absorbance was read at 450nm on a BMG Fluostar Galaxy (BMG Labtechnologies GmbH, Offenburg, Germany). When the biotinylated antibody was used as a second antibody, the detection system was modified to an avidin- horseradish peroxidise conjugate (Fitzgerald Industries International, North Acton, MA, USA).

2.3.1.2. Western blot

Vertical SDS-PAGE was carried out using 10% acrylamide mini gels using the Bio-Rad Miniprotean system (Bio-Rad, Hercules, CA, USA). A 1:10 dilution of running buffer stock was used to fill up the electrophoresis tank to cover the gel completely and 10-15µg of bacterial isolates and 5µg rPsaA were loaded into each well. Bio-Rad KaleidoscopeTM Pre-stained Standard was included in each Western Blot. The gel was run at 60V for 10 min followed by an increase to 100V. The electrophoresis was stopped when the sample dye was close to the bottom of the gel.

Transfer to nitro-cellulose (Life Technologies Inc., Gaithersburg, MD, USA) performed in electroblotting buffer at 120V (constant), 90mA, for 90 min. For antibody probing, nitro-cellulose was initially blocked by incubation in 5% trim-milk TBS Tween overnight at RT.

The nitro-cellulose was then washed 3 times in TBS Tween and incubated with unpurified supernatant of each MAb diluted 1:10 or 1:1,000 polyclonal antiserum for 1 hour on a shaker. Nitro-cellulose was washed 3 times and incubated with the second antibody (1: 10,000 IgG1 rat anti-mouse HRP, BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA, or peroxidise conjugated Affini Pure goat anti-rabbit IgG (H+L), Jackson ImmunoResearch Laboratories Inc., West Baltimore, PA, USA) for 1 hour at RT on shaker. The nitro-cellulose was washed for a final time and the blot developed with TMB precipitating substrate.

2.3.1.3. TMB precipitating substrate for Western blot staining

The nitro-cellulose membrane was washed four times in Tris Tween buffer followed by a final wash in PBS after incubation with the HRP conjugate antibody. The blot was equilibrated in citrate EDTA buffer for 5 min followed by a 10 min equilibration step in 1% dextran sulphate in citrate EDTA buffer. Three washes in citrate EDTA buffer was followed

by the addition of 0.05mg/mL TMB in citrate EDTA buffer and 0.25% H_2O_2. Colour development occurred over 15-20 min. The reaction was stopped by transferring the nitro-cellulose blot into H_2O.

UTILISATION OF PNEUMOCOCCAL DIAGNOSTICS AT A TERTIARY MICROBIOLOGY LABORATORY

CHAPTER 3

LANDSCAPE ANALYSIS OF UTILISATION OF PNEUMOCOCCAL DIAGNOSTICS AT CANTERBURY HEALTH LABORATORIES FROM 2001 to 2011

3.1. Introduction

Chapter 3 is the landscape analysis that provides a background to the remaining chapters of this thesis. It outlines a contemporary picture of the existing practices and utilisation of pneumococcal diagnostic methods at a tertiary hospital laboratory that is likely to reflect the experience of similar laboratories worldwide.

This chapter looks at pneumococcal diagnostic methods employed at Canterbury Health Laboratory (CHL), Christchurch, New Zealand (section 3.2.1). It is important to review and know the value of currently applied diagnostic methods and relate it back to the aim of the thesis: improvement of diagnostic testing methods for invasive pneumococcal disease.

In the introductory section 1.7 the diagnostic methods are listed that are currently employed in microbiology laboratories around the world. The utilisation and limitation of these methods is discussed in detail there. It is important to remember though, that outside the usually applied measures to evaluate the accuracy of a test method, such as sensitivity and specificity, another factor to assess the usefulness of a test is its impact on patient health [295]. This chapter tries to take this into account and looks at how available diagnostic methods were used by clinicians in a tertiary hospital environment.

The following pneumococcal diagnostic methods were used during January 2001 to December 2011 at CHL microbiology unit: sputum microscopy and culture, isolation of pneumococci from blood, cerebrospinal fluid (CSF) and other usually sterile sites, and immunochromatographic antigen detection on urine specimens. Two major changes in diagnostic testing practices took place in the period studied and their impact will be discussed in this chapter. Firstly, the NOW *S. pneumoniae* (Binax Inc., Scarborough, ME, USA) urine antigen detection test was introduced in April 2004, and secondly, inadaequate sputum samples were rejected according to defined criteria since 2007 [230].

The main objectives covered in this chapter are:

1. To document pneumococcal testing patterns in a tertiary hospital laboratory over 11 years.
2. To assess the utilisation of tests for pneumococcus among patients admitted with community-acquired pneumonia.
3. To assess the utilisation of tests for pneumococcus among patients admitted with meningitis.

3.2. Material and methods

3.2.1. Setting

This landscape analysis was performed at Canterbury Health Laboratories (CHL). CHL is a large, publicly funded medical diagnostic reference laboratory in New Zealand with laboratory links and collaborative partners throughout New Zealand and the world. The population of the CDHB catchment area was estimated at 502,700 in June 2011 (Statistics NZ). The estimation of the resident population is based on a census population count of the Canterbury area, Christchurch city and surrounding districts. The resident population represents the catchment population for the CDHB. In 2011 CHL provided the sole diagnostic service for secondary care. Most cases of IPD are likely to have presented at secondary care level, however, it is prudent to assume that a minority of less severe cases of IPD were managed at primary care level and that very severe cases could have resulted in death prior to presentation to Christchurch Hospital. Annual incidences were reported per 100,000 catchment population for the CDHB to allow a comparison with national data.

CHL is an IANZ (International Accreditation New Zealand) accredited (ISO 15189) laboratory and provides a tertiary care laboratory service for the CDHB, including Christchurch Hospital as well as general practices in the community. On average CHL process over 2,500 patient requests a day. The microbiology department provides a service for both the public and private sectors. Specialities include bacteriology, virology, serology, mycology, parasitology and infection control services.

It was anticipated that a high degree of awareness of pneumonia testing methods and protocols would exist among physicians working at Christchurch Hospital based on the fact that community-acquired pneumonia (CAP) studies have been undertaken in New Zealand as far back as 1988 in Waikato [296] followed by several studies conducted at Christchurch Hospital [293, 297, 298]. Respiratory researchers from Christchurch have contributed significantly to the development of the CURB-65 score, which is a clinical prediction score for the mortality in patients over the age of 65 years with CAP [299]. This score is recommended by the British Thoracic Society for the assessment of CAP severity. The "Management guidelines for common medical conditions", published by the Internal Medicine service of the CDHB, are a resource for medical staff and include information regarding investigation and management of CAP patients. There, the recommendations for patients with a CURB-age score 3-5, defined as severe CAP, are to obtain 2 sets of blood

cultures prior to giving antimicrobials, a sputum sample for Gram stain and the use of the urinary pneumococcal antigen test. These tests are recommended alongside radiographic, routine biochemistry and haematology investigations. These recommendations on the testing strategies and management of patients with CAP at Christchurch Hospital have not changed in the last three editions (December 2007 – December 2012).

To assess the isolation rates of *S. pneumoniae* in different specimen types, all requested blood cultures during the period from January 2001 until December 2011 were included in the analysis. As for CSFs, only those with pleocytosis were included in the analysis, as defined in section 3.2.5. The respiratory specimen types submitted to the microbiology unit during the study period were varied, and included sputum, tracheal aspirate, aspirate via endotracheal tube (E.T.T) and "lower respiratory specimen". Specimens that yielded no growth were not included in the data analysis. Duplicates were removed if a patient had more than one specimen taken on the same day, which subsequently grew the same organism.

3.2.2. Data sources

The data for the analyses discussed here were obtained from CHL's Laboratory Information System; the data for the discharge diagnoses over the time period from 2001 to 2011 were obtained from the decision support team at the CDHB. The diagnosis *pneumonia due to Streptococcus pneumoniae* was used as the denominator for discharge diagnoses in patients with pneumococcal disease. The estimated resident population data by selected age groups was provided by Statistics New Zealand. As mentioned in section 3.2.1, the estimated resident population represents the population that CHL provides the diagnostic service for at secondary care level.

3.2.3. Utilisation of tests for pneumococcus among patients admitted with CAP

A "snapshot" analysis of the test utilisation in patients that were admitted to Christchurch Hospital's general medicine and paediatric departments was undertaken. For this purpose the year 2011 was chosen as the most recent complete data set available for analysis. Included in this analysis were all patients with CAP. This was defined as adult patients with a primary discharge diagnosis and radiographic evidence of pneumonia. In children the criteria

to define CAP were different as radiographic evidence was not a prerequisite to be included in the analysis. The data set obtained from CDHB decision support contained a list of patients with the following category codes for diagnoses from the International Classification of Diseases and Related Health Problems, 10th revision (ICD-10), a WHO endorsed classification system: *Influenza due to identified influenza virus, Influenza, virus not identified, Viral pneumonia, not elsewhere classified, Pneumonia due to Streptococcus pneumoniae, Pneumonia due to Haemophilus influenzae, Bacterial pneumonia, not elsewhere classified, Pneumonia due to other infectious organisms, not elsewhere classified, Pneumonia in diseases classified elsewhere, Pneumonia, organism unspecified*. The data was filtered for patients on "general medicine" as well as "paediatric medical" in 2011. Clinical information, including radiographic data, information on the presence or absence of specimens collected (blood culture, sputum culture and urinary antigen test for pneumococcus) and the isolation of pneumococci or the detection of antigen in these specimens was collected via Concerto, a clinical information system employed by the CDHB (Orion New Zealand Ltd., Christchurch, NZ).

3.2.4. Assessment of sputum quality

The quality of a sputum specimen was assessed microscopically. A sample containing <10 squamous epithelial cells (SEC) and >25 polymorphonuclear leukocytes (PMN) per low power field (magnification x100) or ≥10 PMN for each SEC was processed and cultured, all other sputum specimens not adhering to these criteria were rejected and discarded as recommended by Murray [230].

3.2.5. Utilisation of tests for pneumococcus among patients admitted with meningitis

CSFs with pleocytosis were included in the analysis to determine ordering patterns and isolation rates of pneumococci in patients with suspected meningitis. Pleocytosis was defined as having any presence of leukocytes ($x10^6$/L) in adults and children over the age of 4 weeks, and the presence of greater than 30 leukocytes ($x10^6$/L) in newborns up to the age of 4 weeks. All CSFs included in the analysis underwent bacterial culture.

3.2.6. Statistical analysis

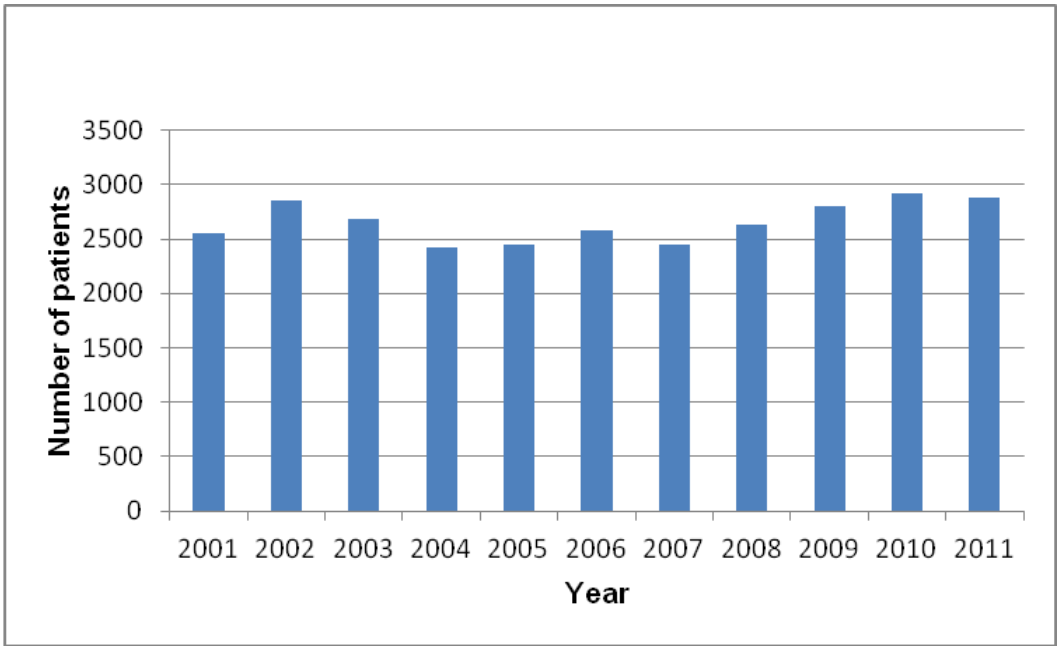
The statistical analyses were performed using Microsoft Excel 2007 for the graphical illustrations and the calculations of mean and standard deviation (SD). The unpaired t-test analysis applied to analyse the effect of the introduction of the urinary antigen test and the sputum rejection criteria was performed using STATA 11.0 (StataCorp, College Station, Texas, USA).

3.3. Results

3.3.1. Summary of discharge diagnoses from 2001 until 2011

Over the 11 year study period, 20,948 patients were admitted and discharged from Christchurch Hospital with the diagnosis of pneumonia. Of these, 1,095 (5.2%) were given the diagnosis of *pneumonia due to S. pneumoniae*. **Figure 3.1** demonstrates the relatively stable numbers of those discharged with the diagnosis of pneumonia (a) and those with the diagnosis of pneumococcal pneumonia (b) during the study period. Of the 684 patients with pneumococccal bacteraemia (2001-2011), 326 (47.0%) patients were discharged with the diagnosis *pneumonia due to S. pneumoniae*. Of those 684 patients 118 (17.3%) were children (0 – 14 years) with pneumococcal bacteraemia, of which 16 (13.6%) were discharged with the diagnosis *pneumonia due to S. pneumoniae*. Eighty one pneumococcal bacteraemia patients were discharged with one of the following diagnoses: *acute bronchitis, bacterial pneumonia, not elsewhere classified, influenza due to indentified influenza virus, influenza, virus not identified, pneumonia in diseases classified elsewhere, pneumonia, organism unspecified, pneumonia due to Haemophilus influenzae*. The last diagnosis was used for one patient only, who had no laboratory evidence of infection with *H. influenzae*. It is therefore assumed that the wrong diagnosis was entered. Nineteen (16.1%) of the children with pneumococcal bacteraemia were discharged with one of the following diagnoses: *pneumonia, organism unspecified, other bacterial pneumonia, pneumonia unspecified, lobar pneumonia unspecified, pneumonia in bacterial diseases classified elsewhere, influenza with pneumonia, influenza virus indentified, viral pneumonia unspecified, influenza with other respiratory manifestation, virus not identified, parainfluenza virus pneumonia*. Overall, 277 patients with pneumococcal bloodstream infections were discharged with a diagnosis other than pneumonia, 83 of those were children < 15 years of age; the specific diagnosis was unavailable for this analysis. Interestingly, the diagnosis *pneumonia due to Streptococcus pneumoniae* was not given to any patients who had pneumococci isolated from respiratory sites, CSF or those with a positive urinary antigen detection test.

a)



b)

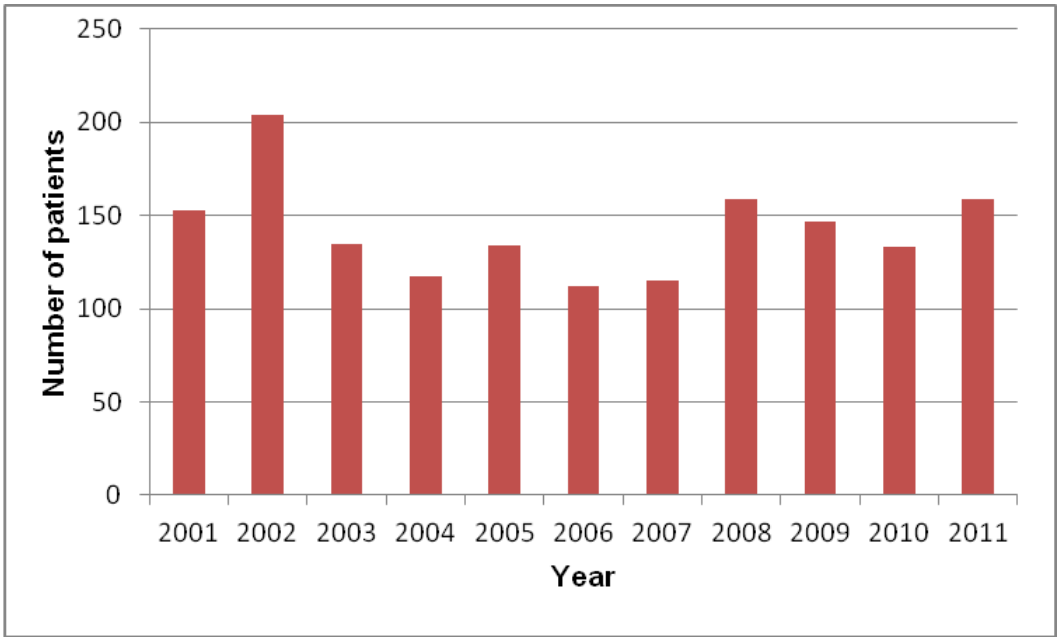


Figure 3.1: a) Number of patients discharged with the diagnosis of pneumonia and b) pneumococcal pneumonia from 2001 – 2011

3.3.2. *S. pneumoniae* isolation rates in blood cultures

The overall number of blood culture (BC) requests processed at the CHL microbiology unit during 2001 until 2011 was 137,924. The total number of those yielding positive growth (real pathogen or contaminant) was 10,445. Of the 137,924 processed BCs 541 (0.4%) grew pneumococci. Of those, 247 (45.6%) were from female patients. The patients' ages ranged from 0-98 years with a mean of 51.5 years. Of the patients with *S. pneumoniae* isolated in their BC, 10 adults (1.8%) were admitted to an intensive care unit.

The age-related population-based rates of isolations of pneumococci in BCs are summarised in **Table 3.1**.

Table 3.1: Annual incidence rates of pneumococci in blood cultures

The rate is given per 100,000 catchment population for the CDHB from 2001 until 2011 in different age groups. The total number of pneumococcal isolates from patients' blood cultures and the total denominator per year in each age group are given in brackets.

Age (years)	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
<2	88.7 (10/ 11,270)	70.7 (8/ 11,310)	53.1 (6/ 11,290)	42.3 (5/ 11,810)	65.8 (8/ 12,160)	32.2 (4/ 12,410)	106.5 (14/ 13,140)	51.7 (7/ 13,530)	29.5 (5 /13,540)	14.8 (2/ 13,520)	0 (0/ 12,720)
2-4	35.2 (6/ 17,040)	29.2 (5/ 17,100)	17.3 (3/ 17,370)	11.3 (2/ 17,700)	33.9 (6/ 17,720)	44.3 (8/ 18,070)	27.1 (5/ 18,420)	26.3 (5/ 19,040)	20.4 (5/ 19,590)	0 (0/ 20,290)	10.3 (1/ 19,450)
5-64	7.9 (28/ 354,650)	7.2 (26/ 361,880)	6.2 (23/ 370,870)	9.8 (37/ 377,770)	3.4 (13/ 383,070)	6.9 (27/ 388,350)	5.6 (22/ 392,150)	5.8 (23/ 395,720)	6.0 (25/ 399,620)	5.4 (21/ 403,190)	7.3 (29/ 397,520)
>65	54.9 (32/ 58,280)	54.2 (32/ 59,070)	63.3 (38/ 59,980)	52.4 (32/ 61,110)	36.8 (23/ 62,510)	20.1 (13/ 64,530)	34.6 (23/ 66,380)	41.4 (28/ 67,650)	39.0 (25/ 69,280)	25.3 (19/ 71,210)	43.8 (33/ 73,000)
All ages	17.2 (76/ 441,200)	15.8 (71/ 449,400)	15.2 (70/ 459,500)	16.2 (76/ 468,400)	10.5 (50/ 475,500)	10.7 (52/ 483,400)	13.0 (64/ 490,100)	12.7 (63/ 495,900)	11.7 (60/ 502,000)	8.3 (42/ 508,200)	12.5 (63/ 502,700)

3.3.3. *S. pneumoniae* isolation rates in CSF

From January 2001 until December 2011 9,127 CSFs were processed at CHL, of those 4,928 showed pleocytosis. Of those CSFs with pleocytosis, 26 (0.5%) CSF grew *S. pneumoniae*. Thirteen (50.0%) isolates were from female patients. Seventeen (65.0%) of these were children, 94.0% were 1 year old or younger. Six (23.0%) of the 26 patients were admitted to intensive care, 1 child and 5 adult patients. **Table 3.2** summarises the population-based isolation rates for *S. pneumoniae* in different age groups over the studied time period.

Table 3.2: Annual incidence rates of culture-confirmed pneumococcal meningitis

The rate is given per 100,000 catchment population for the CDHB from 2001 until 2011 in different age groups. The total number of pneumococcal isolates from patients' CSF and total denominator per year in each age group are given in brackets.

Age (years)	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
<2	0 (0/ 11,270)	26.5 (2/ 11,310)	8.8 (2/ 11,290)	8.5 (1/ 11,810)	8.2 (1/ 12,160)	24.2 (2/ 12,410)	30.4 (4/ 13,140)	7.4 (1/ 13,530)	7.4 (1/ 13,540)	7.4 (1/ 13,520)	0 (0/ 12,720)
2-4	0 (0/ 17,040)	0 (0/ 17,100)	0 (0/ 17,370)	0 (0/ 17,700)	0 (0/ 17,720)	5.5 (1/ 18,070)	0 (0/ 18,420)	0 (0/ 19,040)	0 (0/ 19,590)	0 (0/ 20,290)	0 (0/ 19,450)
5-64	0.3 (1/ 354,650)	0.3 (1/ 361,880)	0.5 (2/ 370,870)	0 (0/ 377,770)	0 (0/ 383,070)	0 (0/ 388,350)	0 (0/ 392,150)	0 (0/ 395,720)	0.2 (1/ 399,620)	0 (0/ 403,190)	0 (0/ 397,520)
>65	3.3 (2/ 58,280)	0 (0/ 59,070)	1.7 (1/ 59,980)	1.6 (1/ 61,110)	1.6 (1/ 62,510)	1.5 (1/ 64,530)	0 (0/ 66,380)	0 (0/ 67,650)	1.4 (1/ 69,280)	0 (0/ 71,210)	0 (0/ 73,000)
All ages	0.7 (3/ 441,200)	0.9 (3/ 449,400)	0.9 (5/ 459,500)	0.4 (2/ 468,400)	0.4 (2/ 475,500)	1.0 (4/ 483,400)	0.8 (4/ 490,100)	0.2 (1/ 495,900)	0.6 (3/ 502,000)	0.2 (1/ 508,200)	0 (0/ 502,700)

3.3.4. *S. pneumoniae* isolation rates in respiratory specimens

Overall, a total of 106,019 respiratory specimens were processed during the study period from January 2001 until December 2011 (79.8% sputa, 9.9% tracheal aspirates, 8.9% lower respiratory specimens and 1.4% E.T.T aspirates) and yielded either growth of a significant microorganism or oropharyngeal flora. Of the 3,490 respiratory specimens growing pneumococci, 87.2% were sputa, 6.3% tracheal aspirates, 6.3% lower respiratory

specimens and 0.2% E.T.T aspirates. The proportion of specimen types and the overall yield of pneumococci in these are listed in **Table 3.3**.

Of the 3,490 isolations of *S. pneumoniae* 1,404 (40.2%) were from female patients. The number of specimens from ICU patients was 289 (8.3%).

Table 3.4 summarises the age-related population-based isolation rates of pneumococci in respiratory specimens.

Table 3.3: Respiratory specimens types processed at CHL from January 2001 to December 2011

Proportion of different respiratory specimen types yielding pneumococcal growth.

	Proportion of pneumococci isolated from total number of respiratory specimens in adults	**Proportion of pneumococci isolated from total number of respiratory specimens in children**
Sputum (n*=84640; 2672)	3044 (3.6%)	81(3.0%)
Tracheal aspirate (n=10526; 450)	221 (2.1%)	26 (5.7%)
Lower respiratory specimen (n=9469; 1982)	219 (2.3%)	71 (3.6%)
Endotracheal tube aspirate (n=1384; 1193)	6 (0.4%)	3 (0.2%)

*n=total number of specimens in adults; children

Table 3.4: Annual incidence rates of pneumococci in respiratory specimens

The rate is given per 100,000 catchment population for the CDHB from 2001 until 2011 in different age groups. The total number of pneumococcal isolates from patients' respiratory specimens and total denominator per year in each age group are given in brackets.

Age (years)	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
<2	8.9 (1/ 11,270)	17.7 (2/ 11,310)	35.4 (4/ 11,290)	42.3 (5/ 11,810)	24.7 (3/ 12,160)	16.1 (2/ 12,410)	0 (0/ 13,140)	7.4 (1/ 13,530)	29.5 (4/ 13,540)	7.4 (1/ 13,520)	23.6 (3/ 12,720)
2-4	23.5 (4/ 17,040)	41.0 (7/ 17,100)	11.5 (2/ 17,370)	19.9 (3/ 17,700)	39.5 (7/ 17,720)	27.7 (5/ 18,070)	27.1 (6/ 18,420)	42.0 (8/ 19,040)	30.6 (6/ 19,590)	39.4 (8/ 20,290)	36.0 (7/ 19,450)
5-64	53.8 (192/ 354,650)	54.2 (196/ 361,880)	42.3 (157/ 370,870)	42.3 (160/ 377,770)	39.1 (150/ 383,070)	32.7 (127/ 388,350)	30.3 (119/ 392,150)	31.1 (123/ 395,720)	27.0 (108/ 399,620)	29.5 (119/ 403,190)	32.9 (131/ 397,520)
>65	389.5 (227/ 58,280)	416.4 (247/ 59,070)	261.7 (157/ 59,980)	270.0 (165/ 61,110)	254.3 (159/ 62,510)	201.4 (130/ 64,530)	210.9 (147/ 66,380)	223.2 (151/ 67,650)	192.0 (133/ 69,280)	160.1 (114/ 71,210)	257.5 (188/ 73,000)
All ages	95.9 (424/ 441,200)	100.3 (452/ 449,400)	69.6 (320/ 459,500)	71.1 (333/ 468,400)	67.1 (319/ 475,500)	54.6 (264/ 483,400)	55.3 (272/ 490,100)	57.1 (283/ 495,900)	50.0 (251/ 502,000)	47.6 (242/ 508,200)	65.4 (329/ 502,700)

3.3.5. *S. pneumoniae* detection rates with the urinary antigen detection test

The urinary antigen detection test (UAT; NOW *S. pneumoniae* by Binax Inc., Scarborough, ME, USA) was introduced at CHL microbiology in April 2004. No age-related rejection criteria for UAT are applied at CHL. Since then 7,393 UAT for the detection of pneumococcal antigen were requested at CHL. **Table 3.5** summarises the detection rates of pneumococcal antigen in urine specimens by the UAT from 2005 until 2011 in different age groups.

Table 3.5: Detection rates (%) of pneumococcal antigen in urine specimens

Specimens tested at CHL from 2005 until 2011 in different age groups. Nominator and denominator data are given in brackets.

Age (years)	2005	2006	2007	2008	2009	2010	2011
<2	66.7 (8/12)	31.3 (5/16)	62.5 (10/16)	66.7 (6/9)	35.7 (5/24)	20.0 (2/10)	0 (0/2)
2-4	100.0 (4/4)	66.7 (4/6)	60.0 (3/5)	100.0 (5/5)	50.0 (4/8)	100.0 (1/1)	33.3 (1/3)
5-64	15.8 (57/361)	18.8 (61/324)	12.6 (38/302)	14.7 (68/463)	16.6 (90/541)	9.1 (53/582)	9.9 (44/445)
>65	16.7 (79/473)	14.9 (61/409)	14.5 (48/330)	16.8 (98/585)	13.2 (96/725)	9.6 (61/633)	8.6 (54/626)
All ages	17.4 (148/850)	17.3 (131/755)	15.2 (99/653)	16.7 (177/1062)	15.1 (195/1288)	9.5 (117/1226)	9.2 (99/1076)

* The UAT was introduced in April 2004, therefore only complete annual datasets from 2005 were included here.

3.3.6. Effect of the introduction of the UAT

The effect of the introduction of the UAT in April 2004 was analysed by looking at the number of patients diagnosed with pneumococcal pneumonia prior and post introduction of the test. The mean monthly number of diagnoses made prior to test implementation was 12.9 with a standard deviation of 8.5 [95% CI 10.1 – 15.6], the mean number of diagnoses made post test implementation was 11.6 with a standard deviation of 6.2 [95% CI 10.3 – 12.9], and the effect of test introduction on the number of patients diagnosed with pneumococcal pneumonia was not statistically significant (p=0.3474). The number of patients discharged with the diagnosis of pneumococcal pneumonia pre and post implementation of the UAT in April 2004 is illustrated in **Figure 3.2**.

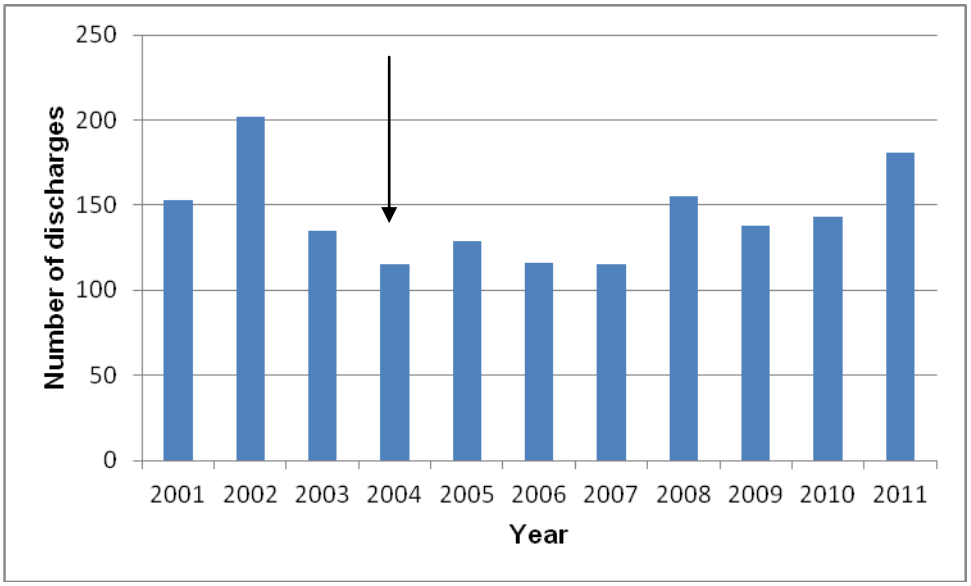


Figure 3.2: Number of yearly patient discharges with the diagnosis of pneumococcal pneumonia

The introduction of the UAT in April 2004 is indicated by an arrow.

3.3.7. Effect of "poor quality" sputum rejection

The monthly distribution of pneumococcal isolates from sputum specimens from 2001-2011 was analysed to study the potential effect of the introduction of sputum rejection criteria in 2007. Analysis was performed including all values from 2001 until 2011 with "pneumococci isolated in sputum specimens". The mean monthly number of pneumococci isolated in sputum specimens prior to the implementation of the sputum rejection criteria was 26.2 with a standard deviation of 12.1 [95% CI 23.4 – 29.0], the mean number of pneumococci isolated in sputum specimens post implementation of the sputum rejection criteria was 19.5 with a standard deviation of 8.3 [95% CI 17.3 – 21.6], (p=0.0004). The implementation of the sputum rejection criteria resulted in fewer isolations of pneumococci. The difference of the mean number of pneumococci isolated in sputum specimens prior and post implementation of the sputum rejection criteria was statistically significant. Sputum rejection was likely to have had a threefold impact: (i) a reduction in number of samples that were tested, (ii) a possible increase in the correct diagnosis of clinically significant pneumococcal disease and (iii) a possible reduction in the number of false positive cases of IPD. The number of pneumococcal isolations from sputum specimens pre and post

implementation of the rejection criteria in 2007 is illustrated in **Figure 3.3**.

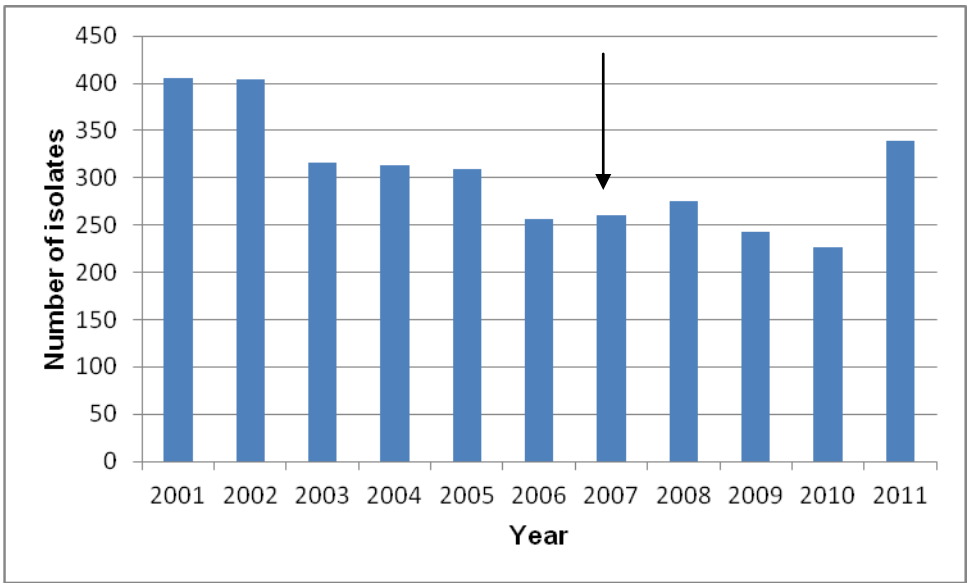


Figure 3.3: Number of yearly pneumococcal isolates pre and post sputum rejection implementation

The introduction of sputum rejection criteria is indicated by an arrow.

3.3.8. Test utilisation in adult and paediatric patients with the discharge diagnosis of pneumonia

In the adult population 996 patients were discharged with the diagnosis of "pneumonia", 468 (47%) were female patients. In the paediatric population a total of 151 children, 83 (55%) female children, were discharged with a diagnosis of "pneumonia" in 2011. The adult ages ranged from 15 to 108 years with a median of 75 years, the children from age 1 month to 14 years with a median of 3 years. **Table 3.6** gives an overview over the different specimen types and the proportion of pneumococcal isolations per specimen type in age groups of patients discharged with the diagnosis of pneumonia in 2011.

Table 3.6: Utilisation of different microbiological investigations in patients discharged with the diagnosis of pneumonia in 2011

Proportions are given in %.

Age (years)	Total number of patients	BC[1] taken (%)	Pneumococcus isolated in BC (%)	UAT taken (%)	Pneumococcus detected by UAT[2] (%)	Sputum taken (%)	Pneumococcus isolated in Sputum (%)
<2	51	22 (43.1)	0	0	0	0	0
2-4	61	22 (36.1)	0	1 (1.6)	0	2 (3.3)	0
5-14	38	16 (42.1)	0	2 (5.3)	0	5 (13.2)	1 (20.0)
15-64	309	191 (61.8)	10 (5.2)	157 (50.8)	22 (14.0)	134 (43.4)	21 (15.7)
>65	687	339 (49.3)	14 (4.1)	218 (31.7)	26 (11.9)	245 (35.7)	33 (13.5)
All ages	1146	590 (51.5)	24 (4.1)	378 (33.0)	48 (12.7)	386 (33.7)	55 (14.2)

[1] BC: blood culture

[2] UAT: urinary antigen test

In the adult population (all adults had radiographic evidence of pneumonia), the greater proportion of specimens was taken from patients older than 65 years. The yield of pneumococci in patients aged 15 to 64 years was marginally greater than that in the older age group (**Table 3.6**) in all different specimens (blood cultures, urinary antigen detection test and sputum specimens). The overall number of pneumococcal isolations from all specimens was 451, which resulted in a yield of 35.1% isolations from all specimen types in 2011. Of those 24 patients who had pneumococcus isolated from blood cultures only 11 had a urinary antigen test performed and only 6 (54.5%) of those detected pneumococcal antigen. Twelve of the 24 patients with pneumococcal bacteraemia had sputum specimens submitted for microbiological investigation and 3 (25.0%) grew pneumococci. Of 157 patients aged between 15 and 64 years who had a UAT performed, 97 patients (62.4%) also had blood cultures taken, 55 (56.7%) had also sputa taken. None of the 55 patients who had the three different

microbiological investigations requested had a positive result in all three. Only one patient of the 97 (1.0%) patients who had both, UAT and blood cultures requested, had a positive result in both tests. Of the 218 patients older than 65 years who had UAT performed, 131 (60.1%) had blood cultures taken. Of the 131 patients with UAT and blood cultures requested 61 (46.6%) had sputa taken. Of the 687 patients in this age group only 61 (8.8%) had the three different microbiological investigations requested. Only one single patient (1.6%) had a positive result in all three tests.

In the group of 22 children aged < 2 years, 19 (86.3%) had radiographic evidence of pneumonia, among 61 children age 2-4 years, 46 (75.4%) had radiographic changes consistent with pneumonia and among the 38 children age 5-14 years 25 (65.8%) had radiographic evidence.

Of the 996 adult patients discharged with the diagnosis of "pneumonia" 136 (13.6%) had pathogens, other than *S. pneumoniae,* identified. **Table 3.7** lists the number of identified pathogens. Ninety three of the 136 patients (68.4%) were 65 years of age or older. Of those 136 patients, 3 (2 with parainfluenza virus type 3, 1 with *M .catarrhalis* identifications) had concurrent pneumococcus isolated in blood culture. Five patients (2 with parainfluenza virus type 3, 2 with *H. influenzae* and 1 with influenza A virus identifications) had pneumococcal antigen detected in the urine, 4 of those also had pneumococcus grown in sputum culture. Nine patients (4 with *M .catarrhalis*, 2 with parainfluenza virus type 3, 2 with influenza A virus and 1 with human metapneumovirus identifications) had pneumococci grown in sputum culture. Only one of the patients (with parainfluenza virus type 3) had pneumococcus isolated in cultures of blood and sputum and antigen detected in the urine. Of the 150 children diagnosed with pneumonia 101 (67.3%) had radiographic confirmation of the pneumonia, of those 51 had blood cultures taken during the episode of the illness. Overall 60 blood cultures were taken in children with pneumonia none of which had pneumococcus isolated. Seventeen (11.3%) children had pathogens, other than *S. pneumoniae*, identified. The majority of other pathogens (76.4%) were viruses (**Table 3.7**), dual viral infections in 3 cases and a viral-bacterial co-infection in one case. The ordering pattern in adults was as follows: 530 patients had BC taken, 379 had sputum cultured, and 375 had the UAT requested and performed. One hundred and eighty seven patients had BC only taken, 228 had BC plus a UAT, and 115 had BC, UAT and sputum cultures. In children the ordering pattern was: BC was taken most frequently (61 BCs). In 53 cases BC were the only specimen taken, in 5 cases BC and sputum were taken for culture, in 2 cases BC and UAT were requested and in only one child all three, BC, sputum culture and UAT, were requested.

Table 3.7: Pathogens other than *S. pneumoniae* identified in the adult and paediatric patients discharged with the diagnosis of "pneumonia"

Identified pathogen	Number of all identifications in descending order of frequency	Number of identifications in adults	Number of identifications in children
Haemophilus influenzae	43	43	0
Legionella spp.	32	32	0
Moraxella catarrhalis	10	10	0
Staphylococcus aureus	8	8	0
Pseudomonas aeruginosa	8	8	0
Escherichia coli	7	7	0
Influenza A virus	6	5	1
Respiratory syncytial virus (RSV)	4	0	4
Parainfluenza virus type 3 (PIV3)	4	3	1
Human metapneumovirus	4	3	1
Rhinovirus	3	2	1
Enterococcus faecalis	2	2	0
Influenza B virus	2	1	1
Mycoplasma pneumoniae	2	0	2
Streptococcus agalactiae	2	2	0
Streptococcus pyogenes	1	1	0
Haemophilus spp.	1	1	0
Klebsiella pneumoniae	1	1	0
Bordetella spp.	1	1	0
Acinetobacter spp.	1	1	0
H. influenzae, rhinovirus	1	0	1
Enterobacter cloacae	1	0	1
M. catarrhalis, H. influenzae	1	0	1
Proteus mirabilis	1	1	0
Pneumocystis spp.	1	1	0
Stenotrophomonas maltophilia	1	1	0
P. aeruginosa, S. aureus	1	1	0
Rhinovirus, adenovirus	1	0	1
RSV, PIV3	1	0	1
RSV, rhinovirus	1	0	1
Serratia marcescens	1	1	0

3.3.9. Test utilisation in adult and paediatric patients with meningitis

It was assumed that most patients with suspected infectious meningitis would have had a CSF taken for microbiological investigation which would demonstrate pleocytosis. It is likely that a small number of patients with suspected infectious meningitis did not have a CSF collected for microbiological investigations. The data available for this analysis did not allow for the differentiation of infectious versus non-infectious CSF pleocytosis. Of the CSFs processed during 2001-2011 4,928 CSFs showed pleocytosis and were cultured. Two hundred and thirty eight had pneumococcal antigen detection test performed (Wellcogen *S. pneumoniae*, Wellcome Diagnostics, Dartford, UK). Fourty three (18.0%) of those were positive for pneumococcal antigen. Nineteen of these positive antigen detection tests (44.2%) belonged to CSFs on children below age 5. All CSFs which had the pneumococcal antigen had also a Gram stain and culture performed. Of these 43 positive pneumococcal antigens in the CSF 26 (60.0%) grew *S. pneumoniae* on culture, one CSF grew *S. oralis*. Sixteen antigen positive CSFs had no growth on culture. Of those all but 5 (68.0%) tested positive for the presence of antimicrobials in the CSF. Eight of the 16 CSFs with no growth on culture (50.0%) had Gram-positive organisms seen on Gram stain and all but one of those had antimicrobials present in the CSF, indicating the presence of non-viable organisms on Gram stain. Of the 26 CSFs that grew *S. pneumoniae* all but 2 (92.0%) had a positive Gram stain demonstrating either Gram-positive cocci or diplococci, 17 (65.0%) had tested positive for the presence of antimicrobials in the CSF. Of all 43 positive pneumococcal antigen CSFs 40 (93.0%) had abnormal protein (normal range: 0.15-0.4g/L) and 34 (79.0%) had abnormal glucose (normal range: 2.8-4.4mmol/L) in their CSF.

Thirty nine (90.0%) of the 43 patients had blood cultures taken at the time of their pneumococcal meningitis. Twenty three (53.0%) of the blood cultures grew *S. pneumoniae*. Of the 16 blood cultures that were negative for pneumococcus, only two had contaminants (one *Bacillus* spp., one coagulase-negative *Staphylococcus*).

3.4. Discussion

This is the first time retrospective data on diagnostic methods for pneumococcal disease were analysed and critically evaluated at the CHL microbiology unit. The annual rates of isolation of pneumococci in the different specimen groups (except for the detection rates in

the UAT) analysed here were presented as per 100,000 catchment population for the CDHB and broken down into separate age-groups. This allowed a direct comparison to nationally published data on invasive pneumococcal disease (IPD) in the population of New Zealand.

In June 2008 the 7-valent pneumococcal conjugate vaccine (PCV-7) was added to the childhood immunisation schedule in New Zealand and from October that year IPD became a notifiable disease. The instigation of the notification programme allowed New Zealand for the first time to provide clearer defined IPD surveillance rates based on IPD notifications rather than solely on laboratory-based surveillance data. As outlined by a published report from the Institute of Environmental Science and Research (ESR) in 2010, the definition for a case of IPD comprises:

1. "The isolation of *S. pneumoniae* from CSF, blood or other normally sterile site; or
2. The detection by nucleic acid amplification test of pneumococcal DNA in CSF, blood or other normally sterile site; or
3. A positive newer-generation *S. pneumoniae* antigen test (ie, Binax NOW) on CSF."[300]

The population-based rates for IPD from 2006-2010 are summarised in **Table 3.8**.

Table 3.8: Annual incidence rates of IPD per 100,000 in New Zealand

The rates are listed for different aggregated age groups from 2006 until 2010 (Adapted from Heffernan *et al.*, 2011 [300]).

Age group (years)	**2006**	**2007**	**2008**	**2009**	**2010**
<2	104.6	96.2	62.9	46.4	29.2
2-4	53.8	53.4	38.0	32.7	20.8
5-64	6.0	6.2	8.5	10.3	7.1
≥65	33.0	35.3	42.0	43.6	39.4
All ages	12.6	13.1	14.8	16.1	12.2

In 2010, the reported annual incidence rate per 100,000 of IPD based on notifications from the Southern region of New Zealand was 10.3, more specifically 8.3 for Canterbury alone. For Canterbury this rate represented a total of 45 case notifications, of which 39 were from patients older than 5 years. The total number of IPD cases notified in New Zealand in 2010 was 535. Four hundred and seventy eight cases were blood culture positive, 20 had a positive CSF culture, 11 had a positive joint culture and 14 had *S. pneumoniae* cultured from other sterile sites. It is of interest to note that no cases were notified on the basis of detection of pneumococcal DNA in molecular tests. As shown in **Table 3.8**, the annual incidence rates most affected by the inclusion of PCV-7 into the childhood immunisation schedule were the rates in the under 2 year and under 5 year age groups. In the elderly the annual incidence rate appears stable. However, when looking at the detection rates of pneumococci in blood cultures and UAT in Canterbury since 2008, a decrease of detection in UAT can be demonstrated while isolation of pneumococci in blood remain stable in the elderly. The decrease in the UAT detection might reflect an overall decrease in IPD in the elderly, but with blood culture rates remaining stable looks unconvincing.

When comparing the data analysed in this chapter with the data presented in **Table 3.8**, the isolation rate of pneumococci in BC per 100,000 showed a consistent drop since 2008 in the less than 2 year old children, presumably as a result of the introduction of PCV-7. The

effect was also noticeable, only to a lesser extent, in the group of children younger than 5 years. The isolation of pneumococci in CSF per 100,000 was at its lowest since 2008 and has remained stable since then. The annual isolation rates of pneumococci in respiratory specimens per 100,000 are highest in patients older than 65 years, which may reflect the greater presence of co-morbidities, such as COPD in that age group. Although the detection rates of pneumococci are high in children less than two years of age, the finding needs to be looked at with caution as it is known that the UAT can produce false-positive reactions due to nasopharyngeal colonisation, particularly in children [252, 253].

Over the study period from 2001 to 2011 two major changes occurred in the microbiological testing strategy at CHL. Firstly, the urinary antigen detection test (UAT) for pneumococci was introduced in April 2004. Following the Gram stain, this was the second test in pneumococcal diagnostics to produce a rapid result on a non-invasive specimen. The data discussed in this chapter demonstrate no statistically significant change in the number of pneumococcal pneumonia diagnosis made after the introduction of the test. However, it is likely that the diagnosis of pneumonia due to *S. pneumoniae* which was used to conduct the analysis to evaluate the effect of test changes (UAT) underestimated the number of patients with pneumococcal pneumonia as suggested by the findings in section 3.3.2. A more standardised approach in assigning a discharge diagnosis would be helpful. The second major intervention was the introduction of sputum rejection criteria in 2007. Here, a significant decrease in the number of pneumococcal isolates from sputum specimens in the time period after the introduction of the rejection criteria for poor specimens in 2007 could be demonstrated. It is likely that the results from "good quality" sputum specimens are more meaningful to clinicians and are able to direct more appropriate clinical management.

The 2011 "snapshot" database analysis for adult and paediatric patients with the discharge diagnosis pneumonia selected out those patients with radiographic evidence of pneumonia. It was interesting to find that the overall yield of pneumococci from blood cultures in adult pneumonia patients was as low as 4.5%. This concurs with published findings that when blood culture is performed to investigate the bacterial aetiology of pneumonia the expected sensitivity is between 3.0 and 30.0% [301, 302]. Only very few of the patients with positive blood cultures, indicating the presence of invasive disease, had concurrent isolation of pneumococci in the sputum or antigen detection in the urine. Again, only less than half of the adult patients with *S. pneumoniae* growing in their BC had UAT requested and performed and only about 50.0% of those had positive antigen detection. This rate of urine antigen positivity in patients with pneumococcal bacteraemia lies well below that reported in other studies [248,

303, 304]. This raises the question of suitability of these tests to diagnose pneumococcal pneumonia when used on their own. Given the doubtful effect of UAT in the diagnosis of pneumococcal pneumonia it is interesting to note that the number of requests of the test increased from 2005 until 2007 and has remained between 1,062 and 1,288 requests per year since. Even more impressive was the lack of BC positivity in children with pneumonia. BC was the most frequent specimen taken in children with pneumonia and none grew pneumococci in the studied group. Blood cultures are, although thought to be an insensitive test for bacterial pneumonia in children, recommended in the unwell child, especially those suspected of "*Staphylococcus aureus* or complicated pneumonia" (guidelines for the diagnosis and management of pneumonia in children: http://www.adhb.govt.nz/starshipclinicalguidelines/_Documents/Pneumonia.pdf; published by Starship Children's Health, Auckland). The number of requests of the UAT was low in children which is not surprising considering the known problem with false-positive reactions in children. Overall, the yield of pneumococci from sputum culture or the rate pneumococcal antigen detection in the UAT was disappointing in both, children and adults.

When other pneumonia-causing pathogens were detected pneumococcus was present in only 2.2% of BC of those cases, most notably in two cases of parainfluenza type 3 infections, but none of the cases with influenza viruses. The majority of adult pneumonia patients who had other pathogens present either had *H. influenzae* or *Legionella* species identified. None of the *Legionella* cases had pneumococcus isolated from blood culture or sputum or antigen detected in the urine, and only 2 of the *H. influenzae* identifications had concurrent isolations of *S. pneumoniae* from sputum. In the paediatric group other identified pathogens were mainly viruses. The presence of co-infections was more frequent than in the adults and RSV featured more dominantly. None of the children had *Legionella* spp. identified. It is worth noting here that the reported diagnostic yield of bacterial pathogens in CAP is poor [305, 306]. Bartlett recommends the move from traditional culture methods to molecular techniques in order to improve the diagnostic yield for recognised pulmonary pathogens [305].

In terms of the overall clinicians' ordering pattern, BC, sputum culture and UAT were rarely ordered together. In adults the most frequent combination of tests is BC plus UAT, in children BC alone is the most frequently requested investigation in pneumonia cases. Interestingly, with increasing age in the three different paediatric age groups the number of radiographically confirmed pneumonia cases declined, either due negative findings on the chest radiograph or due to non-request of a chest radiograph. With only 65.8% of children aged 5-14 years having a radiological diagnosis of pneumonia and a very low yield of

pneumococci isolated from the obtained microbiological specimens, the diagnosis of pneumococcal pneumonia was purely based on clinical grounds.

It is noteworthy that only 53.2% of all adult patients with pneumonia actually had blood cultures taken; a specimen that despite its low sensitivity is invaluable when yielding a pathogen. This is particularly significant when remembering that the "Management guidelines for common medical conditions" published by the Internal Medicine Services of the CDHB recommend that patients with a CURB-age score 3-5, defined as severe CAP, are to have 2 sets of blood cultures taken prior to receiving antimicrobials. In future, it would be of interest to look at clinicians' ordering patterns in specific adult groups, such as elderly, immunocompromised patients or patients with respiratory co-morbidities.

The limitations of the data analysis described here are the retrospective character of the review, the fact that the radiographic review was not done independently, the lack of information as to why some patients did not have certain specimens taken during their hospital stay, and the shortcomings of the ICD-10 coding at Christchurch Hospital.

