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Diagnosis, prognosis and prevention of severe pneumococcal disease

Carl Spindler

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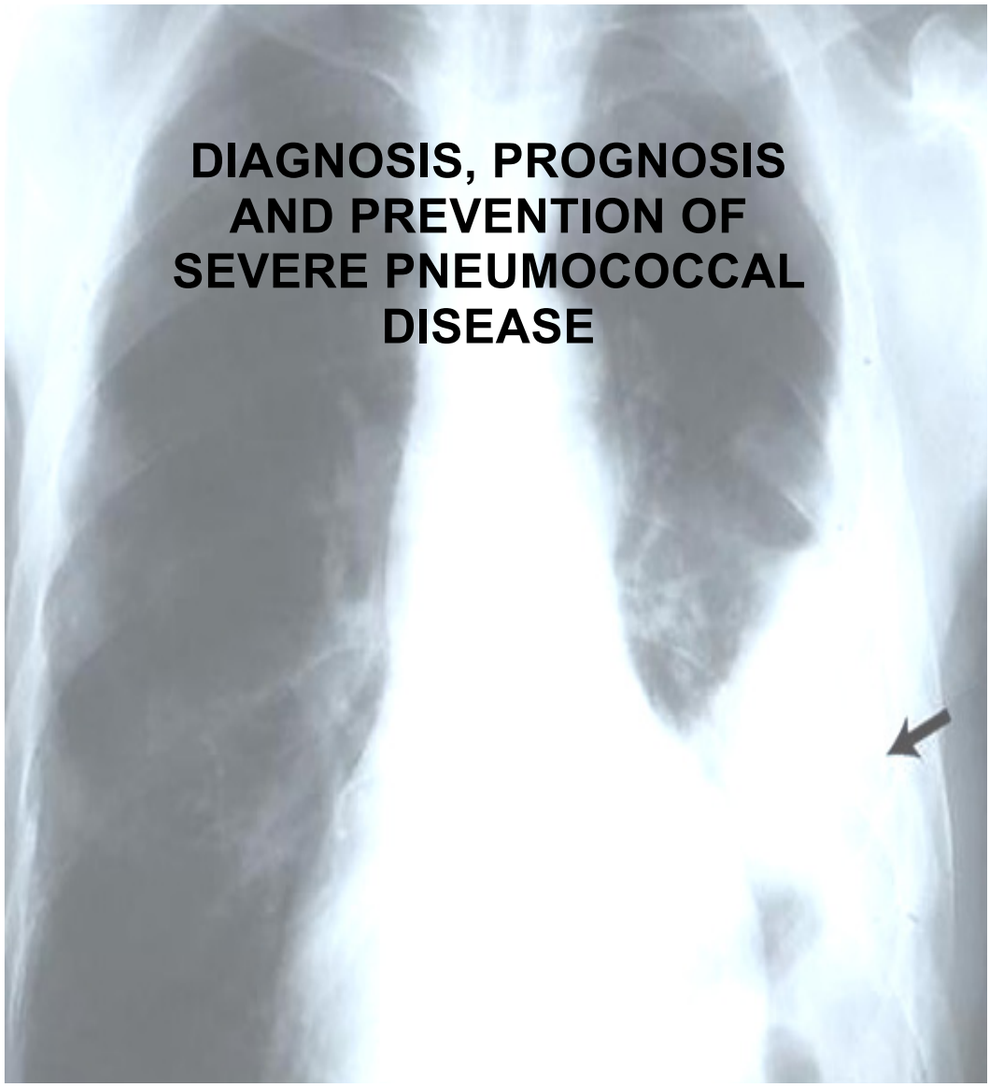
DIAGNOSIS, PROGNOSIS AND PREVENTION OF SEVERE PNEUMOCOCCAL DISEASE



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ABSTRACT

Streptococcus pneumoniae also known as pneumococcus is a major contributor to the disease burden in the world. Infections caused by this bacterium include a wide spectrum of different disease types, from non-severe upper respiratory tract infections to invasive disease forms like bacteremic pneumococcal pneumonia or meningitis, conditions attributed with a high rate of severe disease and mortality. An important feature of this bacterium is that it only causes disease under certain circumstances, and the most common outcome upon contact with this bacterium is transient nasopharyngeal colonization rather than disease from it. Some patient groups such as young children and older people are however at a substantially increased risk of severe disease from the pneumococcus.

The aim of this thesis is to increase the understanding of factors of importance to severe pneumococcal disease.

The first paper addresses the difficulties in obtaining an etiological diagnosis in pneumonias, which potentially would delay an optimal care and treatment of these patients. We were able to demonstrate that our RQ-PCR method targeting specific gene sequences within the bacteria *S. pneumoniae, H. influenzae and M. catarrhalis,* increased the diagnostic yield from sputum samples significantly and also that this method seemed especially valuable in finding the causative bacterium when the patient had received antibiotics prior to specimen sampling.

The second paper addresses the issue of how to identify patients that are at special risk for a severe outcome from pneumococcal pneumonia. We investigated this by analysing the ability of three different severity score systems, initially developed for the assessment of severity in "all cause" community acquired pneumonia, to predict mortality and need of ICU admission in bacteraemic pneumococcal pneumonia. We concluded that these severity scores worked fairly well also in this subgroup of patients, but also identified the CURB-65 as the best score system since it was the most practical to use.

The third paper addresses whether specific pneumococcal properties are able to influence the severity and outcome of invasive pneumococcal disease (IPD). We investigated the relationship between clonal affiliation / serotypes of pneumococci and patient characteristics and outcome of IPD. We concluded that some clones and serotypes seem more prone to cause severe disease among older patients burdened by comorbidities, and are thus behaving like opportunistic pathogens, while other clones and serotype tended to cause milder disease among younger and previously healthy individuals, thus behaving as primary pathogens.

The fourth paper addresses the issue of preventing invasive pneumococcal disease. We investigated the effects of the large scale vaccination campaign with the 23-valent pneumococcal polysaccharide vaccine directed towards all elderly persons in Stockholm County between 1997 and 2001. We studied the effects on incidence and serotype distribution of IPD, and concluded that a substantial decrease in vaccine-type IPD was seen among the vaccinated population, but not among other age groups, and also that no serotype replacement occurred during the 5 year study period

LIST OF PUBLICATIONS

I. M. Kais, **C. Spindler**, M. Kalin, Å. Örtqvist, C.G. Giske. Quantitative detection of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis in lower respiratory tract samples by real-time PCR. Diagn Microbiol Infect Dis. **2006** Jul;55(3):169-78. Epub 2006 Apr19. PMID: 16626914

II. **C. Spindler** , Å. Örtqvist. Prognostic score systems and community-acquired bacteraemic pneumococcal pneumonia. Eur Respir J. 2006 Oct;28(4):816-23. Epub 2006 May 31. PMID: 16737983

III. K. Sjöström*, **C. Spindler***, Å. Örtqvist, M. Kalin, A. Sandgren, S. Kuhlmann-Berenzon, B. Henriques-Normark Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen.Clin Infect Dis. **2006** Feb 15;42(4):451-9. Epub 2006 Jan 17. PMID: 16421787

IV. **C. Spindler**, J. Hedlund, B. Henriques.Normark, Å. Örtqvist. Effects of a large scale introduction of the pneumococcal polysaccharide vaccine among elderly persons in Stockholm Sweden

*Authors contributed equally

LIST OF ABBREVIATIONS

BAL	Bronchoalveolar lavage
CAP	Community acquired pneumonia
CbpA	Choline binding protein A
CD	Cluster of differentiation
CFU	Colony forming units
CIE	Counterimmunoelectrophoresis
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CSF	Cerebro spinal fluid
DID	Department of Infectious diseases
ELISA	Enzyme linked immunosorbent assay
ICU	Intensive care unit
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
LA	Latex agglutination
LRTI	Lower respiratory tract infections
LytA	The major autolysin
MHC-2	Major histocompatibility complex type 2
MIC	Minor inhibitory concentration
MLST	Multi locus sequence typing
MZ	Marginal zone
NOD	Nucleotide binding oligomerisation domain
ODs	Other departments
OPA	Opsonophagocytic killing assay
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PCA	Pneumococcal capsular antigen
PCV	Pneumococcal conjugate vaccine
PFGE	Pulse field gel electrophoresis
Ply	Pneumolysin
PMN	Polymorphonuclear leukocyte
PNSP	Penicillin non-susceptible *Streptococcus pneumoniae*
PPV	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
PSB	Protected specimen brush
PspA	Pneumococcal surface protein A
RQ-PCR	Real time quantitative PCR
SEC	Squamous epithelial cells
TD	T-cell dependent
TI	T-cell independent
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
WBC	White blood cell

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1. INTRODUCTION

1.1 HISTORY, EPIDEMIOLOGY AND GENERAL ASPECTS OF PNEUMOCOCCAL DISEASE

The Pneumococcus is a gram-positive bacterium that can cause a wide variety of infectious conditions in man. Humans are the principle host of the pneumococcus, but there are reports of the pneumococcus causing disease in other animals, such as rodents (Austrian 1981).
The Pneumococcus' full name is *Streptococcus pneumoniae*, and it belongs to the streptococcal family. It was however initially named diplococcus pneumoniae, and it was not until 1974 that it was renamed Streptococcus *pneumoniae* according to its growth in chains in liquid media.
The bacterium was isolated for the first time in 1881 when Pasteur and Sternberg independently of each other injected rabbits with human saliva, and then saw how the rabbits subsequently died of septicaemia caused by pneumococci (Austrian 1981).
Later the same decade, the pneumococcus was recognized as being the primary causative agent of pneumonia, a position that it has withheld up to the present (Austrian 1999).

Worth noting is the vast impact of the pneumococcus in the understandings of cellular biology. It was from the studies of pneumococcal capsular switching in 1928 by Griffith (Griffith 1928), that Avery and colleagues for the first time in 1944 could report on the biologic activity of a nucleic acid, knowledge which would later become one of the cornerstones in molecular genetics (Austrian 1981).
In the pre-antibiotic era (i.e. before 1940) pneumonia was the overall leading cause of death in most countries (Austrian 1999). After the introduction of penicillin and other antibiotics as well as the introduction of modern supportive care, the mortality rates decreased. Despite these medical advances the high rates of mortality and morbidity caused by pneumococcal infections largely remains.

Data on incidence and mortality of pneumococcal disease often rely on estimates, since an etiological diagnosis in most medical conditions caused by the pneumococcus, often is difficult to obtain. The only occasion when one can be certain of the pneumococcus to be the causative agent of a certain disease episode is when the pneumococcus is found in a normally sterile location in the human body such as the blood, the cerebrospinal fluid or synovial fluid. This also means that in order to be able to compare incidence or mortality rates of pneumococcal disease over time or between study sites, one must consider possible discrepancies in when, where and how the sampling procedures were performed. For example the increasing rates of invasive pneumococcal disease (IPD) in Sweden during the past decades, with incidence figures increasing from 5/ 100 000 in 1990 to 15 / 100 000 in 1995 (Hedlund, Svenson et al. 1995; Giesecke and Fredlund 1997) could to some part be explained by an increasing number of blood cultures taken during the same period, although there are in this case other factors that indicate a true increase in incidence of IPD as well.

The most common cause of mortality due to pneumococcal infection is pneumonia, accounting for the majority of fatal cases in both children and adults. While children carry the major disease burden in the developing countries, the elderly are at the highest risk of pneumococcal disease in the developed world. It has been estimated that 1,6 million persons die from pneumococcal disease world wide every year, of those more than 1 million are children below five years of age (WHO 2007).

About 20% of pneumococcal pneumonias are bacteraemic (Ostergaard and Andersen 1993), and in reverse 60-85% of all invasive pneumococcal disease is associated with pneumonia (Kalin, Ortqvist et al. 2000). Pneumococcal bacteraemia occurs frequently in severe pneumococcal disease, and is associated with a mortality of at least 10-20 cases per 100 000 person-years (Ortqvist, Kalin et al. 1993; Feikin, Schuchat et al. 2000; Metlay, Hofmann et al. 2000) although both higher and lower figures have been reported in certain geographic areas and for some ethnic groups.
Compared to invasive disease, the different non-invasive disease forms caused by the pneumococcus (otitis media, sinusitis, bronchitis, etc.) are generally less severe, but considerably more common. Although perhaps not always as serious, the consequences of the non-invasive pneumococcal disease are still vast. In Sweden respiratory tract infections, of which pneumococci represent a major aetiological agent, are among the most common cause of acute illness in all age groups, as well as the most common cause of prescription of antibiotics (Mölstad 2002).

1.2 GENERAL MICROBIOLOGY OF *S. PNEUMONIAE*

S. pneumoniae is a gram-positive lancet shaped bacterium. Typically growing in pairs (as diplococci) or in shorts chains. Pneumococci have an alpha-haemolytic activity through the enzyme pneumolysin, which degrades haemoglobin into a green pigment. As a result the pneumococcus is encircled by a greenish aura during growth on blood agar. The pneumococcus is generally identified in the microbiology department by this alpha-haemolysis together with the presence of optochin sensitivity, but can also be further identified by being catalase negative and soluble in bile salts.

There are several similarities between the pneumococcus and other members of the streptococcal family, such as its general morphology and the structure of the cell wall. There are also many similarities with respect to their genetic composition, which enables transfer of genetic material between different species; this is also the reason for some of the difficulties in distinguishing between the different species in the clinical setting.

The innermost part of the structures that forms the pneumococcus' outer limits is the pneumococcal cell membrane. This is three layered and consists of lipid and teichoic acid. The cell membrane is surrounded by the cell wall, which is composed by peptidoglycan and different carbohydrate polymers (lipoteichoic acid and teichoic acid), which are cross linked and thus forming a very stable structure.

The cell wall also contains a number of important proteins which are discussed under the heading of "1.3.4 Dissemination".

The final layer limiting the pneumococcus from the outside world is the capsule. The capsule is crucial for the pneumococcus' ability to escape from the host defence, and thereby promotes virulence. This is accomplished primarily by concealing the cell wall antigens and proteins from recognition by the host's immune system.

Almost all pneumococci causing human infections are encapsulated. The capsule is made up of units of repeating oligosaccharides, which are synthesized and polymerized within the cytoplasm, and then transported to the surface and covalently bound to the cell wall.

The serotype classification of pneumococci is based on the antigenic properties of the capsule. To date more than 90 different serotypes have been described. According to the Danish classification system, (now the most widely used classification of pneumococcal serotypes) some different types are grouped together according to antigenic similarities. For example serogroup 6 includes the different serotypes 6A, 6B and 6C. All members of the serogroup 6 are genetically closely related to each other, and also demonstrate a high level of antigenic cross-reactivity. This antigenic cross-reactivity is however not as pronounced within all serogroups.

Since the serotypes most likely to cause disease in humans were the first to be classified, they were assigned the lowest numbers. This means that serotypes with low numbers are the most commonly found.

Analysis of the pneumococcal genome have demonstrated that the genes responsible for the different events forming a distinct capsular type are located closely together, and are transcribed as a unit (gene cassette/operon). The fact that these genes are functioning as an operon is probably important for the natural exchange of genetic material (transformation) between different pneumococcal strains. Capsular transformation has been demonstrated to occur both in vivo and in vitro (Dillard, Vandersea et al. 1995; Garcia, Arrecubieta et al. 1997), and is a possible outcome when an operon coding a serotypically different capsule is internalised from an other pneumococcal strain.

1.3 INFECTIOUS ROUTE

In order to cause disease, the pneumococcus must first colonise a mucosal barrier, then be able to cross that barrier in order to disseminate within the host. During this whole process, but in particular when disseminated, the pneumococcus must succeed in circumventing host immunity. These three stages (colonisation, dissemination and host response) are described below.

1.3.1 Colonisation

The first encounter between a pneumococcus and a human usually occurs in the nasopharyngeal cavity of the human. The most probable outcome of this exposure is "innocent" colonization rather than turning ill from it.

Human nasopharyngeal carriage is the principal reservoir of pneumococci and thus the major source of horizontal spread of this pathogen within the community (Gray, Converse et al. 1980). Colonisation of the nasopharynx by the pneumococcus occurs frequently, especially among children. It was shown in a study by Henriques Normark et al. that 60% of preschool children were colonized by one or several serotypes (Henriqus Normark, Christensson et al. 2003), the same figure for adults being lower, 2-3.7 % (Henriques Normark, Christensson et al. 2003; Regev-Yochay, Raz et al. 2004). The differences in colonisation rates are probably due to

the more mature immune system in adults. There is a clear correlation between rising levels of anti-pneumococcal surface antibodies and diminished carriage rates (Syrjanen, Kilpi et al. 2001; Simell, Kilpi et al. 2002). Also, vaccination with conjugated vaccine against pneumococci has resulted in diminished carrier rates of the different serotypes included in the vaccine.
The colonization rates also demonstrate a seasonal variation; pneumococcal carriage is more frequently seen during the cold season. The reason for this remains to be explained, although concomitant viral infections (which are more common during the winter), facilitating pneumococcal adherence to the respiratory epithelium, are generally believed to be involved.
The pneumococci have also been demonstrated to out-compete other frequent colonizers in the upper respiratory tract such as *Hemophilus Influenzae* and *Moraxella catarrhalis*. This is accomplished by the production and release of hydrogen peroxide by the pneumococcus (Regev-Yochay 2006).

The initial episode pf colonization often occurs at the age of 6 months, thereafter acquisition of colonization by a new serotype occurs as often as every four months in infants (Gray, Converse et al. 1980). In early childhood, these first colonising serotypes can often be detected up to 6 months after acquisition, but the time can vary both between serotypes and individuals. In adults, the mean carriage time is shorter, usually 2-4 weeks.

1.3.2 Adherence

The first step the pneumococcus must accomplish is to adhere to the upper respiratory tract epithelium of its host. The exact procedure how this is accomplished is still not fully elucidated. Adherence can be regarded as a major element of virulence. In fact, many of the structures shown to be of importance to adherence also demonstrate properties that are vital for other components of the pneumococcus' ability to be virulent.

Neuraminidase

The pneumococcus binds avidly to the surface of respiratory tract cells, especially up on recognition of sialylated sugars (Krivan, Roberts et al. 1988). It has been suggested that the enzymes neuraminidases (of which the pneumococcus has several types) cleaves terminal sialic acids from glycopeptides on respiratory cells, and thus expose receptors of importance to adherence (Novak and Tuomanen 1999). The fact that respiratory viruses like influenza and parainfluenza demonstrate the same neuraminidase activity further supports the theory that viral infections and pneumococcal infections can interact synergistically.

