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Relationship Between
Nasopharyngeal Carriage
and Acute Otitis Media Due to
Streptococcus Pneumoniae
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To the versatility of life

ABSTRACT

Streptococcus pneumoniae causes a wide variety of diseases, ranging from relatively mild but very common mucosal diseases like otitis media and pneumonia to rarer but severe invasive diseases like bacteraemia and meningitis. These diseases cause a considerable burden worldwide. Nasopharyngeal carriage is a prerequisite for pneumococcal otitis media and viral respiratory infection contributes to its pathogenesis. Nasopharyngeal carriage also comprises the reservoir of pneumococcal strains for their spread in the population. Pneumococcal carriage is most prevalent in children. In planning effective long-term prevention strategies against pneumococcal diseases it is essential to have knowledge on the natural cause of pneumococcal carriage and on how the carriage progresses to disease.

This study describes the natural course of pneumococcal nasopharyngeal carriage in a prospective follow-up of Finnish children from 2 to 24 months of age before the start of pneumococcal vaccinations. Special attention was focused on the dynamics of pneumococcal carriage during health and respiratory infection and development of pneumococcal acute otitis media (AOM).

Almost all children carried pneumococci at least once during the first two year of life and in average the prevalence of carriage was 27%. The frequency of pneumococcal carriage clearly increased with age and with respiratory infection. There was no significant association between pneumococcal carriage and sex or season, but antimicrobial treatment temporarily decreased carriage frequency. During pneumococcal AOM, *S. pneumoniae* and especially the serotype found in the middle ear fluid was practically always found in the nasopharynx, but only half of the serotypes carried during clinical AOM were found to cause the disease. The hazard of carriage acquisition was about three times higher in association with the onset of a new respiratory infection as compared to health. The hazard of pneumococcal AOM media was significantly higher during carriage acquired around the onset of respiratory infection as compared to carriage acquired more than one month before or more than one week after the onset of respiratory infection. Of all pneumococcal AOM events, almost 80% were caused by a

serotype acquired within one month before the sickness onset. Preceding episodes of homologous carriage episodes decreased the hazard of pneumococcal AOM. The pneumococcal AOM hazard during concurrent pneumococcal carriage and respiratory infection did not depend on age.

The natural course of pneumococcal carriage in Finnish children is similar as observed in other industrialised countries. Viral respiratory infection is an important factor for acquisition of carriage and its progression to AOM. Age, respiratory infection and antimicrobial treatment should be considered when the results of carriage studies are interpreted. Nasopharyngeal carriage during AOM can be used as information in monitoring the serotypes causing AOM, and the use of a negative nasopharyngeal finding could be considered in excluding pneumococcal AOM in clinical praxis. Carriage studies are important tools in planning and evaluating methods for preventing pneumococcal AOM.

TIIVISTELMÄ

Pneumokokkibakteeri aiheuttaa laajan kirjon erilaisia sairauksia, alkaen suhteellisen lievistä mutta erittäin yleisistä limakalvojen sairauksista kuten välikorvatulehdus ja pinnallinen keuhkokuume harvinaisempiin mutta vakaviin sairauksiin kuten verenmyrkytys ja aivokalvontulehdus. Näiden sairauksien aiheuttama tautitaakka on maailmanlaajuisesti merkittävä. Nenänielukantajuus on edellytys pneumokokin aiheuttaman välikorvatulehduksen synnylle ja viruksen aiheuttama hengitystietulehdus myötävaikuttaa taudin kehittymiseen. Nenänielukantajuus toimii myös pneumokokkikantojen varastona, jota kautta pneumokokit leviävät väestössä. Pnemokokkikantajuus on yleisintä lapsilla. Tieto pneumokokkikantajuuden luontaisesta kulusta ja siitä kuinka kantajuus etenee taudiksi on välttämätöntä kun pneumokokkitaudeille suunnitellaan pitkällä tähtäimellä tehokkaita ennaltaehkäisymenetelmiä.

Tämä tutkimus kuvaa pneumokokkikantajuuden luontaista kulkua suomalaisilla, lapsilla, joita seurattiin 2 kuukauden iästä 2 vuoden ikään asti ennen pneumokokkirokotusten aloittamista. Erityistä huomiota kiinnitettiin kantajuuden eroihin terveenä ollessa ja hengitystietulehduksen aikana sekä siihen, miten äkillinen pneumokokin aiheuttama välikorvatulehdus kehittyy.