The data available for the analysis of CSFs did not allow for the differentiation of infectious versus non-infectious CSF pleocytosis. Therefore, it cannot be excluded that some of the cases included in the analysis were investigated for a different underlying cause. When analysing the test utilisation in the investigation of patients with meningitis it was interesting to note that although all pleocytotic CSFs had microscopy and culture performed, only 4.8% of those had a pneumococcal antigen requested. The "Management guidelines for common medical conditions" published by the Internal Medicine Services of the CDHB recommends antigen detection tests in adults when the CSF leukocyte count is $> 5 \times 10^6$ cells/L. The quoted rate of 4.8% in this analysis was based on CSFs with a leukocyte count of $> 1 \times 10^6$ cells/L, which makes it likely that the rate would be higher using leukocyte counts of $> 5 \times 10^6$ cells/L as the denominator. Still, it is unlikely that the rate would indicate a strict adherence to the guidelines. The low number of requested antigen detection tests in the pleocytotic CSFs is therefore surprising. Of those that had a pneumococcal antigen test performed, 18.0% were positive. Sixty % of those that were antigen positive also grew *S. pneumoniae* from the CSF. Only one case grew *S. oralis* indicating a false-positive reaction in the pneumococcal antigen detection test. False-positive reactions in bacterial antigen detection assays used on CSF have been described before [307], and, although the Wellcogen assay has not been specifically implied as producing false-positives with the *S. pneumoniae* detection assay, it is very likely that cross-reactions occur, in particular with organisms from the genetically very closely related *S. mitis* group.

Most of the antigen positive CSFs which had organisms present on Gram stain, but did not grow pneumococcus in culture, had presence of antimicrobials in the CSF indicating effective treatment prior to obtaining the CSF. Early treatment of suspected meningitis cases in the community or at presentation in the hospital emergency department prior to laboratory investigations is the likely reason for this. It was reassuring that of all the meningitis cases that had positive pneumococcal antigen 90.0% also had BC taken, of which 53.0% grew *S. pneumoniae*.

Overall this analysis demonstrates that even in an environment with a heightened awareness and research interest of CAP the application of available diagnostic investigations is poor. The one test that is favoured in all age groups is BC, but the yield of pneumococci is low as expected from published data. Only about half the adult patients with positive BC had pneumococcal UAT requested and half of those had antigen detected in the UAT and the recovery of pneumococci in the sputum of these patients was low. It raises the question as to which tests are clinically useful, either in combination or as "stand-alone tests" for the diagnosis of IPD. No single tests seems appropriate as a "stand alone" diagnostic option, and it is likely that requesting a range of microbiological investigations on well timed, "good quality" specimens will provide the best clinical assistance.

It is encouraging to see that both, CSF and BC, are often taken in meningitis cases. This increases the likelihood of identifying the pathogen when the CSF does not grow pneumococcus on culture, while blood does. This could possibly reflect a larger number of organisms in the circulating blood stream. However, this possible explanation is not supported by a publication by Mook-Kanamori who found bacterial titres (cfu/mL) in brain and CSF of inoculated mice at the end-stage of disease to be greater than the titres in blood [308].

The findings of this chapter suggest the need for better utilisation of the currently available testing methods by themselves and in combination. It is also likely, even though the UAT did not have the anticipated positive effect on the diagnosis of pneumococcal disease, that further research in the area of pneumococcal diagnostics and development of improved diagnostic methods will be necessary. The remainder of this thesis will cover some of the aspects by investigating improved diagnostic strategies for the investigation of IPD.

DEVELOPMENT AND EVALUATION OF ANTIGEN DETECTION ASSAYS

CHAPTER 4

DEVELOPMENT AND EVALUATION OF A PNEUMOLYSIN ANTIGEN DETECTION TEST

4.1. Introduction

Pneumolysin (Ply) is a major exotoxin of *S. pneumoniae*. As a cytoplasmic bacterial protein of 53-kDa consisting of 471 amino acid residues it has a complex range of cytotoxic as well as immune-modulatory properties. It is produced by all pneumococcal strains [309].

Ply is grouped within the thiol-activated cytolysins and belongs to the family of cholesterol-dependent cytolysins (CDCs). These are membrane-damaging toxins produced by a variety of Gram-positive bacteria which share similar protein structures and modes of action. The water-soluble monomeric or dimeric toxin form binds to the cholesterol receptor of the host cell membrane. This interaction with the cholesterol receptor leads to formation of large oligomeric transmembrane pores, embedded in the lipid bilayer of the membrane, ultimately causing cell lysis [310]. It is thought that a conformational change takes place after binding to the cholesterol receptor resulting in the transition from monomer to oligomer. The change in secondary structure exposes a hydrophobic domain which can insert into the membrane. Protein dimers form and aggregate to create large ring structures resulting in membrane pores of 30-35nm in diameter [85].

Ply is an important virulence factor of the pneumococcus. Animal studies have demonstrated that mice inoculated with Ply-deficient mutant strains were less likely to succumb to the infection and showed less damage to the alveolar-capilliary barrier compared to wild-type infection, and those mutants were cleared more effectively by the host's immune system [83, 311-313]. During the initial bacteraemic phase of IPD Ply expression appears critical in the process of breaking down the host's inflammatory responses followed by an acute sepsis syndrome in mice [314, 315].

Ply lacks an N-terminal secretion signal sequence, which restricts its release from pneumococci to progress to the stage of bacterial autolysis [316, 317].

Ply has the ability to activate the classical complement pathway in the absence of anti-Ply antibodies by binding IgG Fc with a structural domain that is different from the regions involved in cytolysis [318, 319]. *In vivo*, this leads to a stimulation of inflammatory responses and release of cytokines. The ability of Ply to activate the complement pathway stems from a sequence region shared between Ply and C-reactive protein, an acute phase protein in infection or tissue injury. In Ply the region implicated in membrane binding, complement activation and pore-formation is a highly conserved 11-amino acid Trp-rich loop, which contains a single cysteine and three tryptophan residues [320, 321]. The cysteine is thought to

be an essential component for the cytotoxic effect [322]. However, other regions within the N-terminus have been identified as critical for the process of pore formation [323].

In the early stages of IPD of the respiratory tract, Ply, as shown in culture models, slows ciliary beating of the ciliated bronchial cells and disrupts the bronchial epithelial monolayer [324, 325], resulting in a reduced ability to clear mucus from the lower respiratory tract, promoting bacterial growth. Ply is also responsible for disrupting the alveolar-capillary boundary allowing capillary leakage and flooding of nutritious liquid which encourages bacterial growth and bacterial penetration from the epithelial surface into the pulmonary interstitial space, and from there into the bloodstream [317]. This is supported by histological evidence of pulmonary alveolar oedema and haemorrhage as well as intra-alveolar bacterial propagation in the initial phase of pneumococcal pneumonia [326].

Ply's effects on the host's immune system are variable. Ply has the ability to inhibit phagocytosis and immune cell function through its direct cytotoxic action. Sublytic concentrations of the toxin can inhibit neutrophil and monocyte respiratory burst, chemotaxis, and bacteriocidal activity [327-329] and reduce the production of immune-modulatory lymphokines and immunoglobulins [79].

Ply is immunogenic. IPD produces IgG specific to Ply in 50-85% of adults, particularly in severe cases [330-333]. Immune complexes could be demonstrated in clinical cases with IPD [334] and IgA production has been detected in children with pneumococcal otitis media [335-337]. Murine models have demonstrated that mucosal immunity to Ply can be established [338, 339].

In summary, Ply is a multi-factorial toxin, produced by all pneumococcal strains, with properties that allow it to breach tissue barriers, alter host immune responses and create opportunities for bacterial propagation and dissemination, all of which enhance pneumococcal survival during the development of IPD. Its protein nature allows the development of recombinant antigens and the raising of monoclonal or polyclonal antisera to them. Ply induces local as well as systemic immune responses. These features make Ply a good candidate as a diagnostic target for IPD.

This chapter describes the development of a novel Ply antigen detection assay by a chromogenic detection method, similar to the chemiluminescent sandwich ELISA developed by Cima-Cabal in 2001 [340]. The assay was evaluated with serum specimens from a well characterised adult patient population with CAP. Serum was chosen as the specimen of choice as it is most likely to indicate bacteraemia, invasive disease of the bloodstream.

4.2. Material and methods

4.2.1. Materials

4.2.1.1. Recombinant pneumolysin (Pd-B)

Recombinant pneumolysin (Pd-B) (2mg) was obtained from Prof. James Paton, University of Adelaide, Australia. The protein was identical to native pneumolysin with the exception of mutation reducing its cytotoxicity [341]. The Pd-B was supplied in 50mM sodium phosphate, pH 7.0 with 50% glycerol and azide for preservation. It was aliquotted at 0.29mg/100µL and stored at -20°C.

4.2.1.2. Monoclonal antibody to pneumolysin (PLY-7)

A monoclonal antibody to pneumolysin was obtained from Juan Ramon De Los Toyos Gonzalez, University of Oviedo, Oviedo, Spain. This in-house antibody 2.1D12.61, subsequently named PLY-7 is described in detail by De los Toyos *et al*. [323]. The antibody concentration was 1.5mg/mL. The monoclonal antibody was aliquotted and stored at -80°C.

4.2.1.3. Rabbits for polyclonal antibody production

Two NZ white rabbits were accessed through the Animal Facility at the University of Otago, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand.

4.2.2. Methods

4.2.2.1. Polyclonal antibody production

The rabbits were each inoculated by subcutaneous injection with 150µg Pd-B in 450µL sterile PBS. The antigen was emulsified in 500µL Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Equal parts of the antigenic mixture were administered by subcutaneous injection into both hind-quarters of each animal. Two booster immunisations were administered at one month intervals using incomplete Freund's adjuvant [342].

Rabbit blood obtained from an incision of the auricular artery 10 days following the second booster immunisation was tested against Pd-B to verify polyclonal antibody titres.

4.2.2.2. Purification of polyclonal antiserum

Titres in both polyclonal antisera were comparable and one polyclonal antiserum, "Ivor", was arbitrarily selected to be purified for use in a diagnostic assay. Purification was performed using a Sephadex column. Undialysed rabbit antiserum underwent ion-exchange chromatography on a DEAE-Sephadex A50 column equilibrated with 10mM phosphate buffer, pH 6.5. Unbound material was washed off the column as described by Baumstark [343].

Twenty fractions were collected and checked for their reactivity with anti-rabbit HRP. The specific reactivity of the fractions was tested in an ELISA using pneumococcal lysate and suspension at 5µg/well. The concentration of the pooled fraction was measured at 280nm with the UV-Visual spectrophotometer Cary-50 v3.0 (Agilent, Santa Clara, CA, USA).

4.2.2.3. ELISA optimisation

Initially PLY-7 was selected as the capture antibody with the purified polyclonal antiserum "Ivor" as the second antibody. In order to optimise capture and second antibody concentrations, a two step matrix experiment was performed. Firstly, the microtiter ELISA plate was coated with three different concentrations of monoclonal antibody. The following dilutions of PLY-7 were used: 1:500 (3µg/mL), 1:1,000 (1.5µg/mL) and 1:5,000 (0.3µg/mL) and subsequently the range was extended to 1:10,000 (0.15µg/mL). The use of the microtiter plate in the first part of the matrix experiment is illustrated in **Figure 4.1.** Suspensions and lysates of *S. pneumoniae* and *S. aureus* were made up (sections 2.2.2.10 and 2.2.2.11) and added to the plate coated with PLY-7 in doubling dilutions with a starting concentration of 5µg/well.

Two plates were set up with each of the *S. pneumoniae* and *S. aureus* suspensions, and two with lysates. The suspension/lysate pairs were blocked with assay buffer for 15 min and with x4 assay buffer for 15 min. Incubation times for antigens and antibodies were 1 hour. The detection antibody goat anti-rabbit IgG (H+L) HRP (Jackson ImmunoResearch Laboratories, Inc., West Baltimore, PA, USA) was used at a 1:2,500 dilution.

In the second step of the matrix experiment the microtiter plate was coated with 0.15µg/mL PLY-7, using pneumococcal and *S. aureus* lysates as the antigen at 0.6µg/well. The concentration of the second (polyclonal) antibody was checked in doubling dilutions starting from 1:2000 (0.75µg/mL) (**Figure 4.2**).

After the initial evaluation, serum interference factors led to switching of the antibodies to using the purified polyclonal antibody as the capture antibody. Two concentrations were evaluated: 1:1,000 (1.5µg/mL) and 1:2,000 (0.75µg/mL). The blocking step compared between assay buffer and x4 assay buffer for 15 min as described above. The second antibody, monoclonal PLY-7, was also evaluated in two concentrations: 1:1,000 (1.5µg/mL) and 1:2,000 (0.75µg/mL). The detection antibody HRP rat anti-mouse IgG1 (BD Biosciences, Franklin Lakes, NJ, USA) was used at a 1:2,000 dilution. All graphed data points referring to the ELISA experiments are based on means of duplicates. The variance in all cases of absorbance signal duplicate values was minor, in most cases < 10%.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A **3µg/mL capture**	**5µg/well SPn Suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
B **1.5µg/mL capture**	**5µg/well SPn suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
C **0.3µg/mL capture**	**5µg/well SPn suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
D **Ø capture**	**5µg/well SPn suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
E **3µg/mL capture**	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
F **1.5µg/mL capture**	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
G **0.3µg/mL capture**	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
H **Ø capture**	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048

Figure 4.1: Optimisation of PLY-7 monoclonal antibody concentrations

Layout of the ELISA plate well coating with varying concentrations of PLY-7 as capture antibody against a dilution series of pneumococcal suspension or lysate (positive control) and *S. aureus* suspension or lysate (negative control).

	1	2	3	4	5	6	7	8	9	10	11	12
A 0.15µg/mL capture	750 ng/mL	375 ng/mL	187.5 ng/mL	93.7 ng/mL	46.9 ng/mL	23.4 ng/mL	11.7 ng/mL	5.8 ng/mL	2.9 ng/mL	1.5 ng/mL	0.75 ng/mL	0.375 ng/mL
B 0.15µg/mL capture	750 ng/mL	375 ng/mL	187.5 ng/mL	93.7 ng/mL	46.9 ng/mL	23.4 ng/mL	11.7 ng/mL	5.8 ng/mL	2.9 ng/mL	1.5 ng/mL	0.75 ng/mL	0.375 ng/mL
C 0.15µg/mL capture	750 ng/mL	375 ng/mL	187.5 ng/mL	93.7 ng/mL	46.9 ng/mL	23.4 ng/mL	11.7 ng/mL	5.8 ng/mL	2.9 ng/mL	1.5 ng/mL	0.75 ng/mL	0.375 ng/mL
D 0.15µg/mL capture	750 ng/mL	375 ng/mL	187.5 ng/mL	93.7 ng/mL	46.9 ng/mL	23.4 ng/mL	11.7 ng/mL	5.8 ng/mL	2.9 ng/mL	1.5 ng/mL	0.75 ng/mL	0.375 ng/mL

Figure 4.2: Optimisation of polyclonal antibody concentrations

Layout of the ELISA plate well coating with a constant concentrations (0.15µg/mL) of PLY-7 as capture antibody against 0.6µg/mL pneumococcal lysate (positive control, rows A and B) and *S. aureus* lysate (negative control, rows C and G) and concentrations of polyclonal antibody in doubling dilutions.

4.2.2.4. Spiked serum and plasma preparations

A 0.5 McFarland suspension and lysate of an overnight growth culture of *S. pneumoniae* ATCC 40617 on 5% SBA was made up as described in sections 2.2.2.4 and 2.2.2.5. Plain serum, EDTA and Lithium-Heparin plasma collection tubes were prefilled with pneumococcal suspension and lysate to achieve a final dilution of 1:10 once filled with blood. Blood from a healthy subject was directly collected into the pre-spiked tubes. As expected, moderate haemolysis was observed in the collected blood samples spiked with pneumococcal lysate in bacterial lysis buffer. Samples were centrifuged at 1200 x g for 15 min. The spiked blood samples were analysed in an ELISA coated with 1:10,000 PLY-7 as capture antibody. All samples were run in duplicate in the ELISA. Mean values were used to graph the data.

In the second part of the evaluation with the polyclonal antibody as capture antibody additional spiking of serum samples was performed by adding 7.5µg/well of native pneumococcal lysate to serum of two healthy subjects.

4.2.2.5. Assay precision

The reproducibility of measurements was evaluated on spiked serum and plasma samples as described above, as well as a negative (control) serum sample (un-spiked). All

samples were diluted 1:2 in assay buffer. Monoclonal antibody, PLY-7, was used as capture antibody at 1:10,000 (0.15µg/mL). A calibration curve consistent of Pd-B in doubling dilutions starting at 12.5ng/mL was included in each ELISA plate run. Intra-assay coefficients of variation were determined from 18 replicates of each, EDTA plasma and serum (spiked) and 10 replicates of un-spiked serum. Inter-assay reproducibility of 29 measurements was determined from values obtained on three different plates and time-points of EDTA plasma, Lithium-Heparin plasma and serum (spiked) and un-spiked serum. Intra- and inter-assay coefficients of variation were calculated as for the lower concentration of pneumococcal antigen in the spiked blood samples.

4.2.2.6. Lowest limit of detection

The standard curve was established using Pd-B at a concentration of 0.025µg/mL in doubling dilutions down to 390pg/mL. The minimum detectable Ply concentration was defined as the lowest measurable value that is statistically different from the zero value. Twenty nine zero values from the obtained standard replicates produced a mean absorbance signal of 0.189.

4.2.2.7. Biological limit of detection

The biological limit of detection (BLD) is estimated as the LLD plus 2 or 3 times the standard deviation from a spiked sample approximating the detection limit [344]. For the BLD determination the values obtained from the 1:2 diluted spiked sera were used.

4.2.2.8. Investigation of assay interference factors

To check for the presence of interference factors, sera from two healthy subjects underwent heat treatment at 56°C (complement C1q can cross-link IgG and is heat-labile) in a water bath for 30 min as well as pronase treatment. A 1:2 dilution of pronase (as recommended by the manufacturer for the cryptococcal antigen detection test (Calas® Pronase, Bioscience Inc., Cincinnati, Ohio, USA) was added to the serum and incubated at 56°C for 15 min followed by boiling for 5 min [345]. Recombinant pneumolysin (12.5ng/mL in assay buffer) was included in the treatments to observe any effect caused by either heat or pronase on the target protein.

To investigate the presence of immune complexes, the polyclonal antibody was assayed against Pd-B as well as human albumin and HG-II γ-Globulins, Cohn Fraction II (Sigma-Aldrich, St. Louis, MO, USA) and serum from a healthy subject by agar gel immunodiffusion by Ouchterlony [346].

The specificity of the polyclonal antiserum was investigated by Western Blot. Serum from two healthy subjects (each spiked and unspiked with pneumococcal lysate at 7.5µg/lane) was run in parallel with Pd-B (5-10µg/lane) and crude pneumococcal lysate (7.5µg/lane) and incubated with 1:1,000 polyclonal antibody.

4.2.2.9. Antigen detection in urines

Three CAP patients with only positive Binax NOW *S. pneumoniae* urinary antigen tests and five CAP patients with positive blood cultures, sputum culture and UAT had their urine tested in the Ply ELISA to obtain a preliminary idea of specimen suitability.

4.2.2.10. Western blot analysis

Vertical SDS-PAGE was carried out to check the immunoreactivity of the raised polyclonal antiserum to a range of bacterial lysates as well as approximately 5µg recombinant pneumolyysin (Pd-B) using 10% acrylamide gels with approximate loadings of 5-10µg bacterial isolates. Gel loading of the bacterial strains was based on a visual assessment of acrylamide gel stained with SimplyBlue^{TM} SafeStain (Invitrogen, Carlsbad, CA, USA) separating proteins of crude bacterial lysates. Kaleidoscope^{TM} Prestained Standard (Bio-Rad Laboratories, Hercules, CA, USA) was included as a marker on each gel/blot. The polyclonal antibody concentration used was 1.5µg/mL and the detection antibody, peroxidise conjugated Affini Pure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Baltimore, PA, USA) was used at a 1:2,000 dilution. The Western blot was developed with TMB precipitating substrate (section 2.2.2.3). The bacaterial lysate preparation was performed as described in section 2.2.2.5. The bacterial strains included in the analysis were *S. pneumoniae* (ATCC 49617), *S. pseudopneumoniae* (ATCC BAA-960), *S. oralis* (NZ isolate SID 08/60), *S. mitis* (ATCC 9811), *S. pyogenes* (ATCC 19615), *S. aureus* (ATCC 29213), *Listeria monocytogenes* (ATCC 19111) and *Clostridium perfringens* (ATCC 13124). These strains were chosen either due to their close genetic relationship to *S. pneumoniae* or

due the presence of a Ply-like toxin in those strains. *S. aureus* lysate was included as negative control for the diagnostic ELISA development. Pd-B was included as positive control.

Serum samples from two healthy subjects were included in the analysis, both spiked (7.5µg/lane) and not spiked with pneumococcal lysate.

4.2.2.11. Statistical analysis

The statistical analysis and graphics were performed using Windows Excel 2007 (Microsoft, Redmond, WA, USA) and SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA).

4.3. Results

4.3.1. Polyclonal antisera

Both rabbit antisera, "Ivor" and "Ignus", gave comparable signals to a plate coated with Pd-B in 6M guanidine HCL or in PBS. In both rabbit antisera the titre against Pd-B exceeded 1:100,000.

Antiserum "Ivor" was arbitrarily selected to be used in the development of a diagnostic sandwich ELISA

Twenty fractions were collected and checked for their reactivity with anti-rabbit HRP1:100 and 1:1,000. The specific reactivity of the fractions was tested in an ELISA using pneumococcal lysate and suspension at 5µg/well. Fractions 11, 12 and 13 were selected and pooled on the basis of their highest optical density (OD). The absorbance signal measured at 280nm was 2.0870. An absorbance signal of 1.35 approximates to 1mg/mL of IgG [347]. Sephadex column height was 10cm, diameter 3cm. 7mL of "Ivor" rabbit antiserum was added to 70mL of gel. The estimated concentration of purified polyclonal serum was 1.5mg/mL. The overall yield from 7mL of unpurified antiserum collected in 30mL of pooled fraction was 45mg, or 6.4g/L.

The obtained purified polyclonal antiserum was run on SDS-PAGE using a 10% acrylamide gel to check the purity. The gel showing non-reduced and reduced anti-Pd-B IgG is shown in **Figure 4.3**.

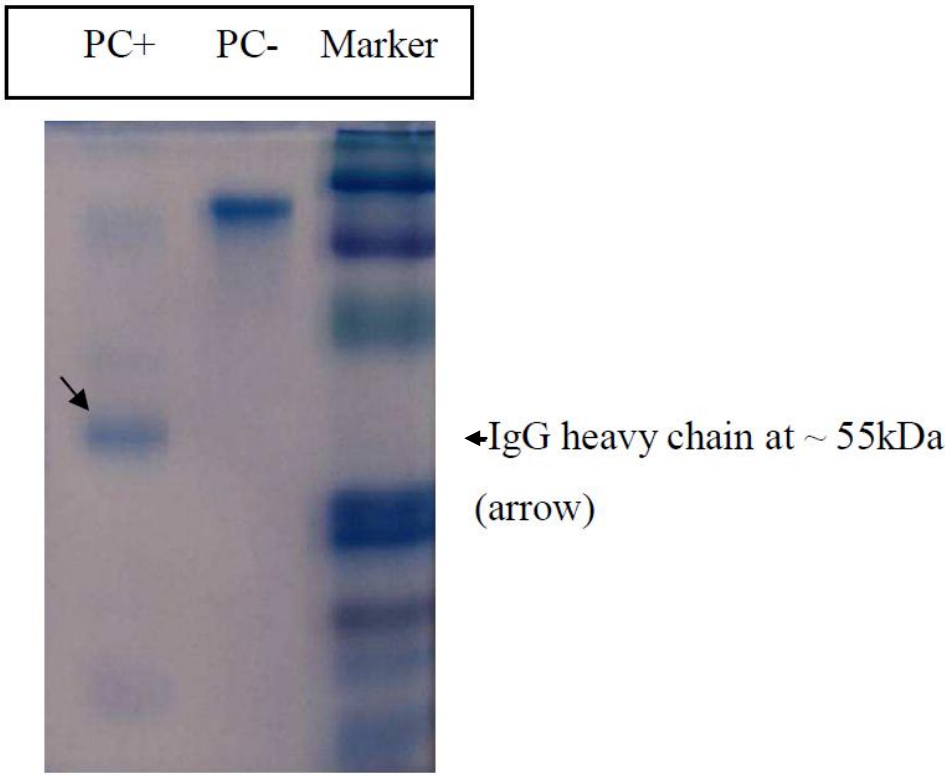


Figure 4.3: Coomassie blue stained protein gel showing anti-Pd-B polyclonal antibody

Reduced (PC+) and non-reduced (PC-) polyclonal IgG against Pd-B. The marker included is a Kaleidoscope™ Prestained Standard.

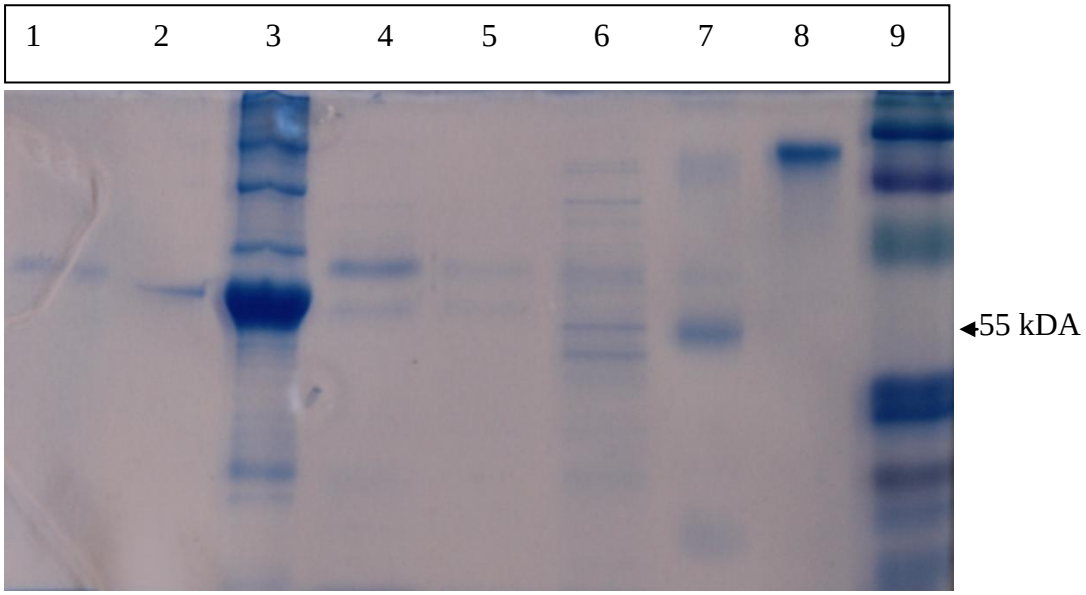
4.3.2. Immunoreactivity of the polyclonal antiserum

The separated proteins of crude bacterial lysates on a 10% acrylamide gel are shown in **Figure 4.4** a) and b). The immunoreactivity to the genetically closely related strains *S. mitis*, *S. pseudopneumoniae* and *S. oralis* as well as non-reactivity to Ply-like toxin harbouring strains (*S. pyogenes*, *L. monocytogenes* and *C. perfringens*) and *S. aureus* is demonstrated in a Western blot (**Figure 4.5**).

A Western blot demonstrating a band for Pd-B and *S. pneumoniae* is shown in **Figure 4.6**.

Lane 2**, Figure 4.7,** demonstrates a non-specific banding pattern rather than the expected single band at the height of 53kDa in a Western blot with polyclonal antiserum against Pd-B. Lane 1 shows a "cleaner" single band at 53kDa with one minor background band where polyclonal antibody reacts against crude pneumococcal lysate containing Ply.

a)



b)

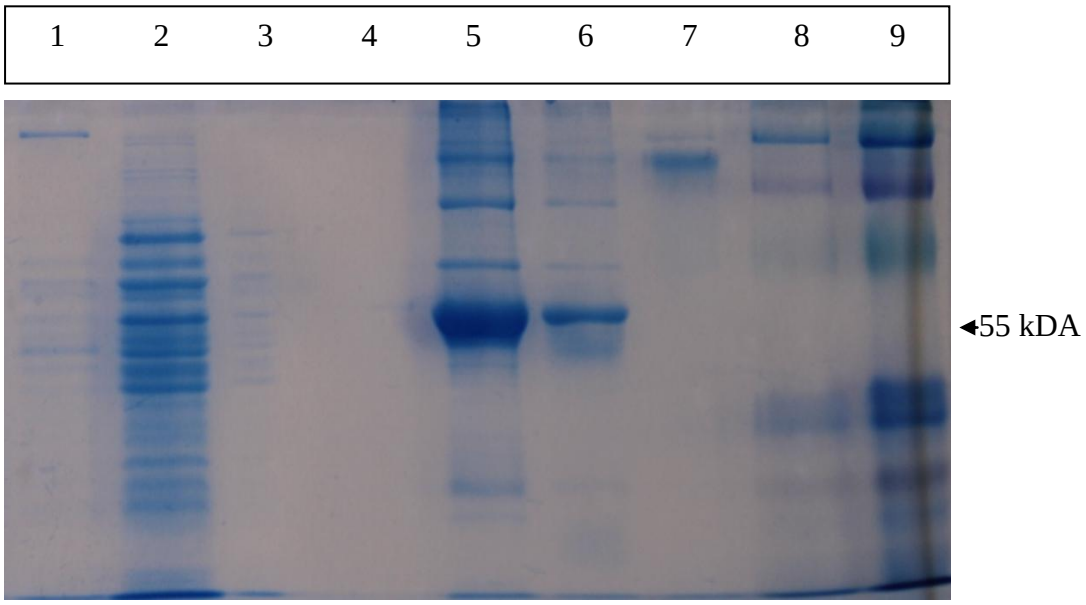


Figure 4.4: Coomassie blue stained proteins of crude bacterial lysates and anti-Pd-B polyclonal antisera

Reduced (PC+) and non-reduced (PC-) polyclonal IgG against Pd-B (as in **Figure 4.3**). a) Lane 1-9: *S. oralis*, *L. monocytogenes*, Pd-B, *S. pyogenes*, *S. aureus*, *C. perfringens*, PC+, PC-, marker. b) Lane 1-9: *S. pseudopneumoniae*, *S. pneumoniae*, *S. mitis*, *S. oralis*, Pd-B, PC+, PC-, marker (spill-over), marker. Thick band demonstrated at ~ 55kDa indicating a reaction of PdB with the anti-PdB polyclonal antiserum at the anticipated molecular weight of Ply.

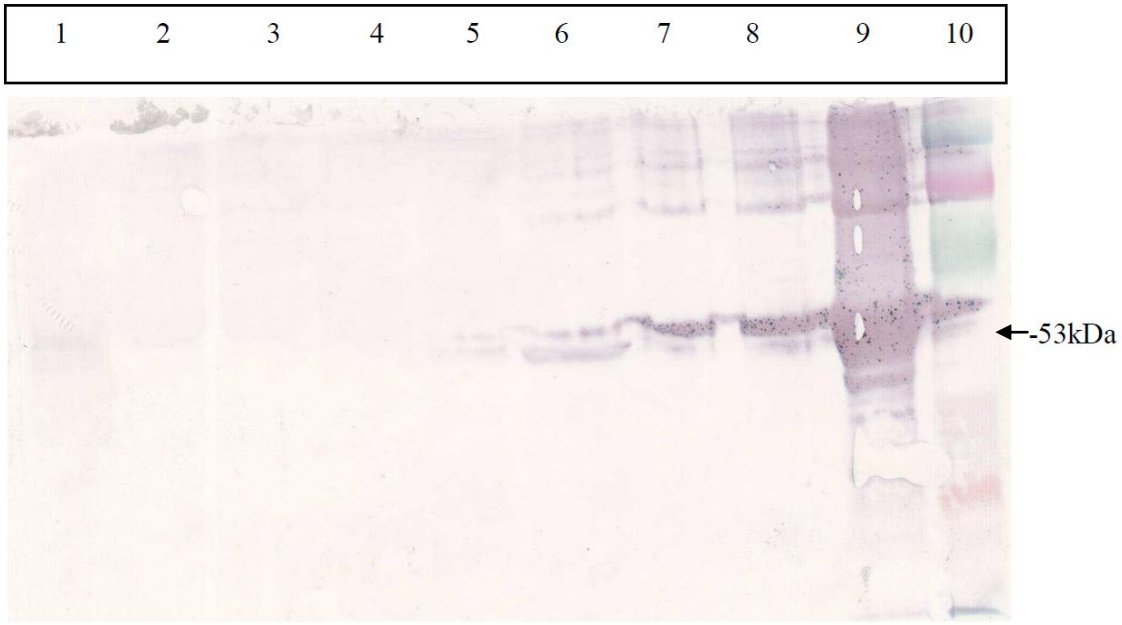


Figure 4.5: Western blot demonstrating bands at 53kDa

No reactivity of polyclonal antiserum with *S. aureus* (lane 1), *S. pyogenes* (lane2), *C. perfringens* (lane 3) and *L. monocytonenes* (lane 4). Recognition of pneumolysin weakly in *S. pseudopneumoniae* (lane 5), *S. pneumoniae* (lane 6), *S. mitis* (lane 7), *S. oralis* (lane 8), and in Pd-B (lane 9) by the purified polyclonal antiserum. Marker in lane 10.

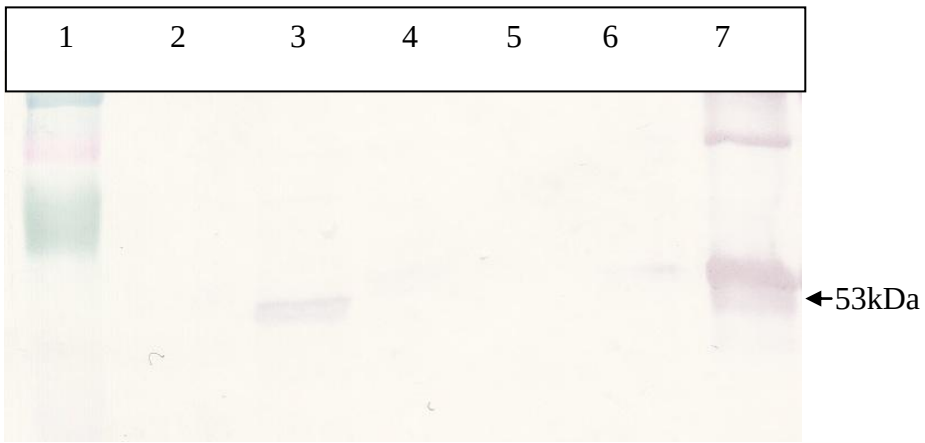


Figure 4.6: Western blot with polyclonal antiserum against Pd-B and bacterial lysates

Lanes: marker (1) and bacterial lysates of *S. aureus* (2) and *S. pneumoniae* (3). Lane 3 demonstrates a single band of polyclonal antiserum reacting with Ply of *S. pneumoniae*. Lane 7 shows a banding pattern against recombinant pneumolysin (1:10 dilution of Pd-B compared to **Figure 4.7**) signalling non-specific reactivity.

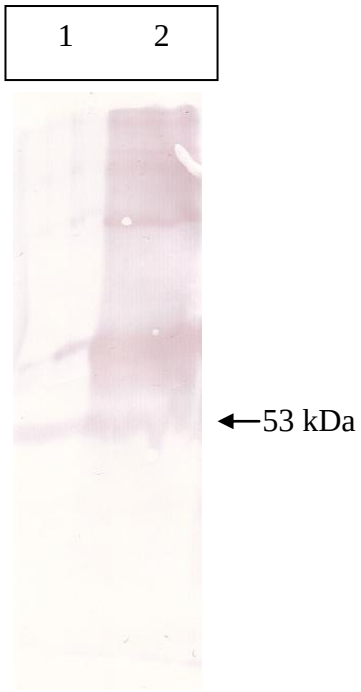


Figure 4.7: Western blot comparison of polyclonal antiserum against Pd-B and lysate

Lane 1: pneumolysin in crude pneumococcal lysate, lane 2: recombinant pneumolysin. A 53 kDa band was detected with the polyclonal antiserum in both lanes. However, more non-specific bands are clearly visible in lane 2.

4.3.3. ELISA optimisation

The first step matrix experiment demonstrated that the blocking step either with assay buffer or x4 assay buffer resulted in similar signal to noise ratios. It was decided to continue to use x1 assay buffer in the diagnostic assay. In the assay using bacterial suspensions as well as lysates, absorbance signals between >1 and <2 were obtained using 1:10,000 (0.15µg/mL) of PLY-7 as capture antibody; these absorbance signals were within the optimal dynamic assay range.

The absorbance readings against *S. aureus* lysates were at the same level as the "background" absorbance of 0.2 or slightly above. However, a signal well above the "background" signal, but below an absorbance value of 1 was observed with *S. aureus* suspensions. This finding was attributed to the protein A affinity purification of the polyclonal antibody. Protein A is a staphylococcal cell wall protein binding to the Fc portion of immunoglobulins, particularly IgG. In *S. aureus* suspension the bacterial cell wall remains intact and protein A is an exposed bacterial surface protein accessible to and therefore expected to bind to the polyclonal antibody purified by protein A. The finding was confirmed

by demonstrating an absorbance signal of 0.5 when testing a 1:4 dilution of Protein A Ceramic Hyper DF (Affinity Chromatography Sorbent, Bioserpa, Life Technologies, France) on a plate coated with 1:5,000 and 1:10,000 PLY-7 and 1:2,000 polyclonal antiserum as the second antibody. The absorbance signal data obtained in the first step of the optimisation using lysate dilutions of *S. pneumoniae* and *S. aureus* are graphed in **Figure 4.8**.

In the second step of the matrix experiment 1:10,000 PLY-7 in PBS was used as capture antibody as well as 2.5µg/well bacterial suspension and lysate, respectively, and polyclonal antiserum was diluted in doubling dilutions starting from 0.75µg/mL as outlined in **Figure 4.2**. Again, *S. aureus* lysate served as the negative control and had an absorbance signal of less than 0.2. The absorbance signal data obtained in the second step of the optimisation are graphed in **Figure 4.9.**

Figure 4.10 demonstrates the signal curve obtained for Pd-B against PLY-7 as the capture antibody. The range of the standard curve from 1:40 to 1: 5,120 to be included in each diagnostic test run was based on this evaluation.

To evaluate the ability of soluble antigen to compete with the bound antigen for the antibody, a competitive assay was set up with pneumococcal lysate coated on the microtiter plate at 1µg/mL in guanidine HCl. The plate was incubated with a dilution series of (1µg/mL in doubling dilutions down to 1:64) of 50µg/well pneumococcal lysate, Pd-B and *S. aureus* lysate (negative control) and immediate addition of 50µg/well 1:2,000 polyclonal antiserum and 1:10,000 monoclonal antiserum. The anti-rabbit and anti-mouse antisera were applied at a 1:2,000 dilution. The chromogenic signal indicated successful competition, although with a marginal curve, with pneumococcal lysate and polyclonal antisera binding. As expected there was no competition observed with the *S. aureus* lysate and the polyclonal antiserum. Interestingly, soluble Pd-B did not achieve competition with the bound antigen on the plate and no absorbance signal was detectable across all soluble antigens with the monoclonal antiserum. The weak signal curve produced with soluble pneumococcal lysate and polyclonal antiserum and the expected result for the negative control are graphed in **Figure 4.11**.

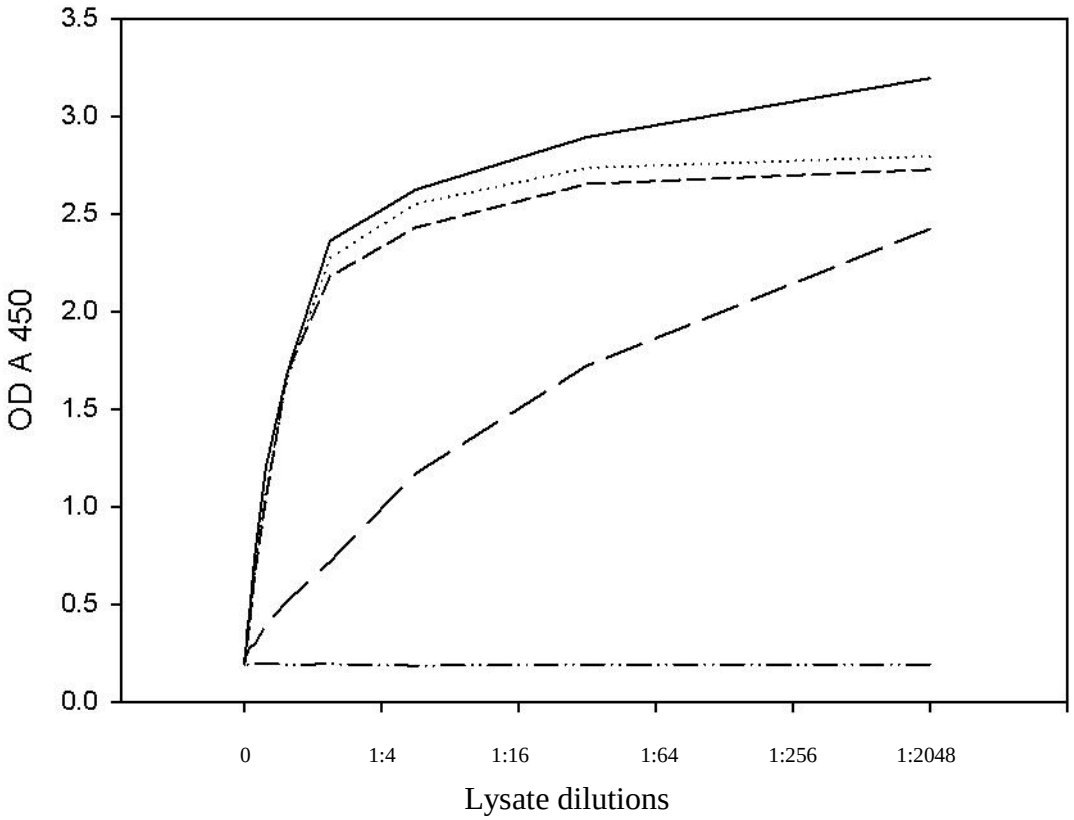


Figure 4.8: Optimisation of the capture antibody of the Ply ELISA

Four different capture antibody concentrations were evaluated: 3µg/mL (straight line), 1.5µg/mL (dotted line), 0.3µg/mL (small dashed line), 0.15µg/mL (large dashed line) on doubling dilutions of pneumococcal lysate. *S. aureus* lysate (double dot dash line) was included as a negative control.

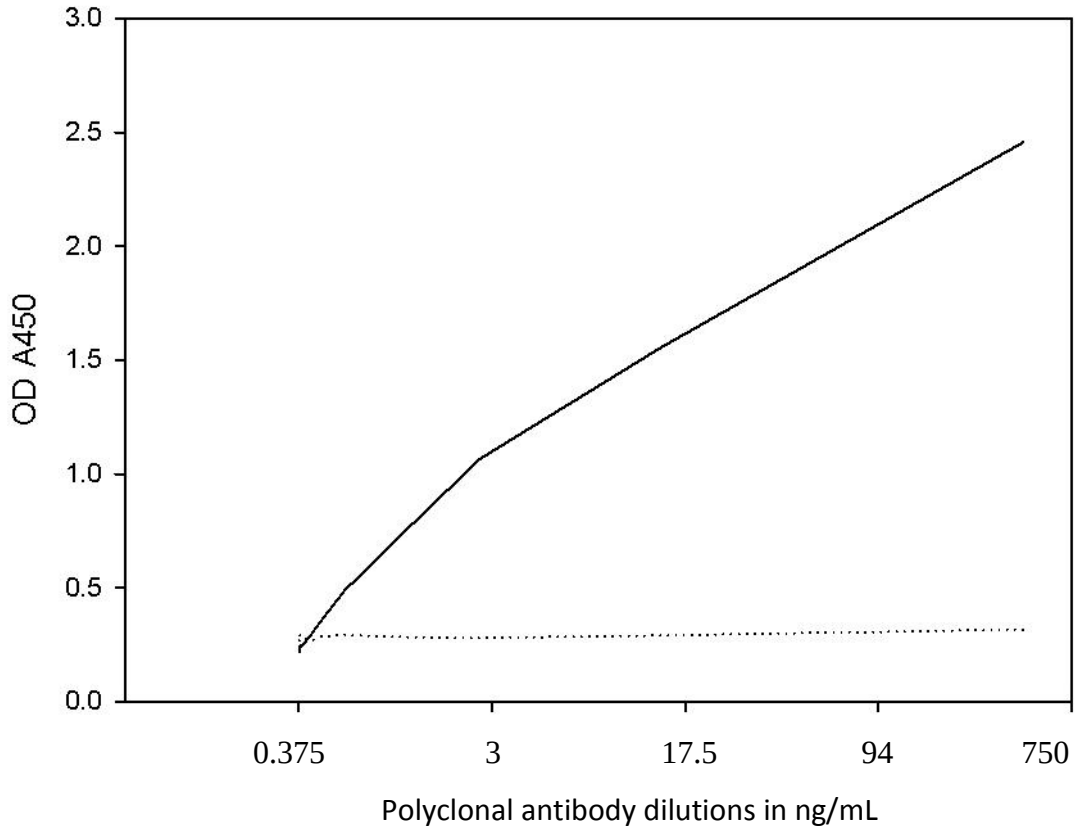


Figure 4.9: Optimisation of the 2nd antibody (polyclonal) of the Ply ELISA

The ELISA plate was coated with PLY-7 at 0.15µg/mL, pneumococcal and *S. aureus* lysates were used at 0.6µg/mL and the polyclonal antibody was diluted out from 1:2,000 (750ng/mL) to 1: 4,096,000 (0.375ng/mL). As expected there was no signal curve for *S. aureus*.

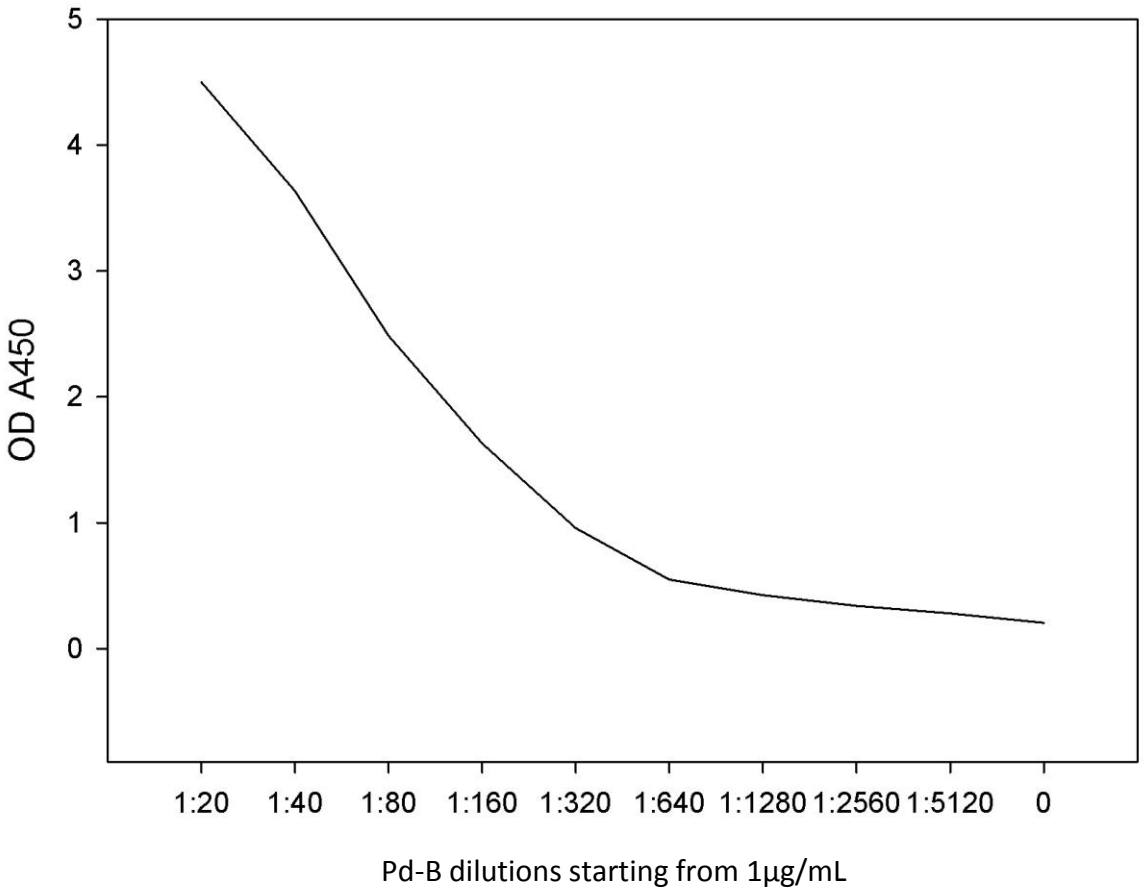


Figure 4.10: Doubling dilution series of Pd-B against PLY-7 as the capture and polyclonal as the 2nd antibody

The Pd-B dilution series was included as a quantitative standard in subsequent ELISA assays.

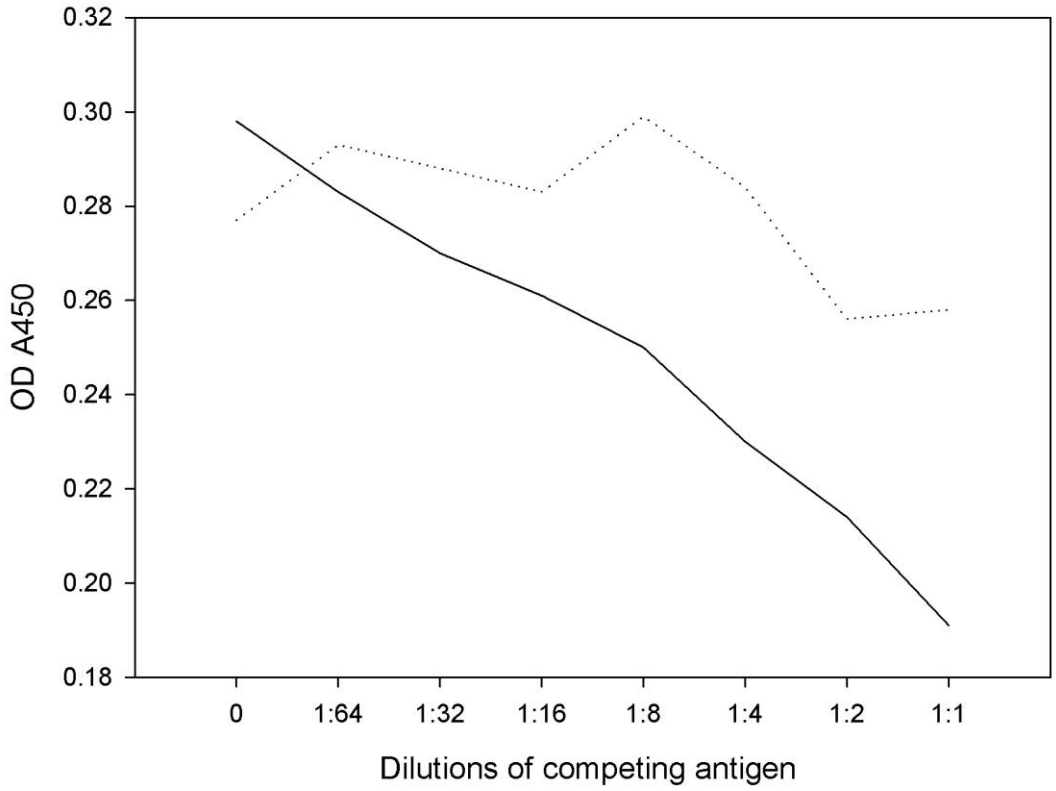


Figure 4.11: Competition of soluble antigen with bound pneumococcal lysate for binding to polyclonal antiserum

Solid line: pneumococcal lysate; dotted line: staphylococcal lysate (negative control). Weak signal curve with competition using polyclonal antiserum. No signals were obtained when using monoclonal antiserum.