Choline binding proteins

Choline is a vitamin related chemical structure that is essential for the growth of several bacteria, including the pneumococcus. Pneumococci attach proteins to the cell wall by bridging them covalently to choline. Many of the surface exposed proteins on a pneumococcus are suggested to be attached by choline bindings. Two examples of such proteins are Pneumococcal surface protein A (PspA) and the major autolysin (LytA). These two proteins are involved in virulence, but not primarily through adherence, and are described further below.

One choline binding protein that has been described to be involved in adherence is the Choline binding protein A (CbpA, also called PspC). This protein forms a bridge with glycoproteins on human respiratory cells. This reaction is however restricted to cytokine activated cells, and has therefore also been suggested to be important for advancing the pneumococcal disease from colonisation to invasion. CbpA-deficient mutants are defective in colonisation of the nasopharynx and fail to bind to various human cells in vitro. CbpA also has been reported to bind secretory IgA and complement component C3.

Phase variation

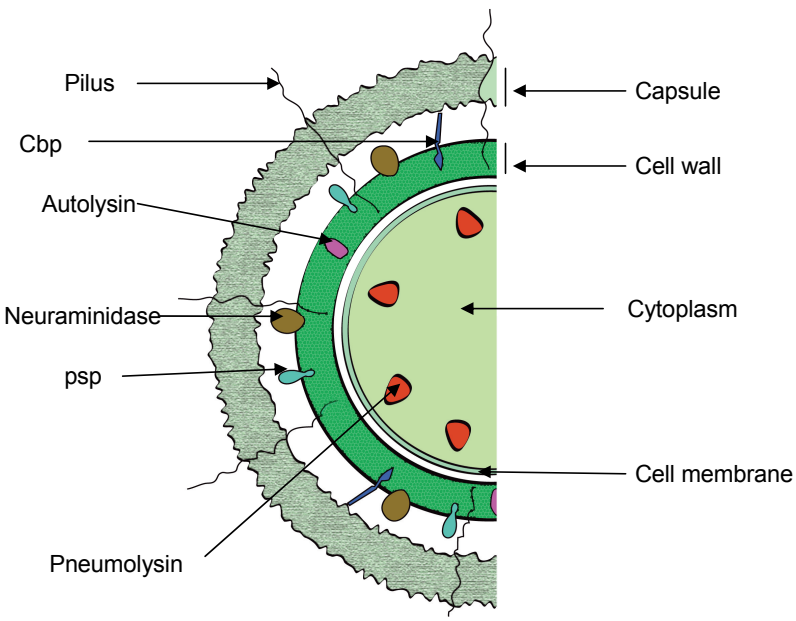
Pneumococci have been shown to have at least two distinctly separated appearances when grown on a transparent medium. These two appearances are referred to as either transparent or opaque. How these different morphological appearances are accomplished remains to be explained, but is generally considered to depend on protein expression and capsular thickness.
Transparent phenotypes have been demonstrated to express a higher amount of neuraminidase, a fact that has suggested this as an explanation for the observed enhanced adhesion of transparent phenotypes in colonisation. Transparent phenotypes also express higher levels of phosphorylcholine in the cell wall. It has been demonstrated that the pneumococcus can bind to airway epithelium cells through a platelet activating factor (PAF) receptor. These receptors are expressed when the cells are stimulated by cytokines like IL-1, and it has been suggested that the pneumococci attach to the airway epithelium by mimicking PAF, which, like the pneumococcus contains phosporylcholine.
Opaque phenotypes have generally been regarded as more virulent, and have been demonstrated to be more prone to cause invasive disease after intraperitoneal challenge of mice (Kim 1998).

Pili

The pili-like structure, recently found to be located on the surface of some pneumococcal strains, has also been demonstrated to be of importance to adherence to epithelial cells. Barochi et al demonstrated that penumococci equipped with pili outcompeted non-pili equipped strains in binding to epithelial cells (Barocchi 2006).

Apart from facilitating adherence, these pili are also thought to evoke a high immune response (IL-6 and TNF-α), thereby causing mucosal inflammation. Such an inflammatory reaction would enhance bacterial clearance and thus be negative from the bacterial point of view. It has however been suggested that an enhanced mucosal inflammation would also facilitate bacterial invasion by other non-pilated strains such as serotype 1, which is rarely seen in carriage, but frequently in invasive pneumococcal disease (Barocchi 2006).

Fig 1 Pneumococcal structures and virulence factors



1.3.3 Invasion

There are two principle procedures that the pneumococcus can use to continue on the way to cause infection in man. The first way is to use the normal air filled channels from the nasopharyngeal cavity to other organs such as the middle ear, the sinuses or the lungs. The most common way for a pneumococcus to cause pneumonia is by aspiration of the pneumococcus from the nasopharyngeal cavity down to the lower airways. The other procedure is direct passage of the pneumococcus through the mucosal barrier in the upper respiratory airways, either into adjacent tissues or directly into the bloodstream. This latter way is regarded to be uncommon (although it has been suggested to be necessary for penetration of the blood-brain barrier in meningitis) and is also referred to as transmigration. The theory behind transmigration is that cytokine activated host cells increase the expression of platelet activating factor (PAF) on the surface. The PAF then works as a docking station for the phosphorylcholine on the pneumococcus, initiating an internalisation process of the bacteria. By this process the pneumococcus is able to penetrate into normally sterile locations (Ring 1998).

When the pneumococci arrive at their final destinations, they will immediately be challenged by the host immune system. The result of this challenge will either lead to clearance of the bacteria (which is probably the most common outcome), or to disease.

In the case of the latter outcome, the bacteria might further disseminate to normally non-infected locations such as the alveoli, the bloodstream or the middle ear.

1.3.4 Dissemination

The procedure of dissemination is ambiguous in the sense that it involves processes that both enhance escape from the immune system as well as to promote an inflammatory response.
By avoiding the immune system, the pneumococcus can continue to survive and grow, however in most cases it will finally be discovered and attacked by the immune system, and will then be in need of an escape route. The escape route in this case is to provoke an inflammatory response which in turn will help to create an opportunity to escape into normally sterile locations such as the blood or the CSF. The inflammatory response will render normally non-permeable tissues and cell barriers permeable in order to summon immunocytes. This is used by the pneumococci to sneak into these normally sterile locations.
As will be discussed below, a great number of pneumococci will probably have to lyse, and thereby release their cytoplasmatic virulence factors, in order to allow a small number of pneumococci to escape by this process. A number of different factors in the pneumococcus are important at this stage, either to escape from the immune system or to create an immune response. The most important of those are listed below.

The Cell wall

The cell wall possesses a number of virulence enhancing factors. Purified peptidoglycan from the pneumococcal cell wall has been demonstrated to induce the same inflammatory response as infection with whole pneumococci (Tuomanen, Rich et al. 1987). The peptidoglycan induces complement activation through the alternative pathway, and induces inflammation though activation of white blood cells and the release of cytokines when recognized by CD14 positive immune cells in the same way that lipo-polysaccharide is recognized in Gram-negative bacteria (Tuomanen, Austrian et al. 1995).

The Capsule

The chemical structure of the polysaccharides in the capsule has been demonstrated to determine the ability of different serotypes to survive in the bloodstream, and probably also to cause invasive disease, an issue that is further discussed in paper III of this thesis.
The most important role in virulence for the capsule is probably accomplished by a direct steric hindrance from the host immune response by camouflaging the underlying adherence structures in the cell wall. The virulent capacity of the capsule however stretches beyond this steric protection, which is demonstrated by differences in ability to cause inflammation among different serotypes. Why different serotypes can trigger the immune system in a dissimilar fashion has not been fully explained, and probably relies on a number of factors. Among them are the ability to activate the alternative pathway of complements, deposition and degradation of complement factors on the capsule, resistance to phagocytosis, ability to induce antibody clearance, and clearance by non-antibody structures such as acute phase proteins (Mufson, Kruss et al. 1974;

Tuomanen, Austrian et al. 1995; van Rossum, Lysenko et al. 2005; Serrano 2006; Nelson, Ries et al. 2007).

PspA

The Pneumococcal surface protein A (PspA) has been shown to be required for full virulence of pneumococci (McDaniel, Sheffield et al. 1991; Briles, King et al. 1996). The PspA contain multiple antigenic epitopes, which can be combined differently, resulting in protein variability. The choline binding part of the protein is attached to the pneumococcal cell membrane (fig 1). The PspA (and probably also PspC as indicated by a recent study by Li. J 2007 (Li, Glover et al. 2007), acts by inhibiting complement activation through both the classical and alternative pathway, and thus inhibits phagocytosis. This mechanism has been suggested to be of great importance in protecting pneumococci from the host immune system especially during invasive disease.

Autolysins

Autolysins are proteins that can cleave bonds of the cell wall. This function is probably indispensable for pneumococci undergoing cell division. These enzymes can however also function as virulence factors in the absence of cell separation. The primary virulence role of autolysins seems to be to release pneumolysin from the cytoplasm by destroying the own cell wall. The major autolysin of pneumococci is an amidase called LytA.

Pneumolysin

Pneumolysin is the most well studied enzyme of the different pneumococcal virulence factors. It belongs to a family of thiol-activated enzymes that are also found in other bacteria (Hirst 2004). Unlike other members of the family, the pneumolysin however lacks a signal peptide, and is therefore kept within the cytoplasm, only released when the bacterium lyses under the influence of autolysins (as mentioned above) or antibiotics. Pneumolysin is lytic to all eukaryotic cells that have cholesterol in their membrane (Hirst 2004). The exact mechanism by how pneumolysin acts is not fully eluded. It has however been suggested to cause lysis by creating a trans-membrane pore in the target cell which results in flooding and haemorrhage of certain cell types.
Pneumolysin demonstrates effects on a wide type of cells, resulting in: lysis of red blood cells, toxic effect on alveolar cells, cytokine stimulation, reduced ciliary movement, activation of the complement system etc.
Since pneumolysin is so important for the virulence of pneumococci, and also has been demonstrated to evoke a relatively high degree of specific immune response, it has been suggested as a candidate antigen for vaccine trials. Immunization with pneumolysin has also been demonstrated to prolong the survival of mice challenged with different serotypes of pneumococci; unfortunately to date no vaccine trials in man with pneumolysin have yet been successful.

1.4 IMMUNE RESPONSE AND INFLAMMATION

The disease caused by the pneumococcus is determined by the virulence of the bacteria and the immune response it evokes. The pneumococcus can trigger the

human immune system in two principal ways. These two components of the human immune system are referred to as the innate immune system and the adaptive immune system. Innate immunity covers a wide range of host defences, including mucociliary clearance, complement activation, macrophages and different sorts of dendritic cells, whereas the adaptive immune system involves the clonal expansion of T and B cells specific to the pathogen.
Although the adaptive- and the innate immune system have been regarded as separate entities, recent data suggests a number of essential interactions between these two systems. In the following headings, however, these systems are described separately.

1.4.1 Innate immunity

Barrier functions

Mechanical clearance of mucus is considered the primary innate airway defence mechanism (Wanner A 1996; Boucher RC 1994). The mucus is produced by the epithelial cells lining the respiratory tract. Trapping and subsequent clearance of the pneumococci as well as other pathogens by mucociliary transport is the primary role of this barrier. Recently, the importance of a so called "chemical shield" in the form of antibacterial peptides, defensines and secretory IgA as well as other antimicrobial substances has been demonstrated to also play a key role in the mucociliary barrier (Bals R 1999; Widdicombe 1995).

Complement system

The complement system consists of a vast number of circulating and membrane bound proteins that can be triggered in a cascade-like fashion upon contact with microbes. The primary role of the complement system is to facilitate opsonisation and subsequent elimination of microbes by binding to them. Some bacteria, but not pneumococci can be lysed directly by complement factors binding to them.
The complement system can be activated in three separate ways:

a) The classical pathway, activated by antibody-antigen complexes or complexes with antigen and other non-antibody complexes (acute phase proteins).
b) The alternative pathway, continuously active at low levels by binding of C3b, but amplified upon contact with foreign surfaces.
c) The lecitin pathway, triggered by lecitin recognition of carbohydrates on bacterial surfaces.

The classical pathway is probably the most important of these three, and specific loss of this pathway has been shown to increase disease severity of pneumococcal disease in animal models (Brown 2002).
Regardless of which pathway that is activated, the determining factor is the deposition of C3 on the bacterial wall, since this is the crucial step in all of the pathways in the elimination of the microbe. As described earlier under the heading virulence factors, the pneumococcus has evolved and developed a number of mechanisms to reduce the amount of C3 deposited.

Pattern recognition receptors

Pattern recognition receptors (PRRs) are receptors that are either bound on the cell surface of host cells, or secreted from certain cells to initiate immune responses. The C-reactive protein is an example of a soluble PRR involved in the classical complement pathway and recognition of pneumococci.
The target molecules that these PRRs can bind to are referred to as pathogen-associated molecular patterns (PAMPs). These PAMPs can be regarded as invariant structures specific to microbes, and they are absent in eukaryotes. One of the most well described PAMP is the lipo-polysaccharide structure found in gram negative cell walls, and the corresponding PAMP in gram positive bacteria like pneumococci is the peptidoglycan structure.
Recently a lot of the research concerning PRRs has been focused on two different types of receptors called Toll-like receptors (TLRs) and nucleotide binding oligomerization domain (NOD) receptors.

Toll-like receptors

The Toll-like receptors are transmembrane proteins. Ten different TLRs have been described in man and in different animal models. Almost all of the TLRs have in fact been shown to be of importance in pneumococcal infections, although TLR-1, TLR-2, TLR-4 and TLR-6 are the ones mostly associated with pneumococcal disease (Paterson and Mitchell 2006; Albiger, Dahlberg et al. 2007).
Upon recognition of its corresponding ligand, the TLR initiates an intra-cytoplasmatic signalling cascade the eventually leads to the production of proinflammatory cytokines and interferons. This signalling cascade is regulated on various levels, resulting in the ability of the innate immune system to in most cases be permissive towards non-pathogenic microflora in various locations, as well as to avoid an uncontrolled immune response that would result in massive tissue damage upon contact with true pathogens such as pneumococci.

Nod receptors

The Nod-receptors are even more recently discovered PRRs. These receptors are situated intra-cytoplasmatically, and are assumed to recognize internalised pneumococci or fragments of pneumococci (Opitz, Puschel et al. 2004).
There are currently a number of other PRRs under investigation for a possible important role in pneumococcal infections. Examples of such proteins are the CD-14 receptor and surfactant proteins.

Cytokines and chemokines

Upon recognition of pneumococci by the innate immune system, a vast number of different cytokines and chemokines are released by different cell types participating in the innate immune response. The cytokines are small glycoproteins that are produced by both immune cells as well as non-immune cells. Pro-inflammatory cytokines like interleukin (IL)-1, IL-6, IL-12 as well as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are expressed in high amounts, and acts by regulating the inflammatory response.

TNF and IL-2 activate macrophages and B-and T-cells by binding to receptors on their surfaces. Reduced expression of TNF-α has for example been demonstrated to lower the amount of neutrophils invading infected lung tissue in pneumonia, with the result of subsequent initiation of invasive disease (Kerr, Irvine et al. 2002).
The chemokines are a subgroup of cytokines that are able to induce chemotaxis, (the recruitment of inflammatory cells to the site of infection). The chemokines are secreted by tissue cells in the event of recognition of microbes such as pneumococci. This secretion results in extravasation of inflammatory cells to the site of infection. These cells are then activated by proinflammatory cytokines, resulting in a cellular response to the infection.

Despite major advances in the understanding of the complex events regulating the immune response in pneumococcal disease the past years, the exact orchestration of the cytokine response in pneumococcal disease remains to be elucidated.
That the regulation of the cytokine cascade needs to be well tuned becomes apparent when the violent cytokine response with subsequent multiorgan failure in septic pneumococcal disease is studied.

1.4.2 Adaptive immunity

The innate immune system plays a key role in the immune response to pneumococcal infections, and as discussed previously it is difficult to make a clear distinction between the innate and the adaptive immune response to pneumococcal infections, since they interact at several levels. The most important feature of the adaptive immune response to pneumococcal infections is the presence of antibodies against capsular polysaccharides. Invasive pneumococci are rapidly cleared by the immune system in the presence of anti-capsular antibodies that opsonize the bacteria..
Since the pneumococci are encircled by a polysaccharide capsule, the only easy accessible antigens are the capsular polysaccharides. Also, since differences in the structure of the polysaccharide capsule determine the serotype, each different pneumococcal serotype can be regarded as a different potential pathogen.
After exposure to pneumococci, the antibody response occur either quickly through activation of resident memory B-cells if there has been a prior encounter with the same serotype, or more slowly through the induction of both mucosal and systemic immunity if the host is naïve to the serotype.
Antigens are normally divided into T-cell dependent (TD), T-cell independent type 1 (TI-1) or T-cell independent type 2 (TI-2). The pneumococcal capsule polysaccharides behave as TI-2 antigens (Lee, Lee et al. 2003). TI-2 antigens can not be presented by major histocompatibility complex type 2 (MHC-2) on antigen presenting cells, and thus do not create a specific T-cell response. Hence, in contrast to TD antigens, TI-2 antigens do not induce classical isotype switches or high-affinity memory B cells. T-cells have however been demonstrated to elicit an immune response despite the lack of antigen processing and presenting by MHC-2. This antibody response is thought to be regulated by interleukins and interferons. IgG2 and IgM are regarded as the most predominant subclasses elicited by capsular polysaccharides (Wu, Shen et al. 2002).
The spleen and the liver have important roles in the adaptive immune response, especially in invasive infections. Normally the liver is the most effective organ clearing antibody coated bacteria, but in the case of pneumococcal infections, the

spleen seems to play a more dominant role. The so called marginal zone in the spleen, an area where the blood passes slowly through tight sinuses, is filled with antibody producing B-cells and phagocytes. This procedure seems especially essential for clearance of only partly opsonized particles like pneumococci or other encapsulated bacteria. Overwhelming pneumococcal disease with a very rapid course has been described in both adults and children who have had their spleen removed for various reasons.

