

# CATCHING THE PNEUMOCOCCUS

Studies focusing on carriage, epidemiology  
and microbiological methods

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***Abstract***

The purpose of this study was to develop sensitive and specific laboratory diagnostic methods for the demonstration of pneumococcal surface antigens or pneumococcus-specific antibodies in clinical samples. The work took account of epidemiological aspects of both pneumococcal disease and nasopharyngeal carriage of pneumococcus.

We first compared the sensitivity of pneumococcal culture and antigen detection methods in nasopharyngeal samples in a developing country setting and then investigated the possibility of improving the sensitivity of the antigen detection by introducing an enrichment step in the procedure. – Further investigations were designed to determine the validity of pneumolysin-specific immune complex bound antibody assay as a tool for diagnosing pneumococcal ALRI in a developing country setting. Finally, we developed an enzyme immunoassay for the detection of pneumococcal capsular polysaccharide antigens, using type-specific antibodies produced in-house in rabbits through immunisation with an in-house-produced pneumococcal whole cell vaccine. The method was tested in nasopharyngeal and middle ear fluid samples.

The first results indicated that antigen detection might be more sensitive than culture in demonstrating pneumococci in URT, particularly in children with prior antimicrobial therapy. Antigen detection is a feasible method for studies on pneumococci in developing countries. For type-specific demonstration of *S. pneumoniae*, detection of pneumococcal antigen after an enrichment step proved a sensitive method that can be applied for epidemiologic study purposes, e.g., in vaccine trials, in areas without ready access to a good microbiology laboratory.

Determination of IC-bound pneumolysin IgG antibodies appears to be a useful method for species-specific diagnosis of pneumococcal infections. The results indicating pneumococcal aetiology in ALRI patients in this study compare well with the best results obtained by the use of lung aspirates. Increasing the number of serial samples improves the sensitivity of the assay, but even two samples provide more positive findings than other methods currently in routine use. Criteria of positivity need to be confirmed in subsequent larger studies with both healthy controls and patients with confirmed pneumococcal disease. It is also important to control the findings in patients with pneumonia of non-pneumococcal origin.

The novel enzyme immunoassay was shown to work well with enrichment culture samples, with an almost 100% sensitivity compared with the culture. Middle ear fluid samples were too diluted for the enzyme immunoassay method used, and only 74% sensitivity compared with culture was achieved. Provided that adequate samples can be obtained, the method will be a useful complement to the current laboratory methods used to diagnose pneumococcal disease.

With the existence of a broad spectrum of microbiological and immunological methods, it is imperative to seek international consensus for standard methods to demonstrate pneumococcus. Otherwise it is very difficult to compare results from different clinical studies. A WHO Working Group recently proposed a standard method for detecting upper respiratory carriage of pneumococcus, but a lot of work remains to be done in other areas of research on pneumococcal infections.

***Keywords:*** acute respiratory infection, antigen detection, capsular polysaccharide, enrichment culture, enzyme immunoassay, immune complexes, nasopharyngeal carriage, pneumococcus, pneumolysin, pneumonia, *Streptococcus pneumoniae*



## Acknowledgements

This study was carried out at the National Public Health Institute (KTL) in Helsinki and in Oulu, in intermittent working periods during 1991–2003. Over the years, I worked in various departments, held several different positions and responsibilities, travelled extensively in Finland and abroad, and gained unique and deep insight into a complex research organisation and an authoritative health policy institution.

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Like most medical research projects today, this research was a collaborative effort. In addition to many departments of the KTL, we interacted with the Research Institute of Tropical Medicine, Manila, University of Queensland, Brisbane, Australia, and Women's and Children's Hospital, Adelaide, Australia, Statens Serum Institut, Copenhagen, and the Aurora Municipal Hospital, Helsinki. The individual collaborators are too numerous to count, but I want to acknowledge the co-authors of the original articles: Raili Haikala, Marilla C. Lucero, PhD, James C. Paton, PhD, Satu Rapola, MD, Sanna Rintamäki, MSc, Eeva Salo, MD, Paula Salo, PhD, Ritva Syrjänen, MD, and Aino K. Takala, MD. My colleagues at the vaccine production unit of the KTL taught me very special practical skills, for which I want to acknowledge Tapani Kuronen, Phil Lic, Rose-Marie Ölander,

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My interest in research work and in microbiology dates back to the 1970s, when I first worked as a curious summer boy at the Department of Medical Microbiology, University of Oulu. My first exposure to laboratory work was guided by Professor Veijo Raunio, MD, and Docent Jorma Ilonen, MD (then a medical student himself). Later on, when I myself was a medical student, my inquiring attitude was further strengthened by the brilliant and inspiring personality of Professor Anja Tiilikainen, MD. She invested heavily in my basic research training, and it gives me special pleasure to be able to deliver – finally – something tangible. Professor Pekka Saikku, MD, encouraged and nourished me in the final stages of the work.

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Helsinki, May 2003

Kari S. Lankinen

## Abbreviations

AHS	$\alpha$ -haemolytic streptococci
ALRI	acute lower respiratory infection
AOM	acute otitis media
ARI	acute respiratory infection
ATCC	American Type Culture Collection
BCG	Bacillus Calmette–Guérin
BHI	brain–heart infusion
CAP	community-acquired pneumonia
CaPS	capsular polysaccharide
CFU	colony-forming unit
CIEP	counterimmunoelectrophoresis
COA	coagglutination
CPS	C-polysaccharide
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
DTP	diphtheria-tetanus-pertussis vaccine
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
EPI	Expanded Programme on Immunization (WHO)
FBS	foetal bovine serum
FinOM	Finnish Otitis Media (Study Group, Cohort Study, Vaccine Trial)
GLM	global linear model
Hi	<i>Haemophilus influenzae</i>
Hib	<i>Haemophilus influenzae</i> , type b
IC	immune complex
IPD	invasive pneumococcal disease
KTL	National Public Health Institute, Finland
LA	latex agglutination
LytA	pneumococcal autolysin
MEF	middle ear fluid
MRC	Medical Research Council (UK)

NPA	nasopharyngeal aspirate
NPS	nasopharyngeal sample (swab)
NPSe	nasopharyngeal sample (processed through enrichment culture)
NS	not significant
OD	optical density
P	probability
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
Ply	pneumolysin
Pnc	pneumococcus
PNG	Papua New Guinea
PsaA	pneumococcal surface adhesin A
PspA	pneumococcal surface protein A
RIA	radioimmunoassay
RITM	Research Institute for Tropical Medicine
RNA	ribonucleic acid
RPM	revolutions per minute
RTI	respiratory tract infection
SGT	serogroup or -type
TNA	transthoracic needle aspirate
URT	upper respiratory tract
WHO	World Health Organization

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## **List of original study reports**

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I–IV.

- I Lankinen KS, Leinonen M, Tupasi TE, Haikala R & Ruutu P (1994) Pneumococci in nasopharyngeal samples from Filipino children with acute respiratory infection. *J Clin Microbiol* 32: 2948-2952.
- II Lankinen KS, Salo P, Rapola S, Salo E, Takala AK & Leinonen M (1997) Pneumococcal capsular antigen detection after enrichment culture: an alternative to culture methods in epidemiologic research. *Am J Trop Med Hyg* 56: 211-5.
- III Lankinen KS, Ruutu P, Nohynek H, Lucero M, Paton JC & Leinonen, M (1999) Pneumococcal pneumonia can be diagnosed by demonstration of pneumolysin antibodies in precipitated immune complexes: a study in 350 Philippine children with acute lower respiratory infection. *Scand J Infect Dis* 31: 155-161.
- IV Lankinen KS, Rintamäki S, Syrjänen R, Kilpi T, Ruutu P & Leinonen M (2003) Type specific enzyme immunoassay for detection of pneumococcal capsular polysaccharide antigens. Submitted.

Some previously unpublished data are also presented.

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# 1 Introduction

Acute respiratory infections are a major cause of morbidity worldwide, with significant mortality associated with acute lower respiratory infections (ALRI). In developing countries, several studies have identified *Streptococcus pneumoniae* and *Haemophilus influenzae* as the major ALRI pathogens. Although *S. pneumoniae* was already discovered more than 120 years ago, the epidemiology of pneumococcal disease still remains incompletely understood.

The problem in research on pneumococcal diagnostic methods is that there is no reliable criterion standard that could be employed as a reference. Frequently suggested as the standard, blood culture is specific, but its sensitivity is low (1, 2), especially in children (3, 4). Antigen detection is similarly complicated by sensitivity problems (5). Serological methods remain largely investigational. They lack international standards and, furthermore, necessitate paired serum samples, which restricts their usefulness in epidemiological studies (6). Therefore, there is a pressing need for improved methods to identify pneumococcal aetiology.

A broad spectrum of diagnostic methods has already been employed in epidemiological and clinical research. Studies utilising lung aspirates have provided perhaps the most accurate data so far, indicating pneumococcal aetiology in 30–60% of all pneumonias (7-10). However, because of the invasive nature of the procedure, lung aspiration has not become clinical routine in diagnosis of respiratory infections. Recently, a Finnish study demonstrated again the high microbial yield with aspiration and showed a relatively low risk of clinically significant adverse events (10).

Bacterial culture is commonly employed in clinical bacteriology, but in developing countries it is not always feasible. In any case, the results of bacterial culture are influenced by a number of factors, such as concurrent antimicrobial treatment or problems in timely transportation of samples to the laboratory for culture. In field trial settings, bacterial culture may not be feasible, especially for *S. pneumoniae*, which dies rapidly if specimens are not processed immediately. Detection of pneumococcal capsular polysaccharide antigens is one alternative, but so far a number of problems have impeded its wider application.

In principle, antigen detection assays have several advantages over bacterial culture. Samples for antigen detection usually do not need any special transport media, and

transport time is not a restriction. Assays can be performed directly after sampling, or at a later date, if more convenient. The methods, such as latex agglutination and counterimmunoelectrophoresis, are relatively simple procedures, requiring only basic-level training. Antigen detection also allows a simple demonstration of carriage of multiple serotypes, much in contrast to conventional culture techniques (11). Antigen detection methods are also less influenced by concomitant use of antimicrobial drugs, a frequent problem in real life (11-14). Thus, a more accurate picture on pneumococcal epidemiology could be achieved by antigen detection.

Precise information on the serotype distribution of *S. pneumoniae* is particularly important for the development of type-specific vaccines. Carriage studies are also an integral part of vaccine trials, because a vaccine that would reduce carriage is expected to provide enhanced protection through herd immunity. Indeed, it has been demonstrated that new *Haemophilus influenzae* type b (Hib) conjugate vaccines can significantly reduce pharyngeal carriage of Hib, thus reducing the spread of infection (15-17). For the new pneumococcal conjugate vaccine, a similar effect has been shown, but the net effect has been complicated by at least partial substitution of the evicted serotypes by other serotypes (18, 19).

However, previous experience with detection of pneumococcal capsular polysaccharide antigens in respiratory samples has been inconclusive (12, 20-22). Antigen detection has usually been significantly less sensitive than bacterial culture, both in carriage studies and as a diagnostic method for pneumococcal infections (12, 20). However, there are also reports that have found antigen detection to be superior to culture (11, 14, 21, 22). In all of these studies, a major problem has been the poor correlation of results obtained by different methods.

The rate of pneumococcal carriage in children in developing countries is extremely high, approaching 100% in several studies (23-25), while e.g. in the USA the carriage rate in preschool children has been only 38% (26). Interesting variation in carriage rates has been reported in children of different ethnic origins in Hong Kong, from 10.8% in Chinese to 55.7% in Vietnamese (27).

In Finland, carriage rates of 20–30% have been reported in children (28, 29). When 329 Finnish children were followed from the age of 2 months to 24 months, carriage was observed to increase gradually with age from 9% to 43% (30). Higher proportions of positive samples are found during AOM (45%–56%), in particular during pneumococcal AOM (97%–100%). Antimicrobial treatment reduces carriage only temporarily (30).

It has been shown that the pneumococcal type found in the blood culture of patients with bacteraemic pneumonia is usually the same as the type present in the upper respiratory tract (URT) (31). Thus, information on pneumococcal serotype distribution derived from the URT of children with acute lower respiratory infection (ALRI) has relevance for the choice of pneumococcal types for inclusion in new vaccines. There are, however, little data on the pneumococcal type distribution in the respiratory tract of healthy or ill children in developing countries (31-33).

It was suggested already in 1983 that both antigens and antibodies could become undetectable in direct diagnostic assays, if they are bound to circulating immune complexes (IC) (34, 35). Antigens and antibodies have been successfully demonstrated in IC in association with several infections of both bacterial (36, 37), viral (38-40), and parasitic origin (41, 42), but so far only a few studies have tested this hypothesis in

pneumococcal infections. These studies have utilised the separation and dissociation of IC with subsequent determination of antibodies to pneumococcal polysaccharide released from the dissociated complexes (43-46).

Demonstration of pneumococcal pneumolysin antibodies in IC has been suggested as a diagnostic method for invasive pneumococcal infection in both adults (47-49) and children (50). Pneumolysin, an intracellular haemolysin, is a logical choice for species-specific diagnosis of pneumococcal disease, because it is produced by 99% of clinical isolates of *S. pneumoniae* (51-53). As a virulence factor of *S. pneumoniae* it is also most probably produced *in vivo* during infection (51).

Study I was undertaken to compare the sensitivity of pneumococcal culture and antigen detection methods in the same samples in a developing country setting. Nasopharyngeal aspirates (NPA) were obtained from Filipino children with ALRI. The findings were also analysed according to whether or not the patients received antimicrobials prior to the sampling. The positive samples provided the useful information on pneumococcal type distribution.

If *S. pneumoniae* is found by culture, it must obviously be present in the nasopharynx, and a concurrent negative result by antigen detection means that the methods presently available are not sufficiently sensitive to demonstrate the bacteria directly from the pharyngeal specimens. In contrast, a corresponding method has been shown to work quite adequately for *H. influenzae* type b (17, 54). Therefore, in Study II we investigated the possibility of improving the sensitivity of pneumococcal antigen detection by introducing an enrichment step in the procedure.

The aim of Study III was to determine the validity of pneumolysin-specific IC-bound antibody (Ply-IC) assay as a tool for diagnosing pneumococcal ALRI in a developing country setting. Sera from Filipino children hospitalised with community-acquired ALRI were compared with those of healthy children participating in a clinical vaccine trial in the same locality. We determined Ply-IC of both IgG and IgM classes and, for comparison, capsular polysaccharide- and C-polysaccharide-specific IC-bound IgG antibodies.

Finally, in Study IV, we developed a competitive enzyme immunoassay for the detection of pneumococcal capsular polysaccharide antigens, using type-specific antibodies produced in rabbits through immunisation with in-house produced pneumococcal whole cell vaccine. The method was tested in nasopharyngeal and middle ear fluid samples.

## 2 Review of the literature

Reviewing published pneumococcal research is a daunting task, as the Medline database currently records more than 14,500 articles published since 1966. In recent years, research efforts have accelerated, and 53% of all reports have been published during the last 10 years (27% of the period covered by the database), 33% during the last 5 years (14% of the period). – The early landmark works of White and Heffron are essential background reading for all pneumococcus researchers (55, 56). Insight to the current knowledge on molecular biology of the pneumococcus can be obtained from a recent book compiled by Alexander Tomasz (57).

Pneumococcus has also been a favourite subject of Finnish researchers in microbiology and infectious diseases, and since the pioneering study of W. Pipping in 1886 at least 55 academic dissertations have been published on the subject (Table 1). Finnish researchers have made major contributions to development of laboratory diagnostic methods (58-60), and, in particular, to the understanding of serological aspects of pneumococcal disease (61-67). Driven by clinical therapeutic problems, extensive studies have been performed on antimicrobial resistance of *S. pneumoniae* and other important pathogens (68-70).

The epidemiology of acute respiratory infections has been thoroughly elucidated, both in adults (71-74), and in children (75-79). Clinical work includes studies on bacteraemic disease, meningitis, peritonitis and pneumonia (80-84). Studies on otitis media and sinusitis are numerous and have focused on epidemiological, bacteriological, therapeutic as well as preventive aspects of the disease (85-98). – Considering the large number of clinical vaccine studies conducted in Finland, surprisingly few theses have emerged on pneumococcal vaccines, most of these focusing on serological aspects (99-103).

Table 1. Finnish academic dissertations related to pneumococcus and pneumococcal disease since 1886.

Year	Author (reference)	Title	University
Studies on pneumococcus and pneumococcal disease			
1886	Pipping W (104)	Studier öfver pneumococcus	Keisarillinen Aleksanterin Yliopisto, Helsinki
1934	Candolin Y (80)	Über die durch Pneumokokken und ihnen verwandte Bakterien verursachte kryptogenetische Peritonitis	Helsinki
1948	Oker-Blom N (61)	On antipneumolysin in serum, particularly in pneumococcal infections and on its relation to antistreptolysin	Helsinki
1987	Koskela M (62)	Antibodies to pneumococcal surface polysaccharides in children: Oulu relation to acute otitis media and to vaccination	Oulu
1995	Nissinen A (68)	Antimicrobial resistance in four major respiratory bacterial pathogens: <i>Streptococcus pyogenes</i> , <i>Streptococcus pneumoniae</i> , <i>Haemophilus influenzae</i> , and <i>Moraxella catarrhalis</i>	Helsinki
1995	Virolainen A-R (63)	Pneumococcal etiology and immune responses to pneumococcal antigens in children with acute otitis media	Helsinki
1998	Manninen, R (69)	Determination of antimicrobial resistance in five major clinical bacterial pathogens	Turku
1998	Jero J (85)	Clinical and bacteriological studies of otitis media in children with special reference to <i>Streptococcus pneumoniae</i>	Helsinki
1999	Kuikka A (81)	Factors associated with the outcome of bacteremia during twenty years (1976–1996): with special reference to bacteremias due to <i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , and <i>Pseudomonas aeruginosa</i>	Helsinki
1999	Nieminen T (64)	Circulating antibody secreting cells and salivary antibodies induced by the capsular polysaccharide of <i>Streptococcus pneumoniae</i> after parenteral immunisation and in acute otitis media	Helsinki
1999	Salo P (59)	Evaluation of synthetic peptides and PCR in the diagnostics of pneumococcal infections	Helsinki
2000	Nuorti P (74)	Epidemiology of invasive pneumococcal disease in adults: implications for prevention	Helsinki
2000	Toikka P (82)	Pneumococcal pneumonia in children: diagnosis and clinical profile	Turku
2001	Rapola S (65)	Antibodies to pneumococcal proteins PspA, PsaA and Ply in children less than two years of age: relation to pneumococcal carriage and acute otitis media	Helsinki
2001	Soininen A (66)	Antibodies to capsular polysaccharides of <i>Streptococcus pneumoniae</i> in children: acquisition and measurement	Helsinki
2002	Pihlajamäki M (70)	Antimicrobial resistance in <i>Streptococcus pneumoniae</i> : with special reference to macrolide resistance	Turku

*Table 1. Continued.*

Year	Author (reference)	Title	University
Studies on otitis media and sinusitis			
1950	Björkwall T (96)	Bacteriological examinations in maxillary sinusitis	Helsinki
1953	Lahikainen EA (105)	Clinico-bacteriological studies on acute otitis media: aspiration of the tympanum as a diagnostic and therapeutic method	Turku
1981	Qvarnberg Y (86)	Acute otitis media: a prospective clinical study of myringotomy and antimicrobial treatment	Helsinki
1982	Luotonen J (87)	Studies on the bacteriology of acute otitis media	Oulu
1982	Pukander J (88)	Occurrence of acute otitis media	Tampere
1982	Sipilä P (89)	Inflammatory cells and bacteria in mucoid middle ear effusion of Oulu patients with secretory otitis media	Oulu
1989	Jousimies-Somer H (106)	Bacteriology of acute maxillary sinusitis and nasal flora in young adults	Helsinki
1989	Savolainen S (98)	Acute maxillary sinusitis in young adults: etiological factors	Helsinki
1990	Alho OP (90)	Acute otitis media in infancy: occurrence and risk factors	Oulu
1990	Karjalainen H (67)	Bacterial antibodies and inflammatory cells in middle ear effusion during the course of acute otitis media	Oulu
1994	Heikkinen T (91)	Development and prevention of acute otitis media in children	Turku
1994	Niemelä M (92)	Risk factors and symptoms of acute otitis media in children	Oulu
1998	Kontiokari T (93)	Xylitol in preventing acute otitis media	Oulu
2001	Palmu A (107)	Tympanometry in diagnosis and follow-up of otitis media in children less than two years of age	Tampere
2001	Ruohola A (94)	Acute otitis media: prevention and treatment	Turku
2002	Tapiainen T (95)	Microbiological effects and clinical use of xylitol in preventing acute otitis media	Oulu
Studies on acute respiratory infections			
1966	Ruosteenoja R (108)	Pulmonary function in pneumonia related to radiological and microbiological observations	Helsinki
1967	Heikel P-E (109)	Postmortal changes of the lung: a roentgenographic, microscopic and bacteriological follow-up study on a pediatric series and on animals with experimental pneumonia	Helsinki
1980	Ståhlberg M-R (76)	The influence of form of day care on occurrence of acute respiratory tract infections among young children	Turku
1981	Kujala P (110)	Smoking, respiratory symptoms and ventilatory capacity in young men: with a note on physical fitness and acute respiratory infections	Oulu
1988	Lehtomäki K (60)	Rapid etiological diagnosis of pneumonia in young men	Helsinki
1990	Korppi M (75)	Role of infection in respiratory distress in children	Kuopio
1991	Jokinen C (71)	Incidence and microbial aetiology of pneumonia in the population of four municipalities in Eastern Finland	Kuopio

Table 1. Continued.