Melkein kaikki lapset kantoivat pneumokokkia vähintään kerran ensimmäisten kahden ikävuoden aikana ja keskimääräinen kantajuusprevalenssi oli 27 %. Pneumokokkikantajuus lisääntyi selvästi iän myötä ja hengitystietulehduksen aikana. Pneumokokkikantajuus ei liittynyt merkittävästi sukupuoleen eikä vuodenaikaan, mutta antibioottihoito vähensi kantajuutta lyhytaikaisesti. Pneumokokin aiheuttaman äkillisen välikorvatulehduksen aikana pneumokokki, ja erityisesti juuri välikorvaeritteestä löytynyt serotyyppi löytyi käytännöllisesti katsoen aina nenänielusta, mutta vain puolet välikorvatulehduksen aikana kannetuista serotyypeistä todettiin kyseisen korvatulehduksen aiheuttajiksi. Tuoreen hengitystietulehduksen alkuvaiheessa lapsilla oli noin kolme kertaa suurempi todennäköisyys saada kantajuus kuin terveenä. Äkillisen välikorvatulehduksen kehittymisen todennäköisyys oli merkittävästi suurempi silloin, kun kantajuus oli

alkanut hengitystietulehduksen alkuvaiheen yhteydessä kuin silloin, jos kantajuus oli alkanut yli kuukausi ennen hengitystietulehduksen alkua tai yli viikko hengitystietulehduksen alun jälkeen. Kaikista äkillisistä välikorvatulehduksista lähes 80 % oli sellaisen serotyypin aiheuttamia, joka oli saatu kuukauden sisään ennen taudin alkua. Aikaisemmat saman serotyypin kantajuusjaksot vähensivät saman pneumokokkiserotyypin aiheuttaman äkillisen välikorvatulehduksen todennäköisyyttä. Välikorvatulehduksen todennäköisyys ei riippunut lapsen iästä, jos pneumokokkikantajuus ja hengitystietulehdus osuivat päällekkäin.

Pneumokokkikantajuuden luonnollinen kulku oli suomalaisilla lapsilla samanlaista kuin muissakin teollisuusmaissa. Viruksen aiheuttama hengitystietulehdus on merkittävä tekijä kantajuuden alkamisessa ja sen etenemisessä äkilliseksi välikorvatulehdukseksi. Ikä, hengitystietulehdus ja antibioottihoito pitää ottaa huomioon kantajuustutkimusten tulosten tulkinnassa. Äkillisen välikorvatulehduksen aikaista nenänielukantajuutta voitaisiin käyttää hyväksi välikorvatulehdusta aiheuttavien pneumokokkiserotyyppien seurannassa ja negatiivisen nielunlöydöksen käyttöä pneumokokin aiheuttaman korvatulehduksen poissulkumenetelmänä voitaisiin harkita. Kantajuustutkimukset ovat tärkeitä välineitä kun suunnitellaan ja arvioidaan pneumokokin aiheuttaman äkillisen välikorvatulehduksen ennaltaehkäisyä.

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals I–IV. In addition, unpublished data and data presented in congress abstracts are presented.

I.   Ritva K. Syrjänen, Terhi M. Kilpi, Tarja H. Kaijalainen, Elja E. Herva, Aino K. Takala. Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Finnish Children Younger Than 2 Years Old. J Infect Dis 2001;184:451-459.

II.   Ritva K. Syrjänen, Elja E. Herva, P. Helena Mäkelä, Heikki J. Puhakka, Kari J. Auranen, Aino K. Takala, Terhi M. Kilpi. The Value of Nasopharyngeal Culture in Predicting the Etiology of Acute Otitis Media in Children Less Than Two Years of Age. Pediatr Infect Dis J 2006; 25:1032-6.

III.   Ritva K. Syrjänen, Kari J. Auranen, Tuija M. Leino, Terhi M. Kilpi, P. Helena Mäkelä. Pneumococcal Acute Otitis Media in Relation to Pneumococcal Nasopharyngeal Carriage. Pediatr Infect Dis J 2005; 24:801-6.

IV.   Kari Auranen, Ritva Syrjänen, Tuija Leino, Terhi Kilpi. Dynamics of pneumococcal carriage, acute respiratory infection and pneumococcal otitis media. Submitted.

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ABBREVIATIONS

AOM	acute otitis media
CI	confidence interval
PI	Bayesian posterior probability (credible) interval
FinOM	Finnish Otitis Media
Hi AOM	acute otitis media caused by *Haemophilus influenzae*
HR	hazard ratio
KTL	National Public Health Institute
MEF	middle ear fluid
MLST	multi locus sequence typing
NPA	nasopharyngeal aspirate
NPS	nasopharyngeal swab
NPV	negative predictive value
OME	otitis media with effusion
OR	odds ratio
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
Pnc AOM	pneumococcal acute otitis media
Pnc carriage	pneumococcal carriage
PPV	positive predictive value
PsaA	pneumococcal surface adhesin A
THL	National Institute for Health and Welfare
WHO	World Health Organisation

1   INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a bacterium recognised as an important pathogen for more than 100 years. Infections caused by pneumococci are still important causes of morbidity and mortality. Our knowledge about pneumococcal infections has increased rapidly during recent decades and there is now a comprehensive body of knowledge on the epidemiology of pneumococcal infections from different parts of the world. Complex interactions between pneumococci, their host organs, and other microbes in their ecological niche—that is the human nasopharynx—have been detected with current methods of molecular characterisation.

