AETIOLOGY IN COMMUNITY-ACQUIRED PNEUMONIA

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ABSTRACT

Background: Although community-acquired pneumonia (CAP) is a common and well-known disease, its microbial aetiology is still not well characterized. During the past few years nucleic acid detection using real-time polymerase chain reaction (PCR) has been developed for detection of many bacterial and viral pathogens causing respiratory tract infections.

Objectives: 1) to estimate the accuracy of the quantitative real-time PCR (RQ-PCR) method for identifying pneumococci in sputum; 2) to determine the aetiology of CAP by implementing new diagnostic PCR techniques combined with conventional methods; 3) to compare CAP patients with a pure bacterial aetiology with those with both bacterial and viral findings regarding severity of illness and length of hospital stay; 4) to study the inflammatory response, especially procalcitonin (PCT) levels, in patients with CAP and the correlation to different respiratory pathogens.

Material and methods: Adults admitted to Karolinska University Hospital were studied during a 12-month period. All patients were tested with an extensive panel of conventional methods and in addition sputum samples were analysed with RQ-PCR for Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis; and nasopharyngeal specimens were analysed with real-time PCR for viruses common in the airways. Serum samples were collected within 24 hours of admission for subsequent measurement of PCT, C-reactive protein, transthyretin and interleukin-6. The pneumonia severity index (PSI) was used to assess the severity of illness.

Results: In sputum samples, culture was significantly positive in 19/128 (15%), whereas a significant concentration of DNA was found with RQ-PCR in 34/127 (27%) cases ($p<0.001$). Seventeen of the 34 RQ-PCR–positive sputum samples were negative by sputum culture, of which 14 were from patients treated with antibiotics prior to sampling. A microbial aetiology was found in 67% of all patients ($n=124$). The most frequently detected pathogens were S. pneumoniae (70 patients [38%]) and respiratory virus (53 patients [29%]). Multiple pathogens were present in 43 (35%) of those with a determined aetiology. The likelihood of getting a score corresponding to PSI classes IV or V was higher in patients with combined bacterial-viral findings than in those with a bacterial pathogen alone (odds ratio 4.98, 95% confidence interval 2.09 – 11.89; $p<0.001$). The median length of hospital stay was seven days among patients with mixed infections and four days among those with a bacterial aetiology alone ($p=0.018$). Median serum concentrations of PCT were higher in patients with bacteraemia than in those without bacteraemia (6.11 µg/L vs. 0.34 µg/L, $P=0.0002$), in those with non-bacteraemic pneumococcal aetiology than in those infected with other classical bacteria (1.18 vs. 0.18, $P=0.038$), in patients with pneumococcal as compared to viral aetiology (2.43 vs. 0.24, $P=0.017$), and in patients with PSI classes 4-5 (2.07) than in those with PSI classes 1-3 (0.52, $P=0.03$).

Conclusions: The sensitivity of sputum RQ-PCR was higher than that of sputum culture, especially after antibiotic therapy had been initiated. By supplementing traditional diagnostic methods with new PCR-based methods, a high microbial yield was achieved. Mixed bacterial-viral infections were frequent and these patients developed severe CAP more often and stayed longer in hospital than those with a bacterial aetiology alone. High PCT seems to be a good marker of invasive as well as severe disease and of pneumococcal aetiology, but for localised bacterial infections caused by other pathogens the test is less sensitive.
LIST OF PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their roman numerals:

Quantitative detection of Streptococcus pneumoniae from sputum samples with real-time quantitative polymerase chain reaction for etiologic diagnosis of community-acquired pneumonia. 

Etiology of community-acquired pneumonia: Increased microbiological yield with new diagnostic methods. 

III. Niclas Johansson, Mats Kalin and Jonas Hedlund. 
Clinical impact of combined bacterial and viral infection in patients with community-acquired pneumonia. 

Procalcitonin levels in community-acquired pneumonia – correlation to aetiology and severity. 
Manuscript
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CAP</td>
<td>Community-acquired pneumonia</td>
</tr>
<tr>
<td>CF</td>
<td>Complement fixation</td>
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<tr>
<td>CFU</td>
<td>Colony-forming units</td>
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<tr>
<td>CIE</td>
<td>Countercurrent-immunoelectrophoresis</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>EIA</td>
<td>Enzyme-immunoassay</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Emergency room</td>
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<tr>
<td>HA</td>
<td>Haemagglutinin</td>
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<tr>
<td>HBoV</td>
<td>Human bocavirus</td>
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<tr>
<td>HCoV</td>
<td>Human coronavirus</td>
</tr>
<tr>
<td>HMPV</td>
<td>Human metapneumovirus</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipoooligosaccharide</td>
</tr>
<tr>
<td>LRTI</td>
<td>Lower respiratory tract infection</td>
</tr>
<tr>
<td>MIF</td>
<td>Microimmunofluorescence</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NAAT</td>
<td>Nucleic acid amplification test</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NPH</td>
<td>Nasopharynx</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>Procalcitonin</td>
</tr>
<tr>
<td>Ply</td>
<td>Pneumolysin</td>
</tr>
<tr>
<td>PSB</td>
<td>Protected specimen brush</td>
</tr>
<tr>
<td>PSI</td>
<td>Pneumonia severity index</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
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</table>
1 BACKGROUND

1.1 HISTORY AND INCIDENCE OF COMMUNITY-ACQUIRED PNEUMONIA

The condition community-acquired pneumonia (CAP) was recognised already in ancient times and has since been described throughout history. In 1830 Laennec was the first to describe the pathological changes of pneumonia. In the late nineteenth century the connection was made between microbes and the development of pneumonia. Friedlander found in 1882/1883 a causal association between bacteria and pneumonia, bacterial pathogens being detected in nearly all of 50 CAP patients studied (Woodhead 2002).

Pneumonia is a common disease throughout the world. The World Health Organisation (WHO) has estimated that about 450 million cases occur every year (Ruuskanen et al. 2011). Prospective population studies have reported an annual CAP incidence of 0.5-1% (Woodhead et al. 1987; Jokinen et al. 1993). The incidence is highest among the very young and the elderly. Of all persons in the western world developing CAP, 22-42% require hospitalisation, and this incidence increases with age (Lim et al. 2009).

1.2 PROGNOSIS OF COMMUNITY-ACQUIRED PNEUMONIA

During the pre-antibiotic era the case fatality rate of CAP was estimated to be between 30% and 40% (Podolsky 2006). Since the introduction of antimicrobial therapy the mortality has decreased, but is still substantial. In a meta-analysis the overall mortality rate among adults was estimated to 13.7% (Fine et al. 1996). However, the rate varies from different clinical settings. The mortality among adults managed in the community is reportedly below 1%, compared to hospitalised adults (4%-14%) and patients admitted to intensive care (>50%) (Lim et al. 2009). The long-term mortality among CAP patients is also higher than in controls (Lim et al. 2009). In Sweden the mortality rate among CAP patients treated at departments of infectious disease is 3.5-6% (Spindler et al. 2012).

The outcome of an acute lower respiratory tract infection depends on the virulence of the organism and the inflammatory response in the lung (Mizgerd 2008). Several pathogens have been linked to the development of severe CAP, such as *Streptococcus pneumoniae*, *Legionella pneumophila*, *Staphylococcus aureus* and Gram negative bacteria (Fine et al. 1996; Rello et al. 2003; Paganin et al. 2004). A number of host factors have also been identified as correlating to poor prognosis, such as age, residence in nursing home, and underlying diseases (File 2003; Mandell et al. 2007; Lim et al. 2009). Univariate studies have suggested that more than 40 different parameters are associated with increased CAP mortality (Lim et al. 2009).
1.2.1.1 Prediction models for assessing severity

No single risk factor is sensitive or specific enough to predict poor outcome. Various scoring systems have therefore been developed to help the clinician to identify patients at risk of poor prognosis at an early stage. The most studied predictive models are the pneumonia severity index (PSI) and the CURB-65 score. The PSI is a comprehensive prediction rule, developed to estimate the mortality risk within 30 days of admission (Fine et al. 1997). Patients are classified into five risk classes. In classes I-III the mortality rate is expected to be less than 1%, in class IV almost 10% and in class V almost 30%.

The CURB-65 score also measures the 30-day mortality risk. It includes five easily measurable parameters (Lim et al. 2000). A simplified version of CURB-65 where only clinical parameters are included (CRB-65) has also been evaluated regarding the 30-day mortality risk (Lim et al. 2009). PSI as well as CURB-65 and CRB-65 scores have been extensively validated and their use gives high prognostic accuracy (Mandell et al. 2007; Aujesky et al. 2008; Lim et al. 2009).

1.3 AETIOLOGY OF COMMUNITY-ACQUIRED PNEUMONIA

A variety of microbes are responsible for the development of CAP. Initial antibiotic treatment is, however, empirical. Therefore knowledge of pathogens causing CAP constitutes the basis for selecting empirical antimicrobial treatment, which has a significant impact on prognosis (File 2003). Many aetiological studies have been performed. Most performed before 1939 concerned lobar pneumonia caused by classical bacteria (Humphrey et al. 1948), and before the introduction of antimicrobial therapy *S. pneumoniae* was estimated to be the causative agent in more than 95% of this kind of pneumonia (Macfarlane et al. 1982). Around World War II when antibiotic treatment started to be available, more attention was paid in the literature to other pathogens, besides classical bacteria, that were also capable of causing CAP. Initially these were categorised as “viral pneumonia” or “atypical pneumonia” (Humphrey et al. 1948). The interest in these kinds of pneumonia was further increased with the discoveries of new respiratory pathogens such as *Mycoplasma pneumoniae* (Mufson et al. 1967), Legionella spp. (White et al. 1981; Macfarlane et al. 1982), and *Chlamydophila pneumoniae* (Fang et al. 1990; Lieberman et al. 1996).

Other factors have also influenced the concern for CAP aetiology. In the late-twentieth century the proportion of elderly people and those living in nursing homes had gradually increased among patients hospitalised with CAP, arousing suspicion that the incidence of pathogens such *S. aureus* and Gram negative enteric bacilli would increase among hospitalised CAP patients (Lim et al. 2001).

The pathogen spectra differ in some ways in all aetiological studies. The many reasons for this include health-care delivery, population factors (age, co-morbidity etc), study factors (use of different aetiological methods, number of samples obtained etc), and whether the study was performed in hospital or elsewhere (File 2003; Lim et al. 2009). There are also regional differences in the pathogen spectra of CAP. For instance Legionella spp. are found more frequently in countries bordering the Mediterranean Sea (Marrie 1998) and *Coxiella burnetii* have been commonly detected in countries such as Spain and Canada (Uzun et al. 1994; Jang et al. 1995).
The British Thoracic Society has in guidelines from 2001 put together the results from aetiological CAP studies performed in different parts of the world (Table 1) (BTS 2001). Similar collections of pathogens were found in most of these studies and collectively, \textit{S pneumoniae} was the most frequently isolated organism.

Table 1.

<table>
<thead>
<tr>
<th></th>
<th>United Kingdom (5 studies n=1137) Mean (%)</th>
<th>Rest of Europe (23 studies n=6026) Mean (%)</th>
<th>Australia &amp; New Zealand (3 studies n=453) Mean (%)</th>
<th>North America (4 studies, n=1306) Mean (%)</th>
</tr>
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<tbody>
<tr>
<td>\textit{S pneumoniae}</td>
<td>39</td>
<td>19.4</td>
<td>38.4</td>
<td>11.3</td>
</tr>
<tr>
<td>\textit{H influenzae}</td>
<td>5.2</td>
<td>3.9</td>
<td>9.5</td>
<td>6.3</td>
</tr>
<tr>
<td>\textit{Legionella}</td>
<td>3.6</td>
<td>5.1</td>
<td>7.5</td>
<td>4.8</td>
</tr>
<tr>
<td>\textit{M catarrhalis}</td>
<td>1.9</td>
<td>1.2</td>
<td>3.1</td>
<td>1.2</td>
</tr>
<tr>
<td>\textit{S aureus}</td>
<td>1.9</td>
<td>0.8</td>
<td>2.9</td>
<td>3.8</td>
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<tr>
<td>Gram negative enteric bacilli</td>
<td>1</td>
<td>3.3</td>
<td>4.6</td>
<td>5.3</td>
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<tr>
<td>\textit{M pneumoniae}</td>
<td>10.8</td>
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<td>14.6</td>
<td>4.1</td>
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<td>\textit{C pneumoniae}</td>
<td>13.1</td>
<td>6.3</td>
<td>3.1</td>
<td>5.9</td>
</tr>
<tr>
<td>\textit{C pittaci}</td>
<td>2.6</td>
<td>1.4</td>
<td>1.4</td>
<td>0.1</td>
</tr>
<tr>
<td>\textit{C burnetti}</td>
<td>1.2</td>
<td>0.9</td>
<td>0</td>
<td>2.3</td>
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<tr>
<td>All viruses</td>
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<td>9.5</td>
<td>10.6</td>
<td>8.9</td>
</tr>
<tr>
<td>\textit{Influenza A and B}</td>
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<td>5.3</td>
<td>6.4</td>
<td>5.9</td>
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<tr>
<td>Mixed</td>
<td>14.2</td>
<td>6.3</td>
<td>19.6</td>
<td>8.5</td>
</tr>
<tr>
<td>Other pathogens</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>8</td>
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<tr>
<td>Unknown</td>
<td>30.8</td>
<td>50.7</td>
<td>31.6</td>
<td>40.7</td>
</tr>
</tbody>
</table>


Among hospitalised CAP patients in Sweden \textit{S. pneumoniae} has also been the dominant finding, followed by \textit{H. influenzae}, \textit{M. pneumoniae} and different respiratory viruses (Fransen et al. 1969; Kalin et al. 1983; Berntsson et al. 1985; Holmberg 1987; Ortqvist et al. 1990; Burman et al. 1991; Stralin et al. 2010).

The majority of aetiological studies concern hospitalised patients. In the investigations of CAP aetiology in outpatients, about the same collection of pathogens has been found as among inpatients (Harrison et al. 1987; Woodhead et al. 1987; Almirall et al. 2000; Lagerstrom et al. 2003) although the incidence of atypical agents in some of these studies is reportedly high (Blanquer et al. 1991; Marrie et al. 1996).

Most aetiological CAP studies are well designed. Nevertheless the aetiology of unknown origin in these investigations is, with a few exceptions (Macfarlane et al. 1982; Lehtomaki et al. 1988; Lieberman et al. 1996; Lim et al. 2001), as high as 30-50%.
Bacterial pathogens in respiratory tract infections

1.3.1 Streptococcus pneumoniae

*Streptococcus pneumoniae* was first described in 1886 by Louis Pasteur and George Sternberg. It is a Gram-positive diplococcus. It is α-haemolytic on sheep blood agar and is distinguished from other streptococci by its sensitivity to optochin (Watson et al. 1995). The pneumococcal cell wall is composed of peptidoglycan and teichoic acid. The cell wall has an inner and an outer layer of C-polysaccharide, a ribitol teichoic acid containing phosphorylcholine and galactosamine. The C-polysaccharide is specific for *S. pneumoniae*. The cell wall is surrounded by a polysaccharine capsule and contains several different proteins (Ellis et al. 1998).

*S. pneumoniae* was already in the late nineteenth century known to be the most common pathogen causing lower respiratory tract infections (Watson et al. 1995), and has remained the leading cause of CAP. In the developed countries pneumococcal CAP occurs in all age groups but is most common in the elderly. This agent is also responsible for a substantial morbidity and mortality, and invasive disease takes place in about 20% of all cases (Kadioglu et al. 2008). A number of risk factors have been associated with pneumococcal disease, such as alcohol abuse, recent influenza infection, lung disease, congestive heart failure, immunodeficiency (diabetes mellitus, haematological malignancies, asplenia, organ transplant recipients, HIV) (Watson et al. 1995). occur

1.3.1.1 Pathogenesis

All pneumococcal disease begins with the establishment of colonisation of the naso-oropharynx (Kadioglu et al. 2008). Colonisation is most common in early childhood. The overall colonisation rate of healthy children is estimated to 20-40% and over 60% in infants and day-care children (McCullers et al. 2001). Colonisation in healthy adults is rather unusual, with a rate of <10%. The ability of the pneumococcus to adhere and to colonise is crucial in the pathogenesis of pneumonia. If no lower respiratory tract infection (LRTI) develops, the colonising bacteria are cleared from the upper airways; but this procedure can take weeks for adults and months for children (van der Poll et al. 2009).

Following adherence and colonisation, *S. pneumoniae* will occasionally cause LRTI. For the development of pneumonia the pneumococci will gain access to the lower respiratory tract through micro-aspiration. The bacteria will then adhere to the respiratory epithelial cells and in a series of events induce an inflammatory response. To enable these events the pneumococcus possesses different virulence factors.
1.3.1.2 Virulence factors

1.3.1.2.1 The Capsule
The polysaccharide capsule possessed by all strains of \textit{S. pneumoniae} is probably the most important virulence factor for the pneumococcus (Mitchell et al. 2010). Currently more than 90 serotypes have been identified (Lynch et al. 2008). The expression of the capsule reduces entrapment in the mucus, allowing the pneumococcus access to the epithelial surface (Kadioglu et al. 2008). It also prevents phagocytosis (van der Poll et al. 2009).

1.3.1.2.2 The cell wall
The pneumococcus undergoes autolysis by the enzyme autolysin. It has been speculated that this is a mechanism for the bacteria to release and exchange genetic material. When the autolysin breaks down the cell wall, strongly proinflammatory fragments are produced (Watson et al. 1995).