Year	Author (reference)	Title	University
1996	Nohynek H (77)	Acute lower respiratory tract infection in children: the ability of microbiological, clinical and radiological methods to detect bacterial infection	Helsinki
1999	Koivula I (72)	Pneumonia in the elderly: risk factors, clinical efficacy of pneumococcal vaccine and long-term prognosis	Kuopio
1999	Säynäjäkangas P (73)	Keuhkokuumeesta aiheutunut sairaalahoito Suomessa 1972–1993	Oulu
2000	Heiskanen-Kosma T (78)	Community acquired pneumonia in childhood: a population-based study on bacterial etiology and risk factors	Kuopio
2001	Vuori-Holopainen E (79)	Etiology and antimicrobial treatment of pneumonia and other common childhood infections warranting hospitalization	Helsinki
Studies on meningitis			
1980	Leinonen M (58)	Immunochemical identification and demonstration of bacterial capsular polysaccharides	Oulu
1986	Valmari P (83)	Bacterial meningitis in children: general considerations, diagnosis and monitoring of treatment response	Oulu
1993	Anttila M (111)	Childhood bacterial meningitis: antimicrobial treatment, recovery and initial follow-up	Helsinki
1993	Roine I (112)	A trial of two simple acute phase reactants in improving prognosis of severe childhood infections in countries with limited laboratory resources	Helsinki
1995	Kilpi T (113)	Childhood bacterial meningitis: various clinical presentations, recovery and adjunctive therapy	Helsinki
1996	Häyrinen J (84)	Capsular polysaccharides of bacteria causing meningitis: antibody-binding properties and cross-reactivity with tissue glycoproteins	Kuopio
Studies on pneumococcal vaccines			
1997	Sankilampi U (99)	Streptococcus pneumoniae and the elderly: an epidemiological and serological study focusing on the potential of the polysaccharide vaccine	Oulu
1998	Honkanen P (100)	Pneumokokki- ja influenssarokotusohjelman toteuttaminen eläkeikäisessä väestössä: rokotushalukkuuteen vaikuttavat tekijät, rokotteiden haittavaikutukset ja pneumokokkirokotteen tuoma lisäteho keuhkokuumeen ehkäisyssä	Oulu
1999	Åhman H (101)	Immune response to pneumococcal conjugate vaccines in infants	Helsinki
2000	Väkeväinen M (102)	Function of antibodies evoked by pneumococcal conjugate and polysaccharide vaccine in adult and infants	Helsinki
2001	Wuorimaa T (103)	Immunogenicity and tolerability of an 11-valent pneumococcal conjugate vaccine	Helsinki

## 2.1 Clinical and epidemiological significance of pneumococcus

Untreated invasive pneumococcal pneumonia has a mean case fatality rate of 80%, ranging from 66% in age group below 30 years up to 93% in persons older than 50 years (114, 115). Serum therapy reduced the mean case fatality rate to 45% (114) and finally, when penicillin therapy became available, only 17% of all patients died (116). The rate, however, remained considerably higher in the age group above 50 years, at 28% (116, 117). For non-bacteraemic disease, the rates are significantly lower (118).

Despite the increased availability of antimicrobials, vaccines and intensive care facilities, the mortality associated with pneumococcal bacteraemia remains essentially unchanged. In a recent study in the UK, the overall mortality was 21% (119). In this adult material, older age, apyrexia, tachypnea, bilateral consolidation, and several biochemical laboratory parameters were associated with higher case fatality. In the Netherlands, mortality in a university hospital was 25.9%, and risk factors for fatal outcome included shock, respiratory insufficiency and pre-existing renal failure (120). – An exceptionally low case fatality rate of 7% has been reported for Sweden 1977–1984 (121).

The significance of pneumococcus is undisputed as a major cause of serious invasive disease, meningitis, pneumonia and septicaemia. However, as a true pathogen the pneumococcus is able to infect most tissues and fluids in the body. More than 30 different clinical conditions have been associated with pneumococcal infection, as summarised below in Table 2. Recent reports on rare or particularly severe disease forms have been characterised by high frequency of antimicrobial resistance in pneumococci (122-127). Pneumococcus also has a potential to cause nosocomial infections (128). Although not a life-threatening condition in itself, acute otitis media can cause serious and chronic complications; in any case, otitis is a major public health problem worldwide (88, 92, 94, 129-135).

In Finland, the annual incidence rate of invasive pneumococcal infections varies from 8.9 through 24.2 to 45.3 per 100,000 children less than 16 years, less than 5 years and less than 2 years of age, respectively (136). The most common clinical entities are bacteraemia without focus, pneumonia and meningitis. In children less than 2 years of age, increased risk for invasive disease is associated with day-care attendance and history of frequent otitis media (137). For those at least 2 years of age, existence of siblings younger than school age indicates an increased risk.

In Finnish adults, the overall incidence of invasive pneumococcal disease is 9.1 per 100,000 per year, but increases from 27.1 through 35.8 to 44.5 per 100,000 in those aged at least 65, 75 or 85 years, respectively (138).

In Stockholm, the incidence of pneumococcal pneumonia in adults was 7 per 100,000 and 5 per 100,000 for bacteraemic disease in the early 1980s (139). From 1988 to 1992, a more than threefold increase in incidence of reported invasive cases was observed in Sweden, from 3.2 to 10.2 per 100,000 inhabitants (140). A similar increase in incidence has been reported separately for southern Sweden (117).

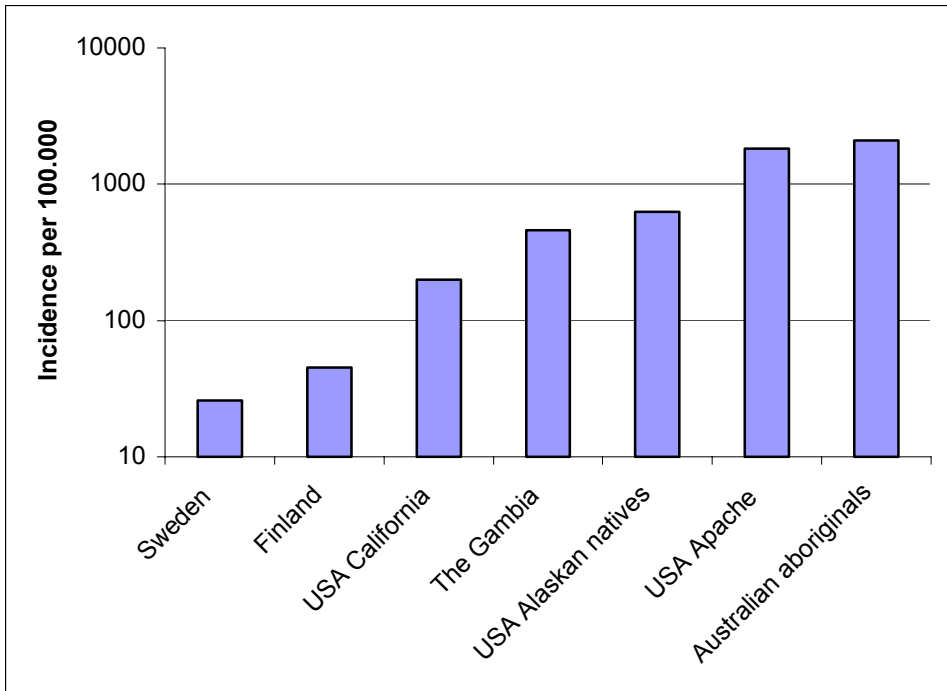
On a global scale, the disease burden caused by pneumococcus is difficult to estimate, and truly scientific attempts have not been made. The WHO has published several reports, which originate from collated expert opinions (141-143). All these indicate

consistently more than 1 million deaths due to pneumococcal pneumonia and meningitis annually in young children, mainly in developing countries.

*Table 2. Clinical spectrum of disease caused by pneumococci.*

Disease	Reported or reviewed by, year of publication	Reference
Abscess, renal	Lee, 1997	(144)
Abscess, testicular	Dobroszycki, 1997	(145)
Abscess, tuboovarian	Sirotnak, 1996; Abalde, 1998	(146, 147)
Aneurysm, mycotic	Brouwer, 1998	(148)
Aneurysm, pseudoaneurysm	Marinella, 1998	(149)
Aortitis	Maclennan, 1997	(123)
Arthritis, septic	Arlievsky, 1998; Bradley, 1998; O'Brien, 1998	(124, 150, 151)
Bartholinitis	Sing, 1998	(152)
Conjunctivitis	Ertugrul, 1997; Barker, 1999; Medeiros, 1998	(153-155)
Corneal infiltrates	Sankaridurg, 1999	(19)
Corneal ulceration	Srinivasan, 1997; Kunimoto, 2000	(156, 157)
Dacryocystitis	Aasuri, 1999	(158)
Empyema, pleural	Hardie, 1996	(159)
Endocarditis, subacute	Garcia-Zamalloa, 1998; Lindberg, 1998	(160, 161)
Endophthalmitis	Cid, 1997; Mulhern 1997; Chan, 1998; Garretson, 1999	(122, 162-164)
Epiglottitis	Berg, 1996; Trollfors, 1998	(165, 166)
Gastritis, phlegmonous	Iwakiri, 1999	(167)
Haemolytic uremic syndrome	Cabrera, 1998; Gray, 2001	(168, 169)
Keratitis	McLeod, 1998; Aasuri, 1999	(158, 170)
Mastoiditis	Antonelli, 1999	(126)
Meningitis	Stanek, 1999	(171)
Meningitis, with purpura	Cnota, 1999	(172)
Osteomyelitis	Arlievsky, 1998; Bradley, 1998	(124, 150)
Otitis media, acute	Luotonen, 1982; Daly, 1999;	(133, 135)
Pacemaker infection	Mezilis, 1997	(173)
Pericarditis	Saenz, 1998; Go, 1998	(125, 174)
Peritonitis	Candolin, 1934; Hemsley, 1998; Sirotnak, 1996; Abalde, 1998; Chuang 1999; Gill, 1999	(80, 146, 147, 175-177)
Pneumonia	Catterall, 1999	(178)
Pseudoaneurysm	Marinella, 1998	(149)
Sacroilitis, septic	O'Brien, 1998	(151)
Septicaemia	Picard, 1998	(179)

Estimates of Greenwood lead to the substantially higher figure of around 2 million deaths (180). The highest incidence is found consistently among children less than 2 years of age, but there is significant variation from country to country, apparently due to genetic reasons (Fig. 1). Mulholland has estimated that 70% of severe pneumonia is of pneumococcal origin (181). Global estimates are lacking for otitis media in children and for pneumonia in adults (182). In industrialised countries, 60–70% of children have at least one episode of acute otitis media by 2 years of age (107, 129).



**Fig. 1. The incidence of invasive pneumococcal disease in children aged less than 2 years. Data are from Sweden (183), Finland (136), California (184), The Gambia (185), Alaskan natives (186), Apache (187) and Australian aboriginals (188). Modified after Greenwood (180).**

### ***2.1.1 Importance of serotypes***

Most wild strains of *S. pneumoniae* have a polysaccharide capsule (189). The capsule is essential for the virulence of the organism, primarily protecting the bacteria against phagocytosis. To date, 90 distinct capsular types (i.e. serotypes) have been described (Table 3), six of them as recently as 1995 (190). Different serotypes possess different capabilities to cause serious disease, and most of them are quite uncommon (189). Some of the types are antigenically related to each other and such related serotypes are combined together in serogroups (189). In this review, unless discussing a specific serogroup or serotype, the abbreviation SGT is used to include both classes. Earlier studies have often reported group level findings, and study results should therefore be compared with caution.

In a meticulous longitudinal analysis of 15 selected years between 1935 and 1974 at Boston City Hospital, Finland demonstrated that prevalence of individual SGT changes over time (191). Only seven SGT were found in every one of the selected years in blood

culture (SGT 1, 3, 4, 7, 8, 14, and 18), and these accounted for 60% of the 1,543 strains identified by type. No single SGT was isolated during all selected years from focal infections.

The distribution of SGT in paediatric cases is different from that in adults (129, 191), although adults with small children acquire pneumococcus more often than adults with school-aged children, and this is usually the type carried by the child (192). In addition, serotypes responsible for bacteraemia in adults and children differ from those isolated from focal infections such as otitis media. Similar findings have been reported from Denmark, with the additional conclusion that the type distribution is also dependent on the age *and* sex of the patient (193). These findings had significant implications for the development of pneumococcal vaccines. The 14-valent vaccine was licensed in the United States in 1977 and the 23-valent vaccine in 1983.

Extending the follow-up in Boston from 1979 to 1982, Barry, Craven and Finland concluded that the six most common paediatric SGT (14, 19, 18, 6, 4 and 9) accounted for 90% of the total paediatric isolates, and the six most common adult SGT (12, 9, 8, 4, 3 and 6) made up 55% of the total adult isolates (194). Prevalence changes were again apparent: six of the eight most common SGT in 1935 (1, 2, 3, 5, 7 and 8) were now replaced by SGT 6, 9, 12, 18 and 19.

Summarising results from several epidemiological studies, Klein concluded that SGT 6, 14, 18, 19, and 23 are responsible for most cases of bacteraemia or meningitis (129). Serotype 14 was the principal cause of invasive disease in several studies. Serogroup 19 was the leading cause of otitis media, serotype 3 was also important, but an infrequent cause of septicaemia or meningitis in children (129). However, different pneumococcal serotypes predominate in different parts of the world. For example, SGT 2 and 25 seldom cause bacteraemia or pneumonia in the United States, but are common causes of disease in Papua New Guinea, and in South Africa (129).

Combining 13 existing datasets, Scott and coworkers obtained a study sample of 7,010 episodes of invasive disease (195). It was again shown that serotype 14 causes bacteraemic disease most frequently (10.5% of all cases), followed by SGT 6, 19, 3, 23, 1, 9, 4, 8, 18, 7 and 5. These 12 SGT accounted for 80.9% of all isolates. More than half of the isolates came from patients at the extremes of life: 34% were aged less than 5 years and 20% were aged 65 years or more. Significant differences were found in serotype distribution as related to age. For example, serotype 1 is associated with progressive decline in relative risk throughout adulthood, but the risk of serotype 3 disease increases progressively, to peak in the seventh decade of life. Serotype 8 is the only other type that has a relative preference for adults. Male to female ratio is 1.8:1, without marked variation in the proportion of males between different serogroups (195).

*Table 3. Type designations and antigenic formulas of 90 types of pneumococci (190).\**

Type	Antigenic formula	Type	Antigenic formula
1	1a	19C	19a, 19c, 19f,7h
2	2a	20	20a, 20b, 7g
3	3a	21	21a
4	4a	22F	22a, 22b
5	5a	22A	22a, 22c
6A	6a, 6b	23F	23a, 23b, 18b
6B	6a, 6c	23A	23a, 23c, 15a
7F	7a, 7b	23B	23a, 23b, 23d
7A	7a, 7b, 7c	24F	24a, 24b, 24d, 7h
7B	7a, 7d, 7e,7h	24A	24a, 24c, 24d
7C	7a, 7d, 7f, 7g, 7h	24B	24a, 24b, 24c, 7h
8	8a	25F	25a, 25b
9A	9a, 9c, 9d	25A	25a, 25c, 38a
9L	9a, 9b, 9c, 9f	27	27a, 27b
9N	9a, 9b, 9e	28F	28a, 28b, 16b, 23d
9V	9a, 9c, 9d, 9g	28A	28a, 28c, 23d
10F	10a,10b	29	29a, 29b, 13b
10A	10a, 10c, 10d	31	31a, 20b
10B	10a, 10b, 10c, 10d, 10e	32F	32a, 27b
10C	10a, 10b, 10c, 10f	32A	32a, 32b, 27b
11F	11a, 11b, 11e, 11g	33F	33a, 33b, 33d
11A	11a, 11c, 11d, 11e	33A	33a, 33b, 33d, 20b
11B	11a, 11b, 11f, 11g	33B	33a, 33c, 33d, 33f
11C	11a, 11b, 11c, 11d, 11f	33C	33a, 33c, 33e
11D	11a, 11b, 11c, 11e	33D	33a, 33c, 33d, 33f, 6a
12F	11a, 12b, 12d	34	34a, 34b
12A	12a, 12c, 12d	35F	35a, 35b, 34b
12B	12a, 12b, 12c, 12e	35A	35a, 35c, 20b
13	13a, 13b	35B	35a, 35c, 29b
14	14a	35C	35a, 35c, 20b, 42a
15F	15a, 15b, 15c, 15f	36	36a, 9e
15A	15a, 15c, 15d, 15g	37	37a
15B	15a, 15b, 15d, 15e, 15h	38	38a, 25b
15C	15a, 15d, 15e	39	39a, 10d
16F	16a, 16b, 11d	40	40a, 7g, 7h
16A	16a, 16c	41F	41a, 41b
17F	17a, 17b	41A	41a
17A	17a, 17c	42	42a, 20b, 35c
18F	18a, 18b, 18c, 18f	43	43a, 43b
18A	18a, 18b, 18d	44	44a, 44b, 12b, 12d
18B	18a, 18b, 18e, 18g	45	45a

*Table 3. Continued.*

Type	Antigenic formula	Type	Antigenic formula
18C	18a, 18b, 18c, 18e	46	46a, 12c, 44b
19F	19a, 19b, 19d	47F	47a, 35a, 35b
19A	19a, 19c, 19d	47A	47a, 43b
19B	19a, 19c, 19e, 7h	48	48a

\* The antigenic formulas represent arbitrary designations of cross-reactions as seen by the capsular reaction.

