UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 479

PCR detection of *Streptococcus pneumoniae* and *Haemophilus influenzae* in pneumonia patients

GUMA M. K. ABDELDAIM

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2009

ISSN 1651-6206
ISBN 978-91-554-7598-7
urn:nbn:se:uu:diva-107931

Dissertation presented at Uppsala University to be publicly examined in Hörsalen, Uppsala University Hospital, Department of Clinical Microbiology, Dag Hammarskjölds Väg 17, Uppsala, Friday, October 16, 2009 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Abdeldaim, G M K. 2009. PCR detection of *Streptococcus pneumoniae* and *Haemophilus influenzae* in pneumonia patients. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 479. 62 pp. Uppsala. ISBN 978-91-554-7598-7.

PCR is a rapid, reproducible method for nucleic acid detection. However, this technology displays significant deficiencies when applied in clinical microbiology. This work's aim was to improve current diagnostics and provide sensitive and quantitative real-time PCRs.

Paper I describes the development of a sensitive and specific quantitative real-time PCR for the detection of *Streptococcus pneumoniae,* based on the Spn9802 DNA fragment. Applied to nasopharyngeal aspirates from 166 pneumonia patients, Spn9802 PCR had a sensitivity of 94% and a specificity of 98%.

In Paper II the performance of a *ply* gene PCR for identification of pneumococcal lower respiratory tract infection (LRTI) was evaluated on bronchoalveloar lavage fluids. At the detection limit 10^3 genome copies/mL, 89% sensitivity but only 43% specificity was achieved.

Paper III shows that *S. pneumoniae* DNA is detectable in plasma from acutely febrile patients. Sensitivities were low (26-42%) for detection of pneumococcal pneumonia, for bacteraemic pneumococcal pneumonia they were 60-70%.

Paper IV describes evaluation of four PCR targets for *Haemophilus influenzae* detection. A real-time PCR based on the P6 gene was developed and applied to 166 CAP patients, using cut-off of 10^4 genome copies/mL the assay had a sensitivity of 97% and a specificity of 96%.

In paper V, the two real-time PCRs presented in papers I and IV were combined with a PCR for detection of *Neisseriae meningitidis*. The analytical sensitivity of this multiplex real-time PCR was not affected by using a mixture of reagents and a combined DNA standard (*S. pneumoniae/ H. influenzae*) in single tubes. Applied to 156 LRTI patients, this PCR had sensitivities over 90% for *S. pneumoniae* and *H. influenzae*, and specificities of 89% and 96%, respectively.

In conclusion, real-time PCR assays are useful for the diagnosis of *S. pneumoniae* and *H. influenzae*. They enable detection after antibiotic installation, and quantification increases the etiological specificity of pneumonia.

Keywords: Lower respiratory tract infections, Pneumonia, *Streptococcus pneumoniae*, *Haemophilus influenzae*, real-time PCR

Guma M. K. Abdeldaim, Department of Medical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

ISSN 1651-6206
ISBN 978-91-554-7598-7
urn:nbn:se:uu:diva-107931 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-107931)

"Glory be to You (God), we have no knowledge except whatever you have taught us. You are the Aware, the Wise!" The Noble Qur'an (Surah 2, versus 32)

To my parents

To my wife and children

To my sisters and brothers

List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Abdeldaim GM**, Strålin K, Olcén P, Blomberg J, Herrmann B. Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of *Streptococcus pneumoniae* target genes, with special reference to the Spn9802 fragment. *Diagn Microbiol Infect Dis* 2008; **60**: 143-150.

II **Abdeldaim GM**, Herrmann B, Korsgaard J, Olcén P, Blomberg J, Strålin K. Is quantitative PCR for the pneumolysin (*ply*) gene useful for detection of pneumococcal lower respiratory tract infection? *Clin Microbiol Infect* 2009; **15**: 565-570.

III **Abdeldaim GM**, Herrmann B, Mölling P, Holmberg H, Blomberg J, Olcén P, Strålin K. Usefulness of real-time PCR applied to plasma samples for detection of pneumococcal pneumonia. *Clin Microbiol Infect* (Accepted for publication).

IV **Abdeldaim GM**, Strålin K, Kirsebom L, Olcén P, Blomberg J, Herrmann B. Detection of *Haemophilus influenzae* in respiratory secretions from pneumonia patients by quantitative real-time PCR. *Diagn Microbiol Infect Dis* 2009; **64**: 366-73

V **Abdeldaim GM**, Strålin K, Korsgaard J, Blomberg J, Herrmann B. Multiplex quantitative PCR for detection of lower respiratory tract infection caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* (Submitted).

Contents

Abbreviations

16S rRNA	16 subunits ribosomal RNA
ATCC	The American Type Culture Collection
BAL	Bronchoalveolar lavage
bexA	Capsule-producing gene
CAP	Community-acquired pneumonia
CCUG	Culture Collection University of Göteborg
CFU	Colony-forming unit
C_t	Threshold cycle
fucK	Fuculokinase synthesing gene
LNA	Locked nucleic acid
LRTI	Lower respiratory tract infection
lytA	Autolysin gene
$MgCl_2$	Magnesium chloride
MLST	Multilocus sequence typing
NAAT	Nucleic acid amplification technique
NCTC	The National Collection of Type Cultures
NPA	Nasopharyngeal aspirates
PCR	Polymerase chain reaction
ply	Pneumolysin gene
rnpB	Rnase P RNA gene
T_a	Annealing temperature
T_m	Melting temperature

Introduction

Lower respiratory tract infections (LRTI) are a persistent and pervasive public health problem. They cause a greater burden of disease world-wide than human immunodeficiency virus infection, malaria, cancer, or heart attacks (1).

Pneumonia can be defined as LRTI with inflammation of the lung parenchyma. It is a potentially severe condition (2). It is the most frequent infectious cause of death in developed countries (3), and is still one of the most important causes of mortality in children especially among those under the age of 5 years. This is even more significant in developing countries (4). In the year 2000 1.9 million children died from acute respiratory infections, 70% of them in Africa and southeast Asia (5). In one study in Sweden the mortality among adult patients with community-acquired pneumonia (CAP) who were treated in departments of infectious diseases was 3.5% (6). In another study from Scandinavia the mortality within 3 months after admission to the hospital was 9% (7).

CAP is the most frequently studied entity of pneumonia. Another important entity is nosocomial pneumonia, in which individuals develop pneumonia within 48-72 hours after admittance to hospital.

Etiology

While a large number of microorganisms can cause CAP, *Streptococcus pneumoniae* is clearly the most prevalent etiologic agent (8). Among CAP patients subjected, during six separate studies (9), to both blood culture and lung puncture culture, *S. pneumoniae* was identified by at least one of the two methods in 48% of the patients.

Haemophilus influenzae is one of the most frequent etiologies of CAP. In two previous studies, culture of transtracheal aspirate was positive for *H. influenzae* in 13% (16/119) (9) and 15% (9/61) (10) of CAP patients subjected to transtracheal aspiration.

Other frequent etiologies of CAP include *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, *Staphylococcus aureus*, and respiratory viruses (Fig. 1.) (8).

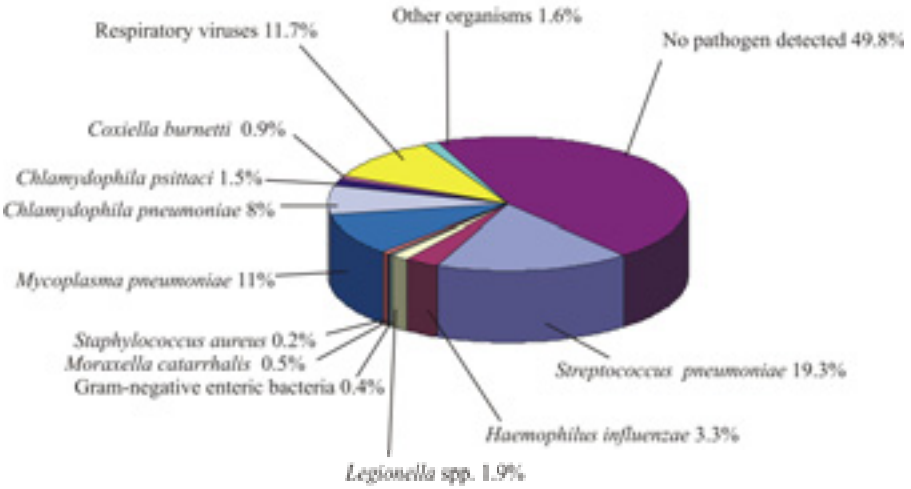

Figure 1. Etiological agents of CAP identified in 9 European studies. Data are presented as percentage means. Figures for hospitalized patients are almost the same (8).

Streptococcus pneumoniae

S. pneumoniae, commonly called the pneumococcus, was first identified in 1881 by Louis Pasteur in France and G. M. Sternberg in the USA. It is both a member of the normal oropharyngeal flora of most people, and an important human pathogen (11).

S. pneumoniae is a species of the genus *Streptococcus*, which comprises almost 50 species that in some cases are difficult to discriminate (12, 13). It is Gram-positive, non-motile and encapsulated. Most strains are facultatively anaerobic, although occasional isolates are strictly anaerobic (11). Sub typing is classically performed using specific typing sera which induce capsular swelling (the quellung reaction). More than 90 distinct capsular polysaccharides have been identified (14). Serotypes differ in invasive capacity, mortality rates, age and geographical distribution and in whether they act as primary pathogen or are more prone to infect persons with underlying disease (15-17). Thus, serotyping is useful for epidemiological purposes and to assess coverage of polyvalent vaccines.

During pneumococcal infections, such as pneumonia, viable *S. pneumoniae* bacteria may enter the blood stream and cause bacteraemia. However, even in cases of non-bacteraemic pneumococcal infections, degraded products from *S. pneumoniae*, such as polysaccharides, may enter the blood stream and circulate for long periods of time (18) and may be excreted in the urine (19).

Haemophilus influenzae

H. influenzae was first documented in 1883 by Robert Koch. During the influenza pandemic of 1889-92, Pfeiffer noted the constant presence of large numbers of small bacilli in the sputum of patients affected by the disease. He had established these organisms in stable subculture by 1889 and in 1893 he argued that the bacillus was the causative agent of the disease. In 1933 Smith, Andrewes and Laidlaw confirmed that the true causative agent was virus, but there still remains a possibility that secondary infection with *H. influenzae* contributed to the high mortality seen in the 1889-92 and 1918-19 pandemics (20). *H. influenzae* is a human-restricted Gram-negative bacterium that is part of the normal nasopharyngeal flora of most humans. It is a pleomorphic Gram-negative rod, aerobic but facultatively anaerobic. Some strains produce a capsule. The capsules are composed of polysaccharides and represent six distinct antigenic types, designated a-f. *H. influenzae* that lack capsular polysaccharides are referred to as nontypeable (20).

Apart from pneumonia, *S. pneumoniae* and *H. influenzae* can cause diseases such as meningitis, bacteraemia, sinusitis and acute otitis media.

Laboratory diagnosis

Microscopic examination

Gram´s staining of sputum specimens is an inexpensive and rapid method that is still in use for determination of the bacterial cause of pneumonia (21). While several authors have outlined important limitations of this tool in terms of sensitivity, reliability, and impact on treatment decisions (22-24), others consider it useful in the initial evaluation of patients with CAP (25-27); hence, the usefulness of Gram´s staining of sputum is still controversial. Furthermore, the yield of Gram stain has proven to be highly dependent on the application of strict criteria by a skilled microbiologist (28, 29).

Bacterial culture

Bacterial culture is an inexpensive method and also provides bacterial strains for further studies, such as testing of sensitivity to antimicrobials and molecular epidemiologic studies. However the yield of fastidious bacteria such as *S. pneumoniae* and *H. influenzae* is very low when virtually any specimen from the respiratory tract is collected after antibiotic therapy (30). Cultures from sterile sites like blood, pleural fluid, or lung puncture may provide definite CAP etiologies and can be considered as reference standards with which the sensitivity and specificity of other techniques are compared. However, the positivity rate of blood culture is low (31). By bronchoscopic techniques, secretions including bronchoalveolar lavage (BAL) can

be collected close to the site of the infection. However, as the bronchoscope passes the pharynx which includes the oropharyngeal flora, it may contaminate the lower respiratory tract (32). Thus, to differentiate between infection and colonisation, a cut-off limits of 10^4 colony-forming units (CFU)/mL is generally used for BAL culture (33). In order to completely exclude the risk of contamination from the pharynx, transtracheal aspiration can be performed (34), but this technique has been associated with complications.

Sputum is a non-invasive lower respiratory tract sample. As sputum samples pass the mouth on their way out, they are always contaminated by the oropharyngeal flora. In order to reduce the risk of false positive sputum culture results, the generally accepted cut-off limit of sputum culture is 10^5 CFU/mL. However, a problem with this sample type is that a substantial proportion of CAP patients cannot produce sputum samples of high quality.

In CAP patients, the lower and upper respiratory tract is normally colonized with the bacterial pathogen, which is responsible for the infection. Thus, culture of nasopharyngeal aspirates (NPA) or swabs from adult patients with pneumonia can be used for detection of *S. pneumoniae* and *H. influenzae* as CAP etiologies in populations with expectedly low carriage rates of these bacteria (35, 36). However, the usefulness of quantitative or semi-quantitative cultures of nasopharyngeal secretions has not been studied to my knowledge.

S. pneumoniae cultured on blood agar produces 1 mm colonies which are round, domed and surrounded by a zone of α-haemolysis. It is differentiated from other α-haemolytic streptococci by its sensitivity to optochin and bile solubility (20). Optochin sensitivity is the most important identification test, and it is commonly used in the clinical laboratory. However, atypical optochin resistant pneumococci have also been reported (37), which has made the definite identification of pneumococci more difficult. Species identification by DNA sequencing may provide a better discrimination (13).