1.3.1.2.3 Proteins involved in virulence
Choline-binding proteins are a family of surface proteins bound to the choline component of the cell-wall lipoteichoic acid (McCullers et al. 2001). \textit{S. pneumoniae} encodes 10-15 such proteins including PspA and autolysin (Kadioglu et al. 2008). The function of PspA appears to be protection against host complement system (Jedrzejas 2001). The major autolysin is LytA. Its main function is cell-wall degradation, which finally leads to cell lysis and cell death (Jedrzejas 2001; Kadioglu et al. 2008; Mitchell et al. 2010).

Pneumolysin (Ply) is a protein located in the cytoplasm. It belongs to a family of cholesterol-dependent, pore-forming toxins produced by more than 20 species of Gram-positive bacteria, and is found in virtually all pneumococcal isolates (Kadioglu et al. 2008; Mitchell et al. 2010). Ply has multiple virulence functions. The toxin has cytotoxic effects on ciliated bronchial epithelial cells (Mitchell et al. 2010) and slows ciliary beating (Jedrzejas 2001; van der Poll et al. 2009). It activates the classical complement pathway despite the absence of specific antibodies, reducing opsonic activity against the pneumococcus (McCullers et al. 2001; Mitchell et al. 2010). Further, Ply stimulates alveolar oedema and haemorrhage and inhibits neutrophil and monocyte respiratory bursts, chemotaxis and bactericidal activity (Jedrzejas 2001; Mitchell et al. 2010).

Neuraminidase is located on the surface of the cell wall, on all strains of pneumococci. The enzyme cleaves terminal sialic acid from glycoproteins, glycolipids and cell-surface oligosaccharides, causing damage to the host. This will unmask potential binding sites for the organism, contributing to increased adhesion (McCullers et al. 2001; Kadioglu et al. 2008; Mitchell et al. 2010).

Hyaluronate is also located on the cell surface. It supports the pneumococcal invasion of tissue by breaking down the hyaluronic acid component of extracellular matrix (Jedrzejas 2001; Mitchell et al. 2010).

Peptide permeases (Psa) are lipoproteins located on the cell wall of pneumococci. PsaA, PsaB and PsaC are involved in the transport of manganese ions. Mutations in PsaA reduce the adherence of pneumococci to lung or endothelial cells (Mitchell et al. 2010).
1.3.2 Haemophilus influenzae

*Haemophilus influenzae* was first isolated and described in 1892 (Levin et al. 1977). In 1933 Fothergill and Wright reported that almost all adults have antibodies against *H. influenzae* (Wallace et al. 1978) and in 1942 the first report of *H. influenzae* pneumonia appeared (Levin et al. 1977).

*H. influenzae* is a small fastidious Gram-negative rod. The cell wall includes an inner cytoplasmic phospholipid bilayer, a peptidoglycan layer, a periplasmic space and an outer membrane. The latter consists partly of lipopolysaccharide, called lipooligosaccharide (LOS) and has proteins inserted which are involved in the adherence to the host cells (Garcia-Rodriguez et al. 2002). Some strains possess a polysaccharide capsule (Ellis et al. 1998). Six different capsular serotypes have been identified, named a to f.

*H Influenzae* is a frequent coloniser of the upper airways. Sixty to 80% of children carry *H. influenzae* strains (Sarangi et al. 2000). The colonisation rate then diminishes in adults (Garcia-Rodriguez et al. 2002), though *H. influenzae* is still evident in the oropharynx. Persons with underlying chronic obstructive pulmonary disease (COPD) have, however, for the most part, increased colonisation with *H. influenzae* in both the upper and the lower respiratory tract, regardless of a possible exacerbation (Leanord et al. 2002). Most strains associated with respiratory tract infections are non-capsulated (Wallace et al. 1978). Underlying co-morbidity, especially chronic lung disease, as well as age and alcoholism, predispose to pneumonia, and to bacteraemia caused by non-capsulated *H. influenzae*, and it is the most common pathogen causing exacerbations in patients with COPD. On the contrary it is a rather uncommon CAP aetiology in previously healthy young persons (Murphy 2003).

1.3.3 Moraxella catarrhalis

For most of the twentieth century *Moraxella catarrhalis* was regarded as a commensal organism of the upper airways. However for the past 30 years it has been clear that it is a pathogen in respiratory tract infections (Enright et al. 1997; Murphy et al. 2009).

*M. catarrhalis* is a Gram negative coccus. The cell wall is composed of two phospholipid bilayers separated by a periplasmic space and a thin peptidoglycan layer. The outer membrane consists partly of lipopolysaccharide, called lipooligosaccharide (LOS), (Ellis et al. 1998), which seems to play an important role in the stimulation of the host inflammatory response (Murphy et al. 2009). Almost 20 adhesins have been identified, with the purpose to support colonisation. Some *M. catarrhalis* strains have pili or fimbriae which may also have a role in the adherence to the epithelial cells (Enright et al. 1997).

The colonisation in the nasopharynx in children is high (up to 75%), and starts as early as at three months of age. In contrast, the carrier rate among adults is only 2-3% (Wood et al. 1996; Enright et al. 1997; Verduin et al. 2002). Although not a common pathogen,
*M. catarrhalis* can cause LRTI in adults. The majority of infections affect patients with underlying diseases, especially COPD, elderly people and those with nosocomial respiratory infections. Pneumonia caused by *M. catarrhalis* tends to be rather mild and bacteraemia is very rare (Enright et al. 1997; Verduin et al. 2002; Murphy et al. 2009).

### 1.3.4 Staphylococcus aureus

*Staphylococcus aureus* is a common pathogen in nosocomial pneumonia. In CAP it is more uncommon, but important due to high morbidity and mortality. *S. aureus* is a Gram positive coccus. The most important virulence factor is the cell wall, including polysaccharide, peptidoglycan, teichoic acid and protein A. Some strains also possess a capsule. These capsules are to some degree antiphagocytic, enhancing colonisation and spread within the host. *S. aureus* also secretes enzymes and toxins, including catalase, hyaluronidase, and lipase, which all contribute to the pathogenesis (Ellis et al. 1998). In addition, some strains also produce a specific exotoxin, Panton-Valentin-Leucocidin toxin, which sometimes results in severe necrotizing pneumonias (Morgan 2007).

Several risk factors for developing *S. aureus* CAP have been identified, including age (the elderly and the very young) chronic pulmonary disease, liver disease, renal impairment, diabetes mellitus, malignancy, immunosuppressive therapy and HIV. CAP caused by *S. aureus* has also been associated with preceding influenza infections (Schwarzmann et al. 1971; Hageman et al. 2006). Reports suggesting that a substantial proportion of *S. aureus* CAP cases are due to meticillin-resistant *S. aureus* have raised concern that this pathogen may have a greater role in the future (Kallen et al. 2009).

### 1.3.5 Gram negative enteric bacilli

Gram negative enteric bacilli are frequent pathogens in nosocomial pneumonia, but, in contrast, they are rather uncommon agents in CAP (Ellis et al. 1998). When detected in CAP cases, the patients are predominantly elderly persons with evident co-morbidities. Gram negative enteric bacilli aetiology is also more common in nursing-home-acquired pneumonia (Janssens 2005).

### 1.3.6 Mycoplasma pneumoniae

*Mycoplasma pneumoniae* was first isolated in 1944 from a sputum culture in one patient with primary atypical pneumonia. This agent was first considered to be a virus until 1961 when the efficacy of antibiotics against this pathogen was clear (Lind et al. 1997; Waites et al. 2004). *M. pneumoniae* is a small microorganism lacking a rigid cell wall. The organism lives in close association with the host’s epithelial cells, preventing it from removal by the host’s mucociliary clearance mechanism. Its organelle consists of a network of adhesions, interactive proteins and adherence accessory proteins that facilitate
attachment to the epithelial cell. It is still unclear how \textit{M. pneumoniae} injures the respiratory epithelial cell, but adhesins seem to be important for cytotoxicity. Hydrogen peroxide and superoxide radicals are known virulence factors of \textit{M. pneumoniae}, inducing oxidative stress in epithelial cells (Ellis et al. 1998; Waites et al. 2004).

\subsection*{1.3.6.1 Epidemiology}

\textit{M. pneumoniae} occurs endemically with epidemic peaks at 3-7-year intervals (Lind et al. 1997; Hammerschlag 2001; Waites et al. 2004). The proportion of \textit{M. pneumoniae} pneumonia cases is to somewhat higher during the summer. It is also able to cause outbreaks in institutional settings (Hammerschlag 2001; Waites et al. 2004). Aetiological studies have found \textit{M. pneumoniae} in up to 40\% of all CAP cases and as many as 18\% of the cases require hospitalisation in children. The incidence is highest among school-aged children with a decline after adolescence. Nevertheless \textit{M. pneumoniae} also occurs in older persons (Waites et al. 2004).

\subsection*{1.3.7 Chlamydophila pneumoniae}

\textit{Chlamydophila pneumoniae} was first isolated from an eye in 1965. In 1986 Grayston et al. isolated the pathogen for the first time from university students with respiratory-tract infections (Grayston et al. 1986). \textit{C. pneumoniae} is a small, strictly intracellular bacterium. It has a trilaminar cell wall but lacks peptidoglycan, which results in a spur-like structure. Asymptomatic carriership has in several studies been observed to be high. The seroprevalence of \textit{C. pneumoniae} may be as high as 40\% in the northern hemisphere. There seem to be cyclical changes in the incidence. Like \textit{M. pneumoniae}, \textit{C. pneumoniae} is able to cause outbreaks in institutional settings (Ellis et al. 1998).

\subsection*{1.3.8 Chlamydia psittaci}

\textit{Chlamydia psittaci} has long been a well-known cause of pneumonia. Its structure is similar to \textit{C. pneumoniae}. It is a zoonos, spread to humans mainly through birds. Nowadays it is seldom found in CAP cases (Ellis et al. 1998).

\subsection*{1.3.9 Legionella}

Since Legionella was first characterised in 1977, it has become a well-known pathogen causing both sporadic and epidemic CAP. It is a Gram negative bacillus. More than 50 different species have been identified, of which half have been associated with human disease (Newton et al. 2010). More than 90\% of clinical cases are caused by \textit{Legionella pneumophila}. Of the at least 15 recognised serotypes, serotype 1 is responsible for over 80\% of cases worldwide (Carratala et al. 2010; Newton et al. 2010). Other species also detected in CAP are \textit{L. bozemanae}, \textit{L. micdadei} and \textit{L. dumoffi}, but these are mostly found in immunocompromised patients. \textit{L. longbeachae} is another species, detected in
Australia and New Zealand in as many as approximately 30% of all Legionella cases (Newton et al. 2010). Legionella possesses many traditional bacterial virulence factors, including lipopolysaccharide, flagella, pili and outer membrane proteins. It is an intracellular pathogen replicating within alveolar cells (Newton et al. 2010).

Legionella species have been detected in all sources of fresh water (Carratala et al. 2010), transmitted primarily through inhalation. A significant increase in incidence of legionellosis during the past few years has been documented (Joseph et al. 2006; Carratala et al. 2010; Newton et al. 2010) and, in contrast to what was earlier thought, Legionella has lately been found to also cause milder disease. The pathogen is also connected with outbreaks where cooling towers are the main suspected sources. Immunosuppression in patients is a well-documented risk factor for Legionnaire’s disease (Carratala et al. 2010).

**Viral pathogens in respiratory tract infections**

In 1933 influenza was the first virus to be discovered. During the 1940s-1960s several other respiratory viruses were identified and recently additional new viral pathogens have been detected. Viruses are a very common cause of respiratory tract infections. Most cause only mild disease in the upper airways, but some may result in LRTI. For the virus to be infective, it must penetrate the host cell and use its replicative machinery to reproduce. To initially attach to the host cell the virus uses specific sialic acid receptors linking to the mucus glycoproteins. The next step is entry of the host cell by pinocytosis, which uncoats the genetic material. Replication of the viral genome then takes place, followed by translation of structural viral proteins. Finally new viruses are released from the cell by budding off from the cellular membrane or by cell lysis (Ellis et al. 1998). Several viruses have been associated with respiratory tract infections, including influenza virus, parainfluenza virus, respiratory syncytial virus, adenovirus, rhinovirus, coronavirus, cytomegalovirus, herpes simplex, measles, and varicella zoster. In the past ten years new respiratory viruses have also been identified, such as metapneumovirus and bocavirus.

**1.3.10 Influenza virus**

Influenza is one of the most common causes of respiratory infection and is also responsible for high morbidity and mortality (Taubenberger et al. 2008). It results in severe illness in 3-5 million people and death in up to 500 000 during epidemic years (Almond et al. 2012). Influenza virus belongs to the family orthomyxoviridae and is a single-strand RNA virus. Of the three types, it is primarily type A and B that cause infections while type C is rarely pathogenic. Influenza A and B contain eight discrete gene segments, each coding for at least one protein. They are covered by projections of three surface glycoproteins: Haemagglutinin (HA), Neuraminidase (NA) and matrix 2 (M2). HA is able to bind to host cells receptors via sialic acid structures, thereafter facilitating
endocytosis (Taubenberger et al. 2008). NA facilitates virus release from the cell with subsequent spread, via enzymatic cleavage of the sialic acid-HA and sialic acid-cell surface bonds. M2 controls the formation of ion channels, allowing the viral genome to enter the host cell (Ellis et al. 1998).

1.3.10.1 Epidemiology and clinical course

Mutations of HA and NA result in yearly outbreaks of influenza. Influenza is most common among the young children and then decreases with age. On the other hand, severe influenza is more frequent among the very young and the elderly (Ellis et al. 1998). Risk factors for influenza include asthma, cardiac disease, immuno-suppression, pregnancy and post-partum states, diabetes mellitus and obesity. Occasionally pandemics occur. Since 1700 there have been a dozen influenza A pandemics. In 1918 the worst pandemic took place when approximately 50 million people died worldwide (Almond et al. 2012). The last pandemic took place in 2009 with influenza A (H1N1) virus of porcine origin. In most influenza gives only a mild self-limiting upper-airway infection. However, it can also be transmitted and replicate in the lower respiratory tract, resulting in bronchitis or pneumonia (Ellis et al. 1998; Taubenberger et al. 2008).

1.3.10.2 Bacterial co-infections

A bacterial co-infection to influenza is common; occurring in up to 20% of all cases with primary influenza infection. Co-infections are often severe and complicated especially in those over 65 years of age or those with cardiac or pulmonary co-morbidities. The most common bacteria in these co-infections are S. aureus and S. pneumoniae followed by H. influenzae (McCullers 2006).

1.3.11 Parainfluenza virus

Parainfluenza virus was first discovered in the late 1950s. It belongs to the paramyxoviridae family. These viruses are both genetically and serologically divided into four types. Further subtypes have been described. The haemagglutinin-neuraminidase protein, the fusion protein and the M protein are three surface-proteins that have been identified to be among those responsible for aggregation to specific host cells (Henrickson 2003).

Parainfluenza virus primarily infects the epithelial cells in the airways and causes both upper and lower respiratory tract infections. It is a common pathogen in respiratory tract infections in children, and serological studies suggest that most children between six and 10 have evidence of past infections. Immunity is incomplete, resulting in recurrent infections later in life (Henrickson 2003). All serotypes can cause clinical infections but serotypes three and four seem to be most common in adults. This organism has also been connected with outbreaks in nursing homes (Marx et al. 1999).
1.3.12 Respiratory syncytial virus

Respiratory syncytial virus (RSV) was first characterised in 1957. It is a single-stranded RNA virus of the paramyxoviridae family. Two subgroups have been identified, A and B. Of the surface proteins located in the envelope, the attachment glycoprotein (G) and fusion protein (F) seem to be most important for connection to respiratory host cells. After replication in the nasopharynx, the virus can spread to the lower respiratory tract (Hall 2001).

RSV is one of the most common agents in airway infections among children, over 90% having serological evidence of a previous RSV infection (Ellis et al. 1998; Bustamante-Calvillo et al. 2001). Infection occurs mainly during autumn/winter/spring. Outbreaks have been reported (Ellis et al. 1998). Mostly the virus causes infection in the upper airways but 25-40% of these infections may well lead to lower respiratory tract infections, and RSV is the main cause of severe respiratory tract infections in infants younger than two years with the development of bronchiolitis (Bustamante-Calvillo et al. 2001; Hall 2001). It has long been known that RSV causes mild respiratory infections in adults, but now there is growing evidence for its significance also in moderate-to-severe LRTI, especially in the elderly and those with underlying diseases (Falsey et al. 2005).

1.3.13 Adenovirus

At least 100 different serotypes of adenovirus have been identified, but only some of these induce human disease. The penton protein fibres, which form part of the icosahedral protein shell, have been identified as a virulence factor, important for the attachment to the host cell (Ellis et al 1998; Baron 1996).

Adenovirus causes infections primarily in the respiratory and enteric tract. Children have the highest susceptibility to this agent, which is estimated to be responsible for two to five percent of acute respiratory infections. However adenovirus has also been associated with infections in adults (Baron 1996). In the majority of cases the infection is localised to the upper airways, but can also cause LRTI, pneumonia included (Ellis et al. 1998). Outbreaks caused by adenovirus are well-documented and in military recruits. Acute respiratory disease has then been the predominant form of adenovirus disease, with adenovirus pneumonia as a frequent complication (McLean 1973; Lehtomaki et al. 1988).