Analysing more than 70 data sets including serotype information on causing invasive pneumococcal disease (IPD), Hausdorff and coworkers discovered that 5–8 and 10–11 SGT comprise at least 75% of pneumococcal isolates from young children and older children/adults, respectively (182). SGT 4, 6, 9, 14, 18, 19, and 23 cause 70–88% of IPD in young children in the United States and Canada, Oceania, Africa, and Europe, and < 65% in Latin America and Asia. The above SGT plus serotypes 1 and 5 cause 80–90% of IPD in each region except Asia (66%). Serotype 1 accounts for > 6% of IPD in each region, including Europe, except the United States and Canada and Oceania. In contrast, several serotypes not present in 7-, 9-, and 11-valent conjugate formulations are significant causes of disease in older children/adults (182).

Furthermore, in all age groups, serotypes 1 and 14 were more often isolated from blood, and SGT 6, 10, and 23 were more often isolated from cerebrospinal fluid (CSF); in young children, SGT 3, 19, and 23 were more often isolated from middle ear fluid (MEF). Serotypes represented in conjugate vaccines were isolated slightly less frequently from CSF than from blood or MEF. Nonetheless, serotypes in the 9-valent conjugate vaccine formulation still covered approximately 75% of pneumococcal isolates from the CSF of young children in Europe and in the United States and Canada (196).

In a multicentre study conducted in nine African countries between 1971 and 1982, 1,504 strains of pneumococci were typed (197). Altogether 34 serotypes were identified; serotypes 1, 6 and 5 accounted for 54.9% of the findings. The 14 most frequent serotypes accounted for 93.6% of all strains. In Nigeria, serotypes 1, 5 and 3 accounted for 64% of all pneumococcal findings in patients with pneumonia and meningitis (198). The same types were found most frequently also in a larger Nigerian study material, which included conditions like conjunctivitis, arthritis and peritonitis (33). In Ethiopia, SGT 14, 19F, 20, 1, 18 and 5 accounted for 76% of cases with pneumococcal meningitis (127). In Rwanda during 1984–1990, 32 serotypes were identified in 383 clinical isolates, SGT 1, 5, 14, 6, 19, 25 and 18 accounting for 64.8% of all isolates (199). SGT 1, 6, 19, 14, 8, 3 and 7 were most prevalent findings in blood and CSF in South Africa during 1979–1986 (200).

In a material comprising more than 300 paediatric and adult patients in India, ten most common serotypes accounted for 70% of the isolates (201). SGT 1, 6, 15, 7, 19 and 5 accounted for 58% of all isolates from children. – In a smaller study in Tamil Nadu, SGT 1, 5, 6 and 7 accounted for 79% of isolates in children and 71% in adults (202). In New Delhi, the top six SGT were 1, 3, 19, 13, 5 and 7, again accounting for 58% of all isolates (203). In Malaysia, the predominant serotypes are 1, 6B, 19B, 19F and 23F (204). In Hong Kong, the majority of isolates belong to SGT 23, 19, 6 and 3 (205).

The serotype distribution in Bangladesh seems quite different from other Asian countries (206, 207). From 1993 to 1997 altogether 362 pneumococcal isolates from

paediatric patients were serotyped in Dhaka, and SGT 7F, 15, 14, 18, 12 and 19 accounted for 52% of the findings (207).

In invasive infections, the serotype is generally the same as in the strain isolated from the nasopharynx, often reported to be the same in all cases (31, 208, 209). However, also less perfect concordance figures have been reported from developing countries, 82% in Kenya (210), 77% in The Gambia (211), and 46% in PNG (212).

In epidemic conjunctivitis, nonserotypeable, noncapsulated (or rough) isolates predominate. Already the early evaluations used epidemiologic factors and the nonserotypeability of the isolates to infer clonality, and Ertugrul and coworkers have subsequently confirmed this using BOX-PCR-based DNA analysis (153).

### ***2.1.2 Antimicrobial resistance***

In his detailed review of the natural history of pneumococcus, Austrian points out that the pneumococcus was probably the first bacterial species recognised as capable of developing antimicrobial resistance, long before the advent of penicillin (213). More recently, Klugman brought to light all historical publications on antimicrobial resistance (214). Ophthochin resistance was described in 1912 in mice (215) and in 1917 in man (216). Sulfadiazine resistance developing during therapy was reported in 1943, and spread of the resistant strain to a second patient, unresponsive to sulfadiazine, was identified (217). The resistant strains could still be cultured from the patients' sputa 2 months after the onset of their illness (217).

The first report of penicillin resistance in pneumococcus was reported in 1967 in Australia (218). This strain was intermediately resistance to penicillin, (MIC 0.6 µg/ml), thus requiring 60 times higher penicillin concentration than the usual susceptible isolates, with MIC levels at 0.01 µg/ml. First truly penicillin-resistant isolates (MIC 4–8 µg/ml) were reported in 1977, and the strains were increasingly multi-resistant (219, 220).

From 1974 to 1984, the reported distribution of penicillin resistant strains became worldwide, and the problem affected equally both developing and industrialised countries (214). Foci with more than 10% of isolates resistant to penicillin were reported in Papua New Guinea, Israel, Spain, Poland, South Africa, and United States (214, 221). Nosocomial transmission of penicillin resistant pneumococci is not a major problem, but has been documented on case report level (128).

The trends in prevalence and spread of antimicrobial resistance have not been consistent, the rates fluctuating up and down (214). However, high rates of resistance seem to be associated with liberal or uncontrolled use of antimicrobials, and the highest rates have been reported in countries where antimicrobials are available over-the-counter without prescription (Table 4) (214).

In Finland, the first report on pneumococcal penicillin resistance isolated from a clinical case was published in 1979 by Sibakov, Herva and Mäkelä (222). Fortunately, rates of penicillin resistance still remain quite low, below 5%, in all Finnish studies, although independent macrolide resistance and multi-resistance have been observed to increase (68-70, 138).

Table 4. Penicillin resistance in developing countries.

Year	Country	Target group	Penicillin resistance	Ref.
1986	Papua New Guinea	neonates	36% of first acquired strains (7 serotypes)	(23)
1991	Papua New Guinea	children	75% of carriage strains (32 serotypes)	(223)
			52% blood	(223)
			22% CSF isolates	(223)
1998	Kenya	adults	27%	(224)
		children with invasive disease	29%	(224)
		children	47% nasopharyngeal isolates	(224)
1999	Ethiopia	children with meningitis	17%	(127)
1984–1990	Rwanda	all pneumococcal isolates	21%	(199)
1979–1986	South Africa	4,766 pneumococcal isolates from blood	increase from 3.8 to 14.1%	(210)
		1,157 isolates from CSF	increase from 6.8 to 14.1%	(210)

Worldwide, high rates of antimicrobial resistance in pneumococcus have increased the clinical and public health importance of pneumococcal infections. At the same time, the phenomenon has accelerated the development of new antimicrobials or therapeutic regimens and, even more, the development of pneumococcal vaccines. So far, the pneumococcus has been able to negotiate new therapies through increased tolerance and through multi-resistance. Most multi-resistant pneumococci belong to a handful of serogroups, including the paediatric types (200, 224, 225). Inclusion of these in the new conjugate vaccines was thought to be an effective solution to the problem, but pneumococcus responded to this challenge by colonisation and infection with other serotypes (18, 19).

## 2.2 Pneumococcal carriage

Nasopharyngeal carriage of pneumococci is more prevalent in children than adults (211). The odds of carrying pneumococci have been reported to be twice as high among children less than 2 years of age than among older children (226). Immunogenic serotypes are acquired less frequently and are carried for shorter periods (227).

In developing countries, carriage rates are quite high almost regardless of infection status of the children (Table 5). Using serial samples taken at 1 to 2 week intervals, Gratten and coworkers showed that 60% of infants in Papua New Guinea acquired *S. pneumoniae* (and *H. influenzae*) already during the neonatal period, and all infants were colonised by both organisms within the first 3 months of life (23, 24). Carriage period for pneumococci ranged from 5 to 290 days (mean, 96 days). The mothers either did not carry pneumococci or *H. influenzae*, or harboured different types than the infants. However, one-third of the mothers subsequently became colonised with the bacteria carried by their babies.

Table 5. Summary of nasopharyngeal carriage rates of pneumococcus in developing countries.

Country	Subject group	Nasopharyngeal carriage, percentage			Ref.
		Infection	No infection	Overall	
The Gambia	Children, < 5 y	85.1/90.1 <sup>a</sup>	76.1	–	(211)
Pakistan	Children, 2–59 m	64.4	51.9	–	(228)
PNG	Infants	60/100 <sup>b</sup>	–	–	(23)
The Philippines	Children, < 5 y	51/62 <sup>c</sup>	–	–	(11)
South Africa	Children < 12 y	–	29.4	29.4	(229)
Uruguay	Children < 5 y	42.1	15.2	–	(230)
Zambia	Children, < 6 y	–	–	71.9	(226)

<sup>a</sup> any infection/invasive pneumococcal disease

<sup>b</sup> neonates/by 3 months of age

<sup>c</sup> bacterial culture/antigen detection

In industrialised countries the picture is different (Table 6). In the 1930s, 50% of German children had had acquired *S. pneumoniae* by 2 weeks of age (231). In the 1980s in Sweden, only 12% of infants were colonised with pneumococci at 3 months, 30% at 7 months, and 32% at 12 to 18 months (232). However, the colonisation rate was influenced by the number of siblings, and day-care contact (through siblings). Quite a similar trend was observed in Finland during 1994–1997 (28). In this study, however, day care was not associated with an increased risk of pneumococcal carriage.

Rosén and coworkers studied pneumococcal carriage in Swedish children under 5 years of age in day care, comparing vaccinated and unvaccinated children (233). Pneumococci were found in 31.9% of all cultures, and there was no difference in carriage between vaccinees and controls. In centres with 45 children or more, the carriage rate was significantly higher than in centres with fewer children. Children younger than 2 years showed the highest carriage rates. Spreading of pneumococci within centres was common, but rather short-lived (233).

Children acquire several different strains over time, and as many as seven different carried stains have been recorded over a 2-year follow-up period in one individual (192). The carried strains may change rapidly as indicated in an eloquent pulsed-field gel electrophoresis study in 6 children (234). A seasonal pattern has also been described, with carriage peak rates occurring during January–March (235). – Recently, a WHO working group has made an effort to draft recommendations for a standard method for detecting upper respiratory carriage of pneumococcus (236).

*Table 6. Summary of nasopharyngeal carriage rates of pneumococcus in industrialised countries.*

Country	Subject group	Nasopharyngeal carriage, percentage			Ref.
		Infection	No infection	Overall	
Finland	Children, 0–2 y	22–45	13–43	9–43	(30)
Sweden	Children, 6–13 y	18–22	10	11.9	(237)
Sweden	Children, 0.5–5 y	–	31.9*	–	(192)
UK	Children	75	50	–	(238)
UK	Parents	35	40	–	(238)
USA	Children	97	94	96	(239)
USA	Children	8	6.5	–	(240)
USA	Children, < 5 y	–	38	–	(26)
	Children, 6–12 y	–	29	–	
	Adolescents, 13–17 y	–	9	–	
	Parents, ≥ 18 y	–	19	–	
USA	Children, 2 mo–18 y	–	20	–	(241)

\* Percentage calculated from the number of samples, not individuals, all of whom were sampled monthly during a 2-year follow-up period.

### ***2.2.1 Nasopharyngeal carriage and immunisation***

Reduction of naso- and oropharyngeal carriage after immunisation has been well documented for *H. influenzae* (17, 242), and a similar effect was logically proposed also for pneumococcus, although early results with polysaccharide vaccine had been negligible in this respect (29, 192). However, initial studies with pneumococcal conjugate vaccine in Israel gave more encouraging findings, reducing significantly nasopharyngeal carriage of vaccine types (243, 244). These findings were challenged by researchers in The Gambia, who found reduction of vaccine types counter-balanced by an increase in non-vaccine serotypes (245).

Later on, analysing 3,646 nasopharyngeal cultures in Israel, Dagan and coworkers could also confirm that the carriage of non-vaccine serotypes increased significantly in vaccinated children (18). The same finding emerged from the Finnish Otitis Media Vaccine Trial, which revealed that both the carriage of non-vaccine serotypes and the incidence of otitis media caused by non-vaccine serotypes increased (19). It is not yet known whether the changes in carriage could result in other changes in the disease pattern. The implication for spreading of antimicrobial resistance to replacing serotypes also remains unclear (18).

### 2.3 *Pneumococcus* and paediatric acute lower respiratory infections

As pointed out by Nohynek, the aetiology of childhood ALRI is still inconclusively documented, mainly because of difficulties in obtaining adequate samples and lack of reliable diagnostic methods (77). Elaborate, hospital-based studies utilising advanced laboratory techniques have been able to elucidate the etiology at best in 43–88% of the cases (246).

Most studies utilising lung aspiration and bacterial culture technique have showed pneumococcal aetiology in 12–88% of all paediatric ALRI cases in developing countries (7). In a review of 11 studies on the aetiology of paediatric pneumonia in developing countries, 314 organisms were isolated from 744 patients when the samples obtained by lung aspiration (247). Pneumococci comprised 42% of the positive isolates.

In the industrialised world, studies employing a panel of different diagnostic methods (but not lung aspiration) have indicated pneumococcal aetiology in 6–41% of the cases (77, 246). In Finland, lung aspiration disclosed the aetiology in 59% of the 34 cases overall, and in 69% of those 26 cases in which a representative sample was obtained, 30% of the samples were positive for pneumococcus (10). Reviewing nine clinical studies, all but one hospital-based, Juvén concluded that *S. pneumoniae* was consistently the most important etiological agent, surpassed only in two studies by *Mycoplasma pneumoniae* (246). Overall, *M. pneumoniae* was identified in 0–41% of the cases. *Haemophilus influenzae* and *Moraxella catarrhalis* are also important pathogens in ALRI, identified in 1–17% and 3–10% of the cases, respectively (246). Apart from the different diagnostic methods employed, variations in the incidence figures can be explained by different case definitions, variation of patient age or ethnic origin, geographic location, season and the epidemic situation.

Mixed pneumococcal and viral infections are common or very common (77, 246). In two Finnish studies, 20–37% of children hospitalised due to ALRI harboured concomitant bacterial and viral pathogens (248, 249). The review by Juvén indicates a range of 3–34% for mixed bacterial and viral infections (246). Mixed bacterial infections have also been described (4). A similar situation was found in a study in adults, 31% of the 91 patients with established aetiology had two or three aetiological agents implicated (250).

### 2.4 *Streptococcus pneumoniae*

*S. pneumoniae* is a facultatively anaerobic Gram positive bacterium, appearing as pairs (diplococci) or short chains of lancet-shaped organisms on microscopy. Under light microscope, pneumococcus has no spores, no vacuoles, no visible granules, no flagella, and it is non-motile (55). Colony morphology on blood agar is typically smooth, glistening, flat and doughnut like. The colony size varies between 0.5 and 1.5 mm. – The phenotype of pneumococcus varies spontaneously, and is expressed, among others, as phase variation of colony opacity (251). Colony opacity seems to correlate with virulence and adherence of pneumococci (252).

Pneumococci have three surface layers: plasma membrane, cell wall and capsule. As in other Gram positive bacteria, the cell wall is composed of peptidoglycan and teichoic acid. A predominant component of teichoic acid is the C-polysaccharide, which is covalently bound to the peptidoglycan layer (189). The most distinctive morphological feature of the pneumococcus is the outermost layer of its cell wall, the capsule (55).

To date, 90 different capsular types have been described for *S. pneumoniae*. Some of the types are antigenically related to each other and such related types are included together in groups (189). The capsular polysaccharides (CaPS) are composed of repeating oligosaccharides, and for most of them the exact chemical structure is known. The extent of cross-reactivity between types within a group may differ, e.g. 6A and 6B are extensively cross reactive, 19F and 19A are clearly less cross-reactive (189). – The type of culture media may affect the expression of the capsular polysaccharide (253). Exceptionally, pneumococcus may express two capsular polysaccharides (189, 254).

C-polysaccharide (CPS) is composed of a pentasaccharide repeating unit with ribitol phosphate linkage, and contains phosphorylcholine (255). Antibodies reactive with the phosphorylcholine determinant are induced in mice and rabbits after immunisation with pneumococci, especially when nonencapsulated mutants are used (256).

Pneumococcal protein antigens include autolysin (LytA), pneumolysin (Ply), pneumococcal surface protein A (PspA) and pneumococcal surface adhesin A (PsaA) (257). Pneumolysin is a toxic intracellular protein, which is released upon autolysis. Ply can interfere directly with the opsonisation, phagocytosis, and killing of pneumococci in the host. The protein antigens are common to virtually all serotypes, and have been shown to elicit immune response in animal studies and in humans (65). As such, they offer an alternative approach for developing new pneumococcal vaccines.

Genetic plasticity plays a central role in the biology of the pneumococcus. This is illustrated not only by the existence of the 90 different capsular types, but also by the rapid emergence of penicillin resistant pneumococcal strains. Natural genetic transformation is essential for this plasticity; capsular types can be switched by intraspecies transformation, while interspecies transformation is responsible for the resistant strains (258).

In serological and antigen detection assays, false positive diagnostic findings are possible due to cross-reactions between pneumococcus and *Escherichia coli*, *Klebsiella* spp. and staphylococci and  $\alpha$ -haemolytic streptococci (259-263). – The molecular biology and pathogenetic aspects of pneumococcus have recently been reviewed elsewhere (57).

## 2.5 Pneumococcal laboratory diagnosis

At least twenty different approaches are available for laboratory diagnosis of pneumococcus. The multitude of methods is conveniently classified into four distinct categories: 1) bacterial culture, usually complemented with simple identification tests; 2) antigen detection with a range of immunoassays; 3) demonstration of genetic material; and 4) serological antibody assays.

Direct microscopic examination of Gram stained specimens has only a limited use in clinical practice, and it does not correlate well with a positive culture result (264). At best, Gram stain was in agreement with 75% of purulent samples, when compared with quantitative culture of expectorates in a Swedish study (265). In Finnish military conscripts, Gram stain of purulent sputum samples was positive in 65% of the patients with definitive pneumococcal pneumonia (250). – However, the negative predictive value of Gram stain appears significant (250, 264, 265), and sputum purulence can be used to differentiate pneumococcal from viral, chlamydial and mycoplasmal pneumonia (250, 266). For these reasons, Gram stain is being increasingly recommended for inclusion in clinical diagnostic protocols (267).