When the polyclonal antiserum was used as capture antibody the concentration of 1:1,000 gave superior signal intensity to 1:2,000. The blocking step resulted in comparable signals when the plate was blocked with assay buffer or x4 assay buffer. The signals when using 1:1,000 or 1:2,000 dilutions of the second antibody, PLY-7, resulted in comparable signals. The dilution 1:1,000 was chosen in subsequent experiments.

4.3.4. Spiked serum and plasma preparations

Due to the limited volumes of patient serum samples available from the 1999/2000 CAP study, plasma was evaluated as a suitable alternative to serum. Both pneumococcal suspension and lysate were used to spike the serum and plasma samples at a dilution of 1:10

at 0.5 McFarland (approximately 10^8cfu/mL). All samples were tested undiluted in the ELISA. Contrary to the finding in section 7.3.3, describing the performance of different blood specimens in the PsaA ELISA assay, where samples spiked with pneumococcal suspension were non-reactive in comparison to lysate, in the pneumolysin assay plasma and serum samples spiked with either suspension or lysate produced comparable signal curves in the ELISA using PLY-7 monoclonal antibody as capture antibody (**Figure 4.12**).

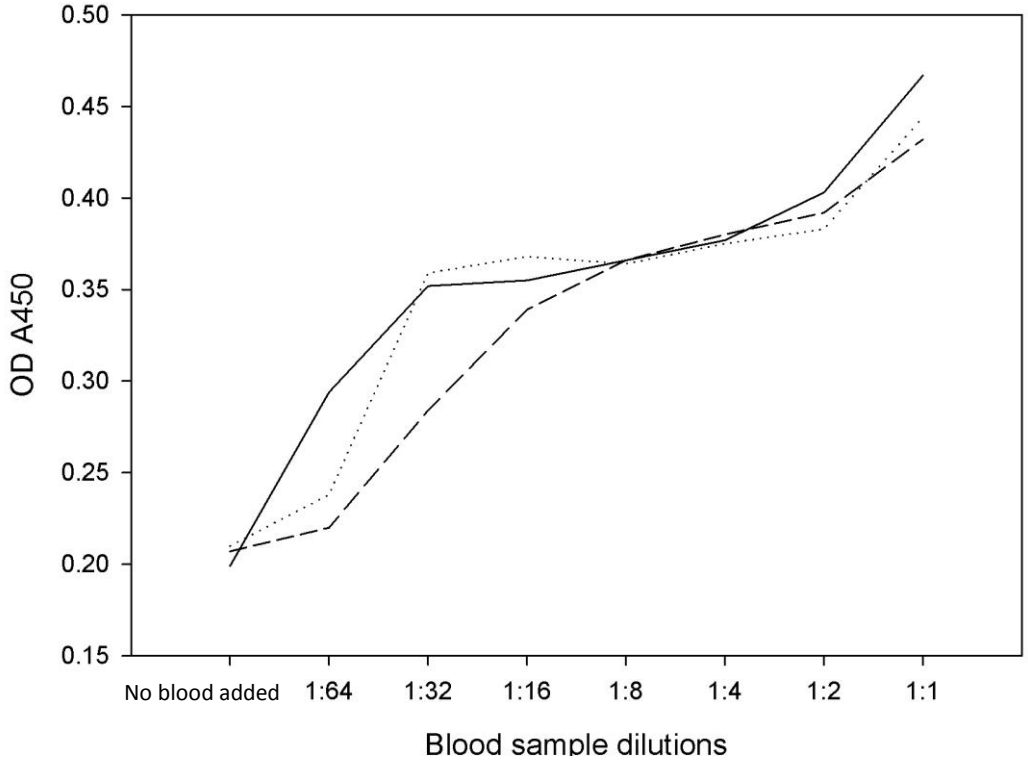


Figure 4.12: Comparison of assay performance of different specimens

Three different specimen types, serum (solid), Lithium-Heparin plasma (dotted) and EDTA plasma (dashed), were compared in an ELISA against PLY-7 as capture antibody. The blood samples had been spiked with pneumococcal lysate in this experiment.

The spiked serum and plasma (EDTA and Lithium-Heparin) samples that gave off a signal with PLY-7 as capture antibody (**Figure 4.12**) produced signals below the limit of detection in an ELISA with polyclonal antibody as capture antibody (1.5µg/mL). The two serum samples of healthy subjects spiked with pneumococcal lysate at 7.5µg/well also read at absorbance signal values below the limit of detection.

4.3.5. Assay precision

The intra-assay coefficient of variation averaged 4.6% and the inter-assay coefficient of variation averaged 8.2%. The values are summarised in **Table 4.1**. It is noteworthy that the serum and plasma samples were tested in a 1:2 dilution due to limited available volume of samples. The serum from one healthy subject, presumed to be free of pneumococcal antigens, produced a very similar absorbance signal to the other samples at 1:2. The low signal in the spiked blood samples was attributed to the low quantity of Ply in the samples. However, the negative serum gave a high absorbance signal reading when compared to the zero values. Blood from a further three presumed healthy individuals was taken and analysed in the ELISA with PLY-7 capturing antigen. All three produced signals indicating the presence of Ply. Against the standard curve of concentrations of Pd-B serum #1 had a high signal equating to >25ng/mL Ply, serum #2 read at 17ng/mL and serum #3 at 5ng/mL. Although the assay precision experiment was aimed at assessing the assays's reproducibility across an ELISA plate and at different time points it also demonstrated an issue of non-specific binding by showing the high absorbance values in the negative serum. These results suggested an interference produced by serum specimens with the PLY-7 capture antibody.

Table 4.1: Precision data for the performance of the Ply ELISA

	EDTA plasma (PL[1]spiked) OD A450[2]	**Lithium-Heparin Plasma (PL spiked) OD A 450**	**Serum (PL spiked) OD A 450**	**Negative serum OD A 450**	**Zero value OD A450**
Intra-assay	n=18	n=18	n=18	n=10	n=10
Mean	0.256	0.251	0.269	0.259	0.172
SD[3]	0.009	0.011	0.018	0.008	0.008
CV (%)[4]	3.5	4.3	6.9	3.3	5
Inter-assay	n= 29	n= 29	n= 29	n= 29	n=29
Mean	0.269	0.272	0.288	0.279	0.189
SD	0.020	0.017	0.019	0.029	0.017
CV (%)	7.3	6.5	7.2	10.5	9.5

[1]PL: Pneumococcal lysate

[2]OD A450: Absorbance signal read as optical density at 450nm

[3]SD: Standard deviation

[4]CV: Coefficient of variation

4.3.6. Lowest limit of detection

The lowest limit of detection (LLD) that is statistically robust was calculated by adding 2 standard deviations (2 x 0.017) to the mean. The resulting absorbance signal representing the LLD was 0.226. The extrapolated value for the Ply concentration representing the LLD was 680pg/mL. However, the serum that was included as being negative for pneumococcal disease (obtained from a healthy subject) showed a mean absorbance signal value of 0.279, well above the defined LLD.

4.3.7. Biological limit of detection

BLD is thought to represent more appropriately the limit of detection in a real sample. The calculated BLD absorbance value was 0.226 plus 3 times the standard deviation of the spiked serum sample values obtained for inter-assay precision. Three instead of 2 standard deviations was chosen to account for the fact that the presumed “negative” serum had a higher

inter-assay variability. The resulting absorbance signal value for BLD was 0.283, with an extrapolated pneumolysin concentration of 1.4ng/mL.

4.3.8. Investigation of serum interference

Sera from healthy subjects showed a significant reduction of signal after pronase treatment, to levels below the limit of detection, but not after heat treatment. Pd-B also showed a reduction of signal to a level below the limit of detection after pronase treatment and a reduction to a signal representing 1.75ng/mL after heat treatment. Although Pronase appears useful to reduce serum interference, it also appears to interact with the antigenic epitope. The different absorbance signals are listed in **Table 4.2**.

The Ouchterlony passive immunodiffusion experiment revealed no precipitation between the polyclonal antiserum and any of the antigens. It is possible that the negative result was influenced by a suboptimal proportion of antibody to antigen.

When assessing the specificity of the polyclonal antiserum against serum spiked, serum unspiked, Pd-B and pneumococcal lysate, a 53kDa band could be demonstrated with Pd-B and pneumococcal lysate in assay buffer, but there was no reaction (other than diffuse background staining) in any of the serum samples (spiked or not spiked with pneumococcal lysate). The result is shown in **Figure 4.13**.

Table 4.2: Absorbance signals of sera and recombinant pneumolysin after pronase and heat treatment

Two healthy subjects (S1and S2) with high absorbance signals in the Ply ELISA using PLY-7 as capture antibody. Both sera underwent treatment with pronase and heat to assess if a reduction of the absorbance signal was possible. Pd-B in assay buffer was included as a control for the effect of the treatments on the protein.

	S1 serum (OD A450)	**S2 serum (OD A450)**	**Pd-B (1:80 in assay buffer) (OD A 450)**
Undiluted	0.652	0.966	1.710
Diluted at 1:2	0.731	0.959	1.241
Pronase treated	0.219	0.198	0.208
Heat treated at 56°C	1.017	1.294	0.567

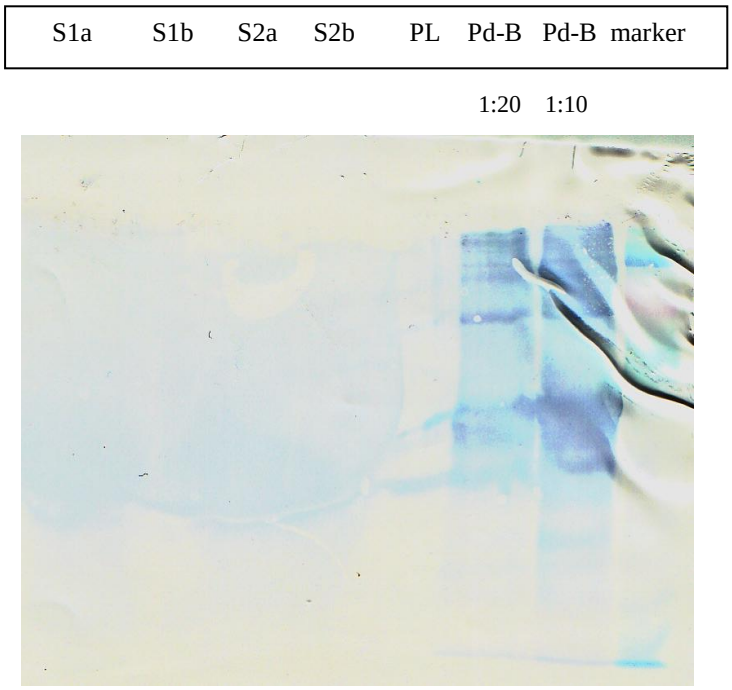


Figure 4.13: Western blot demonstrating specificity of polyclonal antiserum

The polyclonal antiserum was tested at 1:1,000 dilution against the serum of two healthy subjects (S1 and S2) either unspiked (a) or spiked with pneumococcal lysate (b). No bands were visible in the lanes with the serum specimens, but the polyclonal antiserum reacted with pneumococcal lysate (PL) and Pd-B at dilutions 1:10 and 1:20. Marker in lane furthest to the right.

4.3.9. Ply antigen detection in urine

All urines that were tested in the developed Ply antigen detection assay were negative.

4.4. Discussion

The assay described in this chapter was developed for the detection of Ply in well-characterised patient serum samples [293]. The intention was to develop a diagnostic assay that would have a superior sensitivity compared to the current "gold standard" for IPD, blood cultures. The assay was set up similarly to the assay published by Cima-Cabal [260, 340]. The differences in assays were (i) replacing chemiluminescence with a chromogenic detection system and (ii) using a different polyclonal antiserum. The decision not to use chemiluminescence as the detection system was based on the inability to optically cross-check

results in chemiluminescence-based signal detection. A lower sensitivity of the chromogenic assay was anticipated and accepted as a trade off. The polyclonal antibody raised in rabbits for this study posed a problem that led to two different set up formats of the sandwich ELISA. The first design included the PLY-7 monoclonal as the capture antibody and the polyclonal as the second antibody. Due to interference of human serum with the polyclonal the antibodies were switched to using the polyclonal as the capture antibody.

The monoclonal antibody used by Cima-Cabal was, as in this assay, PLY-7, an IgG1 (κ) isotype which had previously been epitope mapped [323]. PLY-7 had been found not to cross-react with *S. suis*, *S. pyogenes*, *L. monocytogenes*, *L. ivanovii* or *C. perfringens*, all producers of a cytotoxin similar to Ply. Cross reactivity to *S. salivarius*, *S. aureus*, *E. coli*, clinical isolates of the viridians streptopcoccal group, *S. agalactiae*, *H. influenzae* and *N. meningitidis* had not observed. *S. mitis* and *S. sanguis* were weakly cross-reactive in the chemiluminescent sandwich ELISA assay [340].

The publication by del Mar García-Suárez *et al.* evaluated the assay on human urine samples, not serum. Since then, there has not been a publication which has evaluated the assay on human serum samples. However, their assay's performance on human urine was compared subsequently with the Binax NOW *S. pneumoniae* urinary antigen test and proved to have lesser sensitivity than the Binax Now assay in adults, but showed potential as a complementary method for diagnosis of pneumococcal infections in children [348]. Although urine is easier to obtain than serum it may not signal invasive disease. It is noteworthy that the Binax NOW *S. pneumoniae* urinary antigen test has been questioned with regards to its clinical usefulness in a publication in 2010. Its implementation showed no substantial benefit economically or with regards to patient outcome in hospitalised patients with CAP [259].

The assay development was complicated by the finding of an unexplained interference when testing serum specimens with the monoclonal PLY-7 as capture antibody, whilst there was no detectable interference when the assay was evaluated on recombinant and native Ply in assay buffer. It is likely that one or more non-specific components of the sera caused non-specific binding to the antibody. An attempt was made to eliminate serum interference with methods known to be effective. En enzymatic method using pronase and heat treatment at 56°C were used. Heat treatment proved unsuccessful and pronase treatment reduced non-specific absorbance signals, but also adversely affected the detection of pneumolysin, eliminating pronase as a potential solution to the interference problem. Capture antibodies were switched from PLY-7 to polyclonal antiserum. This resulted in satisfactory Ply detection in lysates in assay buffer, however, no detection of Ply in serum in samples spiked with known quantities of Ply. The polyclonal antibody was the most likely variable responsible for

the erroneous results and the antibody specificity was investigated by Western Blot. Sera from two healthy subjects were run on a Western blot, spiked with pneumococcal lysate and with no pneumococcal lysate added, together with Pd-B and pneumococcal lysate in assay buffer and incubated with 1:1,000 polyclonal antibody, a 53kDa band was detected with Pd-B and pneumococcal lysate in assay buffer, but there was no reaction in any of the serum samples, either with or without added pneumococcal lysate. This finding, together with the fact that Pd-B was prepared from *Escherichia coli* harbouring a plasmid [341] suggests that blocking of potential receptor sites in serum is taking place. The Pd-B production in *E. coli* has most likely resulted in an antigen that should have been purified prior to antisera production in order to decrease potential for non-specific binding sites to other proteins. The inadaequate purification is demonstrated by the reaction of Pd-B against the polyclonal antibody showing multiple bands in Western blot. The polyclonal epitope which recognises Ply is prevented from specifically binding to its antigen receptor.

The effect of this interference or non-detection could not be corrected by either heat deactivation or pronase treatment of serum. It was decided that this assay was unsuitable for diagnostic purposes on serum samples, it could however, prove useful on other human specimen types, such as plasma, urine, CSF or respiratory specimens.

Of note, a lack of competition was observed with soluble Pd-B and the bound antigen on the plate and no absorbance signal was detectable across all soluble antigens with the monoclonal antiserum. The results were reproducible and an explanation for this was not found. It can only be stated that under the assay conditions described here no competition was observed when using monoclonal antiserum. If all monoclonal antibody would have been bound to soluble antigen and would have been unavailable for binding, there should still have been a signal curve allowing binding to bound antigen in the wells at the lowest concentration of soluble antigen. However, this was not the case and the observation remained unexplained. This is an *in vitro* experiment that is providing artificial conditions. It remains undetermined as to whether the same result would apply *in vivo*.

Three options remained to further develop this assay. One was to develop another polyclonal antiserum raised against purified Ply (recombinant or native) or option two, to obtain the polyclonal antiserum raised by the Spanish group [340], thirdly, the assay could be further evaluated on CSF (representing another invasive specimen), EDTA or Lithium-Heparin plasma and urine specimens. With regards to urine, preliminary data on a very small sample size (section 4.3.9) indicated that the sensitivity would be less than with the Binax NOW *S. pneumoniae* urinary antigen test, which is in line with the findings of del Mar García-Suárez *et al.*. Also, it is noteworthy that the presence of the *ply* gene in urine in the

studied CAP 1999/2000 patients was 1.4% in the 145 urines that underwent *ply* PCR and were used in this analysis (section 9.3.1) and 2% in the publication by Murdoch *et al.* [267]. The preliminary results on urines in the Ply antigen detection test supported the decision to not pursue further development of the Ply antigen detection assay, but instead investigate an alternative antigen target. An antigen detection assay for pneumococcal surface adhesin A (PsaA) was developed (Chapters 5-7).

CHAPTER 5

VERIFICATION OF A PNEUMOCOCCAL SURFACE ADHESIN A PLASMID

5.1. Introduction

Pneumococcal surface adhesin A (PsaA) is a metal-binding lipoprotein with 309 amino acid residues located in the pneumococcal cell wall and forms part of an ABC-type manganese complex [349]. It is thought to be expressed by all pneumococcal serotypes [350]. PsaA belongs to the conserved group of surface and subsurface proteins which act as pneumococcal virulence factors outside the pneumococcal capsule [351]. These proteins facilitate the adherence and integration of the pneumococcus into the host's epithelial cells of the nasopharynx. Sequence homology between PsaA of analysed pneumococcal serotypes is high [350, 352] making it a suitable target in pneumococcal diagnostic test development. The *psaA* gene has been detected in all pneumococcal serotypes with high specificity [95] and gene mutations have effects on pneumococcal virulence [353]. PsaA has been described in the literature as a possible vaccine candidate target and a potential target in the diagnosis of IPD [354].

The protein was first reported when it was identified as an antigen that cross-reacted with a monoclonal antibody raised against a non-encapsulated strain of *S. pneumoniae* [355]. Later, further characterisation revealed a high degree of genetic similarity to other streptococcal proteins, SsaB of *S. sanguis*, FimA of *S. parasanguis* and ScaA of *S. gordonii*, all associated with bacterial adhesion [356] and the possibility of horizontal gene transfer of *psaA* to other related streptococcal species was demonstrated [59].

On the bacterial surface, the protein attaches directly to the lipid of the cytoplasmic membrane and forms a covalent bond between the Cys residue and the diacylglyceryl of the lipid bilayer. Once attachment has taken place, PsaA's signal peptide is cleaved resulting in a variability of N-terminal sequences. This variability might be the cause of a variety of 3-D structures of PsaA, which could potentially explain its dual functionality as an adhesin and a metal ion transport protein [357].

A recombinant plasmid was constructed by Prof. James C. Paton, School of Molecular and Biomedical Science, University of Adelaide, Australia. A commercial product was unavailable. The plasmid contained a fragment of *psaA* of approximately 830 bp cloned into the BamHI/KpnI site of pQE30 vector (Qiagen Inc., Valencia, CA, USA) with ampicillin resistance. This chapter describes work completed to confirm the presence of *psaA* in the plasmid and the subsequent expression of the plasmid construct. Since the plasmid formed the

basis of the production of a protein to which specific antibodies were to be raised it was essential to verify the accuracy of the plasmid.

5.2. Material and Methods

5.2.1. Materials

5.2.1.1. Ampicillin stock solution

Stock solution of ampicillin was made up to the concentration of 125mg/mL. The solution was run through a sterile filter and stored at -80°C.

5.2.1.2. Lauria Bertani (LB) agar

1% (w/v)	tryptone
0.5% (w/v)	yeast extract
0.2M	NaCl
1.5% (w/v)	agar

Adjust to pH 7.0. The solution was autoclaved. Antimicrobials were added aseptically as required once the autoclaved solution had cooled to approximately 55°C.

5.2.1.3. Lauria Bertani (LB) media

1% (w/v)	tryptone
0.5% (w/v)	yeast extract
0.2M	sodium chloride

Adjust to pH 7.0. The solution was autoclaved and amoxicillin stock (above) solution was added aseptically to a final concentration of 100µg/mL when the autoclaved solution had cooled to approximately 55°C.

5.2.1.4. SOC media

2% (w/v)	tryptone
0.5% (w/v)	yeast extract
10mM	NaCl
2.5mM	KCl
10mM	$MgCl_2$
10mM	$MgSO_4$
20mM	glucose

5.2.1.5. Isopropylthio-β-D-galactoside (IPTG)

100mM IPTG (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia) stock solution was made up. Final concentration of 1mM IPTG was used in the method.

5.2.1.6. IMAC buffers

Binding buffer:

5mM	imidazole
50mM	NaCl
20mM	Tris

Adjust to pH 7.9.

Wash buffer:

60mM	imidazole
50mM	NaCl
20mM	Tris

Adjust to pH 7.9.

Elution buffer:

500mM	imidazole

50mM	NaCl
20mM	Tris

Adjust to pH 7.9.

5.2.2. Methods

5.2.2.1. Preparation of samples for the SDS PAGE

The recombinant PsaA obtained from Prof. Paton (section 5.1) was mixed with sample buffer (reducing or non-reducing) at a 1:5 dilution. The samples were incubated at 100°C for 15-20 min prior to loading.

5.2.2.2. Gel electrophoresis

The samples and protein standards (Bio-Rad Laboratories, Hercules, CA, USA; Sigma-Aldrich, St. Louis, MO, USA) were loaded into the gel. The gel was run at 55V, 17mA (14V/cm) for 10 min, then the current was increased to 165V, 30-40mA (20V/cm). Proteins were visualised by gentle agitation in Coomassie Blue stain for approximately 30-60 min. The gel was destained overnight in destaining solution. Gels were dried in the Bio-Rad GelAir drying system (Bio-Rad Laboratories, Hercules, CA, USA).

5.2.2.3. QIAprep for purification of plasmid DNA

The QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA, USA) was used according to manufacturer's instructions to purify high-copy plasmid DNA from 1-5mL overnight cultures of *E.coli* in LB medium.

5.2.2.4. Sequencing of *psaA* plasmid

DNA sequencing was performed on purified DNA in order to confirm the accurate nucleotide sequence of the *psaA* plasmid. Oligonucleotide primers used had the following nucleotide sequence: pQ30-f (5'-TGAGCGGATAACAATTATAATAGATT-3') and pQ30-r (5'-ATGGAGTTCTGAGGTCATTACT-3').

The primer melting temperature was established by using the following formula: (A+T) x2 + (G+C) x4 = Tm (primer melting temperature). The primers were used at a final concentration of 100µM (custom primers, Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia).

PCR pre-mix (for one reaction):

10% (v/v)	buffer with Mg^{2+}
10mmol	dNTPs
50µmol	pQ30-f
50µmol	pQ30-r
0.5% (v/v)	Fast-start enzyme at 5u/µL

10µL purified plasmid DNA was added to 40µL of PCR pre-mix.

Cycling conditions:

Cycling was performed on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) with initial denaturation at 94°C for 6 min, 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min elongation at 72°C, then a final elongation step of 72°C for 5 min, followed by holding at 4°C for 5 min.

Sequencing reaction:

10% (v/v)	purified PCR product
5µmol	primer
5% (v/v)	Big Dye Terminator
20%	sequencing buffer

5.2.2.5. Agarose gel electrophoresis

PCR products were analysed using agarose gel electrophoresis to visualise DNA bands of a specific molecular weight.

The agarose solution was poured into a casting tray and a comb inserted at the anode (positive) end. Once the comb was removed the gel was submerged in 0.5x TBE buffer in the electrophoresis tank. Approximately 8µL of each sample was mixed with 2µL loading buffer

and pipetted into the gel wells. SeeBlue® Pre-Stained Standard (Life Technologies Inc., Gaithersburg, MD, USA) was included in each electrophoresis. The samples were electrophoresed at 10-20 V/cm and the bands visualised on the Gel-Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA).

5.2.2.6. Cloning and expression of *psaA* plasmid

Cloning and expression of the *psaA* plasmid was approved by the Institutional Biological Safety Committee in accordance with the Hazardous Substances and New Organisms (HSNO) Act (Application GMO05/UO011).

The ChampionTM pET Directional TOPO® Expression kit (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia) was used according to the manufacturer's instructions.

The kit uses a strategy to directionally clone a blunt-end PCR product into a vector for high-level expression in *E.coli*. The *psaA* PCR product was cloned into the pET TOPO® vector and then transformed into One Shot® TOP10 *E.coli*. One Shot® TOP10 are chemically competent *E.coli* supplied in the kit. Up to 5 colonies of transformants were analysed via sequencing using the T7 forward and reverse primer included in the kit. Once the correct insert had been confirmed by sequence analysis the clone was frozen at -80°C in glycerol stock solution (0.85ml of bacterial culture in LB broth plus 0.15mL sterile glycerol) for long-term storage.

After purification with Pure LinkTM HQ Mini Plasmid Purification Kit (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia) the *psaA* plasmid DNA was transformed into BL21 Star™ (DE3) One Shot® *E.coli* supplied in the expression kit. The kit supplies a pET TOPO® vector containing the *lacZ* gene encoding β-galactosidase as a positive expression control. The vector also contains the ampicillin resistance gene needed for the selection of transformants. The BL21 Star™ (DE3) One Shot® *E.coli* strain is specifically designed for the expression of genes regulated by the T7 promoter.

Induction of protein expression with IPTG was initiated at 1mM per induction reaction. A non-induced *E. coli* culture was grown up in parallel. Aliquots were removed at times 0, 1 hour, 2 hours, 3 hours and 4 hours post incubation at 37°C. The protein expression was confirmed with gel electrophoresis using a 10% reducing SDS gel.

5.2.2.7. Immobilised metal affinity chromatography (IMAC)

Imidazole is a heterocyclic aromatic organic compound used in the purification of His-tagged proteins in IMAC. By competition, imidazole elutes tagged proteins which are bound to Ni ions, attached to the surface beads in the chromatography column. Excess imidazole passes through the column and displaces the His-tag from nickel co-ordination, hence freeing his-tagged proteins.

The stored bacterial cell pellets were thawed and 200mL re-pelleted and resuspended in 40mL of ice-cold imidazole binding buffer. The bacterial cells were lysed by sonication for 2 min over 3 cycles with 1 min intervals between cycles to cool the samples, and subsequently they were subjected to 3 freeze-thaw cycles. The soluble protein fraction was collected by centrifugation at 8,000 x g for 30 min (4°C). 20mL of extract was loaded onto a 2mL packed resin Ni column, Ni-NTA Purification System (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia). Imidazole was included in the wash and elution buffers to minimise the binding of untagged, contaminating proteins. The column was washed 5 times with wash buffer and the supernatant was saved for SDS-PAGE analysis prior to the washing and after each wash step. Finally, elution buffer was added and the fraction that passed through the column was collected and saved for SDS-PAGE analysis. The fractions were stable at 4°C or at -20°C after adding glycerol for long-term storage.

5.3. Results

5.3.1. Designing primers for the amplification of *psaA*

The recombinant *psaA* plasmid arrived as a 1030 bp fragment cloned into the BamHI/KpnI site of pQE-30 vector (Qiagen Inc., Valencia, CA, USA), which included an ampicillin resistance gene. The vector size was 3,461 bp. The vector has coding sequences for a β-lactamase, a 6xHis-tag, a T5 promoter/lac operator element, a T5 transcription start, and lambda t0 and rrnB T1 transcriptional termination regions (**Figure 5.1**).

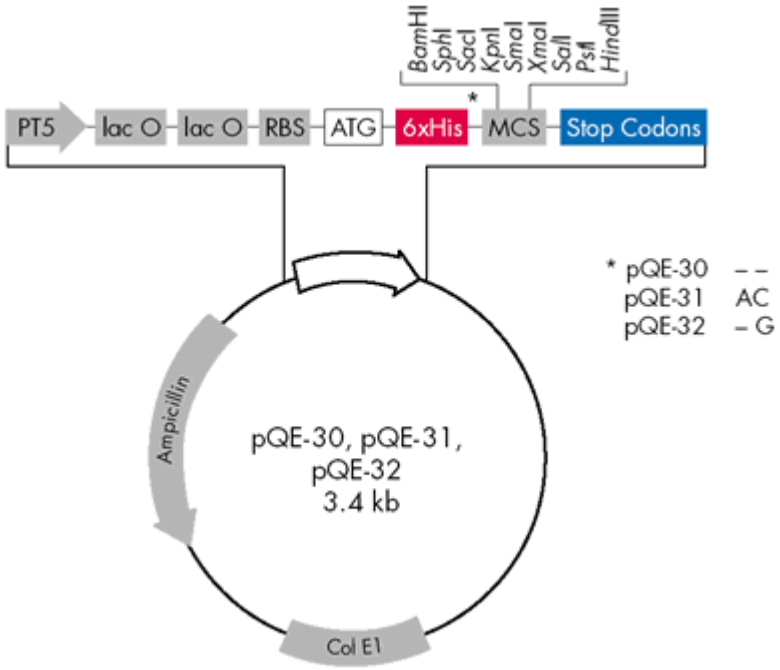


Figure 5.1: pQE-30, pQE-31, pQE-32 vectors: positions of elements

PT5: T5 promoter; lac O: lac operator; RBS: ribosome –binding site; ATG: start codon; 6xHis: 6xHis tag sequence; FXa Recognition Site: Factor Xa Protease recognition site; MCS: Multiple cloning site; Stop Codons: In all 3 reading frames; Col E1: Col E1 origin of replication; Ampicillin: Ampicillin resistance gene (Adapted from http://www1.qiagen.com).

The DNA sequence in **Figure 5.2** depicts the flanking parts of the pQE-30 vector with the inserted *psaA* sequence at the Bam HI/Kpn I restriction site. The sequences of the inner and outer sequencing primers are highlighted (green and blue, respectively) in the *psaA* sequence.

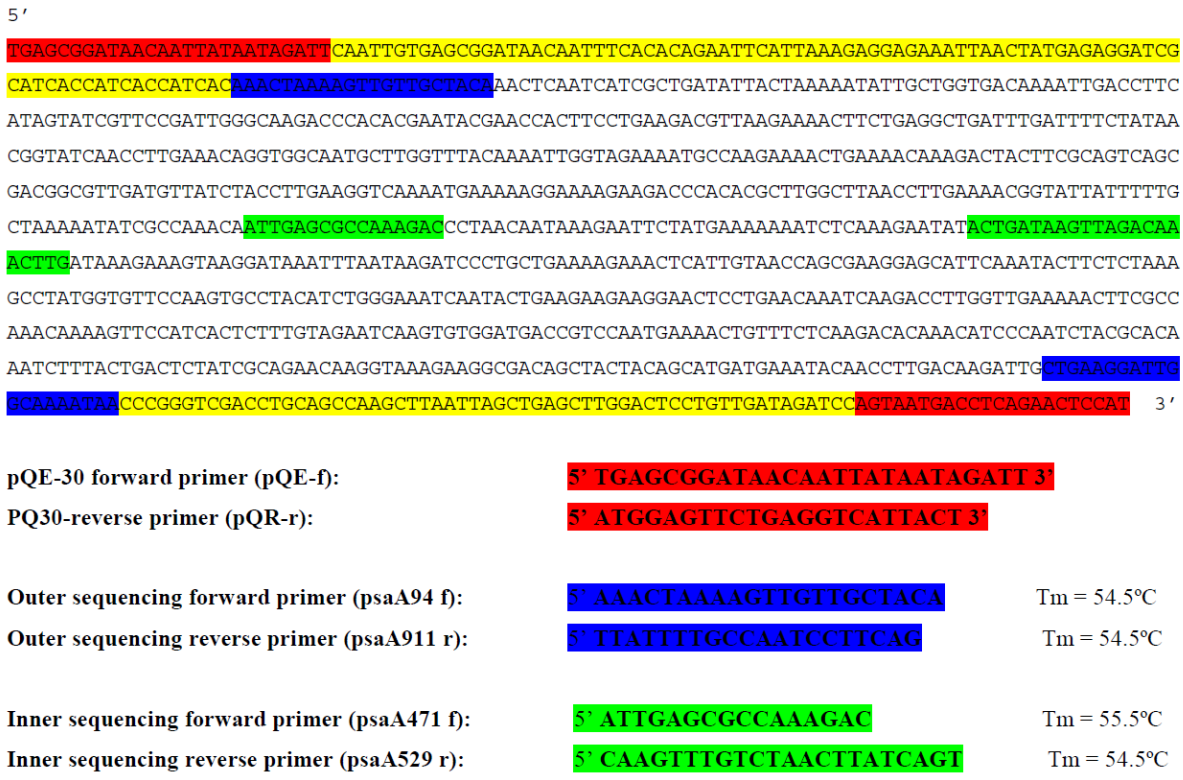


Figure 5.2: pQE-30 plus insert (*psaA*)

The red highlighted sequence marks the forward and reverse primers flanking the sequence belonging to pQE-30. The red plus yellow highlighted sequences are base pairs belonging to pQE-30 up to the cloning site for *psaA*. The recognition site for Bam HI / Kpn I is 5’…GGATC$^{\downarrow}$C…3’ / 3’…C$_{\uparrow}$CTAGG…5’.

The blunt-end PCR product was cloned into pET-TOPO® vector using the pQE-30 forward and reverse primers, and then transformed into One Shot® TOP10 *E.coli*. Transformants 2, 4, 5, 6, 7, 8 were amplified with inner and outer sequencing primers and the PCR products purified prior to sequencing. AccuPrime™ Pfx DNA polymerase (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia) (containing an enzyme preparation with recombinant DNA polymerase from Thermococcus species strain KOD) was used to help ensure high fidelity amplification of the nucleic acid templates by PCR. Forward and reverse sequences were aligned for each PCR product, in order to confirm the correct DNA sequence. Due to the *psaA* gene spanning over 837 bp, two different primer pairs were necessary to cover the whole sequence. The primer pair *psaA*94-f and *psaA*529-r covered the first half of sequence analysis and primer pair *psaA*471-f and *psaA*911-r covered the second half of the psaA sequence with a small overlap between the two sequences. The sequences were aligned with the *psaA* sequence of *Streptococcus pneumoniae* strain R6, accession number AF248230

(GenBank nucleotide search, http://www.ncbi.nlm.nih.gov/). In **Figure 5.3** sequences of the analysed transformants have been aligned. The sequencing alignment confirmed the success of the One Shot cloning, and lead to one PCR product to be transformed into BL21 *E.coli* and subsequently expressed first in a pilot expression, then in a large scale expression.

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
5AWf_2005-                                                                 AAACT
5AWr_2005-                                                                 AAACT
7AWf_2005-                                                                 AAACT
7AWr_2005-                                                                 AAACT
PSA gene        ---------- ---------- ---------- ---------- ---------- -----AAACT
Consensus                                                                  AAACT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
5AWf_2005-      AAAAGTTGTT GCTACAAACT CAATCATCGC TGATATTACT AAAAATATTG CTGGTGACAA
5AWr_2005-      AAAAGTTGTT GCTACAAACT CAATCATCGC TGATATTACT AAAAATATTG CTGGTGACAA
7AWf_2005-      AAAAGTTGTT GCTACAAACT CAATCATCGC TGATATTACT AAAAATATTG CTGGTGACAA
7AWr_2005-      AAAAGTTGTT GCTACAAACT CAATCATCGC TGATATTACT AAAAATATTG CTGGTGACAA
PSA gene        AAAAGTTGTT GCTACAAACT CAATCATCGC TGATATTACT AAAAATATTG CTGGTGACAA
Consensus       AAAAGTTGTT GCTACAAACT CAATCATCGC TGATATTACT AAAAATATTG CTGGTGACAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
5AWf_2005-      AATTGACCTT CATAGTATCG TTCCGATTGG GCAAGACCCA CACGAATACG AACCACTTCC
5AWr_2005-      AATTGACCTT CATAGTATCG TTCCGATTGG GCAAGACCCA CACGAATACG AACCACTTCC
7AWf_2005-      AATTGACCTT CATAGTATCG TTCCGATTGG GCAAGACCCA CACGAATACG AACCACTTCC
7AWr_2005-      AATTGACCTT CATAGTATCG TTCCGATTGG GCAAGACCCA CACGAATACG AACCACTTCC
PSA gene        AATTGACCTT CATAGTATCG TTCCGATTGG GCAAGACCCA CACGAATACG AACCACTTCC
Consensus       AATTGACCTT CATAGTATCG TTCCGATTGG GCAAGACCCA CACGAATACG AACCACTTCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
5AWf_2005-      TGAAGACGTT AAGAAAACTT CTGAGGCTGA TTTGATTTTC TATAACGGTA TCAACCTTGA
5AWr_2005-      TGAAGACGTT AAGAAAACTT CTGAGGCTGA TTTGATTTTC TATAACGGTA TCAACCTTGA
7AWf_2005-      TGAAGACGTT AAGAAAACTT CTGAGGCTGA TTTGATTTTC TATAACGGTA TCAACCTTGA
7AWr_2005-      TGAAGACGTT AAGAAAACTT CTGAGGCTGA TTTGATTTTC TATAACGGTA TCAACCTTGA
PSA gene        TGAAGACGTT AAGAAAACTT CTGAGGCTGA TTTGATTTTC TATAACGGTA TCAACCTTGA
Consensus       TGAAGACGTT AAGAAAACTT CTGAGGCTGA TTTGATTTTC TATAACGGTA TCAACCTTGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355