The marginal zone (MZ) B-cells have been demonstrated to generate a massive IgM response generated by the maturation of the B-cells to plasmoblasts, the first three days after exposure to invasive pathogens. Proper development of the marginal zone and the MZ B-cell compartment does not occur until the age of two years. This delay is probably partly responsible for the inability of infants below two years of age to mount effective immune responses towards pneumococci and pneumococcal polysaccharides (Timens, Boes et al. 1989)

While IgG and IgM class antibodies are very important for the immune response in infection with pneumococci, their protective role in colonization is generally regarded as limited (Obaro, Adegbola et al. 1996), although some recent reports of post vaccination effects of the 7-valent conjugate vaccine on carriage have demonstrated lowered carriage rates (Millar, O'Brien et al. 2006), which would indirectly implicate an effect of IgG and IgM on carriage. The direct Ig protective effect on carriage seems instead to be conferred by IgA subclass antibodies. These antibodies are expressed by B-cells in the mucosal lamina propria, and then transported to the surface of the mucosal cells where they can be secreted as secretory IgA. Luminal secretory IgA is as described previously thought to interfere with pathogen adherence to mucosal epithelial cells (Pilette, Ouadrhiri et al. 2001).

Further, recent studies have also investigated whether T-cells, and especially CD4+ cells, independently of their role in the antibody mediated defence, also have important features for the adaptive immunity. This issue attracted attention when McCool and Weiser (McCool and Weiser 2004) demonstrated that clearance of colonising pneumococci was not affected by the absence of antibodies in a murine model. This issue was further elucidated by Malley et al. (Malley, Trzcinski et al. 2005), who suggested that CD4+ cells are crucial for the acquired immunity against pneumococci, regardless of the antibody production.

1.5 CLINICAL SYNDROMES OF SEVERE PNEUMOCOCCAL DISEASE

1.5.1 Pneumococcal pneumonia

Pneumonia can hardly be considered as *one* disease, since it has so many different appearances, depending on causative agent, severity of disease, site of acquisition, clinical presentation and more. A generally accepted definition of pneumonia would therefore be**:** Inflammation of one or both lungs with consolidation. Pneumonia is frequently but not always due to infection. The infection may be bacterial, viral, fungal or parasitic. Symptoms may include fever, chills, cough with sputum production, chest pain, and shortness of breath.

Streptococcus pneumoniae is the most common identifiable cause of pneumonia leading to hospitalization in adults, and is considered to account for at least half of the almost 1.000.000 pneumonia admissions in the US each year (Musher, Alexandraki et al. 2000). In Sweden, the incidence of pneumococcal pneumonia has been estimated to about 5-10/1000 persons ≥65 years of age (Örtqvist 2001), and a pneumococcal aetiology is considered to account for 32%-54% of all patients

admitted with pneumonia in Sweden (Holmberg 1987; Ortqvist, Hedlund et al. 1990). There are very few studies estimating mortality in pneumococcal pneumonia. This is probably due to the fact that it is difficult to separate bacteraemic pneumococcal pneumonia from non-bacteraemic pneumococcal pneumonia, with very different risks of a fatal outcome. Fine et al. performed a meta-analysis of more than 2000 patients hospitalized for both bacteraemic and non-bacteraemic pneumococcal pneumonia, and noticed a total mortality rate of 8% (Fine 1996). That these epidemiological figures rely on estimates is of course an effect of the fact that a certain/true etiological diagnosis of pneumonia is very difficult to obtain. This issue is further discussed later on.

In order just to reach the lower respiratory tract, the pneumococcus has to escape from the host immune defence at several levels. If the bacteria are successful in reaching the alveolar spaces, they are still continuously confronted with secretory IgA, type specific IgG and other properties of the immune system. The adhesion factors and virulence factors described previously are important at this stage although specific alveolar adhesins have also been described (Tuomanen, Austrian et al. 1995).
The earliest histological step in the development of pneumococcal pneumonia is the flooding of infected alveoli by red blood cells and protein rich exsudate, due to damage and increased permeability of the alveolar-capillary barrier (Tuomanen, Austrian et al. 1995). Also, neutrophils begin to invade the alveolar spaces, and together with the resident B-cells and other dendritic cells they begin to fight the infection. This second wave of immune response with subsequent vaso-permeability and fluid filled alveoli eventually leads to a marked increase of the lung weight after a couple of days. This first stage of pneumonia is referred to as the red hepatisation.
Due to the induction of procoagulant activity on human endothelial cells by the pneumococci, there is also a marked increase in fibrin formation, leading to a reduction of the blood supply to the area. At this stage, the neutrophils are also capable of trapping the pneumococci against the surface of pulmonary cells (AlonsoDeVelasco, Verheul et al. 1995; Tuomanen, Austrian et al. 1995). Because of the intense inflammation and the poor blood flow through the infected part of the lung it will develop a greyish appearance, a stage that is therefore referred to as grey hepatisation.
Resolution of the patophysiological changes in the lung caused by the pneumococci is depending on clearance of pneumococci from the alveoli by phagocytosis from the neutrophils and macrophages. These polymorphonuclear leukocytes (PMNs) have in animal models been demonstrated to be sequestered into capillaries throughout the lung, but migrate into alveoli only at the site of infection (Doerschuk, Markos et al. 1994). Pneumococcal pneumonia has a remarkable ability to heal. Pneumonias caused by bacteria like *Staphylococcus aureus* or *Klebsiella pneumoniae* often heal with abscess formation or scarring, which is a very rare outcome of pneumococcal pneumonias. It has been suggested that immuno modulation by pneumococci somehow de-escalates the immune response, resulting in a decreased production of tissue damaging products such as free oxygen radicals. Also, PMNs seem to readily undergo apoptosis instead of releasing cytotoxins upon contact with pneumococci. These two features would lead to a diminished inflammatory response, hence reducing the risk of severe tissue damage in the lung, but would also potentially promote deeper (i.e. invasive) disease (Haslett 1997).

Although pneumococcal pneumonia is often described as the typical example of a classical pneumonia, the clinical presentation is highly variable. However, a typical presentation often includes a fast onset of disease with fever, chills, pleuritic chest pain and productive cough. Notably, absence of respiratory symptoms are not uncommon, and as many as 50% of patients with bacteremic pneumococcal pneumonia were lacking respiratory symptoms in a study by Örtqvist et al. (Ortqvist, Grepe et al. 1988). Within 48 hours after onset, the chest x-ray often reveals a dense alveolar (often lobar) infiltrate. Bilateral infiltrates are common. The laboratory findings of a pneumococcal pneumonia often demonstrate a significant leukocytosis, and highly elevated CRP levels, although leukopenia and low CRP values are sometimes seen. It has been shown that the ability to predict an etiological causative agent in pneumonia is very low, utilizing clinical parameters and laboratory findings (Woodhead and Macfarlane 1987).

1.5.2 Meningitis

After the implementation of large-scale vaccination programs against encapsulated *Hemophilus Influenzae* type B in most western countries in the beginning of the 1990's, *Streptococcus pneumoniae* is now the dominating aetiological agent of bacterial meningitis, accounting for about 50% of cases. In analogy with pneumococcal pneumonias it is difficult to estimate the true incidence of pneumococcal meningitis because of difficulties in diagnostic procedures. However, the incidence of pneumococcal meningitis does not seem to have varied significantly in Sweden or other western countries during the past decades, with incidence figures of about 0.5-1/100.000 inhabitants; ((SSID) 2004; Lexau, Lynfield et al. 2005; Weisfelt, van de Beek et al. 2006), whereas exact epidemiological data from developing countries are still lacking (Gordon, Walsh et al. 2000). Mortality rates in pneumococcal meningitis have also remained unchanged during the past decades, and a meta analysis performed on 8 studies from 1962-2001 concluded that risk of a fatal outcome from pneumococcal meningitis was 21-28% (Zimmerli 2003).

Pneumococcal meningitis requires the invasion of bacteria into the central nervous system (CNS). The blood-brain barrier, interposing the circulatory system and the CNS is however very efficient in protecting the CNS from infections. The blood-brain barrier consists of endothelial cells that differ from their peripheral counterparts by having tight junctions of a much higher electrical resistance as well as much less pinocytic activity and highly specific transport systems. Despite these protecting factors, the pneumococci seem to use the concept of transmigration through up-regulation of Cbp's and pinocytosis by the PAF-receptor discussed earlier under the heading 1.3.3 "Invasion", to get access to the CNS (Cundell, Gerard et al. 1995). Having entered the CSF, an exponential growth of pneumococci may occur, since the host defences in the CSF seem to be very ineffective against encapsulated bacteria. The reason for this is not fully elucidated, but lack of sufficient complement concentrations as well as low concentrations of type specific immunoglobulins seem to be important (Smith and Bannister 1973; Stahel, Nadal et al. 1997)

A direct causal role of the pneumococcal toxins (i.e. pneumolysin, H_2O_2 and "pneumococcal adherence and virulence factor A") for mortality and for the development of hippocampal neural apoptosis has been documented in experimental meningitis using genetically modulated mutants of pneumococci, whereas neuraminidase- and hyaluronidase deficient strains did not influence the

outcome (Braun, Sublett et al. 2002; Wellmer, Zysk et al. 2002). An association between CSF concentrations of lipoteichoic/teichoic acid and poor outcome in human pneumococcal meningitis has also been documented experimentally (Koedel, Scheld et al. 2002; Gerber, Pohl et al. 2003)
Classical signs of meningitis are fever, neck rigidity, decreased consciousness, and convulsions. These are also the characteristic clinical findings in pneumococcal meningitis and was found in 96%, 55%, 94%, and 12% of cases respectively in a recent study from Denmark (Ostergaard, Konradsen et al. 2005). The same study showed that approximately 30% of cases of pneumococcal meningitis were secondary to an otogenic focus, whereas ~20% were due to a lung focus, ~8% to a sinusitic focus and in ~40% no primary infection focus was found. Routine examination of the CSF for bacteria, WBC including differential counts, and concentrations of glucose, lactate and protein is the primary investigation to diagnose meningitis. Pneumococci can be visualized by Gram staining in ~90% and can be grown from the CSF in 98% of documented cases with pneumococcal meningitis, and in addition a positive blood culture is observed in ~67% of cases (Gray and Fedorko 1992).

1.5.3 Invasive pneumococcal disease

The term invasive pneumococcal disease (IPD) or pneumococcal bacteraemia is usually defined as the presence of pneumococci in normally sterile body locations such as the blood or the CSF, thereby including e.g. both the bacteremic pneumococcal pneumonia and pneumococcal meningitis described above.
The incidence of IPD has been estimated to at least 10-20 per 100.000 people per year in all age groups in the western countries (Örtqvist 2001) and between 30 and 80 per 100.000 people among the elderly (Fedson and Scott 1999). Dramatically higher figures are reported within certain ethnical groups like the native population in Alaska, Navajo Indians in the US and among Australian aborigines (Davidson, Parkinson et al. 1994; Watt, O'Brien et al. 2004).
The reason for this discrepancy in IPD rates between countries and populations is probably multifactorial, but contributing factors probably include variations in blood sampling frequency, socio-economic factors, differences in underlying conditions and variations in serotype distribution (the latter is further discussed under the heading serotype distribution).
In adults, IPD is associated with pneumonia in 60-85%, while meningitis accounts for 5-10%, the remaining few percent being various deep tissue infections such as joint-infections, abscesses, endocarditis etc. There is also a small portion of patients presenting with IPD, without any signs of focal pneumococcal infection. Conversely, in about 20-30% of pneumococcal pneumonias, a positive blood culture for Streptococcus pneumonia can be found, with the same figure for meningitis being ~70%.
The mortality in IPD has remained unchanged the past decades, with most countries reporting mortality rates between 10 and 30% (Mirzanejad, Roman et al. 1996; Kalin, Ortqvist et al. 2000; Yu, Chiou et al. 2003).

The clinical characteristics of invasive pneumococcal disease are of course primarily related to the primary focus of the infection. However, the pneumococcal bacteraemia can trigger the immune system to a violent response described earlier, resulting in a fulminant pneumococcal sepsis. In brief, sepsis is characterised as the

combination of a systemic inflammatory response and the presence of an infection. A systemic inflammatory response is clinically characterised in patients having more than one of the following criteria: fever >38ºC or <36ºC; heart rate >90/min; respiratory rate >20/min or a PaCO2 of <32 mmHg; and a white blood cell count of >12.000 cells /µl or <4.000 cells /µl. If these criteria are fulfilled and the patient also develops hypo-perfusion or organ dysfunction, the sepsis is considered a severe sepsis episode (Levy, Fink et al. 2003).

1.6 FACTORS OF IMPORTANCE TO PROGNOSIS AND SEVERITY OF PNEUMOCOCCAL DISEASE

1.6.1 Factors attributed to the bacterium

A number of bacterial properties have been identified as being important for the severity and outcome of pneumococcal disease. All of the factors that have been identified as important for the virulence of pneumococci will consequently also be important for the prognosis and severity of the disease.

There are however a number of other bacterial factors, to our present knowledge lacking apparent impact on virulence, that nevertheless seem to contribute to the prognosis and severity of pneumococcal disease.

That some serotypes seem more prone than others to cause invasive disease has been known for a long time. Recently, several studies have demonstrated that genetic differences other than those originating serotype, may be important for the severity of disease. Pneumococcal strains not sharing the same genetic properties are hence referred to as belonging to different clones. Several studies have also demonstrated that serotypes with high polysaccharide content are more lethal in murine models of sepsis than serotypes with lower polysaccharide content (Briles, Crain et al. 1992; Weiser and Kapoor 1999). It is however very likely that the genetic background of the recipient strain plays a role since capsule transformed strains do not necessarily acquire the virulence of the donor strain. This issue is further discussed in paper III of this thesis. Another recent example of the importance of such genetic diversity is the finding that clones harbouring the so called pathogenicity islet rlrA in their genome, coding for the pilus-like structure, evoke a much higher inflammatory response than rlrA-negative clones (Barocchi 2006).

Other bacterial factors demonstrated to be of possible importance for the severity and prognosis of pneumococcal disease include antimicrobial susceptibility (Harwell and Brown 2000). In a study by Metlay et al. the impact of penicillin susceptibility on outcome was investigated and found to be of importance. Patients with nonsusceptible infections had a greater severity of illness, although no significant difference in mortality could be detected. This was demonstrated by the incidence of suppurative complications being four times greater among patients infected with nonsusceptible isolates compared with that among patients infected with susceptible isolates (4 of 44 cases [9%] and 3 of 148 cases [2%], respectively (Metlay, Hofmann et al. 2000). There is however still no consensus whether antimicrobial susceptibility has any significant impact on the prognosis of pneumococcal disease, and other studies have failed to demonstrate such a relation (Yu, Chiou et al. 2003).

Serotype distribution

Disease has been documented by almost all known serotypes of *Streptococcus pneumoniae* (Shapiro, Berg et al. 1991). The serotype distribution seems to change over time, demonstrated by the fact that invasive pneumococcal infections caused by any of the serotypes included in the present 7-valent conjugate vaccine (4, 6B, 9V, 14, 18C, 19F, 23F) increased from 15% to 59% between the years 1928 and 1998 in the USA, i.e. before the vaccine was introduced. In contrast, infections caused by serotypes that were the most prevalent ones at the beginning of 20th century (1, 2, 3 and 5) decreased from 71% to 7% in adults, and from 18% to 2% in children. Socio-economic factors, such as crowded living conditions, probably promoted outbreaks of epidemic serogroups, which are seldom seen today (Feikin and Klugman 2002).
There are also distinct differences in the prevalence of serotypes causing disease in different age groups. Types 14, 6B and 19F are among the more prevalent types in children (Poehling, Talbot et al. 2006) while serotypes like 3 and 8 are relatively much more prevalent among the elderly (Scott, Hall et al. 1996). Interestingly, some studies have reported major differences of serotype distribution within very narrow age strata, for example in neonates (less than 1 month old), where 25% of all IPD was caused by serotype 1 (Kaltoft, Zeuthen et al. 2000; Sleeman, Knox et al. 2001).

The capability among serotypes to cause severe disease or carriage seems to be very different. Less than 30 different serotypes account for more than 90% of all invasive disease. In order to compare the invasive disease potential with the potential to only cause carriage, most studies have estimated the invasiveness of prevalent pneumococcal serotypes based on the relative serotype prevalence in invasive and colonizing isolates. The prevalence and rank order of invasive serotypes has directed the selection of target serotypes included in the conjugate vaccine. However, since some serotypes are much more commonly found all together, it is not evident that the serotypes most commonly found in invasive disease, have the highest potential to cause invasive disease (Brueggemann, Peto et al. 2004).
Commonly found serotypes in invasive disease are types: 14, 9V, 3, 23F and 7F (Henriques, Kalin et al. 2000; Feikin and Klugman 2002; Kronenberg, Zucs et al. 2006), although the prevalence demonstrate some regional differences. Serotypes 1 and 4 are other serotypes relatively commonly found in invasive pneumococcal disease, but interestingly almost never found among carriers (Brueggemann, Peto et al. 2004). Conversely, serotypes 6A, 6B, 11A and 19A are frequently found in carriage, but less often in invasive disease (Hausdorff, Feikin et al. 2005).

Although invasive pneumococcal disease is generally regarded as being a severe form of pneumococcal disease per se, different invasive serotypes have been suggested to worsen the outcome of this severe pneumococcal disease. Examples given of such serotypes are serotypes 3, 6B,14,19A,19F and 22F (Henriques, Kalin et al. 2000). Also, other studies have reported high mortality rates for specific serotypes such as serotype 3 (Mufson, Kruss et al. 1974; Ortqvist, Kalin et al. 1993); (Martens 2004).
The concept that not all serotypes with a high potential to cause invasive disease, are accompanied with a similar high risk of a fatal outcome is further discussed in paper III, included in this thesis.

1.6.2 Factors attributed to the host

Common factors predisposing for invasive pneumococcal disease, and thus in this context for severe pneumococcal disease are listed in table 1.

Non-immune system specific factors

The incidence of pneumococcal disease is up to 50 times higher in children below 2 years of age and adults older than 65 years compared to other age groups. The major contributor to this elevated risk of pneumococcal disease probably depends on a suboptimal functioning of the immune system (see next heading). There are however also other factors involved, since comorbidities like malignancies and diabetes mellitus are more common among the elderly.

Alcoholism has been demonstrated to elevate the risk for both pneumococcal pneumonia and severe pneumococcal disease. The effect of alcoholism is probably multifactorial, involving both an impaired gag-reflex as well as decreased neutrophil bactericidal effects (Jareo, Preheim et al. 1996; de Roux, Cavalcanti et al. 2006). Smoking was recently demonstrated to be one of the strongest independent risk factors for invasive pneumococcal disease. The exact pathophysiological mechanism regarding smoking is not fully understood, but probably involves both increased carriage rates as well as impaired muco-ciliary clearance (Nuorti, Butler et al. 2000; Arcavi and Benowitz 2004).