Viability of pneumococci in the usual laboratory media is limited to 24–48 hours, and transport delays therefore lead to poor recovery of organisms. Dorset egg medium has been shown to retain pneumococcal viability up to 30 days at room temperature (268). In sputum samples, pneumococci may remain viable for a couple of days at room temperature, and up to 10 days at 4 °C (269).

### **2.5.1 Bacterial culture**

Worldwide, conventional bacterial culture still remains the most frequently used method for recovering pneumococcus from a variety of clinical specimens. Pneumococcus grows relatively well on regular sheep blood agar, but the addition of gentamicin in the blood agar will improve the recovery rate (270); only one study could not substantiate this observation (271). Horse and goat blood may be used equally well (272). In 1980, Nichols and Freeman suggested the use of a selective agar medium with crystal violet, nalidixic acid and gentamicin supplement, but this modification has not gained popularity (273). Three studies indicated that anaerobic incubation increases the recovery of *S. pneumoniae* (274-276), but comparing aerobic and anaerobic methods in 1,173 respiratory tract specimens, Baesman and Strand found no significant difference between the two techniques (277). Although first suggested to be useful as enrichment media (238), addition of broth cultures to routine culture protocol has not been shown to provide additional pneumococcal isolates or additional benefit for clinical management (278).

Optochin and bile solubility tests are used for identification of suspected colonies (279-281), although results are not always unequivocal (282). Further confirmation may be sought by observing the quellung reaction with Omniserum or by demonstrating the presence of capsule with other immunochemical tests. Further characterisation with antiserum pools or serotype specific antisera will provide the epidemiologically important information on pneumococcal serotype. The interpretation of all confirmatory tests, including the quellung reaction, requires specialised and skilled staff.

For routine bacterial culture of pneumococcus, it is currently recommended by the National Public Health Institute (KTL), Finland that sheep blood agar be supplemented with gentamicin. Normally, four separate  $\alpha$ -haemolytic colonies with different colony morphology are picked up and cultured in four sectors on a blood agar plate. Optochin disks are placed in the middle of each sector. If the colony morphology resembles

pneumococcus but the optochin test is negative, a bile solubility test is used as an additional identification method.

Whenever available, blood culture is routinely used in clinical diagnosis of suspected invasive infections, but for pneumococcus the utility of this method is seriously limited by low sensitivity (1, 2), especially in children in whom it may be as low as 19% (4). More generally, less than 10% of all blood cultures will be positive in paediatric ALRI patients (3, 4, 283, 284). However, the results can be somewhat improved with adequately large sample volumes (285-288), whereas the exact timing between subsequent blood cultures is not important (289). Mermel and Maki have suggested that clinical laboratories should routinely monitor the volume of blood cultured as a quality assurance measure (290). A sample volume of 10–30 ml is recommended for adults, 1–5 ml for children (291, 292).

However, in a study in 161 critically ill new-borns, Neal and coworkers did not find any significant difference in mean volume of blood in positive or negative cultures (mean 0.5 ml per bottle). Nevertheless, the authors concluded that the blood volume from new-borns might still be inadequate for detecting septicaemia (293).

Quantitative blood cultures may be helpful in detection of low concentrations of pneumococci, and of Hib in particular, but this relatively labour-intensive method is not widely used (294, 295). Blind subcultures at 24 hours have been recommended to optimise detection speed of paediatric bacteraemia (296). It is also possible to combine antigen detection with blood culture (297, 298).

Reviewing medical records of 939 paediatric patients with chest radiographic findings consistent with pneumonia, Hickey and coworkers found that blood culture was performed in 409 (44%) of the cases (299). Only eleven cultures (2.7%) grew pathogenic bacteria (10 *S. pneumoniae* and 1 *H. parainfluenzae*). On top of it, medical records revealed no changes in clinical management as a result from the blood culture findings and all patients recovered from pneumonia. The relatively modest conclusion of the authors was that blood cultures are uncommonly positive in outpatients diagnosed with pneumonia, but the results really provide justification to question the utility of blood cultures in management of paediatric pneumonia.

In an investigation on 1,222 children with pneumonia in the Gambia, Banya and coworkers attempted to identify predictors for positive blood culture (284). Analysing 36 different variables, basic demographic characteristics, symptoms, signs, laboratory investigations and management type, they found that high fever was a strong positive predictor of a positive culture. While the overall isolation rate was 7.9%, the rate was 26% in children with a temperature of 39.5 °C or higher. Unfortunately, fever that was that high occurred too infrequently for this to be adopted as a positive selection criterion. Definite conclusions or recommendations could not be drawn.

Various advanced blood culture technologies have been reviewed by Campos, including radiometric broth culture, infrared spectroscopy, biphasic agar/broth culture, lysis direct plating and manometric broth culture (292). The discouraging conclusion is that the many technological advances have not significantly improved the sensitivity of pneumococcal diagnostics (292, 300).

The value of sputum culture in diagnosis of pneumococcal pneumonia remains debatable (209, 275). In the patients with confirmed bacteraemic pneumococcal pneumonia, the isolation rates from sputum samples have varied from 48 to 94%, as

summarised by Drew (275). Drew himself achieved the highest isolation rate by using anaerobic incubation and optochin disk on original plating.

Quantitative sputum culture techniques have been suggested to improve the yields and monitor therapeutic success (301), even to identify bacterial RTI (237), but such methods are not in clinical use.

Kalin and coworkers isolated pneumococci in 27% of 201 purulent sputum samples, from 128 adult patients with CAP; 53% of pretreatment samples were positive, but only 8% of samples obtained after initiation of antimicrobial therapy yielded pneumococci (265). Lehtomäki and coworkers isolated pneumococci in 23% of 98 sputum samples obtained from 106 military conscripts with pneumonia (250). However, sputum culture was positive in 23 of the 32 (72%) patients with definite pneumococcal pneumonia, and in none of the 21 with a presumptive diagnosis.

Holloway and coworkers have suggested that colonising pneumococci could be washed away from sputum samples, while clinically relevant pneumococci will be present in such large numbers that the washing procedure will not affect isolation rates (302). Similar observations have been published by Bartlett and Finegold as well as Kalin (303, 304).

In an attempt to use nasopharyngeal swabs in diagnosis of pneumococcal pneumonia, Hedlund and coworkers achieved a sensitivity of 27% in 261 adult patients with CAP (208). In determination of the bacterial aetiology of acute otitis media, a positive nasopharyngeal culture has relatively low predictive value for *S. pneumoniae* (43%), but the negative predictive value is as high as 99% (305). For nasal culture, the predictive value of finding pneumococci in MEF has been shown to be 49% (306).

It is important to note that for respiratory secretions, the pathogen recovery rates are significantly affected by the selection of sampling site and sampling method, as concluded in a study from 1961 by Box and coworkers (239). In a survey for carriers of *S. pneumoniae*, Robins-Browne and coworkers showed first that pneumococci can be found in 29.4% of nasopharyngeal swabs, but in only 2.6% of throat specimens. The finding was confirmed by Capeding and coworkers, who compared 1,567 nasal and oropharyngeal swab pairs in a Philippine study setting, and showed that pneumococcus is isolated significantly more often from nasal swabs. For *H. influenzae*, a similar difference was not shown (307).

Comparing nasal, nasopharyngeal and oropharyngeal swabs, and nasopharyngeal aspiration in 96 children, Rapola and coworkers concluded that nasopharyngeal aspirates were optimal for the detection of carriage of both *S. pneumoniae* and *H. influenzae*. When nasopharyngeal aspirate is not available, e.g. from children with no obtainable secretions, the nasopharyngeal swab seems optimal for the detection of both *S. pneumoniae* and *H. influenzae* among children younger than 13 months of age. Among older children, similarly, the nasopharyngeal swab seems optimal for the detection of *S. pneumoniae*; however, for *H. influenzae*, the oropharyngeal swab seems optimal (308).

One possible explanation for the above difference is the interesting observation reported by Tano and coworkers (309). They found that  $\alpha$ -haemolytic streptococci (AHS) collected from the tubal orifice inhibit 93% of *S. pneumoniae* strains, while AHS from the adenoid inhibit only 76% of pneumococcal strains *in vitro*.

Excellent research results may be obtained with the use of lung aspirates (310-314), but because of its invasive nature this method has not been generally recommended for

routine use (3). In a developing country setting, the utility of needle aspiration was demonstrated by Silverman and coworkers in 1977: the aetiology of pneumonia could be shown by examination of needle aspirate in 79% of 88 paediatric patients (61% by culture and 18% by Gram-stain only), while CIE and blood culture were positive in only 20% and 11%, respectively (4).

*Table 7. Summary of pneumococcal culture results using needle aspiration sampling technique.*

Patient group, country	Number of samples	Number of positive cultures	Pnc isolates	% of Pnc positive	Ref.
Infants with bronchopneumonia, Egypt	233	182	32	17.6	(310)
Children < 34 months, India	18	6	2	33	(312)
Children < 10 y, USA	28	15	4	26.7	(313)
Infants < 2 y with pneumonia, Chile	505	228	5	2.1	(314)
Children < 10 y, Nigeria	88	54	31	57.4	(4)

An MRC research group in the Gambia has continued to use lung aspirations in research setting (9, 315-317). In a recent review, Greenwood emphasised that approximately 400 aspirates have been taken in recent years without any life-threatening complications although a few children did have haemoptysis, subcutaneous emphysema or pneumothorax, all resolving without intervention (180). Greenwood concluded that in selected cases and in an appropriate setting it is legitimate to use this technique to obtain epidemiological information on the aetiology of pneumonia in children, and in the evaluation of intervention studies such as vaccine trials.

Similar results have also been obtained in Finland by Vuori-Holopainen and coworkers, who emphasised the high microbiological yield of the procedure, and its relatively low risk of clinically significant adverse events (10). In this study, culture, antigen and nucleic acid detection techniques were used. Aspiration disclosed the aetiology in 20 (59%) of 34 cases overall and in 18 (69%) of 26 patients from whom a representative specimen was obtained. The researchers concluded that aspiration should be used if identification of the pathogen outweighs the modest risk of the procedure.

### ***2.5.2 Serotyping of strains***

Today, serotyping of pneumococcal isolates is performed in only a few advanced microbiological laboratories, usually acting as reference laboratories for pneumococcus. Although different serotypes tend to cause different diseases, the serotype information is less important for clinical management of patients, but it is essential for epidemiological surveillance purposes, and for vaccine development and clinical trials.

The classical method for serotyping is quellung reaction, in which cultured isolated are blended with group or type-specific antisera, and the capsular swelling reaction is

observed under microscope (279). Typing is performed using antisera produced by Statens Serum Institut, Copenhagen. The Omniserum, nine pooled sera labelled A through I, 46 types of group antisera and factor sera cover the whole range of 90 types (318). An additional group of five pooled antisera, labelled P through T, can be used together with pools A through F plus H in a chessboard set-up, which allows the typing on nearly 90% of all invasive disease isolates (318, 319).

Several other typing methods have also been developed, including capillary precipitin typing, counterimmunoelectrophoresis (CIEP), latex agglutination and coagglutination (279). In 1997, Fenoll and coworkers published a simple dot blot assay for serotyping of pneumococci (320). In 1988, Waltman and coworkers described a novel typing system based on pneumococcal proteins, but this has not gained wider popularity so far (321).

At KTL, serotyping of pneumococcal isolates is currently performed using CIEP or, for the neutral SGT 7 and 14, latex agglutination. The quellung reaction is used to confirm uncertain results.

### ***2.5.3 Antigen detection***

#### ***2.5.3.1 Capsular polysaccharides***

Latex agglutination of serum samples was shown useful in diagnosing Hib pneumonia in bacteraemic patients, but for pneumococcal pneumonia the sensitivity remained below 40% (322).

Boersma, Holloway and coworkers have done much pioneering work on pneumococcal antigen detection. In 1991, they reported 94% sensitivity for pneumococcal capsular antigen detection from sputum samples in CAP patients (323). They appropriately pointed out that, where positive, the antigen detection remained positive even during antimicrobial therapy, a finding later confirmed conclusively also *in vitro* (324). In another study, 28 (93%) of 30 patients with pneumococcal pneumonia gave positive results in latex agglutination of sputum samples (325). In yet another study, the same investigators showed that CaPS antigen detection from oropharyngeal swabs provided more positive findings than swab culture, but both were still inferior to Gram stain, culture and antigen detection of sputum samples in the same patients (326).

Antigen detection in pleural fluid samples may provide important information in problematic clinical situations. Boersma and coworkers showed antigen detection to be superior to both Gram stain and culture of pleural fluid samples (327). In their Dutch material, pleural fluid was present in 40 to 50% of pneumonia cases, irrespective of aetiology (327). Ruiz-González and coworkers showed that latex agglutination was positive in 17 (94.4%) of 18 transthoracic needle aspirates (TNA) from patients with pneumococcal pneumonia. It is, however, quite unlikely that invasive methods like pleural fluid aspirates or TNA would ever be used routinely on patients with pneumonia, even if the presence of lobar effusion or pleural fluid could be reliably verified by radiography.

Demonstration of pneumococcal capsular polysaccharide antigen in urine in patients with confirmed pneumococcal disease is positive in 60–70% of the patients (328, 329). However, in one study in 31 patients with pneumococcal pneumonia, pneumococcal antigen could be demonstrated in only one patient, yielding a sensitivity of 3% (330). Capeding and coworkers used a modified tube latex agglutination assay and obtained positive findings in 45% of patients with ALRI (331). Sensitivity of the antigen detection may be improved through combining type-specific antigen detection with CPS detection in urine (see Chapter 2.5.3.2).

In two brief reports, Fischer and coworkers pointed out that antigen detection might be useful in avoiding false negative interpretations of blood cultures (297), even when pneumococci have been autolysed (298). Antigen detection with COA or latex agglutination remains positive in spite of partial or complete autolysis.

### 2.5.3.2 *C-polysaccharide*

Detection of CPS in sputum has been attempted by COA and at least two different EIA methods (332-335). Holmberg and collaborators used a sandwich ELISA with monoclonal antiphosphorylcholine antibody and a polyclonal rabbit anti-CPS antiserum. In a material of 147 adult patients with clinical and radiographic evidence for pneumonia, 105 (71%) provided a sputum sample (333). Of the sputum samples from patients who had pneumococci isolated in bacterial culture, 27 of 33 (82%) were positive in CPS detection. Calculated in a subgroup of 34 patients with pneumonia of other known or suspected aetiology, the specificity was 94%. In another report on a subgroup of the same samples, ELISA was compared with LA and COA. ELISA showed the highest sensitivity (95%), but there was no significant difference between the three tests (334).

Parkinson and his coworkers developed an EIA method for quantitation of CPS in sputum samples (335). Of 34 patient sputum samples collected within 24 h of the first radiographic report of pneumonia, 12 grew pneumococcus on culture. By using a cut-off point of 0.5 µg of CPS per ml of sputum, all 12 specimens were positive by EIA (sensitivity 100%). The authors speculated that the test might be used to distinguish between invasive pneumococcal disease and pneumococcal carrier state.

Krook and her collaborators studied the same 105 sputum samples described above and compared CPS-COA test with a commercial COA test kit detecting CaPS (Phadebact; Pharmacia Diagnostics, Sweden). The sensitivity obtained with CPS-COA was 95.8% and with the commercial CaPS-COA kit 83.3%; the specificity was 96.5% and 91.2%, respectively. Thus, compared with the CPS-ELISA developed by the same group, the CPS-COA appeared to offer improved sensitivity in a rapid and simple assay (332).

Yolken and collaborators developed an immunoassay utilising horse antiserum against CPS and biotinylated rabbit antibodies to type-specific pneumococcal capsular polysaccharides (336). CPS antibodies were used in the solid phase and anti-CaPS in the liquid-phase reagent. Testing the system in 25 CSF samples from patients with documented pneumococcal meningitis and 25 samples from patients with meningococcal meningitis plus 16 samples from patients without bacterial meningitis, 100% sensitivity

and specificity was achieved. Despite the results the method has not been adopted into clinical use.

In 1990, Bromberg and coworkers reported on a latex agglutination system for detection of pneumococcal C-polysaccharide in urine (328). CPS in urine was positive in 23 out of 33 patients (70%) with pneumococcal bacteraemia. Thus, used alone CPS detection in urine was unsatisfactory. However, an additional 4 patients had type-specific capsular polysaccharides in urine, adding up a reasonable total sensitivity of 82%. – Apparently, this research line has not been continued, because there are not further publications on the topic.

More recently, several study groups have tested the Binax NOW *Streptococcus pneumoniae* Urinary Antigen Test (Binax; Portland, ME), an immunochromatographic assay detecting CPS. Domínguez and coworkers detected the antigen in 80.4% of patients with pneumococcal pneumonia, with similar sensitivity in both bacteraemic and nonbacteraemic cases, 82.1% and 78.3%, respectively (337). Two other studies obtained similar figures for culture-confirmed cases (338, 339). However, the test has not proved useful in children, as it appears to be positive in nasopharyngeal carriers of pneumococci (340, 341).

#### **2.5.4 Polymerase chain reaction**

Published in 1985, the polymerase chain reaction (PCR) employs the enzymatic amplification of DNA *in vitro* (342). By synthesising many copies of a selected DNA sequence, PCR is capable of exponentially increasing the quantity of the target DNA segment in a sample (343). One PCR cycle consists of three steps: DNA denaturation, primer hybridisation and DNA polymerase extension.

The PCR principle has already been applied to several aspects of pneumococcal diagnostics, including blood, serum, CSF and respiratory samples (Table 8). The pneumolysin gene was independently chosen as a primer target by Karen Rudolph and coworkers and our Finnish group in the early 1990s. More recently, the gene encoding the pneumococcal surface adhesin protein (PsaA), has also been proposed as a target, as this has been confirmed to be present in all 90 serotypes of *S. pneumoniae* (344).

In their first study, Rudolph's group included also a primer for the cell wall autolysin (LytA) protein gene, obtaining sensitivities of 75 and 63% for the pneumolysin and autolysin primers, respectively, in buffy coat fractions of blood samples from patients with blood culture positive pneumococcal pneumonia (345). For whole blood, the sensitivities were below 40% for both primers. – In the Finnish material, positive reactions were obtained from all 20 acute phase sera from patients with blood culture positive pneumococcal pneumonia (346). Differences between the two study findings may be due to e.g. inhibitory factors in blood, or different timing of samples. All three reported methods were highly specific.

More recently, Dagan and his coworkers reported on pneumolysin-PCR method and studying a small number of sera, achieved 100% sensitivity in pneumococcal bacteraemia, but the authors concluded that the high rate of detection of pneumococcal

DNA in healthy controls (35/202 children, 17%) compromises the usefulness of the test in diagnosis of invasive infections, at least in children (347). Menéndez and her collaborators studied sera from 184 patients with CAP, but their study did not include any control groups (348). They could only conclude that PCR produced five times more pneumococcal diagnoses than blood culture alone.

Table 8. PCR assays for pneumococcal diagnostics.