H. influenzae is cultured on blood agar since it contains the so-called "X factor" (haematin) and "V factor" (nicotinamide adenine dinucleotide). The growth on blood agar is poor due to the lack of availability of V factor; however, growth is greatly improved on chocolate (heated blood) agar because more V factor is available (20). Requirement for both X and V factors is used to discriminate between *H. influenzae* and *Haemophilus parainfluenzae* and the lack of hemolysis on blood agar normally distinguishes *H. influenzae* from *Haemophilus haemolyticus.*

Antigen detection

Antigen detection techniques have been used as alternatives to culture. The test can be applied directly to clinical specimens like sputum, urine and cerebrospinal fluid, or to the bacteria culture. The antigens most commonly

analyzed are capsular polysaccharides (38). Several techniques have been used for antigen detection, including coagglutination, counterimmunoelectrophoresis, latex-particle agglutination, enzyme immunoassay and radioimmunoassay, and immunochromatography. Pneumococcal antigen detection in urine is a generally accepted way of establishing pneumococcal etiology in CAP. The commercially available test, NOW *S. pneumoniae* urinary antigen test (Binax), detects C polysaccharide in urine and is widely used. However it is non-specific for use in children (39). The usefulness of antigen detection in specimens other than urine is controversial. While some reporters suggest that antigen detection is a useful diagnostic tool for *S. pneumoniae* and *H. influenzae* pneumonia (40-42), others have questioned the clinical usefulness of such tests, especially when applied to samples from children (39, 43, 44).

Antibody detection

Antibody detection assays are usually used to demonstrate infections caused by virus and atypical bacteria, and no sensitive assays for detection of antibody responses to *S. pneumoniae* and *H. influenzae* are in routine use. To have optimal yield, serologic testing requires a serum specimen collected within the first 5 days after the onset of infection and a second serum specimen collected a number of weeks after the first one (38). Therefore, antibody measurements have no major importance for the choice of antimicrobial therapy.

PCR

PCR methods for detection of *S. pneumoniae* and *H. influenzae* are described on page 21.

Nucleic acid amplification- an overview

Nucleic acid amplification tests (NAAT) have been established as useful tools in the molecular microbiology laboratory. The polymerase chain reaction (PCR) as we know it today was developed by Kary Mullis in the early 1980s (45, 46). PCR is a technique for amplifying DNA sequences *in vitro*. It can amplify a specific sequence of DNA by as many as one billion times and be visualized as distinct bands on agarose gel (47). NAAT includes not only PCR but also alternate technologies, like strand-displacement amplification and transcription-mediated amplification (48).
During the last decade NAAT has become of central importance and is now commonly used for diagnostic purposes.

Real-time PCR

Real-time PCR is a modification of traditional PCR (gel based PCR) and was first described in the late 1990s (49). It has revolutionized the method of diagnosis in clinical microbiology laboratories (50). It is called "real-time PCR" because it allows the scientist to actually view the increase in the amount of DNA as it is amplified. The monitoring of accumulating amplicon in real time has been made possible by the labelling of oligonucleotide probes (i.e TaqMan® probe or Molecular Beacons) or amplicons with molecules capable of fluorescing (i.e SYBR® Green). These labels produce a change in signal following direct interaction with, or hybridization to, the amplicon. The signal is related to the amount of amplicon present during each cycle and will increase as the amount of specific amplicon increases.

The TaqMan® procedure utilizes the 5´-3´ exonuclease activity of the Taq polymerase. The TaqMan® probe is a short oligonucleotide (single stranded DNA) that contains a fluorophore at the 5´ end and a quencher, which keeps the molecule non-fluorescent, at the 3´ end. When the probe binds to the template DNA sequence, the polymerase encounters the probe and cleaves it. Thus, when the fluorophore is released into the solution, it is able to fluoresce (Fig. 2).

Quantitative Real-time PCR

Quantitative Real-time PCR is based on the contention that there is a quantitative relationship between the amount of target nucleic acid present at the start of a PCR assay and the amount of product amplified during its exponential phase. Consequently, the monitoring of PCR product accumulation makes it possible to perform an absolute or relative quantitative analysis of DNA in the samples studied by using standards with known concentrations. However, the accuracy of quantification is dependent on the standards used and sample preparation (51).

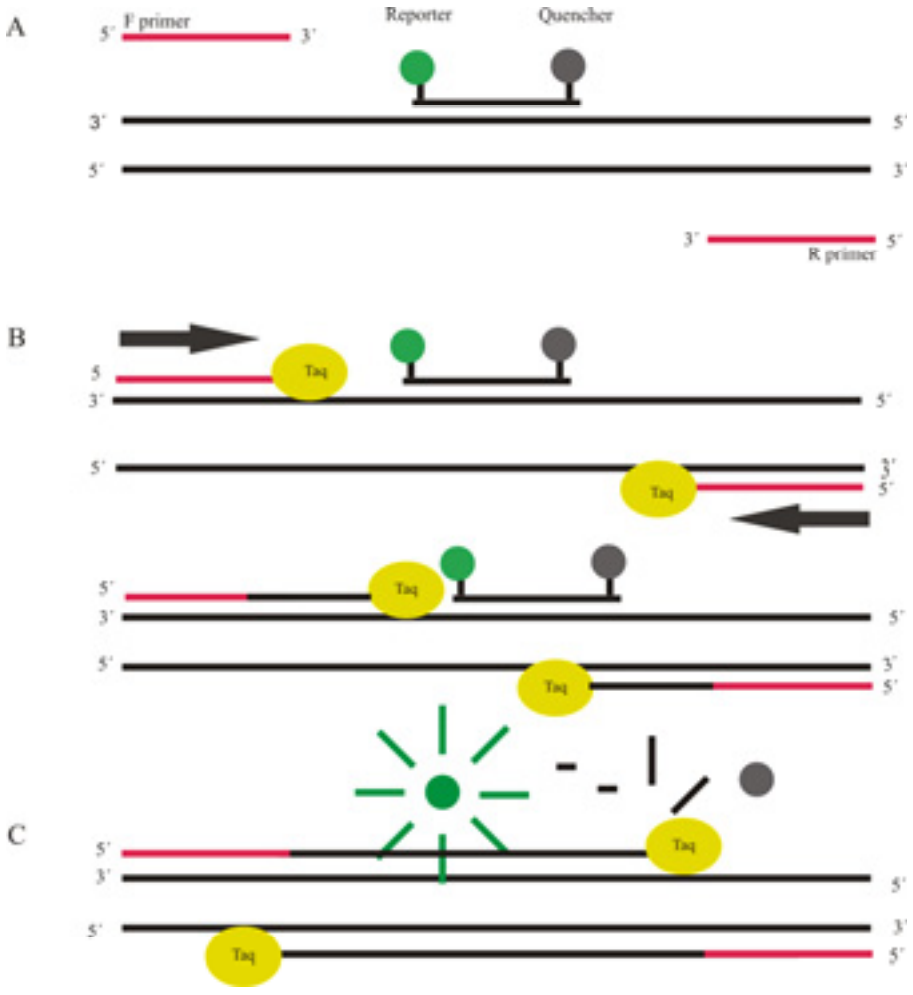

Figure 2. TaqMan® real-time PCR. (A) Initialization: a fluorescent reporter dye and a quencher are attached to the 5'and 3' ends of a TaqMan® probe. (B) Polymerization and strand displacement of DNA: when both reporter and quencher are attached to the probe, reporter dye emission is quenched. (C) Cleavage: during each extension cycle the DNA polymerase cleaves the reporter dye from the probe. Polymerization completed: once separated from the quencher, the reporter dye emits its characteristic fluorescence.

PCR Primer and Probe Design

One of the most important factors in successful PCR is proper primer design. Primers that only amplify one product will provide the best assay sensitivity and specificity. In this study, the chosen target genes for *S. pneumoniae* or *H. influenzae* were first tested by *in silico* exploration using the on-line software BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers and probes were evaluated using the on-line software Oligo Analyzer 3.0 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Probes containing locked nucleic acids (LNAs) were evaluated using the on-line software (http://oligo.lnatools.com/expression/).

When designing primer and probe, the length should normally be between 18 and 30 nucleotides, while the G-C content should be between 20 and 80%. Primer and probe self- and inter-complementation should be avoided. The melting temperature (*Tm*) of the probe should be several degrees above the *Tm* of the primers. Gs on the 5' end of the probe should be avoided and a strand should be selected that gives the probe more Cs then Gs.

Locked nucleic acid (LNA)

Locked nucleic acid (LNA) is a class of nucleic acids containing nucleosides whose major distinguishing characteristic is the presence of a methylene bridge that connects the 2′-oxygen of ribose with the 4′-carbon of the ribose ring (Fig.. 3). This bridge results in a locked 3′-endo conformation, reducing the conformational flexibility of the ribose and increasing the local organization of the phosphate backbone (52, 53). These modifications allow increased stability of the nucleic acid duplexes formed between LNAs and other nucleic acids and does not compromise their sequence specificity (52, 54). Moreover, the water solubility of LNAs is similar to the solubility of DNAs or RNAs. As a result of these useful properties, LNAs can substitute for native nucleic acids in many biological applications (55). Usually, LNA/DNA duplexes have increased thermal stability (3–8 °C per modified base in the oligonucleotide) compared with similar duplexes formed by DNA alone, and this increase in thermal stabilities obtained for LNA oligonucleotides depends on the length of the sequence and the number of LNA nucleotides. When designing probes containing LNA, it is recommended that one LNA is used to every two or three DNA nucleotides (56).
In this study, LNAs were used in order to increase the T_m of the probes (papers **I**, **IV** and **V**).

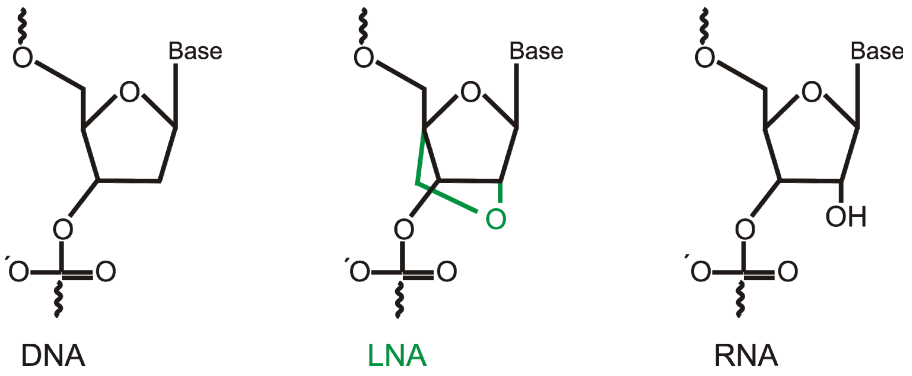

Figure 3. Chemical structure of 2′-*O*,4′-*C*-methylene linked LNA residues.

The concept of species

Bacteria are classified in much the same way as eukaryotes, but there are important differences in genetic transfer systems that include horizontal gene transfer between distantly related taxa and variable rates of recombination. Actually, the concept of a prokaryotic species is not theory-based to the same extent as it is for eukaryotes, but rather it is determined by practical needs and arbitrary judgments. Thus, to enable use of bacteria in clinical medicine, agriculture, food-processing industries and other operational activities, bacterial taxonomy is vital – even though it is quite imperfect.

At present, a prokaryotic species is defined as "a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions" (57). In practice a species is defined as a group of strains that is characterized by a certain degree of phenotypic consistency, showing 70% of DNA-DNA binding and over 97% of *16S rRNA* gene-sequence identity (58). According to this definition, we can decide when two organisms are similar enough in their genotypic and/or phenotypic properties to be given the same name. But even when we use this definition it is still difficult to delineate bacterial species. Many bacteria have genetically determined systems for the uptake and integration of exogenous DNA (59), and allow genetic exchange by transformation and recombination of both intraspecies and interspecies strains. This genetic exchange can lead to enormous strain to strain variation in the gene content within the same species. In a previous study (60), it was

observed that strains of the same species can vary up to 30% in gene content. On the other hand, some strains that show >70% DNA-DNA binding are classified into different species, on the basis of pathogenicity or host range, like strains of *Escherichia coli* and *Shigella* species (61), making the current classification somewhat inconsistent.

Instead of using a single gene such like *16S rRNA* for phylogenetic and taxonomic analysis, several housekeeping genes (usually 7) are used in multilocus sequence typing (MLST) (62). This gives a higher and more stable resolution for species definition but also enables grouping of isolates into major genetic lineages within a species (63). In recent years the rapidly increasing generation of DNA sequence data has made it possible to compare entire genomes of different bacteria strains. This has given rise to new terms such as 'species genome' or 'pan-genome', indicating the total genetic material found in one species, one genus or even all bacteria (64).

In addition to all the phenotypic and genotypic data, consideration of the ecological perspective is necessary to determine the boundaries between strain clusters and the formation of species (65). Thus it is not surprising that the man-made taxonomy comes into conflict with results derived from the complex characteristics of bacteria.

The species *S. pneumoniae*

MLST analysis of housekeeping genes of the closely related species *S. pneumoniae, Streptococcus mitis, Streptococcus oralis* and *Streptococcus pseudopneumoniae* has shown that no single gene could resolve streptococcal species clusters to define how strains should be denoted, but the concatenated sequences defined four separated species clusters (66). *S. pseudopneumoniae* is the most recently defined species in the group (67) and is also the species most closely related to *S. pneumoniae* in evolution (68) as well as regarding virulence properties (68, 69).

In clinical routine diagnostics, robust and user-friendly detection methods are necessary. Differentiation of *S. pneumoniae* from other viridans group streptococci, like *S. mitis* and *S. oralis* has been based on phenotypic characteristics, most commonly by demonstrating optochin susceptibility and/or solubility in bile (sodium deoxycholate) (70). However, optochin resistance and bile-insoluble strains of *S. pneumoniae* have been reported (71, 72). Moreover, commercial biochemical-based tests like Rapid ID 32 Strep systems have shown poor correlation when compared to DNA-DNA hybridization within the viridans group streptococci (73). The commonly used NOW *S. pneumoniae* antigen test (Binax Inc) is also positive for isolates of *S. pseudopneumoniae* (69). Genotyping using *16S rRNA* gene sequencing has limited use for the identification of *S. pneumoniae* because of the high genetic similarity (99%) between species of the viridans group, whereas DNA-

DNA homology studies show only 50 to 60% similarity between members of the viridans group (74).