Practically all children harbour pneumococci in their nasopharynx at least temporarily during the first years of life. Pneumococcal carriage is typically asymptomatic and does not cause any harm. At the same time pneumococci cause a wide range of diseases, mostly in association with or after a respiratory infection. The mildest of pneumococcal diseases develop when the bacteria spread from the nasopharynx to the adjacent organs and cause otitis media or sinusitis restricted to the mucosa. Pneumonia may follow if bacteria are inhaled or aspirated to the lungs. If pneumococci invade the blood vessels through the mucosa, they cause bacteraemia and can spread further to other normally sterile organs. Such invasive disease forms are serious and can be life-threatening without effective treatment. If the bacteria spread through the blood-brain-barrier and cause meningitis, the risks of fatality or permanent disability are especially high.

Pneumococcal diseases are common in all parts of the world. The annual global burden of pneumococcal pneumonia and invasive diseases has been estimated more than 10 million cases and almost one million deaths in children aged less than five years. Young children and the elderly are at the highest risk. Otitis media is very common in children and it therefore consumes extensive financial and health care resources and affects the quality of life of a large population. Otitis media is also the most common cause of antimicrobial treatment in children. Antimicrobial use is associated with a new threat, that of increasing antimicrobial resistance of pneumococci, which may jeopardise the availability of treatments for pneumococcal diseases. The development of effective vaccines for the prevention

of pneumococcal infections offers one solution to this problem. The pneumococcus has developed more than 90 different types of surrounding capsule, i.e. serotypes, which have different disease-causing potentials and induce different immune responses in the human host. The current pneumococcal conjugate vaccines include 7 to 13 different serotypes and are effective in preventing pneumococcal infections caused by the vaccine serotypes. By reducing carriage of the vaccine serotypes, however, vaccination increases infections caused by other serotypes due to the replacement phenomenon.

The main reservoir of pneumococcal strains in the population is asymptomatic nasopharyngeal carriage, which is most prevalent in children. Vaccinating children not only protects them against pneumococcal diseases but also affects pneumococcal transmission in the population through indirect effects, both through herd immunity and replacement of carriage and disease in unvaccinated persons. In addition, the vaccine affects differently the carriage acquisition, carriage prevalence, pneumococcal otitis media and invasive pneumococcal disease. Thus, it is important to gain new knowledge about the elements associated with transmission of pneumococci, acquisition and natural course of pneumococcal carriage, and the progression of carriage to disease. This knowledge is essential for understanding the components of the long-term effectiveness of pneumococcal vaccination, for planning successful vaccination programmes, for specifying the optimal timing of vaccination, and for preventing other factors that enhance disease development.

This thesis is based on data from the Finnish otitis media (FinOM) Cohort Study, conducted in 1994–1997. In the FinOM Cohort Study, children not vaccinated with pneumococcal vaccines were prospectively followed so as to collect data on pneumococcal carriage, respiratory infections and otitis media. The epidemiological study preceded a trial on the efficacy of two 7-valent pneumococcal conjugate vaccines in preventing pneumococcal otitis media and it was particularly designed to collect data on pneumococcal otitis media. Therefore, the study offered a useful opportunity to study the process of pneumococcal carriage and the dynamics of carriage, respiratory infection and otitis media.

2 REVIEW OF THE LITTERATURE

2.1 *Streptococcus pneumoniae* as a bacterium

Streptococcus pneumoniae (the pneumococcus) was isolated for the first time by George Sternberg in 1880 and Louis Pasteur in 1881 (1). It was formerly known as Diplococcus pneumoniae and belongs to the mitis group within the genus Streptococcus. The pneumococcus is a lancet-shaped and mostly encapsulated gram-positive bacterium that grows in pairs or short chains in broth culture. On blood agar, pneumococci form small greyish, mostly mucoid or smooth colonies, surrounded by a greenish zone of partial haemolysis (alpha-haemolysis). Non-capsulated pneumococci produce rough colonies. Pneumococci are easily lysed, while the centre of the colony soon becomes depressed because of autolysis.