Study group	Diagnosis	Sample	No of PCR + /total (%)		Ref.
			Patients with positive blood culture	Controls	
Rudolph <i>et al.</i> 1993	pneumococcal bacteraemia	buffy coat whole blood	6/8 (75) 3/8 (37.5)	1/8 (12.5) 0/6 (0)	(345)
Salo <i>et al.</i> 1995	pneumococcal pneumonia	serum	22/22 (100)	6/100 (6)	(346)
Dagan <i>et al.</i> 1998	pneumococcal bacteraemia	serum	9/9 (100)	35/202 (17)	(347)
Isaacman <i>et al.</i> 1998	pneumococcal bacteraemia	blood	12/21 (57)	16/103 (16)	(349)
Toikka <i>et al.</i> 1999	pneumococcal bacteraemia	plasma, serum and buffy coat	2/4 (50)	0/15 (0)	(350)
García <i>et al.</i> 1999	pneumococcal pneumonia	transthoracic needle aspirate	15/17 (88)	2/23 (0.9)	(351)

PCR methods have been applied in examination of TNA (351, 352). While the results were encouraging – 80–90% of patients with either presumptive or definite diagnosis of pneumococcal pneumonia showed a positive finding in PCR – the usefulness of TNA in clinical practice remains debatable. Several studies have shown that PCR-based assay systems can detect the presence of pneumococcal DNA in a significant percentage of culturally sterile MEF samples (353-355), and Hendolin and coworkers have even developed a multiplex PCR assay for simultaneous detection of the four most frequent bacterial species found in middle ear effusions (356, 357).

Comparing PCR and a commercial RNA hybridisation test (AccuProbe™), with conventional identification methods, Kaijalainen and coworkers concluded that the new methods identified similarly typical optochin sensitive pneumococci, but gave partly controversial results for atypical optochin resistant, noncapsulated isolates (358). Thus, overall, they did not improve the identification of pneumococcus.

Hassan-King and coworkers have applied an autolysin-PCR to improve pneumococcal detection rates in blood culture (359), later extending the method to a multiplex PCR for simultaneous detection of both Hib and pneumococcus (360). Their results suggest that at least some benefit may be obtained by applying the PCR on culture negative samples (359, 360). Similar results have been reported by Rintamäki and others, using a novel PCR modification that employs microwell hybridisation with Europium-labelled probes (361). Recently, a real-time PCR application has been introduced and proved useful in identifying pneumococcus in middle ear fluid samples (362). The method provides means to quantify the amount of bacteria in the sample.

PCR applications for CSF investigations have also been applied in diagnosis of pneumococcal meningitis (363-365). – Finally, du Plessis and coworkers have developed PCR systems to detect penicillin-resistant pneumococcus in clinical samples, based on amplification of the pneumococcal penicillin-binding protein genes *pbp1A* and *pbp2B* (366, 367).

### ***2.5.5 Antibody assays***

Although antibody assays are a generally applied means for diagnosing viral pneumonia, they are not commonly used in diagnosis of bacterial disease. The development of commercial kits has been complicated by the large number of cross-reacting bacterial components (6). Most assays measure response to CaPS antigens (368-373), but more recently CPS and protein antigen assays have also been introduced (374-376). Both EIA and RIA applications are available (371, 377-381), and a novel nitrocellulose-based solid phase multi-antigen immunoassay has also been described (382).

Serological methods will not be very useful in clinical work as long as they necessitate paired serum samples, often taken at 2-week intervals (6). On the other hand, the use of serological methods is well justified in combination with other methods, as a confirmatory test (183). Serological findings can provide valuable additional information in aetiological and epidemiological surveys and clinical vaccine trials (6).

In adults, CaPS induce good antibody response after immunisation. After infection, the response is variable and has not been conclusively shown to be useful for establishing clinical diagnoses. Using 13 to 14 antigens as pools, the sensitivity has varied between 30 and 90% (372, 383). Using a combination of different assays (CaPS, CPS, Ply, and phosphorylcholine) Burman and coworkers found the serological methods valuable in evaluating the significance of positive cultures and COA of samples from the respiratory tract (374). Comparing antigen detection and serological methods in 90 patients with CAP, Boersma and coworkers found that the CaPS antibody assay was as specific (85%) as antigen detection, but the sensitivity remained as low as 50% (323).

In children below 2 years of age, antibody response to capsular polysaccharides is, generally speaking, poor after both infection and immunisation, especially against the most common paediatric types (6, 35, 62, 384-387). – Although children respond poorly to pneumococcal capsular polysaccharides, CPS is able to induce antibody production both in serum and in the middle ear (388-390). The mucosal responses seem to be independent from systemic response (391). – More recently, the specificity of pneumococcal CaPS antibody assays has been questioned, based on extensive studies at the KTL (392). Inhibition studies suggested that some CaPS preparations may contain cross-reactive epitopes or impurities, other than CPS, that are common to many types of pneumococcal CaPS.

Protein antigens, such as Ply, are common to all pneumococcal serotypes and thus appear attractive and simple alternatives for measuring serologic responses (393, 394). Even if the Ply-EIA method has been shown to be sensitive compared with both culture and antigen detection and CaPS antibody assays, some blood culture positive samples

have remained negative in this test. Therefore, Leinonen and coworkers proceeded to test the hypothesis that the antibody responses could be masked by the formation of immune complex of circulating pneumolysin and antibodies (6).

### ***2.5.6 Immune complex assays***

Circulating antigen–antibody complexes or immune complexes (IC) are a result from the host antibody response to an infectious agent. IC containing pneumococcal polysaccharide in patients with pneumococcal pneumonia have been demonstrated in several studies (44-46). Demonstration of pneumococcal antibodies in IC has subsequently been applied in diagnosis of invasive pneumococcal infections in both adults (43, 47-49) and children (50). The methods have been based on demonstration of polysaccharide or pneumolysin antibodies in precipitated IC immune complexes.

In 80 adults with CAP, pneumococcal polysaccharide antibodies could be demonstrated in dissociated IC in 85% of 20 patients with bacteraemic pneumococcal pneumonia, in levels exceeding a cut-off determined in samples from 84 healthy controls (43). Figures for patients with probable pneumococcal pneumonia, patients with pneumonia of non-pneumococcal aetiology, and patients with pneumonia of unknown aetiology, were 36.4%, 16.7% and 41.9%, respectively.

Studying serum samples from 129 adults with CAP, Leinonen and coworkers demonstrated a sensitivity of 97.8% and specificity of 83.4% for this method (47). Another pneumonia study in Finland employed blood and sputum culture, detection of polysaccharide antigens and free and IC bound pneumolysin IgG antibodies to confirm pneumococcal aetiology (48). The IC assay provided most positive results (in 47% of the cases), while the others methods were positive in only 9–24% of the cases.

In 195 children with pneumococcal pneumonia (diagnosed with a panel of antigen and antibody assays), positive IC findings were obtained disappointingly in only 14% of the cases (50). The encouraging finding of this study, however, was that in a control group of 120 healthy children, less than 1% gave positive findings. These findings prompted one of the studies reported in the subsequent chapters. In 129 Kenyan adults with pneumococcal pneumonia the sensitivities of the IC assays was around 20% (395).

## **3 Aims of the research**

### **3.1 Overall aims**

1. To review the current situation in pneumococcal epidemiology and laboratory diagnostics.
2. To develop laboratory diagnostic methods, with which pneumococcal surface antigens or pneumococcus-specific antibodies can be demonstrated in clinical samples with sufficient sensitivity and specificity.

### **3.2 Specific aims**

3. To compare standard bacterial culture with demonstration of pneumococcal polysaccharide antigen in nasopharyngeal samples by latex agglutination and counterimmunoelectrophoresis, focusing on the demonstration of carriage.
4. To improve the sensitivity of antigen detection by adding an enrichment phase to the method, thereby providing the bacteria with an opportunity to multiply, to exceed the threshold limit required by the antigen detection methods.
5. To study the utility of pneumococcus-specific antibodies bound in immune complexes in the diagnosis of pneumococcal disease.
6. To develop an enzyme-linked immunoassay for demonstration of type-specific pneumococcal polysaccharide antigens, and to study its applicability in the diagnosis of pneumococcal disease.

## 4 Materials and methods

### 4.1 Study sites and participating institutions

Study I was performed at the Research Institute for Tropical Medicine (RITM), Manila, between June and October 1984. The study protocol was approved by the Internal Review Board of the institute. The catchment area of the hospital consists of periurban slums and middle-class housing in southern metropolitan Manila. The results were analysed at the National Public Health Institute, Helsinki, Finland.

Study II was performed at the National Public Health Institute, Helsinki, Finland, and the clinical samples we obtained from patients at the Aurora Municipal Hospital, Helsinki, Finland between June 1993 and January 1994. The study protocol was approved by the Internal Review Board of the City of Helsinki Health Services.

In Study III, the ALRI patients were participants in a clinical study performed at the Research Institute for Tropical Medicine (RITM) in Alabang, a periurban area of Manila, from March 1989 to August 1990. Control subjects participated in a comparative *Haemophilus influenzae* type b (Hib) vaccine immunogenicity trial conducted from November 1992 to November 1994 in Cabuyao, Laguna, The Philippines (73). The studies were approved by the ethical review board of both participating institutes, and informed consent was obtained from the parents or the guardian of each participant before enrolment.

The clinical part of Study IV was performed in a special study clinic between April 1994 and August 1999, located at Hervanta Health Centre in Tampere, Finland. The initial method development took place at the National Public Health Institute in Helsinki, and the diagnostic laboratory work and analysis of results was performed at the National Public Health Institute, Department in Oulu (now the Department of Microbiology, Oulu).

## 4.2 Patients and controls

*Study I.* Patients less than 5 years with ALRI were enrolled in the study, with parental consent. The diagnostic criteria for ALRI have been published elsewhere (396). Relevant patient history and clinical findings were all recorded on structured case record forms. Information on the use of antimicrobial agents was based on interviews with the parents.

*Study II.* We aimed at obtaining nasopharyngeal swabs (NPS) from 100 consecutive children less than 8 years of age with symptoms of acute respiratory infection, who attended the paediatric outpatient clinic of the hospital. Although an aetiological diagnosis was not aimed at, this patient group was selected because children with respiratory infections are known to have a high carriage rate of pneumococcus (397). The study site was chosen because at this clinic NPS is a routine test for patients. Information on the use of antimicrobials was recorded during parental interview. Only children who had not received antimicrobials in connection with the present illness, or during the 2 previous weeks, were accepted into the study. In Finland, antimicrobials are available by prescription only and surreptitious use of these drugs is extremely rare. Therefore, screening tests for the use of antimicrobials were not considered necessary.

*Study III (patients).* Filipino children less than 5 years old admitted to the RITM tertiary level referral hospital were prospectively enrolled in the study, if they had cough of less than 3 weeks' duration or difficulty in breathing, and any of the following: 1) tachypnea (respiratory rate  $\geq 50/\text{min}$ ), 2) chest indrawing, 3) inability to drink, 4) cyanosis, or 5) infiltrate on chest X-ray even in the absence of the previously enumerated symptoms. The inclusion criteria were based on the pneumonia definition protocol advocated by WHO at that time (398). Altogether 350 children fulfilled the inclusion criteria and entered the study. Three serum samples were obtained: 1) on admission (baseline, day 1), 2) during hospital stay (day 3), and 3) in convalescent phase (mean  $\pm$  SD, day  $16 \pm 4.8$ , range 8–35). Blood culture was performed at least once for all patients.

*Study III (healthy controls).* A subgroup of 150 Filipino infants were randomly selected from the 200 infants enrolled in the Hib vaccine immunogenicity trial conducted (73). In addition to regular EPI vaccines (BCG, DTP, OPV and measles), the subjects received Hib and hepatitis B vaccines. Serum samples for antibody determinations were obtained either after the primary vaccination series at 16 weeks or 1 month after measles vaccination at 40 weeks. At the time of serum sampling, upper respiratory tract carriage of *S. pneumoniae* was studied by nasal and oropharyngeal swabbing using conventional culture methods (307). The nutritional status of both the healthy children and the patients was assessed by standard methods (399).

*Study IV.* 329 healthy infants born in the Hervanta area, Tampere, Finland, were enrolled in the FinOM Cohort Study between April 1994 and August 1995 and were followed-up from 2 to 24 months of age (400). All infants born or residing in the area were eligible to participate in the study if they were 2 months  $\pm$  2 weeks old and had no prior immunisation with a pneumococcal vaccine. – The children were examined, and the nasopharyngeal samples obtained, at age-scheduled healthy visits at ages 2, 3, 4, 5, 6, 9, 12, and 15 months ( $\pm$  2 weeks) and at age 18 months. A close-out visit took place at age 24 months (400).

### 4.3 Clinical samples

Nasopharyngeal swabs. NPS samples were obtained via the nostril with a calcium alginate tipped aluminium wire swab (Calgiswab 1; Orion Diagnostica, Espoo, Finland). The swab was inserted to a distance equal to that from the child's nose to the outer ear to ensure that the tip was in the nasopharynx. Once in the nasopharynx, the swab was left in place for 5 sec and then gently removed.

*Nasopharyngeal aspirates.* For NPA sampling, a catheter (Vygon) was inserted through the nostril. Suction was applied only after the catheter elicited a vigorous cough reflex, and a sample volume ranging from 0.5 to 3 ml was collected in a sterile mucus trap (Vygon). The same samples were also used for extensive virological investigations, the results of which have been published elsewhere (396).

*Middle ear fluid.* MEF samples were obtained through tympanocentesis whenever AOM was diagnosed.

### 4.4 Bacteriological methods

#### 4.4.1 *Quantitative bacterial culture and Gram staining*

Immediately after specimen collection, a portion of the NPA samples was homogenised with an equal volume of dithiothreitol solution (Sputasol) for 10 min at room temperature (Study I). A Gram-stained smear of the homogenised sample was examined by light microscopy. The bacterial findings were recorded and graded as either predominant (++) or minor (+). Polymorphonuclear leukocytes and epithelial cells were also counted to assess the purulence of the sample (Study I). Samples with  $\geq 25$  polymorphonuclear leukocytes per microscopic field were classified as purulent.

One microlitre each of homogenised NPA samples, undiluted or diluted  $10^{-2}$  or  $10^{-3}$ , was inoculated onto a set of three blood agar plates (without antibiotics, with gentamicin or with bacitracin) and on MacConkey agar plates (Study I). MacConkey plates were used to find gram-negative bacteria, which sometimes may give false-positive reactions in antigen detection of pneumococci. The number of colonies on the plates after 1 to 2 days of incubation at 37 °C was recorded, and the bacteria were identified by standard methods (279). The routine dilution method used in the laboratory allowed the reliable detection of isolates with  $\geq 10^5$  CFU/ml.

In Study II, the NPS samples were cultured on enriched chocolate agar plates and selective sheep blood agar plates containing 5 g/ml gentamicin. The plates were incubated in 5% CO<sub>2</sub> at 36–37 °C in the study clinic, usually overnight, and were transported to the bacteriologic laboratory in Oulu. The plates inoculated on Fridays and Saturdays were incubated in the study clinic until Sunday. In the laboratory, the plates

were examined for *S. pneumoniae* and were incubated further overnight, to reach a total incubation time of 48 h.

To identify *S. pneumoniae*, 4 different  $\alpha$ -haemolytic colonies were tested for optochin sensitivity. A bile solubility test was used if the optochin test was negative but colony morphology was suggestive of the species. The number of colonies was counted from the plate with more abundant growth.

Pneumococcal isolates were serotyped by counterimmunoelectrophoresis and latex agglutination (for types 7 and 14), using antiserum pools and group- and type-specific antisera (401). The isolates of groups 6, 9, 18, 19, and 23 were subtyped by using pneumococcal factor antisera. All antisera were purchased from Statens Serum Institut (Copenhagen, Denmark).

After plating, the swab was also swirled immediately in a 5 ml BHI broth tube (without added antimicrobials) and then inserted in another BHI broth tube with 5  $\mu$ g/ml of gentamicin (Study IV). Both tubes were incubated 24–48 hr at 35 °C in an atmosphere of 5% CO<sub>2</sub> and then stored at –20 °C for antigen detection. Before storage, a control culture was performed: 200  $\mu$ l from each tube was cultured and the pneumococci were identified and typed as described above for direct culture.

#### ***4.4.2 Detection of pneumococcal capsular polysaccharide antigens***

Part of the NPA samples was stored at –20 °C. For antigen detection, the samples were diluted 1:2.5 in phosphate-buffered saline containing 20% inactivated foetal calf serum, 2% Tween 20, and 0.01% merthiolate, sonicated for 1 to 3 min, and heated in a boiling water bath for 5 min. Three different methods were used to detect pneumococcal antigens in the heated samples. 1) Counterimmunoelectrophoresis (CIEP) was performed as described earlier (58). For the primary demonstration of pneumococcal antigens, Omniserum and a mixture of eight type-specific antisera (for the presumably most common types or groups 3, 4, 6, 8, 9, 18, 19, and 23) were used. 2) For the detection of neutral polysaccharides of types 7 and 14, type specific latex reagents were prepared as described earlier (58). 3) In addition, the coagglutination (COA) test (Phadebact Pneumococcus Test; Pharmacia, Uppsala, Sweden) was performed to demonstrate all pneumococcal capsular polysaccharides.

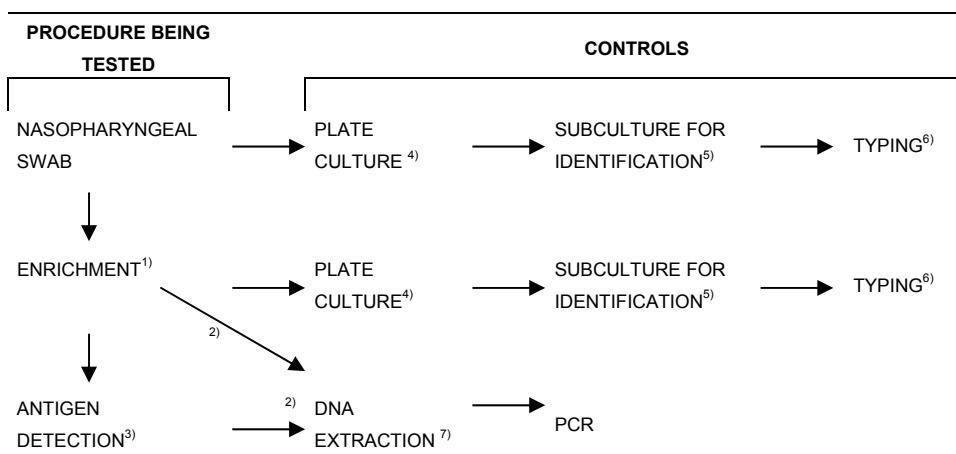
When a positive finding was obtained by CIEP and/or COA, the capsular polysaccharide type was further determined by CIEP, using antiserum pools and the complete selection of type- or group-specific antisera. All pneumococcal antisera were purchased from Statens Serum Institut (Copenhagen).

#### ***4.4.3 Antigen detection after an enrichment culture***

Nasopharyngeal swabs were subjected to enrichment in broth, followed by antigen detection. Brain–heart infusion (BHI) broth with and without gentamicin was tested in

parallel in this step to investigate whether the use of a selective antimicrobial agent would enhance the probability of detecting *S. pneumoniae*, as has been shown for regular culture (270). Several control tests were also performed: 1) direct plate culture of the swabs before enrichment, 2) culture from the BHI broth after enrichment, and 3) polymerase chain reaction (PCR) from the enrichment broth (Fig. 2).