1.3.14 Coronavirus

The two first coronaviruses (CoV) were discovered in the 1960s: HCoV-229E and HCoV-OC43 (Woo et al. 2009). Between November 2002 and July 2003 a CAP outbreak with a previously unknown coronavirus occurred. It was named SARS-CoV. A total of 8098 probable SARS cases from 29 countries and 774 SARS-related deaths
were reported (CDC 2003). Not long thereafter two additional new human coronaviruses were detected in 2004; CoV- NL63 and CoV- HKU1 (Fouchier et al. 2005).

Coronavirus is endemic in humans. It is more common during the winter season. HCoV-229E and HCoV-OC43 have been estimated to be responsible for 5-30% of respiratory tract infections in humans, while a few recent studies have found NL63 in 2-3.6% of samples (Woo et al. 2009). This virus has mostly been associated with relatively mild upper respiratory tract infections, but it is also linked to pneumonia, where age and underlying diseases are identified risk factors.

1.3.15 Rhinovirus

Human rhinovirus (HRV) was first detected in 1956. It belongs to the picornaviridae family and is a single-stranded non-enveloped RNA virus. More than 100 different serotypes have been recognized, for which reason repeated episodes of infection throughout an individual’s lifetime often occur (Louie et al. 2005). These serotypes are classified into three groups (HRV-A, HRV-B, HRV-C), where HRV-A and HRV-B use a specific receptor, ICAM-1, for entry into the host cell (Kennedy et al. 2012). Unlike other respiratory viruses, HRV does not induce cytotoxic effects of the respiratory epithelial cells. However, HRV infection causes disruptions of the epithelial barrier function (Kennedy et al. 2012).

HRV is estimated to cause approximately 1/3-1/2 of all cases of common cold (Hayden 2004; Louie et al. 2005). Although studies indicated 40 years ago that rhinovirus was able to provoke LRTI, the pathogen has been associated predominantly with infections in the upper airways. However several recent reports have shown links between rhinovirus and LRTI (Hayden 2004; Louie et al. 2005). Rhinovirus has also been associated with outbreaks in institutional settings (Louie et al. 2005; Hicks et al. 2006).

1.3.16 Enterovirus

Enterovirus is part of the picornaviridae family. Four species and 108 serotypes have been recognized. Entoviruses cause a wide range of pathologies in children and adults, including respiratory tract infections. Enterovirus is a well-known pathogen in upper-airway infections. However, several reports have also proposed its involvement in the development of LRTI, pneumonia included, in immunocompetent infants and adults (Hohenthal et al. 2008; Andreoletti et al. 2009).
1.3.17 Herpes virus

Herpes simplex virus (HSV) is a double-stranded DNA. Gingivostomatitis, genital infections, ocular infections and encephalitis are the most common manifestations. HSV pneumonia also occurs but is predominantly associated with severely ill and/or immunocompromised patients (Ellis et al. 1998).

1.3.18 Metapneumovirus

Human metapneumovirus (HMPV) was first identified in 2001. It is classified to the paramyxoviridae family and is closely related to both RSV and parainfluenza virus (Fouchier et al. 2005; Walsh et al. 2008). Its envelope consists of three surface glycoproteins (F, SH and G) and the interior is covered by the matrix protein M. The main target of HMPV is the respiratory epithelial cells, where the surface proteins probably are responsible for the attachment (Feuillet et al. 2012).

Since its discovery, metapneumovirus has been detected in countries all over the world in patients with respiratory tract infections (Fouchier et al. 2005). It is a well-recognized pathogen in airway infections among infants and children, with a reported incidence between 5-15% among hospitalised infants. HMPV has also been associated with febrile upper and lower respiratory infections among adults, and fatal pneumonia in immunocompromised patients (Walsh et al. 2008).

1.3.19 Bocavirus

Human bocavirus (HBoV) was first discovered in 2005 and is the most recently detected of all known respiratory viruses. The virus is a part of the parvoviridae family. It was first isolated from a person with pneumonia, and has since been detected worldwide, not only in the airways but also in other locations such as stool, serum, and urine samples (Chow et al. 2008; Jartti et al. 2012). HBoV has been linked to both upper and lower respiratory tract illnesses, including common cold, asthma, bronchitis, bronchiolitis and pneumonia. The pathogenesis is still unknown (Jartti et al. 2012).

1.4 DIAGNOSTIC METHODS FOR AETIOLOGY IN COMMUNITY-ACQUIRED PNEUMONIA

Diagnosis of the microbiological aetiology is a cornerstone in the management of patients with CAP. The technique of culturing specimens on agar media has been available since its invention by Robert Koch in the late nineteenth century (Bartlett 2004). In the early twentieth century many diagnostic methods were widely used in the attempt to reach a microbiological diagnosis and the aetiology was established in almost all CAP cases. One of the reasons for this intensive effort to prove a microbial
cause may have been that the aetiology was at the time almost exclusively represented by *S. pneumoniae*, and the only treatment available in those days was serum therapy. Serotyping of the pneumococcus was therefore necessary (Henrichsen 1999; Bartlett 2004). After World War II the detection rate of a causative microorganism in CAP gradually declined, probably due to the establishment of effective antimicrobial therapy (Bartlett 2011).

Except for urinary antigen tests for *S. pneumoniae* and *L. pneumophila*, and antigen-detection tests for some respiratory viruses, the development of new diagnostic tests for establishing the aetiology of CAP has been slow (File 2011). However, the introduction of nucleic acid amplification tests (NAAT) during the past few years has been a clear advance in microbiological methods for respiratory pathogens.

**Bacterial diagnostic methods**

1.4.1 **Bacterial cultures**

1.4.1.1 **Blood culture**

Blood culture is an important tool when diagnosing hospitalised CAP patients. Specificity is considered to be high since the organism found in the blood is from a sterile location. Sensitivity, however, is low, only about 5-16% yielding a likely aetiological diagnosis (Saranglao et al. 2002; Bartlett 2004). Further, blood cultures cannot be used for detecting atypical pathogens. *S. pneumoniae* accounts for about 65% of all positive blood cultures in CAP (Bartlett 2004), but bacteraemic *S. pneumoniae* cases only represent an estimated 10-20% of all suspected pneumococcal cases (Vernet et al. 2011). Some possible explanations of the low positive yield could be intermittent and low density of the organisms in the blood and prior antibiotic treatment (Vernet et al. 2011).

Blood cultures also have a prognostic significance in CAP, where collection within 24 hours after arrival in hospital has been associated with significantly decreased 30-day mortality (Meehan et al. 1997).

1.4.1.2 **Nasopharyngeal culture**

The relationship between findings of respiratory pathogens in the nasopharyngeal flora and the aetiology of CAP is difficult to interpret. Soon after birth the skin and mucosal sites get colonised with commensal bacteria and then the bacterial flora is in a constant state of flux. The nasopharynx colonisation is by a broad variety of bacteria including potential pathogens as *S. pneumoniae, H. influenzae* and *M. catarrhalis*. In most cases this carriage will cause no clinical symptoms. Yet nasopharyngeal colonisation precedes bacterial invasion of the lower airways, even if only a small percentage develop clinical symptoms of infection (García-Rodríguez et al. 2002).

In contrast to children, the colonisation frequency by potential respiratory pathogens among adults is, then, in general low (about 1%-3%) (García-Rodríguez et al. 2002;
Verduin et al. 2002). Moreover, finding of pneumococci in the nasopharynx is supported by other pneumococcal diagnostic methods in CAP patients (Kalin 1982; Hedlund et al. 1990). These facts would be suggestive that identification of \textit{S. pneumoniae} in the nasopharynx reflects a pneumococcal aetiology of CAP.

1.4.1.3 Gram staining and culture of sputum specimens

Gram staining is one of the oldest methods of detecting organisms infecting the lower respiratory tract. Hans Christian Gram devised his technique primarily not to distinguish between different groups of bacteria, but to be able to observe bacteria more rapidly (Austrian 1960). When strictly purulent specimens have been analysed the sensitivity reportedly ranges between 65\% and 80\% (Bartlett 2004) and specificity for pneumococci between 79\%-100\% (Bartlett 2004; Werno et al. 2008). A meta-analysis of various reports comparing Gram stain with culture results has, however, shown diverse results with a sensitivity of 15\%-100\% and a specificity ranging from 11\% to 100\% (Reed et al. 1996). There have been some concerns with this method; difficulties in obtaining acceptable sputum specimens, smear interpretation requires training, variable criteria for a “positive” smear, diagnostic accuracy mainly for \textit{S. pneumoniae}; and prior antibiotics may reduce the yield (Saranglao et al. 2002). For these reasons, this method is no longer available in all microbiological laboratories.

Culture of expectorated sputum is also an old and well-validated method. The big problem with sputum culture has been the marked contamination of the specimens with bacterial flora colonising the oropharynx. This leads to both false-positive results and false-negative results due to overgrow of potential pathogens (Davidson et al. 1976; Bartlett 2011). Several actions can however be taken to reduce the risk of contaminants; sputum purulence is an important factor. Several studies have shown that sputum samples with >25 polymorphonuclear cells and <10 squamous epithelial cells per low-power field (LPF), decrease the rate of bacteria from the oropharyngeal flora (Murray et al. 1975; Tebbutt et al. 1978) and this correlates well with the findings in simultaneously obtained transtracheal cultures (Geckler et al. 1977). Quantification of sputum culture is also important. Identification of potential pathogens in the specimens in concentrations exceeding $10^6$ CFU/mL reduces the rate of false-positive findings (Bartlett et al. 1978; Guckian et al. 1978). Later studies found that detection of \textit{S. pneumoniae} in sputum cultures with concentrations exceeding $10^5$ CFU/mL also supports a true aetiology (Kalin 1982). Further, with different sputum washing techniques the salivary part will be removed, which also largely eliminates the pharyngeal bacterial flora (Bartlett et al. 1978). Austrian showed that the organism obtained in a representative sputum culture represented the agent responsible for the pneumonia in 93\% of cases compared to subsequent lung aspiration and blood cultures (Bartlett 2011).

Legionella spp requires specialized media for culturing sputum specimens. Even if sputum culture is considered to be the gold standard for detection of Legionellosis, the sensitivity of this method is low (10\%-80\%) and requires three to seven days to be ready (Tronel et al. 2009; File 2011).
Culture of *M. pneumoniae* from respiratory tract specimens requires specialized and expensive growth media, serial blind passages and incubation periods of up to several weeks. Compared to PCR-based methods, the sensitivity has been estimated to be no more than 60% in experienced laboratories. Culture of *M. pneumoniae* is therefore not possible in most laboratories (Waites et al. 2004).

Culture of *C. pneumoniae* requires the use of specific cell cultures and is not generally available (Bartlett 2004). These methods are also time-consuming (Blasi et al. 2009).

### 1.4.1.4 Culture of transtracheal aspirates

One evident problem with sputum cultures is the obvious risk of contaminants from the upper airways. One way to eliminate this risk is to bypass the upper airways by collecting respiratory specimens through transtracheal aspiration (TTA). Studies in healthy medical students proved that the lower airways are sterile (Bartlett 2011). This method was widely used during the 1970s and the microbial yield was high. The false-positive results were in most cases among patients with underlying lung disease. During the 1980s TTA was used less frequently; nowadays hardly at all.

### 1.4.1.5 Culture of bronchoalveolar lavage and protected specimen brush

The use of flexible bronchoscopy to obtain specimens from the lower respiratory tract was introduced in 1979. Samples can be collected through bronchoalveolar lavage (BAL) and a protected specimen brush (PSB). The bacterial concentrations considered significant after dilution are $10^4$ CFU/mL with BAL and $10^3$ CFU/mL with PSB, corresponding to a concentration in a non-diluted specimen of $10^6$ CFU/mL (Bartlett 2011). Antibiotic therapy prior to sample collection significantly affects the results with a high rate of false-negative findings (Meduri et al. 1991). Moreover, risk of contamination from the upper airways has also been reported with this method. Some studies have indicated promising results regarding immune-chromatographic membrane test for *S. pneumoniae* applied on BAL (Jacobs et al. 2005). Bronchoscopy has mostly been used in the intensive care of patients treated with mechanical ventilation.

### 1.4.1.6 Culture of transthoracic needle aspirates

Transthoracic needle aspiration was introduced in 1883 (Bartlett 2011). The most extensive study was reported by Bullowa in 1935 where 1467 patients were examined with this method. Among the 211 patients with bacteraemic pneumococcal aetiology, the culture of the corresponding transthoracic needle aspirate was positive in 78%. The explanation for the false-negative results was improper placement or non-viable microorganisms (Bartlett 2004). This technique is relatively safe, but due to its invasive
nature and concerns about complications, it is used mostly in complicated cases (Werno et al. 2008).

1.4.2 Antigen detection

1.4.2.1 Antigen detection of Streptococcus pneumoniae

Detection of pneumococcal antigen in clinical samples has been investigated for many years, especially in the post-antibiotic era when the need for methods of identifying S. pneumoniae even after start of antimicrobial therapy was obvious. Over the past few decades different methods targeting the capsular polysaccharide antigen of the pneumococcus have been analysed and used (Werno et al. 2008). The four most common immunassays for various body specimens (sputum, serum, urine, cerebrospinal fluid) for antigen detection were countercurrent-immunoelectrophoresis; latex agglutination; staphylococcal co-agglutination; and enzyme-linked immunosorbent assay (ELISA). However hesitations regarding both sensitivity and specificity, and the fact that some of these methods were complicated, have made them less applicable (Farrington et al. 1991).

Recently a rapid immunochromatographic test (ICT) was developed to detect the C polysaccharide which is a part of the pneumococcal cell wall. The antigen test has most of all been evaluated on urine specimens with a reported sensitivity of 70%-80% and a specificity of >90% (Klugman et al. 2008; Bartlett 2011), but optimism is also reported when the test is applied to other body specimens. Its limitations concern low specificity in children and that the test may remain positive several weeks after the infection period. In addition, cross-reactions with some α-haemolytic oral streptococci are possible (Klugman et al. 2008). Other pneumococcal antigens such as pneumolysin have been investigated but are not demonstrably superior to the cell-wall C polysaccharide assay (Bartlett 2011).

1.4.2.2 Antigen detection of Legionella pneumophila

Commercial diagnostic kits using radioimmunoassays (RIA), enzyme immunoassays (EIA) and immunochromatographic assays for detection of soluble Legionella pneumophila serogroup 1 antigen in urine have been available for several years (Murdoch 2003; Tronel et al. 2009). They are rapid, providing a result within 30 minutes. The sensitivity ranges between 70%-100% and the specificity has been estimated to approach 100% (Murdoch 2003; Tronel et al. 2009). One recent meta-analysis including 30 different articles, found a pooled sensitivity of 74% and a pooled specificity of 99.1% (Shimada et al. 2009). One disadvantage is that the test only detects L. pneumophila serogroup 1. Fortunately L. pneumophila accounts for about 90% of all community-acquired cases and serogroup 1 is responsible for 85%-90% of these (Bartlett 2004; Shimada et al. 2009).
1.4.3 Serology

1.4.3.1 Serology for atypical pathogens

Detection of antibodies against *M. pneumoniae* long constituted the basis in the diagnosis of this pathogen. Initially, the compliment fixation test (CF) was the most common method used. Alongside studies showing high sensitivity and specificity there were reports concerning nonspecific reactions as well as cross-reactions with other organ-specific antigens (Kenny et al. 1990). CF has therefore often been replaced by other assays, including indirect immunofluorescence assays, particle agglutination assays and enzyme-linked immunoassays. A fourfold or greater rise in antibody titres or a rise in IgM titres indicates a current or recent infection (Waites et al. 2004).

The current methods for detecting *C. pneumoniae* are microimmunofluorescence (MIF), ELISA and EIA. MIF is however considered to be the reference standard for serodiagnosis. Raised IgM antibodies or a fourfold or greater increase in IgG antibodies suggest an acute or recent infection (Blasi et al. 2009).

Of all the antibody detection tests available for Legionella, indirect immunofluorescence is the standard reference. Fourfold or greater increase of reciprocal antibody titre to ≥128 is considered diagnostic. Only measurement of IgM is unreliable. The method seems to have high specificity but a proportion of people with proven Legionella infection do not have detectable seroconversion (Murdoch 2003).

1.4.3.2 Serology for Streptococcus pneumoniae

Over the years different antigens have been used in serological analysis for *S. pneumoniae*. Pneumococcal surface adhesion A (PsaA) is present in the cell wall of all serotypes and anti-PsaA ELISA has been used to establish a pneumococcal aetiology (Klugman 2008). Further, antibody assays to capsular C-polysaccharides, to type-specific polysaccharides and to pneumolysin have been measured with enzyme-linked immunoassays (EIA) (Klugman et al. 2008; Korppi et al. 2008). Comparison between acute and convalescent serum is necessary. However, not all these assays have been fully validated (Klugman et al. 2008).

1.4.4 Nucleic acid amplification tests

The past few decades have seen major focus on the development of new molecular methods for respiratory pathogens, primarily using the polymerase chain reaction (PCR) technique: this expansion has been an important improvement in the diagnosis of CAP aetiology. PCR-based methods can detect nucleic acid from principally all potential pathogens. They can also detect non-viable organisms; and they are rapid. This has resulted in more frequent detection of different pathogens as well as discoveries of some new respiratory agents. The main questions have concerned
sensitivity and specificity. There are risks of false-negative results due to the presence of PCR inhibitors in the samples. The high sensitivity of PCR also involves a major risk of false-positive results due to contamination by exogenous material or colonizing bacterial flora (Murdoch 2003).