5AWf_2005-      AACAGGTGGC AATGCTTGGT TTACAAAATT GGTAGAAAAT GCCAAGAAAA CTGAAAACAA
5AWr_2005-      AACAGGTGGC AATGCTTGGT TTACAAAATT GGTAGAAAAT GCCAAGAAAA CTGAAAACAA
7AWf_2005-      AACAGGTGGC AATGCTTGGT TTACAAAATT GGTAGAAAAT GCCAAGAAAA CTGAAAACAA
7AWr_2005-      AACAGGTGGC AATGCTTGGT TTACAAAATT GGTAGAAAAT GCCAAGAAAA CTGAAAACAA
PSA gene        AACAGGTGGC AATGCTTGGT TTACAAAATT GGTAGAAAAT GCCAAGAAAA CTGAAAACAA
Consensus       AACAGGTGGC AATGCTTGGT TTACAAAATT GGTAGAAAAT GCCAAGAAAA CTGAAAACAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
5AWf_2005-      AGACTACTTC GCAGTCAGCG ACGGCGTTGA TGTTATCTAC CTTGAAGGTC AAAATGAAAA
5AWr_2005-      AGACTACTTC GCAGTCAGCG ACGGCGTTGA TGTTATCTAC CTTGAAGGTC AAAATGAAAA
7AWf_2005-      AGACTACTTC GCAGTCAGCG ACGGCGTTGA TGTTATCTAC CTTGAAGGTC AAAATGAAAA
7AWr_2005-      AGACTACTTC GCAGTCAGCG ACGGCGTTGA TGTTATCTAC CTTGAAGGTC AAAATGAAAA
PSA gene        AGACTACTTC GCAGTCAGCG ACGGCGTTGA TGTTATCTAC CTTGAAGGTC AAAATGAAAA
Consensus       AGACTACTTC GCAGTCAGCG ACGGCGTTGA TGTTATCTAC CTTGAAGGTC AAAATGAAAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
5AWf_2005-      AGGAAAAGAA GACCCACACG CTTGGCTTAA CCTTGAAAAC GGTATTATTT TTGCTAAAAA
5AWr_2005-      AGGAAAAGAA GACCCACACG CTTGGCTTAA CCTTGAAAAC GGTATTATTT TTGCTAAAAA
7AWf_2005-      AGGAAAAGAA GACCCACACG CTTGGCTTAA CCTTGAAAAC GGTATTATTT TTGCTAAAAA
7AWr_2005-      AGGAAAAGAA GACCCACACG CTTGGCTTAA CCTTGAAAAC GGTATTATTT TTGCTAAAAA
PSA gene        AGGAAAAGAA GACCCACACG CTTGGCTTAA CCTTGAAAAC GGTATTATTT TTGCTAAAAA
Consensus       AGGAAAAGAA GACCCACACG CTTGGCTTAA CCTTGAAAAC GGTATTATTT TTGCTAAAAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
5AWf_2005-      TATCGCCAAA CAATTGAGCG CCAAAGACCC TAACAATAAA GAATTCTATG AAAAAAATCT
5AWr_2005-      TATCGCCAAA CAATTGAGCG CCAAAGACCC TAACAATAAA GAATTCTATG AAAAAAATCT
7AWf_2005-      TATCGCCAAA CAATTGAGCG CCAAAGACCC TAACAATAAA GAATTCTATG AAAAAAATCT
7AWr_2005-      TATCGCCAAA CAATTGAGCG CCAAAGACCC TAACAATAAA GAATTCTATG AAAAAAATCT
PSA gene        TATCGCCAAA CAATTGAGCG CCAAAGACCC TAACAATAAA GAATTCTATG AAAAAAATCT
Consensus       TATCGCCAAA CAATTGAGCG CCAAAGACCC TAACAATAAA GAATTCTATG AAAAAAATCT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
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5AWf_2005-   CAGAGAATAT ACTGATAAGT TAGGACAAAC TTG------- ---------- ----------
5AWr_2005-   C--------- ---------- ---------- ---------- ---------- ----------
7AWf_2005-   CAAAGAATAT ACTGATAAGT TA-GACAAAC TTG------- ---------- ----------
7AWr_2005-   CAAAG----- ---------- ---------- ---------- ---------- ----------
PSA gene     CAAAGAATAT ACTGATAAGT TA-GACAAAC TTGATAAAGA AAGTAAGGAT AAATTTAATA
Consensus    CARAGAATAT ACTGATAAGT TARGACAAAC TTGATAAAGA AAGTAAGGAT AAATTTAATA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
2AWf_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
2AWr_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
4AWf_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
4AWr_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
6AWf_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
6AWr_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
8AWf_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
8AWr_2005-          AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
PSA gene            AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT
Consensus           AAG AATATACTGA TAAGTTAGAC AAACTTGATA AAGAAAGTAA GGATAAATTT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
2AWf_2005-   AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
2AWr_2005-   AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
4AWf_2005-   AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
4AWr_2005-   AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
6AWf_2005-   AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
6AWr_2005-   AATAAGATCG CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
8AWf_2005-   AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
8AWr_2005-   AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
PSA gene     AATAAGATCC CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC
Consensus    AATAAGATCS CTGCTGAAAA GAAACTCATT GTAACCAGCG AAGGAGCATT CAAATACTTC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
2AWf_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
2AWr_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
4AWf_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
4AWr_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
6AWf_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
6AWr_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
8AWf_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
8AWr_2005-   TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
PSA gene     TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA
Consensus    TCTAAAGCCT ATGGTGTTCC AAGTGCCTAC ATCTGGGAAA TCAATACTGA AGAAGAAGGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
2AWf_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
2AWr_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
4AWf_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
4AWr_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTKCGCC AAACAAAAGT TCCATCACTC
6AWf_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
6AWr_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
8AWf_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
8AWr_2005-   ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
PSA gene     ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC
Consensus    ACTCCTGAAC AAATCAAGAC CTTGGTTGAA AAACTTCGCC AAACAAAAGT TCCATCACTC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
2AWf_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
2AWr_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
4AWf_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
4AWr_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAAKTG TTTCTCAAGA CACAAACATC
6AWf_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
6AWr_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
8AWf_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
8AWr_2005-   TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
PSA gene     TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC
Consensus    TTTGTAGAAT CAAGTGTGGA TGACCGTCCA ATGAAAACTG TTTCTCAAGA CACAAACATC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
2AWf_2005-   CCAATCTACG CACAAATCTT TACTGACTCT ATCGCAGAAC AAGGTAAAGA AGGCGACAGC
```



Figure 5.3: Sequence alignment of selected transformants

Base pair mismatches are highlighted in green. The highlighted red sequence signals the finish and start of the first and second half of the *psaA* sequence, respectively.

5.3.2. Protein expression with IPTG

Transformant 4 was chosen for the expression experiment. Quantification of the transformant 4's purified plasmid was obtained using The NanoDrop® ND-1,000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Optical density measurements were carried out at wavelength 260nm and 280nm and the DNA was quantitated to be 212ng/µL. One nanogram (1:200 dilution of transformant 4 in x1 TE buffer) of the purified plasmid was transformed into BL21 Star™ (DE3) One Shot® *E.coli*; the puc19 vector was transformed into BL21 Star™ (DE3) One Shot® *E.coli* as a control for the IPTG expression experiment.

After transformation, the BL21 *E.coli* were grown overnight at 37°C in a shaking incubator. For long term storage 800µL each of BL21 with *psaA* and BL21 with puc19 were transferred into cryotubes and 200µL 100% glycerol was added for stable storage at -80°C.

Two lots of 10mL of LB broth with ampicillin (100µg/mL) were inoculated with 500µL each of the overnight cultures. After shaking incubation at 37°C for 2 hours, the 10mL broth culture was split into two 5mL cultures and 1mM IPTG and was added to one 5mL aliquot. A 500µL aliquot was removed at the time of induction with IPTG for culture containing PsaA non-induced (T0), PsaA induced (T0'), pUC19 non induced (T0) and pUC19 induced (T0'). 500µL aliquots were removed after 1 hour post induction (T1), 2 hours post induction (T2), 3 hours post induction (T3) and 4 hours post induction (T4). The aliquots were named as before T1 and 1', T2 and 2', T3 and 3', T4 and 4', when non-induced or induced, respectively. The aliquots were centrifuged in an Eppendorf 5417C microcentrifuge (Eppendorf AG, Hamburg, Germany) at 20,200 x g for 1 min. The supernatant was discarded and the visible cell pellets frozen at -20°C. The IPTG induced expression was visualised in a 10% reducing SDS gel and demonstrated no expressed protein at the expected Mr of 37 kDa in the pUC19 vector without construct, but increasing amounts of recombinant PsaA in T0-4 with marginally thicker bands when induced with IPTG (T0'-4') (**Figure 5.4**).

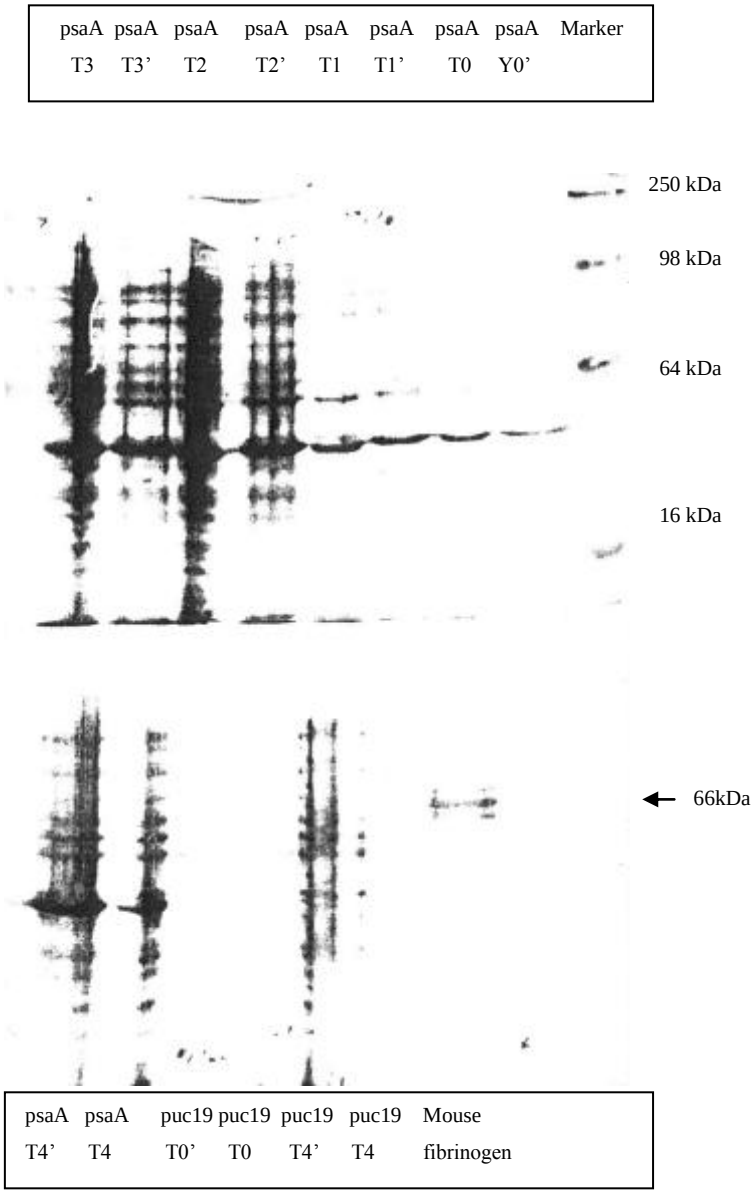


Figure 5.4: IPTG induced expression of *psaA*

T0-4 (non-induced), T0'-4' (IPTG induced) bacterial cell aliquots. pUC19: control for a vector without insert. The top gel shows T0-T3, the bottom gel shows the controls and T4 aliquots. Mouse fibrinogen (Mr ~66 kDa) was added to act as an extra guide for molecular weight. PsaA is at the expected height of 37 kDa.

Large scale expression followed in 1,000mL of LB broth plus ampicillin and 1mM IPTG for induction as per manufacturer's protocol. The cells were harvested 3 hours post induction as in pilot expression. In total, 6.6mL of wet bacterial cell pellet was stored at -80°C.

5.3.3. Verification of PsaA by high performance liquid chromatography (HPLC)

One quarter of the total packed cell pellet (approximately 2mL) was suspended in 10mL 50M NaCl 20mM Tris, pH 8.0, sonicated and freeze-thawed twice. Centrifugation was performed for 5 min at 30,500 x g in SS-34 Sorvall rotor centrifuge (Sorvall DuPont, Newtown, CT, USA).

The supernatant containing approximately 5mg of the 37kDa band (estimated from the SDS Page, **Figure 5.4**) was applied to a 2.5mL column of Ni-NTA His-Bind resin in binding buffer with 5mM imidazole. The break through (280nm) peak was collected and the column washed with wash buffer containing 60mM imidazole. Bound material was collected and dialysed/concentrated to ~5mg/mL.

IMAC was performed as described before (section 5.2.2.8) and the protein was collected. After concentration and dialysis of the collected protein the molecular weight was checked by SDS Page (**Figure 5.5).**

Five, 60 and 70µL samples of the concentrated protein was applied to HPLC C-4 column and solvent B (0.05% trifluoroacetic acid + 60% CH_3CN) gradient was run from 65 to 100% over 20 min. A single major peak was collected and peak crest analysed directly by electrospray ionisation mass spectrometry (ESI MS) (**Figure 5.6**) and measured at 215nm O_2 absorbance units.

Tryptic digest was performed overnight and a tryptic map was obtained confirming the digestion profile of PsaA with the use of BioLynx (Waters Corp., Milford, MA, USA) for peptide mapping.

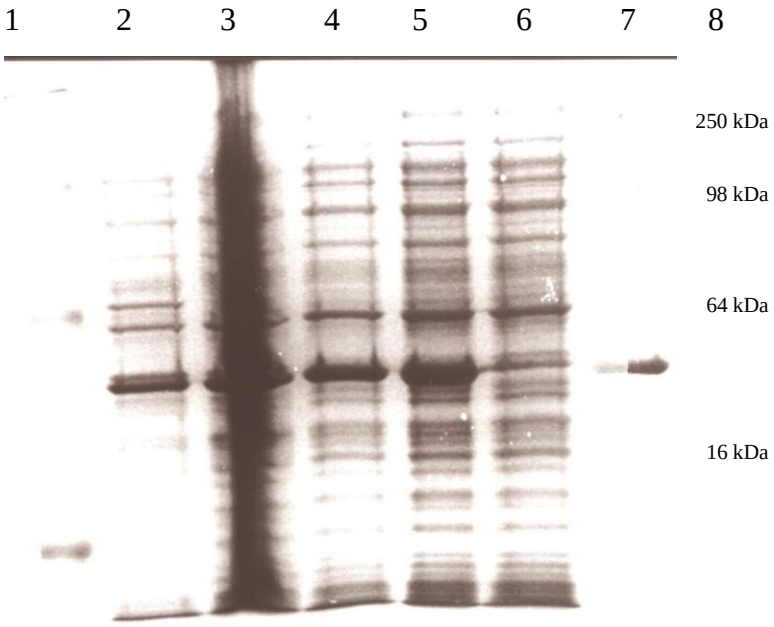


Figure 5.5: PsaA obtained post IMAC

Lane 1: marker, lane 2: T3', lane 3: cell debris, lane 4: 2.5mL supernatant, lane 5: 5mL supernatant, lane 6: unbound fraction, lane 7: collected presumptive PsaA fraction, lane 8: marker kDa approximation.

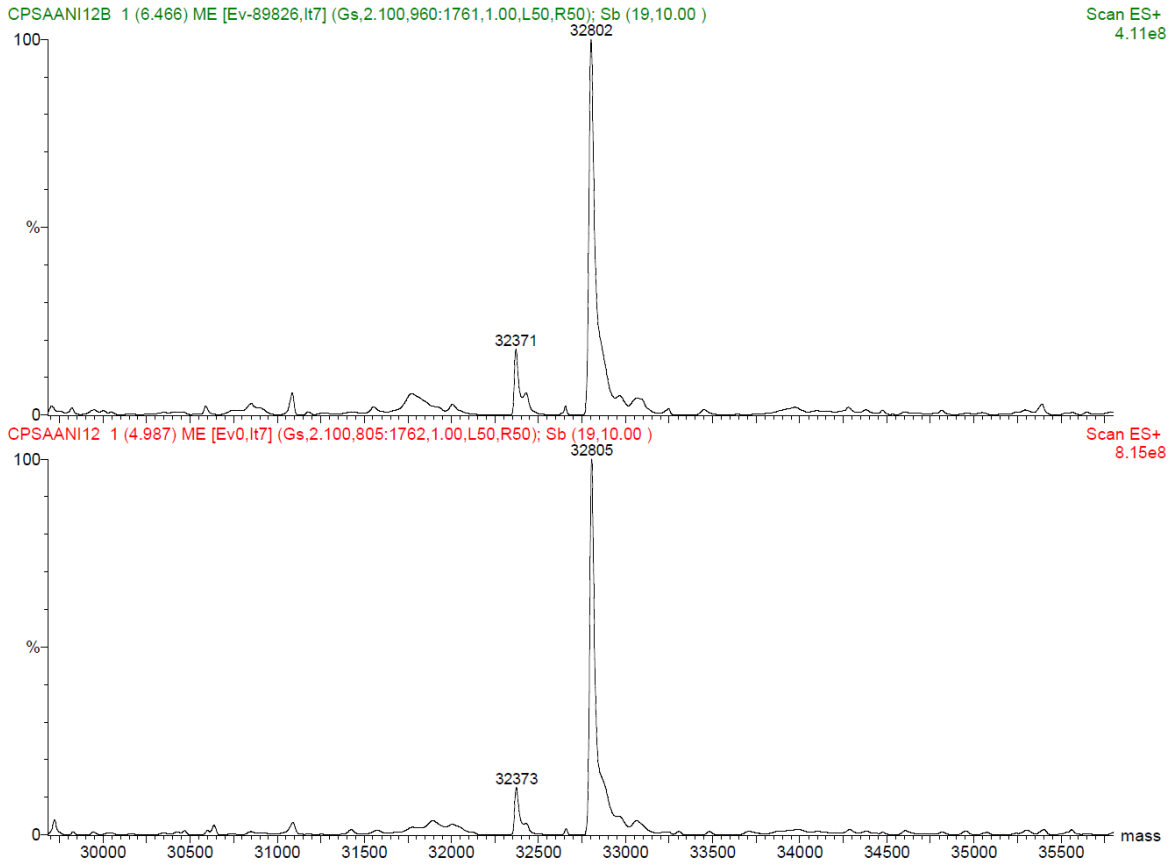


Figure 5.6: Electrospray Ionisation (ESI) mass spectra of recombinant PsaA

5.4. Summary

In this chapter various methods combining molecular techniques with protein biochemistry were used to verify the *psaA* plasmid and to demonstrate that rPsaA as a recombinant protein can be expressed. It was possible to clone a blunt-end *psaA* PCR product into a pQE-30 vector for high level expression of the protein in *E. coli*. Recombinant PsaA was subsequently verified by high performance liquid chromatography and electrospray ionisation mass spectrometry. The rPsaA underwent tryptic digest and mapping and the expected digestion peptide profile was confirmed.

These steps were essential prior to setting up an immunoassay for the detection of native PsaA in patient specimens based on monoclonal and polyclonal antibodies raised to rPsaA. This verification step ensured that the recombinant protein produced by transformed bacteria was based on the expected DNA sequence. This in turn ensures the specificity of the antibodies raised against it.

CHAPTER 6

PNEUMOCOCCAL SURFACE ADHESIN A – MONOCLONAL AND POLYCLONAL ANTIBODY PRODUCTION AND CHARACTERISATION

6.1. Introduction

PsaA is not new as a target for diagnosis of pneumococcal disease. Alongside pneumolysin and autolysin, the pneumococcal surface adhesin gene (*psaA*) has recently been considered as a PCR target for diagnostic tests [281, 358]. Here, PsaA was investigated as a target in an antigen detection assay. Different findings have been reported as to the surface accessibility of PsaA, and therefore the potential binding of antibodies to the protein. In one study the binding of undiluted anti-PsaA monoclonal antibodies (MAbs) could be demonstrated using immunofluorescence [355]. In another study rabbit anti-PsaA polyclonal antibodies failed to bind suggesting that removal of the cell wall might have to occur prior to PsaA being accessible [359]. Overall, however, the theory of surface accessibility is supported by experiments confirming PsaA's role as an adhesin and virulence factor [360, 361].

In this study four MAbs and two polyclonal antibodies raised against recombinant pneumococcal surface adhesin A (rPsaA) were produced and characterised. The specificity of all antibodies was assessed by a combination of immunodot blots, Western blots and ELISAs. The epitopes of the four MAbs were mapped to assess their suitability to be used in a diagnostic sandwich ELISA assay.

6.2. Material and methods

6.2.1. Polyclonal antibody production

Two NZ white rabbits were accessed through the Animal Facility at the University of Otago, Christchurch School of Medicine and Health Science, Christchurch, New Zealand. Polyclonal antisera were raised in the rabbits by primary subcutaneous injection of 20µg recombinant PsaA (rPsaA) in 100µL PBS emulsified in 130µL Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) [342]. Two booster injections were given at one month intervals using incomplete Freund's adjuvant. Serum of centrifuged rabbit tail blood was used to test polyclonal antibody titres in an ELISA against pneumococcal (*S. pneumoniae* ATCC 49617) lysate at a 1:40 dilution and rPsaA.

6.2.2. Immunisation of mice

Male RBF/DN mice were obtained from a breeding colony from the University of Otago, Christchurch, New Zealand. To immunise 2 RBF/DN mice 50µL rPsaA at 1µg/µL was mixed with 200µL PBS and 200µL Freund's adjuvant. The viscous mixture was transferred into a 1mL syringe ready for injection. Each mouse was anaesthetised with halothane and injected intraperitoneally with approximately 225µL of the antigenic preparation (equivalent to 25µg of rPsaA). The second intraperitoneal immunisation followed after 4 weeks.

One month after the second immunisation the mouse antibody response was evaluated in tail blood obtained from each mouse. The serum of centrifuged tail blood was tested in10-fold dilutions in an ELISA against pneumococcal cell lysate (1:500 in 6M guanadine HCl).

6.2.2.1. Cell fusion

Mouse 2, which had the marginally higher antibody response (section 6.3.2), was culled one month after the second immunisation and the spleen was excised aseptically.

Spleen lymphocytes were fused with a mouse myeloma cell line using polyethylene glycol (PEG) (Hybri-Max, Sigma-Aldrich, St. Louis, USA). The myeloma line FOX-NY is deficient in adenine phosphoribosyl transferase (APRT), an enzyme that uses extraneous adenine to produce purines. The enzyme acts as a rescue pathway in case the major purine pathway, involving folic acid reductase, is blocked by folic acid antagonists, such as aminopterin, which also blocks thymidine. Hence, the addition of aminopterin to APRT–negative cells kills the cells even in the presence of thymidine.

When these APRT-negative FOX-NY cells are fused with the spleen cells from immunised RBF/DN mice the Ig heavy chain producing cells are selected for using AAT (adenine/aminopterin/thymidine) because the IG heavy chain gene and the APRT gene are located on the same chromosome following translocation of the APRT gene from chromosome 8.

To optimise the chance for cell fusion a ratio of 5-10:1 of spleen cells to FOX-NY is needed. FOX-NY and spleen cells were combined at a ratio of 1:5, and spun at 1,000 x g for 10 min. The cell membranes were fused with 50% PEG, adding 1mL PEG drop by drop over 1 min. The mixture was placed in a water bath at 37°C for 90 seconds with occasional shaking. After fusion, fresh medium was added drop by drop, increasing the volume gradually up to 30mL while shaking. The 30mL fusion mixture was decanted into 400mL of fresh

Gibco^{TM} RPMI Medium 1640 (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia) to a concentration of ~10^6cells/mL containing 15% (v/v) fetal calf serum. AAT (adenine/aminopterin/thymidine; 75:0.08:16 x 10-6M, respectively) was added. The fused cells were transferred into a 96-well culture plate at 10^5cells/well. Cell growth occurred under standard condition in a 37°C incubator with regular media changes and the cells were regularly checked for viability under a light microscope.

6.2.2.2. Screening of supernatants

Supernatants were screened by ELISA coated with a cell suspension of an overnight growth of *S. pneumoniae* ATCC 49617 at a dilution of 1:5,000 in 6M aqueous guanidine HCl. 100µL of each individual cell culture supernatant was added aseptically to each well. Coating occurred overnight at 4°C and the plate was blocked for 1 h at RT, the solution was removed and replaced with 100µL of culture supernatant/well. Plates were incubated for 2 h at RT, washed 4 times, and then anti-mouse Ig horse radish peroxidase (HRP) added to each well for a further incubation of 1 h at RT. The plates were finally washed and (TMB) substrate solution was added. The reaction was terminated by the addition of 100µL of 1.0M HCl/well and absorbance read at 450nm.

To avoid evaporation losses plates were covered for all steps preceding the addition of substrate. Positive wells were cloned by limiting dilution.

Cell culture supernatants from wells with microscopically visible clones were diluted at 1:100 with sterile PBS and screened with a competitive ELISA using *S. pneumoniae* (ATCC 49617) cell suspension dilution as the competitor. The plate was coated with recombinant PsaA at 1:1,000 in sterile PBS. A second detection antibody was used specific to the primary antibody and a chromogenic signal was read at 450nm.

6.2.3. Protein A purification of the monoclonal antibodies

MAbs to rPsaA were purified from culture supernatants by chromatography on a Protein-A spin column (Nunc ProPur^{TM} Kits, In Vitro Technologies, Auckland, New Zealand). This system allowed the purification of approximately 1mg of mouse monoclonal IgG from 500mL of culture supernatant.

The buffers used for the procedure were buffers A and B.

Buffer A (solid mixture):

1.5M	glycine
3M	NaCl

Loading buffer:

Buffer A in solution (H_2O), pH 9.0

Buffer B:

40mM	citric acid
50mM	NaCl

Adjust to pH 3.2.

In summary, frozen monoclonal antibody stocks were thawed and 29g of buffer A were added per 100mL of supernatant: 116g of buffer A were added to 400mL each of supernatant clones 13A7 and 15D7, 145g of buffer A were added to 500mL of supernatant clones 6A7 and 4D6.

After flushing the protein A affinity column (protein A Sepharose) with loading buffer until baseline absorbance at 280nm was indicated on a fraction collector with a chart recorder, each monoclonal antibody/buffer mixture was run through the column at a set gradient. The IgG was eluted with buffer B and the peak fraction was collected. To regenerate the column in between the purification of the different antibodies it was flushed with 10mL 40% (v/v) methanol, followed by loading buffer to return to baseline absorbance.

6.2.4. Dialysis of purified fraction

The dialysis tubing (Sigma-Aldrich, St. Louis, MO, USA) was prepared by covering it with dd H_2O and boiled in a microwave oven for 2 minutes. One end of an approximately 20cm long piece of membrane was tied into a knot, then filled up with the collected purified fraction and secured at the other end with a knot. The "sausage"-shaped dialysis membrane was placed into a plastic bag filled with polyethylene glycol granules and left for 30 min. The dialysis membrane containing a dehydrated fraction was cleaned from surrounding polyethylene glycol sludge; a fresh knot was tied at the level of the remaining fluid and placed into 0.1M $NaHCO_3$, pH 8.3, on a plate stirrer.

Following extensive dialysis the antibodies were concentrated using the Urifil-10 Concentrator (Millipore, Bedford, MA, USA). The dialysates were placed into sterile vials and the optical density (OD) measured on a Cary 50 instrument version 3.0 at 280nm prior to and post biotinylation. An absorbance signal of 1.35 approximates to 1mg/mL of IgG antibody. The ODs measured against the dialysing buffer as the blank (0.1M $NaHCO_3$) are listed in **Table 6.1**.

Table 6.1: OD measurements

Antibody (designation)	**Measured OD at 280nm**	**Approximated antibody concentration (pre-biotinylation)**	**Approximated antibody concentration (post-biotinylation)**
4D6 (PSAA-4)	0.1687	125μg/mL	220μg/mL
6A7 (PSAA-6)	0.1217	90μg/mL	400μg/mL
13A7 (PSAA-13)	0.3442	255μg/mL	700μg/mL
15D7 (PSAA-15)	0.1990	147μg/mL	180μg/mL

6.2.5. Isotyping of the MAbs

The commercial kit set used for this procedure was Serotec MMT1 (Serotec, Kidlington, Ox, UK). The four MAbs were diluted to a concentration of 1μg/mL in PBS. 150μL of freshly diluted MAb were pipetted into development tubes provided in the test kit. They were incubated at RT for 30 seconds then vortexed. An isotyping strip was placed into each development tube. Within 5-10 min a blue band on the strip indicated which class or subclass the MAb belonged to.

6.2.6. Peptide microarrays

RepliTope^{TM} Microarrays were custom made by JPT Peptide technologies, Berlin, Germany according to Swiss Prot code POA4G2, 309aa. The layout of the microarray slides and spots is shown in **Figures 6.1** and **6.2**, respectively. **Table 6.2** lists the protein amino acid sequence of the individual peptides spotted onto the slides.

Two unpurified synthetic peptides were obtained through Mimotopes PTY Ltd, Victoria, Australia. The first peptide sequence (#146) was SYYSMMKYNLDKI, the second (#134) QDTNIPIYAQIFT. Both peptides had an H- acytelated N-terminus and –OH C-terminus.

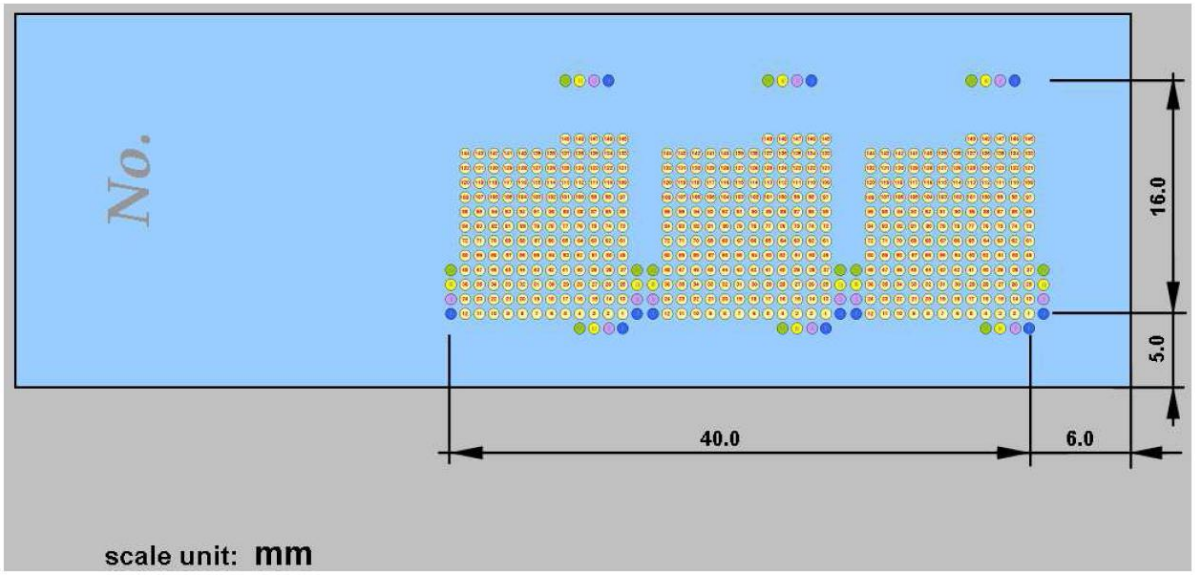


Figure 6.1: Microarray slide layout as provided by the manufacturer (JPt)

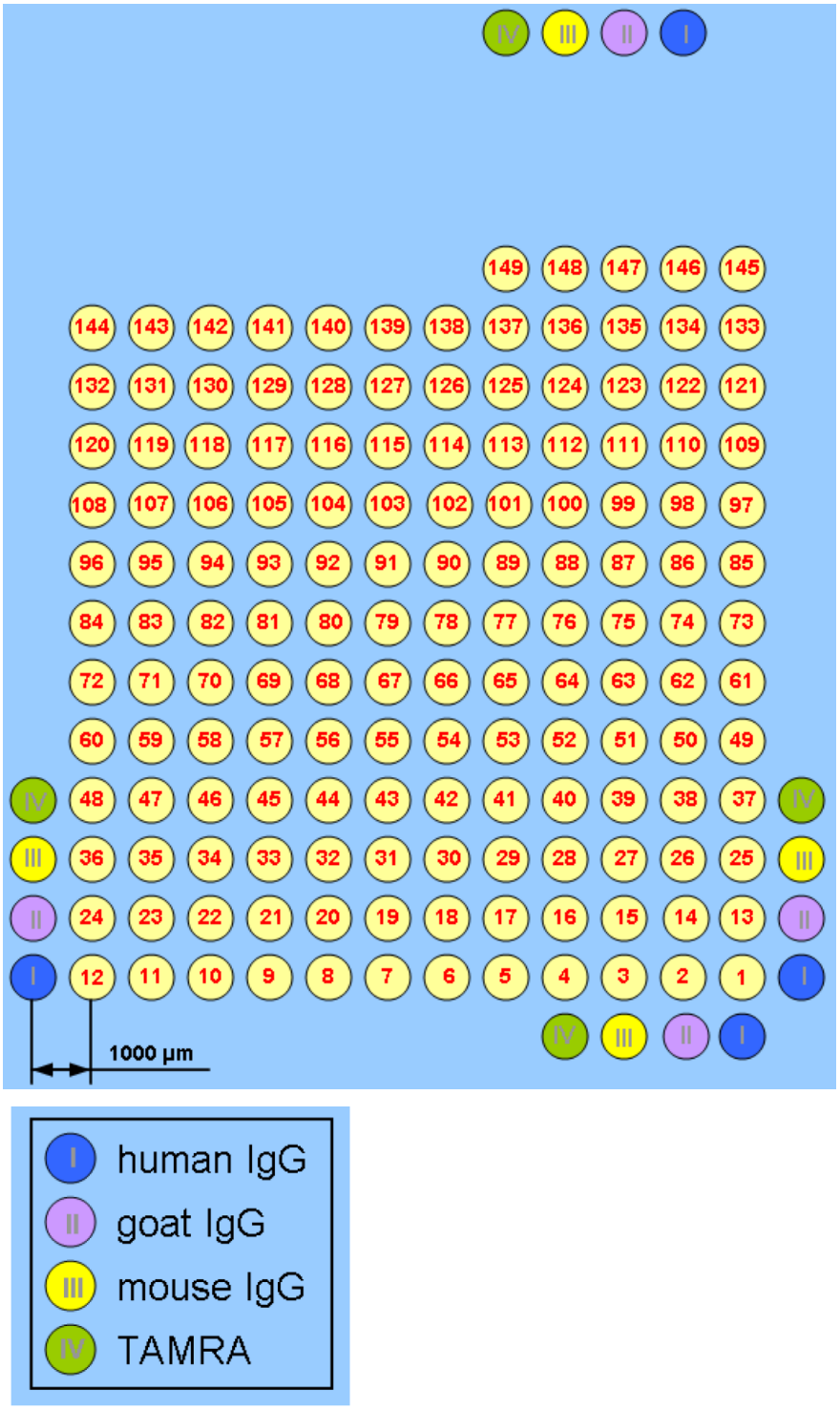


Figure 6.2: Microarray spot layout which occurs in triplicate on each slide

The controls are as illustrated in I-IV.

Table 6.2: Spot numbers and their associated amino acid sequences

Spot No.	Sequence	Spot No.	Sequence	Spot No.	Sequence	Spot No.	Sequence
1	MKKLGTLLVLFLS	39	KKTSEADLIFYNG	77	NIAKQLSAKDPNN	115	EEGTPEQIKTLVE
2	KLGTLLVLFLSAI	40	TSEADLIFYNGIN	78	AKQLSAKDPNNKE	116	GTPEQIKTLVEKL
3	GTLLVLFLSAIIL	41	EADLIFYNGINLE	79	QLSAKDPNNKEFY	117	PEQIKTLVEKLRQ
4	LLVLFLSAIILVA	42	DLIFYNGINLETG	80	SAKDPNNKEFYEK	118	QIKTLVEKLRQTK
5	VLFLSAIILVACA	43	IFYNGINLETGGN	81	KDPNNKEFYEKNL	119	KTLVEKLRQTKVP
6	FLSAIILVACASG	44	YNGINLETGGNAW	82	PNNKEFYEKNLKE	120	LVEKLRQTKVPSL
7	SAIILVACASGKK	45	GINLETGGNAWFT	83	NKEFYEKNLKEYT	121	EKLRQTKVPSLFV
8	IILVACASGKKDT	46	NLETGGNAWFTKL	84	EFYEKNLKEYTDK	122	LRQTKVPSLFVES
9	LVACASGKKDTTS	47	ETGGNAWFTKLVE	85	YEKNLKEYTDKLD	123	QTKVPSLFVESSV
10	ACASGKKDTTSGQ	48	GGNAWFTKLVENA	86	KNLKEYTDKLDKL	124	KVPSLFVESSVDD
11	ASGKKDTTSGQKL	49	NAWFTKLVENAKK	87	LKEYTDKLDKLDK	125	PSLFVESSVDDRP
12	GKKDTTSGQKLKV	50	WFTKLVENAKKTE	88	EYTDKLDKLDKES	126	LFVESSVDDRPMK
13	KDTTSGQKLKVVA	51	TKLVENAKKTENK	89	TDKLDKLDKESKD	127	VESSVDDRPMKTV
14	TTSGQKLKVVATN	52	LVENAKKTENKDY	90	KLDKLDKESKDKF	128	SSVDDRPMKTVSQ
15	SGQKLKVVATNSI	53	ENAKKTENKDYFA	91	DKLDKESKDKFNK	129	VDDRPMKTVSQDT
16	QKLKVVATNSIIA	54	AKKTENKDYFAVS	92	LDKESKDKFNKIP	130	DRPMKTVSQDTNI
17	LKVVATNSIIADI	55	KTENKDYFAVSDG	93	KESKDKFNKIPAE	131	PMKTVSQDTNIPI
18	VVATNSIIADITK	56	ENKDYFAVSDGVD	94	SKDKFNKIPAEKK	132	KTVSQDTNIPIYA
19	ATNSIIADITKNI	57	KDYFAVSDGVDVI	95	DKFNKIPAEKKLI	133	VSQDTNIPIYAQI
20	NSIIADITKNIAG	58	YFAVSDGVDVIYL	96	FNKIPAEKKLIVT	134	QDTNIPIYAQIFT
21	IIADITKNIAGDK	59	AVSDGVDVIYLEG	97	KIPAEKKLIVTSE	135	TNIPIYAQIFTDS
22	ADITKNIAGDKID	60	SDGVDVIYLEGQN	98	PAEKKLIVTSEGA	136	IPIYAQIFTDSIA
23	ITKNIAGDKIDLH	61	GVDVIYLEGQNEK	99	EKKLIVTSEGAFK	137	IYAQIFTDSIAEQ
24	KNIAGDKIDLHSI	62	DVIYLEGQNEKGK	100	KLIVTSEGAFKYF	138	AQIFTDSIAEQGK
25	IAGDKIDLHSIVP	63	IYLEGQNEKGKED	101	IVTSEGAFKYFSK	139	IFTDSIAEQGKEG
26	GDKIDLHSIVPIG	64	LEGQNEKGKEDPH	102	TSEGAFKYFSKAY	140	TDSIAEQGKEGDS
27	KIDLHSIVPIGQD	65	GQNEKGKEDPHAW	103	EGAFKYFSKAYGV	141	SIAEQGKEGDSYY
28	DLHSIVPIGQDPH	66	NEKGKEDPHAWLN	104	AFKYFSKAYGVPS	142	AEQGKEGDSYYSM
29	HSIVPIGQDPHEY	67	KGKEDPHAWLNLE	105	KYFSKAYGVPSAY	143	QGKEGDSYYSMMK
30	IVPIGQDPHEYEP	68	KEDPHAWLNLENG	106	FSKAYGVPSAYIW	144	KEGDSYYSMMKYN
31	PIGQDPHEYEPLP	69	DPHAWLNLENGII	107	KAYGVPSAYIWEI	145	GDSYYSMMKYNLD
32	GQDPHEYEPLPED	70	HAWLNLENGIIFA	108	YGVPSAYIWEINT	146	SYYSMMKYNLDKI
33	DPHEYEPLPEDVK	71	WLNLENGIIFAKN	109	VPSAYIWEINTEE	147	YSMMKYNLDKIAE
34	HEYEPLPEDVKKT	72	NLENGIIFAKNIA	110	SAYIWEINTEEEG	148	MMKYNLDKIAEGL
35	YEPLPEDVKKTSE	73	ENGIIFAKNIAKQ	111	YIWEINTEEEGTP	149	KYNLDKIAEGLAK
36	PLPEDVKKTSEAD	74	GIIFAKNIAKQLS	112	WEINTEEEGTPEQ		
37	PEDVKKTSEADLI	75	IFAKNIAKQLSAK	113	INTEEEGTPEQIK		
38	DVKKTSEADLIFY	76	AKNIAKQLSAKDP	114	TEEEGTPEQIKTL		

Each RepliTope peptide microarray slide contained three identical subarrays with a peptide molecule density within each spot of 150fmol/mm2. A disposable incubation chamber was attached to the slide (Gene-Frame^{TM}, ABgene Ltd., Epsom, KT, UK) as recommended by the manufacturer. The MAb solution was aimed to contain approximately 80µg of MAb per slide. The maximum volume the incubation chamber could hold was 330µL.

The known concentrations of the MAbs were: 685µg/mL for PSAA-13; 400µg/mL for PSAA-6; 220µg/mL for PSAA-4; and 300µg/mL for PSAA-15. Each MAb was diluted to a final concentration of 240µg/mL, except for PSAA-4 which was applied to the slide undiluted.

The MAb solution was carefully pipetted into the incubation chamber and care was taken not to touch the slide surface by the pipette tip in order to avoid artefacts during the fluorescence scanning. A pre-treated polyester cover slip was placed over the adhesive frame avoiding any air bubbles. The slides were incubated at 4°C overnight. Subsequently the slides were washed 5 times for 5 min with dd H_2O on a shaker. Incubation with the fluorescently labelled secondary antibody followed using 1:1,000 anti-mouse Cy3 (Sigma-Aldrich, St. Louis, MO, USA) in assay buffer. The adhesive incubation chamber was carefully removed and the wash step was repeated as described before and the slides dried by washing them in dust free methanol and letting them air dry. Analysis of the slides was performed using a fluorescent scanner, GenePix 4000B (Geneworks Pty Ltd., Hindmarsh, SA, Australia), wavelength setting 635nm with PMT grain of 100 at 10% power and 532nm with PMT grain of 450, at 100% power.

6.2.7. Immunofluorescence

Immunofluorescnce was performed to assess the binding capacity and specificity of the MAb, PSAA-13, that was selected for the development of a diagnostic assay, to *S. pneumoniae*. A microscopy slide with preformed wells was coated with a heavy suspension of *S. pneumoniae* (ATCC 49617). *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *E. faecalis* (ATCC 29212) were used in a heavy suspension to check for cross-reactivity.

The bacterial suspensions were made up in PBS. Ten µL of each bacterial suspension was applied in duplicate onto the slide wells and dried at 37°C. The smears were heat fixed to the slides. Methanol fixation was tried with no added benefit [362]. A 1:10 dilution of PSAA-13 was added at 10µL per slide well and incubated for 30 min at 37°C. The slide was washed

with PBS for 10 min at RT, then incubated with 10µL per slide well 1:100 goat anti-mouse IgG1 (gamma) FITC conjugate (Caltag Laboratories, Invitrogen, Carlsbad, CA, USA) at 37°C for 30 min. A final wash with PBS for 10 min followed. The slides were then air dried and read under UV light at x100 oil immersion and images were taken using the Isis FISH imaging system software (MetaSystems, Altlussheim, Germany).

6.2.8. Synthetic peptides

The synthetic peptides were analysed in a competitive ELISA against pneumococcal suspension at 1:100 in guanidine HCl (approximately 0.078µg of pneumococcal protein per well).

6.2.9. Biotinylation

The dialysates of the four MAbs were split in half. One half had thiomersal added for preservation and was stored at 4°C. The other half was biotinylated [347].

In short, the succinimide ester of biotin is used to bind to free amino groups on the antibody. A solution of N-hydroxysuccinimide biotin is made at 10mg/mL in dimethyl sulfoxide. An antibody solution of at least 1-3mg/mL is made in sodium borate buffer (0.1M, pH 8.8). Any azide present in the antibody solution must be removed prior to coupling. Biotin ester and antibody are combined at a ratio of 25-250µg of ester per mg antibody. The combination is mixed well and incubated at room temperature for 4 hours. Subsequently 20µL of 1M NH_4Cl per 250µg of ester are added and incubated at room temperature for 10 min. Any uncoupled biotin is removed by dialysing the antibody solution against PBS. The dialysis was performed at 4°C for 48 hours.

6.2.10. Bacterial isolates

Except for those indicated as wild type strains, the following streptococcal species and other bacterial isolates were kindly provided by the Institute of Environmental Science & Research Limited (ESR), New Zealand Reference Culture Collection, Porirua, New Zealand: *S. pneumoniae* (ATCC 33400, ATCC 49617, ATCC 49619, Staten Serum Institute, Copenhagen, Denmark serotypes 1N, 2, 3,4L,5,6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10F, 11A, 11B, 11C, 11F L, 12F, 13L, 14L, 15A, 15B, 15C, 15FL, 17A, 17F, 18A, 18B,

18C, 18F, 19A, 19B, 19C, 19F L, 20L, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25L, 33A, 33B, 33C, 33F), *Streptococcus mitis* (ATCC 9811), *Streptococcus oralis* (NZ isolate SID 08/60), *Streptococcus pseudopneumoniae* (ATCC BAA-960), *Streptococcus mutans* (ATCC 25175, strain AHT Fitzgerald, strain KIR Fitzgerald), *Streptococcus sanguinis* (strain OPAI, strain J9, ATCC 10556), *Streptococcus salivarius* (strain 57 I, ATCC 13419, ATCC 7073), *Streptococcus pyogenes* (ATCC 21546, ATCC 12344, ATCC 19615, ATCC 21547, ATCC 49117, NCTC 8322), *Streptococcus agalactiae* (ATCC 12386, ATCC 13813), *Streptococcus anginosus* (NCTC 8037), *Enterococcus faecalis* (ATCC 29212, ATCC 14506), *Pseudomonas aeruginosa* (ATCC 27853), *Bordetella pertussis* (wild type, NZ clinical isolate), *Bordetella parapertussis* (ATCC 15311), *Legionella pneumophila* (ATCC 33152), *Legionella longbeachae* (wild type, NZ clinical isolate), *Staphylococcus aureus* (ATCC 29213), *Arcanobacterium haemolyticum* (NCTC 9697), *Burkholderia cepacia* (ATCC 25416), *Klebsiella pneumoniae* (ATCC BAA-1706), *Haemophilus influenzae* (ATCC 49247), *Haemophilus parainfluenzae* (NCTC 4101), *Escherichia coli* (ATCC 35218), *Enterobacter cloacae* (ATCC 13047), *Enterobacter aerogenes* (ATCC 13048), *Neisseria menigitidis* (CDC f-8), *Corynebacterium diphtheriae* biotype *gravis* (ATCC 19409), *Acinetobacter baumanii* (ATCC 19606).

6.2.11. ELISA

ELISAs were performed as described in section 2.3.1.1. The capture antibody used was either one of the MAbs or polyclonal antiserum, K34 or K35. Capture antigen consisted of rPsaA, cell lysate or cell suspension (*S. pneumoniae* ATCC 49617). When the biotinylated antibody was used as second antibody, the detection system was changed to an avidin- HRP conjugate (Fitzgerald Industries International, North Acton, MA, USA). A negative control (*S. aureus* ATCC 29213) was included in each assay.

6.2.12. Cross-reactivity studies

To assess the specificity of the MAbs and polyclonal antisera they were tested against suspensions of all bacterial isolates in an immunodot blot and against selected isolates in Western blots. Cross-reactivity studies of the four MAbs and the two polyclonal antisera were carried out in an ELISA against lysate and suspension of selected streptococci: *S. mutans* (ATCC 25175), *S. sanguinis* (ATCC 10556), *S. mitis* (ATCC 9811), *S. oralis* (NZ

isolate SID 08/60), *S. salivarius* (ATCC 7073), *S. pseudopneumoniae* (ATCC BAA-960), *S. pyogenes* (ATCC 12344), and *S. agalactiae* (ATCC 13813). *S. pneumoniae* (ATCC 33400) was used as positive control.

6.2.13. Immunodot blot

All bacterial suspensions were brought to a similar concentration 1µg/µL and 2.5µL or 5µL spots were directly dotted on nitrocellulose paper. Once air dried, the nitrocellulose papers were carefully wetted in TBS Tween buffer (15mM Tris, 150 mM NaCl, pH 7.4, 0.1% Tween) and then transferred into 5% trim-milk (0.1% fat) TBS Tween and blocked overnight at RT on a shaker.

After overnight incubation the blots were washed 5 times with TBS Tween and then incubated in MAbs solutions (1:10 MAbs in TBS Tween plus 1% (v/v) BSA) for 1 hour on a shaker at RT. After 5 washes in TBS Tween the second antibody was added at 1:10,000 (IgG1 anti-mouse HRP in 1% BSA TBS Tween) and incubated on shaker for 1 hour at RT. After another 5 washes in TBS Tween and one wash in PBS the blots were equilibrated in citrate-EDTA buffer (10mM sodium citrate and 10mM sodium EDTA pH 5.0) for 5 min, then equilibrated in 1% dextran sulphate in citrate-EDTA for 10 min. After 3 washes with citrate-EDTA the blots were developed with 0.05% TMB 0.25% H_2O_2 in citrate-EDTA. The reaction was stopped with dd H_2O.

6.2.14. Western blot analysis

Vertical SDS-PAGE was carried out using 10% acrylamide mini gels with 10-15µg loadings of bacterial isolates and 5µg rPsaA using the Bio-Rad Miniprotean system. Transfer to nitro-cellulose performed at 120V (constant), 90mA, and was verified by the use of Bio-Rad Kaleidoscope Pre-stained Standard. For antibody probing, nitro-cellulose was initially blocked by incubation in 5% trim-milk TBS Tween overnight at RT. The nitro-cellulose was then washed 3 times in TBS Tween and incubated with unpurified supernatant of each MAb diluted 1:10 in TBS buffer containing 1% (v/v) BSA for 1 hour on shaker. Nitro-cellulose was washed 3 times and incubated with the second antibody (1:10,000 IgG1 anti-mouse HRP in 1% BSA TBS Tween) for 1 hour at RT on shaker. The nitro-cellulose was finally washed and the blot developed with TMB precipitating substrate as described in section 6.2.13.