2.1.1 Structure of *Streptococcus pneumoniae*

The pneumococcal cell surface has three layers: the plasma membrane, the cell wall and the capsule composed of polysaccharides. The plasma membrane and the peptidoglycan backbone of the cell wall act as anchors for the cell wall polysaccharides and surface proteins. Two cell wall polysaccharides, teichoic acid and lipoteichoic acid, are common to all pneumococcal strains. Moreover, several surface proteins are conserved across most pneumococcal strains. The best known of these are the lipoprotein pneumococcal surface adhesin A (PsaA), the enzymes immunoglobulin A-protease and neuraminidases and three choline-binding proteins, pneumococcal surface protein A, major pneumococcal autolysin and pneumococcal surface protein C (PspC, also referred to as CbpA or SpsA) (2,3). Several new proteins and other surface components involved in the adherence and virulence mechanisms have been recognised recently (4,5). A subpopulation of pneumococci produces pilus-like protein structures, able to extend beyond the polysaccharide capsule (6). One of the most intensively studied intracellular structures is pneumolysin, a toxin common to all clinically relevant pneumococcal strains (7). The capsule is the outermost layer of the cell surface and consists of

polysaccharides, which show considerable variation in their chemical composition, amount and antigenic capacity.

2.1.2   Identification of *Streptococcus pneumoniae*

The standard for the clinical diagnosis of pneumococcal infection is growth of viable pneumococci from samples collected from the middle ear fluid (MEF) or normally sterile body sites, such as from blood, cerebrospinal fluid, the pleural cavity or joints. Similarly, the standard method to identify pneumococcal carriage is growth of *S. pneumoniae* from an upper respiratory tract sample. *S. pneumoniae* grows well on plain blood agar, but the use of selective culture agars that include gentamicin or colistin with nalidixic or oxolinic acid suppresses the growth of other organisms and thus increases the sensitivity of recovering pneumococci (8,9). The use of culture selective medium is especially important when culturing samples from the upper respiratory tract, a niche for numerous non-pathogenic and potentially pathogenic bacteria (10). Pneumococcal growth requires carbon dioxide (CO_2). The optimal pH for pneumococcal growth is around 7.2 and the optimal temperature is between 35°C and 37°C. To increase the yield of pneumococci, specimens are sometimes incubated in an enrichment broth prior to culture. After culture, pneumococci are identified by their characteristic colony morphology and differentiated from other alpha-haemolytic streptococci by their susceptibility to optochin and autolysis in contact with bile salt. The differentiation of atypical pneumococci from other alpha-haemolytic streptococci is sometimes difficult (11).

Pneumococci can be identified directly without culture from clinical samples by the immunologic reactions of their antigenic structures with specific antisera or by identifying their genetic material with molecular techniques. These tests may not only detect live bacteria but also killed pneumococci or even fragments of them. These techniques can be used to determine the serotype or to differentiate non-serotypeable pneumococci from other alpha-haemolytic streptococci (11). However, non-cultural identification methods offer limited possibilities for further characterisation of pneumococcal properties, since strains cannot be retained for later analysis (9,12).

Counterimmunoelectrophoresis and different latex agglutination methods have been used to detect capsular polysaccharide antigens, either directly or after enrichment in broth (13). A rapid and easy-to-perform commercial immune-chromatographic test, Binax NOW®, originally developed to detect cell wall

polysaccharide antigens in the urine has recently been adapted to nasopharyngeal and MEF samples (14-16). The antigen detection test seems promising for selected clinical purposes.

Polymerase chain reaction (PCR) tests are based on amplification of nucleic acids of the pneumococcal genes (17,18). PCR tests are increasingly used for research as well as for clinical purposes, although the precise role that they can play has not yet been established (9,19). In general, PCR-based methods are highly sensitive in detecting bacteria even in low densities (20). PCR methods are also valuable in detecting pneumococci in normally sterile body sites during antimicrobial treatment. The disadvantage of PCR methods is that they may also recognise genetic material that has migrated from the adjacent organs of the host or from non-pneumococcal strains that are close relatives to pneumococci, or even airborne contaminants (12). For example, PCR-positive but culture-negative MEF may yield genetic material from nasopharyngeal carriage or previous pneumococcal otitis media (19). Nevertheless, DNA from non-viable bacteria rapidly clears from the MEF, and metabolic activity has been demonstrated even in culture negative but PCR positive MEF samples, and (21). Thus, PCR may detect viable bacteria that fail to grow in culture, for example, due to small amount or dilution of the sample, or biofilm formation (19,22,23). Trzciński et al. detected viable pneumococci in a remarkable proportion of samples that were initially negative by conventional culturing but positive by PCR when they recultured and carefully examined the original samples (24).

2.1.3   Classification of *Streptococcus pneumoniae*

S. pneumoniae are classified into serotypes and serogroups according to the antigenic properties of the capsular polysaccharide (25). The formerly used American nomenclature named serotypes according to their order of discovery. Currently, the Danish nomenclature is used worldwide. It classifies serologically cross-reacting serotypes into serogroups. Of the known 46 groups, some comprise a single known serotype, while some represent two or more immunologically related serotypes. More than 90 serotypes have been distinguished, the exact number depending on the definition used. In addition, some strains cannot be serotyped because they are non-encapsulated. These strains are also called 'rough' strains because of their specific colony morphology.