### 1.4.4.1 PCR in serum specimens

Several studies have evaluated *S. pneumoniae* PCR in serum using the pneumolysin gene. Sensitivity has varied between 29%-100% compared with *S. pneumoniae* found in blood cultures (Smith et al. 2009; Vernet et al. 2011). Where non-bacteraemic *S. pneumoniae* cases have also been included the sensitivity has been lower. Further, positive PCR results have also been detected in control patients, raising questions regarding the specificity of PCR (Murdoch 2004; Werno et al. 2008; File 2011). However, recent studies using the autolysin gene and quantitative data have shown promising results (Rello et al. 2009; Blaschke 2011).

### 1.4.4.2 PCR in respiratory specimens

#### 1.4.4.2.1 PCR in respiratory specimens for detecting *Mycoplasma pneumoniae*

The development and validation of molecular-based tests for detecting *M. pneumoniae* have been going on since the late 1980s. Despite differences in study designs, targeted sequences and primers in the different studies, most techniques are basically the same (Daxboeck et al. 2003; Waites et al. 2004). In general, PCR has enhanced sensitivity and is considerably more rapid than culture (Murdoch 2003; Waites et al. 2004). The correlation between PCR and serology is overall good (Murdoch 2003). However, one study comparing PCR to serology found a high specificity of 98.5% but a sensitivity of only 66.7% (Martinez et al. 2008).

#### 1.4.4.2.2 PCR in respiratory specimens for detecting Legionella spp.

Legionella DNA can be detected from lower respiratory tract specimens with PCR. When testing samples from this site, PCR has proved equal or superior to culture. Whether the findings of PCR-positive and culture-negative samples represent contamination or increased sensitivity of PCR is unknown, but the latter is likely to be a major contributor to the increased yield (Murdoch 2003).

#### 1.4.4.2.3 PCR in respiratory specimens for detecting *Chlamydia pneumoniae*

Although PCR techniques for identifying *C. pneumoniae* have been investigated repeatedly, the results from different studies have often been contradictory. In general, PCR appears to be at least as sensitive as culture. Yet it has not been possible to assess specificity because of the lack of a gold standard as reference method. Reports have also warned of a high risk of contamination when PCR assays have been applied to upper-airways specimens (Dowell et al. 2001; Murdoch 2003; Kumar et al. 2007).
1.4.4.2.4 **PCR in respiratory specimens for detecting classical respiratory pathogens**

During the past few years studies have used PCR methods for detecting potential classical pathogens in respiratory specimens. The main problem with respiratory samples is the potential risk of bacterial contaminants from the upper airways. PCR detection of *S. pneumoniae* and *M. catarrhalis* from nasopharyngeal secretions has revealed high correlation to comparative cultures from the nasopharynx (Greiner et al. 2001; Greiner et al. 2003). However, the relationship between these findings and infections in the lower respiratory tract is unknown.

PCR assays for pneumococci have also been evaluated in sputum specimens. The first generation of qualitative PCR assays using the pneumolysin gene in sputum samples found a high sensitivity (83%-100%) (Murdoch 2003). However, only one of these studies tested throat swabs from both CAP patients and a control group. Similar high positive levels of *S. pneumoniae* PCR were found in both the CAP group (55%) and the control group (58%), suggesting that at least part of the positive sputum PCR results could be due to contamination of the upper-respiratory flora (Murdoch et al. 2003).

Some later studies have investigated the use of quantitative PCR assays in sputum samples for identification of *S. pneumoniae* (Yang et al. 2005; Bayram et al. 2006). Kais et al recently evaluated real-time quantitative sputum PCR (RQ-PCR) in sputum for *S. pneumoniae* using the ply gene, *H. influenzae* using the frdB gene and *M. catarrhalis* using the copB gene in an unselected material of patients with varying forms of respiratory tract infections. There were substantially more significant findings of the three pathogens examined, compared to sputum cultures (Kais et al. 2006).

**Viral diagnostic methods**

1.4.5 **Viral culture**

Viral culture has been considered to be the gold standard for diagnosis of respiratory viruses. In acute viral respiratory tract infections, the virus is usually found in high amounts in the epithelial cells of the upper airways. For viral culture, specimens can therefore be collected either with nasopharyngeal aspiration or nasopharyngeal swabs. As viruses are strictly intracellular pathogens, the specimens are inoculated with specific cell cultures suitable for the viruses to grow in. The disadvantages of this method are that it normally takes 7-10 days or more to achieve a positive result, and not all viruses, e.g. HBoV and picornaviruses, can be cultured (McAdam et al. 2009).

1.4.6 **Antigen detection**

Immunological assays use antibodies to detect viral antigen directly in clinical samples. Two main antigen assays have been developed for detecting respiratory viruses:
immunofluorescence (IF) and ELISA. In comparison with IF, ELISA can also detect cell-free viral proteins. Several immune assays for different viruses have been developed including multiplex testing (Landry 2009). They are efficient, cheap and fast in detection of respiratory viruses. However, their sensitivity and specificity are in general lower than in virus isolation.

1.4.7 Serology

Serology has been extensively used in the diagnosis of viral respiratory infection. Initially the complement fixation test was mainly used, but now most laboratories use ELISA, which is a more sensitive assay. Seroconversion between acute and convalescent samples is diagnostic (Ellis et al. 1998). Since it takes several weeks before a viral aetiology can be revealed, serology is no longer very useful in clinical practice: more sensitive viral methods now are available.

1.4.8 Nucleic acid amplification tests

The gold standard of viral detection has been conventional cell culture. As stated above there are, however, several drawbacks to this method. The development of NAAT for detecting microorganisms has revolutionised the diagnostics of viral aetiology. Several PCR assays have been developed for identifying viral pathogens in respiratory samples (Templeton et al. 2004; Watzinger et al. 2004; Gunson et al. 2005; Gunson et al. 2006; van de Pol et al. 2007; Brittain-Long et al. 2008). Multiplex systems targeting most of the respiratory viruses have also been developed, and have showed a substantial increase in sensitivity compared to conventional methods (Tiveljung-Lindell et al. 2009; Arens et al. 2010) coupled with high specificity (Arens et al. 2010). Due to their superiority, NAAT has in many laboratories replaced the other viral techniques (Tiveljung-Lindell et al. 2009).

1.4.9 Molecular virus screening

The molecular virus screening method is a recently developed technique used in the search for new unknown viruses. The method was first described by Allander et al. in 2005 when human bocavirus was identified (Allander et al. 2005). PooledDNAase-treated nasopharyngeal samples are examined using a random PCR technique. Amplified PCR products are separated and cloned. After large-scale sequencing of the clones, the sequences are evaluated through an automated protocol and compared with known sequences, in the search for new undiscovered viruses (Allander et al. 2005; Chow et al. 2008; Jartti et al. 2012).
1.5 HOST DEFENCE IN RESPIRATORY TRACT INFECTIONS

To develop respiratory tract infection, the microorganism must enter the epithelial surface to be able to start invasive infection. The microbes can gain access to the respiratory tract through inhalation, which is the route for viruses and some intracellular bacteria. Bacteria can also reach the epithelial cells through haematogenous spread. However, the most common path for most bacteria is through colonisation of the upper airways followed by micro-aspiration of bacterial-laden oropharyngeal secretions into the lower respiratory tract. This aspiration occurs regularly in normal hosts while sleeping (Welsh et al. 2001). To eliminate these agents the host defence has a complex, developed, two-part system: the innate immune response and the adaptive immune response.

1.5.1 Innate immune response

1.5.1.1 Mechanical defences

Inspired air usually enters the nose. The nasal hair acts to filter larger particles, particularly those greater than 10 µm and washed out by secretion or expelled by sneezing. The oropharyngeal mucosa is composed of squamous epithelium and normally inhabited by a complex flora of aerobic and anaerobic microorganisms which compete with potential, more invasive, pathogens (Welsh et al. 2001). Further, particles larger than 5 µm are also usually trapped in the tortuous channels of the nasopharynx, the glottis, trachea and bronchioles. These particles are deposited on the airway mucosa, which is covered by a mucociliary blanket. Efficient ciliary function is critical in effectively clearing the overlying mucous secretions (Twigg 1998).

1.5.1.2 Soluble proteins

Bacteria have normally a diameter between 0.5-2 µm which means that they can relatively easily pass the mechanical defence and reach the alveolar space. Particles that pass this barrier are met by a range of soluble proteins with antimicrobial effects in the airway lining fluid. Immunoglobulin A is located primarily in the upper airways, where it seems to have a protective role mostly against viruses. By contrast immunoglobulin G is found in the alveolar space and its function is to opsonise and activate complement (Reynolds 1988). The complement system consists of a variety of proteins and is important in cellular recruitment, opsonisation and lysis. Several other proteins such as peroxidise, lactoferrin, defensin, collectin, ficolin, lysozyme, peroxidise and lactoferrin also have antibacterial functions (Zhang et al. 2000; Boyton et al. 2002; Tsai et al. 2008).
1.5.1.3 Phagocytic defence

When microbes gain access to the lower respiratory airways the phagocytic lung defence plays an important role. The alveolar macrophages constitute the first line of phagocytic defence. Certain microbes have the ability to replicate intracellularly in the epithelial cells (e.g. Legionella spp, Mycoplasma, virus) and are thus resistant to alveolar macrophages. In these cases specific cell-mediated immunity is needed for eradication (Zhang et al. 2000).

If the invading pathogens are too virulent or represent too large a load to be contained by the macrophages alone, the elaboration of macrophages and other lung cells will initiate an inflammatory reaction. Alveolar macrophages activate epithelial cells through production of different cytokines, particularly TNF-α and interleukin-1 (Mizgerd 2008). This stimulation activates the epithelial cells to produce chemokines, colony-stimulatory factors and adhesion molecules to rapidly recruit polymorphonuclear cells to the alveolar space. Activated neutrophils phagocyte and neutralize microbes (Twigg 1998). Further, neutrophils secrete various signals, including TNF-α, IL-1β, IL-6 and chemokines for activation of dendritic cells, and IL-12 for activation of T-cells and Natural killer (NK) cells (Boyton et al. 2002; Mizgerd 2008).

Recognition of viral components starts the activation of various numbers of cytokines, foremost interferon (INF)-α and INF-β, produced mainly by plasmacytoid dendritic (pDC) cells and alveolar macrophages (Kohlmeier et al. 2009). INF initiates the production primarily of NK cells, which enhance cytolysis of virus-infected cells (Kohlmeier et al. 2009) and release INF-γ, leading to recruitment of virus-specific cytotoxic T lymphocytes (Boyton et al. 2002).

Along with the proinflammatory response the host has developed a sophisticated anti-inflammatory system to counterbalance and compartmentalize the immune response to microbial agents, such as IL-1 and IL-10 (Zhang et al. 2000).

1.5.2 Adaptive immune response

Parallel with the activation of neutrophils the adaptive immune response is also stimulated, and here the antigen-presenting cells (APC), chiefly dendritic cells are essential. The antigen-bearing APC migrate towards regional lung-draining lymph nodes and present the antigen to naive T-cells (Welsh et al. 2001). The complex of antigen and class II MHC proteins bind to antigen-naive CD4+ helper T lymphocyte receptors to initiate activation of specific T effector lymphocytes. These activated effector cells then migrate back to the site of antigen deposition in the lung parenchyma where the immune response can be amplified (Welsh et al. 2001). T-lymphocytes produce a variety of cytokines that participate in the inflammatory process (Figure 1). The T-helper cells begin to differentiate into two different subsets, Th1 and Th2.
Figure 1. Generation of specific immune responses in the lung.

Th1 cells produce mainly IL-2 and INF-γ, important primarily for cellular immunity. Cellular immunity is further divided into two parts: delayed-type hypersensitivity reactions which are involved in the control of intracellular microbes such as Legionella, and cytotoxic reactions which are responsible for defence against viral infections (Twigg 1998). These cytotoxic reactions occur through CD8+ T cells by inducing apoptosis of the virus-infected epithelial cells (Kohlmeier et al. 2009). Th2 cells mainly secrete IL-4, IL-5 and IL-10 and are primarily responsible for driving B-cells to produce antibodies. Humoral immunity is mediated by bone-marrow-derived B lymphocytes, and initiated after foreign antigen is bound to the B-cell receptor. In the setting of activated CD4+ T-lymphocytes, differentiation into specific B-lymphocytes then occurs for production of antibodies (Welsh et al. 2001). Immunoglobulin G is the most abundant immunoglobulin in BAL fluid and important in opsonisation and complement binding, which aid phagocytosis (Twigg 1998).

1.6 INFLAMMATORY MARKERS

Many different inflammatory markers have been studied in patients with CAP to investigate the inflammatory response in different conditions. Diagnosing bacterial infections is often challenging because the clinical presentation can be similar to other non-infectious conditions, such as trauma, pancreatitis and vasculitis, and most microbiological methods are not for point-of-care diagnosis. Bacterial infections can also be difficult to distinguish from viral infections based on clinical presentation. Therefore the relationship between inflammatory markers and different pathogens causing CAP has been studied. Many inflammatory markers have also been investigated regarding their correlation to CAP severity.
1.6.1 Cytokines

Several cytokines have been studied in the development of CAP. Some of the better known are the proinflammatory mediators TNF-α, IL-1 and IL-6.

TNF-α has been associated with severe pneumonia (Moussa et al. 1994). However this cytokine is produced at the site of infection and then to varying extents spills over into the circulation (Antunes et al. 2002). Elevated TNF-α level is also strongly associated with severe sepsis (Moussa et al. 1994; Puren et al. 1995).

IL-1 is also produced at the site of infection, not always detected in serum and associated with severe sepsis (Antunes et al. 2002). Also, it peaks early in the inflammatory process.

IL-6 is produced by many cell types, including neutrophils, monocytes/macrophages, T- and B-cells and various stoma cells (Standiford et al. 1999). It has diverse haematological and immunological effects and plays a major part in the acute-phase response (Heinrich et al. 1990). It peaks within 36 hours and is usually measurable in serum in patients with bacterial infections (Standiford et al. 1999). IL-6 has been correlated to CAP severity (Antunes et al. 2002) and raised levels have also been associated with pneumococcal bacteraemia (Lieberman et al. 1997).

The main issue with these proinflammatory cytokines is their short half-life in serum, and that their levels in the blood are very variable, transient and non-specific. TNF-α and IL-1 are present in serum in very low concentrations in most infectious conditions and are often not detectable (Moussa et al. 1994; Christ-Crain et al. 2010).

1.6.2 White blood cells

White blood cells (WBC) have been used widely to investigate bacterial infections and this is also the case in CAP, although few studies have dealt with this subject.

Lethomäki et al showed that if a cut-off value of $10 \times 10^9 /L$ was used the value was exceeded in 67% of the pneumococcus group but only 6% in the virus group (Lehtomaki 1988). A leucocyte count of more than $15 \times 10^9 /L$ and a proportion of neutrophils $>20\%$ has been suggested for a pneumococcal aetiology (Ponka et al. 1983; Fang et al. 1990). However in the Lethomäki study the influenza cases were excluded because of the presumed high frequency of coincidental pneumococcal infection and in the Pönkä and Fang studies no comparison was made to the median WBC among the purely viral cases. Further, overlap of leucocyte counts between pneumococcal and viral infections was marked.

1.6.3 Acute-phase proteins

The hepatocytes in the liver are the major organ for the synthesis of acute-phase proteins. There are many different acute-phase proteins with different tasks (Table 2). The proteins are stimulated during inflammation. The major regulator of acute-phase protein production is IL-6, but IL-1 and TNF-α are also involved in the stimulation. Many acute-phase protein plasma levels, such as C-reactive protein, serum amyloid A, fibrinogen, haptoglobin, α1-antichymotrypsin and α1-antitrypsin, increase during an acute-phase response. Simultaneously, other proteins such as albumin, transthyretin and transferrin concentrations decrease. (Heinrich et al. 1990).
Table 2. Acute-phase proteins

<table>
<thead>
<tr>
<th>Plasma proteins</th>
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<tbody>
<tr>
<td>Albumin</td>
</tr>
<tr>
<td>α2-Globulin</td>
</tr>
<tr>
<td>Gamma globulin</td>
</tr>
<tr>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>Transthyretin</td>
</tr>
<tr>
<td>Haptoglobin</td>
</tr>
<tr>
<td>Haemopexin</td>
</tr>
<tr>
<td>Transferrin</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
</tr>
<tr>
<td>Inter-α-Trypsin inhibitor</td>
</tr>
<tr>
<td>α1-Antichymotrypsin</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
</tr>
<tr>
<td>Complement Factors 3 and 4</td>
</tr>
<tr>
<td>α1-Acid glycoprotein</td>
</tr>
<tr>
<td>C-Esterase inhibitor</td>
</tr>
<tr>
<td>Lysozyme</td>
</tr>
<tr>
<td>C-reactive protein</td>
</tr>
</tbody>
</table>

1.6.3.1 C-reactive protein

C-reactive protein (CRP) together with serum amyloid A shows the highest increases during an acute-phase response (Heinrich et al. 1990; Hansson et al. 1997). CRP is synthesised in the liver during infection and also in many other non-infectious inflammatory conditions. It binds to polysaccharide in pathogens and shares in opsonisation by activating the classical complement pathway (Simon et al. 2004). Secretion begins within 4-6 h after stimulus, doubles every 8 hours, and peaks after about 36 h. After the disappearance of the stimulus the CRP concentration decreases with a half-life of 19 hours (Coelho et al. 2007). In diagnosis, it has been used mainly to separate bacterial infections from viral infections and other non-infectious conditions (Simon et al. 2004). Its ability to predict different pathogens in CAP has, however, been questioned (Hedlund et al. 2000; Simon et al. 2004; van der Meer et al. 2005; Chalupa et al. 2011).