Congestive heart failure is associated with all cause pneumonia, but also with severe pneumococcal disease (Lipsky, Boyko et al. 1986; Watt, O'Brien et al. 2007). The exact mechanisms for this are unknown and surprisingly no such association is seen with ischemic heart disease.

Malignancies, with special reference to leukaemia and lymphomas have been attributed to a near 50% increased risk of a fatal outcome in invasive pneumococcal infections (Bernard 1981; Fernandez Guerrero 2003). This has generally been explained by an impaired immunity and neutropenia.

There is an increased risk of IPD in children and adults with renal allografts, chronic renal insufficiency or nephrotic syndrome (Robinson 2004). Contributing factors to IPD in patients with renal disease include impaired leukocyte function, immunosuppressive drugs in recipients of renal transplants, and hypogammaglobulinemia, and urinary loss of factors B and D of the alternate complement pathway (Robinson 2004).

Patients with different forms of liver disease are also overrepresented in materials of invasive pneumococcal disease. Especially liver cirrhosis seems to elevate the risk of a fatal outcome in invasive pneumococcal disease. The pathophysiological explanation for this is probably multifactorial and includes factors like disruption of the intestinal mucosa because of oedema resulting in impaired hepatic filtration of bacteria. Patients with liver cirrhosis also have a lower number of Kupffer's cells, and alteration in cellular immunity leads to a decrease in the number of neutrophils and impairment of their function (Thulstrup 2000).

The role of the spleen in the immune response was discussed under the heading “adaptive immune response”. An impaired function of the spleen or total asplenia will thus result in diminished clearance of pneumococci from the blood stream. The extremely elevated risk of invasive pneumococcal disease in patients with sickle-cell anaemia has been contributed to splenic dysfunction (Wong, Overturf et al. 1992).

Table 1

COMMON PREDISPOSING FACTORS FOR INVASIVE PNEUMOCOCCAL DISEASE	
Direct immune system related disorders	**Non-direct immune system related disorders**
Immunoglobulin deficiencies	Age
Complement defects	Alcoholism
Leukopenia	Smoking
Immuno-suppressive treatment	Congestive heart failure
HIV	Malignancy
	Diabetes Mellitus
	Liver disease
	Kidney disease
	Asplenia

Immune system specific factors

There are more than 100 known primary immune deficiencies known to enhance the risk of serious pneumococcal infection. For example defects in B-cell function, or suboptimal cellular helper immune function, result in an inadequate antibody response, which enhances the risk of serious pneumococcal infection. Patients with IgG2 subclass deficiency are more prone to develop both pneumonias and invasive pneumococcal disease (Braconier, Nilsson et al. 1984). Selective IgA deficiency has however not been demonstrated to have an impact on severity or invasiveness of pneumococcal disease.
Interestingly, only 5% of children with x-linked agammaglobulinemi develop invasive pneumococcal disease, further strengthening the idea that non-Ig-mediated immune mechanisms also contribute to blood clearance of *S. pneumoniae* (Picard 2003).

Similarly, deficiencies of early complement activation via the classic pathway, complement C3 defects, conditions associated with impaired NF-kappa B activation, and interleukin-1 receptor-associated kinase-4 deficiency have also been associated with an increased risk of severe systemic pneumococcal infection (Picard 2003).
However, patients with primary immune disorders resulting in inhibited neutrophil function or congenital immune deficiencies other than hyperimmunoglobulin E syndrome do not exhibit an increased risk for severe pneumococcal infections compared with the general population.

HIV-infected individuals have a 50- to 100-fold higher incidence of invasive pneumococcal disease than the general population (Nuorti, Butler et al. 2000). After the introduction of the highly active antiretroviral therapy in the mid-1990s, a 50% reduction in invasive pneumococcal disease among patients with AIDS was seen (Heffernan, Barrett et al. 2005).
Mortality in IPD has in previous studies not been shown to be higher among patients with HIV compared to the general population, probably due to a) the fact that B-cell immunity is not primarily affected in HIV and b) a large proportion of these patients are fairly young and without other underlying diseases. However in a recent study when age and severity of illness were corrected for, HIV patients had significantly increased 14-day mortality as compared to non-HIV patients. This poorer outcome was found to be related, in part, to the level of immunocompromise, as indicated by the CD4 count (Feldman, Klugman et al. 2007).

1.7 DIAGNOSIS OF PNEUMOCOCCAL INFECTIONS

Obtaining a reliable etiological diagnosis in infections can often be difficult. Therefore, antimicrobial therapies are often initiated long before the identification of the causative agent. The value of microbiological investigations - especially in community acquired pneumonia - has been questioned because of the low diagnostic yield from the different specimens obtained, but also since a positive microbiological culture quite often does not result in any change of the antimicrobial treatment (Woodhead, Arrowsmith et al. 1991; Sanyal, Smith et al. 1999; Lidman, Burman et al. 2002). Many researchers and clinicians are however still in favour of performing microbiological investigations. Among the different reasons for this is the possibility to choose optimal antibiotic treatment, to study the epidemiology of antimicrobial resistance, as well as to facilitate decisions for further investigations if treatment failure occurs. Also, in the case of pneumococcal infections, a number of fast and fairly reliable tests for the early detection of pneumococcal infections have been developed, aiming to enabling guidance on the first-line antimicrobial treatment. These rapid methods were recently recognised and promoted by the US National Institutes of Health (Mandell, Wunderink et al. 2007).

The lack of a golden standard has made it difficult to evaluate new diagnostic methods for pneumococcal infections. The only way to be certain of the pneumococcus being the causative agent of an infection is the detection of the bacterium in normally sterile locations such as the blood, the CSF or synovial fluid.

Blood / Serum

Blood culture

Blood culture is an important method in the arsenal of diagnostic procedures in pneumococcal disease. While only 25-30% of pneumococcal pneumonias are considered to be bacteraemic (Ostergaard and Andersen 1993; Musher 2000) almost 2/3 of all patients with pneumococcal meningitis display positive blood cultures for pneumococci (Gray and Fedorko 1992).
Although *S. pneumoniae* grows rapidly in most conventional and automated blood culture systems, the production of autolysins (described previously) by the pneumococcus can result in a distortion of the appearance of pneumococci on Gram stain or prevent growth in blood culture.

Other diagnostic methods on blood / serum samples

Antigen tests

A number of different assays for the detection of pneumococcal capsular and cell wall antigens have been developed. Target antigens in those assays have often been either serotype specific capsular antigens, or the non-serotype specific cell wall polysaccharide.
The three mostly used techniques in detection of these antigens have been CIE (counterimmunoelectrophoresis), LA (latex agglutination) or ELISA (enzyme linked immunosorbent assay). Although several investigators have demonstrated a high specificity for these methods, they have unfortunately often been marred with low sensitivity, not reaching above 55% compared to blood cultures (van der Auwera 1983; Dominguez, Andreo et al. 2006).
The Binax NOW *Streptococcus pneumoniae* antigen test (Binax, Inc., Portland, Maine), an immunochromatographic membrane assay that detects the presence of the cell wall polysaccharide antigen was in a recent study used to detect pneumococci in blood culture bottles (Petti 2005). The method was found to have a very high sensitivity, but also demonstrated a number of false positive results due to *Streptococcus mitis*, which are known to be able to cross react with the pneumococcal cell wall polysaccharide (Alonso-Tarrees, Cortes-Lletget et al. 2001).

PCR

Pneumococcal DNA-detection in blood has been investigated during the past decade. The gene targets mostly used in these assays have either been pneumolysin (*ply*) or the major autolysin (LytA). Up to date, none of these PCR's have been routinely used in the clinical setting since most assays, despite specificity of >90%, have been flawed with either too low sensitivity or technical difficulties such as the presence of inhibition in the serum samples (Rudolph, Parkinson et al. 1993; van Haeften, Palladino et al. 2003). Another potential problem with these PCR assays on blood/serum is the detection of pneumococci among healthy controls / carriers, although different studies show conflicting results concerning this (Dagan, Shriker et al. 1998; Lorente, Falguera et al. 2000).
However, PCR assays may be valuable in finding pneumococcal DNA in blood culture bottles where no growth was detected (Sheppard, Harrison et al. 2004).

Serology

Antibodies against several pneumococcal structures can be identified after pneumococcal infections. Older assays include detection of antibodies against capsular polysaccharide (Holmberg, Krook et al. 1985; Burman 1991), as well as against pneumolysin (Kalin 1987), while more recent studies have evaluated antibodies against pneumococcal surface antigen A psaA (Scott 2005). Most assays have however been hampered by a both low sensitivity and specificity, and hence serology for pneumococci in the clinical setting is rarely used nowadays (Leinonen 1994).

CSF

CSF direct microscopy and culture

Gram staining on CSF is an important and fast method for diagnosis of pneumococcal meningitis. Because of the lack of a true golden standard, sensitivity analysis of this method is difficult to assess, but 75-90% of culture positive samples are regarded to be positive also in the direct microscopy assay, especially when the bacterial load is high. This was demonstrated for CSF by LaScolea et al (LaScolea 1984) who showed that 25%, 60% and 97% of CSF specimens with $<10^3$, 10^3-10^4 and $\geq 10^5$ bacteria/ml, respectively, were positive by Gram stain.
CSF culture is usually positive within 1-3 days, and almost all patients with highly suspected pneumococcal meningitis that was not treated with antibiotics prior to sampling, display positive CSF cultures ((SSID) 2004).

Other diagnostic methods on CSF

PCR techniques for detecting pneumococci in CSF are becoming increasingly available. Two recent studies indicate both sensitivity and specificity well above 90% (Tzanakaki, Tsopanomichalou et al. 2005; Van Gastel, Bruynseels et al. 2007). It has been suggested that the better sensitivity in PCR assays of CSF as compared to blood depend on lower amount of inhibiting factors in CSF. In order to perform antibiotic susceptibility testing, look for unexpected pathogens and carry out serotyping as well as other epidemiological typing methods, CSF-culture is still, and probably will remain, the reference method for bacterial meningitis. New PCR assays targeting antibiotic susceptibility issues are currently under development (Kojima, Nakagami et al. 2006). The Binax Now antigen test (initially developed for detection of pneumococcal antigen in urine) has also been evaluated on CSF in pneumococcal meningitis, suggesting both a very high sensitivity and specificity for this test (Marcos, Martinez et al. 2001; Samra, Shmuely et al. 2003).

Sputum

Sputum Gram stain and culture

Although sputum Gram stain with subsequent culture has been used to detect streptococci in LRTIs since the beginning of the 20th century, the value of both

Gram stain and culture of sputum is still questioned. The main argument against the value of these methods is the possible presence of colonizing oral bacterial flora in the samples, making interpretation of the bacterial findings difficult. In order to overcome this problem, the number of squamous epithelial cells (SEC) in ratio to the number of leukocytes in the samples has been used as a marker if the sample contains saliva or is derived from the lower respiratory tract. There is no definite consensus regarding the number of leukocytes / SEC needed to be present in the sample in order to consider it as coming from the lower respiratory tract, and thus as of good quality. However, several studies have concluded that a sample ratio of 5-10 leukocytes/SEC is enough (Kalin, Lindberg et al. 1983; Boersma, Lowenberg et al. 1993).
Another way of limiting the presence of colonizing flora is the technique of washing sputa. With this technique the sputum sample is washed in a filter, resulting in the removal of saliva and contaminating bacterial flora, and thereafter homogenized, Gram stained and cultivated (Kalin 1982; Cao, Ishiwada et al. 2004). Since this technique is very simple, it is somewhat surprising that it does not seem to be much used.
Sputum is often cultured semi-quantitatively or quantitatively, since a high concentration of bacteria is more likely to represent a significant bacterial finding. Although not standardised, a bacterial load of $\geq 10^5$ cfu/ml is often considered a significant finding.
A second limitation with sputum cultures is the problem of sampling. Many patients with respiratory tract infections including those with pneumococcal aetiology are unable to produce a good quality sputum sample. This is especially apparent in children.
For some patients this obstacle can be overcome by letting the patient inhale 3% hypertonic sodium chloride, breathe against resistance, and finally cough by manual assistance by a physiotherapist.
Further, the validity of Gram stain has been demonstrated to be dependant on the experience of the examiner (Fine, Orloff et al. 1991).
Recently several authors have reported very low diagnostic value of sputum culture, and again a discussion whether sputum culture still has any impact on the treatment or care of patients with pneumonias has emerged (Ewig, Schlochtermeier et al. 2002; Garcia-Vazquez, Marcos et al. 2004; Musher 2005). A study by Musher et al. (Musher 2004), however, could demonstrate that among 105 patients with verified pneumococcal pneumonia, 86% had positive sputum cultures, and 80% had Gram stain consistent with *S. pneumoniae*.

Sputum antigen

Several pneumococcal antigen assays for sputum were investigated between 1985 and 1990, and shown to have remarkably high sensitivities (70-95%) and specificities (75-100%) (Holmberg, Holme et al. 1985; Holmberg and Krook 1986; Ortqvist, Jonsson et al. 1989). The same assays previously described for serum (CIE, LA and ELISA) were also tested on sputum. These assays were also shown to perform well on washed sputum as well as samples containing a large amount of saliva, and as expected also performed well if the patient had received antibiotics prior to sampling (Boersma, Lowenberg et al. 1992). Despite the excellent performances of the sputum antigen tests, there are very

few reports of them being used in any clinical settings since the mid 1990s, perhaps due to the arrival of the PCR methodology at the same time.
Pneumococcal antigen was however again shown in a recent study to be a valuable tool for the diagnosis of pneumococcal pneumonia, and was demonstrated to increase the diagnostic yield by 24% when adding this method to ordinary sputum Gram stain and culture (van der Eerden, Vlaspolder et al. 2005). Cross-reactions with oral streptococci may theoretically occur in sputum samples, and this should be considered when interpreting results of the pneumococcal capsular antigen (PCA) test in sputum. However, false-positive PCA results in sputum due to cross-reacting oral flora are unlikely, because the magnitude of antigen produced by these microorganisms is usually inadequate.

Sputum PCR

A number of different PCR assays for the detection of pneumococci and other respiratory tract pathogens are available. The three mostly used assays are conventional PCR for single pathogen detection, multiplex PCR and real-time quantitative PCR.
Conventional PCR assays for the detection of respiratory pathogens in sputum can be an extremely useful tool concerning bacteria that are not normally present in the upper respiratory tract. However when running PCR assays directed towards bacteria like pneumococci and other frequent colonizers, there is substantial risk that a positive result in the assay merely indicates colonization (Murdoch 2004). This was demonstrated by Murdoch et al who received the same amount of positive results among their controls as among their patients with pneumonia, although the sample specimens in this study came from throat swabs (Murdoch, Anderson et al. 2003).
Multiplex PCR allows the detection of several possible pathogens simultaneously. While this method has been evaluated extensively for atypical pathogens like *Mycoplasma pneumoniae*, *Chlamydophilae* species and *Legionella* species, few investigators have included primers also for classical pathogens like *S. pneumoniae*. Strålin et al. however recently compared culture to multiplex PCR for *S. pneumoniae*, *Haemophilus influenzae*, *M. pneumoniae* and *Chlamydophila pneumoniae*. They examined sputum and nasopharyngeal samples from adults with CAP and controls, and found sensitivity ranging from 58 to 100% and specificity ranging from 42 to 100 (Stralin, Tornqvist et al. 2006).
Real-time quantitative PCR (RQ-PCR) uses fluorescent-labelled DNA probes, or inbinding of double stranded DNA, emitting detectable light, which enables direct quantification of the number of gene-copies in the sample. Besides generating quantitative data, the RQ-PCR is also fast since no gel-electrophoresis step is needed, further it is regarded as fairly safe from DNA-contamination since all laboratory procedures occur in a single tube. Several studies have evaluated RQ-PCR in diagnosis of pneumococcal infections (Johansson, Kalin et al.; Apfalter 2005; Kais, Spindler et al. 2006; Chan 2007), using different bacterial cut-offs in order to distinguish true infections from possible colonization.
The RQ-PCR technique is discussed more in detail in paper I, included in this thesis
An important issue in PCR-assays for respiratory tract samples is the specificity of the selected target genes. Traditionally, the target genes mostly used have

been either coding for pneumolysin, lyt A or psaA. In a recent study these three target genes were evaluated against each other (Carvalho Mda, Tondella et al. 2007). The authors concluded that pneumolysin is maybe not a suitable target-gene, since there might be cross-reactivity with other streptococcal species, while lytA and psaA did not demonstrate any such cross reactivity. Other studies have however demonstrated very low cross reactivity for pneumolysin primers (Yang, Lin et al. 2005; Bayram, Kocoglu et al. 2006). The different results obtained for pneumolysin could probably be explained by which part of the pneumolysin gene that is used.

Nasopharyngeal culture

In adult patients with pneumococcal pneumonia, nasopharyngeal cultures can sometimes be an important diagnostic tool, especially when it is difficult to obtain a sputum sample. This method is however mostly used in the Scandinavian countries. Hedlund et al. could demonstrate that the method has a high specificity, although the sensitivity for the detection of pneumococcal pneumonia was only 28% (Hedlund, Ortqvist et al. 1990). The main argument against the method has been the high frequency of pneumococcal carriage in some populations. However, in adults without close contact with small children pneumococcal carriage is very uncommon (< 5%) (Regev-Yochay, Raz et al. 2004), and carriage is also relatively uncommon (<10%) among adults with children in the family (Goldblatt, Hussain et al. 2005).

Other respiratory tract samples

All the above described methods applicable on sputum can and have successfully been used on other materials such as protected specimen brush (PSB), bronchoalveolar lavage (BAL) (Stralin, Korsgaard et al. 2006; Abdeldaim, Stralin et al. 2007) pleural fluid (Utine 2007), trans-tracheal aspiration (Kalinske, Parker et al. 1967) and trans-thoracic needle aspiration (Torres, Jimenez et al. 1990).

Urine antigen tests

Urine antigen tests for *Streptococcus pneumoniae* have become widely used in the clinical setting since their introduction a few years ago. The most commonly used commercially available kit is the Binax NOW *S. pneumoniae* Urinary Antigen Test, which is an immunochromatographic membrane assay (ICT), detecting *S. pneumoniae* C-polysaccharide.
The sensitivity of this urine antigen test has been demonstrated to range between 66 and 82% for patients with pneumococcal pneumonia, depending on which other diagnostic test it was compared to (the higher figure when compared to blood culture) (Dominguez, Gali et al. 2001; Farina, Arosio et al. 2002; Gutierrez, Masia et al. 2003; Roson, Fernandez-Sabe et al. 2004). When the test has been evaluated for patients with invasive pneumococcal disease, a sensitivity level around 80% has been demonstrated (Dominguez, Gali et al. 2001; Murdoch, Laing et al. 2001).