For standard detection of *S. pneumoniae*, a semiquantitative culture was performed on blood agar plates with 5 µg/ml of gentamicin, as described above in section 4.4.1. After plating, the swab was immediately swirled in a 5-ml BHI broth tube (without added antimicrobials) and then inserted in another BHI broth tube with 5 µg/ml of gentamicin. Both tubes were incubated 24–48 hr at 35 °C in an atmosphere of 5% CO<sub>2</sub> and then stored at –20 °C for antigen detection. Before storage, a control culture was performed: 200 µl from each tube was cultured and the pneumococci were identified and typed as described above for direct culture.



**Fig. 2. Flow chart of the study design, Study II. Storage periods were added for practical reasons and can be omitted if antigen detection or typing is feasible on site. 1) media tested: brain-heart infusion broth with and without gentamicin; incubation for 24–48 hr at 35 °C in an atmosphere of 5% CO<sub>2</sub>; 2) storage if necessary for practical reasons; in this study –20 °C; 3) = by latex agglutination and counterimmunoelectrophoresis; 4) = blood agar with gentamicin; 5) blood agar with plate with optochin disk; 6) = by latex agglutination and counterimmunoelectrophoresis of strains stored at –70 °C; 7) = with phenol:chloroform: isoamyl alcohol (25:24:1); 8) = with primers based on the pneumolysin gene sequence. PCR = polymerase chain reaction.**

#### 4.4.4 Antigen detection and serotyping

Pneumococcal capsular polysaccharide antigens in the enrichment broth were demonstrated by latex agglutination (detecting neutral serotypes 7 and 14) and counterimmunoelectrophoresis (detecting all other serotypes), using methods described

previously (401). Pneumococci that gave positive reactions only by Omniserum (a mixture of antisera against all pneumococcal serotypes) or antiserum pools (nine pools with 4–7 serotypes in each pool) were classified as nontypeable. All pneumococcal isolates were also typed by the same methods. All pneumococcal antisera were obtained from Statens Serum Institut (Copenhagen).

#### ***4.4.5 Extraction of DNA and the PCR***

The specimens were prepared as previously described (402). Briefly, 200 µl of enrichment broth was mixed with a solution containing 0.1 M NaOH, 2 M NaCl, and 0.5% sodium dodecyl sulphate. The suspension was incubated for 15 minutes at 95 °C and 200 µl of 0.1 M Tris-HCl, pH 8.0 was added. The DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1; Sigma, St. Louis, MO, USA). Finally, DNA was precipitated with ethanol and dissolved in water.

The selection of oligonucleotide primers was based on the pneumolysin gene sequence (403). The outer primers were 23-mers and amplified a 348-basepair (bp) region of the pneumolysin gene, and the inner primers were 23- and 21-mers amplifying a 208-bp region (346). Amplification and detection were performed as described by Salo and others (346). The generally recommended procedures were used to avoid contamination (103).

### **4.5 Serological methods**

#### ***4.5.1 Precipitation of immune complexes***

Immune complexes were precipitated from 100 µl of serum with 3.5% polyethylene glycol (PEG) by overnight incubation at +4 °C (23, 34, 35). The precipitate was washed twice with 3.5% PEG and dissolved in 100 µl of phosphate buffered saline (PBS) and stored at -20 °C until analysed. Buffer salt concentrations, incubation temperature and pH of the PEG were carefully monitored. Alternatively, the samples could be stored intact and precipitated before immune complex assays.

#### ***4.5.2 Determination of immune complex bound antibodies***

Dissolved immune complexes were diluted 1/100 with PBS containing 10% foetal calf serum. EIA determinations were done separately for the following IC-bound antibodies: IgG and IgM class antibodies to pneumolysin (Ply-IgG, Ply-IgM), and IgG antibodies to a

mixture of 14 pneumococcal capsular polysaccharides (CaPS-IgG) and to C-polysaccharide (CPS-IgG).

For determination of antibodies, microtitre enzyme immunoassay plates (Maxisorb; NUNC, Roskilde, Denmark) were coated overnight at +37 °C with following antigen solutions: 1) pneumolysin (5 µg/ml in PBS) produced in *Escherichia coli*; 2) mixture of 14 pneumococcal capsular polysaccharides (Pneumovax; MSD, West Point, Pa, USA) containing 50 µg/ml of polysaccharide from serotypes 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F and 25; and 3) C-polysaccharide preparation (5 µg/ml) isolated from a pneumococcal mutant strain with C-polysaccharide capsule (C-mutant CSR, SCS-2, clone 1) by the method of Pedersen et al (404).

After incubation, plates were washed three times with PBS containing 0.05% Tween, and 100 µl of diluted dissolved immune complexes was added to the wells in duplicate, and incubated at +37 °C for 2 hrs. After washing as above, the conjugate, alkaline phosphatase-conjugated anti-human IgG (diluted 1/3000; Sigma, St Louis, Mo, USA) or anti-human IgM (Caltag, San Francisco, Ca, USA) was added and incubated at +37 °C for 2 hrs. The plates were then washed three times with PBS-Tween and still once more with distilled water. Finally, the substrate, 1 mg of p-nitrophenylphosphate (Sigma 104) in 1 ml of diethanolamine buffer, pH 9.6, was added, followed by incubation of the plates at +37 °C for 30 min.

The optical density (OD) values were measured at 405 nm by automated ELISA reader (Multiskan MCC/340; Labsystems Oy, Helsinki, Finland). The levels of immune complex bound antibodies were expressed as EIA units (mean OD of duplicates × dilution)

Mean levels of immune complex bound antibodies were first determined in healthy children, and the conventional cut-off limits for normal population were derived from this material by adding 2 SD to the mean values.

## **4.6 Method development in Study IV**

### ***4.6.1 Production of the vaccine***

American Type Culture Collection (ATCC, Rockville, MD, US) strains with distinct capsular specific reactions were used to produce the vaccine for immunisation of rabbits. The seeding dose was grown in BHI medium with serum at +37 °C overnight and the large-scale production took place in serum-free Todd-Hewitt medium (BBL Microbiology Systems, Cockeysville, MD, USA), in a shaker at +37 °C. Bacterial growth was monitored using a Klett densitometer, and the process was stopped with formalin when the maximum growth had been reached, usually in 4 to 7 hours.

After centrifugation at 9000 RPM for 10 minutes the supernate was discarded and the sediment was crushed in a mortar. The resulting paste was resuspended in Sørensen's vaccine buffer solution (20 ml 15 M KH<sub>2</sub>PO<sub>4</sub> + 80 ml 15 M NaPHO<sub>4</sub> + 300 ml 0.9% NaCl).

### ***4.6.2 Production of the antisera***

Serotype-specific antisera were produced in rabbits applying methods and immunisation schedules described elsewhere (405, 406). Briefly, the immunisation was started with a 100 µl intravenous vaccine dose and an injection of 250 µl into both hind footpads. Footpad injections were continued once weekly for 4 weeks, and a booster dose was given after a 4-week interval. The blood was collected after 10 days, and the serum was separated. Immunoglobulins were precipitated after absorption of antisera by ammonium sulphate, and resuspended in 2 ml 0.9% NaCl. After dialysis overnight at +4 °C against 0.9% NaCl, the immunoglobulins were stored in 10 mg/ml solutions at -20 °C.

### ***4.6.3 Absorption of antisera***

Antisera were pre-treated with whole bacterial suspension of pneumococcal mutant strain with C-polysaccharide capsule (C-mutant CSR, SCS-2, clone 1, kindly provided by Dr Jørgen Henriksen, Statens Serum Institute, Copenhagen, Denmark) to remove antibodies specific to C-polysaccharide that could lead to cross-reactions between pneumococcal types (404, 406). Three 1-hour incubations were carried out at room temperature, and after each incubation, bacteria were removed by centrifugation. Absorbed antisera (about 50 µg/ml solution) were stored at +4 °C until tested in EIA. Type 19F antiserum was additionally treated with 23F bacterial suspension because cross-reactivity between these types was observed.

### ***4.6.4 Optimisation of the reaction conditions***

Optimal reagent dilutions were determined by checkerboard titration. The best results were achieved when wells of EIA plates (Maxisorp Nunc-Immuno Plates, Nunc, Roskilde, Denmark) were coated overnight at +37 °C with 3 µg/ml of capsular antigens in PBS. The plates were washed four times (PBS with 0.05% Tween) and blocked for 1 hour at +37 °C with 10% foetal bovine serum (FBS; Gibco BRL, Life Technologies, South America) in PBS. Antisera were used as 1:30,000 to 1:150,000 dilution (depending on type) and peroxidase conjugated anti-rabbit IgG (Sigma, St. Louis, MO) was used in 1:20,000 dilution.

Peroxidase was quantified by measuring peroxydation of o-phenylenediamine dihydrochloride (Sigma) in the presence of H<sub>2</sub>O<sub>2</sub> by measuring the absorbance at 492 nm in ELISA reader (Labsystems Multiskan MCC/340, Labsystems, Finland) after addition of H<sub>2</sub>SO<sub>4</sub> to stop the reaction. Incubation times for competition step and peroxidase conjugated antibody were 2 hours at 37 °C. Colour reaction was stopped after 30 min incubation at room temperature.

#### ***4.6.5 Testing of sensitivity and specificity***

Sensitivity of the competitive EIA was determined by using purified capsular polysaccharide antigens (ATCC). Known amounts of antigen were added to the antiserum dilution. Inhibition was allowed to happen for 1 hour in plastic tubes (in some experiments tubes were kept overnight at +4 °C after this) and 1 hour in the antigen-coated wells of microtitre plate at +37 °C. Antigen dilution curves with purified antigens were determined on each plate. The standard series included 0 ng (= maximum binding), 5 ng and 50 ng/ml of specific capsular antigen and 50 ng/ml or 500 ng/ml of CPS as specificity control. 10% FBS without antiserum was used as the background control.

Cross-reactivity between the targeted serotypes was examined by coating the plates with individual purified antigens and cross-testing them with all the antisera used in the study.

### **4.7 Statistical methods**

Data were analysed with SAS System software (version 6.09) on VAX computer. Statistical significance of differences in rates was calculated by the  $\chi^2$  test. The strength of agreement between two different methods was evaluated by calculating the kappa (K) values for two-way tables (407). The K values were classified as follows: < 0.20, poor; 0.20 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, good; and 0.81 to 1.00, very good agreement (407).

In Study III, descriptive statistics were produced by the univariate procedure of the SAS programme. Statistical significance of differences in rates was calculated by the  $\chi^2$  test and, after logarithmic conversion, t-test was used for detecting difference between means. Pearson correlation coefficient was used for correlation assessments (arithmetic and logarithmic values). Combined effects of multiple variables, such as age and pneumococcal carrier status, were evaluated by General Linear Model (GLM) procedure. This model calculates the effect of several variables into one linear model, providing one probability estimate for the combined effect.

## **5 Results**

### **5.1 Comparison of culture and antigen detection methods in ALRI patients (I)**

#### ***5.1.1 Patients and samples available***

A total of 318 Filipino children with ALRI were enrolled; 135 (42%) patients were admitted to the hospital for treatment. Many patients were severely ill, and 32 patients died after admission. The distribution of the main entry criteria in the study group has been published previously (396). A definite infiltrate on the chest X-ray was seen in 38% of the patients, as judged by coded readings by two paediatric radiologists. In the remaining patients, the diagnosis of lower respiratory involvement was based on clinical signs or symptoms.

Pneumococcal antigen was searched for in 292 samples (92%). These samples constitute the basis for the analysis of pneumococcal type distribution. Both antigen detection and culture results were available for 227 (71%) patients, which is the subgroup for the comparison of the different methods. The distribution of clinical findings in both subgroups was similar to that of the original group of enrolled patients.

#### ***5.1.2 Pneumococcal findings***

By culture, *S. pneumoniae* was found in 115 (51%) of the 227 NPA samples. Pneumococcal antigen was detected in 140 (62%) of the same samples (Table 9). Both methods were positive in 95 (42%) samples. Twenty (17%) of the culture-positive samples were negative in the antigen detection assay. There was no correlation between

negative antigen finding and the number of pneumococcal colonies in culture; actually, more than 90% of the culture positive samples grew more than  $10^6$  bacteria per ml.

*Table 9. Pneumococcal antigen detection compared with pneumococcal culture in 277 nasopharyngeal aspirates.<sup>a</sup>*

Pneumococcal antigen	Pneumococcal culture (number of samples)		Total number of samples
	Positive	Negative	
Positive	95	45	140
Negative	20	67	87
Total	115	112	227

<sup>a</sup> The agreement between the tests is moderate ( $K = 0.43$ ).

Forty-five (32%) of the 140 antigen positive samples were negative by culture. In 17 (38%) of these samples, gram-positive diplococci were seen as the predominant finding in gram-stained smears. Some 98.6% of the samples were considered purulent, with more than 25 polymorphonuclear leukocytes per microscope field.

### ***5.1.3 Effect of antimicrobial agents***

Information on the use of antimicrobial agents within 48 h prior to the enrolment was available from 223 of the 227 ALRI patients; 66 (30%) patients had a history of antimicrobial use (Table 10). Pneumococcal culture was positive significantly less often in the patients with antibiotic therapy (38%) than in those who did not use antibiotics (56%,  $P < 0.05$ ). There was no difference between the two groups in the number of colonies detected in culture. Again, more than 90% of the cultures grew more than  $10^6$  bacteria per ml, and in about 30% of the isolates the colonies were too numerous to count in both groups.

*Table 10. Pneumococcal findings in culture and by antigen detection versus use of antibiotics within 48 h prior to nasopharyngeal sampling.*

Antibiotics within 48 h prior to sampling	Number (%) of patients with given results by			
	Culture		Antigen detection	
	+	-	+	-
Yes, 66 patients (30%)	25(38)	41 (62)	35 (53)	31 (47)
No, 157 patients (70%)	88 (56)	69 (44)	103 (66)	54 (34)
Significance	$P < 0.05$		NS <sup>a</sup>	

<sup>a</sup> NS, not significant

Antigen detection was positive equally often in both patient groups. In culture-positive samples, antigen detection was positive in 83% of specimens if the patient had not received antimicrobial agents and in 80% if the agents had been used. In culture-negative samples, antigen was found in 43% of those not given antibiotics and in 37% of those previously given antibiotics. An analysis of the combined findings of antigen detection

and culture in relation to antimicrobial use (Table 11) showed that when both methods were positive only 22% of the patients had used antimicrobial agents; when both methods were negative, 40% had used antimicrobial agents ( $P < 0.05$ ).

*Table 11. Proportion of patients with preceding antimicrobial therapy in relation to antigen and culture findings.*<sup>a</sup> – Difference between groups A and D is significant ( $P < 0.05$ ).

Pneumococcal findings	Number of patients	% receiving antimicrobial therapy
A. Antigen positive, culture positive	93	22
B. Antigen negative, culture positive	20	25
C. Antigen positive, culture negative	45	33
D. Antigen negative, culture negative	65	40

<sup>a</sup> Information on the use of antimicrobial agents was available from 223 patients.

#### **5.1.4 Gram stain**

As a standard method, Gram stain was also done, but this proved very insensitive and possibly non-specific. Gram-positive diplococci were seen as the predominant finding in 67 of 115 (58%) of the culture-positive NPA samples, in 73 of 140 (52%) of the antigen-positive samples, and in 24 of 67 (36%) of the NPA samples with no other evidence of pneumococcal involvement.

#### **5.1.5 Sensitivity of methods**

In the 292 samples for which antigen detection results were available, COA was the single most sensitive method: it was positive in 144 (85%) of 170 (75%) of these samples, while CIEP was positive in 128 of 170 (75%) of these samples. The two tests had a moderate level of agreement ( $K = 0.59$ ). Latex agglutination was used only for the detection of pneumococcal types 7 and 14. For this purpose, it was invaluable, as only 8 (25%) of the 32 strains of these serotypes gave a definite reaction with both COA and CIEP. Individually, COA detected 78% and CIEP detected 38% of types 7 and 14.

#### **5.1.6 Pneumococcal type distribution**

In 132 of 170 (78%) of the antigen-positive samples, pneumococcal type or group could be identified by CIEP or latex agglutination (Table 12). Two different antigens in the same sample were detected in specimens from 14 patients (8%). Paediatric types or

groups 6, 14, 19, and 23 accounted for 55% and the seven most common types or groups (6, 14, 19, 23, 15, 7, and 11) accounted for 64% of the pneumococcal types or groups identified.

*Table 12. Pneumococcal type or group distribution in 170 Filipino children by age group.a*

Pneumococcal type/group	Total number (%) found	No. found in given patient age group (mo)		
		0–11 (N = 100) <sup>b</sup>	12–24 (N = 111)	25–60 (N = 81)
6	32 (17.4)	7	15	10
14	27 (14.7)	12	9	6
19	27 (14.7)	10	6	11
23	16 (8.7)	7	3	6
15	7 (3.8)	1	3	3
7	5 (2.7)	2	2	1
11	4 (2.2)	1	1	2
9, 13, 21c	9 (4.9)	3	4	2
32, 33, 35, 45d	8 (4.3)	2	5	1
3, 4, 8, 10, 16, 20, 25, 29, 31, 39, 44e	11 (6.0)	3	7	1
Nontypeable	38 (20.6)	14	15	9
Total no. found	184 (100)	62	70	52

a Two types were detected simultaneously in 14 children.

b N = number of NPA samples.

c Three of each type were found.

d Two of each type were found.

e One of each type was found.

## 5.2 Pneumococcal antigen detection after an enrichment culture in ARI patients (II)

### 5.2.1 Patients and samples

Nasopharyngeal swabs were obtained from 101 Finnish children with symptoms of acute respiratory infection. Most of them were also febrile, but none had received any antimicrobial therapy before or at the time of sampling. One patient was excluded because he was over the set age limit (8 years), three samples were not available in the antigen detection phase, and the PCR was not performed for two other samples. Thus, complete evaluation was possible in 95 samples, which constitute the material of this report.

Table 13. *Pneumococcal findings in nasopharyngeal samples from 95 Finnish children by direct culture versus polymerase chain reaction (PCR) and pneumococcal capsular polysaccharide antigen detection after overnight enrichment culture in brain–heart infusion broth without gentamicin.*

Pneumococcal culture vs. antigen detection		Antigen detection <sup>a</sup>		Total
Pneumococcal culture		Positive	Negative	
Positive		27	2 <sup>b</sup>	29
Negative		15	51	66
Total		42	53	95
Pneumococcal culture vs. PCR		PCR		Total
Pneumococcal culture		Positive	Negative	
Positive		28	1 <sup>d</sup>	29
Negative		25	41	66
Total		53	42	95
Antigen detection vs. PCR		PCR		Total
Antigen detection		Positive	Negative	
Positive		41	1	42
Negative		12	41	53
Total		53	42	95

<sup>a</sup> Sensitivity = 93%, specificity = 77%, compared with culture

<sup>b</sup> One serotype 24, one nonencapsulated finding in culture

<sup>c</sup> Sensitivity = 53%, specificity = 98%, compared with PCR

<sup>d</sup> One serotype 23 finding in both culture and antigen detection

<sup>e</sup> Sensitivity = 77%, specificity = 98%, compared with PCR

### 5.2.2 Direct culture of the swabs

*S. pneumoniae* was isolated from 29 (30%) of the bacterial cultures plated directly from swabs (Table 13). In visual assessment of the growth density, seven samples (24%) yielded scanty, nine (31%) moderate, and 13 (45%) yielded abundant growth. The most frequent serotypes or groups found in culture were the paediatric types, representing 74% of the typeable strains (Table 14).