1.6.3.2 Transthyretin

Transthyretin is a serum- and cerebrospinal-fluid-carrier of the thyroid hormone thyroxine (T4) and retinol-binding protein bound to retinol. The liver secretes transthyretin into the blood, and the choroid plexus secretes transthyretin into the cerebrospinal fluid. TTR concentration is decreased during acute lung infection. This protein has not been widely studied in CAP but one study has shown that of nine
different acute-phase proteins analysed transthyretin had the shortest half-life after CRP (Hansson et al. 1997).

1.6.4 Procalcitonin

The hormone calcitonin is synthesised in the C cells of the thyroid gland and K cells in the lung in response to hypercalcaemia. Its biological activity is to lower serum calcium concentration by inhibiting bone resorption. The immature calcitonin has 33 amino acids and is part of a larger 116-amino-acid prohormone, procalcitonin (PCT) (Gilbert 2011).

1.6.4.1 PCT correlation to bacterial infections and other inflammatory diseases

PCT is detected in serum of normal persons but in very low concentrations. In contrast, in response to invasive bacterial infections, plasmodium spp. and some fungi, PCT can be elevated several thousand times (Muller et al. 2001; Christ-Crain et al. 2010; Gilbert 2010). During infection several different parenchymal cells can release PCT, such as liver, kidney and monocytes (Niederman 2008). Investigators have identified PCT as part of a proinflammatory response in the innate immune system to bacterial infection. In vitro studies have observed elevated PCT levels in response to bacterial endotoxin and proinflammatory cytokines as TNF-α, IL-1, IL-6 (Muller et al. 2001; Niederman 2008; Gilbert 2011). Moreover, in animal models of sepsis in guinea pigs, antibodies to PCT increased survival (Gilbert 2010). The exact role of PCT during bacterial infections is, however, still not known. Raised PCT levels have also been observed in non-infectious inflammatory conditions such as pancreatitis, due to biliary obstruction or necrosis: chemical pneumonitis; in burns; in heat stroke; in mechanical trauma and after surgery (Muller et al. 2001). However, in differentiating bacterial infection from non-infective causes of inflammation a meta-analysis concluded that PCT was more sensitive (85% vs. 78%) and more specific (83% vs. 60%) than CRP (Christ-Crain et al. 2010). Synthesis of PCT is detectable within 3–6 hours after onset (Muller et al. 2001; Niederman 2008; Gilbert 2011) and in contrast to transiently classical cytokines, increased PCT levels persists for several days (Muller et al. 2001).

1.6.4.2 PCT correlation to bacterial and viral infections

PCT production is almost completely blocked by γ-INF. γ-INF is a central cytokine for the regulation of the viral host defence, and the entrance of viral pathogens in the lower respiratory tract induces its production. For this reason PCT has been suggested to be superior for discriminating between bacterial and viral infections, and support for this is also found in several clinical studies (Toikka et al. 2000; Ip et al. 2007; Kruger et al. 2009; Schuetz et al. 2009; Chalupa et al. 2011). As PCT has been emphasised as superior for diagnosing bacterial infections, several studies have evaluated PCT-guided algorithms for antibiotic therapy decision-making in patients with lower respiratory tract infections (Christ-Crain et al. 2004; Briel et al. 2005; Christ-Crain et al. 2006; Stolz et al. 2007; Kristoffersen et al. 2009; Schuetz et al. 2009). Most studies used a PCT cut-off of ≤ 0.25 µg/l for withholding antibiotic treatment. In summary, these studies showed no failures as a consequence of following the PCT-guided regime, while overall antibiotic use was reduced.
### 1.6.4.3 PCT correlation to CAP severity

Several studies have investigated the relationship between PCT and severity of CAP measured as mortality as well as different prognostic score systems (e.g. APACHE II, the Pneumonia Severity Index). The main findings in these reports have been that elevated PCT concentrations correlate with increased severity (Boussekey et al. 2005; Masia et al. 2005; Boussekey et al. 2006; Lacoma et al. 2011) and the correlation is better than with other biomarkers (Muller 2007; Kruger et al. 2008).
2 AIMS

The aims of the work reported in this thesis were:

- to estimate the accuracy of a recently-developed RQ-PCR method for identifying pneumococci in sputum, among patients with CAP treated in hospital,

- to determine the aetiology of CAP among patients admitted to hospital and assess the occurrence of mixed infections, by implementing a new diagnostic PCR platform combined with conventional methods,

- to compare patients with a pure bacterial aetiology with those with findings of both bacteria and virus regarding possible differences in the severity of illness and length of stay in hospital, and

- to study the inflammatory response, especially procalcitonin levels, in patients with CAP and the correlation to different pathogens commonly detected in respiratory tract infections. In addition, we wanted to assess the value of PCT and CRP as predictors of the severity of the disease.
3 MATERIAL AND METHODS

3.1 PATIENTS

All patients with CAP admitted to the Department of Infectious Diseases at Karolinska University Hospital in Solna, Stockholm, Sweden, from September 13, 2004, to September 12, 2005 were reviewed for inclusion in a prospective study. A total of 237 patients were eligible for inclusion and finally 184 were included in the study. CAP was defined as clinical signs of acute lower-respiratory-tract infection with onset before admission and presence of new infiltrates on a chest radiograph.

3.2 SPECIMEN COLLECTION

The total number of patients from whom the different types of specimen for microbiological investigation were obtained, is shown in Table 3. On admission, specimens for bacterial cultures from nasopharyngeal secretions, sputum (if the patient was able to produce this), and blood were obtained before starting antibiotic therapy. These samples were collected at the emergency room (ER) by the physician on call and before the patients were included in the study. A total of 61 and 40 patients left sputum samples which were analysed by culture and RQ-PCR, respectively, and 38 patients had their sputum samples analysed by both methods. It is often difficult to obtain sputum samples of good quality at the ER. Accordingly, efforts to get induced sputum specimens were made with the assistance of a respiratory physiotherapist on the ward. Sputum samples were then further analysed by culture (n =113) and RQ-PCR (n = 112), and 103 patients had their sputum sample analysed by both methods (Figure 2). Together with the sputum samples collected at the ER totally, 128 and 126 patients had at least 1 sputum sample analysed by culture and RQ-PCR, respectively, collected at the ER and/or on the ward (Table 3). Sputum samples were also examined for Legionella by culture and/or PCR. Urine samples for pneumococcal and L. pneumophila antigen detection and nasopharyngeal secretion samples for bacterial and viral detection were obtained one day after hospital admission. Blood samples were obtained within 24 hours after hospital admission and 4 weeks later for serological analysis. Serum samples were also collected within 24 hours after admission to hospital in all patients and stored at -70 °C for subsequent measurement of PCT, CRP, transthyretin and IL-6.
Figure 2. Study flowchart: number of patients with specimens collected at the Emergency Room (ER) and on the ward respectively, analysed with different methods.

Table 3. Specimens obtained for microbiological investigations

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of patients tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture</td>
<td>179</td>
</tr>
<tr>
<td>Sputum culture</td>
<td>128</td>
</tr>
<tr>
<td>Sputum RQ-PCR</td>
<td>126</td>
</tr>
<tr>
<td>Sputum culture/PCR Legionella</td>
<td>138</td>
</tr>
<tr>
<td>Nasopharyngeal secretions culture</td>
<td>158</td>
</tr>
<tr>
<td>Nasopharyngeal secretions M. pneumoniae</td>
<td>101</td>
</tr>
<tr>
<td>Nasopharyngeal secretions C. pneumoniae</td>
<td>99</td>
</tr>
<tr>
<td>Urine antigen assays S. pneumoniae</td>
<td>169</td>
</tr>
<tr>
<td>Urine antigen assays L. pneumophila sg 1</td>
<td>168</td>
</tr>
<tr>
<td>Nasopharyngeal secretions viral isolation</td>
<td>157</td>
</tr>
<tr>
<td>Nasopharyngeal secretions viral real-time PCR</td>
<td>156</td>
</tr>
<tr>
<td>Paired blood samples for serology</td>
<td>131</td>
</tr>
</tbody>
</table>
3.3 MICROBIOLOGICAL METHODS

3.3.1 Bacterial diagnostic methods

All sputum samples were examined by microscopy, and samples containing a preponderance of leukocytes and a few squamous epithelial cells were considered acceptable for culture, according to accepted criteria (Bartlett et al. 2000). The number of colony-forming units per millilitre (cfu/mL) was assessed with a semi-quantitative technique (Kalin et al. 1983; Kais et al. 2006). Quantitative cultures of sputum samples and qualitative cultures of nasopharyngeal samples as well as blood samples were performed in accordance with accepted methods and criteria. RQ-PCR targeting the pneumolysin (ply) gene of S. pneumoniae, the fumarate reductase (frdB) gene of H. influenzae, and the outer membrane protein (copB) gene of M. catarrhalis were performed as described by Kais et al (Kais et al. 2006). Respiratory tract samples were also examined for L. pneumophila with culture and/or PCR and for M. pneumoniae and C. pneumoniae with PCR (Welti et al. 2003). Immunochromatographic membrane tests (BinaxNOW S. pneumoniae and BinaxNOW Legionella; Inverness Medical Innovations) were performed on urine samples, for detection of pneumococcal and L. pneumophila antigens. Enzyme-linked immunosorbent assays (Ani Labsystems) were used according to the manufacturer’s instructions for detecting immunoglobulin G (IgG) and IgM antibodies to M. pneumoniae and C. pneumoniae.

3.3.2 Viral diagnostic methods

Virus isolation was performed on all nasopharyngeal secretion samples in accordance with diagnostic practice (Ostlund et al. 2004). For serology, an enzyme-linked immunosorbent assay was used for the detection of IgG antibodies to adenovirus, influenza A and B viruses, parainfluenza 1, 2, and 3 viruses and RSV (Bidwell et al. 1977; Grillner 1987). The detection of adenovirus, enterovirus, human bocavirus, human coronaviruses 229E, HKU-1, NL63, and OC43, human metapneumovirus, influenza viruses A and B, parainfluenza viruses 1–3, rhinovirus, and RSV (A and B) was performed retrospectively using a newly-developed real-time PCR platform (Tiveljung-Lindell et al. 2009). In addition, 76 nasopharyngeal samples, without virus detectable by PCR, and with sufficient volume, were subjected to unbiased sequence-based molecular virus screening as described by Allander et al. (Allander et al. 2005).

3.4 DATA COLLECTION

PSI was used for severity assessment, and information on the 20 different predictors included in the PSI was prospectively registered for all patients. Missing data were considered as normal findings, in accordance with the PSI protocol (Fine et al. 1997). Data on length of hospital stay, mortality and treatment in the intensive care unit (ICU) were collected from the case record forms. Data on seven different vital signs for appropriateness of hospital discharge of patients with CAP (Halm et al. 2002)
were also prospectively collected, as follows: time to temperature \( \leq 37.8 \, ^\circ C \), heart rate \( \leq 100/min \), systolic blood pressure \( > 90 \, \text{mmHg} \), oxygen saturation \( > 90\% \) or \( \text{PaO}_2 \geq 60 \, \text{mm Hg} \), respiratory rate \( \leq 24/min \), normal mental status and normal oral nutrition. In our department all patients with saturation below 90\% receive oxygen therapy. We therefore changed the parameter oxygen saturation \( \geq 90\% \) to cessation of oxygen therapy.

3.5 MEASUREMENT OF BIOMARKERS

Serum CRP was analysed on an Architect Ci8200 analyzer (Abbott Laboratories, Abbott Park, IL, USA). The CRP assay had a total coefficient of variation (CV) of 0.8\% at 8 mg/L and the assay calibrator was traceable to ERM-DA474/IFCC. Serum transthyretin was analysed on a BN Prospec \textsuperscript{TM} nephelometer (Siemens Healthcare Diagnostics, Deerfield, IL, USA) with reagents from the same manufacturer. The assay had total CV of 2.3 \% at 110 mg/L and 2.9 \% at 230 mg/L. The procalcitonin kit was from Roche and run on a Cobas E instrument (Roche Diagnostics, Mannheim, Germany). The total CVs for the procalcitonin assay were 3 \% at 0.48 µg/L and 4 \% at 10.2 µg/L. IL-6 was measured with a sandwich ELISA (DY206, R&D Systems, Minneapolis, MN, USA). The intraassay CV was 5\%.

3.6 DIAGNOSTIC CRITERIA

A microorganism was considered to be of definite aetiological significance if it was cultured from blood, pleural fluid, a protected specimen brush (cut-off, \( \geq 10^3 \) cfu/mL protected specimen brush broth), or bronchoalveolar lavage (cut-off, \( \geq 10^4 \) cfu/mL bronchoalveolar lavage fluid); or if the urine antigen assay for \textit{S. pneumoniae} or \textit{L. pneumophila} was positive. Detection of \textit{L. pneumophila} by culture and/or PCR from sputum was also considered as definite support for the aetiology. In accordance with our previous findings of patients with bacteraemic pneumococcal pneumonia, identification of \( \geq 10^5 \) cfu/mL of \textit{S. pneumoniae} in sputum culture or nasopharyngeal culture was accepted as of probable significance (Kalin 1982; Kalin et al. 1983; Hedlund et al. 1990). Pneumococcal DNA corresponding to \( \geq 10^5 \) cfu/mL by use of RQ-PCR was also considered to be of probable significance (Kais et al. 2006). For other bacteria, identification of \( \geq 10^5 \) cfu/mL in sputum culture (Bartlett et al. 1978) or DNA corresponding to \( \geq 10^6 \) cfu/mL with RQ-PCR for \textit{H. influenzae} and \textit{M. catarrhalis} were considered to be of probable significance. The identification of \textit{M. pneumoniae} and \textit{C. pneumoniae} using PCR, the presence of IgM antibodies, a twofold increase in IgG between acute and convalescent phase samples were all considered to be support. A viral aetiology was considered probable if at least one of the following criteria was met: detection of a virus in a respiratory secretion sample by use of isolation or PCR, or a tenfold increase in IgG titre between acute and convalescent phase samples.
3.7 STATISTICAL METHODS

Paper I

Statistical comparison between groups of subjects was made with Fischer's exact test, 2-tailed. A p value below 0.05 was considered significant. All analyses were performed with the JMP statistical computer program (SAS Institute, Cary, NC).

Paper III

Categorical data were summarized using frequency counts and percentages. Continuous data were presented as mean and standard deviation. The number of days to improvement of vital signs and the length of hospital stay are presented with median values and visualized in bar plots. The Chi-square test and Fisher’s exact test were used in comparisons between patients with findings of bacteria alone and those with bacteria and virus, regarding categorical data. When comparing the groups with scores corresponding to PSI classes IV or V with those in lower classes, odds ratios (OR) and 95% confidence intervals (95% CI) are presented. Since a higher mean score for the co-morbidity part of the PSI score was obtained in patients infected with both bacteria and virus as opposed to those without viral findings (p = 0.013), we also used a modified PSI score without the inclusion of co-morbidity, age, sex and residence in nursing home, but including the remaining 12 predictors, in order to obtain a more pure physiology score. Factorial analysis of variance (ANOVA) with adjustment for co-morbidity was performed when analysing the difference between groups regarding the number of days to improvement of vital signs and length of hospital stay. Distribution of these variables was positively skewed and before the formal analyses the variables were reciprocal-transformed and log-transformed. The Mann-Whitney U-test was used to analyse the PSI score. A p-value of < 0.05 was considered statistically significant.

Paper IV

Categorical data were summarised using frequency counts and percentages. Continuous data were presented as median and range (minimum – maximum). Serum concentrations of IL-6 were transformed to 10 logarithms in order to normalize their distribution. In comparisons between different sub-groups the Mann-Whitney U test was used. P < 0.05 was considered statistically significant.
4 RESULTS AND DISCUSSION

4.1.1 Patient characteristics

The baseline characteristics of the patients are shown in Table 4. The study population consisted of 94 males and 90 females. Mean age was 61.3 years (range, 18–93 years). A total of 74 patients had at least one underlying disease, with malignancy as the most frequent (19%). The average stay in hospital was 7.1 days (range, 1–69 days). At hospital admission, 40 patients (22%) had already been started on antibiotic therapy. Thirteen patients (7.1%) were treated in the ICU and seven (3.8%) died in hospital. Seventy-five patients (41%) were classified as PSI classes IV or V.