6.2.15. Statistical analysis

Vertical line graphs were generated using the graphics software of SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA).

6.3. Results

6.3.1. Rabbit antibody response

Both polyclonal antisera, K34 and K35, showed strong signals against both lysate and rPsaA. The response to rPsaA for both polyclonal antisera was at a titre of >1:100,000.

6.3.2. Mice antibody response

Both intraperitoneally immunised mice produced good antibody responses one month after the second immunisation with recombinant PsaA. The detectable dilution in a chromatogenic ELISA coated with pneumococcal cell lysate (*S. pneumoniae* ATCC 49617) at 1:500 in 6M guanidine HCl in mouse 1 was 1:10,000, in mouse 2 1:100,000.

6.3.3. Supernatant screening

Those wells displaying a visible colour signal on the ELISA screening plates, indicating binding between a potential clone and pneumococcal cells, were assessed microscopically. The results are summarised in **Table 6.3**.

Table 6.3: Screening results of cell culture supernatants

Plate No.	Well No.	ELISA Signal Intensity	No. of clones under light microscopy
2	H8	Medium	Nil
4	D6	Medium	1
6	A7	Medium	4
11	F1	Medium	1
13	A7	Strong	1
13	E1	Low	Nil
15	G9	Strong	Nil
15	D7	Medium	1
15	C8	Strong	1

Cell culture supernatants from wells with microscopically visible clones were diluted at 1:100 with sterile PBS and screened in a competitive ELISA using pneumococcal cell suspension dilutions as the competitor. In principle, unlabeled monoclonal antibodies (1:100 in sterile PBS) was added to pneumococcal cell suspension dilutions (doubling dilutions in sterile PBS) and incubated. The mixture containing possible antibody-antigen complexes and unbound antibodies was then added to the plate which was coated with recombinant antigen (rPsaA) at 1:1,000 in sterile PBS. A wash step removed the unbound antibody from the well. The more antigen was present in the cell suspension, the more antibody bound to it, hence the less antibody is present to bind to the antigen coating the well. A second detection antibody was used specific to the primary antibody and a chromogenic signal was read at 450nm. The weaker the signal, the higher was the antigen concentration in the first step of the assay. Clones 15D7, 6A7, 4D6 and 13A7 produced a curve of decreasing signal intensity indicating binding of the monoclonal to the cell suspension dilution series. These clones were chosen for large scale monoclonal antibody production and purification.

6.3.4. Isotyping of the monoclonal antibodies

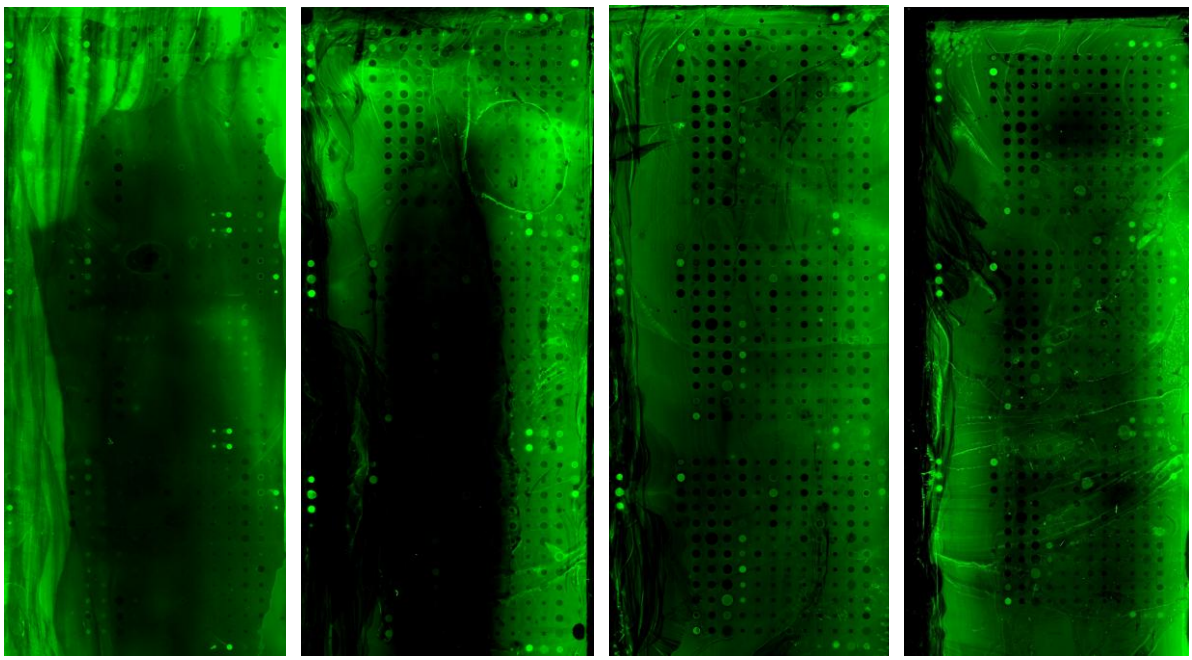
Clones 4D6, 6A7, 13A7 and 15D7 were isotyped as IgG1 kappa monoclonal antibodies. They were designated as PSAA-4, PSAA-6, PSAA-13 and PSAA-15, respectively.

6.3.5. Mapping of antigenic determinants by peptide microarrays

Microarray scanning revealed a number of different spots with different signal intensity for each MAb. PSAA-13 showed fluorescent signal at spots #88, #134-136 and #145-146, PSAA-4 showed signals at #146, PSAA-6 at spots #146, #101-106, and PSAA-15 at #146 and #103-104, with only spot #146 being commonly recognised by all four MAbs. Controls II-IV showed a fluorescent signal, goat IgG, mouse IgG and TAMRA, control I (human IgG) was not recognised (**Figure 6.3**).

Because of their highest measured intensity the peptides represented by spot #146 and #134 were chosen to be synthesised for testing against pneumococcal antibody in solution.

A modelled three-dimensional view of potentially recognised antigenic epitopes could be visualised using UniProtKB/Swiss-Prot software (http://web.expasy.org/docs/swiss-protguideline.html) (**Figure 6.5**).



PSAA-13 @ 80 µg PSAA-4 @ neat PSAA-6 @ 1:2 dilution PSAA-15 @160 µg

Figure 6.3: Microarray images of potentially recognised PsaA peptides

All four MAbs were analysed for their peptide recognition using commercially produced microarray slides. Slide layout as in **Figure 6.1**.

CLUSTAL X (1.81) multiple sequence alignment was performed to demonstrate the position within the PsaA protein sequence of the epitopes identified by fluorescent signal on the peptide microarray. The positions are highlighted in **Figure 6.4**.

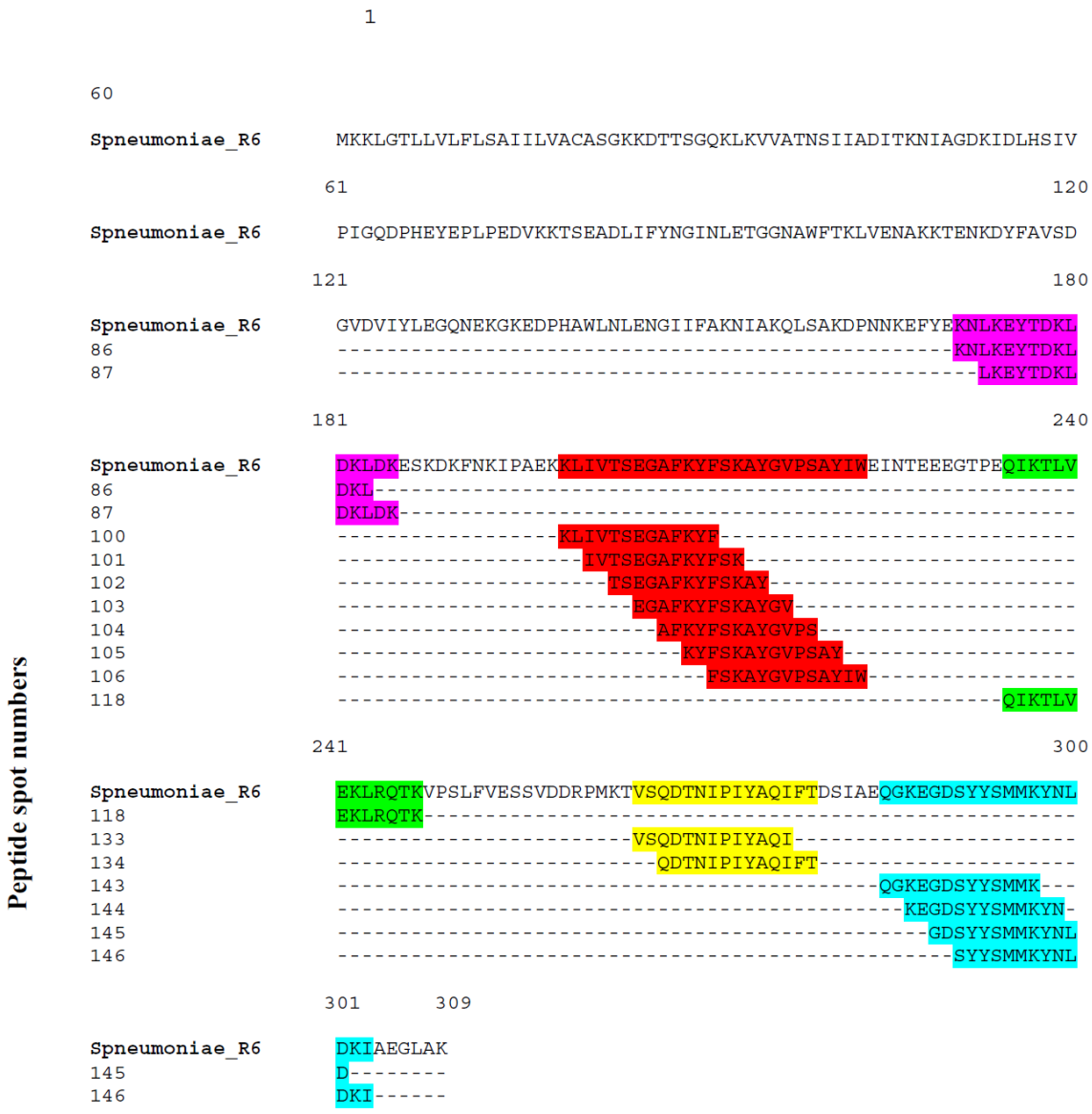
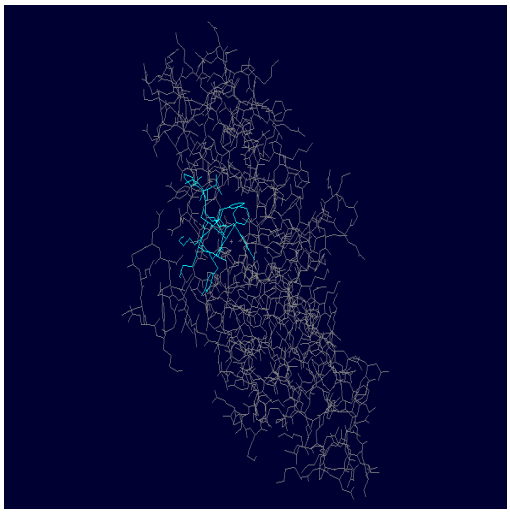
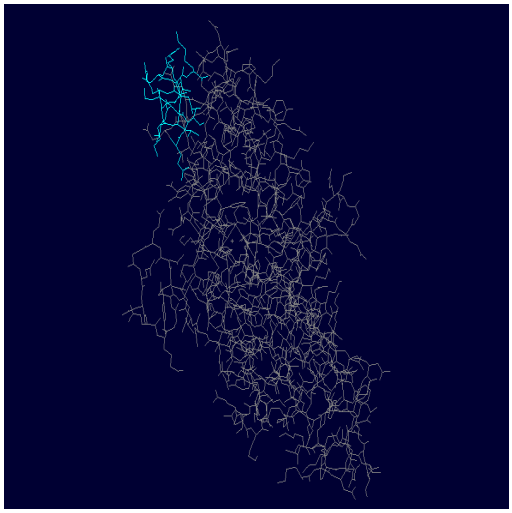


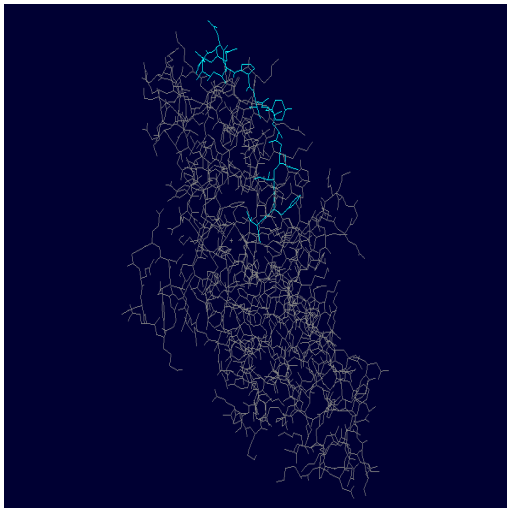
Figure 6.4: Alignment of peptide spots with PsaA protein sequence using CLUSTAL X (1.81) multiple sequence alignment



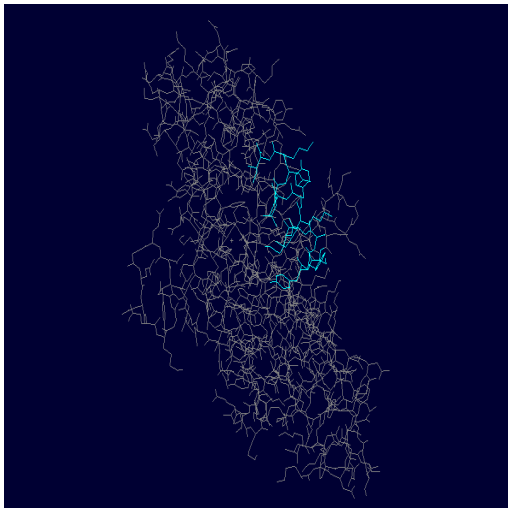
Spot 104



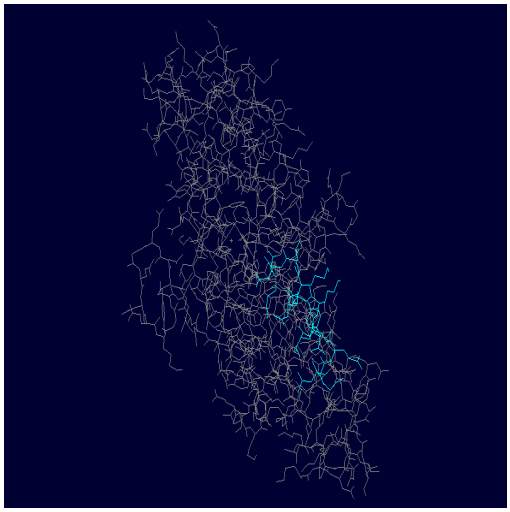
Spot 118



Spot 134



Spot 146



Spot 87

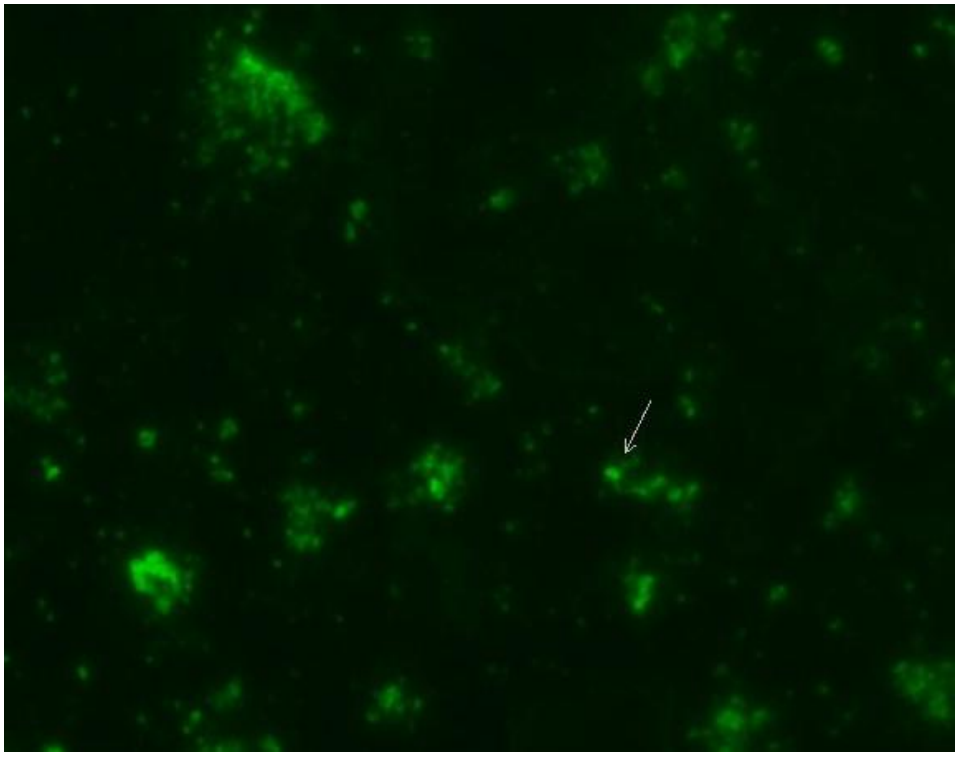
Figure 6.5: Three-dimensional view of possible antigenic epitopes

The microarray epitope spots that were identified as potentially recognised antigenic peptides are highlighted (blue) within the structure of PsaA.

6.3.6. Immunofluorescence

Immunofluorescence proved difficult for several reasons. It was almost impossible to fix intact pneumococcus onto the slide wells. Several approached were unsuccessful: a heavy versus a light suspension, a direct smear versus a suspension in PBS, heat fixation versus methanol fixation and overnight growth from 5% sheep blood (SBA) agar versus a young 6 hour growth in Todd-Hewitt broth in 5% CO_2 at 37°C. The best result was obtained by using an overnight growth on SBA, making a heavy suspension in PBS and using heat fixation. Bright apple green fluorescence was observed for the wells coated with *S. pneumoniae*, although individual bacterial cells of characteristic morphology were difficult to identify. For all other bacterial strains fluorescence was either completely lacking or weak and attributed to background staining. In order to investigate the reason for the lack of identifiable characteristic pneumococcal cells the slides were all washes in PBS and counterstained with a Gram stain. The Gram stain revealed a good cellular layer for all but the pneumococcal smears. In the case of the pneumococcal smears as few as 1 or 2 bacteria were identified per high power field, but the background showed a dense layer of Gram-negative proteinaceous material. Even the individual pneumococci varied in their morphology suggestive of being affected either by osmotic pressure due to the suspension in PBS or the age of the bacteria. Several decolourised pneumococci could be identified and the Gram-negative background most likely represented lysed bacterial cell debris. **Figures 6.6** a) and b) show examples of the immunofluorescent findings suggestive of specific binding of PSAA-13 to *S. pneumoniae* ATCC 49617. It cannot be concluded whether the antibody truly binds to intact pneumococcal cells, but it appears to bind to exposed PsaA once cell lysis has occurred.

a)



b)

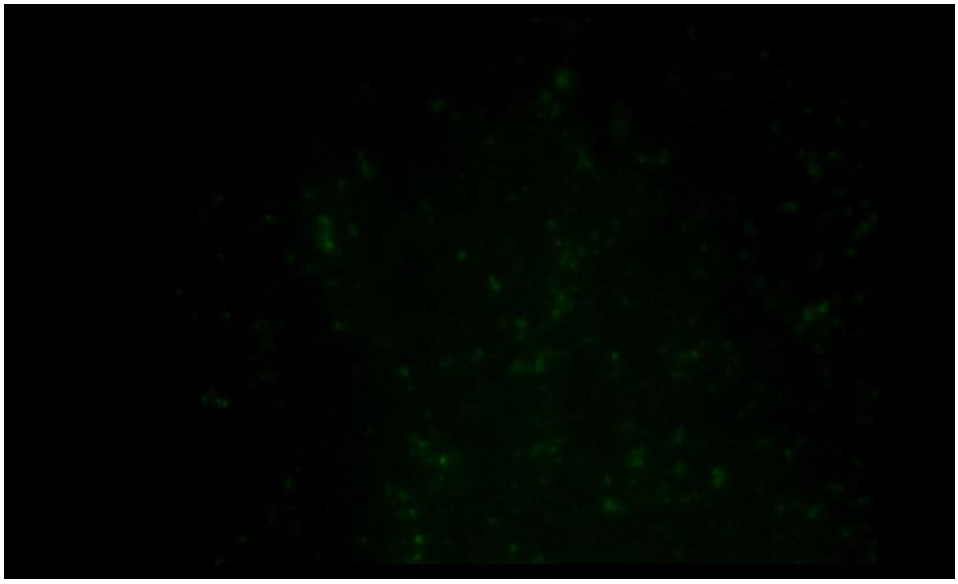


Figure 6.6: Immunofluorescence to demonstrate binding of PSAA-13 to pneumococcus

a) Fluorescent staining (apple green) of *S. pneumoniae* cellular material; possible identification of a bacterial cell (arrow); b) Negative control (*S. aureus* ATCC 29213).

6.3.7. Synthetic peptides

The synthetic peptides, #134 (QDTNIPIYAQIFT) and #146 (SYYSMMKYNLDKI) were analysed at 1mg/mL in assay buffer in a competitive ELISA with PSAA-13 and PSAA-4 (at 1:10 and 1:100 in assay buffer) as competitor against 1:100 pneumococcal suspension in gHCl. No competition occurred.

6.3.8. Immunodot blot analysis

All pneumococcal serotypes included in the testing were recognised by all four MAbs and both polyclonal antisera. **Figure 6.7** a) Illustrates the immunodot blot layout and b) illustrates the reactive dots demonstrated using with PSAA-4 in a pattern representative for all other MAbs and polyclonal antisera. **Table 6.4** illustrates the immunoreactivity of all MAbs and both polyclonal antisera to selected bacteria known to be capable of inhabiting the human respiratory tract either as a commensal or a pathogen. All antibodies reacted strongly against the three pneumococcal ATCC strains. Weak reactions of the MAbs were observed with *L. pneumophila*, *B. cepacia* and *S. pyogenes*. Equally weak reaction of the polyclonal antisera could be demonstrated against *H. influenzae*, *H. parainfluenzae*, *L. longbeachae* and *S. mutans* strain KIR.

a)

1N	2	3	4L	5	6A	6B	7A	7B
7C	7F	8	9A	9L	9V	9N	10A	10B
11A	11B	11F	11C	12f	13L	14L	15A	15B
15C	15F	17A	17F	18A	18B	18C	18F	19A
19B	19C	19F	22A	20L	22F	23A	23B	23F
24A	24B	24F	25L	33A	33B	33C	33F	E. faecalis ATCC 29212

b)

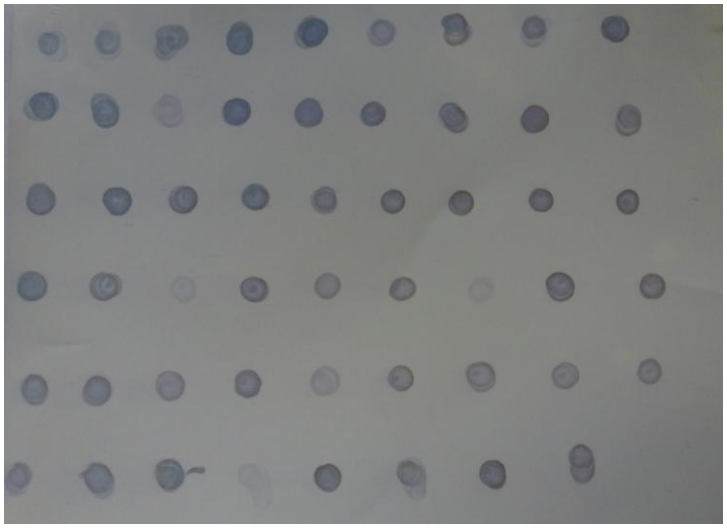


Figure 6.7: Immunodot blot analysis of PSAA-4 against pneumococcal serotypes

a) Layout of individual serotype dots, and b) of immunodot blot reactions. *E. faecalis* ATCC 29212 is included as a negative control and shows no signal.

Table 6.4: Immunoreactivity in immunodot blots of four MAbs and two polyclonal antisera against rPsaA to a selection of bacterial organisms

Organism (ATCC/NCTC No.)	**PSAA-4**	**PSAA-6**	**PSAA-13**	**PSAA-15**	**K34**	**K35**
S. pneumoniae (33400)	+	+	+	+	+	+
S. pneumoniae (49619)	+	+	+	+	+	+
S. pneumoniae (49617)	+	+	+	+	+	+
H. influenzae	-	-	-	-	weak +	weak +
H. parainfluenzae	-	-	-	-	weak +	weak +
L. pneumophila	weak +	weak +	weak +	weak +	-	-
L. longbeachae	-	-	-	-	weak +	weak +
B. cepacia	weak +	weak +	weak +	weak +	-	-
P. aeruginosa	-	-	-	-	-	-
N.meningitidis	-	-	-	-	+	+
E. aerogenes	-	-	-	-	-	-
E. cloacae	-	-	-	-	-	-
S. pyogenes (49117)	-	-	-	-	-	-
S. pyogenes (12344)	weak +	weak +	weak +	weak +	-	-
S. pyogenes (19615)	-	-	-	-	-	-
S. pyogenes (21546)	-	-	-	-	-	-
S. pyogenes (8322)	-	-	-	-	-	-
S. pyogenes (21547)	-	-	-	-	-	-
S. agalactiae (13813)	-	-	-	-	-	-
S. agalactiae (12386)	-	-	-	-	-	-
E. faecalis (29212)	-	-	-	-	-	-
E. faecalis (14506)	-	-	-	-	-	-
E. coli	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-
B. pertussis	-	-	-	-	-	-
B. parapertussis	-	-	-	-	-	-
A. baumanii	-	-	-	-	-	-
S. anginosus	-	-	-	-	-	-
C. diphtheriae	-	-	-	-	-	-
A. haemolyticum	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-
S. mutans (Strain AHT)	-	-	-	-	-	-
S. mutans (Strain KIR)	-	-	-	-	weak +	weak +
S. mutans (25175)	-	-	-	-	-	-
S. salivarius (Strain 57 I)	-	-	-	-	-	-
S. salivarius (13419)	-	-	-	-	-	-
S. salivarius (7073)	-	-	-	-	-	-
S. sanguinis (Strain OPAI)	-	-	-	-	-	-
S. sanguinis (10556)	-	-	-	-	-	-
S. sanguinis (Strain J9)	-	-	-	-	-	-

6.3.9. Western Blot Analysis

Western blot analysis of all four MAbs against pneumococcal (*S. pneumoniae* ATCC 49617) suspension, lysate and rPsaA demonstrated a band at 37kDa (**Figure 6.8**).The specificity of the reactions observed in the immunodot blots was further investigated by Western blot. All four MAbs and K34 and 35 were tested against a selection of pneumococcal serotypes: 3,33C, 9A and 24F. *E. faecalis* 29212 and rPsaA were included as negative and positive controls, respectively. All reacted in the representative fashion demonstrating reactivity with the four selected pneumococcal serotypes (**Figure 6.9**).

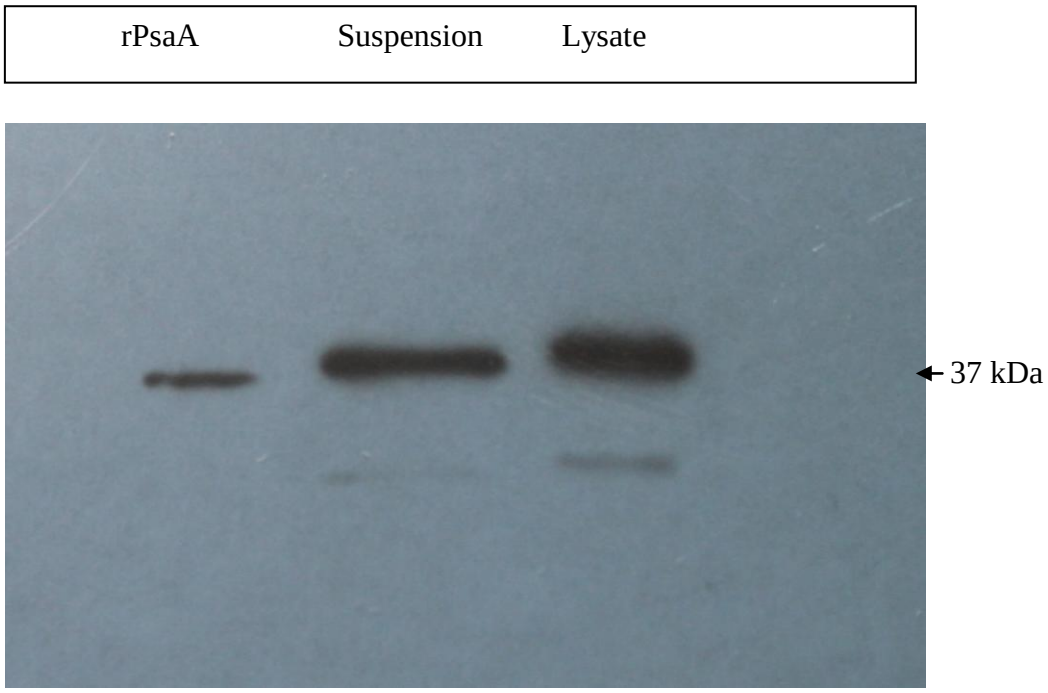


Figure 6.8: Western blot of PSAA-13 against rPsaA and pneumococcal antigen

S. pneumoniae ATCC 49617 was used to produce the pneumococcal lysate and suspension to provide the antigen basis for the Western blot.

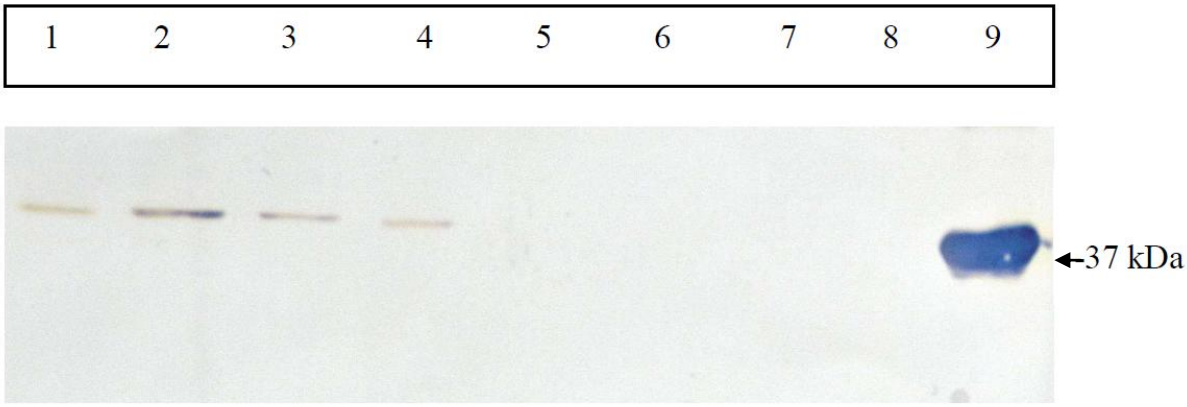


Figure 6.9: Western blot analysis of PSAA-15 against selected pneumococcal serotypes and other bacterial species

Lane 1-4: serotypes 3, 33C, 9A and 24F; lane 5-7: empty; lane 8: *E. faecalis* 29212; lane 9: rPsaA.

To further evaluate specificity against other organisms *S. mitis* ATCC 9811, *S. oralis* NZ isolate SID 08/60, *S. pseudopneumoniae* ATCC BAA-960 were included in the Western blot analysis as they are the most likely species to cross-react with antisera raised against pneumococcal proteins due to their close genetic relationship. They were tested together with those bacterial isolates that had caused weak reactions in the immunodot blots with the MAbs: *B. cepacia, L. pneumophila, L. longbeachae, S. pyogenes* ATCC 12344; rPsaA was included as positive control. All MAbs reacted weakly with *S. pseudopneumoniae* ATCC BAA-960 and rPsaA. No visible reaction could be demonstrated with the other bacterial isolates (**Figure 6.10** a).

The polyclonal sera, K34 and K 35, were tested against *S. mitis* ATCC 9811, *S. oralis* NZ isolate SID 08/60, *S. pseudopneumoniae* ATCC BAA-960, as well as *S. mutans*, strain KIR, *L. longbeachae, N. meningitidis, H. parainfluenzae, H. influenzae*, again using rPsaA as positive control. Visible reactions were observed with *S. oralis* NZ isolate SID 08/60, *S. pseudopneumoniae* ATCC BAA-960 at the expected height of the 37 kDa protein, no visible band was demonstrated with *S. mitis* ATCC 9811 or *S. mutans*, strain KIR. *L. longbeachae, N. meningitidis, H. parainfluenzae* and *H. influenzae* produced a non-specific banding pattern (**Figure 6.10** b).

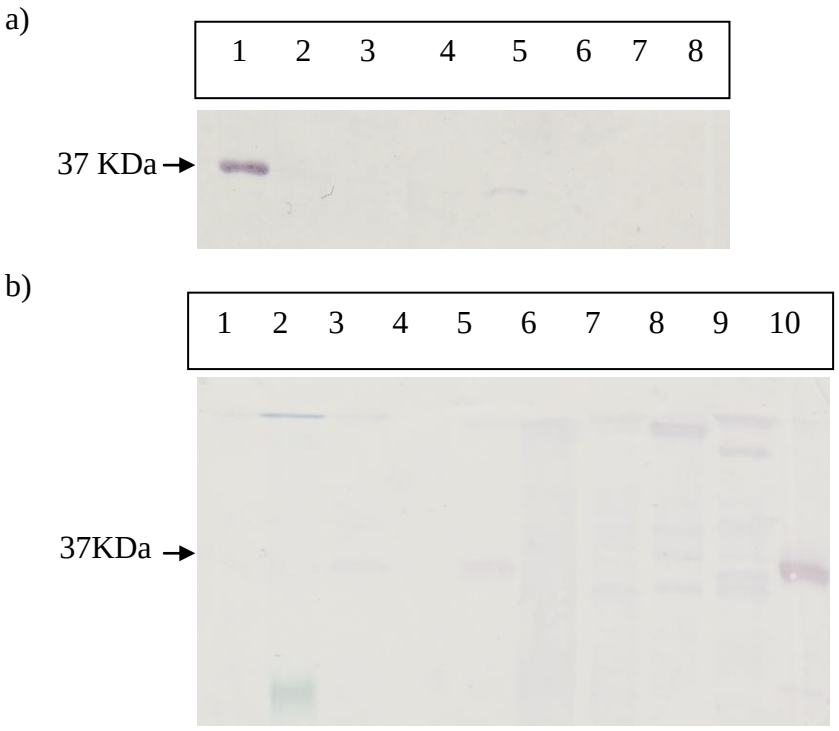


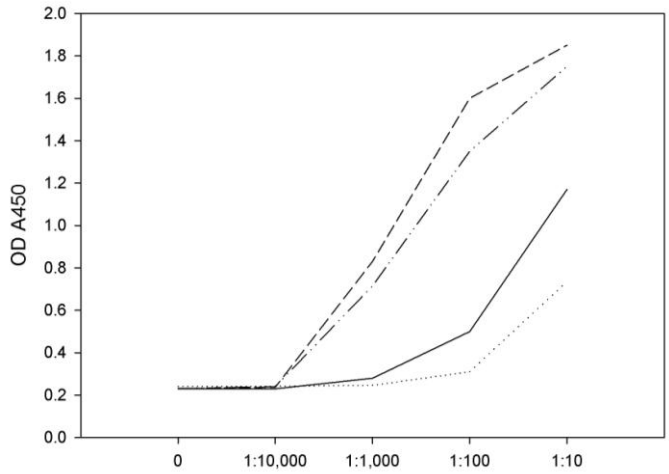
Figure 6.10: Western blot analysis of antibody reactivity to various bacteria

a) Monoclonal antibody (PSAA-4) tested against other bacterial species. Lane 1: rPsaA; lane 2: *B. cepacia*; lane 3: *L. pneumophila*; lane 4: *L. longbeachae*; lane 5: *S. pseudopneumoniae*; lane 6: *S. oralis*; lane 7: *S. mitis*; lane 8: *S. pyogenes* ATCC 12344; b) Polyclonal antiserum (K34) tested against other bacterial species. Lane 1: *S. mutans*, strain KIR; lane 2: marker; lane 3: *S. pseudopneumoniae*; lane 4: *S. mitis*; lane 5: *S. oralis*; lane 6: *L. longbeachae*; lane 7: *N. meningitidis*; lane 8: *H. parainfluenzae*; lane 9: *H. influenzae*; lane 10: rPsaA.

6.3.10. ELISA immune-reactivity studies

All four non-biotinylated MAbs and both polyclonal antisera were tested in an ELISA coated with antigen (10-fold dilutions of pneumococcal lysate and suspension in guanidine HCl) in duplicates. All MAbs produced a signal curve. PSAA-4 and 13 gave the strongest signal, with 13 being slightly stronger. All MAbs produced a stronger signal on suspension than lysate. The curves produced by MAbs supernatant and non-biotinylated purified antibody fractions on ELISA wells coated with 10-fold dilutions of pneumococcal suspension) are shown in **Figures 6.11** a) and b). All four MAbs tested against recombinant PsaA produced a strong signal. A comparison of reactivity of all MAbs' supernatant (SN) and antibodies raised against PsaA provided by the CDC (CDC PC and CDC MC) against capture antigens is shown in **Figure 6.12**. MAb SN (1:20), CDC PC and MC were used at 1:500. The coating of antigens was at 1:100 dilutions.

a)



b)

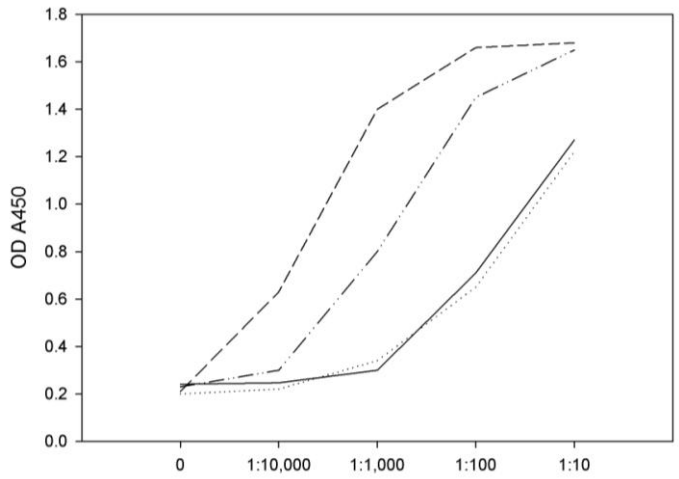


Figure 6.11: Reaction of four MAbs in an ELISA with antigen coated wells

a) Supernatant and b) the non-biotinylated purified fractions of the MAbs (Lines: solid: PSSA-6; dashed: PSAA-13; dotted: PSAA-15; dash-double dot: PSAA-4). Demonstration of decreasing OD A450 with increasing antigen dilutions in the wells. PSAA-13 demonstrates the best absorbance signals.

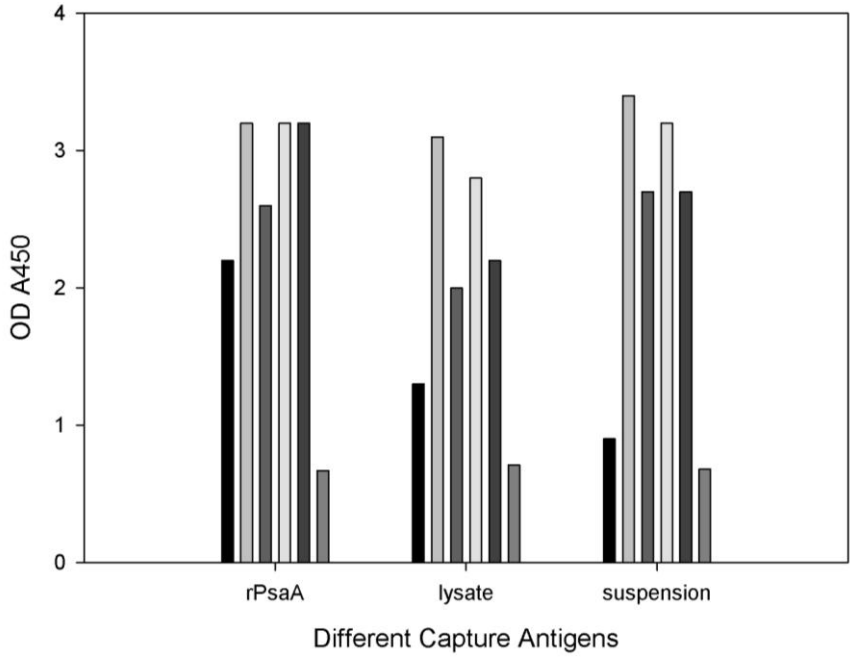
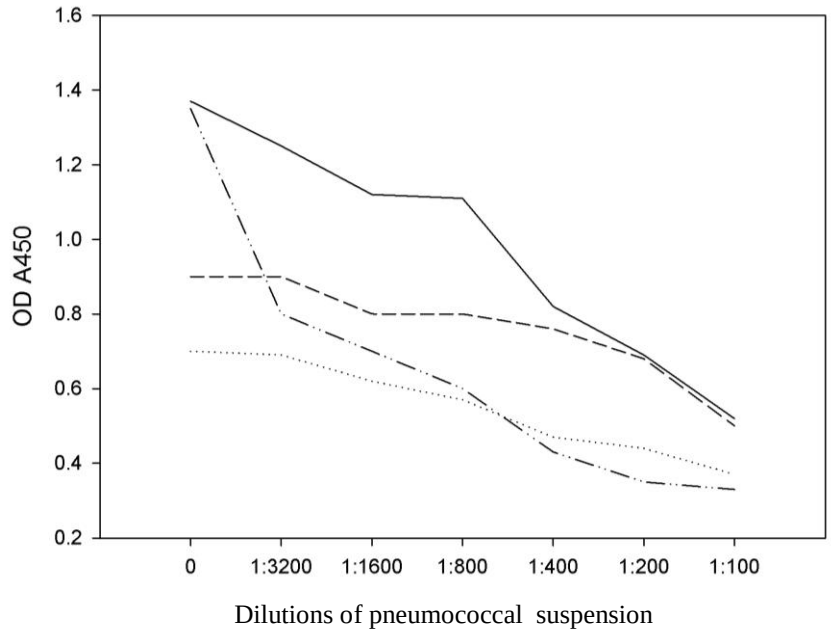


Figure 6.12: Absorbance measured when different antibodies reacted with different antigens

Recombinant PsaA, pneumococcal lysate and suspension were used as capture antigens to test the reactivity of the four Mabs as well as a monoclonal and polyclonal antibody specific to PsaA obtained from CDC. PSAA-13 shows the highest signal against all three antigens. Columns in greyscale from left to right: PSAA-15, PSAA-13, PSAA-6, PSAA-4, CDC MC, CDC PC.

Lysate and suspension coated plates were used for a competitive ELISA using suspension, lysate and rPsaA as competitors. Using suspension as the competitor produced best results for all antibodies on lysate and suspension coating. Recombinant PsaA performed equally well, but lysate did not produce a satisfactory curve. Results with PSAA MAbs and CDC antibodies on suspension coated plates are shown in **Figure 6.13** a) and b), respectively.

a)



b)

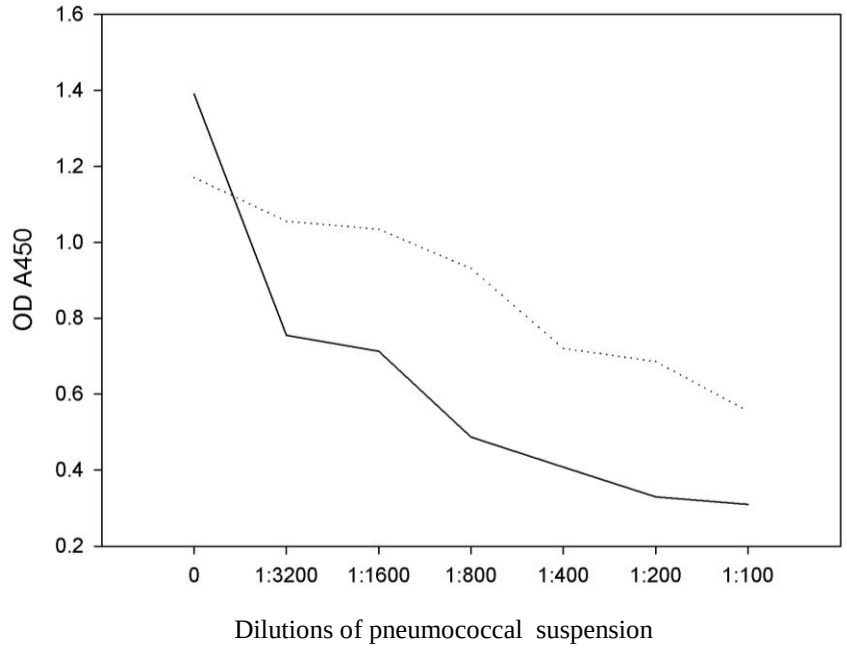


Figure 6.13: Competition ELISA with pneumococcal suspension as the competitor

a) MAb PSAA-13 produced the most convincing competition curve of the four MAbs (Lines: solid: PSSA-4; dashed: PSAA-6; dotted: PSAA-15; dash-double dot: PSAA-13). b) The polyclonal CDC antibody performed better than the monoclonal antibody (solid line: CDC PC; dotted line: CDC MC).

After biotinylation the performance of the MAbs was checked against lysate as capture antigen. The result is summarised in **Figure 6.14**. Performance of PSAA-13 is superior to other MAbs.

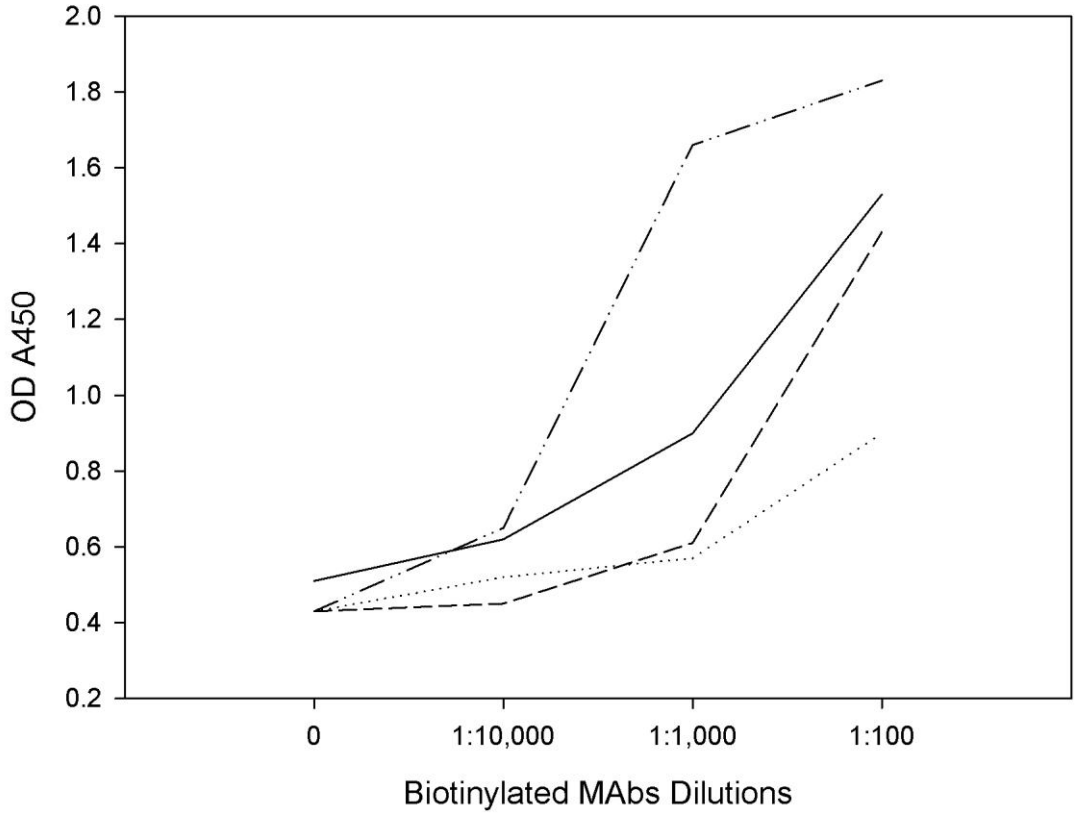


Figure 6.14: Reactivity of biotinylated MAbs with pneumococcal lysate

Pneumococcal lysate acted as capture antigen. Biotinylated PSAA-13 reacted strongest with the antigen as demonstrated by the higher absorbance signals at lower dilutions. Lines: solid: PSSA-4; dashed: PSAA-15; dotted: PSAA-6; dash-double dot: PSAA-13.

Non-biotinylated PSAA-13 was used for coating at a dilution of 1:300 in PBS. Pneumococcal lysate 2-fold dilutions were added followed by all 4 biotinylated MAbs (1:100). As anticipated there was no reaction with biotinylated PSAA-13, but a good signal curve with PSAA-4, less so with PSAA- 6 and 15 (**Figure 6.15**). The experiment was repeated with PSAA-4, 6 and 15 as capture antibodies, but neither PSAA-6 nor 15 produced a signal curve. Best results were achieved with biotinylated PSAA-4 on non-biotinylated PSAA-13.

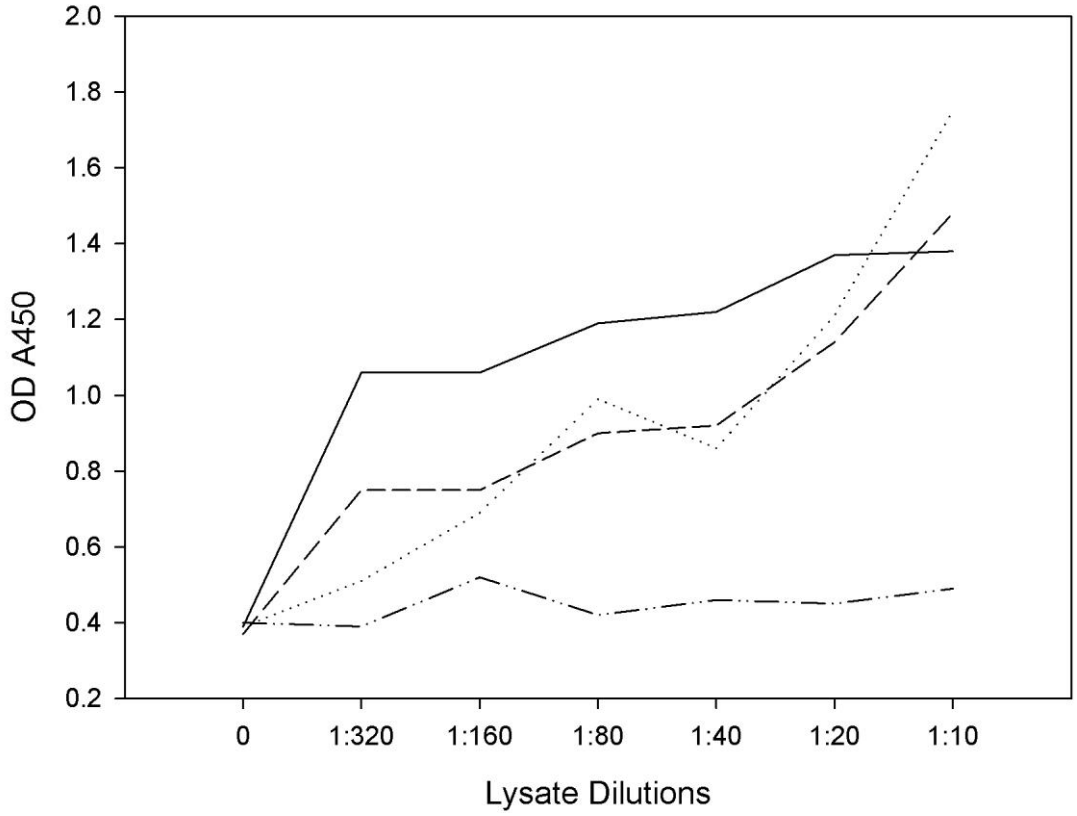


Figure 6.15: Biotinylated MAbs tested against non-biotinylated PSAA-13

Best absorbance signal curve was obtained with PSAA-4 against PSAA-13 as capture antibody. As expected, no signal curve observed with biotinylated PSAA-13. Lines: solid: PSSA-6; dashed: PSAA-15; dotted: PSAA-4; dash-double dot: PSAA-13.

Both polyclonal antisera reacted strongly with lysate and suspension, but produced a stronger signal using suspension and in a dilution curve of 10-fold dilutions from 1:10 to 1:1,000. Overall the signal was stronger on suspension for both, MAbs and polyclonals indicating either better access to the epitope or a higher concentration of epitope in suspension. The best signal obtained on lysate occurred at a 1:100 dilution and not at 1:10 and this was a consistent finding (**Figure 6.16**).

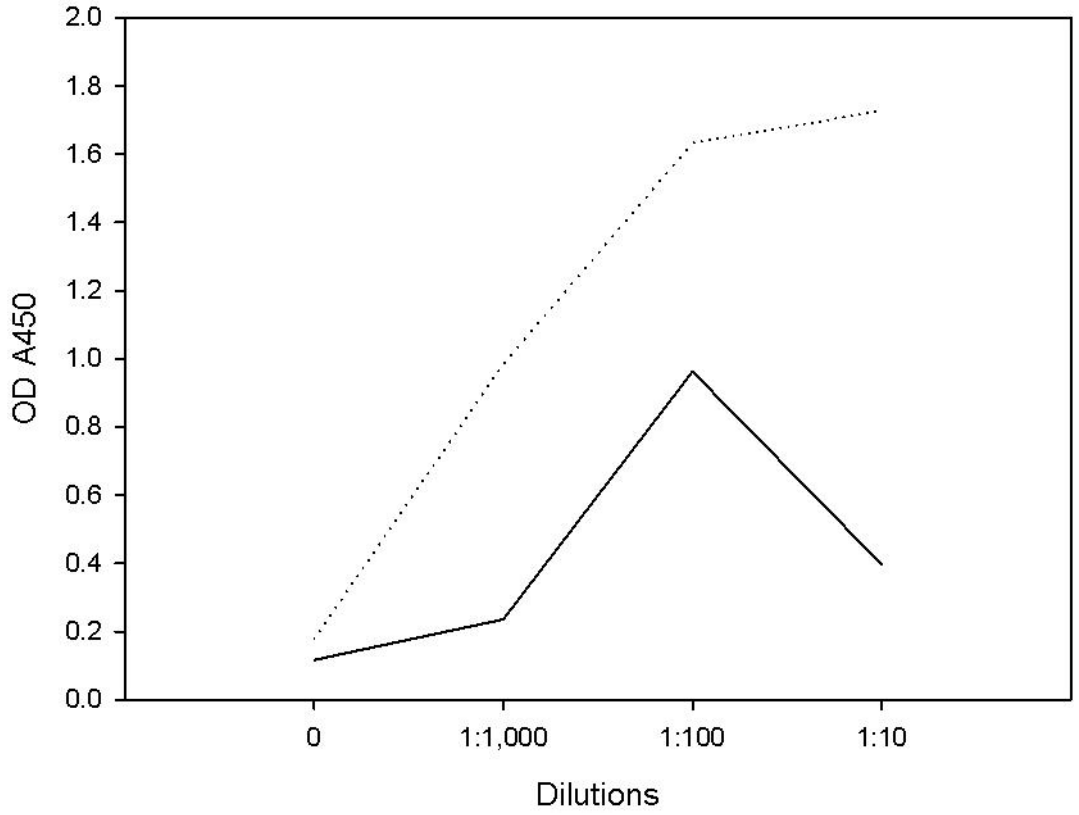


Figure 6.16: Comparison of reactivity of polyclonal antiserum to pneumococcal lysate and suspension

Reactivity of polyclonal antiserum K 34 against pneumococcal lysate (straight line) and suspension (dotted line) dilution is graphed and demonstrates higher absorbance signals with suspension and overall lower signals for lysate. Additionally the signal at 1:100 is superior to 1:10 with lysate.

6.4. Discussion

Antigen detection tests as well as serological studies to aid the diagnosis of IPD have been of variable success in the past [258, 259]. In the present study four MAbs and two polyclonal antisera were produced against rPsaA. They were characterised with the intention to assess their suitability for a diagnostic antigen detection assay. With regards to being a suitable antibody for pneumococcal antigen detection in cases of IPD the antibody should provide the following prerequisites: (i) binding to preferably all different pneumococcal serotypes, (ii) highly specific binding (minimal or no cross-reactivity with other closely related streptococcal species) and (iii) binding to antigen in specimens from normally sterile sites, including serum.

All four MAbs isotyped as IgG1 kappa and underwent peptide microarray analysis to identify the potential antigenic epitope. Two epitopes that showed the highest fluorescence on the triplicate microarrays were selected and peptides were purchased for further testing. The arrays were spotted in triplicate on each slide and contain overlapping peptides, derived from the amino acid sequence of an antigen, in this case PsaA [363]. The PsaA sequence is synthesised as linear peptides of 13 amino acids each covalently bound to a continuous cellulose membrane by the C-terminus [364, 365].

The inherent issue with these specifically manufactured peptide arrays in identifying antigenic epitopes is that they are meant to mimic native protein, but conformational change when bound to a solid-phase is likely. Antigenic epitopes can be linear (continuous) within one part of the antigen structure, usually no more than 15 amino acids in length, or they can be discontinuous (conformational) and stretch over several binding regions. The latter will form a composite antigenic epitope that is unlikely to be obvious on a linearised peptide array. Even if part of the composite epitope reacts with the synthetic peptide on the array the affinity of binding with the MAb might be too low to be picked up as a signal in an ELISA. Therefore the lack of reactivity of the two selected synthetic peptides that were manufactured and tested in solution in a competitive ELISA against the four MAbs does not necessarily exclude MAb binding to some partial epitope. In fact, peptide #134 covers partially the sequence of peptide P4, which is considered part of a functional epitope of PsaA mediating adherence to nasopharyngeal epithelial cells [97, 361]. Our results signal that the MAbs are unlikely to be produced against a linear 15-mer antigenic epitope, however, they may well target at least part of the functional epitope of PsaA, which spans over at least 28 amino acids (residues 251-278) [361].

Analysis of the MAbs and polyclonal antibodies in immunodot blots, Western blots and ELISA revealed the unequivocal specific binding of the four MAbs, and at the very least the PSAA-13 epitope is distinct from the others.

As previously mentioned, the pneumococcal suspension produced a higher signal at the same protein concentration than the pneumococcal lysate. One plausible explanation is that the epitope is more accessible in the intact organism and which is its preferred conformational state. The lower signal observed in lysate at 1:10 than at 1:100 could be the result of optimal antigen-to-antibody ratios where the "prozone-effect" of excess antigen results in restricted antibody binding sites. However there was no increase in signal at dilutions of 1:20 and 1:50 of lysate which should rule out this option. Another possible explanation is that as the lysate was obtained using Triton X 100 solubilisation antigen adsorption to the wells of the microtitre plate could be compromised by the presence of this detergent when the antigen is more concentrated whereas on dilution the effect of residual or antigen bound Triton X 100 is lessened.

The observation of the lower signal at higher lysate concentration was reproducible and unaffected by variations in other potential sources of errors in ELISA which are typically quite low [366].

Specificity studies in immunodot blots and Western blots suggest that the MAbs and the polyclonal antisera recognise all pneumococcal serotypes included in currently available vaccines. Although only a small selection of pneumococcal serotypes was confirmed by Western blot to produce a specific band to the 37kDa rPsaA it is likely that these MAbs recognise most, if not all pneumococcal serotypes. Signal intensity in the immunodot blots and the subsequent Western blot, albeit consistency of bacterial suspension concentration, was variable suggesting that some serotypes might have stronger affinity to the MAbs than others. Further studies are warranted. The only species other than pneumococcus that the MAbs reacted with by Western blotting was *S. pseudopneumoniae*. It is somewhat surprising that there was no cross-reactivity to *S. mitis* and *S. oralis* as this has been described before [367]. However, an unequivocal interpretation of this finding cannot be made due to the limited number of available typed isolates of these bacterial strains. In addition, despite the attempt to standardise bacterial concentrations across all strains in the suspensions and lysates used it is likely that neither measuring the protein concentrations by Bradford assay nor measuring the optical density will ensure more than an estimate of bacterial concentration. The possibility of variation in concentration could also account for detection or non-detection of some species in the different immune assays. The polyclonal cross-reactivity in the Western blot analysis to *S.*

oralis and *S. pseudopneumoniae* is not surprising, but the lack of reaction to *S. mitis* is. Again, testing of further type strains of these species is warranted.

The results of the cross-reactivity studies performed during this project are consistent with the genetic similarity of some of the organisms. The signal produced by PSAA-13 against the suspension of *S. pseudopneumoniae* in the ELISA and Western blot suggests not only the presence of PsaA, but a similar conformational epitope leading to binding, but likely at a lesser affinity than to *S. pneumoniae*. The other streptococcal species either lack sufficient sequence homology at the PsaA sequence or have a different epitope structure and therefore lack demonstrable binding in an ELISA. Both polyclonal antisera gave high signal in the immunodot blots to all three ATCC strains of *S. pneumoniae* tested as well as weak reactions to *S. mutans* (strain KIR) and *L. longbeachae*, *H. parainfluenzae* and *H. influenzae* (**Table 6.4**). These weak reactions in the immunodot blot were confirmed as non-specific by Western blot. However, Western blot demonstrated a cross reactivity with *S. pseudopneumoniae* and *S. oralis* (**Figure 6.10** b)). The non-specific nature of the reactivity of the polylonal antisera to other bacterial species can be explained by the fact that both, antigen and antibody are multivalent and less specific than monoclonal antibodies. However, this non-specific response does not necessarily preclude their use in a diagnostic assay, for example as a capture antibody.

S. mitis, *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, *S. cristatus*, *S. oralis*, *S. infantis*, *S. peroris* and *S. pneumoniae* as well as *S. pseudopneumoniae* are frequent members of the commensal nasopharyngeal flora and have a close genetic relationship [220, 283, 368]. A blast nucleotide sequence search revealed 100% sequence homology for *psaA* for *S. pneumoniae*, *S. oralis*, *S. mitis*, *S. sanguinis*, *S. infantis* and 89% for *S. gordonii* and *S. equi* subspecies *zooepidemicus* and 87% for *S. uberis* (http://blast.ncbi.nlm.nih.gov). The fact that *S. pseudopneumoniae* has not come up as an organism with sequence homology at *psaA* level during the blast search is likely due to the lack of nucleotide sequence submissions of this relatively newly described organism.

In conclusion, MAbs as well as polyclonal antibodies have successfully been raised to rPsaA. The MAbs appear to have a selective specificity to *S. pneumoniae* and one tested strain of *S. pseudopneumoniae*. The reactivity to *S. pseudopneumoniae* and lack of reactivity to *S. mitis* and *S. oralis* warrants further investigation. Determination of the sensitivity of the antibodies in a sandwich ELISA and investigation into the performance of the antibody binding in serum specimens are described in Chapter 7. Specimens other than serum were determined to be outside the scope of this study, their evaluation would be of future interest.

CHAPTER 7

DEVELOPMENT AND EVALUATION OF A PNEUMOCOCCAL SURFACE ADHESIN A ANTIGEN DETECTION TEST

7.1. Introduction

As already alluded to in the introduction of Chapter 6, questions were raised by Jedrzejas [103] as to the surface accessibility of PsaA and hence its accessibility for antibodies. Russell *et al.* had demonstrated reactivity of a MAb with pneumococcal PsaA [355] in an immunofluorescence experiment, but Johnston *et al* could only show binding of antibodies to PsaA if the bacterial cell wall was removed first [359]. Both researchers used different experimental designs. In the light of the protein's importance in bacterial adherence to host cells and virulence [352, 353, 369] it is more than likely that PsaA is exposed at the bacterial surface. It is known that the protein is immunogenic [370] and convalescent antibody responses in humans rise after patients recover from IPD [371]. In his experiments, Johnson immunised mice with multi-antigenic peptides (MAPs) made with the putative binding domains of PsaA followed by challenge with selected pneumococcal serotypes. He reported a reduction in nasopharyngeal carriage for the selected serotypes and high antibody titers to PsaA and the MAPs. These experiments supported the earlier finding that intranasal inoculation of mice with PsaA in combination with PspA elicited protection against pneumococcal carriage [372].

In humans, high anti-PsaA antibody titers were found to be present in young children following pneumococcal colonisation [373] and specific PsaA antibody responses could be demonstrated in infants after AOM [374].

PsaA's common occurrence in all known pneumococcal serotypes as well as its ability to elicit antibody responses in carriers as well as in symptomatic individuals makes it a potential candidate for vaccine as well as for diagnostic test development [354].

As described in Chapter 6, the antibodies raised against rPsaA appeared suitable for use in a diagnostic assay for the detection of native PsaA in patient specimens. As differentiating between colonisation and IPD is considered a major problem in pneumococcal diagnostics, it was decided to evaluate the assay on a specimen type most likely to be indicative of IPD if positive. Hence, the assay was evaluated on serum and plasma of patients with CAP.

7.2. Material and Methods

7.2.1. Materials

7.2.1.1. Recombinant PsaA

Recombinant pneumococcal surface adhesin A (rPsaA) (section 6.2) was used to raise monoclonal and polyclonal antisera.

7.2.1.2. Monoclonal antibody to rPsaA

Monoclonal antibodies to rPsaA were obtained by immunising male RBF/DN mice as described in section 6.2.2. The monoclonal antibodies were aliquotted and stored at -80°C.

7.2.1.3. Polyclonal antibodies

Two NZ white rabbits were immunised to raise polyclonal antisera to rPsaA as described in section 6.2.1.

7.2.1.4. Phosphate buffer

6.9mM NaH_2PO_4 x H_2O

3.1mM Na_2HPO_4 anhydrous

Adjust to pH 6.5.

7.2.1.5. Patient samples

Three hundred and four patient serum or plasma samples were tested in the PsaA ELISA. Out of those 298 were serum samples and 6 plasma samples (since there was no serum available for testing on those patients). The specimen had been collected during a CAP study from July 1999 until July 2000 (section 2.2.2.12).

7.2.2. Methods

7.2.2.1. Purification of polyclonal antibody

One polyclonal antibody, K34, was selected arbitrarily to be purified for use in the diagnostic assay (both polyclonal antisera showed strong signals against pneumococcal lysate and rPsaA and the titre against rPsaA exceeded 1:100,000).

Purification was performed through use of a Sephadex column. Undialysed rabbit antiserum was subjected to ion-exchange chromatography on a DEAE-Sephadex A50 column equilibrated with 10mM phosphate buffer, pH 6.5. The unbound material was washed off the column and predominantly contained IgG [343].

Column height was 13cm, diameter 3cm. Ten mL of rabbit K34 serum was added to 90mL of gel.

Seven fractions were collected and checked for their reactivity with anti-rabbit HRP. Subsequently the specific reactivity of the fractions was tested in an ELISA using pneumococcal suspension (ATCC 49617) at 7.5µg/well and *S. aureus* suspensions (ATCC 29213) at 7.5µg/well as a negative control.

The selected fractions to be used in the development of a diagnostic assay were pooled and the concentration measured at 280nm (Cary 50 v 3.0).

7.2.2.2. ELISA optimisation

Polyclonal K34 antiserum was selected as the capture antibody to be coated on the microtiter plate. The selection of the polyclonal, versus one of the four MAbs as capture antibody was based on the fact that Mabs supernatant due to the presence of 10% fetal calf serum was unsuitable for coating to ELISA plate wells and purification of polyclonal antisera proved less complicated than large scale purification of the MAbs. In order to determine the optimal capture antibody and second antibody concentrations, a two step matrix experiment was performed. In the first step, the microtiter plate was coated with three different concentrations of polyclonal antibody as outlined in the diagram in **Figure 7.1**. *S. pneumoniae* and *S. aureus* suspensions and lysates were made up as described in sections 2.2.2.10 and 2.2.2.11 and were added at 5µg/well and then diluted out in doubling dilutions.

Two plates were set up with each *S. pneumoniae* and *S. aureus* suspensions and two with lysates and the suspension/lysate pairs were blocked with assay buffer for 15 min and

with x4 assay buffer ("super-block") for 15 min. Incubation times for antigens and antibodies were consistently kept at 1 hour. The detection antibody HRP-conjugated rat anti-mouse IgG1 (BD Biosciences, Franklin Lakes, NJ, USA) was used at a 1:2,000 dilution.

In the second step of the matrix experiment, the microtiter plate was coated with a constant amount of polyclonal antibody, either 8µg/mL for use of suspensions (2.5µg/well) as the antigen or 1µg/mL when using lysates (0.3µg/well). The concentration of the second antibody was checked in doubling dilutions starting from 1:10 (**Figure 7.2**). The graphed data are based on means of absorbance value duplicates.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A 8µg/mL capture	**5µg/well SPn Suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
B 4µg/mL capture	**5µg/well SPn suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
C 1µg/mL capture	**5µg/well SPn suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
D Ø capture	**5µg/well SPn suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