### 5.2.3 Enrichment followed by antigen detection

Antigen detection from enrichment broth without gentamicin resulted in nine more positive findings than antigen detection from enrichment culture with this antibiotic. For clarity, only results from the broth without gentamicin are presented in this section. Antigen detection from the enrichment broth yielded 42 positive findings (45% more than culture:  $P > 0.001$ ). There were two culture-positive and antigen-negative samples: one

of these grew *S. pneumoniae* that was nonencapsulated, and thus expected to remain negative for antigen detection.

Again, the most frequent serotypes or groups were the paediatric types 19, 6, 14, and 23, representing 73% of the typeable antigen findings (Table 14). In six patients (6%), two pneumococcal types could be demonstrated simultaneously, the additional findings being one each of serotypes or groups 3, 4, 7, and 23, and two of serogroup 6. Serotypes or groups from culture isolates were identical in 26 of the 27 cases (96%) that were simultaneously positive (Table 14). One sample from which serotype 24 was isolated remained nontypeable by antigen detection. Seven of the 115 antigen-positive, culture-negative findings gave positive reactions only by Omniserum or antiserum pools.

Table 14. Type/group distribution of pneumococcal isolates and antigen-positive findings in 95 nasopharyngeal samples.<sup>a</sup>

Pneumococcal serotype or group	Direct culture	Antigen detection
4	1	2
6	4	5
7	0	0
11	1	1
14	4	5
15	3	3
19	8	9
21	0	1
23	4	5
24	1	0
35	1	2
Nontypeable	2 <sup>b</sup>	9
Total	29	42
Same type/group	26	26
Different type/group	1	1
Additional, typeable	1	8
Additional, nontypeable	1	7

<sup>a</sup> In six patients, two pneumococcal types/groups were detected, the second finding being one each of serotypes or groups 3, 4, 7, and 23, and two of serogroup 6; these are not included in the figures of the table.

<sup>b</sup> Includes one nonencapsulated isolate.

### 5.2.4 Control tests

When enrichment broths were cultured and subcultured, the same 29 samples as in direct culture plus two additional ones were positive for *S. pneumoniae*. The presence or absence of gentamicin in the enrichment broth did not affect the results; in only one case was the culture from the enrichment tube with gentamicin negative when that from the tube without gentamicin was positive. – This left 13 cases in which the enrichment antigen detection results were positive without any evidence of *S. pneumoniae* by culture.

We therefore sought further evidence of *S. pneumoniae* in these samples by using the PCR for the pneumolysin gene in the enrichment cultures (Table 13).

Overall, pneumococcal DNA could be demonstrated in 53 samples by the PCR (24 positive findings more than culture;  $P < 0.001$ ). The PCR results were positive in 28 of the 29 samples in which direct culture had shown *S. pneumoniae*, and also in 41 of the 42 samples that had been positive by antigen detection after enrichment culture, including all samples with nontypeable findings. One culture-positive, antigen-positive sample (serogroup 23) remained negative in the PCR assay, probably because of inhibitory factors in the sample (408).

### ***5.2.5 Sensitivity of the methods***

If only culture-proven findings are considered true positives, antigen detection was 93% sensitive and 77% specific. However, antigen detection missed only one of the culture positive samples since the other isolate associated with an antigen-negative sample was nonencapsulated. Taking this into account, the sensitivity of the antigen detection compared with bacterial culture was 96%.

It also seems justified to calculate the test performance parameters using the PCR as baseline, even if this is not accepted as the criterion standard. Compared with the PCR, the sensitivity of antigen detection was 77% and its specificity was 98%. In the same comparison, the sensitivity of direct culture was as low as 53% and its specificity was 98%.

## **5.3 Immune complex assay for pneumolysin antibodies in ALRI patients (III)**

### ***5.3.1 Healthy controls***

Serum samples for IC-bound antibody assays were available from all 150 Filipino children; 34 samples had been obtained at a mean ( $\pm$  SD) age of 16 ( $\pm$  1.4) weeks (range 14–21 weeks), and 116 samples at 40 ( $\pm$  4.6) weeks (range 37–44 weeks, with two outliers sampled late at 57 and 83 weeks). 61% of the infants were boys. None were malnourished.

Arithmetic mean levels ( $\pm$  SD) of IC-bound Ply-IgG, Ply-IgM, CaPS-IgG and CPS-IgG antibodies were 302 ( $\pm$  168), 216 ( $\pm$  114), 162 ( $\pm$  121), and 234 ( $\pm$  236) EIA units, respectively. The resulting cut-off values for positivity (mean +2SD) are shown in Table I. Altogether 20 healthy children (13%) had one or more values exceeding the cut-off limits, the proportion varying from 3 to 7% in different assays (Table 15).

IC-bound antibody levels did not correlate with age (Pearson correlation coefficients for logarithmic values: Ply-IgG 0.012, Ply-IgM 0.039, CaPS-IgG 0.136, CPS-IgG 0.093). There were no significant differences between means of the two sex groups or of the two age groups (16 and 40 weeks) for any of the IC-bound antibodies assayed.

Pneumococcal carriage was significantly more common at 40 weeks than at 16 weeks (83 vs. 53%,  $P < 0.001$ ), but there were no differences in IC-bound antibody levels with respect to pneumococcal carrier status. Age and pneumococcal carrier status had no combined effect on antibody findings in GLM analysis. Of the 20 healthy children with values exceeding the cut-off limit, 18 (90%) were carriers of pneumococcus at the time of serum sampling, as compared with 96 (73%) of the 130 children who were negative in all IC assays ( $P = 0.12$ ).

*Table 15. Values for pneumococcus-specific immune complexes in sera from 150 healthy children*

Value	Immune complexes specific to			
	Ply-IgG <sup>a</sup>	Ply-IgM	CaPS-IgG <sup>b</sup>	CPS-IgG <sup>c</sup>
Mean (EIA units)	302	216	162	234
2 SD	336	228	242	472
Cut-off (mean + 2 SD)	600	450	400	700
Number of samples exceeding the limit (%)	11 (7)	5 (3)	6 (4)	7 (5)

<sup>a</sup> Ply = pneumolysin

<sup>b</sup> CaPS = capsular polysaccharide

<sup>c</sup> CPS = C-polysaccharide

### 5.3.2 ALRI patients

1 to 3 serum samples were available from 349 of the 350 enrolled Filipino children (all 3 samples for 190, 2 samples for 109, and only 1 sample for 50 patients; of these 334 were baseline samples, 262 in-patient and 242 convalescent-phase samples). Mean ( $\pm$  SD) age of the patients was 18 ( $\pm$  14) months (range 0–59 months), 72% were less than 2 years old. 59% were boys. Consistent with the diagnostic criteria and typical of the tertiary level referral hospital, the patients were severely ill; 51 of the 350 patients died (case fatality rate 15%). Assessed by weight for age 91%, height for age 67% and weight for height 79% of the patients were malnourished. Altogether 20 (6%) patients had a positive blood culture, including 9 positive for pneumococcus. According to parental interview, 55% of the children had received antibiotics before coming to hospital.

The findings on IC-bound antibodies in each sample were first evaluated on the basis of the cut-off level derived from the control population. Second, IC kinetics in individual patients were also evaluated to identify changes in IC-bound antibody levels during illness (changes calculated from first to second, first to third, and second to third sample). Evaluation of kinetics was possible in 299 patients, with two or three samples available.

The results were also analysed by two patient subgroups, those with only two first samples (N = 248) and those with a complete three-sample series available (N = 190).

Positive rates for IC-bound Ply-IgG in the baseline, in-patient and convalescent sample were 25%, 25% and 34%, respectively. Of the 248 patients with the first two samples available 90 (36%) exceeded the cut-off limit for Ply-IgG in at least one of the samples. For the 190 patients with a complete three-sample series the corresponding value was 97 (51%). Only 4–6% of the samples or 3–14% of the patients were positive in Ply-IgM, CaPS-IgG and CPS-IgG assays (Table 16). Age did not affect the results in any of the assays.

In 21% of the 299 patients IC-bound Ply-IgG level doubled between the first and third sample and a 2-fold decrease was observed in 15%. Overall, 2-fold or greater increases and/or decreases in Ply-IgG were observed in 100 (33%) of the 299 patients (Table 17). Most cases with 2-fold changes (76/100) also had high levels of IC-bound Ply-IgG antibodies (Table 17). Only 24 cases were positive by 2-fold increase or decrease criteria, but had low antibody levels (14% of all positives).

Only 22 (9%) of 248 patients demonstrated significant changes in Ply-IgG levels between the two first samples. By contrast, 77 (40%) of the 190 patients with a complete three sample series showed 2-fold or greater changes (Table 16).

If a 2-fold or greater change is set as an additional criterion of positivity, and the findings are combined with the number of patients exceeding the cut-off limit, then 166 (55%) of 299 patients were found positive in Ply-IgG assay (Table 17). For the subgroup with the first two samples available, the combined positivity criterion was met by 96 (39%) of 248 patients; for the patients with a complete three-sample series, 115 (60%) of 190 scored positive (Table 16). – The use of the 2-fold increase or decrease criterion did not result in significant improvement in Ply-IgM, CaPS-IgG or CPS-IgG assays (4–24% positive), and even the combination of the two criteria did not change the situation much (9–26% positive).

To reflect real-life situations, where the number of samples available per patient varies very much, we also analysed the results of all available samples, regardless of which sample or samples were missing. In this analysis, 156 (45%) of 349 patients exceeded the cut-off limit for Ply-IgG, and when this was combined with the 100 patients with 2-fold or greater changes, 180 (52%) of 349 scored positive for Ply-IgG.

There were nine patients whose blood culture grew *S. pneumoniae*. Three of them had only one serum sample available, and one of these was Ply-IgG positive by the cut-off limit criterion. Five of the remaining six patients (83%) were positive in Ply-IgG assay by cut-off limit (N = 3) and/or 2-fold changes (N = 4) criteria.

### 5.3.3 Method reproducibility

To test reproducibility of the test, 10% of the samples were prepared and tested twice on separate days. The correlation of the two results was very good (correlation coefficients for Ply-IgG, CaPS-IgG, CPS-IgG were 0.810, 0.736 and 0.769, respectively; Ply-IgM assays not repeated).

Table 16. *Pneumococcus-specific immune complexes in 838 sera from 349 children with ALRI. Three samples were obtained: on admission (N = 334), during hospital stay (N = 262) and in convalescent phase (N = 242).*

Classification of findings	Samples analysed No. of patients	Immune complex type			
		Ply-IgG <sup>a</sup>	Ply-IgM	CaPS-IgG <sup>b</sup>	CPS-IgG <sup>c</sup>
Mean ( $\pm$ SD), EIA units	All available N = 349	544 ( $\pm$ 418)	239 ( $\pm$ 127)	214 ( $\pm$ 327)	256 ( $\pm$ 323)
Patients exceeding the cut-off limit (%)	First sample N = 334	85 (25)	11 (3)	16 (5)	10 (3)
	Two first N = 248	90 (36)	18 (7)	22 (9)	16 (6)
	All three N = 190	97 (51)	22 (12)	26 (14)	19 (10)
Patients with $\geq$ 2-fold changes (%)	Two first N = 248	22 (9)	11 (4)	15 (6)	19 (8)
	All three N = 190	77 (40)	28 (14)	29 (15)	46 (24)
	Exceeding the limit or with $\geq$ 2-fold changes (%)	Two first N = 248	96 (39)	24 (9)	25 (10)
	All three N = 190	115 (60)	38 (20)	29 (19)	50 (26)

<sup>a</sup> Ply = pneumolysin

<sup>b</sup> CaPS = capsular polysaccharide

<sup>c</sup> CPS = C-polysaccharide

Table 17. *Pneumolysin-IgG-specific immune complex findings in ALRI patients according to cut-off limit and 2-fold change criteria (based on the 299 patients with two or three samples available).*

$\geq$ 2-fold change criterion <sup>a</sup>	Cut-off limit criterion (%)		
	Positive	Negative	Total
Positive	76 (25)	24 (8)	100 (33)
Negative	66 (22)	133 (45)	199 (67)
Total	142 (47)	157 (53)	299 (100)

<sup>a</sup> 2-fold or greater increase or decrease from first to second, first to third, or second to third sample.

## 5.4 Free antibodies and immune complexes in healthy Filipino children

In an unpublished study, we outlined the natural history of immune complexes *in vivo* and studied the development of pneumococcus-specific immune complexes in Filipino children during the first year of life, comparing the findings with free antibody levels.

Sera were obtained from 235 infants enrolled in a Hib vaccine immunogenicity trial conducted from November 1992 to November 1994 in Cabuyao, Laguna, The Philippines (409). In addition to regular EPI vaccines (BCG, DTP, OPV and measles), the subjects received Hib and hepatitis B vaccines. Serum samples for antibody determinations were obtained either after the primary vaccination series at 7, 12, 16, 20, 40 and 44 weeks of age. At the time of serum sampling, upper respiratory tract carriage of *S. pneumoniae* was studied by nasal and oropharyngeal swabbing using conventional culture methods (307).

We precipitated the immune complexes from six serial serum samples and determined pneumolysin, capsular polysaccharide and C-polysaccharide antibodies bound in these complexes using the methods described above in Chapters 4.5.1 and 4.5.2. For comparison, free antibodies against pneumolysin and C-polysaccharide were also determined. – Samples for determination of both immune complex bound and free antibodies were available from 131 to 235 subjects, depending on the time point of sampling (Table 18). Altogether 1,183 samples were analysed; 62% of the samples were from boys. – The frequency of pneumococcal carriage increased from 38% at 7 weeks to 85% at 44 weeks of age (Table 18).

### 5.4.1 Immune complexes

All immune complex types showed a general increasing trend from 7 to 44 weeks of age (Fig. 3). Correlation analysis showed that the increase does correlate with age ( $P < 0.0001$  for all three types), but this correlation is weak (Pearson correlation coefficients: Ply-IgG 0.326, CaPS-IgG 0.130, CPS-IgG 0.166). High levels of immune complex bound antibodies were detected in only about 1 % of the samples. No sex difference was detected. – In nasopharyngeal carriers of pneumococci, high immune complex levels were detected very infrequently. Findings were similar for all types of immune complexes determined, and there was no trend for persisting immune complexes in carriers. However, higher IC-bound pneumolysin levels at 16 and 40 weeks were associated with higher carriage rates at preceding sampling points, but not at the current sampling point. No association could be shown between carriage of *H. influenzae* type b and levels of pneumococcus-specific immune complexes.

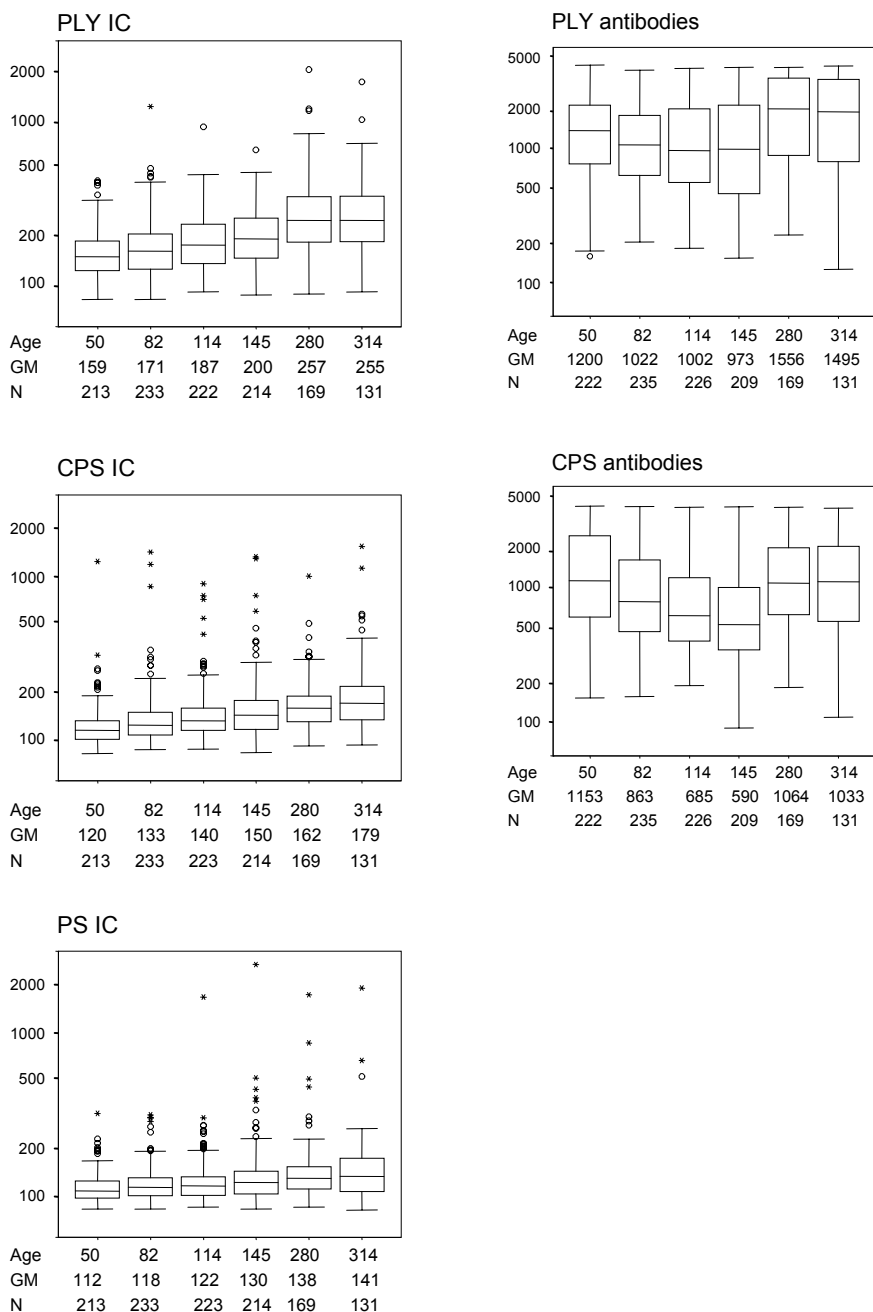
*Table 18. Nasopharyngeal carriage of pneumococci and immune complex (IC) findings in 235 healthy Filipino children.*

Age (weeks)	7	12	16	20	40	44
N	213	233	222	214	169	131
Pneumococcal carriage positive	38%	53%	62%	72%	80%	85%
Ply-IC <sup>a</sup> > 600	0	0	0	1	5	6
CPS-IC <sup>b</sup> > 700	1	2	2	3	1	2
CaPS-IC <sup>c</sup> > 400	0	0	1	4	4	3

<sup>a</sup> Ply = pneumolysin; <sup>b</sup> CPS = C-polysaccharide; <sup>c</sup> CaPS = capsular polysaccharide

### **5.4.2 Free antibodies**

Pneumolysin and C-polysaccharide antibody levels showed a declining trend from 7 to 20 weeks, mainly reflecting dynamics of maternally derived antibodies. The levels had risen to the initial starting level or above that between 20 and 40 weeks of age (Fig. 3). – Correlation of IC-bound and free antibodies was poor.