For decades, the standard for serotyping pneumococci after their identification in a culture has been the Neufeld test, also called the Quellung or capsular swelling test. The test was first described already in 1902 (26). It is based on microscopic visualisation of the capsular swelling and agglutination of cells in reaction to adding specific antisera on a pure pneumococcal culture (25). The method requires time, resources and a well-trained staff to interpret the results. Alternative serotyping methods such as counterimmunoelectrophoresis and latex agglutination tests (13,27) have been developed to reduce the work load. Currently, PCR-based assays to identify serotype-specific sequences within pneumococcal capsular genes are increasingly used for pneumococcal serotyping.

The importance of simultaneous carriage of multiple serotypes has become obvious in planning the treatment and prevention strategies for pneumococcal diseases. Multiple carriage poses a special challenge for serotype detection. Sparse growth of the minor strains in a cultured sample may be masked by abundant growth of the major strains (28), especially if only one or a few colonies are picked for serotyping. Up to about 60 colonies should be picked to reach 95% probability in detecting the minority type if it comprises only a small percentage of the total growth (29). Since serotyping of multiple colonies is time and resource intensive, easier and less expensive methods for detecting multiple pneumococcal serotypes have been developed, such as the colony blot assay (30), the immunoblot method (31), the latex sweep method (32) and direct demonstration of pneumococcal capsular polysaccharides after enrichment culture by the Quellung test or electro-immuno-assay (33). Novel PCR-based methods seem precise and effective. Especially simultaneous amplification of multiple sequences in a single reaction—a process referred to as multiplex PCR—is promising in reducing the cost and workload in identifying multiple carriage (34).

The structure of the capsular polysaccharide is only one expression of the *S. pneumoniae* genome. Several advanced molecular typing techniques have been developed for detailed characterisation of pneumococcal strains and their clonality. Multilocus sequence typing (MLST), in which allelic profiles of pneumococcal strains are obtained by sequencing internal fragments of multiple house-keeping genes is currently widely used (35). Other methods used for molecular characterisation of pneumococcal isolates include multilocus enzyme electrophoresis, penicillin-binding protein profile analysis, pneumococcal surface protein A typing (36), pulsed-field gel electrophoresis (PFGE) (37), restriction fragment end labelling (38) and ribotyping (39). The first whole genome sequences of pneumococcal strains were published in 2001 (40,41). The achievement enabled

exact identification and comprehensive comparison between different pneumococcal strains. The genetic methods have offered substantially increased possibilities for understanding the characteristics and functions of pneumococcal virulence factors in their interaction with host cells.

Molecular characterisation has revealed that pneumococcal strains expressing the same serotype relatively often belong to otherwise diverse clones (42). Although rare, it is also possible that pneumococci of a single clonal type express different serotypes (42), a phenomenon known as capsular switching.

2.2 Establishment of carriage and the pathogenesis of pneumococcal infection

S. pneumoniae gains entry into the human host by adhering to the mucosal epithelium of the respiratory tract. Pneumococci often remain in the nasopharynx as commensals without causing symptoms or any apparent harm to the host. The condition of harbouring pneumococci in the upper respiratory tract is called 'carriage' or 'colonisation'. Even asymptomatic carriage can be considered as infection if it causes an antigen-antibody response. Occasionally the bacteria spread from the nasopharynx to the middle ear cavity through the Eustachian tube or to the paranasal sinuses to cause otitis media or sinusitis, respectively. Pneumococci can also be inhaled as aerosols or aspirated into the alveoli of the lungs, causing pneumonia. Local infection may progress to systemic infection by transfer of bacteria into the circulation via pulmonary capillaries through the vascular endothelial cells. The pathogenesis of pneumococcal infection depends on the virulence of the bacteria, i.e. their capacity to adhere to and invade the host cells and escape clearance by the host defence mechanisms.

2.2.1 Adherence of pneumococci to human cells

In the human respiratory tract *S. pneumoniae* first encounters the mucosa covered by a mucus layer and epithelial cells. A healthy mucosa inhibits bacterial attachment physically by the washing action of the saliva, the cough reflex, and the viscous mucus layer which acts as a physical barrier and transfers the bacteria outwards from the body by aid of beating movements of cilia attached to the mucosal surface. The mucosa also produces antibacterial substances that kill bacteria or

inhibit their growth (43). These include lysozyme, lactoferrin and antimicrobial surfactant proteins. The ample commensal flora on the respiratory tract protects the mucosa by interfering with growth and invasion of pathogenic bacteria.