The species *H. influenzae*

The genus *Haemophilus* is characterized by a promiscuous ability to exchange genetic material by transformation and recombination, both between strains within species (75-77) and between species (78). This genetic exchange can lead to high strain to strain variation in the gene content within the same species. Thus, delineation of *H. influenzae* is still reported as an unresolved challenge when MLST, phenotyping and detection of marker genes was used (79). However, intraspecies genetic characterization with high resolution of both encapsulated and noncapsulated *H. influenzae* strains has been achieved by MLST (80, 81).

Differentiation of *H. influenzae* from closely related species, such as *Haemophilus haemolyticus* and *Haemophilus aegyptius,* is difficult. The commonly used X- and V- growth factors are not reliable, since all three of these species are dependent on both X- and V- factor (82). The beta-hemolytic phenotype of *H. haemolyticus* is routinely used to distinguish *H. haemolyticus* from *H. influenzae*. However, non-hemolytic *H. haemolyticus* strains have been reported (77, 83). Furthermore, DNA-DNA hybridization and multilocus sequence analysis have shown high levels of similarity between *H. influenzae* and *H. aegyptius*, which make it questionable whether *H. aegyptius* merits consideration as a separate species (79, 84, 85). Automated phenotyping, including broad biochemical panels, has been shown to identify most strains to correct *Haemophilus* species, but problems still remain and more extensive strain collections are needed for evaluation (86, 87).

PCRs used for the detection of *S. pneumoniae* and *H. influenzae*

Several PCRs have been developed for the detection of *S. pneumoniae* and *H. influenzae* (88-96). However, the use of PCR has two main problems. Firstly, both *S. pneumoniae* and *H. influenzae* can colonize the pharynx in the absence of disease (asymptomatic colonization). This is also a problem for culture diagnostics, but it is more pronounced when using sensitive nucleic acid amplification tests. Secondly, nonspecific reactions can occur when target genes are also harboured by closely related bacteria species from oropharyngeal flora. In the diagnosis of *S. pneumoniae,* species such as *S. mitis* and *S. oralis* may cause an unspecific reaction (96-98). Similarly, it

is difficult to obtain specific detection of *H. influenzae* in the presence of some other *Haemophilus* species (84, 99).

The *ply* and *lytA* genes have been mainly used as targets for detection of *S. pneumoniae* by PCR (100). However, *S. mitis* and some related viridans streptococci have been shown to occasionally harbour the *ply* and *lytA* genes (96). Two recent studies (101, 102), suggest that the *lytA* gene is a more suitable gene target than the *ply* gene, as PCR was negative for *lytA* in all tested strains of non-pneumococcal streptococci (n=50 and 51, respectively), whereas the *ply* PCR was positive for 9 and 16 strains, respectively.

Two other interesting PCR targets in *S. pneumoniae* are the genes coding for the pneumococcal surface antigen A (*psaA*) (91) and the penicillin-binding protein 2b (*PBP2b*) (103), but these genes have also been identified in some viridans streptococci (104, 105).

Several PCR assays have been developed for the detection of *H. influenzae* (88, 90, 92-94). The *bexA* gene has been used in assays for encapsulated *H. influenzae* (88). However, it has been reported that capsular strains (a, e, and f) were not detected by the *bexA* PCR (106). The IS*1016* gene has also been used to detect the encapsulated *H. influenzae* (107). But, St. Geme *et al*. found approximately 11% of non-capsulated *H. influenzae* from pharyngeal carriage isolates to be positive for IS*1016* (108). The *16S rRNA* and *P6* genes have been used as targets, as they are present in both encapsulated and non-encapsulated strains (109, 110). However, specificity problems regarding these two genes have been reported (94, 109, 110), especially with closely related species like *Haemophilus parainfluenzae*, *H. aegyptius* and *H. haemolyticus*.

In this study we tested several target genes for the detection of *S. pneumoniae* and *H. influenzae* and we found that some of them are more specific, but all have limitations which are in concord with the species concept.

Evaluation of diagnostic tests

A diagnostic test for an infectious agent can be used to demonstrate the presence or absence of infection. Demonstrating the presence of the infecting organism is often crucial for effective clinical management. To be useful, diagnostic methods must be accurate, simple and affordable for the population for which they are intended. The basic performance characteristics of the test under evaluation for its ability to distinguish infected from uninfected individuals are: clinical sensitivity, that is, the probability that a truly infected individual will test positive; and clinical specificity, that is, the probability that a truly uninfected individual will test negative. The classical way to test clinical sensitivity and specificity is to compare the results of the designed test with the results of the gold standard method using a 2 by 2 table, as shown in figure 4. Ideally, the gold standard

provides error-free detection, which means that it does not have any false-negative or false-positive results. For most, if not all conditions in clinical medicine, a gold standard that is without error is not available (111, 112), therefore researchers use the best available method to determine the presence or absence of the target condition, and such a method is referred to as the reference standard rather than the gold standard (113). Researchers evaluating the diagnostic accuracy of a test often encounter situations where the reference standard is less sensitive than the evaluated test. Therefore they use alternative methods to evaluate the new test and discuss the strengths and limitations of that particular method (114, 115).

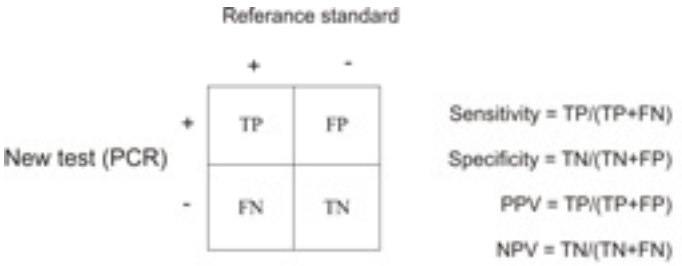

Figure 4. Accuracy measures using 2 by 2 table. TP, true positive results; FP, false positive results; FN, false negative results; TN, true negative results; PPV, positive predictive value; NPV, negative predictive value.

Alternative methods to evaluate the new diagnostic test

Discrepant analysis

This is an attempt to identify the truly positive patients that the reference standard misses. In discrepant analysis the false-positive samples that were PCR positive but negative by culture are subjected to additional testing by one or more tests. If any one of these additional tests yields a positive result, then the original PCR test positive result is considered to be true positive and the original culture negative result is considered a false negative (116). The strength of this method is that it is straightforward and easy to use without statistical expertise. But it has limitations because the verification pattern is dependent on the new test results and it provides the status (117) of the target condition for those who are re-tested, but not for those not re-tested, which is usually the majority.

Composite reference standard

When there is no single gold standard, the results of several imperfect tests can be combined to create a composite reference standard. In this method the imperfect tests are applied to all patients and if one of the reference tests is positive, the target condition is considered to be present (positive). This

method is simple and easy to understand, and the researcher can combine several sources of information to evaluate whether the target condition is present or not. Unlike the discrepant analysis, the application of the second reference standard is independent of the new test results. The limitation of this method is that the inclusion of more than two reference tests in the composite reference standard may then obscure the final definition of the disease.

Latent class analysis

Latent class analysis is a statistical technique which can be used when no gold standard is available. It combines the results of several test methods, such as PCR, cell culture and antigen detection, to estimate the true but unknown sensitivity and specificity of each method as well as the prevalence of the target condition. These unknown parameters are called latent variables, as opposed to the measured or manifest variables, such as the number of patients which are positive in all tests, the number of patients which are negative in all test methods etc, i. e. frequencies. The latent variables are estimated using standard statistical techniques such as maximum likelihood. The strength of this method is that it is objective. However the estimates can be biased if the number of test methods is three or less. This is because the tests are assumed to be independent, which cannot be tested statistically in this case (the independence assumption might of course be true if the tests are based on completely different methods such as cell culture and PCR).

In this study the PCR assays in papers **I** and **IV** were evaluated by discrepant analysis. In paper **V** the Spn9802 target was evaluated by a composite reference standard and the *P6* target was evaluated by discrepant analysis. This resulted in increased specificity and a higher number of pneumonia cases with defined etiology.

Aims

- To develop real-time PCR assays for specific detection of *S. pneumoniae* and *H. influenzae* and to estimate the cut-off level which can differentiate between disease-causing infection and colonization.

- To evaluate the specificity of a quantitative pneumolysin (*ply*) gene PCR applied to clinical specimens for identification of pneumococcal LRTI.

- To evaluate the performance of the real-time PCR in detecting *S. pneumoniae* DNA in blood samples from acutely febrile pneumonia patients.

- To develop and evaluate a sensitive and specific quantitative multiplex real-time PCR for detection of *S. pneumoniae, H. influenzae* and *Neisseria meningitidis* as pathogens in respiratory and cerebral infections.

Materials and methods

Clinical specimens

To evaluate PCRs for detection of *S. pneumoniae* and *H. influenzae* a total of 166 nasopharyngeal aspirates from adult CAP patients (median age 71 years) and from 84 adult controls (median age 69 years) without respiratory symptoms were used. These specimen collections are described in detail in papers **I** and **IV**.

To test the performance of a quantitative *ply* PCR for detection of *S. pneumoniae*, (paper **II**) and to evaluate the mutiplex real-time PCR for the detection of *S. pneumoniae* and *H. influenzae* (paper **V**), a total of 156 BAL fluids from LRTI patients (median age 63 years), and from 31 adult controls (median age 64 years) were used. The adult controls were consecutively collected, underwent bronchoscopy for suspected malignancy and they did not have pulmonary infection.

In paper **III**, plasma samples from 92 patients (median age 70 years) with CAP, and 91 controls (median age 67 years), were tested to evaluate rapid real-time PCR assays for the detection of *S. pneumoniae* from plasma.

DNA preparation

DNA preparation is one of the most important steps when performing quantitative PCR. The presence of inhibiting substances in the sample may lead to a complete absence of amplification products or have a quantitative effect (51).

The DNA of bacterial strains used for assay optimization and specificity evaluation in papers **I** and **III** was purified by phenol-chloroform extraction of bacteria harvested in exponential growth phase, after culture on blood agar at 37°C in 5% carbon dioxide.

DNA from the nasopharyngeal aspirates used for assay evaluation was purified by the Qiamp DNA mini kit (Qiagen, Hilden, Germany) (papers **I** and **IV**), whereas DNA from BAL fluid was purified by the automatic MagNa Pure LC DNA-Isolation System (Roche Diagnostics) (papers **II** and **V**). DNA from plasma samples was purified by the automatic NucliSens easyMAG instrument (Biomérieux, Marcy l'Etoile, France) (paper **III**).

PCR methods used in our study

For detection of *S. pneumoniae* we developed a real-time PCR based on the sequence of the Spn9802 DNA fragment described by Suzuki *et al* (118). The sensitivity and specificity of the Spn9802 PCR was compared with the autolysin (*lytA)* gel-based PCR as described by Strålin *et al* (94) (paper **I**). The quantitative real-time PCR for *ply* was used as described by Corless *et al*. (88), except that 3.5 mmol/L $MgCl_2$ was used instead of 5.5 mmol/L and that the elongation time was 40 s instead of 1 min (papers **II** and **III**).
For detection of *H. influenzae* we developed two real-time PCRs, one based on the sequence of outer membrane protein *P6*, and the other based on the sequence of RNase P RNA gene (*rnpB*) (paper **IV**). The specificity of the real-time *P6* PCR was compared with *16S rRNA* gel-based PCR as described by Strålin *et al.* (94), *rnpB* real-time PCR, and capsule-producing gene (*bexA*) real-time PCR as described by Corless *et al*. (88), see above. Samples which were negative by culture for *H. influenzae* but positive by *P6* PCR were further tested by *fucK* PCR as described by Meats *et al*. (81). For the detection of *N. meningitidis ctrA* PCR was used as described by Corless *et al*. (88), see above.

To enable multiplex detection we developed a PCR assay where the Spn9802 PCR for *S. pneumoniae* (paper **I**), the *P6* PCR for *H. influenzae* (paper **IV**) and the *ctrA* PCR for the detection of *N. meningitidis* (88) were combined (paper **V**).

All clinical samples and reference strains were run on a Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia).

Optimization

To have a successful PCR, the annealing temperature (T_a), magnesium chloride ($MgCl_2$), primers and probe concentrations must be optimized.

Annealing temperature is one of the most important parameters that needs to be adjusted in the PCR reaction. It is defined as the temperature at which the single stranded primer will specifically bind to the template sequence. To optimize T_a, a PCR instrument (iCycler™, BioRad) with gradient temperature was used. The PCR products were tested on 2% agarose gel electrophoresis. The temperature that yielded a strong bright band without primer dimer was chosen.

Primer concentration is another parameter that must be considered to minimize the risk of amplification artifacts. All combinations of forward and reverse primers at 900, 600, 300, and 100 nM were tested. The lowest concentrations that resulted in a strong band were chosen.

Another important parameter is the $MgCl_2$ concentration. PCRs with different concentrations of $MgCl_2$ (1.5 mM, 2.5 mM, 3.5 mM, and 4.5 mM) were run to find the optimum concentration. The PCR products were tested on 2% agarose gel electrophoresis. The concentration that yielded a strong bright band without primer dimer was chosen.

Once T_a, primer concentration and $MgCl_2$ are optimized, the next step is to optimize the probe concentration. Three concentrations of probe were tested (400 nM, 200 nM, and 100 nM). The lowest concentration that gave a low threshold cycle (C_t) value and strong fluorescence was chosen.

Sensitivity, specificity and reproducibility of PCR assays

The detection capacity of a quantitative real-time PCR assay is commonly expressed as the analytical sensitivity. In this study two experiments (for each PCR) were performed with serial dilutions of target DNA (5 to 500-600 genome copies per reaction) in carrier tRNA (1µl/mL) and 2 to 4 tubes of each dilution.

The specificity of the assays was determined by testing a collection of reference strains and clinical isolates.

Moreover, the clinical sensitivity and specificity were evaluated by testing clinical samples and results from the new PCR assays were compared with a reference method as well as with other published PCRs.

The reproducibility of quantification was evaluated by testing DNA preparations with known concentrations (duplicates of 500, 2,000 and 10,000 genome copies per PCR reaction) in five consecutive runs.