**Fig. 3. Immune complex bound anti-pneumococcal antibody levels and free antibody levels in Filipino children during the first year of life.**

## 5.5 Enzyme immunoassay for capsular polysaccharide antigens (IV)

### 5.5.1 Sensitivity and specificity for purified antigens

Sensitivity of the assay with purified polysaccharide antigens varied from 5 to 100 ng/ml when the value of 85% or less of the maximum optical density (OD) was considered as positive result (Table 19), and from 5 to 500 ng/ml, if the value of 60% or less of maximum OD was considered as the true positive result. The strongest inhibition was attained by 1 h incubation in +37 °C, after which the tubes were left overnight to +4 °C before transfer into the wells of antigen-coated EIA plates. Table 19 also shows that only minor inhibition, 0 to 13%, was seen with purified CPS preparation.

Cross-reactivity between the types included in this study was only seen between types 19F and 23F. When absorption with 23F bacterial suspension was completed, type 19F antiserum was specific only for type 19F.

*Table 19. Sensitivity of the assay with purified polysaccharide antigens. 85% or less of the maximum optical density was considered as positive result.*

Serotype	Sensitivity (ng/ml)	Inhibition (%) by CPS 500 ng/ml
4	5	9
6B	50	1
9V	100	13
14	50	2
18	50	0
19F	5	0
23F	50	18

### 5.5.2 Nasopharyngeal samples

Table 20 shows the type-specific EIA findings compared with culture of the 100 nasopharyngeal samples (processed through enrichment culture, NPSe). The overall sensitivity of the assay for NPSe samples was 99% compared with culture. EIA missed only one culture-positive sample in which types 18C and 6B were found by culture, but EIA detected only type 6B. Specificity of the assay was 93%, because 70% of types 6A culture-positive samples were 6B positive by EIA. If both 6A and 6B findings are considered as specific results, the actual sensitivity would be 95% and specificity 100%.

Cut-off point was set at 60% or less of the maximum OD to avoid any cross-reactivity. This was an easy decision, because inhibition of the positive samples in EIA was so strong that with most of the samples the OD levels were nearly at the same level than background.

### 5.5.3 Middle ear fluid samples

Determination of the cut-off point turned out to be quite difficult when MEF antigen negative samples were studied. In the end, an empirical approach seemed to be only reasonable way. Cut-off point was set to 85% or less of the maximum OD level; the level when no antigen is added to the control reaction. For 550 middle ear fluid (MEF) samples only 74% sensitivity was achieved compared with the culture, partly explained by the necessary great dilution of the samples (Table 21).

Table 20. Antigen detection results in nasopharyngeal samples processed through enrichment culture.

Counterimmuno-electrophoresis type (N = 100)	Type identified in the enzyme immunoassay							
	4	6B	9V	14	18C	19F	23F	Negative
4	7	0	0	0	0	0	0	0
6A	0	7	0	0	0	0	0	3
6B	0	10	0	0	0	0	0	0
14	0	0	0	10	0	0	0	0
9N	0	0	0	0	0	0	0	7
9V	0	0	9	0	0	0	0	0
18C	0	1 <sup>a</sup>	0	0	7	0	0	0
19F	0	0	0	0	0	10	0	0
23F	0	0	0	0	0	0	10	0
Negative	0	0	0	0	0	0	0	19

<sup>a</sup> sample was positive in CIEP both 18C and 6B, but EIA identified only type 6B as positive

Table 21. Antigen detection results in diluted middle ear fluid samples.

Type identified in counterimmuno-electrophoresis (N = 550)	Type identified in the enzyme immunoassay							
	4	6B	9V	14	18C	19F	23F	Negative
4	0	0	0	0	0	0	0	0
6A	0	13 <sup>a</sup>	0	0	0	0	0	3
6B	0	3	0	0	0	0	0	4
14	0	0	0	5	0	0	0	0
9V	0	0	0	0	0	0	0	0
18C	0	0	0	0	0	0	0	0
19F	0	0	0	0	0	18	0	2
23F	0	0	0	0	0	1	28	13
Other	1	0	0	0	0	1	1	27
Negative	2	2 <sup>b</sup>	2	0	3	6	0 <sup>c</sup>	419

<sup>a</sup> in one sample also types 19F and 18C were found

<sup>b</sup> one 6B, one 6B + 4

<sup>c</sup> 5 apparently non-specific positive results

## 6 Discussion

The purpose of this study was to develop laboratory diagnostic methods with which pneumococcal surface antigens or pneumococcus-specific antibodies can be demonstrated in clinical samples with sufficient sensitivity and specificity. The efforts were motivated both by clinical diagnostic needs and needs for epidemiological study tools, in particular for clinical vaccine trials. We built the study plan on the extensive research experience on the pneumococcus in our study group. Over the years, we had become convinced that antigen detection methods are useful in pneumococcal laboratory diagnosis. The puzzling research question was, why were the results of antigen detection and bacterial culture never in sufficient agreement for the clinician to be able to rely on one or the other. We wanted not only to rework old methods, but also wanted to look for innovative new solutions to the diagnostic problems of pneumococcus.

In Study I we investigated the presence and type distribution of pneumococci in the upper respiratory tract of 318 Filipino children with ALRI, comparing immunological antigen detection methods to bacterial culture. The results demonstrate that antigen detection may be more sensitive than culture in detecting pneumococci in URT, particularly in children with prior antimicrobial therapy. – We did not type the strains isolated in culture, but it has been demonstrated earlier in our laboratory by using same methodology as in the present study that the typing findings obtained by antigen detection match very well those obtained from typing of cultured strains (410).

The data show that pneumococcal capsular polysaccharides are present in amounts detectable by routine immunological methods in 83% of nasopharyngeal aspirates from which pneumococci can be cultured corresponding to amounts of  $\geq 10^5$  CFU/ml. When the antigen was found, it could be identified with type/group-specific antisera in 78% of the cases. The results obtained by COA and CIE had a moderate level of agreement, but CIE is clearly less useful in the detection of neutral CaPS of types 7 and 14.

40% of the NPA samples from which no pneumococci grew in culture contained pneumococcal antigen. In about 40% of these samples Gram-positive diplococci were also found as the predominant bacteria in Gram-stained smears. Several plausible reasons exist for the antigen-positive, culture-negative findings. Although culture was performed promptly after obtaining the samples, the viability of the bacteria may in some cases have been lost before culturing. Various host defence mechanisms (e.g. antibody, complement,

phagocytic activity) operating during infection may also have killed the bacteria already before the samples were taken. Use of antimicrobials prior to the sampling obviously impairs the culture results (265). Culture isolation rate for pneumococcus was significantly lower if the patient had used antibiotics during the preceding 48 hours. In all these cases, it is expected that bacterial antigens can remain detectable after the bacteria are no more viable. Indeed, our earlier studies have shown that 33% of culture-negative middle ear exudates also contain pneumococcal antigen (401).

Could the antigen-positive but culture-negative results be false positive findings? Polysaccharides cross-reactive with certain pneumococcal types do occur e.g. in enteric bacteria (261). *Escherichia coli* or Klebsiella spp. were actually isolated from 20% of the antigen-positive, culture-negative samples. However, it is unlikely that these would be the rare cross-reactive *E. coli* or Klebsiella capsular types among about 200 possible types. Cross-reactions are possible also with  $\alpha$ -haemolytic streptococci (259, 411), but recent evidence suggests that this is important in saliva and not in nasopharyngeal samples (20). In the present study, other respiratory pathogens and non-pathogens were isolated with comparable frequency in all four subgroups, with *H. influenzae* as the most frequent pathogen (30–40% of the samples).

The value of pneumococcal antigen finding in NPA for aetiological diagnosis has been under much debate. Earlier, it was considered to correlate well with the clinical diagnosis of bacterial pneumonia (412, 413). More recent data, however, demonstrates that pneumococcal finding in the upper airways does not indicate pneumococcal infection in the lower airways (410). On the other hand, if pneumococcus is not present in NPA, pneumococcal aetiology is unlikely (410) and if pneumococcus is cultured from blood in patients with invasive disease, the same type is present in the URT (31).

Carriage of pneumococci is very common in children (235, 270), especially in developing countries, where the carriage rate in healthy children can be close to 100% (11, 23-25, 211, 226, 228-230). On the other hand, there are reports suggesting that the amount of antigen in sputum samples due to carriage is too low for immunochemical detection (20, 414). Our results indicate that at least in nasopharyngeal aspirates from Filipino children, the bacteria are present in sufficiently high numbers to allow antigen detection by the methods used.

In Study II we sought to further improve the sensitivity of antigen detection methods, because earlier studies comparing culture and antigen detection gave inconclusive results. The goal was achieved by adding a simple enrichment step before the antigen detection procedure. In contrast with earlier reports (12, 20-22), the results of culture and antigen detection were in excellent agreement for culture positive samples, but antigen detection also showed *S. pneumoniae* in 23% of the culture negative samples. Pneumococcal DNA could be detected by polymerase chain reaction in all 15 antigen-positive, culture-negative samples. Compared with conventional culture, the sensitivity of antigen detection was high.

Based on these findings, we recommend a simple application of antigen detection after the enrichment step for field epidemiology purposes. The samples can be collected directly into enrichment broth tubes, which can be incubated at 37 °C or, especially in the tropics, at ambient temperature. Because continued viability of bacteria is not necessary after initial growth, and because polysaccharide antigens are known to be very stable, immediate testing or preservation by freezing is not necessary.

In Study III we evaluated the usefulness of pneumococcus-specific immune complex determination in diagnosis of pneumococcal pneumonia in Filipino children with ALRI. The results were compared with those from healthy children from the same geographical area. Pneumolysin-specific immune complexes were found frequently in ALRI patients. The results indicate a far higher proportion of pneumococcal aetiology than obtained by blood cultures, comparable with the highest positivity rates of up to 50–60% seen with lung aspirates in studies of severe ALRI in developing countries (7, 9), and in Finland (10).

Previously, the determination of antibodies to pneumococcal capsular polysaccharides dissociated from IC has been identified as one possible method in pneumococcal diagnostics (43-46). Our earlier studies in Finnish adults with pneumonia have indicated that the determination of pneumolysin antibodies from IC could be an even more valuable diagnostic tool, as 100% of blood culture positive patients had IC-bound Ply-IgG, while none of the healthy controls tested positive (47). In healthy Finnish children, IC-bound Ply-IgG has been found in less than 1% (50).

In the present study, we focused on children in a developing country setting, where pneumonia in under 5-year-olds is a significant public health problem. In four different IC assays, 3–7% of healthy Filipino children had levels exceeding the cut-off limit for positivity. These could naturally be viewed as false positive findings, but this proportion is also a direct consequence of the way in which the cut-off limit was set (mean +2 SD). Nevertheless, they may not be false positives in a biological sense but may rather reflect previous contact with pneumococcus, either as a coloniser or as a cause of a previous infection.

By the age of 40 weeks, more than 80% of all healthy children were oropharyngeal carriers of pneumococcus, but there were no differences in IC-bound antibody findings with respect to pneumococcal carrier status. Thus, pneumococcal carriage seems an unlikely cause for positivity. – In principle, antigens of the colonising bacteria could enter blood circulation through the mucous membranes, but according to the present data this is not the case with the protein antigen pneumolysin. One reason for this could be that it binds readily to cholesterol in cell membranes (51).

We consider previous pneumococcal infection a more plausible explanation for the positive findings in the healthy children. This is also supported by our previous studies showing that in most patients pneumococcus-specific IC remain detectable for at least 40 days after pneumococcal pneumonia (47). Respiratory infections are common in the Filipino setting, but we do not have medical history of our control group that is accurate enough to allow for conclusions on previous pneumococcal disease.

In ALRI patients, the IC-bound Ply-IgG antibody assay provided significantly more positive findings than the other assays. The mean Ply-IgG values in ALRI patients differed markedly from those in the healthy controls, and 51% of the patients with a complete three-sample series had values exceeding the cut-off limit in one or more serum samples. Although 72% of the patients were less than 2 years old, the age groups of healthy children and patients did not match perfectly. Considering the possible bias that might be induced by this difference, it was reassuring that we found no association between age and IC-bound antibody levels or positive findings within either study group.

IC kinetics in ALRI patients did not follow a rigid pattern, and both increases and decreases in IC-bound antibody levels were detected over time. These findings are,

however, well explained by natural differences in antigen load (level of antigenemia), the level of pre-existing antibodies, and the difficulty of ascertaining the exact duration of the illness. Thus, the detected changes seem to reflect the natural dynamic antigen–antibody interaction *in vivo*. The role of maternal antibodies could be assessed in a separate study (see discussion below).

We also measured IC-bound antibodies to 14 pneumococcal capsular polysaccharides and C-polysaccharide, but these were detected in only a very small percentage of the patients. However, in the age group studied, antibody responses to many polysaccharide antigens, especially to the paediatric pneumococcal types, are known to be poor (385, 415), which makes the formation of immune complexes less likely.

An obvious limitation of the pneumolysin IC assay is its inability to provide information of the pneumococcal serotypes or groups, which is essential in epidemiological studies pertaining to the development of pneumococcal vaccines. For these purposes, the type-specific diagnosis still needs to be obtained by other methods. However, for diagnostic purposes in clinical practice, species-specific diagnosis is sufficient.

Diagnostic methods that require three serum samples are not very useful in clinical practice, especially in developing countries. In this study, 25% of the samples were positive in the first sample by cut-off criterion. If also the second (in-patient) sample was taken into account, 36% of the patients exceeded the Ply-IgG cut-off limit, but only 9% showed 2-fold or greater changes. With both criteria combined, altogether 39% of the patients scored positive. Based on our results, 3 days appears a reasonable interval for repeat samples.

In unpublished studies, we also investigated the natural development of pneumolysin-specific immune complexes and antibodies in healthy Filipino children during the first year of life. The dynamics of free PLY and CPS antibodies reflected the decline of maternal antibodies. All IC bound antibodies showed an increasing trend, but high levels of IC bound antibodies were detected in very few samples. Levels of free and IC bound antibodies did not correlate with nasopharyngeal carriage of pneumococcus. The findings in healthy children are essentially different from patients with confirmed or suspected pneumococcal disease obtained in Study III. Taken together, the findings and earlier clinical studies indicate that the method can be used in diagnosis of pneumococcal infections.

In Study IV, we developed a sensitive EIA method for the demonstration of pneumococcal capsular polysaccharides from nasopharyngeal samples (NPS). Types 4, 6B, 9V, 14, 18C, 19F and 23F were selected for this study, because they are the types included in the first heptavalent pneumococcal conjugate vaccine, which was used in the Finnish Otitis Media Vaccine Trial.

The antigen detection results for NPS were consistent with bacterial culture results, except that both serotypes 6A and 6B were detected by EIA method for 6B, which can be easily explained by their closely related antigenic formulas. Interestingly, in vaccine trials, the 6B conjugate vaccine has also been shown to afford cross-protection from 6A infection (20) and, thus, from the practical point of view, the detection of both 6A and 6B polysaccharides with one EIA test can actually be beneficial in clinical efficacy trials. If type-specific finding is needed, the types can be differentiated with capsular quellung test

directly from enrichment cultures. MEF samples were too diluted for the EIA method used, and only 74% sensitivity compared with culture was achieved.

The enrichment culture step is essential for demonstration of capsular polysaccharide in NPS samples. When several antigens are searched for, the sample volume becomes a critical factor in the diagnosis. By enrichment culture this problem can be avoided and, furthermore, no primary plate cultures are needed – only overnight incubation at +37 °C – a major advantage in field trials. Incubated broth cultures can be stored at –20 °C and transferred as batches to the laboratory.

## 7 Summary and conclusions

Understanding the public health importance on *Streptococcus pneumoniae* has prompted active microbiological and epidemiological research work internationally during the last decade, not least due to the promising prospects of pneumococcal conjugate vaccines. The work has been complicated by the fact that for both clinical and epidemiological purposes, the criterion standard for pneumococcal diagnostics is still missing. Lung aspirates produce a high microbiological yield but the perceived risk of complications of this invasive procedure restrains its wider clinical use. Blood culture, although specific, is not useful, because its sensitivity in children remains very low. In many countries, surreptitious use of antimicrobial agents is also quite common, reducing the reliability of culture methods. Therefore, we felt compelled to focus on new non-culture methods for pneumococcal laboratory diagnosis.

The results of Study I indicate that antigen detection is more sensitive than culture in demonstrating pneumococci in URT, particularly in children with prior antimicrobial therapy. It is a feasible method for studies on pneumococci in developing countries.

In an effort to further improve the sensitivity of antigen detection, we introduced in Study II an enrichment phase before antigen detection and compared the results with direct bacterial culture, using nasopharyngeal swabs from children with symptoms of acute respiratory infection. For type-specific demonstration of *S. pneumoniae*, detection of pneumococcal antigen after an enrichment step was shown to be a sensitive method that can be applied for epidemiologic study purposes, e.g., in vaccine trials, in areas without ready access to a good microbiology laboratory.

Determination of IC-bound pneumolysin IgG antibodies appears to be a useful method for species-specific diagnosis of pneumococcal infections. The results indicating pneumococcal aetiology in ALRI patients in Study III compare well with the best results obtained by the use of lung aspirates. Increasing the number of serial samples improves the sensitivity of the assay, but even two samples provide more positive findings than other methods currently used. Criteria of positivity need to be confirmed in subsequent larger studies of both healthy controls and patients with confirmed pneumococcal disease. It is also important to control the findings in patients with pneumonia of non-pneumococcal origin. Elaborate study designs are needed, because mixed infections are common.

The negative IC findings from healthy children provide supportive evidence for Study III and other earlier paediatric studies, which showed positive results in children with acute lower respiratory infection. In adults with pneumonia, specificity problems have been encountered, and further validation attempts are required.

Finally, a novel competitive enzyme immunoassay was developed in Study IV. The method is sensitive, and will be a useful complement to the laboratory methods used to diagnose pneumococcal disease.

Thus, with the completion of the study project three new methods are available for the laboratory diagnosis of the pneumococcus. This achievement needs to be put into perspective with more than twenty alternative methods that are already available. We did not aim to develop just another method, but were driven by practical needs of our fellow field researchers. Thus the most important goals were improved sensitivity, specificity, combined with simplicity. The simple enrichment step improved significantly the usefulness of antigen detection methods. Detection of specific antibodies bound in immune complexes is a promising new study line that should promote several further studies. The novel competitive enzyme immunoassay is sensitive, and particularly well suited for processing of large number of samples. All methods can be used to obtain more detailed etiological and epidemiological information on pneumococcal disease.

The selection of the most appropriate methods for pneumococcal laboratory diagnosis still remains difficult. The decision must be based on practical considerations, availability of laboratory services and microbiological expertise. The setting is definitely important: clinical services should prioritise rapid methods, while epidemiological research can resort to more elaborate and time-consuming methods. Sensitivity and specificity must not be compromised, and if they are, it is advisable to use several methods to complement each other.

With the existence of a broad spectrum of microbiological and immunological methods, it is imperative to seek international consensus for standard methods to demonstrate pneumococcus. Otherwise it is very difficult to compare results from different clinical studies. The recently published WHO Working Group proposal for a standard method for detecting upper respiratory carriage of pneumococcus is an encouraging development in the right direction, but a lot of work still remains to be done in other areas of research on pneumococcal infections.

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