Table 4. Baseline characteristics of patients with community-acquire pneumonia admitted to hospital

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients n=184 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean age</td>
<td>61.3 years</td>
</tr>
<tr>
<td>Male/female</td>
<td>94 (51) / 90 (49)</td>
</tr>
<tr>
<td>nursing home</td>
<td>10</td>
</tr>
<tr>
<td>any co-morbidity</td>
<td>74 (40)</td>
</tr>
<tr>
<td>COPD*/chronic bronchitis</td>
<td>21 (11)</td>
</tr>
<tr>
<td>heart failure</td>
<td>12 (7)</td>
</tr>
<tr>
<td>cerebrovascular disease</td>
<td>16 (9)</td>
</tr>
<tr>
<td>malignancy</td>
<td>35 (19)</td>
</tr>
<tr>
<td>liver disease</td>
<td>7 (4)</td>
</tr>
<tr>
<td>renal failure</td>
<td>0 (0)</td>
</tr>
<tr>
<td>antibiotics prior to hospital admission</td>
<td>40 (22)</td>
</tr>
<tr>
<td>mortality rate</td>
<td>7 (3.8)</td>
</tr>
<tr>
<td>treatment in the intensive care unit</td>
<td>13 (7.1)</td>
</tr>
<tr>
<td>PSI* Class I</td>
<td>27 (15)</td>
</tr>
<tr>
<td>PSI* Class II</td>
<td>46 (25)</td>
</tr>
<tr>
<td>PSI* Class III</td>
<td>36 (20)</td>
</tr>
<tr>
<td>PSI* Class IV</td>
<td>50 (27)</td>
</tr>
<tr>
<td>PSI*Class V</td>
<td>25 (14)</td>
</tr>
</tbody>
</table>

*a COPD= Chronic obstructive pulmonary disease

b PSI= Pneumonia severity index
4.1.2 Evaluation of sputum RQ-PCR for *Streptococcus pneumoniae* in comparison to other methods

4.1.2.1 Pneumococcal aetiology diagnosed by different methods

In 70 patients (38%), *S. pneumoniae* was identified by one or more methods (Table 5). A total of 19 patients (15%) produced at least one sputum specimen with significant growth (≥10⁵ CFU/mL) of *S. pneumoniae*. *S. pneumoniae* DNA (corresponding to ≥10⁵ CFU/mL) with sputum RQ-PCR was detected in 34 patients (27%) (*P* < 0.0001 compared with sputum culture). In 15 of the sputum-culture-positive patients (79%), sputum was collected at the ER. Of the 34 sputum RQ-PCR-positive patients, 17 were negative in corresponding sputum culture and one patient was not cultured (Table 5). Fourteen of these 17 (82%) patients had been treated with antibiotics before sputum sampling. All these 14 specimens were obtained by sputum induction with assistance from a respiratory physiotherapist. Support for the significance of the RQ-PCR findings in the culture-negative sputum specimens was obtained by blood culture in 7/17 (41%).

4.1.2.2 Findings of pneumococci with different methods among patients with complete sample collection

Thirty-five patients had a complete collection of samples examined, that is, cultures from blood and nasopharyngeal secretions, urine antigen assays, and sputum samples collected at the ER, analysed both by culture and RQ-PCR (Table 6). A pneumococcal aetiology was established for 21 (60%) of these patients, among whom RQ-PCR were positive on sputum samples in 17 (81%). Of the 21 patients with established pneumococcal aetiology support from other diagnostic methods was found in 19 cases. Two patients were positive in RQ-PCR as the only method. Thus, in this group, compared with the other methods, the RQ-PCR sensitivity was 15/19 (79%) and the specificity 14/16 (88%). All 13 culture-positive sputum specimens were significantly positive by RQ-PCR.
Table 5. Distribution of pneumococcal findings with different methods from patients with pneumococcal aetiology (n = 70)

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of tested patients</th>
<th>Positive findings n (%)</th>
<th>Findings of pneumococci with ≥1 additional method</th>
<th>Blood culture</th>
<th>Urine antigen assays</th>
<th>Sputum culture ≥10^7 CFU/mL</th>
<th>Sputum RQ-PCR corresponding to ≥10^7 CFU/mL</th>
<th>NPH culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture</td>
<td>179</td>
<td>27 (15%)</td>
<td>22</td>
<td>17/25*</td>
<td>6/21*</td>
<td>13/19*</td>
<td>16/23*</td>
<td></td>
</tr>
<tr>
<td>Urine antigen assays</td>
<td>169</td>
<td>33 (20%)</td>
<td>25</td>
<td>17/31*</td>
<td>7/20*</td>
<td>13/20*</td>
<td>19/29*</td>
<td></td>
</tr>
<tr>
<td>Sputum culture</td>
<td>128</td>
<td>19 (15%)</td>
<td>18</td>
<td>6/19*</td>
<td>7/18*</td>
<td>16/19*</td>
<td>14/18*</td>
<td></td>
</tr>
<tr>
<td>Sputum RQ-PCR</td>
<td>126</td>
<td>34 (27%)</td>
<td>28</td>
<td>13/34*</td>
<td>13/33*</td>
<td>16/34*</td>
<td>22/32*</td>
<td></td>
</tr>
<tr>
<td>NPH culture</td>
<td>158</td>
<td>42 (27%)</td>
<td>35</td>
<td>16/41*</td>
<td>19/39*</td>
<td>14/34*</td>
<td>22/33*</td>
<td></td>
</tr>
</tbody>
</table>

* The denominator represents the number of patients with positive findings in column 3 that also were tested with the other methods.

Table 6. Distribution of pneumococcal findings with different methods in patients with complete collection of cultures from blood and nasopharyngeal secretions, urine antigen assays, sputum sample collected at the ER and analysed by culture as well as RQ-PCR (n=35).

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive findings n (%)</th>
<th>Findings with ≥ 1 additional method n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture</td>
<td>7 (20%)</td>
<td>6</td>
</tr>
<tr>
<td>Urine antigen</td>
<td>7 (20%)</td>
<td>6</td>
</tr>
<tr>
<td>Sputum culture</td>
<td>13 (37%)</td>
<td>13</td>
</tr>
<tr>
<td>Sputum RQ-PCR</td>
<td>17 (49%)</td>
<td>15</td>
</tr>
<tr>
<td>NPH culture</td>
<td>13 (37%)</td>
<td>13</td>
</tr>
</tbody>
</table>
4.1.2.3  Proven pneumococcal aetiology—correlation between different methods of analysing respiratory secretions

According to our definitions, a pneumococcal aetiology was proved in 43 patients with positive blood culture and/or urine antigen detection. Significantly, positive results by sputum culture and RQ-PCR were found in 9/29 (31%) and 16/27 (59%) ($P < 0.001$) respectively of these cases (Table 7). All these sputum samples were collected within 24 h after starting antibiotic treatment.

Table 7. Correlation between culture and RQ-PCR to diagnose pneumococci in sputum in patients with definite pneumococcal aetiology and varying exposure time to antibiotics (n=43).

<table>
<thead>
<tr>
<th>Patients with pneumococcal findings</th>
<th>No exposure to antibiotics prior to sampling</th>
<th>Exposure to antibiotics &lt; 24 hours prior to sampling</th>
<th>Exposure to antibiotics &gt; 24 hours prior to sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum culture $\geq 10^5$ cfu/mL</td>
<td>9/29 (31%)</td>
<td>5/12*</td>
<td>4/10*</td>
</tr>
<tr>
<td>Sputum RQ-PCR (amount corr to $\geq 10^5$ cfu/mL)</td>
<td>16/27 (59%)</td>
<td>8/10*</td>
<td>8/10*</td>
</tr>
</tbody>
</table>

*The denominator represents the number of patients with samples collected during this time interval

4.1.2.4  Findings of pneumococci with different methods in relation to exposure of antibiotics

Figure 3 illustrates positive pneumococcal findings with different methods before and after initiation of antibiotic therapy, and includes sputum samples obtained at the ER as well as those obtained on the ward. In 46 patients, two sputum samples were examined by culture, and in 26 patients two specimens were examined with RQ-PCR. Samples collected before exposure to antibiotic therapy were positive by sputum culture, sputum RQ-PCR and nasopharyngeal culture in 29%, 51%, and 31% of the cases, respectively. The corresponding numbers after start of antibiotic therapy were 4%, 19%, and 11%, respectively.
Figure 3. Findings of pneumococci with different methods and the correlation with exposure to antibiotic therapy when samples were collected. The figures above the bar charts represent number of patients tested with the respective methods.

Several earlier studies have verified the findings of pneumococci in sputum cultures by other methods, indicating high specificity (Macfarlane et al. 1982; Kalin et al. 1983; Burman et al. 1991). Similar findings were made in the present study where sputum-positive cultures were confirmed by other methods in 95% of cases.

On the contrary the sensitivity of sputum culture has often proved relatively poor, and in a meta-analysis performed by Skerrett et al the mean yield of sputum cultures was 56% (Skerrett 1999). One reason for this is the difficulties in obtaining sputum samples from a considerable part of CAP patients (Bartlett 2004; Musher et al. 2004); and earlier reports regarding the effect of sputum induction by hypertonic saline on specimen quality have shown conflicting results (Noseda et al. 1994; Chuard et al. 2001). In our study we showed, however, that the frequency of sputum samples could be markedly increased by sputum induction with assistance from a respiratory physiotherapist.

It has also been noted that previous antibiotic treatment clearly reduces the possibility to achieve a culture-positive sputum sample (Kalin et al. 1983; BTS 1987; Ortgvist et al. 1990; Lim et al. 2001; Saranglao et al. 2002; Bartlett 2004), and this was also the case in the present study. However, when studying patients with proven pneumococcal aetiology and no antimicrobial therapy prior sampling, only 5/12 sputum samples showed pneumococci. This is in contrast to previous studies. Kalin et al. found sensitivity for sputum culture of 70% in bacteraemic patients when high-quality washed sputum without exposure to antibiotics was studied, and in a corresponding
way Musher et al. found a sputum culture sensitivity of 93% (Kalin 1982; Musher et al. 2004). In the present study no washing procedure was performed which could perhaps contribute to the low pneumococcal yield in sputum cultures.

PCR has most often been found to be more sensitive than cultures but the main challenge of regular PCR methods from airway samples has been the difficulties in distinguishing colonisation from infection (Murdoch 2004). This obstacle could potentially be overcome by the use of a quantitative PCR assay. In the present study the yield with sputum RQ-PCR was twice as high as with sputum culture, in the whole study population as well as among those with a proven pneumococcal aetiology. PCR was most of all superior to sputum culture when antibiotics had been initiated. It also afforded the highest pneumococcal yield among those patients with perfect samples as well as in the whole study population (together with NPH culture). Further, compared to other conventional pneumococcal methods, RQ-PCR showed both high sensitivity and high specificity, supporting a cut-off of $10^5$ CFU/mL. Similar results were also found by Yang et al. when they compared sputum RQ-PCR, using the pneumolysin gene, with sputum culture for identification of pneumococci. They found that, based on the ROC curve analysis, the maximal sensitivity and specificity for the PCR assay was a cut-off corresponding to approximately $3.7 \times 10^4$ genomic equivalents/mL. At this threshold the sensitivity was 90% and the specificity 80% (Yang et al. 2005). Aetiological pneumonia studies using RT-PCR applied on BAL and transthoracic needle puncture found *S. pneumoniae* in a considerable part of the cases where analysis with conventional methods gave negative results (Ruiz-Gonzalez et al. 1999; Apfalter et al. 2005). It is therefore not surprising that the pneumococcal findings with sputum RQ-PCR in the present study increased the total yield of *S. pneumoniae*.

False-positive PCR results from respiratory specimens are not always due to the high sensitivity of the method, resulting in difficulties to distinguish colonisation from true infection. Such results can also be due to problems of specificity. Initially the pneumolysin gene was widely used in PCR studies as a target gene for pneumococci. More recently, however, it has become clear that the ply gene also can be detected in Viridans-group streptococci, raising questions regarding the specificity of using the ply gene for *S. pneumoniae* (Klugman et al. 2008; Werno et al. 2008; Blaschke 2011). Pneumococcal DNA in PCR reactions applied on respiratory specimens has been found in a substantial proportion of asymptomatic persons (Murdoch et al. 2003; Abdeldaim et al. 2008). Moreover, in a comparison between different target genes, the lytA gene, the PsaA gene and the Spn9802 gene fragment have all been tested favourably regarding specificity compared to the ply gene (Carvalho Mda et al. 2007; Abdeldaim et al. 2008; Abdeldaim et al. 2010). The ply gene was used in the present study, possibly calling into question the specificity of our analysis. However, our analysis was quantitative, and any amount of DNA from *S. mitis* would probably be low. Moreover, previous data of the negative results from throat samples in healthy control group support a good specificity (Kais et al. 2006), and the positive findings in the present study were confirmed using other diagnostic methods. However, in future studies, it will be important to take into account the choice of primers.

Blood culture provided a diagnosis in 15%, which is comparable to previous reports (Werno et al. 2008).
As stated above the sensitivity of urine antigen detection for pneumococci has been estimated to 70-80% (Bartlett 2011). Similar findings were made in the present study when only the bacteraemic pneumococcal cases were analysed (65%). However, among the patients with non-invasive pneumococcal aetiology, where the positive sputum RQ-PCR results were also included, the sensitivity was only 40% which indicates that the urine antigen is most useful in invasive cases.

Nasopharyngeal culture is easy to obtain and pneumococcal findings correlate well with the aetiology of the pneumonic process (Kalin 1982; Hedlund et al. 1990). In the present study NPH culture, together with sputum RQ-PCR, was the method that performed with the highest yield for \textit{S. pneumoniae}.

As stated above qualitative nasopharyngeal PCR assays are more sensitive than culture but cannot separate asymptomatic carriage from infection. One way to deal with this is to use quantitative nasopharyngeal PCR assays, which have in some recent studies shown promising results (Abdeldaim et al. 2008; Albrich et al. 2012).

### 4.1.3 Aetiology of community-acquired pneumonia

#### 4.1.3.1 Microbiological aetiology of CAP

A definite or probable microbial aetiology of CAP was established for 124 (67%) of the 184 patients when RQ-PCR assays for \textit{S. pneumoniae}, \textit{H. influenzae}, and \textit{M. catarrhalis} from sputum samples and the PCR platform for respiratory viruses were added to conventional methods. In contrast, the microbial yield was 60\% (110 of 184 patients) when only conventional methods were used. A bacterial aetiology was found for 106 patients (58\%) (Table 8). The most frequently detected bacteria were \textit{S. pneumoniae} (70 patients [38\%]), \textit{M. pneumoniae} (15 patients [8\%]), and \textit{H. influenzae} (9 patients [5\%]). A viral pathogen was identified for 53 (29\%) of the 184 patients (Table 9). The most common viral findings were influenza virus (14 patients [8\%]) and rhinovirus (12 patients [7\%]).

#### 4.1.3.2 Diagnostic yield with different bacteriological diagnostic methods

The contribution of the different methods to the determination of aetiology is illustrated in Tables 8 and 9. Blood cultures provided a microbial diagnosis for 31 (17\%) of 179 patients. A probable diagnosis was established with sputum culture for 33 (26\%) of 128 patients, which increased the diagnostic yield by an additional 22 cases. Positive findings with sputum RQ-PCR for \textit{S. pneumoniae}, \textit{H. influenzae}, and \textit{M. catarrhalis} were obtained for 42 (33\%) of 126 patients (1 patient tested positive for both \textit{S. pneumoniae} and \textit{H. influenzae}), which increased the diagnostic yield by an additional 15 cases.
Table 8. Bacterial yield in the study population (n=184) and the contribution of different methods to the aetiology with respect to their different specificity. Additional patients diagnosed by the respective method

| Pathogen                        | Total number of positive findings any method (%) | Blood culture n=179 | Culture pleura fluid n=13 | Urine antigen assays n=168 (LP) | Culture BAL b / protected specimen brush n=12 | Culture and/or PCR c from sputum sample for LP d n=138 | Culture and PCR from respiratory sample for TB d n=18 | Sputum culture n=128 | RQ-PCR spatum n=126 | Culture nasopharyngeal secretions n=158 | PCR nasopharyngeal secretions n= 99 (CP) 101 (MP) e
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumoniae</td>
<td>70 (38)</td>
<td>27</td>
<td>16</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>15 (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>9 (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>7 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>4 (2)</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>3 (1)</td>
<td></td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>2 (1)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus milleri</td>
<td>1 (0,5)</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nocardia cyriacigeorgica</td>
<td>1 (0,5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusobacterium necrophorum</td>
<td>1 (0,5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>2 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total amount of bacterial findings</td>
<td>115</td>
<td>31</td>
<td>2</td>
<td>18</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>22</td>
<td>15</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

Note. E.g. for *S. pneumoniae* an additional 16 cases were diagnosed by urinary antigen test that were not diagnosed by blood culture, another 10 cases by sputum culture that were not diagnosed by blood culture or urinary antigen test, etc.

a LP= *Legionella pneumophila*. SP= *Streptococcus pneumoniae*
b BAL= Bronchoalveolar lavage
c PCR= Polymerase chain reaction
d TB= *Mycobacterium tuberculosis*
e CP= *Clamydophila pneumoniae*. MP= *Mycoplasma pneumoniae*
4.1.3.3 Diagnostic yield with different viral diagnostic methods

Eight cases were detected by viral isolation. Serology accounted for 21 positive findings, in 1 case for both the influenza and the parainfluenza virus. The PCR results of the nasopharyngeal secretion samples of 35 patients were positive, and five of these also had serum samples that tested positive. Influenza virus was the most common finding (i.e., 14 [8\%] of 184 patients) (Table 9). Nine different viral agents were identified. No viral sequences were identified by molecular virus screening in the set of PCR-negative samples.