Reproducibility of multiplex PCR

The reproducibility of multiplex PCR was evaluated by testing 73 BAL samples and DNA preparations with known concentrations, as above (paper **V**). PCRs with primer/probe reagents in both monoplex (one tube for each PCR) and multiplex (one tube for all PCRs) configurations were tested in parallel. We also tested the reproducibility of quantification with positive control DNA of *S. pneumoniae* and *H. influenzae* in separate tubes and combined in a single tube. In addition, the reproducibility of combined standard of DNA from *S. pneumoniae*, *H. influenzae* and *N. meningitides* was evaluated in the same way (data not shown).

DNA sequencing

In previous studies, *rnpB* sequence analysis has been shown to be useful in species identification of streptococci (13, 119). Therefore, in paper **I**, the identification of detected *Streptococcus* species was analysed in clinical samples that were positive by PCR and negative by culture according to the mentioned studies.

Results and discussion

Paper I

Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of *Streptococcus pneumoniae* target genes, with special reference to the Spn9802 fragment

In this study quantitative real-time PCR was developed using primers and a TaqMan® probe complementary to sequences in the Spn9802 fragment (118). It is essential to test the analytical sensitivity and specificity of the PCR on microorganisms *in vitro* prior to application to clinical samples. When serial dilutions of target DNA with known concentrations were tested, the Spn9802 assay was able to detect 10-60 genome copies per reaction tube (Table 1).

Table 1. Analytical sensitivity of the Spn9802 assay

No. of reactions[a]	Genome copies per reaction tube	No. of reactions with detected target
6	600	6/6 (100%)
8	60	8/8 (100%)
8	20	5/8 (62.5%)
8	10	4/8 (50%)
8	5	0/8 (0.0%)

[a] Data derived from 2 experiments with 3 to 4 reactions of each dilution.

When the specificity of the Spn9802 assay was tested on 59 bacterial strains, representing 44 species and including 9 clinical isolates of *S. mitis* and *S. oralis*, all species were negative, except *S. pseudopneumoniae*.

Evaluation of the Spn9802 assay was performed by analysis of 166 CAP patients. *S. pneumoniae* was identified in 68 cases by Spn9802 PCR, in 61 cases by *lytA* PCR and in 50 cases by culture. Of the 50 culture-positive cases, 49 were positive by Spn9802 PCR, whereas 47 were positive by *lytA* (Table 2). Among the 84 controls *S. pneumoniae* was identified in 10 cases by Spn9802 PCR, in 4 cases by *lytA* PCR and in 2 cases by culture.

Table 2. Detection results for 166 nasopharyngeal aspirates (pneumonia cases) using culture and 2 different PCR methods

Culture	*lytA* with gel detection	Spn9802 real-time PCR	No. of samples
+	+	+	47
+	-	+	2
+[a]	-	-	1
-	+	+	9
-	+	-	5
-	-	+	10
-	-	-	92

[a] In the case where *S. pneumoniae* was detected only by culture, there was weak growth (+) of *S. pneumoniae* but abundant growth of *H. influenzae* in the sample.

The presence of *S. pneumoniae* as normal flora in the upper respiratory tract (asymptomatic colonization) is problematic for both culture and PCR. Since the concentration of bacteria in the respiratory tract is higher during infection than during colonization (120, 121), quantification of the bacteria is a way to solve this problem. Real-time PCR provides a rapid quantification that may be used to distinguish between infection and colonization. In the present study the cut-off 10^4 DNA copies/mL for positive result with Spn9802 provided a high specificity, without significant reduction in the sensitivity (Fig.. 5). Another study using quantitative PCR on sputum samples of CAP patients found a detection limit of 3.7×10^4 DNA copies/mL to be appropriate for identification of pneumococcal pneumonia (98).

In a previous study (118), the target gene Spn9802 was shown to be more specific than the *ply* gene for the detection of *S. pneumoniae*. Here we show that species identification by *rnpB* sequence analysis was useful for discrepant analysis of clinical samples that were positive by Spn9802 PCR and negative by culture. Among 29 such samples, 21 yielded *rnpB* sequences of *S. pneumoniae*. That means the use of discrepant analysis to obtain an expanded standard resulted in an increased specificity and a higher number of identified pneumonia cases.

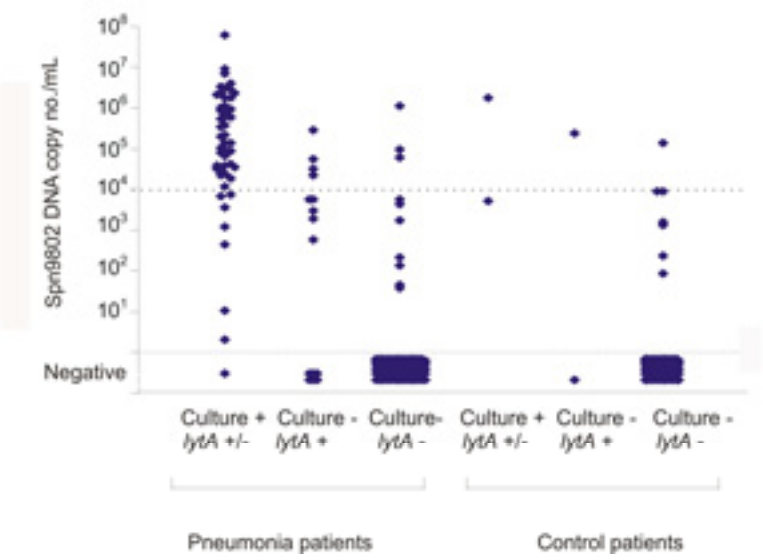

Figure 5. *S. pneumoniae* DNA copy number per milliliter with the quantitative real-time Spn9802 PCR applied to nasopharyngeal aspirates from 166 pneumonia patients and 84 controls, related to culture and *lytA* PCR results.

Paper II

Is quantitative PCR for the pneumolysin (*ply*) gene useful for detection of pneumococcal lower respiratory tract infection?

One of the most commonly used PCR targets for the detection of *S. pneumoniae* is the *ply* gene (98, 122-124), encoding for the pathogenicity factor pneumolysin. When serial dilutions of target DNA with known concentrations were tested, the *ply* assay was able to detect 20 genome copies per reaction tube. Among 109 LRTI patients with negative reference standard and negative *lytA*-based PCR, the *ply* PCR was positive in 51 patients (47%) while results were negative in blood culture, urinary antigen test, BAL culture and *lytA* PCR. Of the 103 LRTI patients who had taken antibiotic prior to sample collection, the combined reference standard was positive in 11 cases, *lytA* was positive in 32 cases, and *ply* was positive in 58 cases. The performance analysis of the quantitative *ply* PCR showed that a cut-off of 10^7 genome copies/mL was required for an acceptable specificity (90%), but that resulted in a low sensitivity (53%).

Our results clearly show that the *ply* gene is not an adequate target for detection of *S. pneumoniae*. Although this conclusion was apparent almost ten years ago (96), *ply* has been used in several studies in the following years (97, 101, 102, 125-128). We therefore found it important to highlight the problem when evaluating the Spn9802 PCR. The summary of these studies is shown in Table 3. When the primer pairs of the mentioned studies were plotted on the *ply* gene as shown in figure 6, it was clearly indicated that non-specific PCR detection is not restricted to a single target sequence in the *ply* gene. Thus our results confirm previous reports that *ply* is a non-specific target for detection of *S. pneumoniae*. Furthermore, it has been suggested that quantification of the *ply* gene could discriminate cases with clinical pneumonia diagnosis from persons that were colonized with *S. pneumoniae* (90, 98). However, our study shows that this is not the case. Figure 7 shows the quantitative results of Spn9802 PCR compared with *ply* PCR in samples from LRTI patients where the reference standard tests and the *lytA* PCR were negative. Assuming the Spn9802 PCR assay represents true positivity it is obvious that the *ply* PCR gives rise to many false positive cases.

In a recent study (129), mutations in the *ply* gene was reported. The impact of these mutations on different *ply* PCR methods was analyzed by *in silico* analysis. No nucleotide substitutions were found in the primers and probe sequences used in our study and originally described by Corless *et al*. (88), as well as in the method of Whatmore *et al*. (96). In the method used by Salo *et al*. (130) and Murdoch *et al*. (127), two nucleotide substitutions were found, one in the reverse inner primer in the 4^{th} position from the 3´end, which may reduce the detection capacity, and another substitution close to the 5´end in the outer primer, which probably has no effect. The

same nucleotide substitution that affected the reverse inner primer of the method of Salo *et al*. also affected the reverse primer in the method used by Saukkoriipi *et al*. (131) and Greiner *et al*. (97).

Table 3. Results of *ply* PCR on strains identified as *Streptococcus* species other than *S. pneumoniae* or atypical *S. pneumoniae*

Author and year	Reference No.	Bacterial strains (No.)	Origin	*ply* PCR
Whatmore *et al*. 2000	(96)	Atypical oral *streptococci* (9)	Clinical isolates	All positive
		Putative atypical *pneumococci* (16)	Clinical isolates	All positive
Greiner *et al*. 2001	(97)	*S. gordonii* (1)	ATCC 12369	Positive
		S. oralis (1)	ATCC 10557	Positive
		S. anginosus (1)	Clinical isolates	Positive
		S. constellatus (1)	Clinical isolates	Positive
		S. mitis (1)	Clinical isolates	Positive
		S. mutans (1)	Clinical isolates	Positive
		S. salivarius (1)	Clinical isolates	Positive
		S. sanguis (1)	Clinical isolates	Positive
Verhelst *et al*. 2003	(128)	Optochin-resistant alpha-hemolytic pneumococci-like *Streptococcus* species (49)	Clinical isolates	19 positive by *ply* and negative in other PCRs 5 positive by *ply* only
Messmer *et al*. 2004	(101)	Atypical *streptococci* (16)	Clinical isolates	8 positive
Neelman *et al*. 2004	(102)	*S. mitis* (32)	Clinical isolates	31 positive
		Streptococcus species (18)	Clinical isolates	10 positive
Kaijalainen *et al*. 2005	(126)	*S. mitis* (9)	Clinical isolates	All positive
Carvalho *et al*. 2007	(125)	Pneumococcus- like *viridans streptococci* (11)	Clinical isolates	All positive

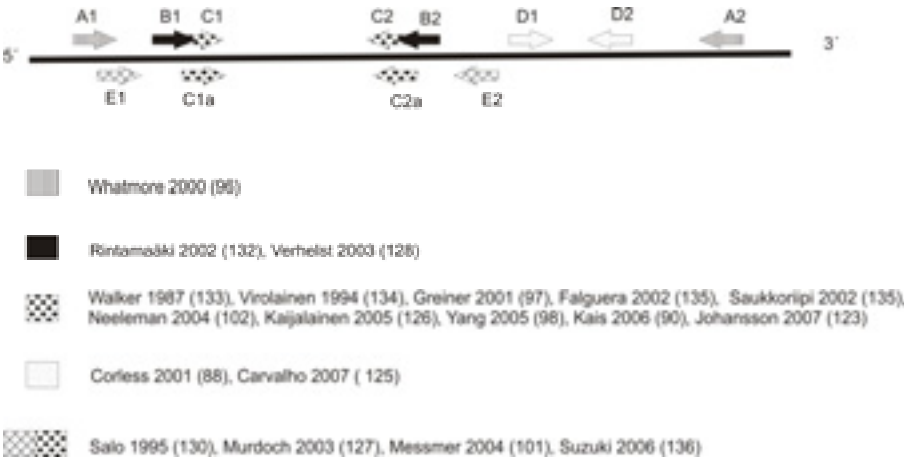

Figure 6. Schematic view of *ply* gene primer sequences used in different studies.

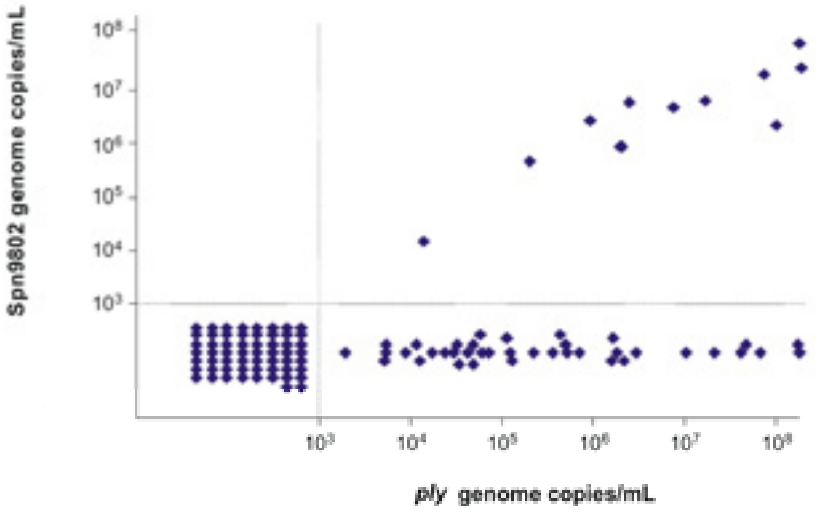

Figure 7. Quantitative results of Spn9802 PCR compared with *ply* PCR applied to bronchoalveolar lavage (BAL) fluid in patients with lower respiratory tract infection. All available reference tests and the *lytA* PCR were negative.

Paper III

Usefulness of real-time PCR for *lytA*, *ply*, and Spn9802, applied to plasma samples to detect pneumococcal pneumonia

Diagnosis of pneumococcal pneumonia is hindered by the lack of a highly sensitive and specific 'gold standard' method. Identification of the bacterium in blood culture provides a definite diagnosis and can serve as an indicator of disease severity. However, the positivity rate of blood cultures rarely exceeds 10% in CAP (137), and can be below 1% if blood samples are obtained during antimicrobial treatment (138). In this study we evaluate the performance of PCR of three different gene targets of *S. pneumoniae* (pneumolysin, *ply*; autolysin, *lytA* and the DNA fragment Spn9802) on 92 plasma samples from patients (median age 70 years) and on 91 plasma samples from controls (median age 67 years), in order to identify a PCR assay that could be used to detect *S. pneumoniae* DNA in blood samples from acutely febrile patients. Among the 92 CAP patients, *S. pneumoniae* was identified by blood culture in 10 cases (11%), by *lytA* PCR in 10 cases (11%), by Spn9802 PCR in 11 cases (12%), by *ply* PCR in 17 cases (18%), and by urinary antigen test in 24 cases (26%).