The specificity of this test has been demonstrated to reach almost 100% in several studies (in adults) (Dominguez, Gali et al. 2001; Murdoch, Laing et al. 2001; Smith, Derrington et al. 2003). The pneumococcal urinary antigen test

remains positive after initiation of antibiotic therapy. In a study by Smith et al., the authors suggested that 83% of patients remained positive after 3 days, and 73% after a week (Smith, Derrington et al. 2003). The fact that many patients remain positive in this test for a long time, also when treated with antibiotics, is appealing in the acute clinical setting, but could of course also be a potential reason for a false positive test. However, since false positive results seems to be quite rare it has been suggested that a positive urine antigen result provides a very probable, albeit not definite, pneumococcal aetiology (Stralin in press).

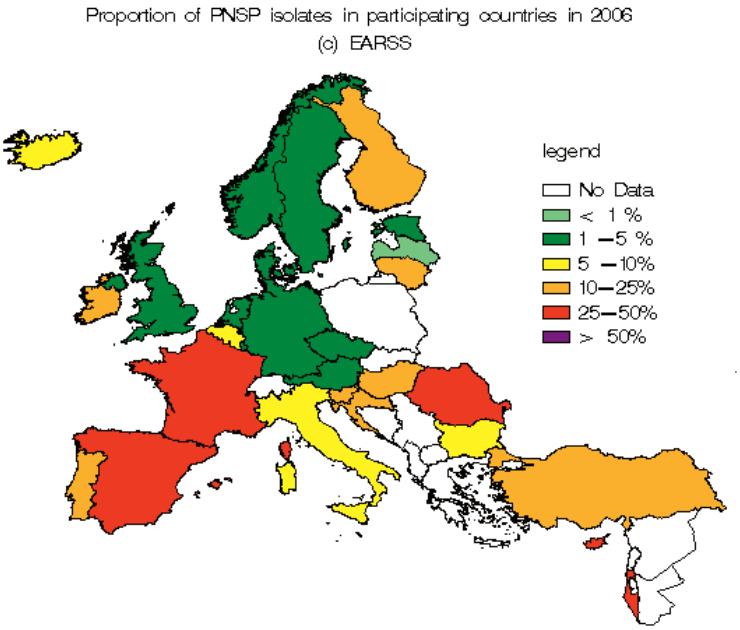
1.8 TREATMENT OF PNEUMOCOCCAL DISEASE

Antibiotic resistance and antibiotic treatment

Until 1967, no pneumococcal isolates with decreased sensitivity to penicillin were described (Hansman and Bullen 1967). Penicillin resistance has since then steadily increased in the world, although major regional differences are present, with figures ranging from 4-62% only within Europe (Reinert, Reinert et al. 2005). Multi-drug resistant strains (resistant to three or more different antibiotics) were observed as early as 1977 in South Africa, and many countries are now reporting prevalence figures of multi-drug resistant strains as high as 30% (Lynch and Zhanel 2005). The prevalence of pneumococcal strains with decreased susceptibility to penicillin in Europe is demonstrated in figure 2.
In Sweden the figures for penicillin non-susceptible *Streptococcus pneumoniae* (PNSP) (defined as all isolates within the groups I: intermediately susceptible and R: resistant) have generally been very low, which has been suggested to be at least partly attributed to the relatively low consumption of broad-spectrum antibiotics in Sweden

Resistance to penicillin and other members of the beta-lactam antibiotic family is caused by changes in the affinity to one or more of the six different penicillin binding proteins in the bacterial cell wall. Clinical isolates of pneumococci have been demonstrated to contain mosaic PBP genes, resulting from genetic transformation from other pneumococci or streptococcal species (Tomasz 1995).
Isolates with “mosaic” PBP genes have also been demonstrated to contain other mosaic genes which would explain why PNSP isolates often are resistant to other antibiotics as well (Pletz, Welte et al. 2007). Since antibiotic resistance among pneumococci often is the result of genetic recombination events, it is not surprising that serotypes that are frequently found in carriage seem more prone to harbour antibiotic resistance. The most commonly found PNSP serogroups in Sweden between 1997 and 2003 were in descending order 9, 19, 14, 23, and 6 (Hogberg, Ekdahl et al. 2006).
Fast evolution of antibiotic resistance within certain geographic areas among pneumococci has been demonstrated to be attributed to the expansion of specific clones (Sjöström 2007).

Figure 2 Proportion of PNSP isolates in Europe 2006



Despite the magnitude of penicillin-resistant isolates, almost no cases of bacteriologic failure following therapy of these cases of pneumococcal pneumonia with penicillin or ampicillin have been described (Feldman 2004). Since the recommended dosing of benzylpenicillin in pneumococcal disease often is as high as 3g 3-4 times daily, antibiotic concentrations well above the MICs for pneumococci are often reached, and are therefore likely to remain active also against resistant pneumococci with a MIC of 4 µg/mL or less (Feldman 2004). Importantly this discussion is not applicable for pneumococcal meningitis, since much lower antibiotic concentration is reached in the CSF.

Hence, while penicillin seems to remain the most appealing treatment option for pneumococcal infections, also in the case of low-grade resistant strains, more caution is probably warranted for some of the cephalosporins. In a prospective, observational international study of 844 hospitalized patients with pneumococcal bacteraemia, Yu and colleagues evaluated the impact of concordant versus discordant empiric antibiotic therapy on 844 patients hospitalized for bacteraemic pneumococcal pneumonia. Discordant therapy with penicillins or cefotaxime, was not associated with a fatal outcome, but discordant therapy with cefuroxime was associated with a significantly increased mortality (Yu, Chiou et al. 2003).

Resistance to macrolides was described already in the 1960s. More than 40% of pneumococcal strains from southern Europe are resistant to macrolides, the same figure in Scandinavia being below 10% (Pallares, Fenoll et al. 2003). Furthermore, macrolide

resistance is frequently detected among penicillin-resistant strains. Recent data are indicating that discordant therapy with macrolides is clearly associated with treatment failure (Daneman, McGeer et al. 2006).
Resistance to fluoroquinolones is also increasing, especially to levofloxacin (Doern, Richter et al. 2005), but has surprisingly not yet reached the same high levels as in many other bacterial species. The reason for this could be that quinolones are not normally used in children. Resistance to quinolones occurs by mutation in the genes that encode for DNA gyrase and topoisomerase IV or by efflux mechanisms (Pallares, Fenoll et al. 2003), and has been demonstrated to be spread by the expansion of certain pneumococcal clones (Klugman 2003).
Resistance to other antibiotic classes like tetracyclines and co-trimoxazole is occurring with diverse prevalence in different regions, but as with the other antibiotics described above, this is predominantly found among penicillin-resistant isolates (Lode 2007).

1.9 PREVENTION OF PNEUMOCOCCAL INFECTIONS

As demonstrated in the introduction of this thesis, pneumococcal infections constitute a major disease burden in the world. It is therefore not surprising that large efforts have been made to try to prevent pneumococcal infections by vaccination.
The first large vaccine trial against pneumococci was conducted in 1911. The vaccine used was a whole-cell vaccine, and was followed a few decades later by the first polysaccharide vaccines, only including four serotypes. However, very little interest was given to pneumococcal vaccines the following decades, probably because of the arrival of antibiotics.
Fortunately new interest for preventing pneumococcal disease appeared again, and a 14-valent polysaccharide vaccine was licensed in the US 1977, followed shortly after by today's 23-valent polysaccharide vaccine (PPV-23) in 1983.
Because of the poor immunogenicity of the PPV-23 in children, new efforts were made to find better pneumococcal vaccines for children, and a number of different vaccine candidates have been evaluated in recent years, culminating in the introduction of a 7-valent conjugate vaccine in the late 1990's. The excellent performance of this vaccine, together with a fear of too few serotypes being included, has led to an increased interest from the pharmaceutical industry, and both a 10-valent and a 13-valent conjugate vaccine will probably be introduced on the market shortly.
Further, vaccine studies aiming to find a common vaccine antigen for all pneumococci, by evaluating a number of different pneumococcal virulence factors, are under way.

1.9.1 Pneumococcal Polysaccharide vaccine

The 23-valent pneumococcal polysaccharide vaccine (PPV-23) contains 25µg of each of the ingoing 23 different purified capsular polysaccharides. The serotypes included in the vaccine (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), were in most cases chosen on the basis of their relative distribution in ability to cause invasive disease. These serotypes account for approximately 90% of all invasive disease in the world. Some of the serotypes demonstrate high cross-reactivity within their serogroup (groups 6 and 15), and thus

only one of the serotypes from these groups was included (6B, 15B), whereas less cross reactivity is seen within other serogroups (groups 9 and 19), resulting in the inclusion of multiple serotypes within these serogroups.

As has been discussed previously, the PPV-23 does not offer a good protection for pneumococcal disease in young children, largely because of delayed maturation of specific subsets of B-cells. A good antibody response is however usually detected to some of the included serotypes also in infants (types 3, 4, 8, 9N and 18C) (Koskela, Leinonen et al. 1986), while the rest of the serotypes included in the vaccine does not evoke a good enough response. Further, even when a good antibody response is elicited (primarily by IgG2 and IgG4), the antibody levels decrease rapidly within a few months (Bernatoniene and Finn 2005). Although antibody responses to PPV in adults are generally much better, they still seem to vary, shown especially by a large inter-individual variability in antibody production to certain serotypes (Musher, Watson et al. 2000).
Several assays to measure antibody response to polysaccharides have been developed, most utilizing ELISA or other enzyme immunoassays (Musher, Watson et al. 2000). These assays have demonstrated that antibodies of all subclasses are detectable following vaccination. The first antibodies appear already within a week (IgM and IgG), but IgG levels sometimes do not peak until after 2-3 months. Among the different Ig subclasses, IgG2 antibodies increase the most after polysaccharide vaccination.
The problem with measurement of antibody response with ELISA following vaccination is that although a quantitative antibody response can be measured, it does not say anything about the quality of the response. In order to overcome this, several assays measuring antibody functional activity have been investigated, including flow cytometric opsonophagocytic killing assays (OPAs), as well as animal challenging models (Musher, Johnson et al. 1990). The problem with the latter has however been that not all pneumococcal serotypes seem to cause disease in animal models.
Although there seems to be a correlation between the quantity of the serotype specific antibodies and the quality of the same antibodies for many serotypes (Goldblatt, Hussain et al. 2005), such a correlation seem to be less valid for others. Because of the marked individual variation among the included serotypes in the ability to induce a protective degree of immune response among different persons, it has been suggested that the vaccine should be considered as 23 different vaccines rather than just one vaccine, in terms of vaccine efficacy (Ortqvist, Henckaerts et al. 2007). Further, it has been demonstrated that the functional activity of the antibodies tend to decline with increasing age (Romero-Steiner, Musher et al. 1999), which may have important clinical implications, since the elderly are at special risk for pneumococcal infections. On the other hand the antibody response after vaccination is satisfactory in most elderly patients (Sankilampi, Honkanen et al. 1996).
There is a slow but steady decline in serotype specific antibodies, and prevaccination levels are generally reached within 5-10 years (Fedson 2003). Very few studies have addressed the issue of revaccination (Artz, Ershler et al. 2003), and although it is widely recommended there is no international consensus. In general, the second vaccine dose seem to induce a much weaker immune response than the first dose, and does not generate an anamnestic response (Jackson, Benson et al. 1999; Torling, Hedlund et al. 2003). Data suggesting revaccination after 5 years have recently been extrapolated from studies on splenectomised patients (Cherif, Landgren et al. 2006).

The PPV-23 is recommended in the US and in most European countries for adults who are at risk for pneumococcal infections because of age, comorbidities or immunosuppression. In the US, vaccine coverage levels for PPV-23 among non-institutionalised residents > 65 years of age have been reported to increase from 46 % in 1998, to presently exceed the 60 % level that was the national target level in 2000. In Sweden the vaccine has been in use in large scale vaccination programs since 1998. Despite the extensive use of this vaccine, the protective role of the vaccine other than for invasive pneumococcal disease is still a matter of debate. The reasons for the ongoing controversy on whether PPV-23 offers any protection against pneumococcal pneumonia, pneumococcal disease in general or all cause pneumonia, is primarily due to the difficulties in obtaining an etiological verification of pneumococcal pneumonia. Further, since most western countries have implemented vaccination programs with PCV7 (discussed further below), the distinction between protective effects obtained from PPV-23 and herd effects from PCV7 will be utterly difficult to assess in new prospective studies.
An indirect measurement of how difficult it has been to effectively demonstrate the efficacy of PPV-23 is that no less than five large meta-analyses have addressed this issue the past years. While there is overwhelming scientific support for the protection of PPV on invasive pneumococcal disease, reaching protective levels of 40-70%(Dear, Holden et al. 2003; Jackson, Neuzil et al. 2003; Andrews, Counahan et al. 2004; Melegaro and Edmunds 2004; Vila-Corcoles, Ochoa-Gondar et al. 2006), several studies including a Cochrane review have failed to demonstrate significant protective effects of the PPV against pneumococcal pneumonia, all cause pneumonia, or death by pneumonia (Cornu, Yzebe et al. 2001; Dear, Holden et al. 2003).
On the other hand, several well conducted observational studies, have demonstrated significant effects on preventing pneumococcal pneumonia (Vila-Corcoles, Ochoa-Gondar et al. 2006), and hospital admission for pneumonia(Christenson, Hedlund et al. 2004), as well as length of stay among patients hospitalized for pneumonia (Fisman, Abrutyn et al. 2006).
Further, recent studies have confirmed previous observations that PPV may not only lower mortality in pneumococcal disease (Christenson, Hedlund et al. 2004; Vila-Corcoles, Ochoa-Gondar et al. 2005), but might also diminish the risk of severe septic episodes and ICU admission in pneumococcal disease (Johnstone, Marrie et al. 2007).

Several studies have also demonstrated that vaccination of elderly persons, and individuals with augmented risk for pneumococcal, with PPV is cost effective, also if the possible protective effect on pneumococcal pneumonia and all cause pneumonia is not accounted for (Ament, Baltussen et al. 2000; Melegaro and Edmunds 2004).

1.9.2 Conjugate vaccines

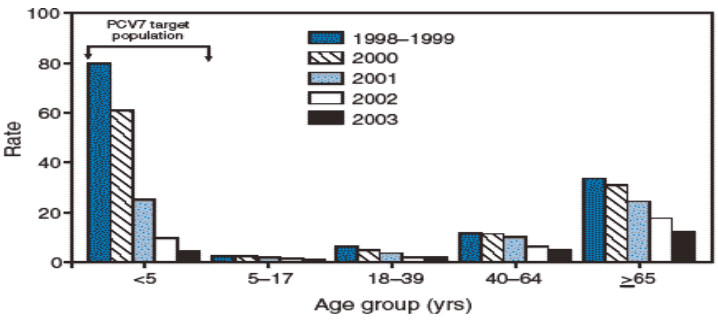
In polysaccharide vaccines, antigens interact directly with surface receptors on B cells in a T-cell independent fashion, with the resulting weak generation of antibody stimulating clones. However, when the polysaccharide is coupled to a protein carrier, the antigen is fragmentized and internalized, enabling presentation on the surface by

MHC class 2. This elicits a strong stimulation of the generation of specific antibody producing B-cell clones.
The 7-valent pneumococcal conjugate vaccine (PCV7) includes seven purified capsular polysaccharides of *S. pneumoniae* (4, 6B, 9V, 14, 18C, 19F, 23F). Each of the polysaccharides is coupled to a non-toxic diphtheria protein analogue. A 9-valent vaccine (PCV9), used for study purposes but not marketed, also included serotype 1 and 5, and in the coming 10-valent vaccine (PCV10) serotype 7F has been added to these nine polysaccharides.
The prevalence and thus the theoretical coverage of the 7 different serotypes included in the vaccine has been demonstrated to comprise 89 % of pneumococcal disease in North America and Australia, and 78% in Europe (due to higher prevalence of especially serotypes 1 and 8 in Europe), while the coverage has been estimated to be lower in Africa (67%) and Asia (43%) (Eskola 2004).
In contrast to the PPV, the PCV has been demonstrated to have an unarguable effect on overall disease burden of pneumococcal disease. This effect largely relies on the decrease of vaccine type IPD. After the large scale introduction of PCV7 in the US, the overall incidence of IPD was demonstrated to decrease from 30.2 to 13.1 per 100 000 ($p<0.0001$) in a study from Atlanta, USA by Stephens et al. (Stephens, Zughaier et al. 2005). Similar observations have been made also in other studies from the US (Whitney, Farley et al. 2003).
The most dramatic effects have been seen in young children, in whom the incidence of IPD associated with serotypes contained within PCV7 decreased by 94 %, between 1998/99 and 2003. Although there was a statistically significant increase of non-vaccine types in the same material, it was overshadowed by the 20 fold greater decrease in vaccine type IPD
PCV7 and PCV9 has also been demonstrated to be effective in preventing all cause pneumonia in infants, although this effect is more modest (20%) as compared to IPD (Lucero MG 2004).
As can be seen in fig 3 the protective effects are however not restricted to the vaccinated population. The mechanism by which protection is conferred to non-vaccinated age groups is called herd immunity, and is resulting from the reduced rates of colonisation and subsequent transmission of pneumococci in the whole society, since the vaccinated population is also the major contributor to transmission. The incidence of IPD among persons >50 years of age was demonstrated to decline from 41 to 29 / 100.000 between 1998 and 2003 (PCV7 introduced in children in 2000) (Lexau, Lynfield et al. 2005), however other studies have failed to confirm significant protection from PCV among elderly persons with comorbidities or from pneumococcal meningitis (Lockhart, Hackell et al. 2006).
An important issue with PCV is the possible emergence of serotype replacement. Since the most widely used PCV is only 7-valent, and proven to be effective also against nasopharyngeal colonization, an ecological niche could probably appear for non-vaccine serotypes. This has also been demonstrated within certain populations. Hicks et al. demonstrated an increase in non-vaccine type IPD among children below five years of age, from 16.3 to 19.9 cases per 100.000, and a corresponding increase among adults ≥65 years of age from 27 to 29,8 cases per 100.000, before and after the introduction of PCV7 (Hicks, Harrison et al. 2007). As discussed before this increase can be considered negligible compared to the vast decrease in vaccine type IPD, however much larger serotype replacement effects have also been demonstrated. In a

recent study among American natives in Alaska, Singleton et al. could demonstrate that although vaccine type IPD among children < 2 declined with an expected 67%, the decline of overall IPD stopped at 41% since an almost doubling of non-vaccine type IPD was seen during the study period (2001-2003 vs. 2004-2006) (Singleton, Hennessy et al. 2007). The non-vaccine types particularly responsible for the increase in both these and other studies have been serotypes 15, 19A and 33F.