If pneumococci survive the host defence mechanisms in the mucus, they may adhere to the epithelial cells of the respiratory mucosa. Adherence is mediated by binding between the bacterial surface structures and their target cells throughout the human respiratory tract. The epithelial cells express glycoconjugate receptors bearing specific carbohydrates on their surface, such as sialic acid residues, oligosaccharides and glycosaminoglycans, and pneumococci initially bind loosely to these receptors (44,45). However, for effective adherence, colonisation and disease development, pneumococci need stronger binding to protein receptors like the polymeric immunoglobulin receptor or keratin 10 (46,47). On the bacterium side, several choline-binding proteins, enzymes and other structures of the cell surface are essential in the adhesion of bacterial ligands to their receptors (4,48-51).

Recent molecular research has remarkably increased our knowledge about the versatility of the pneumococcal surface components and adherence molecules as well as the host structures involved in the adhesion process (5,44,52). It is now known that pneumococci not only directly bind to the host receptors but also have a complex dynamic interplay with several host proteins. This interaction facilitates adherence and subsequent internalisation of pneumococci into the host cells. During the initial contact between pneumococci and the host cells, pneumococcal enzymes reduce the viscosity of the mucus, inhibit the ciliary function, provide nutrients to the bacteria, destroy immunoglobulin activity and inhibit the opsonophagosytosis by human neutrophils. An important mechanism for pneumococci to interact with the host cells is to modify the host structures that contain sialic acid and sugars and expose the target cell receptors for adherence (52). For more effective colonisation, pneumococci need adherence molecules, surface proteins and so-called 'microbial surface components recognising adhesive matrix molecules'. Pneumococci either react directly with the target receptors, or recruit host proteins in the extracellular matrix and serum to act as molecular bridges in attachment of the bacteria to the target cells and in promoting their migration through the cell barriers (5,45).

A special mechanism facilitating pneumococcal attachment to the target cells is phase variation, in which bacteria undergo spontaneous and reversible changes in their phenotypic expression between transparent and opaque colony morphologies (53). The transparent variants, expressing less capsule and increased or altered surface proteins and cell-wall carbohydrate-containing structures, show increased

adherence to the human epithelial cells (48,53). A reduction in the amount of capsule may be a necessary step for efficient colonisation, as a thinner capsule allows greater exposure of pneumococcal cell surface molecules important for interaction with the host cells and adherence (54). However, experimental research in mouse models has shown that some amount of capsule is required for effective colonisation (54,55). Furthermore, the pneumococcal polysaccharide capsule is important for the initial survival of the bacterium on the mucosal membrane, one mechanism being its negative charge (55).

2.2.2 Invasion by pneumococci and development of pneumococcal infection

To cause disease, pneumococci must be internalised into the epithelial cells, migrate across the epithelial barrier and survive in an environment with versatile host protective mechanisms. Mucosal diseases, like otitis media and pneumonia, develop when pneumococci multiply and cause cell damage in the mucosal sites of the human body. For development of invasive disease, e.g. bacteraemia and meningitis, pneumococci need to be translocated through the vascular endothelium and gain access to the blood stream, the cerebrospinal fluid, the lung parenchyma or other normally sterile body sites.

In general, pneumococcal adherence appears to be benign until the host cells are activated by inflammatory cytokines (56,57). The host cell activation increases the number of receptors and induces alterations in the cell surface (56-59). Cell activation also starts a signalling mechanism needed for translocation of bacteria into and through the cells (60). Both pneumococcal and viral structures may be involved in the cell activation and the up-regulation of the host cell receptors (46,60,61).

Inflammation has a major role in the virulence of pneumococci (3,57,62). The inflammatory process includes an influx of neutrophils and destruction of the structure and function of the host cells (59). The pneumococcal cell wall components induce activation of a wide array of cytokines, enzymes and the complement, which leads to strong inflammation (62). Several pneumococcal proteins contribute to the development of inflammation and cell damage, and inhibit the host defence mechanisms (4,52,63). Many adhesive surface proteins and enzymes are multifunctional and promote bacterial transport across the host epithelial and endothelial cells (5,46,52,60). An important pneumococcal virulence

factor is pneumolysin, an intracellular toxin, which enhances the host inflammatory process and impairs the function and viability of the host cells (7,56). Pneumolysin may also cause direct host cell damage (7). Pathogenesis is promoted by pneumococcal autolysins, which cause destruction of pneumococci, thus enhancing the release of cell wall components and internal toxins from dying bacteria and further increasing the inflammation. This process may be facilitated by antimicrobial treatment (64). In addition, the pneumococcal pilus-like structures augment the inflammatory process.

Although the versatile pneumococcal structures are known to participate in the pathogenesis of pneumococcal disease (65), the polysaccharide capsule is still considered the most important virulence factor of *S. pneumoniae*. The capsular polysaccharides do not elicit inflammatory response and they do not appear to be toxic (59). The main role of the capsule in the virulence of pneumococci is to protect the bacteria from phagocytosis by polymorphonuclear leucocytes and thus enhance their survival in the human organism. Pneumococci survive better in the blood and exhibit greater systemic virulence in the opaque phase than in the transparent phase of their morphology, probably mostly because of the thicker capsule (53). Pneumococcal virulence depends on both the amount and the chemical composition of the capsule and it varies across different serotypes (66). Differences in the pneumococcal capsule type were found to have significant effects on pneumococcal growth in a mouse model, both in the nasopharynx and in the lungs (67). However, both the capsule type and the genetic background were important, and their joint influence varied with the site of infection (67).