E 8µg/mL capture	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
F 4µg/mL capture	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
G 1µg/mL capture	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
H Ø capture	**5µg/well *S. aureus* suspension/lysate**	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048

Figure 7.1: Optimisation of polyclonal antibody concentrations as capture antibody

Layout of the ELISA plate well coating with varying concentrations of polyclonal antibody against a constant concentration of pneumococcal suspension or lysate (positive control) and *S. aureus* suspension or lysate (negative control).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	PSAA-13	**PSAA-13**	**PSAA-13**	**PSAA-13**	**PSAA-13**	**PSAA-13**	**PSAA-6**	**PSAA-6**	**PSAA-6**	**PSAA-6**	**PSAA-6**	**PSAA-6**
A 8µg/mL or 1µg/mL capture	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
B 8µg/mL or 1µg/mL capture	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
C 8µg/mL or 1µg/mL capture	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
D Ø capture	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
E 8µg/mL or 1µg/mL capture	**PSAA-15** 1:10	**PSAA-15** 1:20	**PSAA-15** 1:40	**PSAA-15** 1:80	**PSAA-15** 1:160	**PSAA-15** 1:320	**PSAA-4** 1:10	**PSAA-4** 1:20	**PSAA-4** 1:40	**PSAA-4** 1:80	**PSAA-4** 1:160	**PSAA-4** 1:320
F 8µg/mL or 1µg/mL capture	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
G 8µg/mL or 1µg/mL capture	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
H Ø capture	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320

Figure 7.2: Optimisation of MAbs concentrations as 2nd antibody

Layout of the ELISA plate well coating with a constant concentration of polyclonal antibody as capture antibody (either 8 or 1 µg/mL or no capture antibody as in rows D and H) against pneumococcal antigen (in rows A, B, D, E, F, and H) and *S. aureus* antigen (rows C and G), either as suspension or lysate. The 2nd antibody concentrations were varied dilutions of the four Mabs.

7.2.2.3. Spiked serum and plasma preparations

A 0.5 McFarland suspension of *S. pneumoniae* ATCC 49619 was made up from an overnight growth. Suspensions and lysates were obtained as described in Chapter 2. Blood was taken from a healthy subject into plain serum tubes as well as EDTA and Lithium-Heparin tubes which were filled to the recommended volume. The samples were then

immediately spiked with pneumococcal suspension or lysate at 1:100 and 1:10, respectively. The spiked samples were mixed before clotting could occur in the plain tubes.

Samples were centrifuged at 1200 x g for 15 min. When spiked with bacterial lysate a moderate red tinge was observed in both serum and plasma samples due to the expected lysis of a proportion of erythrocytes by the lysis buffer (section 2.2.2.11). The obtained spiked serum and plasma samples were analysed at in an ELISA coated with 8µg/mL or 1µg/mL of polyclonal antibody as capture antibody.

7.2.2.4. Assay precision

The reproducibility of measurements were evaluated on spiked serum and plasma samples as described in section 7.2.2.3 as well as a negative serum sample (un-spiked). All samples were diluted 1:2 in assay buffer. Polyclonal antibody was used as capture antibody at 8µg/mL. A calibration curve of rPsaA in doubling dilutions starting at 0.025µg/mL was included in each ELISA plate run. Intra-assay coefficients of variation were determined from 12 replicates of each, EDTA plasma and serum (spiked) and un-spiked serum. Inter-assay reproducibility of measurements was determined from values obtained on three different plates and time-points of Lithium-Heparin plasma and serum (spiked) and un-spiked serum. The variability of number of replicates included in the evaluation and the use of Lithium-Heparin plasma instead of EDTA plasma in the inter-assay determination was due to the limited volumes of available samples.

7.2.2.5. Lowest limit of detection

The standard curve was established using rPsaA at a concentration of 0.025µg/mL in doubling dilutions down to 390pg/mL. The minimum detectable PsaA concentration was defined as the lowest measurable value that is statistically different from the zero value.

7.2.2.6. Statistical analysis

The statistical calculations were performed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA).

7.3. Results

7.3.1. Polyclonal antisera

Both polyclonal antisera, K34 and K35, showed strong signals against pneumococcal lysate and rPsaA. For both antisera the titre against rPsaA exceeded 1:100,000.

K34 was arbitrarily selected to be used in a diagnostic assay and post-purification fractions 1 to 7 showed strong absorbance signal against anti-rabbit IgG HRP (1:2,000) at 1: 100, 1:1,000 and 1:10,000 at 450nm. The signal was >1 at 1:100. The fractions were then tested against pneumococcal and *S. aureus* suspensions. Highest signal was obtained with fractions 1-4 with fractions 2 and 3 with the highest signals being pooled and measured. K34 pooled fractions 2 and 3 measured an OD at A280 of 1.29. The known OD A280 for 1mg/mL IgG is 1.35. The total volume of fractions 2 and 3 amounted to 20mL. At 1mg/mL the total yield from the purification process (10mL of unpurified serum) was 2g/L.

The obtained purified polyclonal antiserum was run on a Western blot to verify purity. The gel showing non-reduced and reduced anti-rPsaA IgG is shown in **Figure 7.3**.

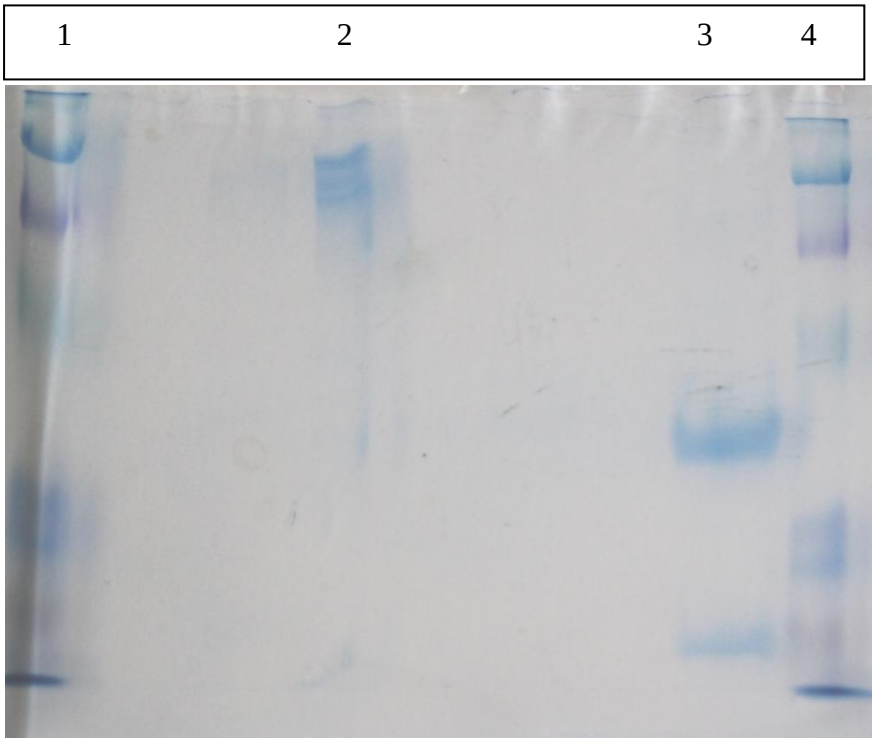


Figure 7.3: Purity check of polyclonal antiserum against rPsaA on SDS PAGE gel

Lanes 1and 4: molecular weight marker, lane 2 and 3: non-reduced and reduced polyclonal IgG against rPsaA, respectively.

7.3.2. ELISA optimisation

The first step of the optimisation demonstrated that the blocking step with either assay buffer or x4 assay buffer resulted in similar signal to noise ratios. It was decided therefore to continue to use x1 assay buffer in the diagnostic assay. In the assay using bacterial suspensions absorbance signal of >1 was obtained using 8µg/mL of polyclonal antibody as the capture antibody, when using lysate the polyclonal antibody concentration could be dropped to 1µg/mL to achieve comparable absorbance signals. The absorbance signals obtained for the first step of the matrix optimisation are graphed in **Figure. 7.4**. The absorbance signal values against *S. aureus* suspensions and lysates were at the same level as the "background" (< 0.2). In the second step of the optimisation 8µg/mL and 1µg/mL of polyclonal antibody as capture antibody were used as well as 2.5µg/well bacterial suspension and lysate, respectively, as outlined in **Figure 7.2.** PSAA-13 gave the strongest signal of all four MAbs, followed by PSAA-4, PSAA-15 and PSAA-6 (**Figure 7.5**). Again, *S. aureus* served as the negative control and had an absorbance signal of <0.2.

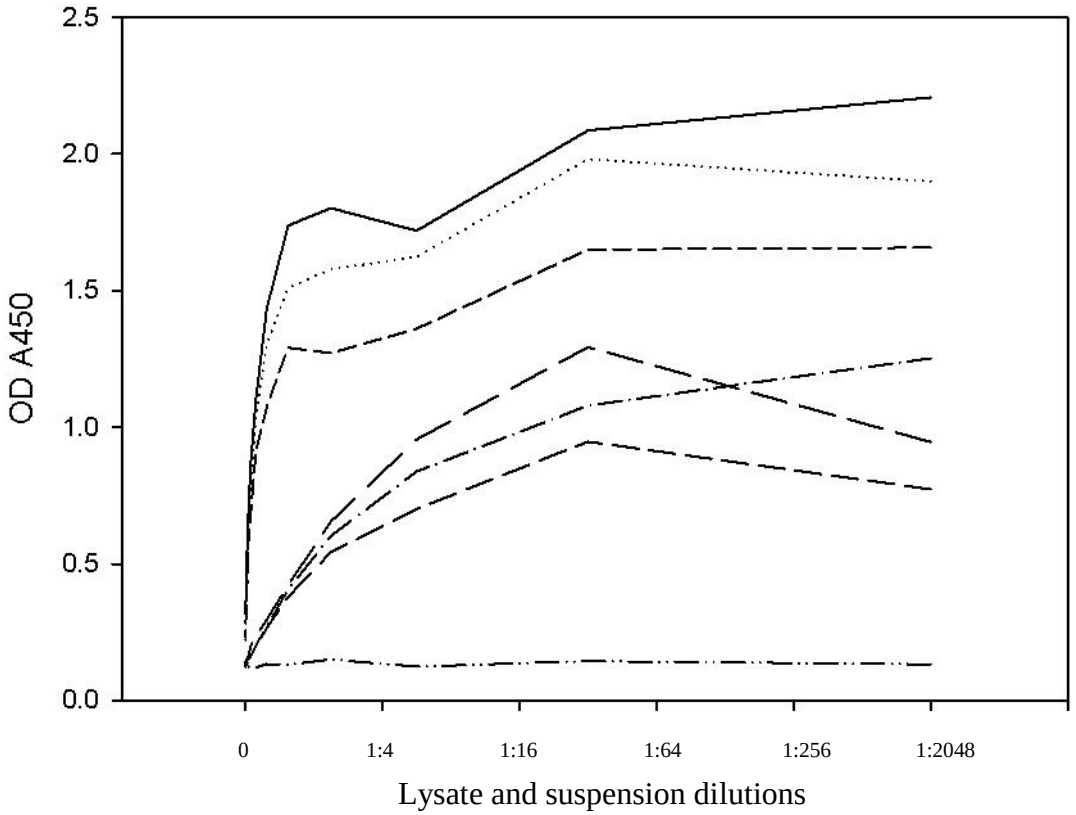


Figure 7.4: Optimisation of the polyclonal capture antibody of the PsaA ELISA

Three different capture antibody concentrations were evaluated against pneumococcal lysate dilutions: 8µg/mL (straight line), 4µg/mL (dotted line), 1µg/mL (small dashed line), and against pneumococcal suspension dilutions: 8µg/mL (large dashed line), 4µg/mL (dot dash line), 1µg/mL (medium dashed line). *S. aureus* lysate (double dot dash line) was included as a negative control. Higher values overall were obtained using lysate dilutions (top 3 curves).

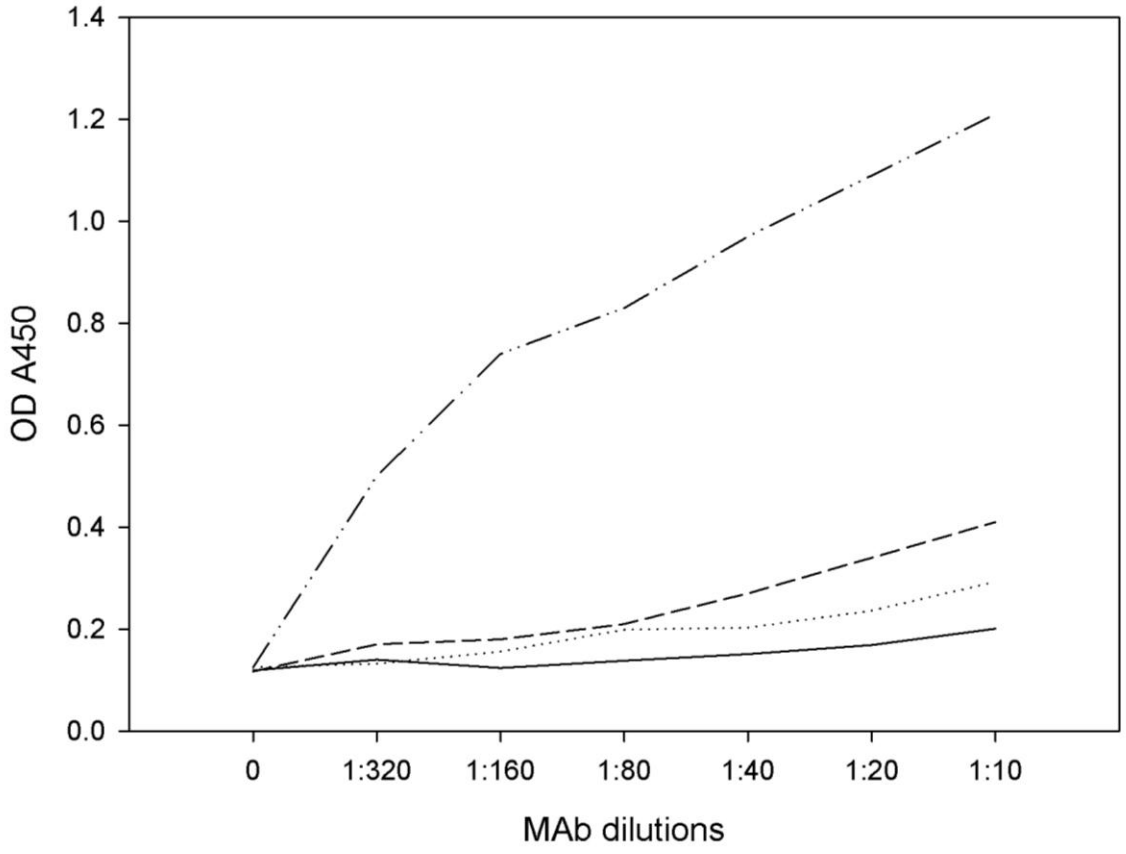


Figure 7.5: Performance of MAbs against pneumococcal lysate in the ELISA optimisation

PSAA-13 performs superior to the other three Mabs. The capture polyclonal antibody concentration used was 1µg/mL. The experiment was performed using pneumococcal lysate as the antigen. Lines: solid: PSSA-6; dashed: PSAA-4; dotted: PSAA-15; dash-double dot: PSAA-13.

In a competition ELISA, the plate was coated with 1µg/mL of pneumococcal lysate in guanidine HCl and incubated with a dilution series (1µg/mL, 1:10, 1:20 in doubling dilutions to 1:160) of 50µL/well pneumococcal lysate, suspension, rPsaA and *S. aureus* lysate as negative control and immediate addition 50µL/well of 1µg/mL polyclonal antibody and 1:10 dilution of PSAA-13.

Anti-mouse and anti-rabbit detection antibodies were added at 1:2,000. The chromogenic signal showed successful competition with the rPsaA for both, polyclonal and monoclonal antisera. *S. aureus* demonstrated a lack of competition as expected. The lack of competition with pneumococcal suspension and lysate was attributed, at least partly, to the crudeness of the preparation (pneumococcal lysate) that was coated to the plate. The competition curve is shown in **Figure 7.6**. **Figure 7.7** shows the reaction of rPsaA with two

different polyclonal concentrations, 8µg/mL or 1µg/mL, as capture antibody. The second antibody used was 1:10 PSAA-13. It demonstrates its suitability as a calibration curve in a quantitative diagnostic assay. There was little difference in signal intensity between 8µg/mL or 1µg/mL capture antibody concentrations. However, it is likely that lower concentrations of native PsaA are present in patient samples; therefore, the higher concentration of 8µg/mL was chosen to be used in the diagnostic assay to increase sensitivity.

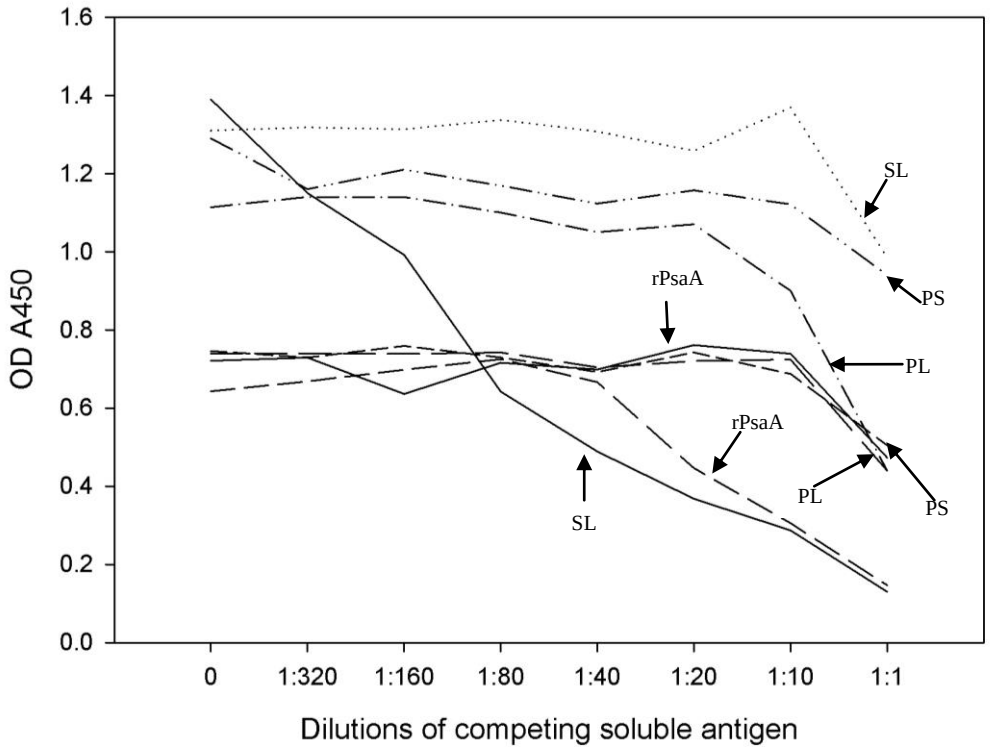


Figure 7.6: Competition experiment with pneumococcal lysate as the capture antigen

Various competitors binding with the monoclonal antibody (PSAA-13) as well as the polyclonal antibody raised against rPsaA. SL: staphylococcal lysate; PL: pneumococcal lysate; PS: pneumococcal suspension (lines for polyclonal antibody binding: dotted= SL; dash-double dot= PS; dash-single dot= PL; solid= rPsaA; lines for MAb binding: solid= SL; small dashed= PS; large dashed= PL; medium dashed= rPsaA).

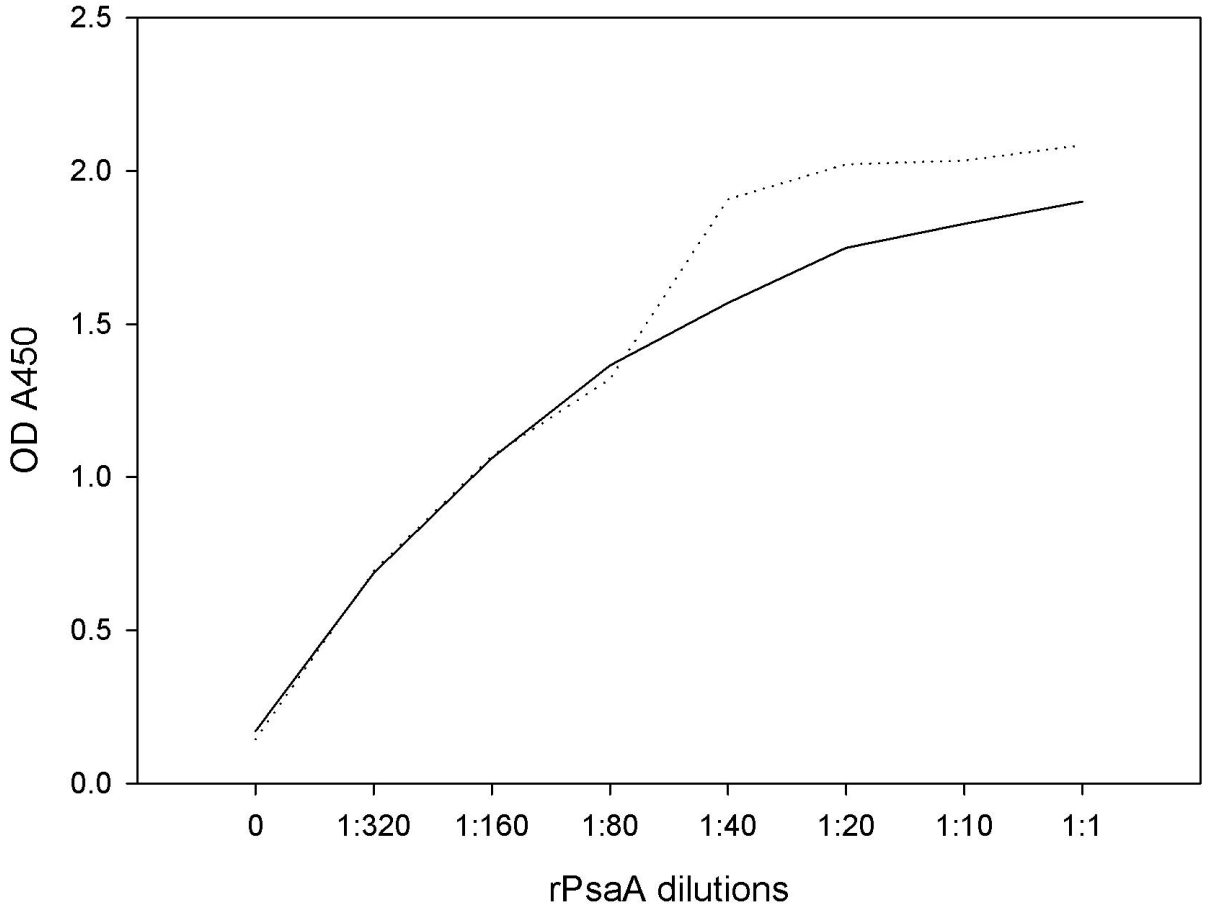


Figure 7.7: rPsaA in a sandwich ELISA

Capture antibody concentrations were 1µg/mL rPsaA (dotted line) and 8µg/mL rPsaA (solid line); doubling dilutions of recombinant psaA were used as the antigen and the second antibody was PSAA-13 diluted1:10. The curve indicates that doubling dilutions of rPsaA are suitable to include as the standard curve in the sandwich ELISA assay.

7.3.3. Spiked serum and plasma preparations

Due to the limited volumes of patient serum samples available from the 1999/2000 CAP study plasma was evaluated as a suitable alternative to serum. When pneumococcal suspension was used to spike the blood samples no signal was detected in the ELISA. This was attributed to the loss of intact bacterial organisms into the cellular fraction after centrifugation and perhaps to the lower concentration of bacterial antigen when a 1:100 dilution of 0.5 McFarland (approximately 10^8cfu/mL) bacterial suspensions was used. When bacterial lysate was used to spike the blood samples at 1:10 performance of plasma EDTA gave a better signal compared to Lithium-Heparin tubes, possibly due to the loss of antigen into the gel fraction of the Lithium-Heparin tubes. Spiked EDTA plasma gave a better signal

than spiked serum. The signal curves are shown in **Figure 7.8**.

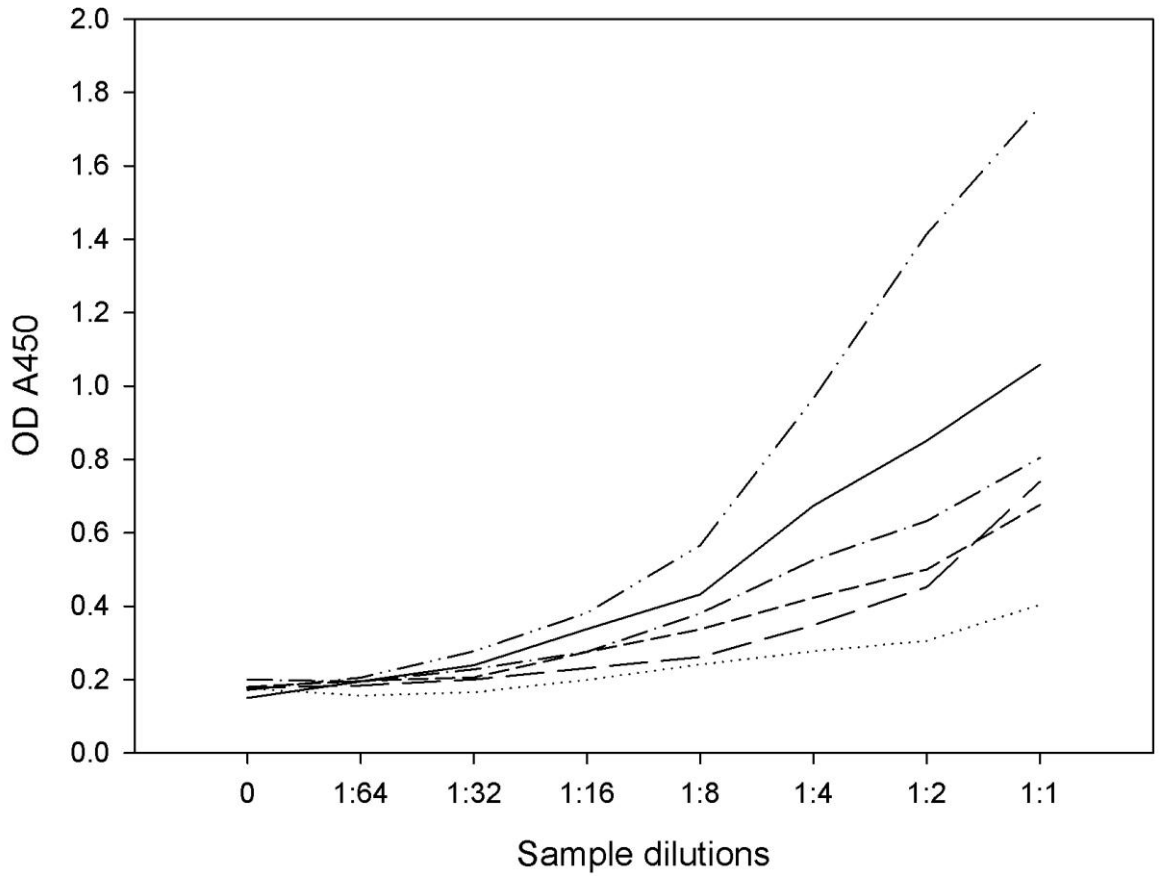


Figure 7.8: Performance of spiked EDTA and Lithium-Heparin plasma and serum with 1µg/mL (1) or 8µg/mL (2) capture antibody concentrations

Lines: solid: plasma EDTA 2; dotted: plasma Lithium-Heparin 2; small dashed: serum 2; dash-double dot: plasma EDTA 1; large dashed: plasma Lithium-Heparin 1; dash-single dot: serum1.

7.3.4. Assay precision

The intra-assay coefficient of variation averaged 17.3% and the inter-assay coefficient of variation averaged 14.7%. The values are summarised in **Table 7.1**.

Table 7.1: Precision data for the performance of the PsaA ELISA

	EDTA plasma (PL[1]spiked) OD A450[2]	Lithium-Heparin Plasma (PL spiked) OD A450	Serum (PL spiked) OD A450	Negative serum OD A450
Intra-assay	n =12	N/A	n =12	n =12
Mean	0.92	N/A	0.38	0.18
SD[3]	0.13	N/A	0.09	0.02
CV (%)[4]	13.90	N/A	26.30	11.80
Inter-assay	N/A	n=17	n=36	n=32
Mean	N/A	0.28	0.40	0.17
SD	N/A	0.03	0.06	0.03
CV (%)	N/A	11.70	16.20	16.25

[1]PL: Pneumococcal lysate

[2]OD A450: Absorbance signal read as optical density at 450nm

[3]SD: Standard deviation

[4]CV: Coefficient of variation

7.3.5. Lowest limit of detection

Eighteen zero values from the obtained standard replicates produced a mean absorbance of 0.17. The lowest limit of detection that is statistically robust was calculated by adding 2 standard deviations (2x 0.03) to the mean. The resulting absorbance representing the lowest limit of detection was 0.22. The extrapolated value for the PsaA concentration was 271.92pg/mL.

7.3.6. Testing of patient samples

All 304 samples were initially tested at a 1:2 dilution because of the limited sample volume and to minimise a possible “matrix effect” (the term “matrix” is referring to the

complex components of a sample, here serum or plasma). However, later testing of undiluted samples did not confirm the presence of a matrix effect in 4 of 6 positive samples. Absorbance values of undiluted and 1:2 diluted samples were comparable.

All samples demonstrating either a visible chromogenic signal or with an absorbance value extrapolated to ≥ 270pg/mL were re-tested undiluted. A sample was declared negative if the absorbance value did not reproduce at re-testing or the average value of the absorbance read in duplicate was less than 270pg/mL. All readings of the included negative serum samples, diluted or undiluted, were well below 270pg/mL.

A total of only 6 serum samples produced an absorbance value being extrapolated to a PsaA concentration of ≥ 270pg/mL. Out of those 6 sera only 4 were reproducible when re-tested undiluted. It is difficult to say whether matrix effect, sample interference, or pipetting variation played a role in the non-reproducibility of the 2 positive samples. It was not possible to re-test as there was not enough sample volume left. Both samples had a PsaA concentration around the cut-off value when tested at 1:2. **Figure 7.9** shows two by two contingency tables comparing the PsaA ELISA performance to the *ply* PCR in plasma and WBC as well as to conventional diagnostic methods, blood and sputum cultures and the pneumococcal UAT. It is of interest to note than none of the patients with detectable PsaA in their serum in the ELISA had detectable *ply* DNA in their blood samples. And vice versa, the patients with *ply* in their plasma and WBC had no PsaA detected in the serum. Against blood culture the sensitivity of the PsaA ELISA was 0%, specificity was 98.0 %, PPV 0% and NPV 95.9%, against sputum culture the sensitivity of the PsaA ELISA was 1.7%, specificity was 96.6%, PPV 20% and NPV 66.5%, and against the UAT the sensitivity of the PsaA ELISA was 2.8%, specificity was 98.9 %, PPV 50% and NPV 72.9%. A summary of the PsaA concentrations and relevant patient information is shown in **Table 7.2**.

a)

PsaA ELISA

Plasma PCR (*ply*)	**0**	**1**	**Total**
0	290	6	296
1	4	0	4
Total	294	6	300

b)

PsaA ELISA

WBC PCR (*ply*)	**0**	**1**	**Total**
0	294	6	300
1	4	0	4
Total	298	6	304

c)

PsaA ELISA

Blood culture	**0**	**1**	**Total**
0	259	5	264
1	11	0	11
Total	270	5	275

d)

PsaA ELISA

Sputum culture	**0**	**1**	**Total**
0	115	4	119
1	58	1	59
Total	173	5	178

e)

PsaA ELISA

UAT	**0**	**1**	**Total**
0	188	2	190
1	70	2	72
Total	258	4	262

Figure 7.9: Two by two contingency tables comparing the PsaA ELISA to qualitative PCR and conventional diagnostic methods for IPD

Evaluation of the PsaA ELISA against a) Plasma *ply* PCR, b) WBC *ply* PCR c) blood culture, d) sputum culture, e) UAT (urinary antigen detection test). The patient samples used for the evaluation were from the CAP study 1999/2000.

Table 7.2: Summary of the six patients with positive results in their PsaA ELISA

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Patient Age and Gender (mean age in study: 64 years)	M 80	F 76	F55	M 90	M 87	F 86
PsaA ELISA (ng/mL)	0.33	3.50	0.33	1.62	0.29*	0.37*
Diagnosis	RSV (CF[5])	Influenza A (DFA and culture positive)	Influenza A (high acute titre in CF, declining in convalescent sample)	No pathogen isolated	Parainfluenza (CF)	Parainfluenza (CF)
Urine Antigen[1]	+[2]	-[3]	-	NT[4]	NT	+
***S. pneumoniae* in Sputum Culture**	+	NT	-	-	-	-
***S. pneumoniae* in Blood Culture**	NT	-	-	-	-	-
***lytA* PCR in serum (cfu/mL)**	$1.19x10^4$	-	-	-	-	-
***lytA* PCR in sputum (cfu/mL)**	$4.89x10^6$	NT	-	-	$1.44x10^6$	$6.84x10^4$
***lytA* PCR in urine (cfu/mL)**	-	-	-	NT	NT	$6.22x10^2$
CAP study antimicrobial Use: Yes: n = 88, No: n = 216	-	-	-	-	-	+
Smoker (CAP study: ex smokers: n = 152, current smokers: n = 58, non smokers: n = 95)	Ex	Ex	Ex	-	Ex	-
Severity scores (PSI/CURBage)[6]	130/3	96/1	55/1	120/4	117/3	136/4
Duration of symptoms in Days mean in study: 7.34 days)	2	3	28	2	1	5

* Not reproducible in repeat test with undiluted serum

[1] NOW *S. pneumoniae* immunochromatographic antigen test (Binax, Portland, ME, USA)

[2] +: Positive

[3] -: Negative

[4] NT: Not tested

[5] CF: Complement fixation

[6] PSI: Pneumonia Severity Index [375]; CURBage score [299]

7.4. Discussion

In terms of novel pneumococcal diagnostics, in particular rapid immunological assays, nothing has come onto the commercial market since the introduction of the NOW *S. pneumoniae* urinary antigen detection test (Binax Inc. Portland, ME, USA) in the early 2000s, which detects C-polysaccharide, a pneumococcal cell wall component. Various issues have been identified as problems with the test performance, including lack of specificity in children [253] and test positivity in nasopharyngeal carriage [251]. The practical usefulness of antigen detection tests, however, their rapidity and ease of use, assured their ongoing attractiveness as a diagnostic tool.

PsaA as a target antigen offered the advantage of being present in all pneumococcal serotypes, as well as having proven immunogenicity. Serum and plasma as specimen types are more invasive to collect than a urine specimen, but it would be expected that patients who will be hospitalised due to CAP will have blood specimens taken early in the assessment process.

The aim of this evaluation of the PsaA antigen detection by sandwich ELISA was to assess its suitability as a rapid diagnostic tool and its clinical usefulness.

It was encouraging to find that either serum or plasma were suitable specimens. However, when serum and plasma were spiked with a high dose of pneumococcal lysate signal intensity differences were observed. Lithium-Heparin plasma performed worst, which limits the test to either EDTA plasma or serum. It is unclear why EDTA plasma demonstrated the strongest signal in the ELISA.

Another interesting observation was the fact that while optimising the ELISA it became apparent that lysate gave a stronger signal than suspension which was contrary to the findings in Chapter 6. Re-measurement of the concentration of lysate and suspension used in the experiments of Chapter 6 revealed a 10-fold higher concentration of suspension (data not shown). This in itself could explain the differences observed. However, it is also important to point out that the measurement of intact pneumococcus is a challenge. It is likely that the bacteria are affected osmotically by suspending them in PBS or water as was alluded to in section 6.3.6, they also lyse due to their own production of autolysin whilst in the stationary growth phase. Measurements performed with the Bradford assay (protein concentration) and measurements of the optical density (OD) at a certain growth phase of the bacteria are two options to obtain a concentration or a value to standardise testing. It was, however, observed, that the values obtained in a Bradford assay and those obtained by measuring the OD of the same substance do not necessarily correlate in a linear fashion. Based on these expected variations it is not possible to draw conclusions about the superiority of suspension or lysate,

although it is clear that lysed bacteria will be recognised by the antibodies. For diagnostic purposes this is important as most patients present to hospital several days post onset of disease (mean duration of symptoms in the patient cohort studied was 7.34 days), a stage where the presence of autolysed bacteria is certain.

The overall performance of the PsaA ELISA was disappointing. It showed no correlation of detection of PsaA with blood culture, sputum culture positivity or urinary antigen detection. One patient had been received antimicrobials prior to having blood samples taken. However, the number of patients with positive PsaA ELISA tests in serum is too small to draw conclusions about the impact of antimicrobials on the performance of the test. Interestingly, all but one patient belonged to the advanced age group, on average 15 years above the mean age of the study population, and severity scores were high in the PSI score.

Positive results in the 6 patients are unlikely to be attributed to cross-reactivity with other bacteria. Only two patients had other bacteria isolated in their sputum, one with *H. influenzae*, the other with *P. aeruginosa*, none of which showed potential cross-reactivity (Chapter 6).

The correlation of the PsaA positive ELISA results with the *lytA* PCR results from different body sites is poor. It is again impossible to draw definite conclusions, but it appears the number of organisms intact or lysed in the patients' blood has been low. Only one patient had detectable *lytA* in the serum ($1.19x10^4$cfu/mL). With the current assay's limit of detection being 270pg/mL, the assay is likely to lack the required sensitivity to detect low numbers of pneumococcal antigen in the blood of patients.

Due to the lack of sensitivity, detection of PsaA in blood samples is not currently a useful tool in pneumococcal diagnostics. Further investigation into the assay's suitability when used on other specimen types, in particular urine, is necessary. The suitability of the Binax NOW *S. pneumoniae* antigen detection assay in urine as a diagnostic tool for IPD in adults has been investigated in a large number of studies since its introduction in the early 2000s. Its benefits and limitations have become clearer over time due to its continued use in the clinical setting, which is largely a result of the lack of alternative diagnostic methods. Urine, as opposed to invasive specimens, such as blood, CSF and fluids from usually sterile sites, cannot unequivocally confirm the presence of invasive disease. The potential detection of PsaA in urine was not pursued in this thesis because of the project's particular focus on tests that can reliably differentiate between colonisation and invasive disease. Testing of the assay on urine specimens is, however, identified as necessary and considered as a future development. The current patient cohort investigated in this study unfortunately did not have

nasopharyngeal swabs or aspirates available. The lack of these specimens will prevent a clear analysis of the usefulness of the test in non-invasive specimens for the diagnosis of IPD. Again, future directions to evaluate the assay should take this into account. It seems prudent to apply future test evaluations to a different patient population including specimens that are indicative of nasopharyngeal colonisation. Lastly, for complete rigor another cohort, such as children admitted with CAP, would need to be tested and assay modifications, such as conversion of the current HRP-based chromogenic detection system to chemiluminescence should be trialled to asses if sensitivity of the assay can be improved.

DEVELOPMENT AND EVALUATION OF A QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ASSAY

CHAPTER 8

DEVELOPMENT OF A QUALITATIVE AND QUANTITATIVE PNEUMOCOCCAL AUTOLYSIN REAL-TIME POLYMERASE CHAIN REACTION

8.1. Introduction

The *lytA* gene encodes a pneumococcal amidase, autolysin, which is expressed as a 318 amino acid protein with a molecular weight of approximately 36kDa. Bacterial autolysins are enzymes that affect the peptidoglycan structure of the cell wall and subsequently cause cell lysis. Pneumococcal LytA requires the presence of choline residues in the teichoic acids of the cell wall for its activity [376]. LytA was thought to have been the sole factor for the release of pneumococcal pneumolysin, this role, however, was pulled into question [377]. It appears that the amount of extracellular LytA regulates the onset of bacterial lysis [378]. *LytA* was first used as a target to detect pneumococcus in 1991 [379] in a DNA probe assay. In 1994, it was used in a PCR detecting pneumococcal DNA in blood cultures [380].

Part 3 of this thesis looks at a quantitative real-time polymerase chain reaction (PCR) assay, its usefulness as a diagnostic tool compared to previously employed diagnostic assays as well as its potential as a marker of disease severity. The advantage of a real-time assay as opposed to a conventional PCR lies in the time-saving omission of a post-amplification step, which also decreases the chances of contamination and it has a greater dynamic range, allowing greater target concentration variations [281]. Above all, when applied to the diagnosis of respiratory viruses, it has been shown that the sensitivity of real time surpasses that of conventional assays [381]. *LytA* as a PCR target has been shown to reliably distinguish between *S. pneumoniae* and *S. pseudopneumoniae* [382]. A publication by Carvalho *et al.* in 2007 [281] investigated three novel real time PCR assays targeting the pneumococcal autolysin (*lytA*), pneumolysin (*ply*) and pneumococcal surface adhesin A (*psaA*). The assays all showed excellent sensitivity, with that of the *lytA* and *ply* assay being superior to *psaA*. *LytA* and *psaA* showed greater specificity than *ply*. Amplification of *S. pseudopneumoniae* was identified as a problem with the *ply* assay. On the basis of these published findings, *lytA* was the chosen gene target for a quantitative real-time PCR assay, which was a modification of the *lytA* assay published in 2007 [281].

8.2. Material and Methods

8.2.1. Primers and probes

The primers and probes used to set up this assay are summarised in **Table 8.1**. Primers and probe were obtained from TIB MOLBIOL GmbH, Berlin, Germany, according to their published sequence [281] and in their modified form (*lytA*-CHL probe). The published probe sequence by Carvalho had the 6-carboxyfluorescein (FAM) label attached to the 5' end of the oligonucleotide; but the terminal nucleotide was a guanine (G) which is known to quench fluorescence. To improve the performance of the TaqMan probe the sequences were modified by

1. Reversing the probe sequence so the probe is now on the complementary strand; 5' terminal nucleotide is now a cytosine (C) (*lytA*-CHL probe).
2. The probe was modified by the removal of the 5' G nucleotide (*lytA*-TIB probe).

Table 8.1: Real-time PCR primers and probes

Oligonucleotide	**Sequence**
lytA-CDC forward	5'-ACGCAATCTAGCAGATGAAGCA-3'
lytA-CDC reverse	5'-TCGTGCGTTTTAATTCCAGCT-3'
lytA-CDC probe	5'-6FAM-GCCGAAAACGCTTGATACAGGGAG-BBQ-3'
lytA-CHL probe	5'-6FAM-CTCCCTGTATCAAGCGTTTTCGGCA-BBQ-3'
lytA-TIB probe	5'-6FAM-CCGAAAACGCTTGATACAGGGAG-BBQ-3'

8.2.2. PCR reaction mix and thermocycling conditions

The PCR reaction mixes for *S. pneumoniae lytA* single-plex and duplex (including ART inhibition control) assays are listed in **Table 8.2**. The final concentrations of the forward and reverse *lytA*-CDC primers were modified from 200nM to 625nM in our assay and the probe concentration was modified from 200nM to 250nM. This modification was necessary considering that multiple PCR targets co-amplifying in a single reaction can create competition [383]. Higher primer and probe concentrations were used to improve the performance of the duplex real-time PCR reaction and ensure that a higher concentration of the *lytA* target would not be affected by the inhibitor control (ART) target (see section 8.2.5), which in turn can decrease the overall sensitivity of the assay. It is noteworthy, that real-time

multiplex amplification has been demonstrated to be comparable to single-plex at no compromise to the assay performance [384].

Cycling conditions were as recommended by Carvalho [281] using the two-step TaqMan protocol (**Table 8.3**).

Table 8.2: PCR reagents used in the single-plex and duplex assays

Reagents	Single-Plex x1 reaction (µL)	Duplex x1 reaction (µL)
H_2O	4.50	2.75
ABI 2x mixture*	12.50	12.50
10µM *lytA*-CDC forward	1.25	1.25
10µM *lytA*-CDC reverse	1.25	1.25
10µM *lytA*-CHL probe	0.50	0.50
10µM ART forward	X	0.75
10µM ART reverse	X	0.75
10µM ART probe (VIC) †	X	0.25

*2x Universal TaqMan® Gene Expression Master Mix (Applied Biosystems, Life Technologies, Foster City, CA, USA)

†VIC: 5' dye label

Twenty microlitres of the PCR reaction mixture was aliquotted in a 96-well PCR reaction plate (Applied Biosystems, Life Technologies, Foster City, CA, USA) and 10µL of DNA was added and DNA amplification performed on ABI 7500 real-time thermocycler (Applied Biosystems, Life Technologies, Foster City, CA, USA) using the cycling conditions as listed above.

Table 8.3: PCR cycling conditions

Step	Temperature	Time	Cycle Number
Hold	50 ^{o}C	00:02:00	1
Denaturation	95 ^{o}C	00:10:00	1
Amplification	95 ^{o}C	00:00:15	50
	60 ^{o}C*	00:01:00	

*Acquisition of fluorescence

8.2.3. Performance of probes

The performance of the *lytA*-CDC probe versus the modified *lytA*-CHL and *lytA*-TIB probes was assessed in parallel by real-time PCR amplification using the conditions as stated in **Tables 8.2** and **8.3.** *S. pneumoniae* (ATCC 49619) template DNA was used to evaluate the performance of the probes using the crossing point, height and slope of the amplification curve for the *lytA* CDC probe as a benchmark.

8.2.4. Analytical sensitivity

To determine the lower limit of detection (LLD) of this assay *S. pneumoniae* (ATCC 49619) was grown on 5% SBA and a 0.5 McFarland suspension was made. Ten-fold dilutions were prepared in phosphate buffered saline pH 7.5 (PBS) and performed from the starting point of 0.5 McFarland (approximately 10^8cfu/mL) measured with an Oxoid turbidometer (Oxoid, Basingstoke, Hampshire, UK). A bacterial colony count was performed in triplicate on the 10^3cfu/mL suspension and confirmed the accuracy of the concentrations. Real-time PCR was performed to determine linearity over the bacterial dilution serious and to determine the LLD.

8.2.5. Inhibitor control

An artificial sequence (ART) PCR was included in the *S. pneumoniae* quantitative real-time PCR assay. ART primers and probe are listed in **Table 8.4**. They were included at a lower concentration (see **Table 8.2**) to prevent competition between the 2 targets and allow preferential amplification of the *lytA* target. The ART forward template sequence is as follows:

5′AGCGGTGACGCATGCCTTCCAGACACCTCCAACACCAAGTGTTTCAGGCTGCGCGACTCT
TTTGCTGGCTTCGCCATGGTGGAGAAGACGCTCGTTCCGACGCCTGGGCACAGGCTACCCAC
CAAGAAGCAAACTGTAGCGTGAGAATGTCTCCTTTG-3′.

The artificial DNA sequence for inhibitor PCR control template (ART) was designed based on the sequence in GenBank (Accession Number: U17140). The amplified product is 158 bp in length.

Table 8.4: Inhibitor control (ART) primers and probe

Oligonucleotide	Sequence
ART forward	5'-AGCGGTGACGCATGCCTTCCA–3'
ART reverse	5'–CAAAGGAGACATTCTCACGCTACAGTT-3'
ART probe	5'–VIC-AACACCAAGTGGCCTTTCAGGCTGCGCGACT-TAMRA-3'

8.3. Results

8.3.1. Primers and probe design

It was noted that the *lytA*-CDC probe contained guanine at the first position on the 5'-end, which strongly absorbs fluorescence [385]; therefore the probe sequence was changed to a FAM-labelled *lytA*-CDC reversed probe, using a reverse strand modification of the CDC probe (**Table 8.1**). The change in the probe sequence to the complementary strand also increased the cytosine amount compared to guanine amount, again leading to higher fluorescent signals (**Figure 8.1**).

8.3.2. Comparison of *lyt*A-CDC probe and its modification

Figure 8.1 demonstrates that the *lytA*-CDC probe has a lower ΔRn (reporter signal) compared to the *lytA*-CHL reverse probe; however, the best PCR efficiency was demonstrated with the *lytA*-TIB probe. The main reason for not selecting the *lytA*-TIB probe was that it would have required significant work-up to determine if the change in sequence would have affected the PCR specificity. Therefore, the *lytA*-CHL probe was selected for its superior PCR efficiency compared to the *lytA*-CDC probe as shown by the better exponential slope of the real-time curve. The slope of the PCR real-time curve indicates the PCR efficiency; a steeper curve is closer to a PCR efficiency of 2.0 (amplification product doubles every cycle) and therefore improves the accuracy of quantification PCR (qPCR). All 3 probes provided the same crossing threshold (C_T) value which indicates that they have the same analytical performance.

Of note: A *psaA* probe was included in the initial evaluation of probe performances. However, it was decided not to pursue the development of a quantitative *psaA* PCR assay; the *psaA* probe performance had the lowest PCR efficiency and lowest fluorescence compared to the other probes (**Figure 8.1**).

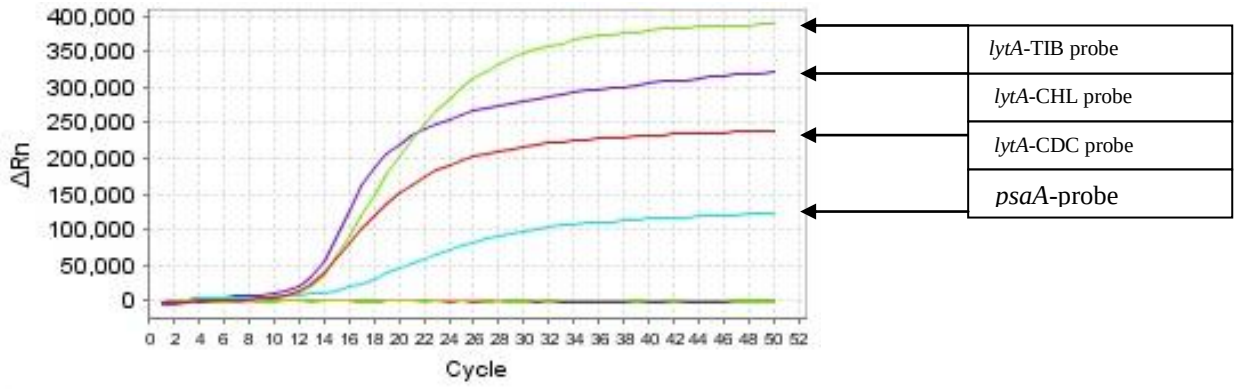


Figure 8.1: Increased fluorescence signal with the modified *lytA*-CHL probe

8.3.3. BLAST alignment of primers and probe

Nucleotide sequence BLAST (Basic Local Alignment Search Tool) search of *S. pneumoniae* (Accession Number: AJ243414), *S. mitis* (Accession Number: AJ252191) and *S. pseudopneumoniae* (Accession Number: AM113495) were performed in http://blast.ncbi.nlm.nih.gov and alignment was performed using CLC DNA workbench 5.0.2 (CLC Bio, Aarhus, Denmark). The aligned sequences and the position of *lytA* primers and probe are shown in **Figure 8.2.**

The BLAST alignment for each oligonucleotide for streptococcal *lytA* gene showed 100% agreement for all *S. pneumoniae* sequences in GenBank except for one strain (Accession Number: AJ252194) which had a single nucleotide mismatch for the primer *lytA*-CDC forward see **Figure 8.3.**

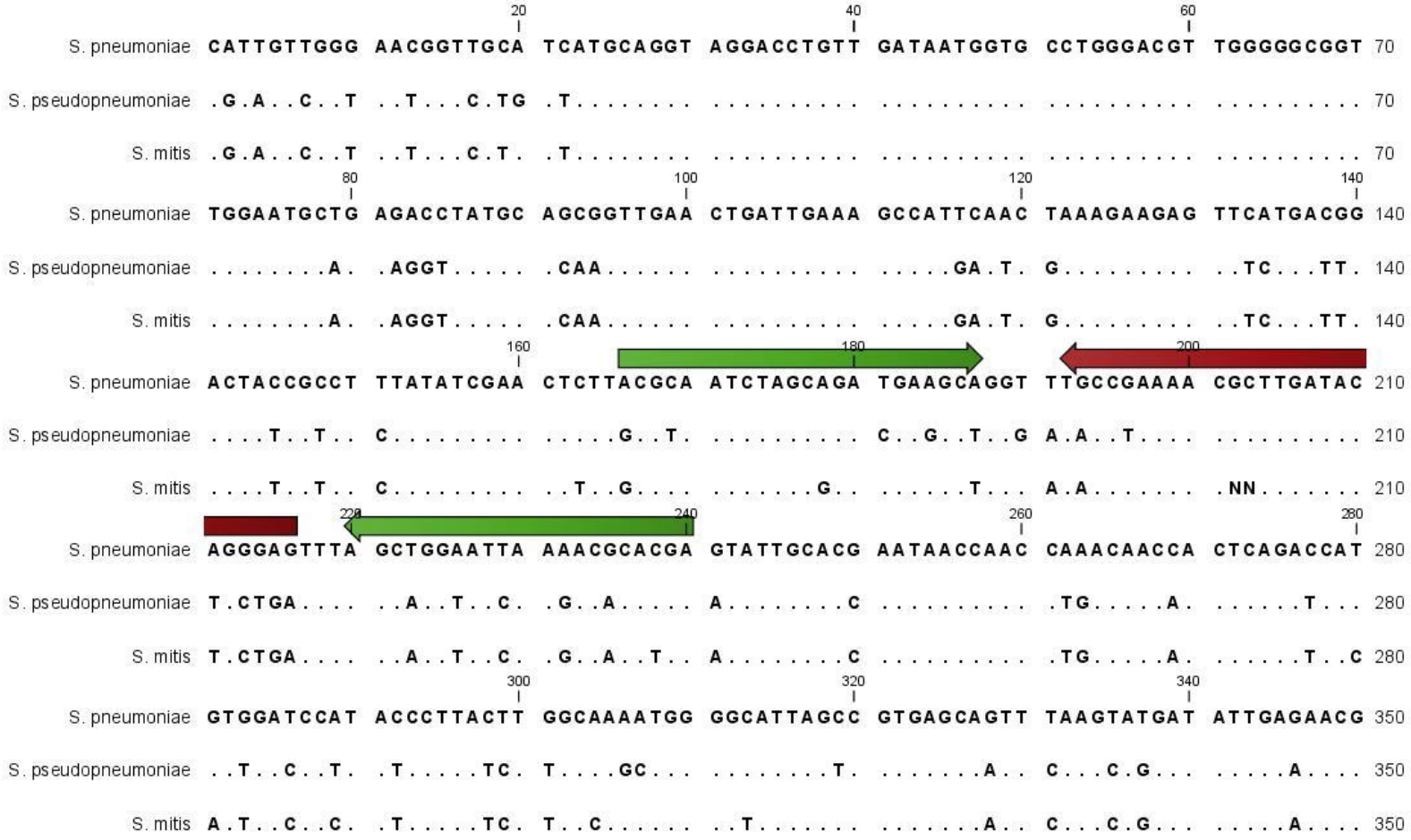


Figure 8.2: Alignment of bacterial nucleotide sequences

The positions of the *lytA*-CDC forward and reverse primers (green) and the *lytA*-CHL reverse probe (magenta) are marked with arrows.