Table 9. Viral yield in the study population (n=184) and the contribution of different methods to the aetiology with respect to their different specificity. Additional patients diagnosed by the respective method

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Total number of positive findings, any method (%)</th>
<th>Culture nasopharyngeal secretions n=157</th>
<th>Serology n=131</th>
<th>PCR a nasopharyngeal secretions n=156</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza virus</td>
<td>14 (8)</td>
<td>3</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>12 (7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>7 (4)</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Parainfluenzavirus</td>
<td>7 (4)</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>4 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metapneumovirus</td>
<td>4 (2)</td>
<td>1</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>3 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes simplex 1 virus</td>
<td>2 (1)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterovirus</td>
<td>1 (0.5)</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total amount of viral findings</td>
<td>54 (29)</td>
<td>8</td>
<td>20</td>
<td>26</td>
</tr>
</tbody>
</table>

Note. - For example, for Influenza virus an additional seven cases were diagnosed with serology which were not diagnosed with virus isolation and another four cases by PCR from nasopharyngeal secretions, not diagnosed with either.

* PCR= Polymerase chain reaction

4.1.3.4 Mixed infections

Conventional diagnostic methods identified two potential pathogens for 20 (11\%) of the 184 patients and three pathogens for two patients (1\%). PCR testing for S. pneumoniae, H. influenzae, M. catarrhalis, and respiratory viruses increased the total diagnostic yield to 42 (23\%) and four (2\%) patients with two and three pathogens, respectively. Of the 106 patients with a definite or probable bacterial aetiology, 42 (40\%) presented with mixed infections of which 35 (33\%) were a viral co-pathogen. When S. pneumoniae was identified, a co-pathogen was found in the samples of 34 of 70 patients: six bacterial pathogens (three of which were isolates of H. influenzae) and 29 viral agents (seven of which were isolates of influenza virus and seven isolates of rhinovirus). Of the 53 patients with documented viral findings, two-thirds (i.e., 35 patients) had at least one additional pathogen identified, of whom 29 (83\%) had S. pneumoniae isolated.
4.1.3.5 Microbiological yield with different methods among patients with complete sample collection

Thirty-eight patients had complete samples: blood, sputum, and nasopharyngeal secretion samples for culture; sputum samples analyzed with RQ-PCR for *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*; nasopharyngeal specimens analyzed by use of PCR; serological testing for *M. pneumoniae*, *C. pneumoniae*, and viruses common in the respiratory tract; and urine antigen assays for detection of pneumococcal and *L. pneumophila* antigens. None of these patients had been given antibiotics before admission to hospital (Figure 4).

According to our diagnostic criteria, a microbial aetiology was established for 34 (89%) of the 38 patients. Multiple pathogens were detected in the samples of 12 patients (32%). Thirty-one patients (82%) received a diagnosis of bacterial infection. For 24 patients (63%), *S. pneumoniae* was the most common bacterial finding, and a second microbial agent was found for 12 (50%) of the 24 patients; of these 12 patients, 10 (83%) had a respiratory virus infection. Thirteen (34%) of the 38 patients received a diagnosis of viral infection. For 10 (77%) of these 13, a bacterial pathogen (predominantly *S. pneumoniae*) was also found.

Figure 4. Total etiology among the patients with complete samples collected (n=38) in percent and occurrence of mixed infections.
Establishing a microbial diagnosis for patients with CAP is challenging. In the present study, aetiology was found in two-thirds of all cases, and the yield increased to ~90% for patients with complete samples collected and no antibiotics given prior to hospital admission. The yield improved by adding to the conventional procedures RQ-PCR testing of sputum samples for *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* and real-time PCR testing of nasopharyngeal secretion samples for 15 respiratory viruses.

*S. pneumoniae* has always been the most common bacterial finding in CAP, but since the introduction of antimicrobial therapy the proportion of pneumococcal findings in aetiological studies performed has varied widely between 7.6-76% (Lim et al. 2009; Bartlett 2011). In 1981 White et al in found evidence for a pneumococcal aetiology in only 11.5% of hospitalised patients with CAP (White et al. 1981). However, of all patients where no pathogens could be identified, 75% had either received antibiotics before entering hospital, or were unable to produce any sputum for culture. In contrast, MacFarlane et al, the year after, in 1982 identified a pneumococcal aetiology in 76% of hospitalised CAP patients (Macfarlane et al. 1982). In that study sputum samples for microbiological analysis were obtained from almost all patients and cultures and antigen-detection were also done from post-mortem lung tissue. In addition most of the pneumococcal cases were identified by CIE in sputum samples using polyvalent pneumococcal anticapsular serum, and as stated above, this method has later been questioned regarding its specificity due to reports concerning cross-reaction with α-streptococci (Farrington et al. 1991).

Evidence for an *S. pneumoniae* diagnosis in different Swedish studies has varied between 32-54% (Kalin et al. 1983; Berntsson et al. 1985; Holmberg 1987; Ortvist et al. 1990; Burman et al. 1991; Stralin et al. 2010). According to a recent review article from Bartlett, there is a tendency that the fraction of pneumococcal findings in aetiological studies has gradually decreased over the past 70 years (Bartlett 2011). In the present study *S. pneumoniae* accounted for 38% of the whole CAP aetiology, findings similar to other Swedish studies. However, when only those with complete diagnostic samples and no antibiotics prior sampling were studied, approximately two-thirds fulfilled the criteria for a pneumococcal diagnosis. These findings support earlier suggestions that pneumococci cause a majority of CAP cases which have shown negative results using conventional methods (2001); (Ruiz-Gonzalez et al. 1999; Lim et al. 2001) and that *S. pneumoniae* is still responsible for more than half of all CAP cases.

*H. influenzae* was found in 5% of the whole study population, a number comparable to other studies (Mandell et al. 2007; Lim et al. 2009). The only methods in the present study to detect this pathogen were sputum samples analysed by culture (n=4) and by RT-PCR (n=6). *H. influenzae* is a common coloniser of the upper airways especially after antibiotic treatment (Kalin et al. 1983; Burman et al. 1991), and the interpretation of *H. influenzae* findings in respiratory-tract samples has therefore often been difficult. In some CAP studies only findings of *H. influenzae* from blood or pleura have therefore been counted as proven aetiology (Wallace et al. 1978).

*M. catarrhalis* has been identified in earlier studies as a pathogen in 1.2-3.1% (BTS2001). The present study showed similar results. Using sputum RQ-PCR did not result in an increased detection rate of *M. catarrhalis*, which supports previous reports of a low total incidence regarding CAP due to *M. catarrhalis*. 
The incidence of *M. pneumoniae* varies broadly in different CAP studies (Lehtomaki et al. 1988; Lieberman et al. 1996). Many epidemiologic studies of CAP have however showed an incidence between 5-15% (von Baum et al. 2009) which is comparable to the 8% in the present study. One explanation to the marked variation in incidence is the occurrence of *M. pneumoniae* in cyclical epidemic peaks, but probably it also depends on what population has been studied and what diagnostic methods that have been used (Hammerschlag 2001).

*C. pneumoniae* is reportedly a frequent pathogen in CAP cases admitted to hospital with an incidence of 3.1-13.1% (2001; Lim et al. 2001). This organism has also been claimed to be a common pathogen in mixed infections (Lim et al. 2001). In the present study no cases with *C. pneumoniae* were found. Previous retrospective serological studies in Sweden have shown conflicting results, with incidences of *C. pneumoniae* ranging from 1.3% (Kragsbjerg et al. 1992) to 12.2% (Sundelof et al. 1993). Örtqvist et al. and Burman et al. found no cases with *C. pneumoniae* (Örtqvist et al. 1990; Burman et al. 1991). One possible reason for the absence of *C. pneumoniae* in the present study might be due to a combination of regionally and yearly variation of the incidence. Further, the clinical relevance of *C. pneumoniae* in respiratory tract infections has also been debated. Finnish studies have associated mixed infections with *C. pneumoniae* with severe CAP, while other studies have found the opposite (Lim et al. 2001). Moreover the diagnostically assays available and the interpretation of their results have not been sufficiently validated and standardised, for which reason questions have been raised regarding their sensitivity and specificity (Dowell et al. 2001; Howard et al. 2005; Kumar et al. 2007).

During the past few years there has been increased interest in viruses causing respiratory infections, and there are probably many reasons for this. New respiratory viral pathogens have been discovered, new emerging (Angeles Marcos et al. 2006) severe viral epidemics, such as SARS, avian influenza A (H5N1) and the 2009 pandemic influenza A (H1N1) have occurred and, not least, since the introduction of new, rapid and sensitive molecular viral methods the possibility to detect respiratory viruses has increased considerably.

In the present study support for a viral aetiology was found in one-third of the patients. The new real-time PCR platform provided a viral diagnosis for 35 (22%) of the 156 patients tested, and the addition of this method to the conventional microbial techniques improved the yield by nearly 50%. In the past few years several other CAP studies, with implementation of RT-PCR for viruses, have been performed, and all have showed similar findings with increased viral yield (Oosterheert et al. 2005; Templeton et al. 2005; Angeles Marcos et al. 2006; Saïto et al. 2006; Diaz et al. 2007; Hohenthal et al. 2008; Jennings et al. 2008; Johnstone et al. 2008; Diederer et al. 2009; Lieberman et al. 2010). The increased viral yield with RT-PCR in these studies has been due mainly to viruses difficult to detect with conventional methods, such as HRV, enterovirus, HMPV and HBoV.

Comparable to the present study, Templeton et al. and Jennings et al. also combined different viral diagnostics (i.e. viral isolation, viral serology, virus RT-PCR) in their studies and found a viral yield in 56% and 29% of all cases respectively (Templeton et al. 2005; Jennings et al. 2008). When only the patients with complete samples in the present study were analysed, a viral agent was identified in 34% of all cases, the same proportion that was found in the study by Jennings et al. (Jennings et al. 2008).

Samples for viral detection with RT-PCR are for the most part collected from the upper airways, and the interpretation of these viral findings has been complex.
Although respiratory viruses have been linked mainly to upper-airway infections in adults, an increasing number of studies have reported viral involvement also in LRTI (Ruuskanen et al. 2011; Cesario 2012; Jartti et al. 2012). Further, in a recent review of the prevalence of respiratory viral findings for asymptomatic subjects, available reports suggest that the prevalence is infrequent (~ 5%), and that the persistence of viral nucleic acids is rather brief even though definitive conclusions not could be drawn given the lack of prospective studies (Jartti et al. 2008).

Taken as a whole, these data indicate that a majority of the viral findings in the present study had clinical relevance, although their role in CAP has to be further elucidated.

The literature differs widely regarding findings of more than one pathogen in CAP. On average the prevalence of mixed infections in previous studies has been around 10% (BTS 1987; Woodhead et al. 1987; BTS 2001). In the present study mixed infections were found in the samples of one-third of the 124 patients with a determined aetiology as well as in the samples of those patients with complete sampling. In our study, *S. pneumoniae* was the most common bacterial agent in mixed infections, and a co-pathogen was found in one-half of the cases, the majority (i.e., 83%-85%) being viral agents. The majority of our CAP patients with a viral finding also had a bacterial aetiology.

Other recent CAP studies, using RT-PCR for viral detection, have also found mixed bacterial-viral infections but their yield was, except for one study (Templeton et al. 2005), generally lower than ours (Angeles Marcos et al. 2006; Saito et al. 2006; Diaz et al. 2007; Jennings et al. 2008; Johnstone et al. 2008; Diederen et al. 2009). The reason for this could probably be the improved bacteriological methods we used. Our data tally with those from a recent study by Lim et al (Lim et al. 2001) and indicate that the incidence of mixed infections in CAP has previously been underestimated.
4.1.4 Clinical impact of combined bacterial and viral infections in patients with CAP

Of 106 patients with a bacterial aetiology, 35 (33%) presented with a viral co-infection as well. When *S. pneumoniae* was identified, a viral co-pathogen was found in the samples of 29 of 70 patients (41%). The characteristics of patients with only bacterial infections vs. those with mixed bacterial-viral infections are shown in Table 10.

Table 10. Baseline characteristics among CAP patients with mono- vs. mixed infections.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with a bacterial aetiology alone n=71 (%)</th>
<th>Patients with a bacterial and viral aetiology n=35 (%)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean age</td>
<td>55.8 / 61.9</td>
<td>34 (48) / 37 (52) / 16 (46) / 19 (54)</td>
<td>0.11</td>
</tr>
<tr>
<td>male/female</td>
<td>3 (4) / 1 (3)</td>
<td>2 (3) / 4 (11)</td>
<td>0.83</td>
</tr>
<tr>
<td>nursing home</td>
<td>2 (3) / 4 (11)</td>
<td>2 (3) / 4 (11)</td>
<td>1</td>
</tr>
<tr>
<td>any co-morbidity</td>
<td>8 (11) / 4 (11)</td>
<td>2 (3) / 4 (11)</td>
<td>0.03</td>
</tr>
<tr>
<td>COPD/chronic bronchitis</td>
<td>3 (4) / 2 (6)</td>
<td>9 (13) / 10 (29)</td>
<td></td>
</tr>
<tr>
<td>heart failure</td>
<td>3 (4) / 2 (6)</td>
<td>9 (13) / 10 (29)</td>
<td></td>
</tr>
<tr>
<td>cancer</td>
<td>2 (3) / 2 (6)</td>
<td>2 (3) / 4 (11)</td>
<td></td>
</tr>
<tr>
<td>liver disease</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>renal failure</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>antibiotics prior to hospital admission</td>
<td>11 (15) / 6 (17)</td>
<td>11 (15) / 6 (17)</td>
<td>0.83</td>
</tr>
<tr>
<td>mortality rate</td>
<td>1 (1) / 2 (6)</td>
<td>1 (1) / 2 (6)</td>
<td>0.25</td>
</tr>
<tr>
<td>treatment in the intensive care unit</td>
<td>7 (10) / 5 (14)</td>
<td>7 (10) / 5 (14)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

4.1.4.1 Patients in PSI classes IV and V

The likelihood of having a score corresponding to PSI classes IV or V was higher in patients with both a bacterial and a viral agent than in those with a bacterial pathogen alone (OR, 4.98; 95% CI 2.09-11.89; *P* < 0.001) (figure 5). The same pattern was seen when only patients with *S. pneumoniae* infections with or without viral co-infection, respectively, were considered (OR, 3.52; 95% CI 1.30-9.56; *P* = 0.013) (Figure 5). A higher mean score for the co-morbidity part of the PSI score was obtained in patients infected with both bacteria and virus than in those without viral findings (11.4 vs. 5.1 points, *P* = 0.013). Scores based on the remaining 12 predictors, after exclusion of co-morbidity, age, sex and nursing-home living, were, however, still higher for patients with mixed infections than for those with purely bacterial infections in the entire material (32.7 vs. 16.2, *P* = 0.004) as well as among *S. pneumoniae*-infected patients (32.9 vs. 17.1, *P* = 0.019).
Figure 5. Proportion of patients in PSI classes IV or V among patients with a pure bacterial aetiology and among those with a mixed bacterial - viral aetiology. The figures above the bar charts represent the number of patients with an established microbiological aetiology.

4.1.4.2 Length of hospital stay

Three patients with an established microbiological aetiology died during their hospital stay. The median length of hospital stay among the remaining 70 patients with a pure bacterial diagnosis was four days, compared to seven days ($P = 0.018$) among the 33 patients with mixed bacterial–viral infections. The difference in length of hospital stay between the two patient cohorts remained significant ($P = 0.038$) after adjustment for co-morbidity (table 10). Time to improvement of respiratory rate and time to discontinuation of oxygen therapy were significantly longer in patients with identification of both virus and bacteria, and there was also a tendency to longer time to defervescence among these patients ($P = 0.087$) (Figure 6).
Our patients infected with both bacteria and virus generally presented with a more severe disease according to the PSI classification than those with a bacterial aetiology alone. The increase in disease severity in mixed infections was also seen if only patients with a pneumococcal aetiology were considered. Also, when we analysed PSI data modified by exclusion of age, sex, nursing home residence and co-morbidity, the significant difference in disease severity remained between the two groups.

Further, when CRB-65 was used as a severity score, the likelihood of an increased risk of death, corresponding to a CRB-65 score $\geq 2$, was higher in patients infected with both a bacterial and a viral agent than in those with a bacterial pathogen alone (OR 4.97, 95% CI 1.65 – 14.91; $P = 0.002$). The difference in CRB-65 score between these two patient cohorts remained significant ($P = 0.015$) after adjustment for co-morbidity (Table 10). That mixed infections may affect the severity of the disease was also supported by findings of a longer hospital stay in these patients, which may at least partly be explained by a prolonged time to defervescence, improvement of respiratory rate and cessation of oxygen therapy.

Few other studies using PCR-based methods have investigated the relationship between mixed infections and clinical outcome. Correlation between mixed infections and severity has shown conflicting results. While two studies discovered an association between combined bacterial-viral infections and severity according to PSI IV and V (Templeton et al. 2005; Jennings et al. 2008), two others did not (Angeles Marcos et al. 2006; Johnstone et al. 2008). Diaz et al. found that mixed infections were associated with prolonged length of hospital stay (Diaz et al. 2007).
Among the present patients with a microbiological aetiology study only 12 were treated in the ICU and no more than three patients died in hospital. Thus, this study was too small to identify any potential differences between the patients with a bacterial diagnosis. However, another recent study found increased mortality among patients with mixed bacterial-viral infections (Diederen et al. 2009).