Table 4 shows the microbiological tests of the individual CAP patients with positive blood culture and/or positive PCR. Among the 10 with bacteraemic pneumococcal pneumonia, 6 proved positive by all three PCR methods, while 2 were negative with all three PCR methods. Five specimens were Spn9802 positive in low copy numbers (200-2000 copies/ mL) but negative by culture. In four of these cases *S. pneumoniae* was also detected by urinary antigen test or sputum culture.

Among 91 control patients, *ply* PCR was positive in 8 cases (9%); blood culture, *lytA* PCR and Spn9802 PCR were all negative. Additionally urinary antigen test was positive in 2 cases (2%), and NPA culture was positive in 4 cases (4%). Of the 8 cases which were positive by *ply* PCR, 1 case was positive by both urinary antigen test and NPA culture, and 1 was positive by NPA culture only.

The diagnostic performance of the three PCR assays in the CAP cases is shown in Table 5. The sensitivities for detection of pneumococcal pneumonia were low (26-42%), although the specificities were high for Spn9802 (98%) and *lytA* (100%). To detect bacteraemic pneumococcal pneumonia, sensitivities were higher (60-70%), and specificities remained high for Spn9802 (94%) and *lytA* (99%). Consequently, Spn9802 and *lytA* had high positive predictive values for pneumococcal pneumonia and high negative predictive values for bacteraemic pneumococcal pneumonia.

Table 4. CAP patients with positive blood culture and/or positive PCR for *Streptococcus pneumoniae* applied to blood samples

Sex, age	Blood culture result	*lytA* PCR (copies/mL)	Spn9802 PCR (copies/mL)	*ply* PCR (copies/mL)	*S. pneumoniae* urinary antigen test	Sputum culture for *S. pneumoniae*	Culture of nasopharyngeal aspirate for *S. pneumoniae*
F, 83	*S. pneumoniae*	9×10^8	1×10^{10}	4×10^9	+	ND	*ND*
F, 79	*S. pneumoniae*	2×10^8	3×10^9	1×10^9	+	ND	ND
M, 91	*S. pneumoniae*	6×10^2	2×10^4	2×10^4	+	ND	+
F, 89	*S. pneumoniae*	2×10^2	2×10^4	1×10^4	+	ND	+
M, 54	*S. pneumoniae*	3×10^2	5×10^3	8×10^3	+	+	+
F, 74	*S. pneumoniae*	2×10^2	1×10^3	1×10^3	+	ND	ND
F, 84	*S. pneumoniae*	8×10^1	0	0	+	+	+
F, 57	*S. pneumoniae*	0	0	3×10^2	+	ND	+
M, 31	*S. pneumoniae*	0	0	0	+	ND	+
F, 85	*S. pneumoniae*	0	0	0	+	ND	–
M, 23	–	1×10^2	6×10^3	1×10^4	+	–	+
F, 78	–	0	2×10^3	6×10^1	+	ND	ND
M, 58	–	0	6×10^2	2×10^2	+	–	+
M, 74	–	0	2×10^2	6×10^2	–	+	–
M, 31	–	0	4×10^2	1×10^2	–	–	+
M, 87	–	0	0	9×10^2	+	ND	+
F, 77	–	0	0	3×10^2	+	+	+
M, 46	–	0	0	6×10^2	ND	ND	–
F, 69	–	0	0	5×10^2	–	ND	–
M, 51	–	0	0	3×10^2	–	–	–
M, 75	–	0	0	2×10^2	–	–	–
F, 32	–	0	0	2×10^2	–	ND	–

+, positive; –, negative; ND, not done

As shown in this study the sensitivity of the PCR assays was low among both pneumococcal pneumonia and pneumococcal bacteraemia; similar figures have been reported in previous studies (139-141). This is likely due to the sample volume used in the DNA extraction (400 µL plasma), and 5 µL (corresponding to 80 µL original plasma) of the purified DNA was applied in the PCR. The use of such a small volume limits the detection capacity, especially in cases of low-grade bacteraemia. In comparison, the blood volume in the four blood culture bottles was approximately 40 mL (4 x 10 mL), corresponding to about 20-25 mL of plasma.

In paper **II** we have shown that *ply* PCR applied to respiratory secretions is non-specific for detection of *S. pneumoniae*. In the present study the *ply* gene PCR presumably also caused false positivity in blood, a locality that is assumed to be "sterile".

Table 5. Performance of PCR for three different gene targets of *Streptococcus pneumoniae* applied to EDTA blood samples from 92 pneumonia patients

Reference group	PCR gene target	Sensitivity [a]	Specificity [b]	Positive predictive value [c]	Negative predictive value [d]
Pneumococcal pneumonia [e]	*ply*	42 (13/31)	90 (55/61)	68 (13/19)	75 (55/73)
	Spn9802	32 (10/31)	98 (60/61)	91 (10/11)	74 (60/81)
	lytA	26 (8/31)	100 (61/61)	100 (8/8)	73 (61/84)
Bacteraemic pneumococcal pneumonia	*ply*	70 (7/10)	85 (70/82)	37 (7/19)	96 (70/73)
	Spn9802	60 (6/10)	94 (77/82)	55 (6/11)	95 (77/81)
	lytA	70 (7/10)	99 (81/82)	88 (7/8)	96 (81/84)

[a] Reported as percentage (number with positive PCR/number with the defined pneumonia diagnosis).
[b] Reported as percentage (number with negative PCR/number without the defined pneumonia diagnosis).
[c] Reported as percentage (number with the defined pneumonia diagnosis / number with positive PCR).
[d] Reported as percentage (number without the defined pneumonia diagnosis / number with negative PCR).
[e] *S. pneumoniae* detected by blood culture, culture of representative sputum, or urinary antigen test.

Paper IV

Detection of *Haemophilus influenzae* in respiratory secretions from pneumonia patients by quantitative real-time polymerase chain reaction

In this study, we developed a specific and sensitive quantitative real-time PCR using the outer-membrane protein *P6* as a target gene for detection of *H. influenzae.* The *P6* assay was able to detect <30 genome copies per reaction tube, when serial dilutions of target DNA with known concentrations were repeatedly tested to identify the detection capacity of the assay (Table 4).

Table 4. Analytical sensitivity of *P6* real-time PCR

No. of reactions	Genome copies per reaction tube	No. of reactions with detected target
6	100	6/6 (100%)
6	30	6/6 (100%)
3	10	2/3 (66%)
7	5	4/7 (57%)
3	3	1/3 (33%)

The specificity of the *P6* PCR, in addition to three other PCR methods (*rnpB*, *16S rRNA* and *bexA*), was tested on DNA from 29 bacterial strains representing 11 species and including 7 clinical isolates of *H. parainfluenzae* (Table 5). The *P6* assay detected all capsular and non-capsular *H. influenzae* strains, and was more specific than *rnpB* and *16S rRNA* PCRs. However, the closely related species *H. aegyptius* and *H. haemolyticus* were also detected, and this result was similar to a previously published *P6* assay (110). The *bexA* PCR was specific (except for *H. parahaemolyticus*), but could not detect four capsular types (a, e, f and one b strain) and none of five non-capsular *H. influenzae* strains. This result was in concordance with a previous report (106). As shown in Table 5 all four target genes had limitations for diagnostic use. The obvious specificity problem for *H. influenzae* PCRs is mainly explained by the frequent genetic exchange by transformation and recombination in the *Haemophilus* genus (59, 75-77).

Table 5. Bacterial strains used in specificity test

Culture collection		Bacterial species	Capsular type	PCR assay			
CCUG	ATCC			*P6*	*16S rRNA*	*rnpB*	*bexA*
3715 T	33389	*Aggregatibacter aphrophilus*†		-	-	-	-
25716 T	11116	*H. aegyptius*		+	+	+	-
31340		*H. cryptic genospecies*		+	-	+	-
12834 T	33390	*H. haemolyticus*		+	-	+	-
6881		*H. influenzae*	*a*	+	+	+	-
23946	9334	*H. influenzae*	*b*	+	+	+	-
15195		*H. influenzae*	*b*	+	+	+	+
6879		*H. influenzae*	*c*	+	+	+	+
6878		*H. influenzae*	*d*	+	+	-	+
6877		*H. influenzae*	*e*	+	-	+	-
15435		*H. influenzae*	*f*	+	+	-	-
45442		*H. influenzae bio var I*	Non-capsular	+	+	+	-
23945 T	33391	*H. influenzae bio var II*	Non-capsular	+	+	+	-
45156		*H. influenzae bio var II*	Non-capsular	+	+	+	-
35407		*H. influenzae bio var III*	Non-capsular	+	+	+	-
36704		*H. influenzae bio var V*	Non-capsular	+	+	+	-
11096		*H. intermedius subsp. intermedius*		+	+	+	-
32367		*H. intermedius subsp. intermedius*		+	+	+	-
3716 T	10014	*H. parahaemolyticus*		-	-	-	-
51599		*H. parahaemolyticus*		-	+	-	+
8259		*H. parainfluenzae*		-	+	-	-
12836 T	33392	*H. parainfluenzae*		-	+	-	-
44486		*H. parainfluenzae*		-	+	-	-
44743		*H. parainfluenzae*		-	+	-	-
45191		*H. parainfluenzae*		-	+	-	-
7596		*H. parainfluenzae*		-	+	-	-
48703 T	NCTC 13334	*H. pittmaniae*		-	+	+	-
10787 T	33393	*Aggregatibacter segnis**		-	-	-	-
46700		*H. segnis*		-	-	-	-
Clinical isolates (7)		*H. parainfluenzae*		-	+ (5 of 7)	+ (2 of 7)	-

T type strain
† Formerly *H. aphrophilus*
* Formerly *H. segnis*

In nasopharyngeal aspirates from 166 CAP patients, *H. influenzae* was identified by culture in 40 cases, and tested positive by gel-based *16S rRNA* PCR in 43 cases, and by *P6* PCR in 59 cases. Of the 40 culture positive cases, 39 were positive by *P6* PCR, while 36 cases were positive by *16S rRNA* PCR. Moreover, 20 cases were culture negative but positive in *P6* PCR, of which 13 also proved positive by *rnpB* PCR and 6 were confirmed by *16S rRNA* PCR. To further test the specificity of the *P6* PCR on the 20 cases that were culture negative and *P6* PCR positive, an additional examination was performed by *fucK* PCR, an assay not designed for maximal analytical sensitivity but for genotyping of isolates. Out of 20 cases, 18 were positive by *fucK* PCR.

Among the 84 control patients, *H. influenzae* was identified by culture in 1 case, by *P6* PCR in 7 cases, by *rnpB* PCR in 8 cases, and by *16S rRNA* PCR in 4 cases. Furthermore, the 6 samples that were culture negative but positive in *P6* PCR were also all positive by *fucK* PCR. *H. influenzae* can be found as a normal flora in the upper respiratory tract system (asymptomatic colonization). This colonization is problematic for both culture and PCR diagnostics. As mentioned above (paper **I**) quantitative real-time PCR provides rapid quantification that may help to distinguish between infection and colonization. In this study we calculated the sensitivity and specificity of the *P6* PCR at a detection limit of the PCR system itself and at a cut-off level of 10^4 DNA copies/mL. As all culture positive patients had *P6* DNA determined at $>10^4$ DNA copies/mL, no change in the sensitivity was seen if the cut-off limit was raised to 10^4 DNA copies/mL. However, the specificity increased from 84.1% to 96.0% (Table 6).

Table 6. Sensitivities and specificities of *P6* PCR compared to culture for detection of *H. influenzae* in nasopharyngeal aspirates from pneumonia patients

P6 PCR cut-off (genome copies/mL)	Sensitivity[a]	Specificity[b]	Positive predictive value[c]	Negative predictive value[d]
Detection limit of the PCR	97.5 (39/40)	84.1 (106/126)	66.1 (39/59)	99.0 (106/107)
$\geq 10^2$	97.5 (39/40)	84.9 (107/126)	67.2 (39/58)	99.0 (107/108)
$\geq 10^3$	97.5 (39/40)	88.1 (111/126)	72.2 (39/54)	99.0 (111/112)
$\geq 10^4$	97.5 (39/40)	96.0 (121/126)	88.6 (39/44)	99.0 (121/122)

[a] Reported as percentage (number of positive PCR/number with positive culture).
[b] Percentage (number of negative PCR/number with negative culture).
[c] Percentage (number of positive culture/ number with positive PCR).
[d] Percentage (number of negative culture/ number with negative PCR).

Paper V

Multiplex quantitative PCR for detection of lower respiratory tract infection caused by *Streptococcus pneumoniae* and *Haemophilus influenzae*

In this study, the quantitative Spn9802 PCR for the detection of *S. pneumoniae* (paper **I**), was combined with the *P6* PCR for the detection of *H. influenzae* (paper **IV**) and the *ctrA* PCR for the detection of *N. meningitidis* (88) in a multiplex PCR format. It is well known that *N. meningitidis* is not a respiratory pathogen, but *S. pneumoniae* and *H. influenzae* are common causes of both respiratory infections and of meningitis. Therefore it was reasonable to include the important meningitis pathogen *N. meningitidis* in a multiplex PCR useful for both situations. The multiplex PCR was evaluated on 156 BAL samples from patients with LRTI and 31 BAL samples from controls. As shown in Table 7, the analytical sensitivity and quantification was not affected by using a combined mixture of reagents and a combined DNA standard (*S. pneumoniae/H. influenzae*) in single tubes. From 156 patients, *S. pneumoniae* and *H. influenzae* were detected by culture and urinary antigen test in 21 and 31 cases, respectively. These pathogens were identified by the multiplex PCR in 52 and 72 cases, respectively (Table 8).

To evaluate a highly sensitive test like real-time PCR with a less sensitive method like culture is problematic and even more difficult if patients are treated with antibiotics before sampling. In this study the Spn9802 target was evaluated by a composite reference standard composed by *lytA* PCR, urinary antigen test and culture. From evaluation of the *P6* PCR discrepant analysis was used by applying the *fucK* PCR on samples which were culture negative but *P6* PCR positive. This resulted in increased specificity and a higher number of pneumonia cases with defined etiology, as shown in Table 9. In this study a cut-off limit of 10^5 DNA copies/mL for positive Spn9802 and *P6* PCRs yielded a high specificity but somewhat reduced the sensitivity. Similar results have been seen in previous studies (33, 142, 143).