```
>emb|AJ252194.1|  Streptococcus sp. partial lytA gene for autolysin, strain COL20
Length=900

 Score = 34.2 bits (17),  Expect = 5.7
 Identities = 20/21 (95%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1    ACGCAATCTAGCAGATGAAGC  21
            ||||||||||||| ||||||||
Sbjct  306  ACGCAATCTAGCGGATGAAGC  326
```

Figure 8.3: Single nucleotide mismatch for the *lytA*-CDC forward primer in *lytA* gene BLAST alignment

One strain of *S. pneumoniae* (Accession Number: AJ252194) shows a single adenine to guanine mismatch.

8.3.4. Analytical sensitivity and standard curve

The 10-fold dilutions were tested in the quantitative assay and the lowest limit of detection (LLD) was 1.8 x10^1 (18)cfu/mL (**Figure 8.4**). The corresponding crossing threshold (C_T) values are listed in **Table 8.5**. The accuracy of the "front end" value (1.8), which was determined by using the average bacterial colony counts of triplicate plates, can be questioned. It is likely that non-viable organisms are present in the dilution series and that a greater number of organisms (> 18cfu/mL), in fact, is detected. Based on the value of 18 cfu/mL of bacterial suspension at the LLD, a volume of 200µL was extracted and eluted in 60µL, resulting in an estimate of 0.6cfu/10 µL (DNA volume used per PCR reaction). The real-time PCR standard curve has linearity over the tested bacterial dilutions and bacterial loads of 1.8x10^2cfu/mL could be detected with acceptable precision and reproducibility under routine clinical laboratory conditions (lowest limit of quantification) (**Figure 8.5**). This level of detection was consistent with that described by Peters *et.al.* [271]. At the level of 1.8x10^2cfu/mL, interassay variation was determined by looking at the reproducibility of the crossing thresholds. The coefficient of variation (CV) was calculated to be 1.68%.

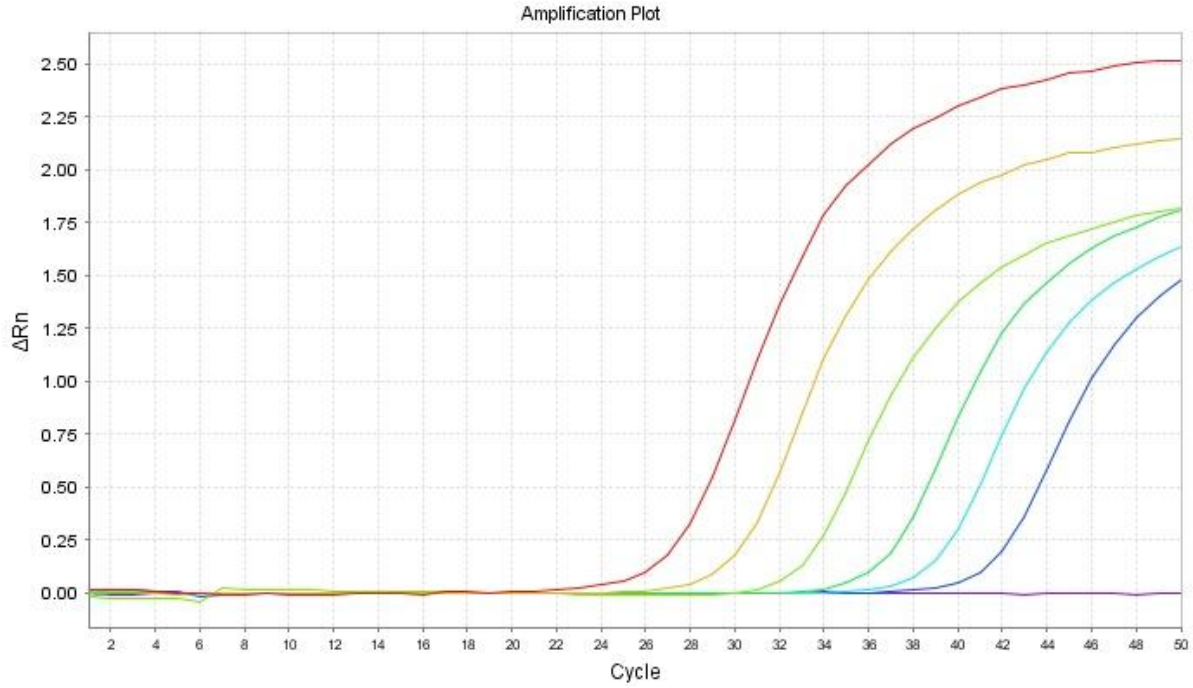


Figure 8.4: Determination of the lowest limit of detection

Ten-fold dilutions starting at 1.8 x10^6cfu/mL (red) to 1.8 x10^1cfu/mL (blue). A "no template" control was included (purple).

Table 8.5: C_T values corresponding to the 10-fold dilutions used for the standard curve of the quantitative assay

SPN cfu/mL	C_T value
1.8 x10^6	27.65
1.8 x10^5	30.61
1.8 x10^4	33.93
1.8 x10^3	37.49
1.8 x10^2	39.80
1.8 x10^1	42.47
NTC*	0

* No template control

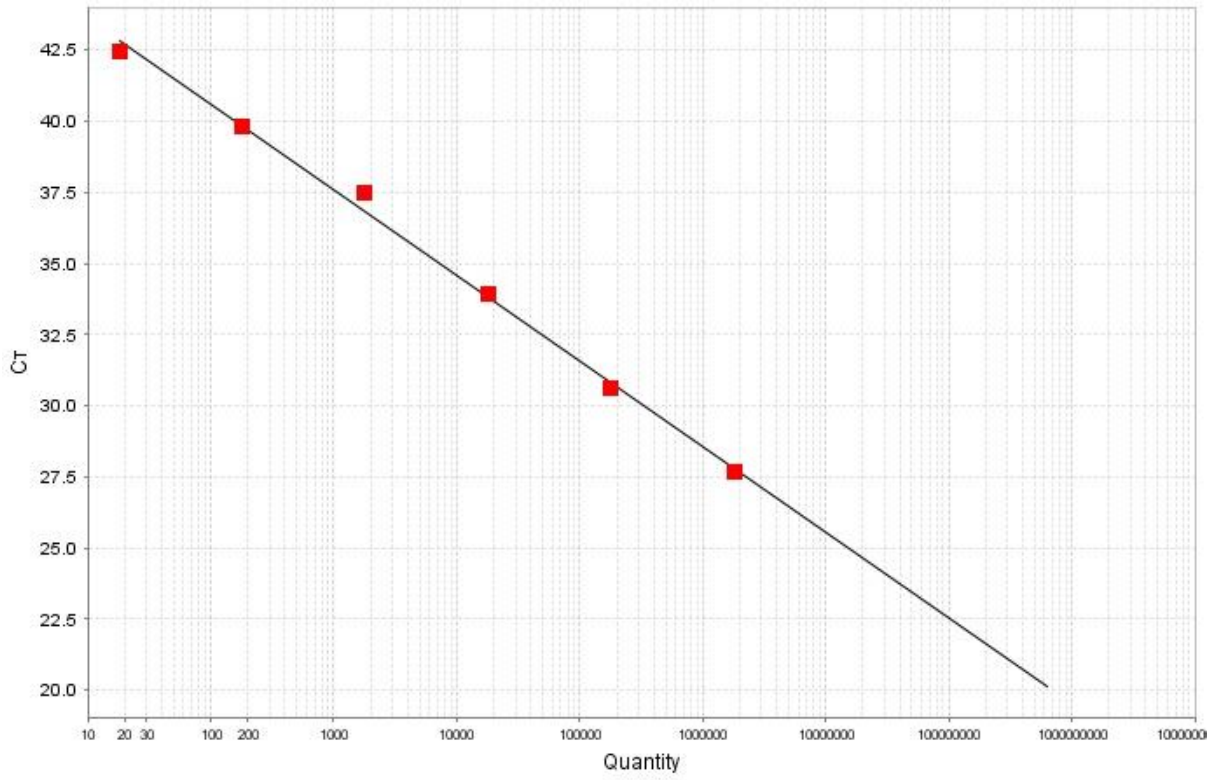


Figure 8.5: The real-time PCR standard curve has linearity over the tested bacterial dilutions

The *Y*-intercept was 46.6. The R^2 value was 0.9. It indicates the closeness of fit between the standard curve regression line and individual C_T data points. An R^2 value of 1 indicates a perfect fit. The slope was -3.0. It was used to calculate the PCR efficiency, which was 115.1% [386]. PCR efficiency of 100% indicates precise doubling of the gradient of the curve.

8.3.5. Effect of inhibitor control on analytical sensitivity

The effect of an inclusion of a PCR inhibitor control (ART) on the analytical sensitivity of the duplex assay was evaluated by performing *S. pneumoniae* single-plex and duplex assays in parallel. The cycle thresholds (C_T) for *S. pneumoniae* concentrations of 10^6 and 10^7 were unaffected by the addition of the ART template (**Table 8.6**). The addition of the inhibitor control template did not lower the analytical sensitivity of the PCR.

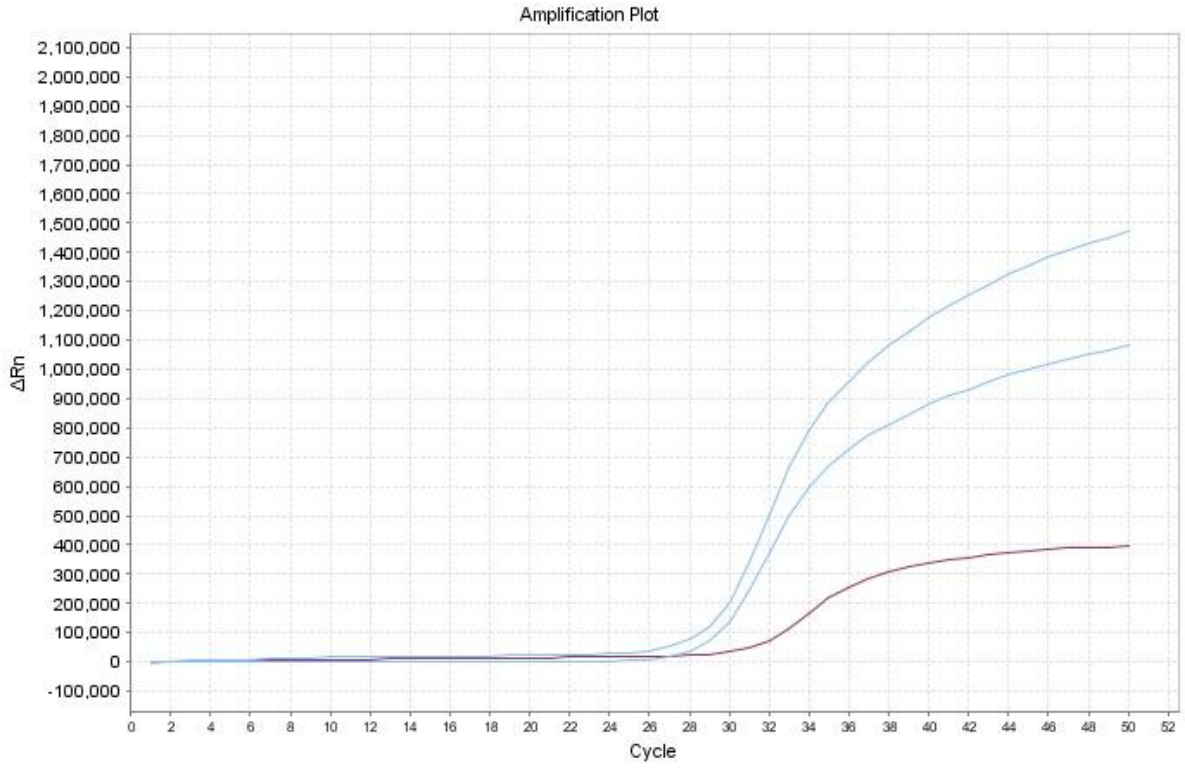


Figure 8.6: Single-plex real-time PCR amplification of 10^6cfu/mL *S. pneumoniae* DNA (top blue line) and duplex PCR including ART amplification

The duplex real-time amplification plot shows decreased fluorescence of the 10^6cfu/mL *S. pneumoniae* amplification (bottom blue line). ART template (10^{-7} dilution) amplification curve is shown in purple. The C_T values for single-plex and duplex *S. pneumoniae* DNA amplification are almost identical.

Table 8.6: Comparison of C_T values for *S. pneumoniae* in a single-plex versus a duplex assay including the Art template for inhibition control

Art template (concentration)	***S. pneumoniae* 10^7cfu/mL (C_T)**	***S. pneumoniae* 10^6cfu/mL (C_T)**
10^{-9}	25.50	28.70
10^{-10}	25.60	28.50
10^{-11}	25.60	28.50
0 (single-plex)	25.20	28.70

8.4. Summary

The real-time PCR assay for the detection of *lytA* was based on a published assay. However, this assay was modified in parts for this study. The modifications included (i) using a reverse strand complementary probe to the published CDC probe, (ii) using a modified primer concentration compared to the publication, (iii) including an inhibitor control in the quantitative assay, (iv) including a PCR reaction mixture with Rox dye fluorochrome to check the PCR efficiency across the 96 well plate in the quantitative assay, and lastly, including a standard based on established bacterial concentrations (in cfu/mL) to achieve quantitative results. PCR efficiency was excellent and indicated a near perfect doubling per cycle. The quantitative limit of detection that was reproducible in the clinical setting was established at 180cfu/mL. This lowest limit of detection is well below the level of bacterial concentration (1000cfu/mL) that is associated with increased septic shock development in adults [387].

CHAPTER 9

EVALUATION OF THE QUANTITATIVE REAL-TIME PCR ASSAY

9.1. Introduction

As previously stated, effective clinical management and surveillance of IPD is hampered by the difficulty to accurately diagnose infections caused by *S. pneumoniae.* The currently available tests have either a low sensitivity to diagnose IPD or they fail to differentiate between *S. pneumoniae's* presence as a coloniser or an invasive pathogen [217]. An ongoing issue with the evaluation of new diagnostic molecular-based assays is the lack of a "gold standard". Historically blood culture has been used as the "gold standard", but lacks sensitivity and specificity in the diagnosis of IPD and hence causes underestimation of the PCR performance as previously highlighted [388].

Two recent publications supported the clinical usefulness of bacterial load measurements. The first demonstrated the association of a high pneumococcal load in the nasopharynx with radiologically confirmed pneumonia and pneumococcal virulence in children [272] and the second evaluated the use of serial quantitative PCR as a monitoring tool for the management of pneumococcal intracranial sepsis [389]. Both strongly suggest that quantitative diagnostic approaches hold clinical value. In the case of measuring the pneumococcal load in the nasopharynx an association of higher bacterial load with radiologically confirmed pneumonia could be established which in turn should lead clinical management in terms of antimicrobial therapy. Decreasing bacterial load in aspirated intracranial fluid has been positively correlated with clinical improvement and hence, potentially prevents further surgical intervention. Additionally, Rello *et al.* was able to demonstrate that a certain breakpoint (1000cfu/mL) in whole blood pneumococcal load of patients with CAP is associated with severe disease and can assist the clinicians as a tool for severity assessment of patients [387].

The previous Chapter 8 has focussed on the development of a quantitative real-time PCR assay targeting the pneumococcal *lytA* gene. In this chapter the assay will be compared to other diagnostic methods. The diagnostic test results obtained for the CAP study in 1999/2000 [293] were used to compare and evaluate the performance of the quantitative *lytA* real-time PCR assay. The assay was also assessed for its clinical usefulness, particularly its use as a marker of disease severity.

9.2. Materials and methods

9.2.1. Subjects

Serum, sputum and urine specimens of patients with community-acquired pneumonia were analysed. Details of the patient group are summarised in section 2.2.2.12. Prior to culture the quality of sputum specimens was assessed according to accepted criteria [230].

9.2.2. Sample nucleic acid extraction

All patient samples had been stored at -80°C since 1999/2000. Between 0.5 and 1mL of thawed serum, sputum and urine underwent DNA extraction using the Promega DNA extraction kit (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. The extraction was performed on the automated M2000sp platform (Abbott Laboratories, Abbott Park, IL, USA).

9.2.3. Qualitative RT-PCR amplification

Real-time PCR assays were performed in a final volume of 30µL containing 10µL DNA on the ABI 7500 RT-PCR system (Applied Biosystems, Life Technologies, Foster City, CA, USA) using MicroAmp® Optical 96-well reaction plates and adhesive covers (Applied Biosystems, Life Technologies, Foster City, CA, USA). The concentrations of primers and probes are listed in **Table 8.2**. The master mix used was 2x Universal TaqMan® Gene Expression Master Mix (Applied Biosystems, Life Technologies, Foster City, CA, USA). Each sample was tested in a duplex RT-PCR, targeting *lytA* as well as ART to control for inhibition. 1µl of 10^{-7} ART template was added to each reaction.

Pneumococcal *lytA* DNA was detected using *lytA*-CDC forward primer and reverse primer as previously described [281] and FAM-labelled *lytA*-CDC reversed probe, using a reverse strand modification of the CDC probe used in previous publications [281]. A positive *S. pneumoniae* extraction and a "No Template" control (molecular grade water) were included in each PCR run. Analysis of qualitative results was performed using ABI 7500 software v 2.0.1.

Pneumolysin (*ply*) PCR had been performed on various specimens, including sputum, white blood cells , plasma and urine as described by Murdoch *et al.* [267] and the data related to the detection of *S. pneumoniae* in this assay was included in the analysis.

9.2.4. Quantitative RT-PCR amplification

All positive samples underwent a quantitative RT-PCR assay using the same PCR conditions and *lytA* primers and probes. An internal control (ART template) was not included at this stage. However, 50x Rox dye fluorochrome was included in each run to verify PCR efficiency across the optical 96-well plate.

The standard curve was set up using 10-fold dilutions of a known concentration of *S. pneumoniae* established by colony plate count. The standard included 6 concentrations from $1.8x10^6$ to $1.8x10^1$ colony forming units (cfu)/mL.

Analysis of quantitative data was performed using the ABI 7500 software v 2.0.1.

9.2.5. Statistical analysis

Data were entered into a Microsoft Excel 2007 database (Microsoft, Redmond, WA, USA) and analysed using Stata/IC 11.0 (StataCorp, College Station, Texas, USA). Dichotomous variables were analysed with McNemar Chi square test for paired data and continuous variables with the Wilcoxon rank sum test. Statistical significance was set at a *P*-value of <0.05. Logistic regression analysis was used to assess independent associations of variables with disease severity. Five different measures of severity were assessed: (1) CURBage score [299] greater than 2, (2) Pneumonia Severity Index (PSI) risk [375] class IV and V, (3) intensive care unit admission, (4) in-hospital death and (5) hospital stay greater than 5 days. The other variables included in each model were: *lytA* PCR results (binary variable or grouped as 0, 1-1,000, 1,001-10^5 and $>10^5$ cfu/mL), age (years), male sex (binary variable), duration of symptoms prior to admission (days), previous or current smoker (binary variable), and antimicrobial use prior to admission (binary variable). For all logistic regression analysis, likelihood ratio tests were used to compare models with and without interaction terms.

9.3. Results

9.3.1. Comparison of qualitative *lytA* PCR assays with other diagnostic tests

Statistically significant differences were noted between the performance of *lytA* PCR and all culture-based comparator tests. *S. pneumoniae lytA* was detected in all but two sputum specimens by PCR that were culture positive and additionally in 51 culture-negative sputum specimens. The difference in results from both tests was statistically significant ($p<0.000$). PCR detected *S. pneumoniae* in 6 sera of blood culture positive patients ($p=0.020$) and in only 4 urines of 69 patients with positive urinary antigen tests ($p<0.000$). The comparison data is summarised in **Table 9.1**.

Table 9.1: Comparison of qualitative *lytA* PCR results with those of other diagnostic tests on the same sample types

a) Sputum PCR vs. culture

		LytA PCR on sputum	
		Negative	Positive
Sputum culture	Negative	97	51
	Positive	2	46

b) Serum PCR vs. blood culture

		LytA PCR on serum	
		Negative	Positive
Blood culture	Negative	246	17
	Positive	5	6

c) Urine PCR vs. urinary antigen

		LytA PCR on urine	
		Negative	Positive
Urine antigen	Negative	185	2
	Positive	65	4

NB: Statistically significant differences were noted between all three comparisons (a: $p<0.000$; b: $p=0.020$; c: $p<0.000$)

9.3.2. Comparison of qualitative pneumolysin and autolysin PCR assays

The data obtained from the previous study [293] using pneumolysin (*ply*) PCR on sputum, white blood cells (WBC), plasma and urine samples [267] was compared with the *lytA* PCR on the same patient sputum, serum and urine isolates, bearing in mind that the isolates stored in 1999/2000 at -80°C had undergone several cycles of freezing and thawing and could have been affected by degradation.

The results comparing the above mentioned tests are tabulated in two by two contingency tables (**Figure 9.1**).

The *ply* PCR that was used as the reference test against the *lytA* PCR had previously been used on the study population and had been validated against reference "gold standards" for *S. pneumoniae* detection (blood culture, sputum culture, and urinary antigen detection). In those comparisons the *ply* PCR positivity rate in blood samples was low (10.0 and 24.0%, respectively, in patients with pneumococcal bacteraemia), whereas *ply* PCR positivity rates in sputum and throat swabs were high in specimens positive for pneumococcus by conventional diagnostic methods [267].

Against *ply* PCR the *lytA* PCR sensitivity and specificity in sputum were 63.1% and 97.7%, respectively. Positive predictive value (PPV) and negative predictive value (NPV) were 98.9% and 44.0%, respectively. For urine *lytA* PCR sensitivity and specificity were 50.0% and 97.2%, respectively, with a PPV of 50.0% and a NPV of 99.3%. When compared to *ply* PCR in plasma, the *lytA* PCR in serum had a sensitivity and specificity of 75.0% and 92.5%, respectively with a PPV of 12.0% and a NPV of 99.6%. When compared to *ply* PCR in WBC, the *lytA* PCR in serum had a sensitivity and specificity of 25.0% and 91.9%, respectively with a PPV of 4.0% and a NPV of 98.9%. Twenty four and 22 *ply* negative specimens in WBC and plasma, respectively, were positive in the *lytA* PCR. It is, of course, of limited value to compare different PCR targets in similar, but essentially different specimen types (serum versus plasma and WBC).

With high NPVs in all four comparisons the test could be useful as a clinical tool for the exclusion of pneumococcal disease. Low sensitivity, however, renders it less likely to be a useful screening test in the clinical setting.

a)

Sputum PCR (*ply*)

Sputum PCR (*lytA*)	**0**	**1**	**Total**
0	44	56	100
1	1	96	97
Total	45	152	197

p <0.000

b)

WBC PCR (*ply*)

Serum PCR (*lytA*)	**0**	**1**	**Total**
0	275	3	278
1	24	1	25
Total	299	4	303

p =0.293

c)

Plasma PCR (*ply*)

Serum PCR (*lytA*)	**0**	**1**	**Total**
0	273	1	274
1	22	3	25
Total	295	4	299

p=0.002

d)

Urine PCR (*ply*)

Urine PCR (*lytA*)	**0**	**1**	
0	139	1	140
1	4	1	5
Total	143	2	145

p=0.068

Figure 9.1: Comparison of positive and negative results by autolysin (*lytA*) and pneumolysin (*ply*) qualitative PCR

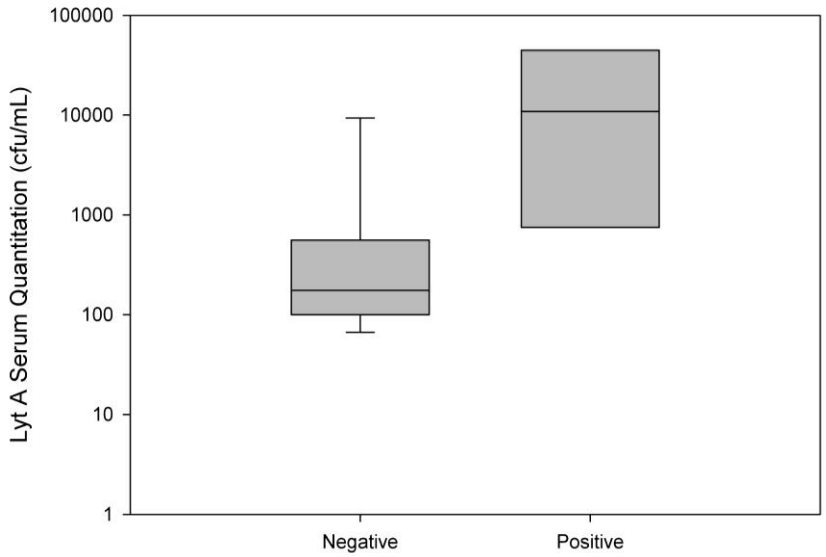
The data is presented in two by two contingency tables. The various specimens used in the comparison are: a) sputum; b) serum and WBC; c) serum and plasma; d) urine.

9.3.3. Quantitative *lytA* PCR on clinical samples

For PCR-positive specimens, the bacterial loads were higher in sputum specimens (median 8.55×10^5 (IQR 4.70×10^4 – 4.69×10^6) copies/mL) than either serum (median 180 (IQR 165 – 8970) copies/mL) or urine (median 623 (IQR 510 – 650) copies/mL).

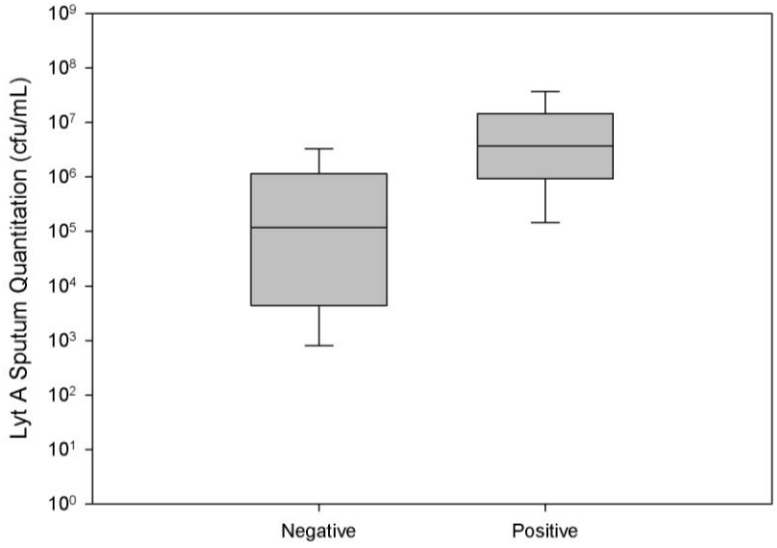
Figure 9.2 shows the bacterial load in all PCR-positive specimens by specimen type and by results of other diagnostic tests. Of note, bacterial loads were statistically significantly higher in culture-positive compared to culture-negative sputum specimens and in serum from patients with positive blood cultures compared to those with negative blood cultures. Positive and negative urine antigen detection tests were compared to bacterial loads in the *lytA* PCR assay, but did not show any correlation of higher bacterial loads and positive antigen test in the urine. Only six data points were analysed (data not shown).

a)



Detection of *S. pneumoniae* in Blood Culture

b)



Detection of *S. pneumoniae* in Sputum Culture

Figure 9.2: Bacterial load in *lyt*A PCR-positive specimens

a) In serum from blood culture positive and negative patients. b) Bacterial load in *lytA* PCR-positive specimens in sputum from sputum culture-positive and negative patients. The whisker boxplots show the median, 25th and 75th percentiles, and 10th and 90th percentiles (the 10th and 90th percentiles are not shown when <10 data points are available).

9.3.4. *Lyt*A PCR results and antimicrobial use

Detection of *S. pneumoniae* by PCR in sputum was statistically significantly less common in patients who reported antimicrobial use prior to hospitalisation and specimen collection (OR 0.26 (95% CI 0.l3 to 0.52), $p<0.000$). There was no association between prior antimicrobial use and PCR positivity in either serum (OR 0.43 (95% CI 0.15 to 1.33), $p=0.130$) or urine (OR 2.55 (95% CI 0.50 to 13.00), $p=0.240$) specimens. There were also no statistically significant differences in bacterial loads with antimicrobial use for each of the three specimen types (data not shown).

9.3.5. Association of PCR results with disease severity

Overall, 71 patients had a CURBage score greater than 2, 146 were PSI risk class IV or V, 8 were admitted to the intensive care unit, 11 died in hospital, and 160 had a hospital stay greater than 5 days. **Table 9.2** shows the association between qualitative PCR results and other pneumococcal diagnostic tests (blood culture, sputum culture, urinary antigen) with disease severity after adjusting for age, gender, duration of symptoms, smoking history, and prior antimicrobial use. The inclusion of an interaction term between sputum pneumococcal PCR and smoking significantly improved the model for severe disease as assessed by PSI risk class. No other interaction terms significantly improved any of the models. **Table 9.3** shows the results for the same models run with quantitative PCR results showing the association between pneumococcal load and disease severity.

Detection of pneumococcus in serum by PCR was associated with four markers of severe disease, and with three markers (CURBage score, PSI risk class and ICU admission) there was an increased odds of severe disease with increasing bacterial load. Detection of pneumococcus in urine was associated with severe disease with the CURBage score only, while detection in sputum was only associated with severe disease as assessed by PSI risk class in patients who had never smoked. Inclusion of additional variables for the detection of other viral or bacterial pathogens did not significantly change any of these findings (data not shown).

Table 9.2: Association between qualitative *lytA* PCR results, disease severity and other pneumococcal diagnostic tests

Outcome	Specimen and test	OR (95% CI)	*p* value
Severe disease (CURBage score 3-5)	Serum *lytA* PCR	2.91 (1.07 to 7.96)	0.040
	Sputum *lytA* PCR	1.59 (0.73 to 3.43)	0.240
	Urine *lytA* PCR	14.00 (1.72 to 114.02)	0.010
	Blood culture	3.71 (0.96 to 14.37)	0.060
	Sputum culture	1.21 (0.52 to 2.81)	0.660
	Urinary antigen	2.78 (1.35 to 5.73)	0.006
Severe disease (PSI risk class)	Serum *lytA* PCR	6.85 (1.99 to 23.52)	0.002
	Sputum *lytA* PCR (previous/current smoker)	2.54 (1.05 to 6.17)	0.040
	Sputum *lytA* PCR (never smoked)	0.14 (0.01 to 1.38)	0.090
	Urine *lytA* PCR	2.12 (0.22 to 20.52)	0.520
	Blood culture	2.52 (0.42 to 15.18)	0.310
	Sputum culture	1.65 (0.69 to 3.96)	0.260
	Urinary antigen	1.88 (0.88 to 4.01)	0.100
ICU admission	Serum *lytA* PCR	10.48 (2.23 to 49.34)	0.003
	Sputum *lytA* PCR	5.32 (0.59 to 47.56)	0.140
	Urine *lytA* PCR	*	
	Blood culture	19.72 (3.23 to 120.21)	0.001
	Sputum culture	1.12 (0.20 to 6.40)	0.900
	Urinary antigen	2.41 (0.57 to 10.20)	0.230
Death	Serum *lytA* PCR	1.75 (0.20 to 15.73)	0.620
	Sputum *lytA* PCR	0.93 (0.12 to 6.87)	0.940
	Urine *lytA* PCR	*	
	Blood culture	3.46 (0.33 to 36.53)	0.300
	Sputum culture	*	
	Urinary antigen	3.31 (0.69 to 15.84)	0.130
Length of admission >5 days	Serum *lytA* PCR	9.92 (2.96 to 33.28)	<0.001
	Sputum *lytA* PCR	0.84 (0.42 to 1.68)	0.630
	Urine *lytA* PCR	2.66 (0.32 to 22.00)	0.360
	Blood culture	11.02 (1.22 to 99.63)	0.030
	Sputum culture	0.76 (0.36 to 1.60)	0.480
	Urinary antigen	1.37 (0.72 to 2.63)	0.330

*Convergence not achieved

Table 9.3: Association between pneumococcal load and disease severity

Outcome	Specimen	Pneumococcal load	OR (95% CI)	*p* value
Severe disease (CURBage score)	Serum	0	1.00	
		1-1000 cfu/mL	1.53 (0.42 to 5.57)	0.520
		1001-10^5 cfu/mL	9.80 (1.76 to 54.50)	0.009
Severe disease (PSI risk class)	Serum	0	1.00	
		1-1000 cfu/mL	5.22 (1.30 to 21.01)	0.020
		1001-10^5 cfu/mL	13.58 (1.55 to 118.86)	0.020
	Sputum (previous/ current smoker)	0	1.00	
		1-1000 cfu/mL	0.35 (0.03 to 4.47)	0.420
		1001-10^5 cfu/mL	4.68 (0.97 to 22.49)	0.050
		>10^5 cfu/mL	2.73 (1.01 to 7.36)	0.050
	Sputum (never smoked)	0	1.00	
		1-1000 cfu/mL	0.02 (0.00 to 1.35)	0.070
		1001-10^5 cfu/mL	0.40 (0.02 to 9.36)	0.570
		>10^5 cfu/mL	0.10 (0.00 to 1.40)	0.090
ICU admission	Serum	0	1.00	
		1-1000 cfu/mL	8.44 (1.26 to 56.58)	0.030
		1001-10^5 cfu/mL	13.55 (1.95 to 94.16)	0.008
Length of admission >5 days	Serum	0	1.00	
		1-1000 cfu/mL	12.90 (2.87 to 58.01)	0.001
		1001-10^5 cfu/mL	6.15 (0.96 to 39.50)	0.060

NB: pneumococcal loads in urine specimens were not included in this analysis as all were <1000 cfu/mL.

9.4. Discussion

The aim of this study was to establish whether quantitative pneumococcal PCR is useful in the clinical setting to assist in the diagnosis of IPD in adults with CAP.

It is known that a combination of traditional culture-based microbiological methods and the additional use of molecular-based assays increases the diagnostic yield in patients with CAP, and it has been demonstrated through the use of molecular assays that *S. pneumoniae* can be a frequent pathogen [390].

The limitations of traditional culture methods alone in diagnosing IPD have led to the development of numerous molecular assays, targeting various genes of the pneumococcus, most commonly pneumolysin (*ply*), autolysin (*lytA*) and more recently Spn9802 [391].

LytA gene was chosen as the target in this study because it is thought to be present in most, if not all pneumococcal serotypes, and also because quantitative *ply*-based PCR did not demonstrate clinical usefulness [392]. It is known however, that non-pneumococcal strains can harbour the *lytA* gene, causing potentially false-positive results [281, 393].

The main finding of the work discussed in this chapter was that detection of pneumococcal DNA in serum is associated with severe disease in adults with CAP and, for some markers, increased bacterial load was associated with increased severity. An association between bacterial load and disease severity for sputum specimens was not observed, except for one group of patients; previous or current smokers had an increased risk of being in the higher PSI risk classes if pneumococcal DNA was detected in sputum. There was an association between pneumococcal DNA in urine and disease severity as assessed by the CURBage score, but with no other markers. The significances of the latter two findings are unclear, may represent chance findings and need to be replicated in other patient populations.

The findings from serum PCR in this study are consistent with other recent reports. Among Spanish adults with CAP, high pneumococcal loads in blood were associated with increased risk of both septic shock and mortality [387]. This study also found that there was a marked increase in mortality above a bacterial load of 10^3cfu/mL. The relatively small number of deaths in our study will have limited our ability to demonstrate any associations with mortality. Pneumococcal load in blood has also been associated with the development of systemic inflammatory response syndrome in adults with CAP [271]. In children with

meningitis and pneumonia, pneumococcal load was also associated with mortality and inflammatory markers [280].

The use of PCR to as a diagnostic tool for pneumococcal disease has had mixed results. Qualitative PCR has been useful for detection of pneumococcal DNA in blood specimens, especially from children [265, 394], but detection in respiratory specimens has been difficult to interpret because of the inability to differentiate colonisation from infection [262, 267]. The choice of PCR assay has a demonstrated effect on results. In a previous study we used a different assay that targeted the pneumolysin (*ply*) gene [267]. This assay has since been shown to have relatively low specificity [281, 368, 382, 395], especially compared with pneumococcal *lytA*-based PCR assays [268, 391], and the use of pneumolysin-based quantitative PCR assays have been questioned with regards to clinical usefulness [392]. Additionally, when compared with blood culture as the "gold standard", *ply* PCR in WBC and plasma had low sensitivities of 10% and 24%, respectively [267]. In our study the *p* values were calculated and showed statistical significance in the comparison between the *ply* and *lytA* PCRs on sputum and between serum and plasma. Starting from the hypothesis that both tests are different in performance, the *p* values indicate that the chances of the tests to be the same in performance are minute. Looking at the sensitivities of the *lytA* PCR against *ply* PCR the performance is disappointing and the *p* values confirm this. However, it is also important to remember that the number of data points was small and serum and plasma is not an exact specimen type match. On the basis of that, only limited conclusions can be drawn and it is doubtful that the statistical significance demonstrated is clinically useful.

The *lytA* gene was chosen as the target for the quantitative assay in our study. This gene is thought to be present in most, if not all, pneumococcal serotypes, acknowledging that occasional non-pneumococcal strains may also harbour the *lytA* gene [281, 393].

In the present study PCR detected *S. pneumoniae* in almost all culture-positive and in many culture-negative sputum specimens reflects the increased sensitivity of PCR. In contrast, serum specimens from 5 of 11 patients with positive blood cultures were PCR-negative and an additional 17 serum specimens from blood culture-negative patients were PCR positive. These results may reflect a combination of increased sensitivity of PCR and the low-level intermittent nature of pneumococcal bacteraemia with occasional sampling errors causing false negative results. It is also worth noting here that blood cultures and serum samples for PCR testing were not necessarily taken at exactly the same time points, thus potentially accounting for the discrepant results between culture and PCR. We also detected pneumococcal DNA in relatively few urine specimens. Although PCR is generally thought to

be less affected than culture by prior antimicrobial treatment, we noted that detection of *S. pneumoniae* by PCR in sputum (but not other specimens) was less common in patients who reported antimicrobial use prior to specimen collection.

Others have reported that plasma and pleural fluid bacterial loads are lower after prior antimicrobial use [396]. The application of quantitative PCR enabled the estimation of pneumococcal burden in clinical specimens. In general, the findings were not surprising, with pneumococcal load being considerably higher in sputum specimens than in positive serum or urine specimens. In addition, bacterial loads were higher in culture-positive compared to culture-negative sputum specimens and in serum from patients with positive blood cultures compared to those with negative blood cultures.

The limitations of this study are the incompleteness of sputum, serum and urine sets for all patients, and the relatively small number of PCR-positive serum and urine samples. The relatively small number of patients who were admitted to the intensive care unit or who died limits the ability to analyse these variables as markers of disease severity. Another limiting factor of the study was the use of serum instead of whole blood. The latter specimen type has the theoretical advantage of also capturing intracellular organisms, but was unavailable to test in our study. In addition, sample deterioration may have occurred through storage resulting in false negative results. Finally, although throat and nasopharyngeal swabs were collected at the time of the CAP study in 1999/2000, these specimens were not available at the time of this study and prevented us from evaluating the utility of measuring pneumococcal load particularly in nasopharyngeal specimens. Recent data has shown some interesting associations between nasopharyngeal pneumococcal load and the presence of radiologically confirmed pneumonia and viral co-infection in children [272] as well as a link between pneumococcal density in the nasopharynx and the likelihood of pneumococcal pneumonia diagnosis in adult patients [266].

The strengths of this study are the use of specimens from a very well-characterised group of adults with CAP. The findings add to an increasing body of evidence suggesting that determination of pneumococcal load has clinical utility. Higher pneumococcal load in serum and, possibly, urine was associated with increased disease severity in adults with pneumonia. Further work is needed to determine whether measuring pneumococcal load in respiratory specimens from adults will differentiate colonisation from coincidental carriage.

Furthermore, the study strengthens the view that it is unlikely that a single diagnostic marker or marker of disease severity is within diagnostic reach. Diagnosing IPD and

differentiating IPD from pneumococcal colonisation in patients with CAP remains a challenge.

EVALUATION OF MALDI-TOF MS AS AN IDENTIFICATION TOOL

CHAPTER 10

MALDI-TOF MS: DIFFERENTIATION OF THE PNEUMOCOCCUS FROM NON-PNEUMOCOCCAL STREPTOCOCCI OF THE MITIS GROUP

10.1. Introduction

The diagnosis of invasive pneumococcal disease (IPD) is often complicated by the difficulty of differentiating between colonisation and invasive disease. Not only can *S. pneumoniae* be detected in non-invasive specimens, but diagnosis is also complicated by the fact that the pathogen belongs to a group of closely related commensal streptococci, the *S. mitis* group. This group includes 12 species according to 16S rRNA sequence-based analysis [286]. The analysis of 16S rRNA and housekeeping gene sequences shows that *S. pneumoniae* is most closely related to *S. pseudopneumoniae*, *S. mitis* and *S. oralis* [397]. Phylogenetic clustering analysis based on concatenated partial sequences has shown a cluster to include type strains of *S. pneumoniae*, *S. pseudopneumoniae* and *S. mitis*, whereas a second cluster included the type strain of *S. oralis*. A third, more diffuse cluster contained the type strain of *S. infantis* [398]. Bacteria of this streptococcal group, which inhabit the same ecological niche, are naturally competent for genetic transformation and it is thought that exchange of genetic material by homologous recombination was an important factor in their evolution [62, 399-401]. As a consequence, many physiological and molecular traits, which are used for the identification of *S. pneumoniae*, are shared by other members of the group [368, 402]. Currently, molecular targets used to differentiate between these species, other than 16S rRNA sequencing, include the *groESL* genes, encoding for highly conserved heat shock proteins [58], *recA*, a gene for the subunit of the bacterial recombinase [286], and some housekeeping genes such as *sodA* [220, 397, 403], *gdh* [404] and *rpoB* [405].

All species belonging to this group can reside as commensals in the human nasopharyngeal tract and can sometimes become invasive pathogens. The presence of any of the members of the *S. mitis* group in blood cultures can be clinically significant, in particular in immunocompromised patients. The treatment strategy of the streptococci other than *S. pneumoniae* differs due to their high penicillin resistance rates [406]. Secure differentiation between these species represents a common challenge for the routine microbiology laboratory and misidentifications occur with all routinely employed identification systems [368, 407, 408].

Recently, matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) techniques have been introduced as a routine microbial identification tool [409-413]. The identification of pathogens is based on the generation of mass spectra from whole bacterial cells or cell extracts, which are compared to a reference library of well

characterised isolates by means of whole spectrum similarity measures [414]. Commercially available systems provide reliable identification for the majority of clinically relevant species. Nevertheless, their algorithms regularly fail at differentiating between *S. pneumoniae* and its closest relatives [287]. On the other hand, multiple studies have shown ways to increase the discriminatory power of mass spectrometry at the species level [291, 412, 415, 416].

In this study the potential ability of MALDI-TOF MS to differentiate between the pneumococcus and other closely related streptococci was investigated as a "proof of concept".

10.2. Material and methods

10.2.1. Bacterial isolates

Two *S. pneumoniae* type strains (ATCC 33400 and 49169), four *S. pneumoniae* serotypes (types 6B, 19A, 19B, and 14; Staten Seruminstitut, Copenhagen, Denmark), and one strain of *S. pseudopneumoniae* (ATCC BAA-960, type strain), one isolate of a non-pneumococcal strain of the *S. mitis* group (#1) and one strain of *S. oralis* (NZ isolate SID 08/60) were provided by the New Zealand Reference Culture Collection at ESR (Institute of Environmental Science & Research, Porirua, New Zealand) and included in the analysis. All other strains were clinical isolates that had been collected and stored at -80°C at Canterbury Health Laboratories, Christchurch, New Zealand. Of those, 8 were clinical *S. pneumoniae* blood culture isolates (2 each of serotypes 6B, 19A, 19B, and 14, serotyped by ESR), and 17 were clinical isolates identified as non-pneumococcal isolates belonging to the *S. mitis* group by ESR by non-molecular methods. Thirteen were clinical isolates of *S. pseudopneumoniae* (226/432, 490/944, 499/962, 785/1491, 773/1479, 169/320, 474/913, 747/1438, 705/1359, 554/1066, 22/57, 35/48, 83/152) that had been identified on the basis of their variable optochin sensitivity (resistance in 5% CO_2 and susceptibility in ambient air), bile insolubility, DNA probe hybridization test positive (AccuProbe *Streptococcus pneumoniae*, GenProbe, San Diego, CA, USA), NOW *S. pneumoniae* immunochromatographic antigen test (Binax, Portland, ME, USA) positive, pneumolysin gene (*ply*) positive by PCR and capsule negative (Quellung test); most of these *S. pseudopneumoniae* isolates identified as *S. mitis* or *S. oralis* using the Rapid ID32 Strep identification kit (BioMérieux, Marcy l'Etoile, France) [221].

10.2.2. Sequence-based identification

The limited ability of current phenotypic identification procedures to differentiate the *S. mitis* group has been well documented and it is recommended to use DNA sequencing of conserved genes such as the *rpoB* gene[405], *sodA* [417] or the *recA* gene [286]. Definitive sequence-based identification was sought for all study isolates presumably identified as *S. pseudopneumoniae*, or non-pneumococcal isolates belonging to the *S. mitis* group by conventional methods.

The bacterial recombinase A gene (*recA*), which has been shown to allow phylogenetic differentiation within the *S. mitis* group [286], was chosen as the target gene. Bacterial lysis was performed as described in section 2.2.2.11. The DNA was extracted from isolated colonies on 5% sheep blood agar by inoculating 500µL of a detergent based buffer (unpublished) and then boiling for 10 minutes. Cellular debris was removed by centrifugation and 5µL of DNA extract (supernatant) was added to 20µL of a PCR reaction mixture containing 1x PCR reaction buffer with $MgCl_2$ (2.0mM final concentration), 100µM of dNTP (Roche Diagnostics, Indianapolis, Indiana, USA), 1.0µM of each primer recA-2F [5'-GCTT(T/C)ATCGATGC(C/T/G)GA(G/A)CA-3'] and recA-5R [5'-GTTTCCGG(G/A)TT(A/T/G)CC(G/A)AACAT-3'] (Invitrogen New Zealand Ltd.**,** Mulgrave, Victoria, Australia) [286], and 1.25 U of FastStart Taq polymerase (Roche Diagnostics, Indianapolis, Indiana, USA). PCR cycling parameters included an initial denaturation for 10 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, and a final extension for 5 min at 72°C. Amplicons were purified with High pure PCR product purification kit (Roche Diagnostics, Indianapolis, Indiana, USA) following the manufactures instructions. The partial *recA* gene was sequenced using primers recA-2F and recA-5R and Big Dye terminator chemistry v3.1 (Invitrogen New Zealand Ltd.**,** Mulgrave, Victoria, Australia). The DNA sequencing products were analysed on ABI genetic analyser Avant 3130xL (Invitrogen New Zealand Ltd.**,** Mulgrave, Victoria, Australia). The DNA sequences were analysed by web-based alignment at http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi and the cut-off for *recA*-based species identification was set at 99% sequence similarity.

10.2.3. MALDI-TOF MS

Bacterial isolates were grown on 5% sheep blood agar at 37°C with 5% CO_2. Bacteria were harvested with a 10µl loop on several different time points. The two pneumococcal ATCC isolates and all *S. pseudopneumoniae* isolates were harvested for mass spectrometry at times 10, 16, 26 and 32 hours, all other bacterial isolates were harvested at times 20 and 26 hours. The bacterial colonies were suspended in 300µl of sterile water, vortexed as per manufacturer's instructions, then mixed with 900µl of 100% ethanol, vortexed again and centrifuged at 7550 x g for 2 min at room temperature. The supernatant was discarded and the ethanol was removed by a second centrifugation and a 5 min drying time of the pellet at room temperature. The pellet was resuspended in 50µl of 70% formic acid and then 50µl of acetonitrile were added mixed with the suspension, followed by centrifugation at 7550 x g for 2 min. One µl of each bacterial extract was spotted onto a ground steel target (Bruker Daltonik GmbH, Bremen, Germany) and air dried for 15 min. Each spot was overlaid with 1µl of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) and allowed to air dry for 15 min prior to MALDI-TOF MS. The method described above was also trialled on blood culture broth that indicated the presence of streptococci on Gram stain. Additionally, a selection of strains was prepared as direct bacterial smears transferred on to the ground steel plate plus the addition of 1µl of 70% formic acid per spot prior to the addition of the matrix solution.

Spectrum acquisition was performed on a Microflex LT benchtop instrument with the flexControl 3.3 software (Bruker Daltonik GmbH, Bremen, Germany) in linear positive mode at a laser frequency of 60 Hz over a mass range of 2,000-20,000 Da. Voltages of ion source 1 and 2 were set at 20 and 18.13kV, respectively. Spectra were accumulated from 160 laser shots per spot. Between 20 and 24 good quality spectra were obtained for each bacterial extracts. A bacterial test standard (Bruker Daltonik GmbH, Bremen, Germany) was included on each ground steel target for instrument calibration.