Figure 3

Rate * of vaccine type IPD before and after introduction of PCV7 by age group and year – Active Bacterial Core Surveillance, Unite States 1998-2003© CDC, USA 2003



* Per 100,000 population.
† For each age group, the decrease in VT IPD rate for 2003 compared with the 1998–1999 baseline is statistically significant (p<0.05).

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2 AIMS OF PRESENT INVESTIGATION

General aims

To increase the understanding of how and why certain individuals become severely ill from pneumococcal disease. The identification of such factors attributed to both patient characteristics and to bacterial properties, will hopefully lead to a reduced mortality and morbidity from severe pneumococcal disease.

Specific aims

Paper I — To investigate the accuracy of a quantitative real time PCR (RQ-PCR) assay for the detection of *Streptococcus pneumoniae, Hemophilus influenzae and Moraxella catarrhalis* in lower respiratory tract samples, and to evaluate the potential benefit from this method in increasing the diagnostic yield in lower respiratory tract infections.

Paper II — To evaluate the accuracy of three score systems: the pneumonia severity index (PSI); CURB-65; and modified American Thoracic Society rule for predicting intensive care unit (ICU) need and mortality due to bacteraemic pneumococcal pneumonia.

Paper III — To investigate the possible relationship between bacterial properties (clonal affiliation and / or serotype) of pneumococci, to severity of disease, as well as to investigate whether co-morbidities and age was associated with any particular serotype / clone.

Paper IV — To analyse the impact of vaccination with the 23-valent pneumococcal polysaccharide vaccine (PPV-23) on the incidence of invasive pneumococcal disease and the serotype distribution in the elderly population of Stockholm County. We also wanted to compare the findings among the elderly with those from other non-vaccinated age-groups in Stockholm, as well as with those from a region in southern Sweden where no similar vaccination campaigns were made.

3 METHODS

3.1 LABORATORY METHODS (I-IV)

RQ-PCR

Polymerase chain reaction (PCR) is a method for detecting specified sequences in the genome or genome products. The idea is to amplify the sought DNA sequence in sufficient amounts to be able to detect it. Ordinary PCR assays are however only qualitative, meaning that they are able to detect a specified DNA sequence, but are unable to demonstrate the amount of the DNA sequence that was detected.
In real-time quantitative PCR (RQ-PCR), the method used in paper I of this thesis, the amount of DNA-molecules formed, is monitored during the course of the reaction by registration of the amount of fluorescent light emitted.
A detailed description of the RQ-PCR assay is given in paper I.

Serotyping

Serotyping is a method by which pneumococcal isolates can be characterised as belonging to one out of over 90 possible serotypes. Serotyping was performed with gel diffusion using 46 type- or group specific typing serum samples, obtained from the World Health Organization (WHO) Collaborating Centre for Reference and Research on Pneumococci at Statens Seruminstitut (Copenhagen, Denmark).
Isolates that could not be typed or belonged to a group that included several types were examined by capsular reaction testing (Quellung test) and / or gel diffusion with type specific antisera obtained from the same WHO collaborating Centre.

Pulse field gel electrophoresis (PFGE)
PFGE is a molecular typing method that can separate pneumococcal isolates from each other according to their genetic profile, enabling the grouping of isolates into specific clones. PFGE differs from conventional electrophoresis assays in the ability to run large DNA fragments such as an entire bacterial genome. The bacterial genome is cut into large fragments by restriction enzymes, and then embedded into an agarose gel that is subjected to a multidirectional electric field that switches its polarity over time. This results in the separation of the large DNA-fragments, enabling visualisation of them on the gel by staining of ethidium-bromide (Hermans 1995).

Multi locus sequence typing (MLST)
Multilocus sequence typing (MLST), is an other molecular typing method that makes use of rapid sequencing technology to uncover variants in a number of conserved genes (called housekeeping genes), for the purpose of characterizing, subtyping, and classifying members of bacterial populations. One of the advantages of MLST over other molecular typing methods is that sequence data are portable, and thus comparable between laboratories and have led to the creation of global databases that allow for exchange of molecular typing data via the Internet (Enright and Spratt 1999).

Box-fingerprinting
This was the third molecular typing method used in this thesis. In this method, highly conserved intergenic DNA sequences, flanked by different amount of repetitive DNA, are separated by gel electrophoresis. The fragments are subsequently transferred to a nylon membrane by Southern blot, and then hybridised with DNA probes, allowing genetic differentiation between isolates. This method has commonly been replaced by PFGE, but is still used (Hermans 1995).

3.2 PATIENT POPULATIONS (I-IV)

Study I

Study I is primarily a method evaluation study. Nevertheless a retrospective analysis of a limited number of clinical parameters among the individuals, from whom the sample specimens were derived, was performed.

A total of 203 consecutive respiratory tract samples from patients with various suspected lower respiratory tract infections as well as samples from patients without suspected lower respiratory tract infections were obtained, including 106 sputum, 51 protected specimen brush [PSB], and 46 BALs

For 14 patients (6.9%), all of them from intensive care units, proper data could not be obtained. According to the patients charts, the primary reason for sending in a sample specimen to the microbiology department included 89 cases of community acquired pneumonia, 27 cases of nosocomial pneumonia, 19 cases of bronchitis or acute exacerbation of COPD, as well as 54 cases with various non-infectious pulmonary conditions, such as suspected lung tumor or fibrosis.

Study II+III

All adult patients (n=114) with community-acquired invasive pneumococcal disease (IPD) and radiography-verified pneumonia who were admitted to the Karolinska University Hospital, Solna, Sweden, between December 1998 and January 2001 were included in the study. Most patients (n=86) were included prospectively on admission to the Dept of Infectious Diseases, as a part of an international multicentre study on the effect of antibiotic resistance on the outcome of IPD. A total of 26 patients had been treated in other departments, and were included retrospectively. In addition, we also included two patients retrospectively who had been treated in the Dept of Infectious Diseases during the study period, but by mistake had not been included on admission. Clinical charts and laboratory parameters were analysed and recorded, enabling calculations of the different severity scores (see below). Clinical characteristics and pneumococcal isolates were available for all patients.

Study III

All patients and corresponding pneumococcal isolates from study II (n=114) were included in this study as well. However we also included 354 patients and pneumococcal isolates from a previous clinical multicenter study of IPD performed during 1993-1995. This study included adult patients from 5 different countries (Canada, the United Kingdom, Spain, Sweden, and the United States) and aimed at defining the influence of prognostic factors in patients with community-acquired pneumococcal bacteremia (Kalin, Ortqvist et al. 2000). Clinical characteristics and pneumococcal isolates were available for all patients.

Study IV

All cases of invasive pneumococcal disease (n=1887), identified through records from the microbiological laboratories, in all age groups at two study-sites, Stockholm County and Skåne County, Sweden, during 1997-2001. Demographic data on all study persons was obtained and registered together with the results of the serotyping procedures.

The total population at the two study sites, Stockholm County and Skåne County amounted to 2.7 million persons, comprising 30% of the total population in Sweden,.

3.3 SEVERITY SCORES

In this thesis a number of different severity scores are used in study II-IV. The aim of a severity score is usually to help the clinician to assess the seriousness of a medical condition. Most frequently it is used as a basis for deciding the level of care needed on admission, but can also serve as a mean of assessing a medical course of events. In this thesis four different severity scores are used. Three of the scores are designed to assess severity in community acquired pneumonia, the Pneumonia Severity Index (PSI), the CURB-65 score and the modified American Thoracic Society score (mATS). The forth score the Acute Physiologic and Chronic Health Evaluation (APACHE-II) score is severity score that is widely used in intensive care unit settings to assess severity of disease regardless of cause.

Pneumonia Severity Index (PSI) adapted from the original publication by Fine et al (Fine, Auble et al. 1997).

Step 1: Is the patient at low risk (class I) based on history and physical examination and not a resident of a nursing home, as well as <50 years of age, and lacking coexisting conditions and physical examination findings listed below?

No: Go to step 2 Yes: outpatient treatment is recommended

Step 2

Patient characteristics	**Points assigned**
Demographic factors	
Age in years	
Males	Age
Females	Age-10
Nursing home resident	+10
Coexisting conditions	
Neoplastic disease	+30
Liver disease	+30
Congestive heart failure	+10
Cerebrovascular disease	+10
Renal disease	+10
Initial physical examination findings	
Altered mental status	+20
Respiratory rate >30 breaths / minute	+20
Systolic blood pressure < 90 mmHg	+20

Temperature < 35ºC or ≥ 40ºC	+15
Pulse ≥ 125 beats / minute	+10
Initial laboratory findings	
pH < 7.35	+30
Blood urea nitrogen > 10.5 mmol / L	+20
Sodium < 130 mmol / L	+20
Glucose ≥ 13.9 mmol /L	+10
Hematocrit < 0.30	+10
PaO2 < 8 kPa or sat.O2 < 90%	+10
Pleural effusion	+10
Total	

30-Day Mortality Data by Risk Class			
Total score	Risk class	Treatment	Estimated mortality %
None (see step 1)	I	Outpatient	0.1
< 70	II	Outpatient	0.6
71-90	III	Outpatient	0.9-2.8
91-130	IV	Inpatient	8.2-9.3
>130	V	Inpatient	27.0-29.2

The CURB-65 score adapted from the original publication by Lim et al.(Lim, van der Eerden et al. 2003).

One point is awarded to each element present, for a total possible maximum score of 5

1. **C**onfusion (new disorientation in person, time or place)
2. **U**rea, or blood urea nitrogen (BUN) level above 7 mmol/L
3. **R**espiratory rate ≥ 30 breaths/min
4. **B**lood pressure, < 90 mm Hg systolic or ≤ 60 mm Hg diastolic
5. Age ≥ **65** years

30-Day Mortality Data by Risk Class		
Risk class	Treatment	Estimated mortality %
0	Outpatient	0.01
1	Outpatient	1.7
2	Outpatient (consider inpatient)	9
3	Inpatient	16
4	Inpatient	37
5	Inpatient	20*

* low numbers and very broad confidence interval

The modified American Thoracic Society (mATS) rule adapted from the original publication by Ewig et al. (Ewig, Ruiz et al. 1998).

The definition of severe pneumonia (need to be admitted to an ICU) relies on a number of minor and major criteria. The criteria for severe pneumonia are fulfilled by having ≥2 minor criteria or > 1 major criterion.

Minor Criteria	**Major criteria**
PaO2 / Fi O2 < 250	Need for mechanical ventilation
Multilobar pneumonia	Septic chock
Systolic blood pressure < 90 mmHg	

The Acute Physiologic and Chronic Health Evaluation (Apache II) score adapted from the original publication by Knaus et al. (Knaus, Draper et al. 1985).

	4	**3**	**2**	**1**	**0**	**1**	**2**	**3**	**4**
Temperature (ºC)	≥41	39-40.9		38.5-38.9	36-38.4	34-35.9	32-33.9	30-31.9	≤29.9
Mean arterial pressure (mmHg)	≥160	130-159	110-129		70-109		50-69		≤49
Heart rate	≥180	140-179	110-139		70-109		55-69	40-54	≤39
Respiratory rate	≥50	35-49		25-34	12-24	10-11	6-9		≤
If FIO_2≥0.5; O_2	≥67	47-66	27-46		<27				
If FIO_2<0.5; PaO_2					>9.3	8.1-9.3		7.3-8.0	<7.3
Arterial pH	≥7.7	7.6-7.69		7.5-7.59	7.33-7.49		7.25-7.32	7.15-7.24	<7.15
S-Sodium (mmol/l)	≥180	160-179	155-159	150-154	130-149		120-129	111-119	≤110
S-Potassium (mmol/l)	≥7	6-6.9		5.5-5.9	3.5-5.4	3-3.4	2.5-2.9		<2.5
S-Creatinine (µmol/l) double if renal failure	≥600	300-599	180-299	130-179	50-129		≤49		
Hematocrite (%)	≥180		150-179	140-149	90-139		61-89		≤60
WBC ($x10^9$/l)	≥40		20-39.9	15-19.9	3-14.9		1-2.9		<1
Glasgow coma scale	See specified below								
Chronic health eval.	See specified below								
Age									

Glasgow coma scale (GCS):

GCS	15	14	13	12	11	10	9	8	7	6	5	4	3
Apache II	0	1	2	3	4	5	6	7	8	9	10	11	12

Chronic Health Evaluation (CHE):

1 point assigned for each of the following co-morbidities (0-5 points) (see original publication for definitions)1. Liver disease, 2. Circulation disorder, 3. Respiratory disease, 4. Renal disease, 5. Immune deficiency 2 points extra assigned if the above resulted in elective surgery
5 points extra assigned if the above resulted in non-elective surgery

Age:

Age	**≥75**	**65-74**	**55-64**	**45-54**	**≤44**
Apache II	**6**	**5**	**3**	**2**	**0**

4 RESULTS AND DISCUSSION

4.1 PAPER 1

The aim of Paper I was to investigate whether a new diagnostic method (RQ-PCR) for the detection of *Streptococcus pneumoniae* and two other common respiratory tract pathogens (*Hemophilus influenzae* and *Moraxella catarrhalis*) in lower respiratory tract samples would increase the diagnostic yield of these samples.
We developed an RQ-PCR protocol with a primer directed to the gene coding for pneumolysin (ply) for *S. pneumoniae*, the gene coding for a specific outer membrane protein (copB) for *M. catarrhalis* and the gene coding for furumate reductase iron-sulphur gene B (frdB) for *H. influenzae*
We were able to detect bacterial DNA over a linear range between 10^4-10^8 CFU/ml. The specificity of the assay was tested with a number of different bacterial species together with a number of throat samples from healthy lab workers, as well as analysis of melting curves.
The RQ-PCR assay was tested and compared to ordinary quantitative culture on 203 consecutive lower respiratory tract samples (sputum, bronchoalveolar fluid (BAL) and protective specimen brush (PSB)). Significant pathogen finding as defined by culture $\geq 10^5$ CFU/ml or RQ-PCR DNA, corresponding to $\geq 10^5$ CFU/ml was found in 45/203 (22.2%) samples with culture and in 68/203 (33.5%) with RQ-PCR, corresponding to a 51% relative increase in pathogen finding with RQ-PCR. The largest increase was seen for *H. influenzae* where an absolute increase of 13/203 samples was seen with RQ-PCR (Table 2).

Table 2

Comparison of significant pathogen findings with culture ($\geq 10^5$ CFU/mL) and RQ-PCR (corresponding to $\geq 10^5$ CFU/mL)

RQ-PCR findings	**Culture findings**			
	S. pneumoniae	***H. influenzae***	***M. catarrhalis***	**None**
S. pneumoniae	12/203 (5.9)	–	–	11/203 (5.4)[a]
H. influenzae	–	16/203 (7.9)	–	19/203 (9.4)[b]
M. catarrhalis	–	–	8/203 (3.9)	2/203 (1.0)
None	2/203 (1.0)[a]	6/203 (3.0)[c]	1/203 (0.5)	141/203 (69.5)

Percent of all samples enclosed in parentheses.

[a] Positive in the *atpA* assay. Inhibition not present.

[b] Positive in the *16S rRNA* assay, 15/19.

[c] Positive in the *16S rRNA* assay, 6/6. Inhibition present in 2/6 samples.

A subset of the patients (n=135) suffered from lower respiratory tract infections as judged by the clinician sending in the specimen. The number of positive findings in this group of patients was 59/135 (43.7%). Fifty-one of these samples were positive in the RQ-PCR assay, whereas 32 samples were positive according to culture. One third of the patients that were positive in the RQ-PCR assay but negative in culture had been treated with antibiotics prior to sampling (Table 3). A total number of 8 patients in this group were negative according to RQ-PCR, but had positive cultures. The two samples with negative RQ-PCR and positive culture for pneumococci, were both positive in the second RQ-PCR when we chose a primer for another pneumococcal target gene (atpA), indicating that our first assay failed in detecting a presumable pneumococcus, and that no true inhibition was present.

Table 3

Comparison of significant pathogen findings with culture (≥10^5 CFU/mL) and RQ-PCR (corresponding to ≥10^5 CFU/mL). Percent of all samples enclosed in parentheses.

Cases with LRTI

RQ-PCR findings	**Culture findings**			
	S. pneumoniae	***H. influenzae***	***M. catarrhalis***	**None**
S. pneumoniae	9/135 (6.7)	–	–	10/135 (7.4)[a]
H. influenzae	–	16/135 (11.9)	–	14/135 (10.4)[b]
M. catarrhalis	–	–	8/135 (5.9)	1/135 (0.7)
None	2/135 (1.5)[a]	5/135 (3.7)[c]	1/135 (0.7)	75/135 (55.6)

[a] Positive in the *atpA* assay. Inhibition not present.

[b] Positive in the *16S rRNA* assay, 13/14.

[c] Positive in the *16S rRNA* assay, 5/5. Inhibition present in 2/5 samples

As discussed in the introduction, the lack of a golden standard becomes very obvious, when evaluating a novel method for the detection of respiratory tract samples. Concerning pneumococci, this can partly be overcome by comparing the findings with the number of different available assays for detecting pneumococci in different sample specimens (blood culture, urine antigen, sputum antigen etc). However, for bacteria like

H. influenzae and *M. catarrhalis*, there are no such comparative models available, and thus the results of a novel detection assay must be interpreted with care. In our assay we chose the amount of DNA corresponding to $\geq 10^5$ CFU/ml as a cut of level for defining a significant finding for all three bacteria. While this is a fairly accepted level of significance concerning pneumococci, there is much more uncertainty whether this level can be directly applied to *H. influenzae* and *M. catarrhalis*.
An important question is of course if it was relevant to look for these bacteria. The pneumococcus is considered as an indisputably relevant pathogen in lower respiratory tract infections. The relevance for *H. influenzae*, and in particular *M. catarrhalis* as important pathogens in respiratory tract infections is however less clear. It is therefore not surprising that many studies have evaluated different PCR assays for the detection of pneumococci in different sample specimens, while very few studies have been focused on the detection of *H. influenzae* and *M. catarrhalis*.