In this study *N. meningitidis* was detected as normal flora in 7 samples from the patient group and in 3 samples from controls. Our study on respiratory samples does not enable evaluation of PCR detection of *N. meningitidis*, but the *ctrA* primer pair in our multiplex assay has been shown to reliably detect meningococci in cases of bacterial meningitis in other studies (88, 144-146). This indicates that our multiplex assay can also be useful in the diagnosis of bacterial meningitis caused by *S. pneumoniae*, *H. influenzae* or *N. meningitidis*.

Table 7. Comparison of using PCR reaction mix with a single DNA standard and oligos for one target organism versus duplex DNA target standard and oligos for three target organisms

Oligos for a single target			Oligos for three targets				
DNA standard copy number of target DNA (number of reactions)	Mean *Ct* value	Mean measured copy number (log10)	DNA standard *S. pneumoniae* and *H. influenzae* copy number of each target DNA	Mean *Ct* value	Mean measured copy number (log10)	Δ *Ct*	Δ copy number (log10)
Spn 10000 (5)	27.7			27.8		0.1	
Spn 2000 (5)	30.2			30.4		0.2	
Spn 500 (7)	32.7			32.4		-0.3	
Hi 10000 (5)	23.8			23.7		-0.1	
Hi 2000 (5)	26.4			26.4		0.0	
Hi 500 (7)	28.6			28.5		-0.1	
Spn (23 clinical samples)	27.7 ± 7.6	3.9 ± 1.8		28.2 ± 7.6	3.8 ± 2.0	0.5	-0.1
Hi (50 clinical samples)	24.1 ± 10.7	3.9 ± 2.8		24.7 ± 7.6	3.8 ± 3.0	0.6	-0.1

Ct = Cycle of threshold; Spn = *S. pneumoniae*; Hi = *H. influenzae*

Table 8. Results of tests for *Streptococcus pneumoniae* and *Haemophilus influenzae* in 156 patients with lower respiratory infection

A. *Streptococcus pneumoniae*

BAL culture	Blood culture	Urinary antigen test	Spn9802real-time PCR	*lytA* PCR	Number of samples
+	+	-	+	+	1
+	-	-	+	+	8
+	-	-	+	-	1
-	+	+	+	+	3
-	+	-	+	+	2
-	-	+	+	+	4
-	-	+	+	-	1
-	-	-	+	+	17
-	-	-	+	-	12
-	-	-	-	+	9
-	-	+	-	-	1
-	-	-	-	-	97

B. *Haemophilus influenzae*

BAL culture	*P6* real-time	*lytA* PCR	Number of samples
+	+	+	26
+	+	-	2
+	-	+	1
+	-	-	2
-	+	+	32
-	+	-	8
-	-	+	14
-	-	-	71

Table 9. Sensitivities and specificities of multiplex real-time PCR for detection of *Streptococcus pneumoniae* and *Haemophilus influenzae* in bronchoalveolar lavage from 156 adults with lower respiratory tract infection

Species	Reference test	Detection limit of the assay		Cutoff 10^5 copies/mL	
		Sensitivity	Specificity	Sensitivity	Specificity
S. pneumoniae	BAL culture, blood culture and urinary antigen test	95% (20/21)	75% (101/135)	90% (19/21)	80% (108/135)
	BAL culture, blood culture and urinary antigen test + *lytA* PCR	91% (43/47)	89% (97/109)	79% (37/47)	95% (104/109)
H. influenzae	BAL culture	90% (28/31)	65% (81/125)	81% (25/31)	85% (106/125)
	BAL culture + *fucK* PCR*	93% (69/74)	96% (79/82)	63% (47/74)	100.0% (82/82)

* *fucK* PCR was performed in the PCR positive and culture negative samples

Conclusions

- The Spn9802 PCR is sensitive and specific for detection of *S. pneumoniae* and *S. pseudopneumoniae*.
- The *ply* PCR is not specific for the detection of *S. pneumoniae*, and quantification does not appear to be clinically useful.
- The Spn9802 and *lytA* PCRs are useful for rapid detection of bacteraemic pneumococcal pneumonia
- The *ompP6* real-time PCR is sensitive and specific enough for identification of *H. influenzae* in respiratory secretions.
- Quantification enables discrimination between disease causing infection and commensal colonization by *S. pneumoniae* and *H. influenzae*.
- The multiplex format of the PCR assay enables diagnosis of *S. pneumoniae* and *H. influenzae* in single tubes which leads to reduction in reagent costs and labor time.
- The PCR assay enables detection after antibiotic treatment has been installed.

Future directions

My work has focused on development and use of quantitative real-time PCR for detection of two major respiratory tract pathogens. The importance of specific target genes, the value of quantification and the possibility to design multiplex assays have been high-lighted.

Concrete follow up tasks from my study are:

- To evaluate the quantitative real-time PCR for *S. pneumoniae* and *H. influenzae* on respiratory samples from children to find a cut-off limit by which disease causing pathogens and commensal organisms may be separated.
- To improve the detection of *H. influenzae* by a new real-time PCR based on the *fucK* gene.
- To evaluate the multiplex PCR for detection of *S. pneumonie*, *H. influenzae* and *N. meningitidis* in liquor samples from persons with suspected bacterial meningitis.

There are also several other challenges waiting ahead. Molecular methods are still expensive compared to conventional culture of bacteria and do not allow antibiotic resistance determination. Quantification can be improved by easier and more robust methodology. Broader detection platforms that provide answer on the medical question: “Which microorganism is causing disease in this patient?” are called for.

The rapidly increasing amount of genetic information provides possibilities to compare entire genomes and thereby identify better targets for detection of pathogens. In addition there has been a constant development of detection techniques in recent years. When array technologies, DNA sequencing and new unconventional techniques become cheaper and even more sensitive and robust they can be combined with bioinformatics and lead to powerful diagnostics in clinical routine laboratories.

Acknowledgements

There is no doubt that these years have been tough, interesting and fun at the same time. I have truly learned a lot and I feel proud of the final result. I have been fortunate in my supervisors, colleagues, family and friends; all have contributed, in different ways, to the completion of my thesis. However, some people deserve special mention.

Björn Herrmann, thank you very much for being my supervisor during my PhD study period! Thank you for your patience, endless encouragement and confidence in my capability as a scientist. Your positive view of situations, especially in stressful moments, has given me the inspiration to reach my dream. Thank you so much for not giving up on me!

Jonas Blomberg, my co-supervisor, thank you for accepting me as a PhD student, for generously sharing your vast knowledge in molecular diagnostics and for handing over the main supervision to Björn, and for your constant help and support during my study.

Krostofer Strålin, my co-supervisor, your critical way of thinking, good observations and knowledge helped me a lot, thank you.

My co-authors for the papers in this thesis: Per Olcén, Paula Mölling, Hans Holmberg, Jens Korsgaard and Leif Kirsebom, thank you all for the fruitful collaboration.

Many thanks go to the Libyan Government, represented by the Higher Education Section, for financial support during the study period. Thanks also to the People's Bureau of Libya, especially Faraj El-Montasser and Sedeeq El-shaeby for always being willing to help. I would like to thank the National Center for the Prevention and Control of Communicable and Endemic Diseases, for giving me the chance to go abroad and continue my study.

Many thanks go to Fuad Bahram, who designed the cover of my thesis, for his patience and for constantly trying very hard to do what I wanted.

I would especially like to thank Eva Hjelm for her kindness and for introducing me to Jonas. Eva Haxton for her limitless help and support. Bengt Kalin, Kristina Vincentsson and Akofa Mc-Kwashie for technical assistance during the study period.

My present (and former) colleagues: Christina Öhrmalm, Magnus Jobs, Ronnie Eriksson, Yajin Song, Nahla Mohamed, Amal Elfitory, Lijuan Hu, Egle Aukstuoliene, Patric Jern, Ylva Molin, Markus Klint, Marie Edvinsson, Åsa Innings, Petra Edqvist, Kristofer Severinson, Linus Kristersson, Patrik Ellström, Hong Yin and Katarina Wallmenius – thank you for support, answered questions, interesting discussions, cakes, company at lunch breaks, and everything else we've done during my years here.

Special thanks to Farid Benachenhou; we have had the kind of friendship that most people never find. Sultan Golbob, thank you for your help, supporting and sharing many good times. Shaman Muradrasoli, our friendship has always been the kind that lets us talk and tell each other anything. In addition I would like to thank Farid and Christina for careful reading and comments on my thesis.

I would like to thank all the staff at the clinical microbiology laboratory for the friendly work environment.

I am also sincerely grateful to all the people who have reminded me that there is a real world outside the lab. Nizar Enwaji, we have shared a million memories, some sad and many happy ones, and opened those special places in the heart, thank you. Osama Swisi, Mohsen El-kharam, Jamal Abo Baker, Khalid Nadeem, Mohamed Elghali, Ali El-Mabsoot and Kheilfa El-Tarhony, thank you all for your friendship which has made me feel less homesick. Usama Hejazi and Mohamed Eissa, thank you for all the interesting discussions. Ibraheem Bozakok and Khalid Elmajbery, thank you very much for helping me when I came to Uppsala for the first time.

Many thanks go to my brothers, my sisters and their respective families for your support, encouragement and never-ending faith in me. Thanks too to my brothers and my sisters in law, it feels good to know that you are there and that you care.

My parents, Moussa and Fatema, never for one second ceased to encourage me and believe in me; how could I possibly give up with support like that? I just can't thank you enough, but I hope you know that already! My mother in law, Fatahea, your love and support, your coming to Uppsala to take care of my son when Samira and I were so busy, is really appreciated, thank you.

My children Maram, Rouaa and Moussa - you light my way, gave me writing breaks while you used my computer and share my interest in animals. I am very lucky to have you in my life.

My wonderful wife, Samira, thank you for your never ending love, support and encouragement during all these years, and for your patience and for taking care of everything even though you were busy with your own PhD study. We have been told that having two PhD students under one roof is a recipe for disaster. But it has been fantastic.

Above all, I thank my God Allah (Glory to him) for helping me to successfully complete my study and for surrounded me by people who have given me love and support during my whole life.

References

1. **Mizgerd JP**. Lung infection--a public health priority. *PLoS Med* 2006;**3**(2):e76.
2. **Fine MJ, Smith MA, Carson CA, Mutha SS, Sankey SS, Weissfeld LA, et al.** Prognosis and outcomes of patients with community-acquired pneumonia. A meta-analysis. *JAMA* 1996;**275**(2):134-41.
3. **Niederman MS, Mandell LA, Anzueto A, Bass JB, Broughton WA, Campbell GD, et al.** Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. *Am J Respir Crit Care Med* 2001;**163**(7):1730-54.
4. **Nascimento-Carvalho CM**. Etiology of childhood community acquired pneumonia and its implications for vaccination. *Braz J Infect Dis* 2001;**5**(2):87-97.
5. **Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C**. Estimates of world-wide distribution of child deaths from acute respiratory infections. *Lancet Infect Dis* 2002;**2**(1):25-32.
6. **Hedlund J, Ortqvist A, Ahlqvist T, Augustinsson A, Beckman H, Blanck C, et al.** Management of patients with community-acquired pneumonia treated in hospital in Sweden. *Scand J Infect Dis* 2002;**34**(12):887-92.
7. **Lidman C, Burman LG, Lagergren A, Ortqvist A**. Limited value of routine microbiological diagnostics in patients hospitalized for community-acquired pneumonia. *Scand J Infect Dis* 2002;**34**(12):873-9.
8. **Woodhead M**. Community-acquired pneumonia in Europe: causative pathogens and resistance patterns. *Eur Respir J Suppl* 2002;**36**:20s-27s.
9. **Ostergaard L, Andersen PL**. Etiology of community-acquired pneumonia. Evaluation by transtracheal aspiration, blood culture, or serology. *Chest* 1993;**104**(5):1400-7.
10. **Burman LA, Trollfors B, Andersson B, Henrichsen J, Juto P, Kallings I, et al.** Diagnosis of pneumonia by cultures, bacterial and viral antigen detection tests, and serology with special reference to antibodies against pneumococcal antigens. *J Infect Dis* 1991;**163**(5):1087-93.
11. **Murray PR, Baron EJ**. *Manual of clinical microbiology*. 9th ed. / editor-in-chief, Patrick R. Murray; editors, Ellen Jo Baron. [et al.]. ed. Washington, D.C.: ASM; [Oxford: Blackwell, distributor]; 2007.

12. **Haanpera M, Jalava J, Huovinen P, Meurman O, Rantakokko-Jalava K**. Identification of alpha-hemolytic streptococci by pyrosequencing the *16S rRNA* gene and by use of VITEK 2. *J Clin Microbiol* 2007;**45**(3):762-70.
13. **Innings A, Krabbe M, Ullberg M, Herrmann B**. Identification of 43 *Streptococcus* species by pyrosequencing analysis of the *rnpB* gene. *J Clin Microbiol* 2005;**43**(12):5983-91.
14. **Henrichsen J**. Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol* 1995;**33**(10):2759-62.
15. **Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, Spratt BG**. Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. *J Infect Dis* 2004;**190**(7):1203-11.
16. **Sandgren A, Sjostrom K, Olsson-Liljequist B, Christensson B, Samuelsson A, Kronvall G, et al.** Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. *J Infect Dis* 2004;**189**(5):785-96.
17. **Sjostrom K, Spindler C, Ortqvist A, Kalin M, Sandgren A, Kuhlmann-Berenzon S, et al.** Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. *Clin Infect Dis* 2006;**42**(4):451-9.
18. **Coonrod JD, Drennan DP**. Pneumococcal pneumonia: capsular polysaccharide antigenemia and antibody responses. *Ann Intern Med* 1976;**84**(3):254-60.
19. **Stralin K, Kaltoft MS, Konradsen HB, Olcen P, Holmberg H**. Comparison of two urinary antigen tests for establishment of pneumococcal etiology of adult community-acquired pneumonia. *J Clin Microbiol* 2004;**42**(8):3620-5.
20. **Greenwood D, Slack RCB, Peutherer JF**. *Medical microbiology: a guide to microbial infections: pathogenesis, immunity, laboratory diagnosis, and control*. 15th ed. / edited by David Greenwood, Richard C.B. Slack, John F. Peutherer. ed. New York; Edinburgh: Churchill Livingstone; 1997.
21. **Miyashita N, Shimizu H, Ouchi K, Kawasaki K, Kawai Y, Obase Y, et al.** Assessment of the usefulness of sputum Gram stain and culture for diagnosis of community-acquired pneumonia requiring hospitalization. *Med Sci Monit* 2008;**14**(4):CR171-176.
22. **Ewig S, Schlochtermeier M, Goke N, Niederman MS**. Applying sputum as a diagnostic tool in pneumonia: limited yield, minimal impact on treatment decisions. *Chest* 2002;**121**(5):1486-92.
23. **Garcia-Vazquez E, Marcos MA, Mensa J, de Roux A, Puig J, Font C, et al.** Assessment of the usefulness of sputum culture for diagnosis of community-acquired pneumonia using the PORT predictive scoring system. *Arch Intern Med* 2004;**164**(16):1807-11.
24. **Theerthakarai R, El-Halees W, Ismail M, Solis RA, Khan MA**. Nonvalue of the initial microbiological studies in the management

of nonsevere community-acquired pneumonia. *Chest* 2001;**119**(1):181-4.