The involvement of the platelet-activating factor receptor in the development of pneumococcal invasive disease was recognised in the 1990s (58). The activated receptor mediates adherence to and translocation through human cells, either by direct binding or by initiating a signalling cascade that leads to host inflammation and bacterial invasion (58-60). However, inhibition of platelet-activating factor receptors has been shown to reduce pneumococcal infection only partly, indicating that other receptors are also needed in pneumococcal invasion (60). A well-known receptor capable for the uptake of pneumococci in the respiratory epithelial cells and their transport through the epithelial barrier is the polymeric immunoglobulin receptor, which binds to the pneumococcal protein PspC (46,68). The laminin receptor promotes the initial adhesion of pneumococci to the human microvascular brain cells and may thus initiate the passage through the blood-brain-barrier (69).

2.2.3 Interactions between pneumococci and other nasopharyngeal bacteria

The nasopharynx is a common niche for a range of non-pathogenic species as well as *S. pneumoniae* and other potential bacterial pathogens such as *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*. Interactions between different species in the establishment of carriage and development of disease are not known well, and the complex mechanisms of their mutual competition and synergism are currently being intensively studied. Understanding these mechanisms is important for the prevention and treatment of pneumococcal diseases since between-species competition offers an opportunity for other species to replace those eliminated by vaccination or antimicrobial treatment. One species may also benefit if another produces enzymes that reduce antibiotic activity or if the species gains antimicrobial resistance by horizontal gene transfer (70).

Non-pneumococcal alpha-haemolytic streptococci inhibit pneumococcal growth in vitro and negative associations between these commensals and pneumococci have been observed in vivo (71,72). A number of studies have shown a negative correlation between the growth of pneumococci and *S. aureus* in the nasopharynx (20,73,74).

In contrast, there are conflicting results regarding bacterial interactions between *S. pneumoniae* and *H. influenzae*. Both negative (75-77) and positive (20,74,78,79) associations have been observed between these bacteria in the nasopharynx of children. Experimental and in vitro studies provide evidence for both synergistic and competitive interactions between these two species (80,81). Concurrent colonisation of epithelial surfaces by these species induces synergistic pro-inflammatory responses (80). Several mechanisms of the mutual competition between *S. pneumoniae* and *H. influenzae* over the ecological niche have been suggested, including competition for nutrients and receptors (82). In animal experiments, *H. influenzae* has been associated with decreased frequency and density of pneumococcal growth (83,84). *S. pneumoniae* has been shown to inhibit the in vitro survival of *H. influenzae* through the production of hydrogen peroxide and neuraminidase (81,85). *H. influenzae* for its part activates the complement-dependent killing of pneumococci (83). *H. influenzae* also enhances carriage-induced antibody production against pneumococcal proteins, at least in children vaccinated with a pneumococcal conjugate vaccine (86).

The complexity of between-species interactions was demonstrated in a study in which colonisation by *H. influenzae* was negatively associated with *S. pneumoniae*

during upper respiratory infection, but when *H. influenzae* and *M. catarrhalis* colonised the host simultaneously, both were positively associated with *S. pneumoniae* (75). Colonisation with *M. catarrhalis* has also been shown to increase the risk of acute otitis media (AOM) in children colonised with either *S. pneumoniae* or *H. influenzae* (76). *H. influenzae* out-competed a non-virulent strain of *S. pneumoniae* in experimental co-colonisation in mice, resulting in selection of more virulent pneumococcal strains (87). Viral upper respiratory infection may also affect the relative occurrence of different nasopharyngeal bacteria (72).

2.2.4   Biofilm formation

One possible explanation for the conflicting findings on between-species interactions is biofilm formation. Biofilms are complex three-dimensional structures, comprising bacteria from one or several species, hyper-adhesively attached to the cell surfaces and encased within a self-produced extracellular matrix (88). Bacteria organised in biofilms interact with each other using intercellular messenger molecules and form special structures for the delivery of nutrients and removal of metabolic waste products (23). In biofilms, bacteria elicit weaker inflammatory responses and are more resistant to host defences and other environmental challenges, including desiccation and antimicrobial treatment, as compared to planktonic strains, i.e. strains not organised in a biofilm (89-91). Bacteria living in biofilms may be undetectable with conventional culture methods but detectable with PCR-based methods (22,23)

In animal models, pneumococcal biofilm formation has been associated with enhanced adhesive capacity, persistent carriage and decreased virulence (89-91). Concordantly, pneumococci in biofilms have expressed high proportions of transparent phenotypes, fit for colonisation, along with decreased amount of capsule, enhanced production of adhesins and decreased production of pneumolysin (90). The presence of *H. influenzae* has been observed to increase pneumococcal biofilm formation in vitro and in vivo (92), but this may be strain-specific and affected by factors associated with the host (93). Although it has been suggested that pneumococcal biofilm formation enhances survival of bacteria and prolongs colonisation rather than promotes invasive disease, biofilms have been observed in association with a variety of infections as well (22,92,94).