There is major evidence of synergistic pathogenesis between influenza and, especially, *S. pneumoniae*. Influenza virus influences the host to predispose for adherence, invasion, and induction of disease by pneumococci. Epithelial injury is however not the only synergistic mechanism. Influenza virus also impairs the innate immune mechanisms with altered neutrophil and macrophage function and defective cytokine responses to *S. pneumoniae* (McCullers 2006; Giamarellos-Bourboulis et al. 2009; van der Sluijs et al. 2010; Nakamura et al. 2011). During typical influenza epidemic years both hospitalisation and death are more frequent due to secondary bacterial pneumonia than to the primary viral infection, and combined influenza-bacterial infections are usually more severe than isolated bacterial infections (McCullers 2006; van der Sluijs et al. 2010).

Support from studies for similar interactions between other viral and bacterial agents is, however, on the whole lacking. Yet, there are some few reports. Studies regarding HRV have shown that rhinovirus predisposes to adherence of pneumococci in the mucous membrane of the trachea (Ishizuka et al. 2003) and it has also been reported that HRV exposure impairs immune responses to bacterial products in human alveolar macrophages (Oliver et al. 2008).

Detection of multiple pathogens in respiratory tract infections will probably be reported more frequently in the future due to the more sensitive diagnostic methods now available. This emphasizes the need for studies on the interaction between bacteria and virus.

### 4.1.5 Procalcitonin levels in community-acquired pneumonia – correlation to aetiology and severity

Of our 184 patients 124 had a definite or probable microbiological aetiology. Serum samples were collected on admission in 65 of these cases and analysed for inflammatory biomarkers (Table 11). The median length of hospital stay was five days (range 2–69 days). Five patients (7.6%) were treated in the ICU and two (3.1%) died in hospital. In 57 patients a bacterial aetiology was found, and in 21 of these a significant viral finding was also made. In eight patients a viral pathogen was found alone (Table 12).
Table 11. Baseline characteristics of 65 patients with community-acquired pneumonia admitted to hospital with serum specimens obtained.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients n=65 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age</td>
<td>58.3 years</td>
</tr>
<tr>
<td>Male/female</td>
<td>33 (51) / 32 (49)</td>
</tr>
<tr>
<td>nursing home</td>
<td>3 (5)</td>
</tr>
<tr>
<td>any co-morbidity</td>
<td>26 (40)</td>
</tr>
<tr>
<td>COPD/&gt;chronic bronchitis</td>
<td>12 (18)</td>
</tr>
<tr>
<td>heart failure</td>
<td>2 (3)</td>
</tr>
<tr>
<td>cerebrovascular disease</td>
<td>6 (9)</td>
</tr>
<tr>
<td>malignancy</td>
<td>14 (22)</td>
</tr>
<tr>
<td>liver disease</td>
<td>1 (2)</td>
</tr>
<tr>
<td>renal failure</td>
<td>0 (0)</td>
</tr>
<tr>
<td>antibiotics prior to hospital admission</td>
<td>8 (12)</td>
</tr>
<tr>
<td>mortality rate</td>
<td>2 (3)</td>
</tr>
<tr>
<td>treatment in the intensive care unit</td>
<td>5 (8)</td>
</tr>
</tbody>
</table>

*a COPD= Chronic obstructive pulmonary disease

Table 12. Findings of microorganisms among the patients with biomarkers analysed (n=65)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of bacterial findings *</td>
<td>59 (91)</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>36 (55)</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>4 (6)</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Streptococcus milleri</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Fusobacterium necrophorum</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Anaerobe mixed flora</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Total number of viral findings *</td>
<td>30 (46)</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>9 (14)</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>7 (11)</td>
</tr>
<tr>
<td>Parainfluenzavirus</td>
<td>7 (11)</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Metapneumovirus</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>
4.1.5.1 Correlation of PCT and CRP to bacteraemia and severity

The bacteraemic patients had significantly higher median concentrations of PCT as well as CRP in comparison to those without bacteraemia (Table 13). The median values of CRP in patients within PSI classes 1-3 and 4-5 respectively were similar: 225 mg/L (range 35.4-456 mg/L) versus 205 (24-435) (P=0.58). In contrast, the median PCT concentration was higher in PSI classes 4-5 (2.1 µg/L, 0.03-150 µg/L) than in PSI classes 1-3 (0.52, 0.01-57, P=0.03). The PCT values were low in non-bacteraemic patients; median 0.2 (0.01-20.9) in PSI classes 1-3 and 0.88 (0.03-150) in PSI classes 4-5 compared to levels noted in those with bacteraemia, 4.4 (0.48-57) and 7.9 (2.1-26.8) respectively with low and high PSI (Figure 7).

Figure 7. Correlations of median serum concentrations of procalcitonin and C-reactive protein to Pneumonia Severity Index (PSI) and bacteraemia (n = 65).

*PCT = Procalcitonin  
CRP = C-reactive protein  
The bar charts represent the median values. The figures above the bar charts represent the numbers of patients.
### Table 13. Inflammatory biomarkers correlated to microbial aetiology

<table>
<thead>
<tr>
<th></th>
<th>Bacteria (n=57)</th>
<th>Bacteria (n=8)</th>
<th>P value*</th>
<th>Totaly (n=16)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. pneumoniae</strong> (n=22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procalcitonin* (µg/L)</td>
<td>1.18 (0.01-20.10)</td>
<td>0.18 (0.01-150)</td>
<td>0.038</td>
<td>6.10 (0.48-57)</td>
<td>0.0002</td>
</tr>
<tr>
<td>C-reactive protein* (mg/L)</td>
<td>212 (24.0-317)</td>
<td>192 (104-297)</td>
<td>0.55</td>
<td>281 (120-456)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Transthyretin* (mg/L)</td>
<td>82.5 (24.2-171)</td>
<td>94.3 (31.1-295)</td>
<td>0.32</td>
<td>46.5 (29.0-126)</td>
<td>0.02</td>
</tr>
<tr>
<td>Interleukin 6* (log10)</td>
<td>2.63 (1.20-4.40)</td>
<td>2.65 (1.80-4.18)</td>
<td>0.86</td>
<td>2.60 (1.98-4.29)</td>
<td>0.008</td>
</tr>
<tr>
<td>Interleukin 6* (µg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.65 (1.20-4.42)</td>
<td>2.10 (1.70-3.50)</td>
<td>0.042</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values within parentheses denote 95% confidence interval.
a Procalcitonin, C-reactive protein, Transthyretin and Interleukin 6 results are presented as median values. The values within the brackets refer to the range.

b 5 Haemophilus influenzae, 4 Moraxella catharralis, 2 Legionella pneumophila, 1 Streptococcus pyogenes, 1 anaerobic mixed flora, 1 Streptococcus milleri

c P values refer to the comparison of CAP cases with S. pneumoniae vs. other bacteria except for M. pneumoniae.

d All patients with non-bacteraemic aetiology including M. pneumoniae (n=5).

e 14 S. pneumoniae, 1 Staph aureus, 1 Fusobacterium necrophorum

f P values refer to the comparison of CAP cases with non-bacteraemic vs. bacteraemic aetiology.

g P values refer to the comparison of CAP cases with bacterial vs. viral aetiology.

h Interleukin 6 was analysed in 12 of these 14 patients

i Interleukin 6 was analysed in 38 of these 41 patients

j Interleukin 6 was analysed in 54 of these 57 patients
4.1.5.2 Biomarkers in non-bacteraemic patients

In non-bacteraemic patients the median PCT level was higher in the group with pneumococcal aetiology than in those with *M. pneumoniae* infection (1.18 vs. 0.034, *P*=0.009) and those with pneumonia caused by other respiratory-tract bacteria (1.18 vs. 0.18, *P*=0.038) (Figure 8). No significant difference was observed between patients with non-bacteraemic pneumococcal aetiology and *M. pneumoniae* diagnosis regarding CRP (212 vs. 113, *P*=0.25), transthyretin (83 mg/L vs. 64 mg/L, *P*=0.82) or IL-6 (2.6 pg/mL vs. 2.1 pg/mL, *P*=0.18). Neither was there any significant difference concerning biomarkers other than PCT between patients with non-bacteraemic pneumococcal disease vs. those with pneumonia caused by other bacteria (Table 13).

PCT levels did not differ significantly between patients with any bacterial infection and isolated viral disease (Table 13), but was significantly higher in patients with pneumococcal aetiology than in those with viral infection (2.4, range 0.01-26.8 vs. 0.24, range 0.04-6.5, *P*=0.017) (Figure 8). Except for IL-6, the other biomarkers seemed not to be differently affected by bacterial vs. viral agents. Interestingly, none of the biomarkers studied differed significantly in patients with a single pathogen identified as compared to those with support for a mixed bacterial-viral aetiology (Table 14).
Table 14. Correlation of inflammatory biomarkers to patients with findings of bacteria alone and to those with mixed infections

<table>
<thead>
<tr>
<th></th>
<th>Bacteria alone (n=36)</th>
<th>Bacteria and virus (n=21)</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Procalcitonin</strong>&lt;sup&gt;a&lt;/sup&gt; (µg/L)</td>
<td>0.41 (0.01-57)</td>
<td>2.1 (0.07-150)</td>
<td>P = 0.078</td>
</tr>
<tr>
<td><strong>C-reactive protein</strong>&lt;sup&gt;a&lt;/sup&gt; (mg/L)</td>
<td>221 (83.6-456)</td>
<td>242 (24.0-435)</td>
<td>P = 0.35</td>
</tr>
<tr>
<td><strong>Transthyretin</strong>&lt;sup&gt;a&lt;/sup&gt; (mg/L)</td>
<td>80.1 (24.2-295)</td>
<td>57.0 (32.6-113)</td>
<td>P = 0.27</td>
</tr>
<tr>
<td><strong>Interleukin 6</strong>&lt;sup&gt;a&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt;)&lt;sup&gt;c&lt;/sup&gt; (pg/mL)</td>
<td>2.60 (1.20-4.29)</td>
<td>2.82 (1.81-4.42)</td>
<td>P = 0.11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Procalcitonin, C-reactive protein, Transthyretin and Interleukin 6 results are presented as median values. The values within the brackets refer to the range.

<sup>b</sup> P values refer to the comparison of CAP cases with a bacterial aetiology vs. bacterial and viral aetiology.

<sup>c</sup> Interleukin 6 was analysed in 33 of these 36 patients

Microbiological methods currently available have not been sensitive and rapid enough to be useful in clinical practice. Therefore, several different biomarkers have been evaluated and used to differentiate bacterial infections from viral and non-infectious conditions by reflecting the inflammatory response.

Previous studies have identified significantly higher levels of CRP and PCT in CAP cases with bacteraemia (Hedlund et al. 2000; Menendez et al. 2011), and found an association between elevated PCT and CAP severity (Boussekey et al. 2006; Muller et al. 2007; Lacoma et al. 2011), results corroborated by our study. However, high PCT levels in our severely ill patients seemed to be induced primarily by the fact that bacteraemia was more common in these patients (Fig 7). Also in lower PSI classes bacteraemia was associated with high PCT. Since bacteraemic patients had significantly higher PCT values, regardless of PSI class, than non-bacteraemic patients, it seems difficult to draw conclusions regarding severity from a single PCT value.

In prior CAP studies PCT levels were low in patients with infections caused by atypical pathogens, (Hedlund et al. 2000; Masia et al. 2005; Kruger et al. 2009; Menendez et al. 2011), except for *L. pneumophila* (de Jager et al. 2009; Haeuptle et al. 2009; Menendez et al. 2011), which is consistent with results from our study. When studying the non-bacteraemic cases with findings of classical bacteria, pneumococcal infection was associated with sevenfold higher PCT levels than infection caused by other bacteria, indicating an especially strong inflammatory response to *S. pneumoniae*, also in non-invasive disease. On the contrary, PCT levels in patients with non-invasive infection caused by other classical bacteria were similar to those in patients with only viral findings. Moreover, no significant difference was found in median PCT levels between patients with any bacterial aetiology and those with isolated viral infection, but the PCT
concentration was 10 times higher in those with proved pneumococcal disease than in those with viral findings alone. These data suggest that PCT is a good marker for systemic infections and for a pneumococcal aetiology, but not for localised respiratory infections caused by less virulent bacteria.

Most other studies have focused only on evaluating PCT as a marker to distinguish bacterial from viral infections in CAP, and they have shown conflicting results. Some have observed increased PCT levels in bacterial CAP compared to viral (Ip et al. 2007; Kruger et al. 2009; Menendez et al. 2011), while others have not (Masia et al. 2005; Gilbert 2011). Overall, compared to these studies, the microbiological methods used were more comprehensive and the aetiology better characterized in the present study.

The immune response in patients with combined bacterial-viral infections has not been thoroughly studied before. In the present study there was no tendency towards PCT-inhibition in the presence of virus among the mixed-infection cases, indicating that PCT production was influenced mainly by the bacterial component. Nor was there an increased inflammatory response to mixed bacterial-viral infections measured by PCT as well as CRP, transthyretin nor IL-6, as a significant explanation of the increased clinical severity noted in mixed infections (Templeton et al. 2005; Jennings et al. 2008; Diederen et al. 2009; Johansson et al. 2011). Importantly, in the present study we investigated only the systemic inflammatory response. Several observations have indicated that the inflammatory response to invasion of pathogens is compartmentalized predominantly to the lung (Dehoux et al. 1994; Monton et al. 1999; Deng et al. 2005). Future studies should therefore also investigate the local immune response so as to further increase our understanding of the relationship between different pathogens and inflammatory responses in CAP.
5 SUMMARY OF MAJOR FINDINGS

- The possibility to establish a pneumococcal aetiology was increased when sputum samples were analysed with RQ-PCR. The sensitivity of this method was higher than that of sputum culture and improved if sputum specimens obtained with saline induction and assistance from a respiratory physiotherapist were examined.

- The total microbial yield was high and by supplementing traditional diagnostic methods with new PCR-based techniques for both the most common bacteria and a number of respiratory viral agents, a higher microbial yield was achieved.

- *S. pneumoniae* was the leading causative agent, accounting for more than one-half of the cases with a determined aetiology, and probably for more than 50% of the aetiology in patients with CAP requiring admission to hospital.

- There was support for a viral aetiology in one-third of all patients.

- Mixed infections were frequent, with the combination of *S. pneumoniae* and a respiratory virus being the most common finding.

- Patients infected with a virus and a bacterial pathogen more often developed severe CAP and had longer hospitalisation than those with a bacterial aetiology alone.

- In patients with CAP, high PCT seemed to be a good biomarker of severe and invasive disease and of pneumococcal aetiology, but for localised bacterial infections caused by other pathogens the test seemed less sensitive.

- Mixed viral-bacterial aetiology was not associated with increased inflammatory response, as measured with PCT, CRP, transthyretin and interleukin-6.
6  FUTURE PERSPECTIVES

A stated above, since the introduction of antimicrobial agents for the treatment of CAP, there has been a gradual decline in the establishment of a microbial aetiology (Bartlett 2011). The reasons for this are probably many. Many of the microbiological methods available lack enough sensitivity or specificity, and are too slow in proving the causative agent. The development of broad-spectrum antibiotic therapy has also reduced the need for identification of the specific pathogen responsible for the infection. In addition, the striving for shorter lengths of hospital stay due to the gradual reduction of hospital resources has emphasized the need for antibiotics initiation immediately when patients are admitted to the emergency room. Most patients will therefore be treated empirically. The decision as to whether a patient should be started on antimicrobial therapy relies foremost upon clinical judgement, where different biomarkers often have great influence.

There are however several drawbacks. There is a major risk of over-consumption of, not least, broad-spectrum antibiotics, with economic importance. With the use of unnecessary broad antibiotics, there is also a risk of resistance development and adverse events in patients.

The development of nucleic acid amplification tests has clearly improved our understanding of respiratory infections. But would more sensitive and rapid microbiological methods result in more pathogen-directed treatment? In a randomized controlled trial between 2002-2005 consisting of hospitalised patients with LRTI, all patients were tested from the upper airways with RT-PCR for respiratory viruses and atypical pathogens. The PCR results were, however, reported immediately only to the physicians in charge in the intervention group. The implementation of RT-PCR increased the aetiological yield from 21% to 43%, but there was hardly any reduction in antibiotic use, duration of hospital stay or treatment cost (Oosterheert et al. 2005). The authors’ own interpretation of the results was that physicians were reluctant to discontinue antibiotic treatment on the basis of a positive viral finding with RT-PCR, before the bacterial culture results were available, which took longer. The ongoing development of new rapid and sensitive bacteriological PCR-based methods could perhaps be an answer to this problem. Whether these tests will gain a clinical impact depends probably chiefly on the future possibility to use them for point-of-care diagnosis.

Another central object is to further establish the significance of virus in the development of CAP: its role in mixed bacterial-viral infections is of special importance. These new diagnostic tools can perhaps be supportive in this effort, which could imply innovations in both the use of antibiotics and the improvement of new antiviral therapies.

Establishing a microbiological diagnosis will, despite the introduction of new molecular methods, probably remain challenging. Therefore the need for development and evaluation of biomarkers and their correlation to the aetiology in CAP will continue to be essential. PCT may contribute but its utility has to be further evaluated in future aetiological CAP studies including sensitive microbiological methods. Other biomarkers have also been introduced and studied, such as human neutrophil lipocalin (HNL), which have shown promising results here (Xu et al. 1995; Fjaertoft et al. 2005). Some authorities have also suggested that the combination of several biomarkers
increases both sensitivity and specificity in predicting the aetiology of CAP. It is hoped that this is an accurate approach in our effort to further improve the management of these patients.
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