The role of *Moraxella catarrhalis* as an important pathogen in lower respiratory tract infections remains a matter of debate. The carriage rate of *M. catarrhalis* in children has been demonstrated to be as high as 75% (Varon, Levy et al. 2000), while the same figure for adults is very low (1-3%) (Verduin, Hol et al. 2002). *M. catarrhalis* is a modestly important pathogen in upper respiratory tract infections in children, but is not regarded as an important pathogen of lower respiratory tract infections in healthy adults. However, *M. catarrhalis* has been demonstrated to be an important pathogen also in adults on certain occasions; 1. in persons with chronic obstructive pulmonary disease (COPD) (Pollard, Wallace et al. 1986; Curran, Coyle et al. 2007), 2. in pneumonia in the elderly (Carr, Walsh et al. 1991; Murphy 2000), and 3. in nosocomial pneumonia (Murphy 2000; Verduin, Hol et al. 2002). Further, patients with pneumonia caused by *M. catarrhalis* appear generally to be burdened with underlying co-morbidities and malnutrition, and are therefore probably at high risk for a severe pneumonia. A correct etiological diagnosis of their pneumonia would thus be desirable. The few published studies on the detection of *M. catarrhalis* in respiratory tract sampled have all evaluated different gene-targets in their assays, in most cases resulting in both high sensitivity and specificity (Greiner, Day et al. 2003; Curran, Coyle et al. 2007).

The role of *Hemophilus influenzae* in lower respiratory tract infections is not as controversial as compared to *M. catarrhalis*, although it is primarily recognized as being important in patients with COPD. Since the introduction of large vaccination campaigns against encapsulated *H. influenzae* type B, respiratory tract infections are primarily caused by non-typeable *H. Influenzae*. Non-typeable *H. influenzae* is the most common cause of exacerbations of COPD and bronchiectasis and also causes infections in patients with cystic fibrosis (Murphy 2003)
Also for *H. influenzae*, a number of target genes have been used in the different conventional PCR assays described. We initially had difficulties in finding a suitable target gene demonstrating low homology with other *Haemophilus* spp., but finally chose frdB after suggestion by Professor Moxon in Oxford.

Concerning the detection of pneumococci, a number of different target genes have been evaluated. The two most widely used target genes in both conventional PCR and RQ-PCR assays have been pneumolysin (ply) and the major autolysin (lytA) (Messmer, Sampson et al. 2004; Apfalter, Stoiser et al. 2005; Yang, Lin et al. 2005; Carvalho Mda, Tondella et al. 2007). Although assays for both these target genes have been demonstrated to have excellent sensitivity, the somewhat lower specificity has by some authors been regarded as problematic. It is well known that other streptococcal strains

(especially Streptococcus mitis and oralis) can harbour the genes coding for ply and lytA (Whatmore, Efstratiou et al. 2000), and misclassification has also been demonstrated to occur, especially in conventional PCR assays (Suzuki, Yuyama et al. 2006). However, it should be mentioned that most researchers criticising the ply target gene of having too low specificity have assessed ply in conventional PCR assays. Among the many apparent advantages of RQ-PCR as compared to conventional PCR assays is the ability to run smaller gene probes, enabling a higher degree of specificity since it can be set to bind less avidly in the presence of point mutations., Further, the analysis of melting curves has been demonstrated to be able to differentiate between pneumococci and other species harbouring the same target genes (Kaijalainen, Saukkoriipi et al. 2005). Despite this, ply may not be an optimal target gene, and new target genes with both high sensitivity and specificity (spn9802 and spn9828) have recently been evaluated and demonstrated to perform well (Suzuki, Yuyama et al. 2006).
The RQ-PCR method described in this article has recently been evaluated in a prospective study on the aetiology of community acquired pneumonia (Johansson, Kalin et al.). The sensitivity was again found to be higher as compared to sputum culture, and the RQ-PCR also performed well in terms of specificity when compared to several other aetiological sampling methods. This RQ-PCR method has since 2005 been modified. Our microbiology department now use a probe-based detection system, and are evaluating spn9802 instead of ply as target gene for *S. pneumoniae*.

Summarized, the RQ-PCR assay substantially increased the number of significant pathogen findings of the 3 examined pathogens. A high quantity of bacterial DNA from lower respiratory tract specimen probably represents a clinically significant infection with the respective pathogen, supported by the clinical data presented in this study. The method seems especially valuable when patients have been treated with antibiotics prior to sampling. Furthermore, the method seems to be useful on all types of respiratory tract specimens.

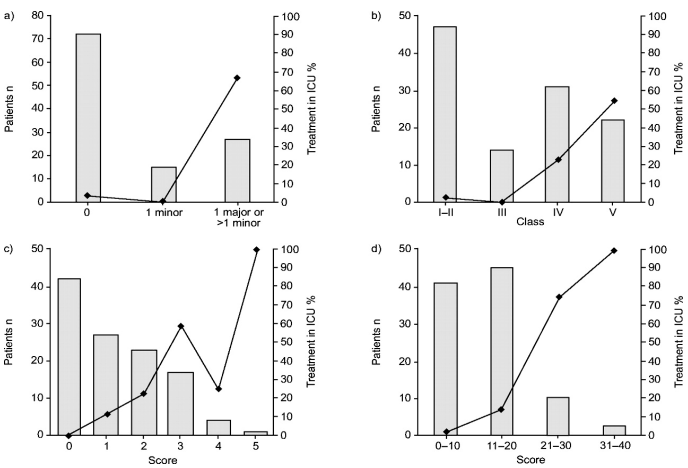
4.2 PAPER II

The aim of study II was to evaluate the accuracy of the three predictive score systems (PSI, CURB-65 and mATS) for predicting mortality and need of ICU-care in bacteremic pneumococcal pneumonia.
All adult patients with bacteremic pneumococcal pneumonia, admitted to Karolinska University Hospital, Solna during 1999 and 2000 were included in the study. In total 75% of the patients had underlying co-morbidities, 13% presented with septic chock and 18% required treatment in the ICU. The total mortality in the patient material was 11%.
We used the Apache II score as reference method, and could in agreement with other publications (Ewig, Ruiz et al. 1998; Marrie 2007) demonstrate that this score correlated directly with the need of ICU treatment. Only 2% of the patients with an Apache II score below 11 required ICU treatment, whereas the corresponding figure for patients with Apache II scores > 20 was 70-100%. The Apache II score also correlated well with mortality, and no patient with a score below 14 on admission died. The Apache II score has also recently been demonstrated to outperform the CURB-65 score in predicting mortality in pneumonia caused by MRSA (Kollef, Reichley et al. 2007).
The three evaluated score systems all showed good accuracy in predicting a fatal outcome, with an area under the receiver operating characteristics curve (ROC) between 0.83 and 0.85, and could predict need of ICU care with fairly good accuracy as demonstrated by high sensitivities and specificities.

The sensitivity / specificity of the three tests for predicting a fatal outcome was 100 / 60% (PSI), 62 / 86% (CURB-65) and 85 / 84% (mATS), and the corresponding figures for predicting ICU-care were 95 / 64% (PSI), 71 / 87% (CURB-65) and 90 / 90% (mATS). The performance of the different score systems with reference to ICU admission and fatal outcome is demonstrated in Figures 4 and 5.

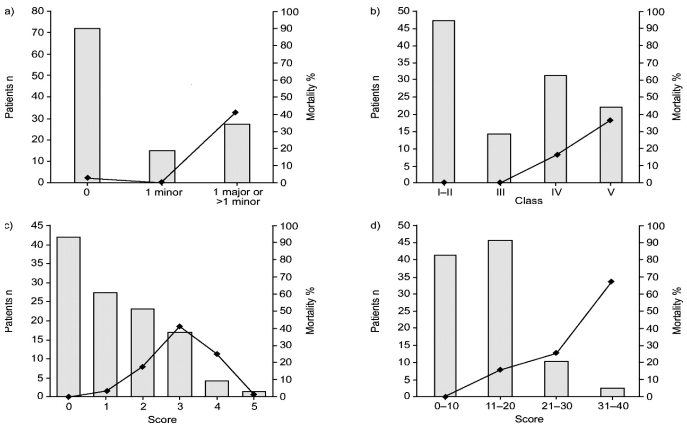
We also investigated if any single factors within the different score systems seemed to be independently important for a severe outcome in bacteremic pneumococcal pneumonia. This was performed by multivariate analyses, using the different severity scores as the continuous variable. The only two factors that turned out to be independently correlated to a fatal outcome were the respective severity score, and whether the patient had been treated in the department of infectious disease (DID) or in other departments (OD). In the logistic regression analyses, the odds ratio (OR) of a fatal outcome was ~10 for patients treated in OD as compared to those treated in DID in all 4 tested score systems. The actual mortality in those treated in DID was 4/88 (4.5%), while 9/26 (34.6%) of those treated in OD died. Although patients treated in ODs were generally more severely ill, as reflected by higher Apache II scores, and had a higher burden of cancer among them, major differences in mortality remained also after comparing patients within the higher score strata (Table 4).

Figure 4



Intensive care unit admission rates within different score strata for a) modified American Thoracic Society rule; b) pneumonia severity index; c) CURB-65 (confusion; urea >7 mM; respiratory rate ≥ 30 breaths·min^{-1}; blood pressure <90 mmHg systolic or ≤60 mmHg diastolic; aged ≥65 yrs old); and d) Acute Physiologic and Chronic Health Index-II score. ▒: number of patients; ◆: intensive care unit admission.

Figure 5



Case fatality rates within different score strata for a) modified American Thoracic Society rule; b) pneumonia severity index; c) CURB-65 (confusion; urea >7 mM; respiratory rate ≥30 breaths·min^{-1}; blood pressure <90 mmHg systolic or ≤60 mmHg diastolic; aged ≥65 yrs old); and d) Acute Physiologic and Chronic Health Index-II score. ▒: number of patients; ◆: mortality.

Table 4

Case-fatality rates (CFR) in patients treated for severe bacteremic pneumococcal pneumonia in the department of infectious diseases (DID) vs. those treated in other departments (OD).

	PSI IV–V, n (%)		CURB-65 3–5, n (%)		mATS >1minor/≥1 major, n (%)	
	Patients	CFR	Patients	CFR	Patients	CFR
DID	33/88 (38) ***	4/33 (12)**	14/88 (16)#	3/14 (21)¶	18/88 (21)#	4/18 (22)**
OD	20/26 (77) ***	9/20 (45)**	8/26 (30)#	5/8 (63)¶	9/26 (35)#	7/9 (78)**

: DID *versus* OD, p<0.01; *: DID *versus* OD, p<0.001; #: DID *versus* OD, nonsignificant; ¶: DID *versus* OD, p = 0.08

The main objective for a severity score system in pneumonias can be to either detect low risk patients that are not in need of in-hospital care. This would potentially lower the costs for treating pneumonias in general and thus allow allocation of resources to those who need it more. Several recent papers have addressed this issue and concluded that many of the pneumonia patients being admitted to hospitals could probably have been treated as out patients (Riccioni, Di Pietro et al. 2005; Davydov, Ebert et al. 2006).

The second objective for a severity score (which was the focus of this study) would be to detect the patients with a high risk of either having or developing a severe

pneumonia, and thus being at high risk for severe complications and/or a fatal outcome.

In this study we demonstrated that all the tested score systems, initially developed for the assessment of "all cause" community acquired pneumonia, could be used in defining severity in bacteremic pneumococcal pneumonia. The case fatality rates within the different score strata in the present study were however much higher than demonstrated in previous studies on "all cause" pneumonia (Roson, Carratala et al. 2001; Lim, van der Eerden et al. 2003; Ewig, de Roux et al. 2004), and also in comparison to a recent study only including patients with "severe" pneumonia (Espana, Capelastegui et al. 2006). The reason for this could be that invasive pneumococcal disease is more severe than most forms of CAP, as well as that invasive pneumococcal pneumonia often is found among elderly persons with comorbidities, already at risk for a fatal outcome.

The PSI was originally intended to identify patients at a low risk of mortality, but has later been demonstrated to also accurately predict patients at high risk of a severe disease and a fatal outcome (Ewig, Kleinfeld et al. 1999; Roson, Carratala et al. 2001; van der Eerden, de Graaff et al. 2004). A limitation of the PSI is the high impact of age in the risk stratification in this system, leading to possible underestimation of severity of illness among young patients lacking co-morbidities (Ewig, de Roux et al. 2004). In contrast to this PSI was recently shown to accurately identify high-risk pneumonia patients who need aggressive management strategies (Buising, Thursky et al. 2006).

The mATS rule was adopted by the American Thoracic Society in 2001. The primary objective of this score is to identify patients with severe community acquired pneumonia. Several studies evaluating the mATS rules have found it to accurately predict both mortality and need of mechanical ventilation in CAP (Angus, Marrie et al. 2002; Ewig, de Roux et al. 2004; Riley, Aronsky et al. 2004). A possible limitation of the mATS for predicting need of ICU care is however that the major criteria "need of mechanical ventilation or inotropic support" identify patients that are already in the ICU rather than predict those who will need it.

The CURB-65 score, included in the British Thoracic Society guidelines, was in this study advocated as the best score system for predicting a severe outcome in bacteremic pneumococcal pneumonia, due to its performance in terms of sensitivity and specificity, but also because it was very easy to use. The CURB-65 score has been demonstrated to accurately predict both mortality and need of ICU admission in several studies on CAP (Ioachimescu, Ioachimescu et al. 2004; Buising, Thursky et al. 2006; Yan Man, Lee et al. 2007). The CURB-65 score system has the past years been shown to perform very well also after a modification by omitting the Urea component of the system. This modified system called CRB-65 has been shown to perform as accurate as the CURB-65 in predicting mortality and a severe outcome (Bauer, Ewig et al. 2006; Capelastegui, Espana et al. 2006). The CRB-65 was implemented in the Swedish Guidelines for management of community-acquired pneumonia 2007 (SSID 2007)

The difference in mortality depending on which ward the patient was admitted to, infectious diseases (DID) or other departments (OD), is important. Although it is very likely that pneumonia patients generally are treated better in an infectious disease ward, a setting where both the knowledge and interest in these patients is high, it is not likely that this would explain the entire difference in mortality. A number of factors could contribute to the more severe outcome for the subset of patients treated in other departments. One such could be factors associated with the infecting agent, e.g. the serotype or the clonal type of the pneumococcus. We did note a difference in

the serotype distribution among the patients treated in the DID as compared to OD, but the small number of patients treated in OD made this aspect difficult to study, since so many different serotypes were involved. This issue is further discussed in paper III. Another factor, that we unfortunately were unable to study because this data was not recorded, was the timing of the first dose of antibiotics administered. This has been demonstrated to be important for the outcome (Houck, Bratzler et al. 2004), and could potentially differ between different departments. We did demonstrate that patients treated in ODs were more severely ill, but the increased severity could not be demonstrated to be attributed to any individual factor in the statistical analyses. Further, patients within the same severe score-strata (in all the tested score systems) in DID vs. OD also demonstrated a major difference in case fatality rates. This implies that these severity scores may not be optimal tools when used as means to balance severity within and between study populations. The Apache II score although maybe too complicated to use in the emergency room setting, is probably the best score for such balancing of severity.

Summarized, we demonstrated in paper II that all score systems were useful for predicting the need for intensive care unit admission and mortality due to bacteremic pneumococcal pneumonia. The CURB-65 was considered the best system since it was very easy to use. Significant differences in mortality were noted among patients with similar severity scores, emphasizing the importance of thorough clinical assessment in addition to calculating severity scores.

4.3 PAPER III

The aim of paper III was to investigate the correlation of serotypes and clonal types of the pneumococci with disease severity and clinical characteristics of the patients. All patients and clinical isolates from study II as well as number of isolates from a previous study, amounting to 494 patients and clinical isolates were included in this study.
All pneumococcal isolates were serotyped and further characterized with molecular typing methods. Three different molecular typing methods were used, PFGE, Box-fingerprinting and MLST. The Box fingerprinting and PFGE has been shown to have approximately the same discriminatory power (Hermans 1995). A total of 173 isolates were analysed with Box-fingerprinting, 247 isolates with PFGE and 105 isolates with MLST.
The different typing methods were able to characterise isolates into clonal groups (clones), both within and between the different serotypes, meaning that several clones could be found within a serotype, but also that isolates within a clone could have different serotype affiliations. The serotypes and clones were further stratified into groups according to their ability to cause invasive disease. This method was adopted from Brueggemann et al. (Brueggemann, Griffiths et al. 2003), enabling the calculation of odds ratios (OR) for different serotypes and clones. An OR >1 indicates high invasive disease potential, while OR < 1 indicates low invasive disease potential and OR~1 indicates medium invasive disease potential.

Clinical parameters and Apache II scores were recorded for all patients, and then analysed together with the results of the molecular typing methods.
We found that serotypes 1 and 7F (regarded as having a high invasive disease potential) was mostly seen among younger patients without co-morbidities, and although being among the 7 most prevalent serotypes in the material, they did not

cause any severe disease, reflected by low APACHE II scores, and was not associated with any fatal cases (Table 5). In contrast, serotypes 3, 6A, 6B, 11A and 19F (regarded to have a low invasive disease potential) were mostly seen among older patients with underlying diseases. These serotypes also seemed to be correlated to severe disease, since a majority of patients with these serotypes had high APACHE II scores, and mortality rates of 20-33% (Tables 5 and 6). That the risk of severe pneumococcal disease was correlated to serotype / clonal type and not only to patient factors, was demonstrated by serotypes with low invasive disease potential giving high APACHE II scores and a high mortality rate also among younger patients without co-morbidities.

Table 5

Serotypes, case-fatality rates, and underlying disease among patients with invasive pneumococcal disease, by invasive disease potential.

Invasive disease potential	Serotypes	Proportion (%) of cases with fatality	Proportion (%) of patients with underlying disease
High	1, 7F	0/63 (0)	27/63 (43)
Medium	4, 9V, 14, 18C	16/167 (10)	111/163 (68)
Low	3, 6A, 6B, 8, 19F, 23F	31/146 (19)	106/142 (75)
Total	…	47/376 (13)	244/368 (66)

NOTE. Invasive disease potential was determined according to the findings of Brueggeman et al. All data were not available for all patients.

We therefore concluded that serotypes with a high invasive disease potential behave as primary pathogens, while serotypes with low invasive disease potential, primarily seem to cause disease in older people with co-morbidities, and can thus be regarded as opportunistic pathogens.