25. **Kuijper EJ, van der Meer J, de Jong MD, Speelman P, Dankert J**. Usefulness of Gram stain for diagnosis of lower respiratory tract infection or urinary tract infection and as an aid in guiding treatment. *Eur J Clin Microbiol Infect Dis* 2003;**22**(4):228-34.
26. **Musher DM, Montoya R, Wanahita A**. Diagnostic value of microscopic examination of Gram-stained sputum and sputum cultures in patients with bacteremic pneumococcal pneumonia. *Clin Infect Dis* 2004;**39**(2):165-9.
27. **Roson B, Carratala J, Verdaguer R, Dorca J, Manresa F, Gudiol F**. Prospective study of the usefulness of sputum Gram stain in the initial approach to community-acquired pneumonia requiring hospitalization. *Clin Infect Dis* 2000;**31**(4):869-74.
28. **Fine MJ, Orloff JJ, Rihs JD, Vickers RM, Kominos S, Kapoor WN, et al.** Evaluation of housestaff physicians' preparation and interpretation of sputum Gram stains for community-acquired pneumonia. *J Gen Intern Med* 1991;**6**(3):189-98.
29. **Hirschmann JV**. The sputum Gram stain. *J Gen Intern Med* 1991;**6**(3):261-3.
30. **Bartlett JG, Mundy LM**. Community-acquired pneumonia. *N Engl J Med* 1995;**333**(24):1618-24.
31. **Campbell SG, Marrie TJ, Anstey R, Dickinson G, Ackroyd-Stolarz S**. The contribution of blood cultures to the clinical management of adult patients admitted to the hospital with community-acquired pneumonia: a prospective observational study. *Chest* 2003;**123**(4):1142-50.
32. **Kirkpatrick MB, Bass JB, Jr.** Quantitative bacterial cultures of bronchoalveolar lavage fluids and protected brush catheter specimens from normal subjects. *Am Rev Respir Dis* 1989;**139**(2):546-8.
33. **Dalhoff K, Braun J, Hollandt H, Lipp R, Wiessmann KJ, Marre R**. Diagnostic value of bronchoalveolar lavage in patients with opportunistic and nonopportunistic bacterial pneumonia. *Infection* 1993;**21**(5):291-6.
34. **Bartlett JG**. Diagnostic accuracy of transtracheal aspiration bacteriologic studies. *Am Rev Respir Dis* 1977;**115**(5):777-82.
35. **Hedlund J, Stralin K, Ortqvist A, Holmberg H**. Swedish guidelines for the management of community-acquired pneumonia in immunocompetent adults. *Scand J Infect Dis* 2005;**37**(11-12):791-805.
36. **Stralin K, Tornqvist E, Kaltoft MS, Olcen P, Holmberg H**. Etiologic diagnosis of adult bacterial pneumonia by culture and PCR applied to respiratory tract samples. *J Clin Microbiol* 2006;**44**(2):643-5.
37. **Aguiar SI, Frias MJ, Santos L, Melo-Cristino J, Ramirez M**. Emergence of optochin resistance among *Streptococcus pneumoniae* in Portugal. *Microb Drug Resist* 2006;**12**(4):239-45.

38. **Cheesbrough M**. *Medical laboratory manual for tropical countries*. Doddington: M. Cheesbrough; 1984.
39. **Dominguez J, Blanco S, Rodrigo C, Azuara M, Gali N, Mainou A, et al.** Usefulness of urinary antigen detection by an immunochromatographic test for diagnosis of pneumococcal pneumonia in children. *J Clin Microbiol* 2003;**41**(5):2161-3.
40. **Nunes AA, Camargos PA, Costa PR, Campos MT**. Antigen detection for the diagnosis of pneumonia. *Pediatr Pulmonol* 2004;**38**(2):135-9.
41. **O'Neill KP, Lloyd-Evans N, Campbell H, Forgie IM, Sabally S, Greenwood BM**. Latex agglutination test for diagnosing pneumococcal pneumonia in children in developing countries. *BMJ* 1989;**298**(6680):1061-4.
42. **Stralin K**. Pneumonia severity related to detection of pneumococcal polysaccharides in urine and serum. *Scand J Infect Dis* 2007;**39**(6-7):645-8.
43. **Dowell SF, Garman RL, Liu G, Levine OS, Yang YH**. Evaluation of Binax NOW, an assay for the detection of pneumococcal antigen in urine samples, performed among pediatric patients. *Clin Infect Dis* 2001;**32**(5):824-5.
44. **Navarro D, Garcia-Maset L, Gimeno C, Escribano A, Garcia-de-Lomas J**. Performance of the Binax NOW *Streptococcus pneumoniae* urinary antigen assay for diagnosis of pneumonia in children with underlying pulmonary diseases in the absence of acute pneumococcal infection. *J Clin Microbiol* 2004;**42**(10):4853-5.
45. **Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H**. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol* 1986;**51 Pt 1**:263-73.
46. **Mullis KB, Faloona FA**. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1987;**155**:335-50.
47. **Bartlett JMS, Stirling DS**. *PCR protocols*. 2nd ed. ed. Totowa, NJ; [England]: Humana Press; 2003.
48. **Gill P, Ghaemi A**. Nucleic acid isothermal amplification technologies: a review. *Nucleosides Nucleotides Nucleic Acids* 2008;**27**(3):224-43.
49. **Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ**. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* 1997;**22**(1):176-81.
50. **Mackay IM**. Real-time PCR in the microbiology laboratory. *Clin Microbiol Infect* 2004;**10**(3):190-212.
51. **Bustin SA**. *A-Z of quantitative PCR*. La Jolla, Calif.: International University Line; 2004.
52. **Braasch DA, Corey DR**. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chem Biol* 2001;**8**(1):1-7.

53. **Pfundheller HM, Lomholt C**. Locked nucleic acids: synthesis and characterization of LNA-T diol. *Curr Protoc Nucleic Acid Chem* 2002;**Chapter 4**:Unit 4 12.
54. **Latorra D, Arar K, Hurley JM**. Design considerations and effects of LNA in PCR primers. *Mol Cell Probes* 2003;**17**(5):253-9.
55. **You Y, Moreira BG, Behlke MA, Owczarzy R**. Design of LNA probes that improve mismatch discrimination. *Nucleic Acids Res* 2006;**34**(8):e60.
56. **Sun BW, Babu BR, Sorensen MD, Zakrzewska K, Wengel J, Sun JS**. Sequence and pH effects of LNA-containing triple helix-forming oligonucleotides: physical chemistry, biochemistry, and modeling studies. *Biochemistry* 2004;**43**(14):4160-9.
57. **Stackebrandt E, Frederiksen W, Garrity GM, Grimont PA, Kampfer P, Maiden MC, et al.** Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* 2002;**52**(Pt 3):1043-7.
58. **Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, et al.** Opinion: Re-evaluating prokaryotic species. *Nat Rev Microbiol* 2005;**3**(9):733-9.
59. **Smith HO, Gwinn ML, Salzberg SL**. DNA uptake signal sequences in naturally transformable bacteria. *Res Microbiol* 1999;**150**(9-10):603-16.
60. **Konstantinidis KT, Tiedje JM**. Genomic insights that advance the species definition for prokaryotes. *Proc Natl Acad Sci U S A* 2005;**102**(7):2567-72.
61. **Bergey DH, Holt JG**. *Bergey's manual of systematic bacteriology*. Baltimore, Md.: Williams & Wilkins; 1984.
62. **Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al.** Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* 1998;**95**(6):3140-5.
63. **Cooper JE, Feil EJ**. Multilocus sequence typing-what is resolved? *Trends Microbiol* 2004;**12**(8):373-7.
64. **Bentley S**. Sequencing the species pan-genome. *Nat Rev Microbiol* 2009;**7**(4):258-9.
65. **Fraser C, Alm EJ, Polz MF, Spratt BG, Hanage WP**. The bacterial species challenge: making sense of genetic and ecological diversity. *Science* 2009;**323**(5915):741-6.
66. **Hanage WP, Fraser C, Spratt BG**. Sequences, sequence clusters and bacterial species. *Philos Trans R Soc Lond B Biol Sci* 2006;**361**(1475):1917-27.
67. **Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho Mda G, Steigerwalt AG, et al.** Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. *J Clin Microbiol* 2004;**42**(10):4686-96.

68. **Kilian M, Poulsen K, Blomqvist T, Havarstein LS, Bek-Thomsen M, Tettelin H, et al.** Evolution of *Streptococcus pneumoniae* and its close commensal relatives. *PLoS One* 2008;**3**(7):e2683.
69. **Harf-Monteil C, Granello C, Le Brun C, Monteil H, Riegel P**. Incidence and pathogenic effect of *Streptococcus pseudopneumoniae*. *J Clin Microbiol* 2006;**44**(6):2240-1.
70. **Facklam R**. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin Microbiol Rev* 2002;**15**(4):613-30.
71. **Munoz R, Fenoll A, Vicioso D, Casal J**. Optochin-resistant variants of *Streptococcus pneumoniae*. *Diagn Microbiol Infect Dis* 1990;**13**(1):63-6.
72. **Obregon V, Garcia P, Garcia E, Fenoll A, Lopez R, Garcia JL**. Molecular peculiarities of the *lytA* gene isolated from clinical pneumococcal strains that are bile insoluble. *J Clin Microbiol* 2002;**40**(7):2545-54.
73. **Kikuchi K, Enari T, Totsuka K, Shimizu K**. Comparison of phenotypic characteristics, DNA-DNA hybridization results, and results with a commercial rapid biochemical and enzymatic reaction system for identification of viridans group streptococci. *J Clin Microbiol* 1995;**33**(5):1215-22.
74. **Kawamura Y, Whiley RA, Shu SE, Ezaki T, Hardie JM**. Genetic approaches to the identification of the mitis group within the genus *Streptococcus*. *Microbiology* 1999;**145 (Pt 9)**:2605-13.
75. **Kroll JS, Moxon ER**. Capsulation in distantly related strains of *Haemophilus influenzae* type b: genetic drift and gene transfer at the capsulation locus. *J Bacteriol* 1990;**172**(3):1374-9.
76. **Kroll JS, Moxon ER, Loynds BM**. Natural genetic transfer of a putative virulence-enhancing mutation to *Haemophilus influenzae* type a. *J Infect Dis* 1994;**169**(3):676-9.
77. **Mukundan D, Ecevit Z, Patel M, Marrs CF, Gilsdorf JR**. Pharyngeal colonization dynamics of *Haemophilus influenzae* and *Haemophilus haemolyticus* in healthy adult carriers. *J Clin Microbiol* 2007;**45**(10):3207-17.
78. **Kroll JS, Wilks KE, Farrant JL, Langford PR**. Natural genetic exchange between *Haemophilus* and *Neisseria*: intergeneric transfer of chromosomal genes between major human pathogens. *Proc Natl Acad Sci U S A* 1998;**95**(21):12381-5.
79. **Norskov-Lauritsen N, Overballe MD, Kilian M**. Delineation of the species *Haemophilus influenzae* by phenotype, multilocus sequence phylogeny, and detection of marker genes. *J Bacteriol* 2009;**191**(3):822-31.
80. **Erwin AL, Sandstedt SA, Bonthuis PJ, Geelhood JL, Nelson KL, Unrath WC, et al.** Analysis of genetic relatedness of *Haemophilus influenzae* isolates by multilocus sequence typing. *J Bacteriol* 2008;**190**(4):1473-83.

81. **Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, et al.** Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. *J Clin Microbiol* 2003;**41**(4):1623-36.
82. **Brooks GF**. *Jawetz, Melnick & Adelberg's medical microbiology*. 24th ed. / George F. Brooks. [et al.]. ed. New York; London: McGraw-Hill Medical; 2007.
83. **Murphy TF, Brauer AL, Sethi S, Kilian M, Cai X, Lesse AJ**. *Haemophilus haemolyticus*: a human respiratory tract commensal to be distinguished from *Haemophilus influenzae*. *J Infect Dis* 2007;**195**(1):81-9.
84. **Casin I, Grimont F, Grimont PA**. Deoxyribonucleic acid relatedness between *Haemophilus aegyptius* and *Haemophilus influenzae*. *Ann Inst Pasteur Microbiol* 1986;**137B**(2):155-63.
85. **Hedegaard J, Okkels H, Bruun B, Kilian M, Mortensen KK, Norskov-Lauritsen N**. Phylogeny of the genus *Haemophilus* as determined by comparison of partial *infB* sequences. *Microbiology* 2001;**147**(Pt 9):2599-609.
86. **Rennie RP, Brosnikoff C, Shokoples S, Reller LB, Mirrett S, Janda W, et al.** Multicenter evaluation of the new Vitek 2 *Neisseria-Haemophilus* identification card. *J Clin Microbiol* 2008;**46**(8):2681-5.
87. **Valenza G, Ruoff C, Vogel U, Frosch M, Abele-Horn M**. Microbiological evaluation of the new VITEK 2 *Neisseria-Haemophilus* identification card. *J Clin Microbiol* 2007;**45**(11):3493-7.
88. **Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB**. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol* 2001;**39**(4):1553-8.
89. **Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ**. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. *J Clin Microbiol* 1997;**35**(11):2854-8.
90. **Kais M, Spindler C, Kalin M, Ortqvist A, Giske CG**. Quantitative detection of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in lower respiratory tract samples by real-time PCR. *Diagn Microbiol Infect Dis* 2006;**55**(3):169-78.
91. **Morrison KE, Lake D, Crook J, Carlone GM, Ades E, Facklam R, et al.** Confirmation of *psaA* in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification and diagnosis. *J Clin Microbiol* 2000;**38**(1):434-7.
92. **Ohkusu K, Nash KA, Inderlied CB**. Molecular characterisation of *Haemophilus influenzae* type a and untypeable strains isolated simultaneously from cerebrospinal fluid and blood: novel use of quantitative real-time PCR based on the cap copy number to determine virulence. *Clin Microbiol Infect* 2005;**11**(8):637-43.