10.2.4. Analysis of mass spectra

Analysis of the mass spectra was performed using the spectrum view of flexAnalysis and Biotyper 3.0 software (Bruker Daltonik GmbH, Bremen, Germany).

All spectra underwent smoothing and baseline subtraction according to standard settings deposited in the flexAnalysis MBT-Standard.FAMS method. Afterwards, peaks that facilitated the differentiation between *S. pneumoniae* and other *S. mitis* group organisms were identified by visual comparison of all spectra in flexAnalysis as well as in the gel view representation in Biotyper 3.0. In order to identify single peaks the "zoom function" in flexAnalysis was used. This allowed the verification of individual peak presence or absence. Of note, the gel view represents the spectral peaks in a banding pattern. Visually it might simplify the demonstration of specific spectral "fingerprints". The presence (+) or absence (-) of these discriminatory peaks in the mass spectra of each isolate was noted in a peak profile (peak presence was assumed if a peak was observed in all spectrum replicates of a given isolate). Peak intensities were not included in the analysis. Each peak profile was additionally cross-checked by visual examination in the gel view of MALDI Biotyper 3.0. Isolate grouping by peak profile was compared to conventional identification results. Sequences were reported to GenBank (www.ncbi.nlm.nih.gov/GenBank/) and the nucleotide sequence accession numbers are listed in **Table 10.1**.

10.3. Results

10.3.1. Reference identification of clinical isolates

This study aimed to identify MALDI-TOF peak signals that discriminate *S. pneumoniae* from closely related *S. mitis* group isolates. Reference identification of study isolates was achieved using a polyphasic approach [418] requiring consistency of *recA* sequencing result and biochemical identification by Rapid ID32 Strep identification kit (BioMérieux, Marcy l'Etoile, France) for species level identification. Nine of the 33 non-pneumococcal study isolates showed inconsistent findings and were thus classified as *S. mitis* group (**Table 10.1**, first column). The sequences that obtained 99% sequence similarity by *recA*-based sequencing were submitted to GenBank.

10.3.2. Identification of discriminatory *m/z* values

The bacteria extracted with the standard 70% formic acid method produced spectra with reduced reproducibility. It was therefore decided to proceed with the analysis on spectra obtained from bacteria using the extended extraction procedure described in section 10.2.3.

The attempts to obtain spectra from direct specimens (e.g. from blood culture broth), rather than culture isolates, was unsuccessful. This was attributed to the low number of organisms present in the blood culture broth leading to a failure to obtain peak profiles post extraction of the broth (a bacterial pellet could not be visualised post extraction as recommended by the manufacturer).

Discriminatory peaks were detected at *m/z* values 2625, 2911, 2937.5, 5253, 5824, 5877, and 6955. Peak position variation between acquisitions despite calibrations should not exceed 500 ppm, which translates to about +/-3 Da at *m/z* 5000 (flexAnalysis 3.3 User Manual; Bruker Daltonik GmbH, Bremen, Germany). The discriminatory *m/z* positions listed here were determined by calculating the mean from 30 values per peak obtained by the flexAnalysis peak detection algorithm. Spectra obtained in quintuplicate and at different time points during bacterial growth showed only a minor degree of variation regarding the presence or absence of these peaks. Three peak pairs (*m/z* 5877 and *m/z* 2937.5, *m/z* 5824 and *m/z* 2911, *m/z* 5253and *m/z* 2625) showed a high degree of correlation and are assumed to each represent differently charged states of a single protein.

Analysis of peak profiles with respect to isolate reference identification demonstrated that characteristic peak patterns could be attributed to different streptococcal species (**Table 10.1**). *S. pneumoniae* isolates consistently showed presence of the correlated peak pair *m/z* 2937.5 and *m/z* 5877 and absence of peaks at *m/z* 2625, 2911, 5253, 5824, and 6955. Of notice, none of the non-pneumococcal isolates exhibited a peak profile associated with the *S. pneumoniae* isolates in this study (MALDI profile 1 (MP) 1).

S. pseudopneumoniae isolates were also characterised by the presence of the correlated peak pair *m/z* 2937.5 and *m/z* 5877. In addition, correlated peaks at *m/z* 2625 and *m/z* 5253were consistently found (MP2).

Isolated peaks at *m/z* 2911, 5824 and 6955 were frequently observed in the spectra of *S. oralis* isolates (MP4). Additional profiles were recorded for non-pneumococcal isolates 22/57 (MP3), SMIT 1, SORA 7 and SORA 9 (MP5), SMIT 7 (MP6). A summary of the MALDI profiles and the associated streptococcal species is given in **Table 10.2**. A complete peak profile of *S. pneumoniae* (ATCC 33400) is shown in **Figure 10.1**. Examples of the “zoomed

in" analysis of individual peaks in flexAnalysis are given in **Figure 10.2**. **Figures 10.3** a) and b) illustrate what is seen in gel view.

Figure 10.4 shows a MSP (main spectral projection) dendrogram representation of hierarchical cluster analysis including the strains analysed in this study and streptococcal strains in the Bruker database. The dendrogram represents the relative distance between the listed strains in terms of their protein spectra similarity. The degree of similarity of spectra is somewhat arbitrary since there is no established "cut off" level as there is with phylogenetic analysis based on DNA sequences. The MSPs show a degree of proteome relatedness within the species clusters.

10.3.3. Sequencing results

All but one of the *S. pseudopneumoniae* strains matched *S. pseudopneumoniae* when aligned with sequences on NCBI BLAST. It was the only isolate slightly deviating from the MALDI profile typical for *S. pseudopneumoniae* missing a peak at *m/z* 2937.5. Three out of 7 presumed *S. mitis* clinical isolates produced sequencing results consistent with *S. oralis* and a matching MALDI profile. The other 4 presumed *S. mitis* isolates aligned with a variety of different streptococcal sequences, but not with *S. mitis*. Only one presumed *S. mitis* (SMIT 7) isolate sequenced as *S. mitis*, its MALDI profile being unique (MP6). All except one isolate in the presumed *S. oralis* group sequenced as *S. oralis*. The sequences that obtained 99% sequence similarity by *recA*-based species identification were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/) and the assigned accession numbers are included in **Table 10.1**. The *recA* sequence-based identifications listed in the table without an accession number signal less than 99% sequence similarity.

Table 10.1: MALDI profiles and *recA* sequencing result of different streptococcal species

Isolate	*recA* Sequence (GenBank accession number)	Conventional Identification[c]	*m/z* Value[a]							MALDI Profile
			2625	2911	2937.5	5253	5824	5877	6955	
S.pneumoniae ATCC 33400	*S.pneumoniae*	*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae ATCC 40619	*S.pneumoniae*	*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 6B (type strain)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 19A (type strain)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 19F (type strain)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 14 (type strain)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 6B (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 19A (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 19F (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 14 (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 6B (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
*S.pneumoniae*19A (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 19F (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pneumoniae 14 (clinical isolate)		*S.pneumoniae*	-	-	+	-	-	+	-	MP1
S.pseudopneumoniae BAA-960	*S.pseudopneumoniae*	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 226/432	*S.pseudopneumoniae* (JQ957541)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 490/944	*S.pseudopneumoniae* (JQ957514)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 499/944	*S.pseudopneumoniae* (JQ957524)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 785/1491	*S.pseudopneumoniae* (JQ957517)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 773/1479	*S.pseudopneumoniae* (JQ957519)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 169/320	*S.pseudopneumoniae* (JQ957515)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 474/913	*S.pseudopneumoniae* (JQ957520)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 747/1438	*S.pseudopneumoniae* (JQ957516)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2

S.pseudopneumoniae 705/1359	*S.pseudopneumoniae* (JQ957518)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 554/1066	*S.pseudopneumoniae* (JQ957522)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 35/48	*S.pseudopneumoniae* (JQ957523)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S.pseudopneumoniae 83/252	*S.pseudopneumoniae* (JQ957521)	*S.pseudopneumoniae*	+	-	+	+	-	+	-	MP2
S. mitis group[b] *22/57*	*S. mitis/ S.pneumoniae*	*S. pseudopneumoniae*	+	-	-	+	-	+	-	MP3
S. mitis group[b] *#1*	*S. oralis*	*S. mitis*	-	+	-	-	+	-	+	MP4
S. mitis group[b] *(SMIT 1)*	*S. sanguinis*	*S. mitis*	-	-	-	-	-	-	+	MP5
S. mitis group[b] *(SMIT 2)*	*S. oralis* (JQ957525)	*S. mitis*	-	+	-	-	+	-	+	MP4
S. mitis group[b] *(SMIT 3)*	*S. oralis* (JQ957526)	*S. mitis*	-	+	-	-	+	-	+	MP4
S. mitis group[b] *(SMIT4)*	*S. mitis/S. pneumoniae*	*S. mitis*	-	-	-	-	-	-	+	MP5
S. mitis group[b] *(SMIT 5)*	*S. oralis* (JQ957527)	*S. mitis*	-	+	-	-	+	-	+	MP4
S. mitis group[b] *(SMIT 6)*	*S.pseudopneumoniae*	*S. mitis*	-	+	-	-	+	-	+	MP4
S. mitis (SMIT 7)	*S. mitis*	*S. mitis*	+	-	+	-	-	+	+	MP6
S. oralis SID 08/60	*S. oralis* (JQ957528)	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 1)	*S. oralis* (JQ957529)	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 2)	*S. oralis* (JQ957530)	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 3)	*S. oralis* (JQ957531)	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 4)	*S. oralis* (JQ957532)	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 5)	*S. oralis* (JQ957533)	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 6)	*S. oralis* (JQ957534)	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 7)	*S. oralis* (JQ957535)	*S. oralis*	-	-	-	-	-	-	+	MP5
S. mitis group[b] *(SORA 9)*	*S. sanguinis*	*S. oralis*	-	-	-	-	-	-	+	MP5
S. oralis (SORA 10)	*S. oralis*	*S. oralis*	-	+	-	-	+	-	+	MP4
S. oralis (SORA 11)	*S. oralis* (JQ957536)	*S. oralis*	-	+	-	-	+	-	+	MP4

[a] "-" absence of peak, "+" presence of peak at a given *m/z* value

[b] *S. mitis* group other than *S. pneumoniae*

[c] The conventional identification performed by ESR was based on non-molecular methods

Table 10.2: MALDI profiles and their associated streptococcal species

Larger strain collections of the listed species need to be evaluated to confirm the consistency of the MALDI profiles.

MALDI Profile	Streptococcal species
MP1	*S. pneumoniae*
MP2	*S. pseudopneumoniae*
MP3	Non-pneumococcal isolate*
MP4	*S. oralis*
MP5	*S. sanguinis*
MP6	*S. mitis*

*This isolate identified as *S. pseudopneumoniae* by non-molecular methods and as *S. mitis/S. pneumoniae* by *recA*-based sequencing. However, sequencing similarity was <99% and the sequence was not submitted to GenBank.

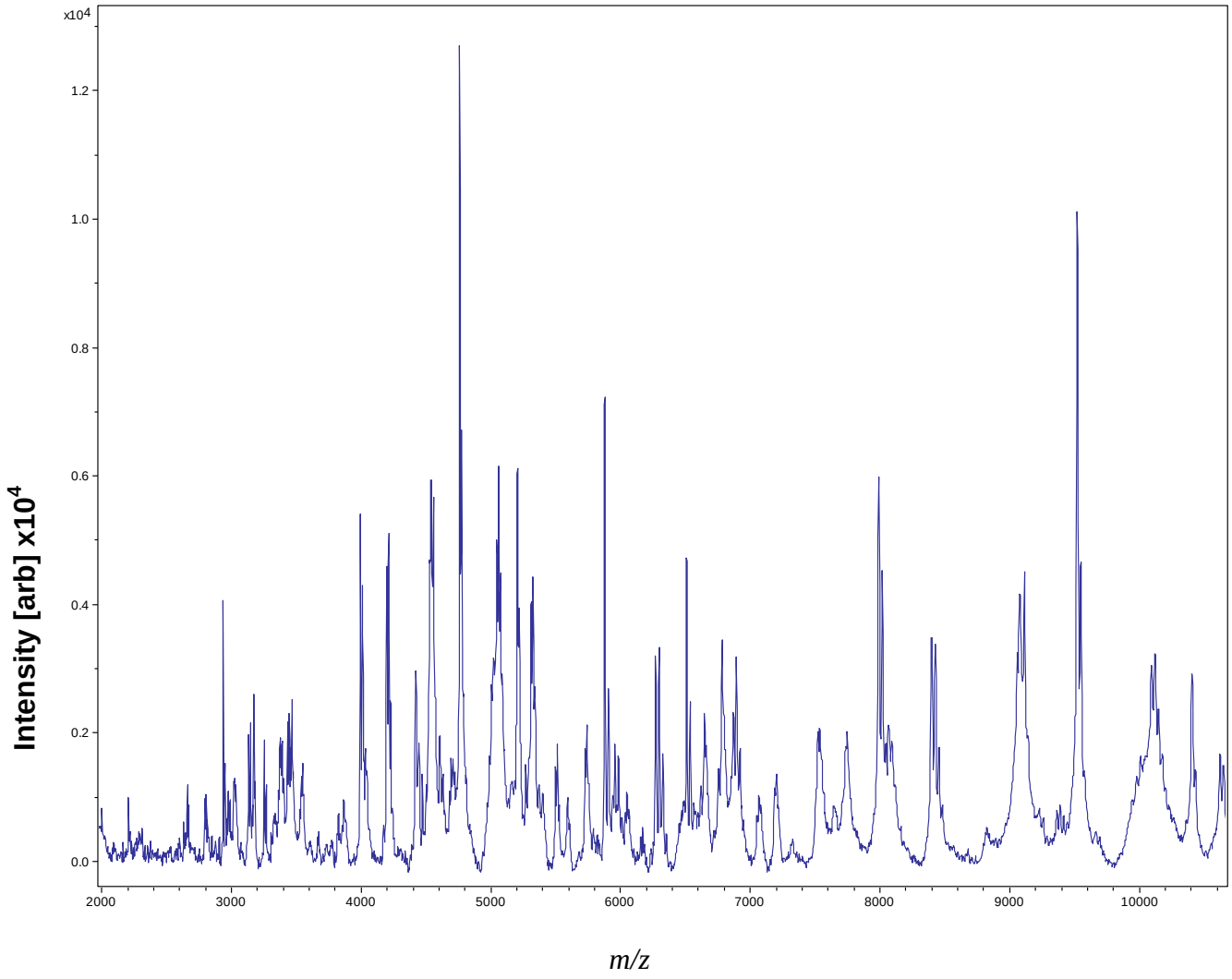
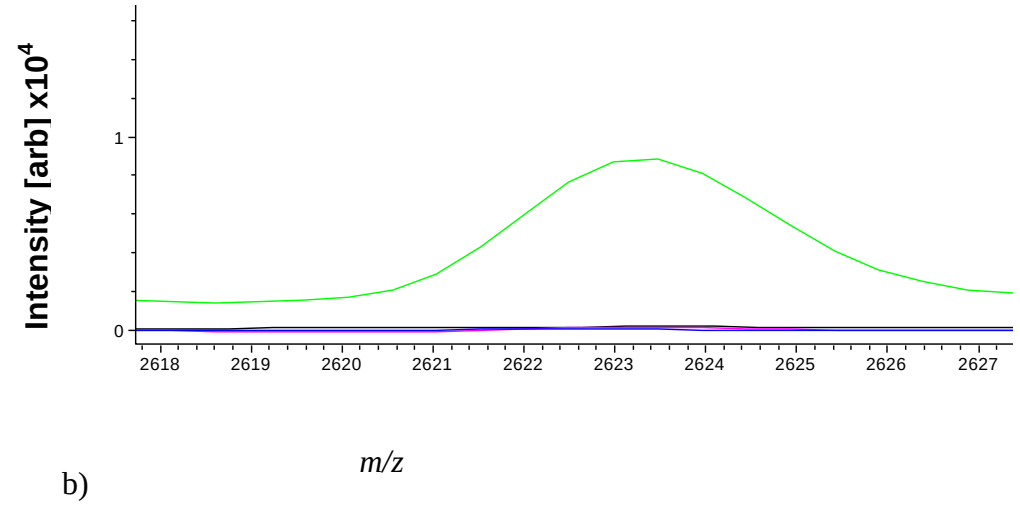
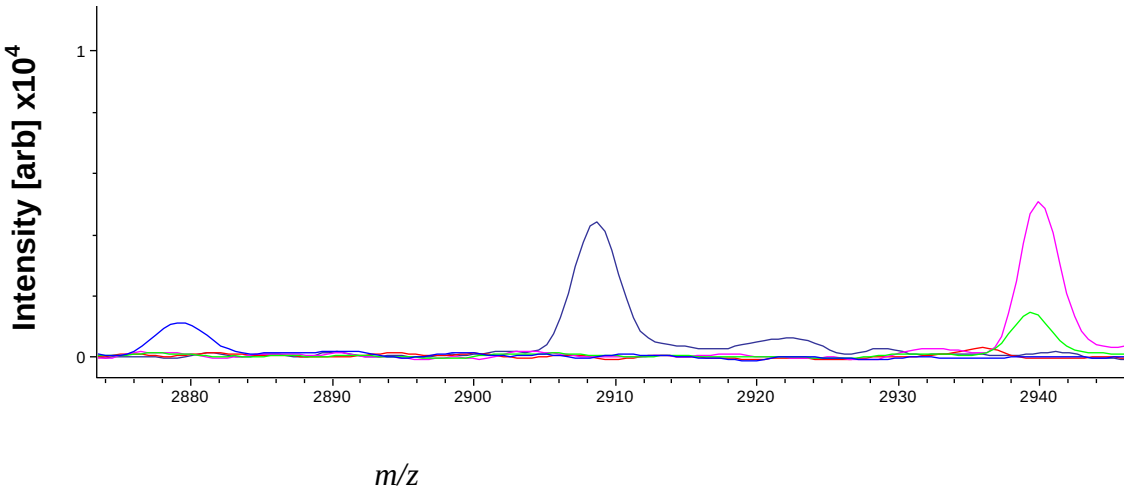


Figure 10.1: Complete protein peak profile of *S. pneumoniae* (ATCC 33400)

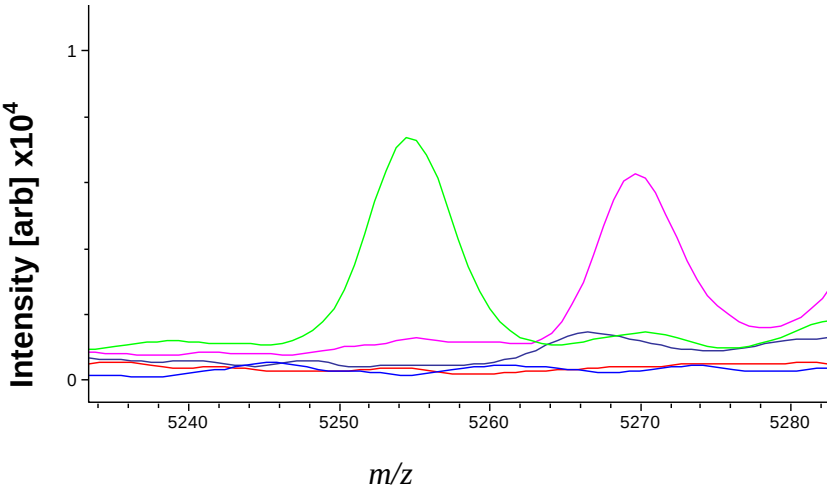
a)


Intensity [arb] x10^4
1
0
2618
2619
2620
2621
2622
2623
2624
2625
2626
2627
m/z

b)


Intensity [arb] x10^4
1
0
2880
2890
2900
2910
2920
2930
2940
m/z

c)


Intensity [arb] x10^4
1
0
5240
5250
5260
5270
5280
m/z

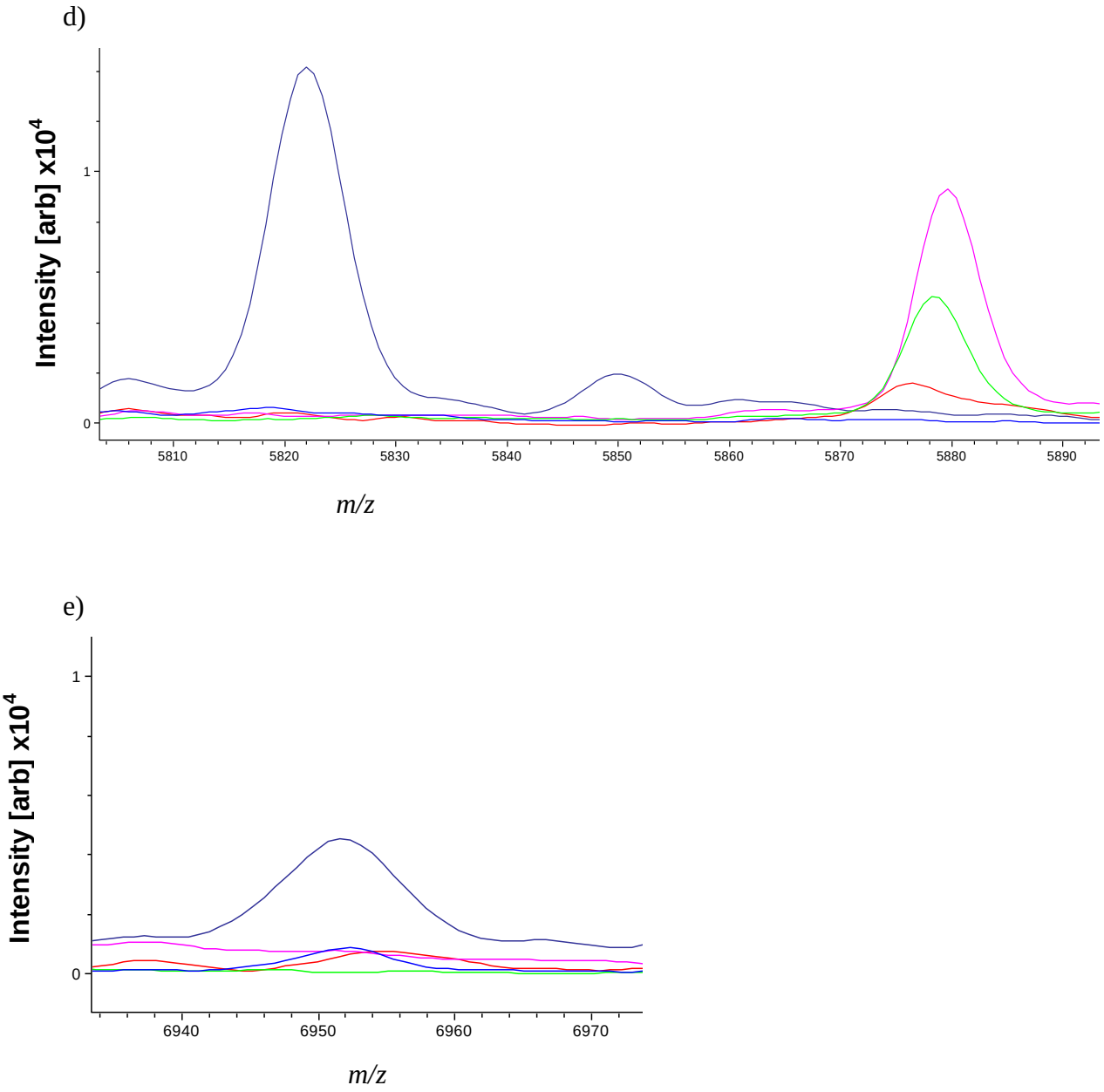


Figure 10.2: Individual *m/z* as they are visible on the flexAnalysis software

Spectra are overlaid to ensure consistency with measured intensities. The chosen spectra were from strains with sequence confirmed identification. *S. pneumoniae* (pink), *S. pseudopneumoniae* (green), *S. oralis* (black), *S. sanguinis* (blue) and *S. mitis* (red). A) *m/z* 2625, B) *m/z* 2911and 2937.5, C) *m/z* 5253, D) *m/z* 5824 and 5877, E) *m/z* 6955.

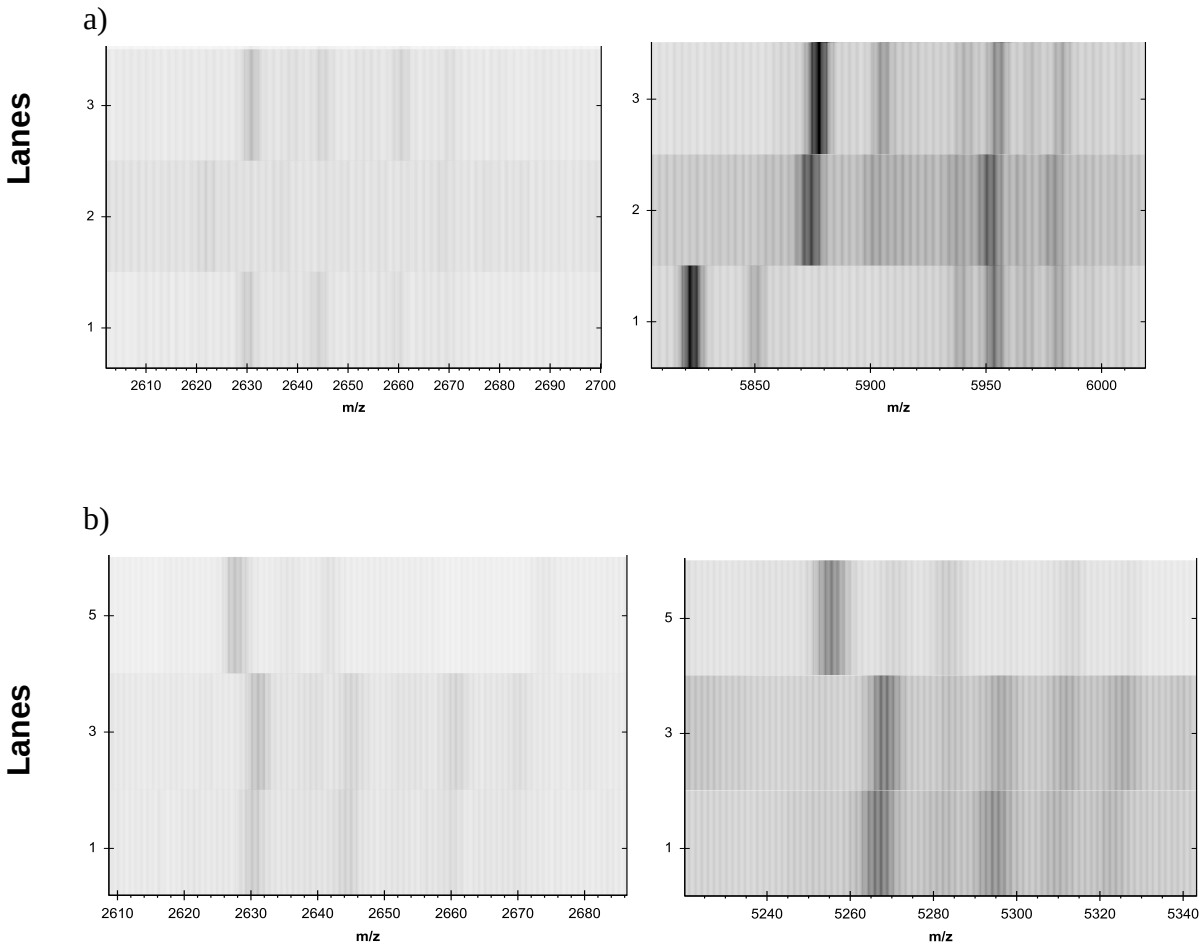


Figure 10.3: Gel view representation of spectral peaks at defined *m/z*

a) Lane 1: *S. pneumoniae*, lane 2: *S. pseudopneumoniae*, lane 3: *S. oralis*; b) lane 1: *S. pseudopneumoniae*, lane 2: *S. pseudopneumoniae*, lane 3: *S. oralis*.



Figure 10.4: Main spectral projection (MSP) dendrogram

The dendrogram demonstrates a hierarchical cluster analysis based on spectral similarities of recorded MALDI spectra from the Bruker database and those analysed in this study.

10.4. Discussion

MALDI-TOF MS was first proposed as a tool for bacterial identification in 1996 [419]. The basis of MALDI-TOF MS for bacterial identification and species differentiation is the rapid detection of the mass-to-charge (*m/z*) ratio of a bacterial isolate and the provision of a unique fingerprint. The characterisation of molecules is based on their molecular mass (*m*), the charge (*z*) and the resulting ratio (*m/z*) as well as the relative intensity of the signal. The idea to use *m/z* of closely related bacterial species for their differentiation is not a new concept.

A publication in 2000 suggested that mass spectra of spore cells of the *B. cereus* group allow differentiation from other Bacillus species [420], streptococci of the mutans group were successfully differentiated by MALDI-TOF MS in 2005 [421] and viridians streptococci have been successfully discriminated by MALDI-TOF MS in 2007 [291]. By now, dedicated mass spectrometry systems are established for routine species identification in many laboratories that achieve discrimination of even closely related species by the comparison of spectrum fingerprints acquired within minutes from tiny amounts of colony material to exhaustive reference databases. Still, current algorithms produce variable results for streptococci of the *S. mitis* group and misidentifications occur frequently in particular for *S. pneumoniae*, *S. mitis* and *S. oralis* [290]. This is of importance considering that all members of the *S. mitis* group can be pathogens and their presence in blood cultures, in particular in immunocompromised patients, can be clinically significant and often requires treatment strategies that differ from *S. pneumoniae* due to high penicillin resistance rates in the *S. mitis* group [406]. In contrast to misidentifications reported from evaluations of commercial MS-fingerprinting systems of *Brucella* spp., *Pseudomonas* spp. and *Acinetobacter* spp., the weakness of these systems in differentiating species from the *S. mitis* group differs. In a study by Williamson *et al.*, clustering by whole spectrum similarity allowed for the correct identification and recognition of isolates belonging to a *S. pneumoniae* conjunctivitis outbreak, but failed to separate non-outbreak pneumococcal isolates from *S. mitis* and *S. oralis* strains [412]. This failure could be result of true limitations of phylogenetic resolution of whole spectrum similarity measures employed by MS-fingerprinting systems. However, this analysis here has identified seven discriminatory peaks on a limited set of isolates. These peaks have significant interspecies variability to assist with species differentiation with the *S. mitis* group.

Here, strains that were species typed by conventional microbiological methods were compared with MALDI profiles based on a subset of discriminatory peaks. Six distinct

MALDI profiles emerged. *S. pneumoniae* grouped consistently in MP1 and no non-pneumococcal isolates demonstrated peak patterns that could be assigned to this profile. *S. pseudopneumoniae* grouped in MP2 and *S. oralis* in MP4. The other non-pneumococcal species belonging to the *S. mitis* group demonstrated peak profiles assigned to MPs 3, 5 and 6. These MALDI profiles are not derived from the algorithms used in the Bruker database nor have they been referred to in the literature before. However, a recent publication has referred since to one of the peaks identified as part of the work contributing to this thesis [422]. At this stage they are based on the analysis of very few isolates and only represent enough data for a "proof of concept." In order to develop an algorithm that allows the introduction of these MALDI profiles into the routine diagnostic setting it is essential to analyse a large number of isolates of each species of the *S. mitis* group.

It is likely that the currently available methods, including single target sequencing, are still insufficiently discriminatory for the *S. mitis* group. To find characteristic MALDI-TOF peaks distinguishing between *S. pneumoniae* and non-pneumococcal isolates belonging to the *S. mitis* group is encouraging. It is conceivable that the incorporation of these findings into existing MALDI-TOF typing software might facilitate the reliable identification of these species under routine conditions.

The identification and speciation of *S. mitis* group strains from a direct clinical specimen like blood cultures would be a further advance in the utilisation of MALDI-TOF MS. The findings in this chapter are based upon the species differentiation of cultured isolates. In culture, some of the species of the *S. mitis* group have characteristic morphological features that aid in the diagnosis, in particular *S. pneumoniae*. These include, in part, colony morphology and haemolysis. When investigating a positive blood culture broth, the only immediate information available is the Gram stain, which indicates the presence of Gram-positive cocci resembling streptococci. At this point, correct species identification by MALDI-TOF MS, in particular of streptococci of the *S. mitis* group would facilitate correct and prompt patient management. The limitation of applying MALDI-TOF MS to direct specimens such as blood culture broth lies in the low number of streptococci of the *S. mitis* group present once the broth is "flagged" as positive by automated blood culture instruments. This is different for Gram-negative organisms or Gram-positives like *S. aureus*. With those organisms large enough numbers of bacteria are present to allow successful bacterial protein extraction and subsequent spectral analysis. In future, this problem might be overcome for the *S. mitis* group by developing protocols to increase the volume of blood that can be processed in a streamlined fashion with the recommended extraction method.

In summary, MALDI-TOF MS is a rapid tool for bacterial identification based on characteristic spectral protein profiles. This study has demonstrated its potential usefulness in differentiating streptococci from an inherently difficult, genetically closely related group (*S. mitis* group), but a much larger number of genetically well characterised streptococcal species needs to be analysed to confirm the robustness of the suggested MALDI profiles. It is therefore important to recognise that the findings discussed in this chapter are preliminary and represent "proof of concept".

Until then, visual check in flexAnalysis or the Bruker Biotyper gel view for the presence of seven peaks could aid the differentiation between *S. pneumoniae* and other *S. mitis* group species.

CHAPTER 11

CONCLUSION AND FUTURE PERSPECTIVE

11.1. Conclusion

Undoubtedly, *S. pneumoniae* is one of the most important human pathogens. Therefore, it is no surprise that the lack of diagnostic methods with appropriate sensitivity, specificity and cost-effectiveness, have led to frustrations among clinicians which have ultimately contributed to the discouragement of pursuing a microbiological diagnosis in current CAP guidelines [423]. Having to resort to empirical therapy because of lack of appropriate diagnostic methods impacts negatively on targeted antimicrobial therapy of individual patients and also hinders the ability of epidemiologists to accurately assess population disease burden or evaluate the efficacy of interventions such as immunisation programmes or novel therapeutic strategies. With this in mind the objective of this thesis was to develop and evaluate novel diagnostic approaches with a focus on the specificity of the test and the ability to implicate *S. pneumoniae*, when detected, as the causative pathogen.

Urine antigen detection tests have been shown to be of some clinical benefit in the diagnosis of pneumococcal pneumonia [217, 247]; however, they lack sensitivity and specificity to be used on their own. The reported sensitivity of the test ranges from 77% to 88% and specificity from 67% to 100% for pneumococcal pneumonia [247, 424]. They can be false-negative in cases with positive blood cultures for *S. pneumoniae*, they can cross-react with other viridians streptococci and they can remain positive for weeks post disease onset. Thus, the test needs to be used in conjunction with other tests. The two antigen-based detection tests developed and evaluated in this study demonstrated similar issues. The pneumolysin (Ply) detection assay was in principle modelled on an assay developed by a Spanish group [340], using the same monoclonal antibody, but a different polyclonal antibody raised against recombinant pneumolysin (Pd-B) expressed in *E. coli*. The resulting interference of the polyclonal antibody with human serum, most likely due to non-specific antigenic epitopes against *E. coli* proteins as well as Pd-B, rendered the assay unsuitable for diagnosis of IPD in serum. Although it is conceivable that this assay may be adaptable to other human specimens, such as respiratory specimens or urine, this was not explored further. The reason behind choosing serum as the specimen for the evaluation was based on the objective to decrease the chance of detection of *S. pneumoniae* in cases of pneumococcal nasopharyngeal colonisation as has been recommended in a recent publication [358]. Furthermore, the general principle used in traditional microbiology diagnostics is to choose the “optimal” specimen for the test. For pneumococcal bacteraemia the “optimal” specimen

would be blood representing the primary site of infection, for pneumococcal pneumonia it would be tissue from the affected pulmonary area. Of course obtaining the "optimal" specimen is not always possible. However, in the case of bacteraemia, blood or serum is a relatively easy to obtain specimen and should be viewed as preferred over specimens from sites that are, although possibly useful, still further removed from the site of infection. In this study, serum and plasma were the only blood specimens used. No whole blood was available. The limited comparison of the performance of plasma versus serum in the ELISAs indicated that they would perform comparably.

In contrast to the experience with the Ply assay, the antigen test to detect pneumococcal surface adhesin A (PsaA) showed more promise. The assay did not demonstrate interference with serum, but, unfortunately, showed poor correlation with other diagnostic methods when results were compared in a well characterised group of adults with CAP. The explanation for this was most likely a lack of analytical sensitivity of the assay. Specificity was assessed by extensive cross-reactivity studies and demonstrated selective reactivity of the monoclonal antibody to *S. pneumoniae* and weakly to *S. pseudopneumoniae*. The lack of reactivity to other members of the *S. mitis* group, in particular *S. mitis* and *S. oralis*, was encouraging. Despite this finding, it is important to remember the possibility of gene transfer to species other than *S. pneumoniae [59]*. Even with this assay and its assured specificity detection of PsaA will not necessarily indicate the presence of *S. pneumoniae.*

It is, however, likely that this assay would be of clinical utility, given increased sensitivity, but further evaluation with a larger patient population, including children with CAP is mandatory.

The need for rapid molecular diagnostic tools for the diagnosis of respiratory pathogens has been widely recognised [425]. For *S. pneumoniae*, PCR-based detection relies on the amplification of gene(s) that are species-specific and unique to the pneumococcus, which is exceedingly difficult. One of the most widely used targets is pneumolysin, but has now been somewhat discredited due to poor sensitivity and specificity as a marker of IPD [263, 264, 267]. In a review of molecular methods to diagnose lower respiratory infections Murdoch stated that the sensitivities for PCR in blood compared to blood cultures as the "gold standard" range from 29% to 100% in adults and 57% to 100% in children. The range of sensitivities was wide even despite similar PCR protocols being used in most studies. In respiratory samples the positivity rates for PCR increased to 68% to 100% in patients with pneumonia. The study did address the issue of sputum samples being PCR positive in colonised individuals and the fact that pneumolysin as a PCR target can be present in other,

non-pneumococcal, viridians streptococci [261]. A recent publication has addressed the role of quantitative assays in differentiating between colonisation and disease. A cut-off value of ≥8000 copies/mL of *lytA* has been proposed to signal pneumonia [266]. Other targets have been identified as potentially performing with higher specificity, the gene fragment Spn9802 [395], *psaA* [354], and *lytA* [426]. In a comparison of three targets on patients with bacteraemic pneumococcal pneumonia (Spn9802, *lytA* and *ply*) *lytA* demonstrated the highest specificity, although the detection of Spn9802 also showed promise, whereas *ply* was shown to lack sensitivity [281]. A publication from 2008 indicated the usefulness of Spn9802 in a quantitative PCR assay. A cut-off value of 10^4 DNA copies/mL was proposed for clinically significant cases [393]. *LytA* was chosen in this study as the gene target for a quantitative PCR assay. The results showed an association of higher bacterial load in serum and, possibly, urine with increased disease severity in the studied adult CAP group of patients. The cut-off value associated with severe disease was 10^3-10^5 cfu/mL for *lytA* PCR. With *lytA* being a single copy gene this cut-off value sits within the range of the values referred to above. The association of severity of disease with bacterial load reflects the most significant finding of Chapter 9. Unfortunately nasopharyngeal swabs were not available for this patient population and, hence, it was impossible to look further into the rates and bacterial load of pneumococcal carriage in the patients overall, and those with severe disease, especially since *lytA* load of ≥8000 copies/mL in the nasopharynx might be sensitive and specific enough to distinguish colonisation from invasive disease in pneumococcal CAP cases [266]. It is likely that quantitative PCR assays, lead at present by *lytA* as a target, will take a front line in pneumococcal diagnostics as they appear to correlate well with disease severity and will help to distinguish the carrier from the patient with IPD. However, they also come with inherent problems. With the lack of a reliable, sensitive "gold standard" to diagnose IPD it is difficult to validate assays. Additionally, variability of individual assay performance makes it hard to standardise cut-offs which are consistent across laboratories.

It seems likely that the quantitative PCR assays will have a definite place in the diagnostic field, but probably not as a single assay. A more accurate diagnosis may be achieved in supplementing results from a molecular assay with traditional diagnostic methods [390] or the detection of inflammatory markers. Procalcitonin has been looked at in this context, but results are inconclusive [427].

The introduction of MALDI-TOF MS has revolutionised bacterial identification in the routine diagnostic laboratory. Previously used identification methods that would have taken

potentially several days of manual work-up have been replaced by a method which is rapid (it takes hardly longer than performing a Gram stain) and extremely cost-effective.

Mass spectrometry has been a routine diagnostic tool in the biochemistry laboratory for some decades and was first applied to bacteria in 1975 [428]. Its entry into routine microbiology was largely made possible by the development of a robust bacterial reference strain database over the last decade or so. Additionally to accumulating a large reference strain database it was crucial to develop an analysing software able to handle large amounts of complex data which was identified as challenge about 10 years ago [429, 430]. Early attempts resulted in the development of MUSE^{TM} (Manchester Metropolitan University Search Engine). The software enabled the MALDI-TOF MS user to rapidly access a summary of the total spectral information including mass and intensity [431]. In 2007, Friedrichs reported a MALDI-TOF MS-based method in which the classification of the spectra rested on a statistical algorithm, SVM (support vector machines) [291]. The concept of SVM was first presented by Vapnik [432]. SVM algorithms have their place particularly in bioinformatics where pattern recognition and discrimination are the objectives. Currently, two different database strategies have evolved. One represents a large database holding a large number of peaks for each reference strain, not focusing on a species-specific pattern of a limited number of peaks, which is the basis for the second strategy. The latter appears to be less affected by growth conditions of the bacterial strains tested [409] and represents the basis to the Bruker Biotyper software.

The idea of looking into MALDI-TOF's ability to differentiate between the species of the *S. mitis* group was mainly driven by clinical need. It is of high importance to differentiate species in order to allow clinicians to give targeted antimicrobials and be aware of the species-dependent risk of penicillin resistance [406]. It was also encouraging that this had been looked at earlier on with some success [291].

It was found that *S. pneumoniae*, *S. pseudopneumoniae* and *S. oralis* produce characteristic and reproducible peak patterns, although it is necessary to confirm this finding with a much larger quantity of well molecularly characterised organisms. They were able to be visually defined with relative ease in flexAnalysis and in gel view of the Biotyper software. *S. mitis*, however, posed a greater problem. This study was limited in so far as only one of the available *S. mitis* strains confirmed as *S. mitis* by *recA* sequencing. Although it demonstrated a different profile from the other species, the reproducibility of this finding would need to be confirmed with a far larger group of organisms that have been unequivocally identified as *S. mitis* by molecular means. It is worth mentioning here that

sequencing of one gene target only (here *recA*) might be insufficient to differentiate the species of the difficult *S. mitis* group reliably. It is also important to remember that GeneBank is an open access databank which is susceptible to holding incorrect identifications.

Still, the findings in this study were encouraging and if applicable to the routine microbiological work-up would assist patient management by identifying non-pneumococcal organisms with possible resistance to the first-line pneumococcal treatment, penicillin, early and avoid unnecessary changes of antimicrobial therapy.

The limitations of the studies for this thesis were the testing of a single, albeit well characterised group of adults with CAP. The samples were collected in 1999/2000 and had been stored at -80°C since then. The samples, however, had been thawed and refrozen on several occasions which might have impacted on the sample integrity, which could have led to antigen and/or DNA degradation. The sample set was incomplete in parts and lacked the collection of nasopharyngeal swabs (NPS) in the patient group. Testing of NPS would have clarified the colonisation status of the CAP patients and would have enabled us to investigate a potential relationship of bacterial load in the nasopharynx and the development of IPD. Furthermore, this study has not investigated the usefulness of the testing strategies included in a set of children with CAP. It is therefore advisable to evaluate the antigen-based detection tests and the molecular-based assay ideally with a prospective case control study, this time including the collection of NPS. Limitations of the MALDI-TOF MS investigation were the limited number of analysed organisms. The reproducibility of the findings will need to be confirmed on a larger scale, which was outside the scope of this work.

It seems fair to say that a solution to the past and present challenges of pneumococcal diagnostic strategies has not been found. However, progress has been made. The findings from this study highlight the difficulties related to sensitivity and specificity inherent in antigen-detection based assays. If they will take a place in pneumococcal diagnostics it will be as a supplemental investigation, not as an exclusive one. The same applies to molecular-based assays. In fact, rather than focussing on *S. pneumoniae* alone, other pathogens might be included in the initial testing of samples, because multiplexing of PCR targets to cover a wide range of respiratory pathogens speeds up the diagnostic process and aids patient management. Syndrome-based testing, like multiplex PCR for CAP, is likely to finds its place in the future microbiology laboratory.

Lastly, in the near future MALDI-TOF MS will become a tool found in most laboratories in the developed world. It will replace the traditional identification of a large number of commonly isolated organisms. It is of utmost importance to focus in more detail on

the protein profiles of closely related bacterial species, of which the *S. mitis* group is only one example. It looks promising that with the further software development accurate species differentiation will be possible. If so, it would replace a costly and time-consuming method that requires skilled scientist input (sequencing). Two methods were not discussed or evaluated in this thesis: diagnostic DNA or peptide microarrays and whole-genome sequencing. As Vernet *et al*. has stated in a recent article looking at unmet needs in pneumococcal diagnostics, methods based on bead technology, microarrays as well as deep sequencing are promising to be useful in determining pneumococcal genotypes and resistance profiles in a single assay [433]. Microarray assays have already been designed to determine pneumococcal serotypes [288, 434, 435]. The method shows promise as a cost-effective monitoring tool, particularly for vaccine-related serotypes. Other applications of microarray technology include transcriptional analysis of gene expression associated with protective immunity against IPD and transcriptome signatures in children with pneumococcal AOM [436, 437]. Although they are likely to play an important part in pneumococcal diagnostics, their entry into the routine laboratory lies some years ahead.

11.2. Future perspective

In summary, these are the suggested future projects that would represent a natural progression from this work:

- Assessment of the described diagnostic assays on paediatric patients with IPD. The collected patient specimens would include sputum, whole blood and urine for culture, molecular-based assays as well as antigen detection tests. Additional specimens would include nasopharyngeal swabs or aspirates to assess the bacterial load in the patients' nasopharynx as well as in specimens from other normally sterile sites, e.g. CSF or joints.
    - Ideally these specimens would be processed real-time or batch tested after having been stored at -80°C, avoiding exposure to recurrent freeze-thaw cycles.
    - As was done in the 1999/2000 CAP study, the patient cohort's demographics and their clinical specifics, such as comorbidities, smoking history, duration of

symptoms, admission to ICU, outcome of clinical episode, would need to be collected.

- It would be of significant value to screen the respiratory specimens of such a cohort for the presence of other bacterial, but in particular viral pathogens. Although that work has been done in part on 1999/2000 cohort, the data on concurrent organisms that were present was based on quantitative PCR, serological and viral culture assays, the latter lacking the required sensitivity. Newer multiplex PCR assays with a wider range of viruses and greater sensitivity are now available and can be set up as quantitative assays.

- Rather than just focussing on one molecular target, such as *lytA*, a quantitative multiplex real-time PCR assay could be developed which would include several *S. pneumoniae* gene targets. A review of gene expression profiles during IPD might assist in the appropriate selection of such targets.
  - DNA microarray analysis could provide an alternative diagnostic platform to real-time PCR assays, allowing the simultaneous screening for numerous pneumococcal gene targets.
- Pneumococcal serotype data was available for the CAP 1999/2000 cohort on BC isolates only. Again, in an ideal cohort, all pneumococcal isolates (from invasive and non-invasive) sites would be serotyped, hence providing invaluable information about the serotypes present in invasive specimens and the nasopharynx from the same patient as well as giving an epidemiological overview of the serotypes currently circulating in Canterbury, New Zealand.
- The antigen detection tests developed during this study could be modified by (i) increasing their sensitivity using chemiluminescence as a detection system, (ii) changing the polyclonal antibody against pneumococcal pneumolysin to increase its specificity and reduce the issue of non-specific binding, which rendered the assay discussed in Chapter 4 clinically not useful, (iii) evaluating purified monoclonal antibodies as capture antibody for the PsaA ELISA to increase sensitivity and specificity of the assay and (iv) evaluating these assays on other clinical specimens, foremost urine and CSF.
  - If the two antigen detection assays would have demonstrated acceptable sensitivity and specificity, it would have been worth considering the set up of a rapid immunochromatographic test strip targeting both antigens.

- MALDI-TOF MS is a very promising tool for the differentiation of species within the *S. mitis* group. A large scale evaluation of the robustness of the method on rigorously characterised streptococcal strains is necessary. The characterisation of the strains will have to include conventional methods as well as a variety of molecular assays, mainly targeting a range of house-keeping genes.
    - MALDI-TOF MS could be modified by using different matrix solutions allowing for detection of a range of proteins exceeding the current range of 2000-20000 Da. Another possible way of achieving this would be to look at different mass spectrometry instruments that would allow a better discrimination of peptide fingerprinting over a larger range of peptide sizes.
    - Finally, development of improved MALDI extraction methods would increase the sensitivity for peptide profiling from direct specimens, such as blood cultures. This could allow rapid species identification of the inherently difficult *S. mitis* group isolates leading to targeted, appropriate patient management.

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