Further, we found that serotypes with a high invasive disease potential were highly genetically related, whereas serotypes with low invasive disease potential were more genetically diverse (Table 6). Only two different clones of serotype 1 were detected, and these two clones belonged to the same clonal cluster (only differing by one allele), according to MLST. The biggest clone in the study (54 isolates), STO 9V-A, included isolates from 4 different serotypes (9V, 14, 19F and 24A). MLST analysis of this clone suggested that all isolates belonged to the same clonal cluster.

Table 6

Major pneumococcal clones found by means of molecular typing and their association with clinical parameters.

Sequence type	Clone	Serotype	No. of isolates	Isolates obtained from patients with underlying disease, %	Percentage of patients, by APACHE II score			Case fatality rate
					<12	12–24	>24	
306	STO 1-A	1	17	24	82	18	0	0
227	STO 1-B	1	8	38	63	38	0	0
180	STO 3-A	3	21	67	33	67	0	43
260	STO 3-B	3	9	67	33	44	22	22
232	STO 3-C	3	3	33	67	33	0	0
205	STO 4-A	4	24	38	67	33	0	8
247	STO 4-B	4	4	75	75	25	0	25
1378	STO 6A-A	6A	4	100	25	50	25	25
138	STO 6B-A	6B	7	57	14	86	0	29
176	STO 6B-B	6B	6	83	17	83	0	0
191	STO 7F-A	7F	34	53	74	24	3	0
53	STO 8-A	8	10	70	50	50	0	10
156/162/838/1184	STO 9V-A	9V/14/19F/24A	54	70	56a	42a	2a	11
62	STO 11A-A	11A	12	100	8	83	8	25
124	STO 14-A	14	41	76	37	49	15	15
13	STO 14-B	14	4	75	50	50	0	0
9	STO 14-C	14	5	60	50b	50b	0	0
36	STO 23F-A	23F	8	75	50	50	0	13
977	STO 23F-B	23F	5	100	0	100	0	0
440	STO 23F-C	23F	5	100c	40	60	0	0
439	STO 23F-D	23F	3	50d	67	33	0	33
33	STO 23F-E	23F	3	100	67	33	0	0
81	STO 23F-F	23F	5	80	40	60	0	20
37	STO 23F-G	23F	3	100	100	0	0	33

NOTE. Only clones with >2 isolates are included.

aData are based on 52 of 54 isolates. bData are based on 4 of 5 isolates. cCalculated on the basis of 4 of 5 isolates. dCalculated on the basis of 2 of 3 isolates.

One of the things that we wanted to investigate with this study was whether capsular types, clonal types or both contributed the most to a severe outcome in invasive pneumococcal disease. Regrettably, due to the limited number of patients infected with a given clonal type, we were unable to fully answer that question.

That certain serotypes contribute to a severe outcome in IPD has been demonstrated in a number of previous studies (Mirzanejad, Roman et al. 1996; Henriques, Kalin et al. 2000; Martens 2004), where serotypes 3 and 5 have been mostly associated with fatal cases. A major difficulty in most studies addressing this issue has however been to

avoid the impact of host factors also contributing to a fatal outcome. In the study by Henriques at al. (Henriques, Kalin et al. 2000), higher case fatality rates were found to be attributed to serotype 3, suggesting that this was the reason for the relatively low mortality in IPD seen in Sweden (where serotype 3 was rarely found), as compared to other countries. In contrast, Alanee et al. (Alanee, McGee et al. 2007) found no association between pneumococcal serotypes and severity of disease or mortality in IPD and concluded that host factors were probably more important determinants of severity and mortality in IPD than bacterial factors. The results of the present study, however, strongly support that bacterial factors are of major importance to the outcome of IPD. The most important argument in favour of this was our finding that also previously healthy individuals became severely ill and had a high risk of a fatal outcome from certain pneumococcal serotypes.

Although some studies have demonstrated that pneumococcal serotype switching can turn low virulent isolates into highly virulent strains (Kadioglu, Taylor et al. 2002), there is mounting evidence that other genetic factors including clonal affiliation contribute to the pneumococcus ability to cause severe disease (Nabenzahl 2004) (Sandgren, Sjostrom et al. 2004; Hanage, Kaijalainen et al. 2005; Leimkugel, AdamsForgor et al. 2005). In this study we could demonstrate that there were differences among clones within a given serotype with respect to their ability to cause severe disease and disease among patients with comorbidities, although the number of isolates within a given clone was small.

Another recent advance in the understanding of factors of importance to pneumococcal virulence is the description of the pathogenicity islet rlrA, coding for a pilus-like structure (Barocchi 2006). The pneumococcal pilus has been demonstrated to be involved in adhesion, colonisation and invasion in animal models of infection, as well as being important for virulence by giving rise to a strong immune response. In line with the findings of the present study, that some serotypes / clonal types seem to induce relatively mild invasive disease, clones of serotype 1 and 7F seem to lack the ability to form such a pilus, due to the absence of the rlrA islet.

The rank order of pneumococcal serotypes found among invasive isolates in children in the US and Canada was the determinant factor of which serotypes to be included in the PCV. The present 7-valent PCV does not include highly invasive serotypes like 1 and 7F. According to the findings of the present study, the lack of these serotypes in the vaccine would however not have a major impact on mortality from IPD among adults, since very few deaths were associated with these serotypes. In contrast, a study from Ghana (Leimkugel, AdamsForgor et al. 2005) demonstrated that only 6% of the pneumococcal meningitis cases among children would be covered by the PCV7, the majority of cases being caused by a serotype 1 clone. The serotype 1 clone described in Ghana, was not at all related to any of the serotype 1 clones described in our material, which further demonstrates the differences in ability to cause severe disease among different clones.

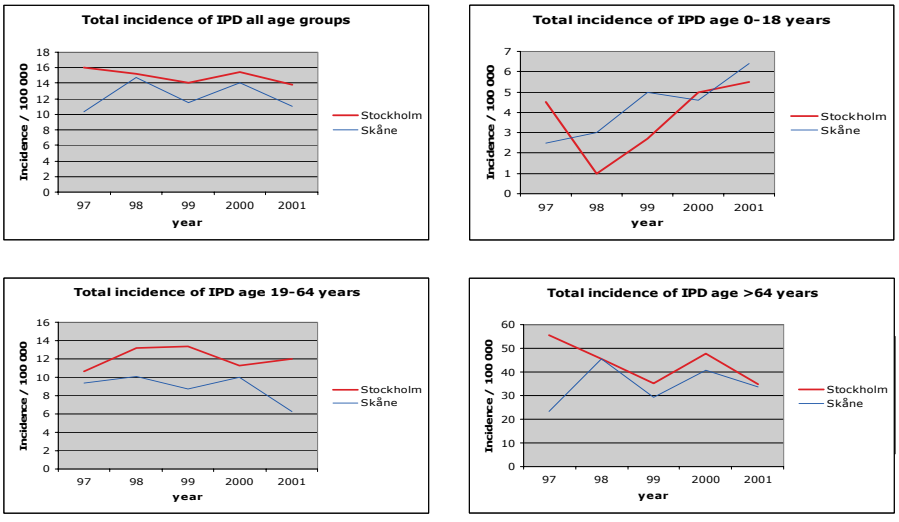
Further, in view of the high capability of pneumococci to exchange genetic material and capsular type, it is possible that opportunistic serotypes and clones not included in the vaccine might become much more prevalent both in carriage and in IPD following vaccination.

4.4 PAPER IV

The aim of paper IV was to investigate the effects of a large-scale vaccination campaign with pneumococcal polysaccharide vaccine (PPV) directed towards all persons ≥ 65 years of age, in terms of incidence of IPD in this age group and other age groups, as well as to investigate the effects on serotype distribution.
All cases of invasive pneumococcal disease in Stockholm County and Skåne County, Sweden, during 1997-2001 were included in the study. Stockholm county was the first county in Sweden to launch a vaccination campaign (October 1998), offering PPV at a reduced cost, together with the influenza vaccine, for all inhabitants >65 years of age. No similar vaccination campaign with PPV was launched in Skåne County during the study period. The vaccine coverage among the elderly in Stockholm was 36% at the end of the study period. A corresponding figure for the vaccination coverage in Skåne could not be obtained, but was estimated to be at least 4-5 times lower, according to regional sales- and distribution statistics for the vaccine during the period.

Of the 1887 included patients with IPD, 1325 came from Stockholm and 562 from Skåne. In Stockholm, the total incidence of IPD was 15.8/100.000 inhabitants in 1997, one year before the introduction of the campaign, and 13.8/100.000 at the end of the study period, in 2001 (Fig 6a). No significant changes in the incidence of IPD occurred in the non-elderly populations during the study period, whereas the incidence in the vaccinated age group of 65+ declined from 55.3/100.000, in 1997, to 34.7/100.000, in 2001 (Fig 6 b-d). When we compared the incidence of IPD between 1997-1998 and 2000-2001, there was an approximately 20% decline of IPD in the 65+ age group between these two time points corresponding to a lowered incidence of 9.3/100.000 inhabitants (95% CI: 0.66 to 17.11; p= 0.034).
No statistically significant trends in terms of decline or increase in incidence in any of the age groups could be seen in the Skåne material.

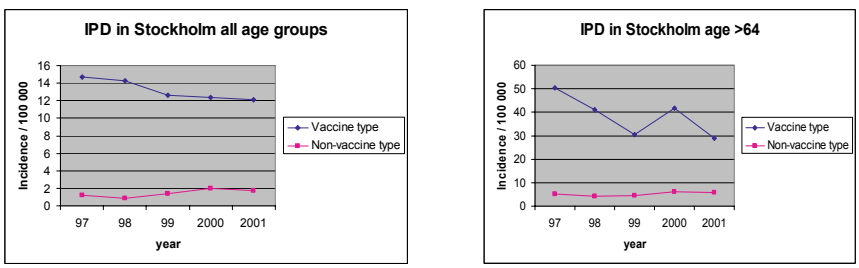
Figure 6
Incidence / 100 000 inhabitants of Invasive Pneumococcal Disease in Stockholm County and Skåne during 1997-2001, concerning (a) all age groups, (b) age 0-18 years, (c) age 19-64 years, (d) age ≥ 65 years



The serotype distribution stratified for age could only be assessed for the Stockholm area (Figure 7). Among persons ≥ 65 years of age, a reduction of vaccine type IPD, from 50.3 to 28.9/100 000 occurred between 1997 and 2001. This corresponded to a yearly decline of 17% (OR 0.814, 95% CI 0.721-0.919) of the risk for infection with a vaccine type strain. The incidence of non-vaccine type IPD was stable throughout the study period in this age group. No statistically significant trends in terms of vaccine type IPD or non-vaccine type IPD was seen in any of the other age groups.

Figure 7

Incidence / 100 000 inhabitants of Vaccine type and non-vaccine type IPD in Stockholm County during 1997-2001, concerning (a) all age groups, (b) ≥ 65 years



The main finding of this study was that a large scale vaccination program with PPV among the elderly in Stockholm County led to a substantial decrease in the total incidence of IPD in this age group, while the incidence remained unchanged among non-vaccinated age-groups in Stockholm and in Skåne where vaccination was rarely performed. The decline of IPD among the elderly in Stockholm was due to a significant decline in the proportion of vaccine serotypes and was of such a magnitude that a tendency to a lowered incidence of IPD in the whole population was observed.

To our knowledge, this is the only study to have investigated the impact on incidence of IPD after a vaccination campaign with PPV. This makes it difficult to relate our findings to other studies. Since the incidence of IPD is known to naturally fluctuate over time, it would have been appealing to study the yearly incidence data over a longer time period than the 5 years presented in this study. Unfortunately we could not obtain data on IPD until 1997, since pneumococcal blood isolates were not reported by the microbiology departments before that year. In order to compensate for this possible bias, we compared the two first years, 1997-1998, with the two last years, 2000-2001, of the study period. The result of this comparison between those two time-periods demonstrated that the incidence of all IPD among the elderly declined by nearly 20%. In a study of the hypothetical effect of PPV on the incidence of IPD in the 65+ age group in the U.S. it was calculated that PPV would reduce the incidence of IPD by 10.6% (sensitivity analysis 6.7%-12%) at a vaccination coverage level of 50% (Fry, Vaccine 2002). The larger decline of 20% seen in our study, however, corresponds well to a 36% vaccination coverage in a previously vaccine "naïve" population with a 50% effectiveness of PPV.

The lack of significant changes in non-vaccinated age-groups, together with the decline in the incidence of IPD among the elderly after the introduction of PPV in Stockholm, suggest that PPV does not give rise to indirect effects, i.e. herd immunity. This was not unexpected, since it is generally accepted, based on old studies with the 8- and 14-valent vaccines that the polysaccharide vaccines do not affect nasopharyngeal carriage (Herva, Luotonen et al. 1980; Wright, Sell et al. 1981).
Several studies have shown a lowered incidence of IPD in the elderly after the impementation of PCV7 in children in the U.S. As discussed before, the mechanism through which this is achieved is herd immunity, which is the result of a reduction of the circulating pool of pneumococci in society. The effect by PCV also on pneumococcal carriage in children is essential for the origin of herd immunity. PCV7 was introduced in Sweden in the end of 2001, but since almost no doses were distributed during that year it is highly unlikely that PCV7 could have influenced the results of the present study.

Although the decrease in IPD, also among the elderly, after the introduction of PCV7 in the U.S. has been impressive, recent studies on serotype replacement indicate that caution is warranted, and that PPV still has an important role in the prevention of pneumococcal disease.
A number of non-vaccine types have been seen to increase more than others (i.e. 19A, 33F and 3) (Hicks, Harrison et al. 2007). The expansion of serotype 19A is especially worrisome since this serotype has been found to be associated with several antibiotic nonsusceptible clones (Pelton 2007). Since serotype 19A is very common among carriage, it is not surprising that reports on clones previously covered by the PCV7, have been demonstrated to acquire the 19A capsular type through capsular switch (Beall, McEllistrem et al. 2006). Serotype 19A is now the predominant serotype in IPD among children, and the second most dominant serotype among adults in the US.
These recent findings further strengthens the argument that we shall continue to vaccinate the elderly with PPV

5 THE MAJOR FINDINGS

- The novel RQ-PCR assay described in paper I, significantly increased the diagnostic yield of *S. pneumoniae, H. influenzae and M. catarrhalis* in lower respiratory tract samples compared to traditional culture.

- RQ-PCR thus increases the possibility of obtaining a rapid etiologic diagnosis in lower respiratory tract infections, enabling fast guidance for therapeutic options, also in patients already taking antibiotics.

- Severity scores originally developed for the assessment of severity of "all cause" community acquired pneumonia can be used also for predicting mortality and need for ICU admission in bacteremic pneumococcal pneumonia. The CURB-65 (or CRB-65) seems to be the most suitable score system, since it is the one that is most easy to use.

- Caution is warranted when using severity score systems for predicting mortality and severe disease in bacteremic pneumococcal pneumonia, since mortality rates may differ substantially among patients also within severity score strata. The result of a severity score must therefore always be accompanied with a thorough clinical investigation.

- Both capsular type and serotype seem to be involved in the potential of the pneumococcus to cause severe disease.

- Some pneumococcal clones and serotypes behave as "primary" pathogens, primarily infecting previously healthy persons, and do not lead to a severe disease. Other pneumococcal serotypes and clones have a more "opportunistic" behaviour, primarily infecting older people with comorbidities, and tend to cause a more severe disease with a high risk of a fatal outcome.

- The large scale vaccination campaign with PPV-23 directed to the elderly people in Stockholm, led to a substantial decrease in the incidence of IPD in this age group, while no changes in the incidence of IPD with respect to other age groups were seen.

- The decline of IPD among the elderly was of such a magnitude that a tendency to a lowered incidence of IPD in the whole population was observed. We found that this lowered incidence was due to a significant decline in the proportion of vaccine serotypes causing IPD in the 65+ age group.

- No serotype replacement with non-vaccine serotypes was noted after the vaccination campaign with PPV-23.

6 CONCLUDING REMARKS

The work presented in this thesis is intended to contribute to the knowledge of diagnosis, prognosis and prevention of pneumococcal disease. Despite the impressive amount of research on pneumococci and pneumococcal disease having been conducted during the past 100 years, pneumococcal disease still constitutes a major burden of morbidity and mortality in the world.

Efforts to both identify patients at risk of severe pneumococcal disease and the subsequent optimised treatment of them can and should be improved. An initial step in this would be to create better methods for identifying the etiological agent in patients with pneumonia. In view of the increasing figures on decreased antibiotic susceptibility for many respiratory tract pathogens, guided antibiotic therapy will probably become increasingly important in the near future. A major obstacle in guided antibiotic therapy is the difficulties in obtaining a good respiratory tract specimen. New PCR methods on blood are currently under investigation, and would of course be an appealing alternative if they could be demonstrated to have good sensitivity. Other means of increasing the detection of respiratory tract pathogens possibly include bronchoscopy on wider indications.

In order to identify patients at high risk for a severe outcome in CAP, a number of different severity scores addressing both disease specific conditions and generic conditions have been investigated, the latter being used predominantly in ICU settings. The vast number of different suggested scores implies that none of these are perfect in identifying patients at high risk for a severe outcome. Also, since conditions of care differ substantially between different sites, the choice of severity score should therefore be based on the local health care setting. The most important issue concerning severity scores is of course if they lead to improved outcomes, an issue which has not been convincingly determined, and thus needs to be studied more in detail.

The reduction of pneumococcal disease seen after the large-scale implementation of PPV in Stockholm indirectly confirms the effectiveness of the vaccine against IPD. Even more impressive is the decline of incidence of IPD seen in many age groups in the U.S. after the introduction of PCV7 in the children vaccination programme. However, as discussed previously, a continuing surveillance of pneumococcal isolates including clonal affiliations and serotypes is highly warranted. Large-scale vaccinations against bacteria that constitute a natural colonizing flora can be seen as a vast ecological experiment. Although serotype distribution always have been changing with time, the low, but steadily increasing figures on serotype shifting being demonstrated within just a couple of years after the introduction of PCV7 are worrisome. In view of the data presented in paper III in this thesis, such serotype shift, also driven by antibiotic pressure, could possibly lead to an increase of more virulent and antibiotic resistant serotypes and clones in society. Future vaccine candidates such as vaccines against pilated pneumococci would provide a better coverage of virulent pneumococcal strains, but as there are more than 90 known serotypes of pneumococci, serotypes of which we have limited knowledge since they are rarely seen today, could possibly increase and occupy this vacant niche. Further, other types of bacteria that are now outcompeted by pneumococci in the nasopharyngeal cavity could also potentially become more important as pathogens.

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