93. **Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, et al.** Measuring nasal bacterial load and its association with otitis media. *BMC Ear Nose Throat Disord* 2006;**6**:10.
94. **Stralin K, Backman A, Holmberg H, Fredlund H, Olcen P**. Design of a multiplex PCR for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* to be used on sputum samples. *APMIS* 2005;**113**(2):99-111.
95. **Wellinghausen N, Wirths B, Franz AR, Karolyi L, Marre R, Reischl U**. Algorithm for the identification of bacterial pathogens in positive blood cultures by real-time LightCycler polymerase chain reaction (PCR) with sequence-specific probes. *Diagn Microbiol Infect Dis* 2004;**48**(4):229-41.
96. **Whatmore AM, Efstratiou A, Pickerill AP, Broughton K, Woodard G, Sturgeon D, et al.** Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: characterization of "Atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes. *Infect Immun* 2000;**68**(3):1374-82.
97. **Greiner O, Day PJ, Bosshard PP, Imeri F, Altwegg M, Nadal D**. Quantitative detection of *Streptococcus pneumoniae* in nasopharyngeal secretions by real-time PCR. *J Clin Microbiol* 2001;**39**(9):3129-34.
98. **Yang S, Lin S, Khalil A, Gaydos C, Nuemberger E, Juan G, et al.** Quantitative PCR assay using sputum samples for rapid diagnosis of pneumococcal pneumonia in adult emergency department patients. *J Clin Microbiol* 2005;**43**(7):3221-6.
99. **McCrea KW, Xie J, LaCross N, Patel M, Mukundan D, Murphy TF, et al.** Relationships of nontypeable *Haemophilus influenzae* strains to hemolytic and nonhemolytic *Haemophilus haemolyticus* strains. *J Clin Microbiol* 2008;**46**(2):406-16.
100. **Gillespie SH**. The role of the molecular laboratory in the investigation of Streptococcus pneumoniae infections. *Semin Respir Infect* 1999;**14**(3):269-75.
101. **Messmer TO, Sampson JS, Stinson A, Wong B, Carlone GM, Facklam RR**. Comparison of four polymerase chain reaction assays for specificity in the identification of *Streptococcus pneumoniae*. *Diagn Microbiol Infect Dis* 2004;**49**(4):249-54.
102. **Neeleman C, Klaassen CH, Klomberg DM, de Valk HA, Mouton JW**. Pneumolysin is a key factor in misidentification of macrolide-resistant *Streptococcus pneumoniae* and is a putative virulence factor of *S. mitis* and other streptococci. *J Clin Microbiol* 2004;**42**(9):4355-7.
103. **Ubukata K, Asahi Y, Yamane A, Konno M**. Combinational detection of autolysin and penicillin-binding protein 2B genes of *Streptococcus pneumoniae* by PCR. *J Clin Microbiol* 1996;**34**(3):592-6.
104. **Dowson CG, Coffey TJ, Kell C, Whiley RA**. Evolution of penicillin resistance in *Streptococcus pneumoniae*; the role of *Streptococ-*

cus mitis in the formation of a low affinity *PBP2B* in *S. pneumoniae*. *Mol Microbiol* 1993;**9**(3):635-43.
105. **Jado I, Fenoll A, Casal J, Perez A**. Identification of the *psaA* gene, coding for pneumococcal surface adhesin A, in viridans group streptococci other than *Streptococcus pneumoniae*. *Clin Diagn Lab Immunol* 2001;**8**(5):895-8.
106. **Sam IC, Smith M**. Failure to detect capsule gene *bexA* in *Haemophilus influenzae* types e and f by real-time PCR due to sequence variation within probe binding sites. *J Med Microbiol* 2005;**54**(Pt 5):453-5.
107. **Kroll JS, Loynds BM, Moxon ER**. The *Haemophilus influenzae* capsulation gene cluster: a compound transposon. *Mol Microbiol* 1991;**5**(6):1549-60.
108. **St Geme JW, 3rd, Takala A, Esko E, Falkow S**. Evidence for capsule gene sequences among pharyngeal isolates of nontypeable *Haemophilus influenzae*. *J Infect Dis* 1994;**169**(2):337-42.
109. **Backman A, Lantz P, Radstrom P, Olcen P**. Evaluation of an extended diagnostic PCR assay for detection and verification of the common causes of bacterial meningitis in CSF and other biological samples. *Mol Cell Probes* 1999;**13**(1):49-60.
110. **van Ketel RJ, de Wever B, van Alphen L**. Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification. *J Med Microbiol* 1990;**33**(4):271-6.
111. **Rutjes AW, Reitsma JB, Coomarasamy A, Khan KS, Bossuyt PM**. Evaluation of diagnostic tests when there is no gold standard. A review of methods. *Health Technol Assess* 2007;**11**(50):iii, ix-51.
112. **Valenstein PN**. Evaluating diagnostic tests with imperfect standards. *Am J Clin Pathol* 1990;**93**(2):252-8.
113. **Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al.** The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Ann Intern Med* 2003;**138**(1):W1-12.
114. **Gustafson P**. The utility of prior information and stratification for parameter estimation with two screening tests but no gold standard. *Stat Med* 2005;**24**(8):1203-17.
115. **Harel O, Zhou XH**. Multiple imputation for correcting verification bias. *Stat Med* 2006;**25**(22):3769-86.
116. **Hadgu A**. The discrepancy in discrepant analysis. *Lancet* 1996;**348**(9027):592-3.
117. **Miller WC**. Can we do better than discrepant analysis for new diagnostic test evaluation? *Clin Infect Dis* 1998;**27**(5):1186-93.
118. **Suzuki N, Seki M, Nakano Y, Kiyoura Y, Maeno M, Yamashita Y**. Discrimination of *Streptococcus pneumoniae* from viridans group streptococci by genomic subtractive hybridization. *J Clin Microbiol* 2005;**43**(9):4528-34.
119. **Tapp J, Thollesson M, Herrmann B**. Phylogenetic relationships and genotyping of the genus *Streptococcus* by sequence determina-

tion of the RNase P RNA gene, *rnpB*. *Int J Syst Evol Microbiol* 2003;**53**(Pt 6):1861-71.

120. **Chastre J, Fagon JY, Bornet-Lecso M, Calvat S, Dombret MC, al Khani R, et al.** Evaluation of bronchoscopic techniques for the diagnosis of nosocomial pneumonia. *Am J Respir Crit Care Med* 1995;**152**(1):231-40.
121. **Rouby JJ, Martin De Lassale E, Poete P, Nicolas MH, Bodin L, Jarlier V, et al.** Nosocomial bronchopneumonia in the critically ill. Histologic and bacteriologic aspects. *Am Rev Respir Dis* 1992;**146**(4):1059-66.
122. **Bayram A, Kocoglu E, Balci I, Filiz A, Eksi F**. Real-time polymerase chain reaction assay for detection of *Streptococcus pneumoniae* in sputum samples from patients with community-acquired pneumonia. *J Microbiol Immunol Infect* 2006;**39**(6):452-7.
123. **Johansson N, Kalin M, Giske CG, Hedlund J**. Quantitative detection of *Streptococcus pneumoniae* from sputum samples with real-time quantitative polymerase chain reaction for etiologic diagnosis of community-acquired pneumonia. *Diagn Microbiol Infect Dis* 2008;**60**(3):255-61.
124. **Saravolatz LD, Johnson L, Galloway L, Manzor O, Pawlak J, Belian B**. Detection of *Streptococcus pneumoniae* colonisation in respiratory tract secretions of military personnel. *Clin Microbiol Infect* 2007;**13**(9):932-6.
125. **Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, et al.** Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. *J Clin Microbiol* 2007;**45**(8):2460-6.
126. **Kaijalainen T, Saukkoriipi A, Bloigu A, Herva E, Leinonen M**. Real-time pneumolysin polymerase chain reaction with melting curve analysis differentiates pneumococcus from other alpha-hemolytic streptococci. *Diagn Microbiol Infect Dis* 2005;**53**(4):293-9.
127. **Murdoch DR, Anderson TP, Beynon KA, Chua A, Fleming AM, Laing RT, et al.** Evaluation of a PCR assay for detection of *Streptococcus pneumoniae* in respiratory and nonrespiratory samples from adults with community-acquired pneumonia. *J Clin Microbiol* 2003;**41**(1):63-6.
128. **Verhelst R, Kaijalainen T, De Baere T, Verschraegen G, Claeys G, Van Simaey L, et al.** Comparison of five genotypic techniques for identification of optochin-resistant pneumococcus-like isolates. *J Clin Microbiol* 2003;**41**(8):3521-5.
129. **Kirkham LA, Jefferies JM, Kerr AR, Jing Y, Clarke SC, Smith A, et al.** Identification of invasive serotype 1 pneumococcal isolates that express nonhemolytic pneumolysin. *J Clin Microbiol* 2006;**44**(1):151-9.

130. **Salo P, Ortqvist A, Leinonen M**. Diagnosis of bacteremic pneumococcal pneumonia by amplification of pneumolysin gene fragment in serum. *J Infect Dis* 1995;**171**(2):479-82.
131. **Saukkoriipi A, Leskela K, Herva E, Leinonen M**. *Streptococcus pneumoniae* in nasopharyngeal secretions of healthy children: comparison of real-time PCR and culture from STGG-transport medium. *Mol Cell Probes* 2004;**18**(3):147-53.
132. **Rintamaki S, Saukkoriipi A, Salo P, Takala A, Leinonen M**. Detection of *Streptococcus pneumoniae* DNA by using polymerase chain reaction and microwell hybridization with Europium-labelled probes. *J Microbiol Methods* 2002;**50**(3):313-8.
133. **Walker JA, Allen RL, Falmagne P, Johnson MK, Boulnois GJ**. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. *Infect Immun* 1987;**55**(5):1184-9.
134. **Virolainen A, Salo P, Jero J, Karma P, Eskola J, Leinonen M**. Comparison of PCR assay with bacterial culture for detecting *Streptococcus pneumoniae* in middle ear fluid of children with acute otitis media. *J Clin Microbiol* 1994;**32**(11):2667-70.
135. **Falguera M, Lopez A, Nogues A, Porcel JM, Rubio-Caballero M**. Evaluation of the polymerase chain reaction method for detection of *Streptococcus pneumoniae* DNA in pleural fluid samples. *Chest* 2002;**122**(6):2212-6.
136. **Suzuki N, Yuyama M, Maeda S, Ogawa H, Mashiko K, Kiyoura Y**. Genotypic identification of presumptive *Streptococcus pneumoniae* by PCR using four genes highly specific for *S. pneumoniae*. *J Med Microbiol* 2006;**55**(Pt 6):709-14.
137. **Chalasani NP, Valdecanas MA, Gopal AK, McGowan JE, Jr., Jurado RL**. Clinical utility of blood cultures in adult patients with community-acquired pneumonia without defined underlying risks. *Chest* 1995;**108**(4):932-6.
138. **Grace CJ, Lieberman J, Pierce K, Littenberg B**. Usefulness of blood culture for hospitalized patients who are receiving antibiotic therapy. *Clin Infect Dis* 2001;**32**(11):1651-5.
139. **Dominguez J, Gali N, Matas L, Pedroso P, Blanco S, Gimenez M, et al.** PCR detection of *Streptococcus pneumoniae* DNA in serum samples for pneumococcal pneumonia diagnosis. *Clin Microbiol Infect* 2001;**7**(3):164-6.
140. **Sheppard CL, Harrison TG, Morris R, Hogan A, George RC**. Autolysin-targeted LightCycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. *J Med Microbiol* 2004;**53**(Pt 3):189-95.
141. **Smith MD, Sheppard CL, Hogan A, Harrison TG, Dance DA, Derrington P, et al.** Diagnosis of *Streptococcus pneumoniae* infections in adults with bacteremia and community-acquired pneumonia: clinical comparison of pneumococcal PCR and urinary antigen detection. *J Clin Microbiol* 2009;**47**(4):1046-9.

142. **Kahn FW, Jones JM**. Diagnosing bacterial respiratory infection by bronchoalveolar lavage. *J Infect Dis* 1987;**155**(5):862-9.
143. **Thorpe JE, Baughman RP, Frame PT, Wesseler TA, Staneck JL**. Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. *J Infect Dis* 1987;**155**(5):855-61.
144. **Fernandez-Rodriguez A, Alcala B, Alvarez-Lafuente R**. Real-time polymerase chain reaction detection of *Neisseria meningitidis* in formalin-fixed tissues from sudden deaths. *Diagn Microbiol Infect Dis* 2008;**60**(4):339-46.
145. **Gray SJ, Trotter CL, Ramsay ME, Guiver M, Fox AJ, Borrow R, et al.** Epidemiology of meningococcal disease in England and Wales 1993/94 to 2003/04: contribution and experiences of the Meningococcal Reference Unit. *J Med Microbiol* 2006;**55**(Pt 7):887-96.
146. **Taha MK, Alonso JM, Cafferkey M, Caugant DA, Clarke SC, Diggle MA, et al.** Interlaboratory comparison of PCR-based identification and genogrouping of *Neisseria meningitidis*. *J Clin Microbiol* 2005;**43**(1):144-9.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 479

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-107931

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2009