2.3 Immunity related to pneumococcal carriage and infection

In addition to the physical and biochemical host defence mechanisms of the mucosa, humans have a versatile complex of immunological defence mechanisms to protect themselves against pathogenic microbes. The immune system can be classified into different overlapping and co-operating subsystems. The innate (unspecialised) immune system is not pathogen-specific and therefore it is independent of previous contacts with pneumococci. The adaptive (specialised) system is based on development of protection mechanisms after recognition of pneumococcal structures. The innate immunity operates through leucocytes and circulating proteins (the latter mechanism occasionally referred to as 'humoral innate immunity'). Adaptive immunity has been divided in two components. First, antibody-dependent (humoral) immunity refers to recognition of different polysaccharide or protein molecules (antigens) by the host and production of antigen specific antibodies. The antibodies bind to their target antigens and start a process aiming at destruction of the bacteria and preventing their attachment and invasion. Second, the adaptive cell-mediated immunity operates through leucocytes and does not involve antibodies. In the case of mucosal infections, the immune system first operates at the mucosal level. The systemic immunity operates through the circulation in the whole body.

2.3.1 Immunological responses to pneumococcal carriage and acute otitis media

If pneumococci escape the physical and biochemical host defence mechanisms of the upper respiratory tract mucosa, the bacteria invade the tissue beneath the epithelium and stimulate the mucosal innate immune reaction. After the pneumococcal structures have been recognised by the host receptors, the release of cytokines and other chemical mediators recruits leucocytes into the upper respiratory mucosa. This leucocyte migration has an important role in the clearance of pneumococcal carriage (95). Inflammation and cell damage further accelerate the process of the innate immune response.

In the systemic innate immune reaction, the pneumococcal structures, facilitated with host proteins like the C-reactive protein, trigger complement activation, which results in a cascade of biochemical reactions producing molecules that cover (opsonisate) the bacteria. The opsonisation makes the bacteria easier to

be embedded (phagocytised) and killed by polymorphonuclear leucocytes and macrophages. The proteins produced by the complement cascade also enhance recruitment and activation of additional cells and molecules participating in the process. Furthermore, the inflammatory vasodilatation enhances the migration of phagocytic leucocytes from vessels into the infected tissue. Although very quick, the innate immunologic mechanism is relatively ineffective, as pneumococci have many mechanisms to escape clearance by complement activation (96). As a link between the innate and adaptive immunity, dendritic cells and macrophages process the pneumococcal antigens and present them to leucocytes responsible for the development of adaptive immunity.

The adaptive immune system targets pneumococcal infections specifically and is developed after pneumococcal strains have been encountered by the host. The best known adaptive immune mechanism is production of antibodies against the polysaccharides of the pneumococcal capsule and a number of other pneumococcal antigenic structures. The importance of adaptive cell-mediated immune mechanisms independent of antibodies has been recognised only recently and is less well understood (97,98)

At the mucosal level, the antibody production takes place in the so-called 'mucosa-associated lymphoid tissue' beneath the epithelium. The pneumococcal polysaccharide and protein antigens induce production of mucosal antibodies, transported back to the mucosal surfaces and secreted. The majority of these secretory antibodies belong to the immunoglobulin A class. Their main function is to prevent bacterial attachment and invasion into the mucous membrane by binding to the bacterial adhesins and blocking their interaction with the receptors in the epithelial cells.

Systemic antibodies against pneumococcal antigens circulate in the blood. They are produced in the lymph nodes and in the spleen and opsonise bacteria for phagocytosis and interfere with the function of different pneumococcal structures.

The capsular polysaccharides induce serotype specific antibody production. The production is independent of T-cells, as polysaccharide antigens can bind directly to B-cells and activate them to proliferate and differentiate to antibody-producing plasma cells. The antibody response to purified polysaccharide antigens is short-lived and does not induce immunological memory (99). According to a current concept, however, T-cells and B-cells interact in the production of antibodies against the capsular polysaccharides during natural encounters between pneumococci and the host, most likely because the capsular polysaccharides in living pneumococci are presented by leucocytes to the immune system together

with the protein and lipid components of the cell wall (100,101). The main function of anti-capsular antibodies is to participate in opsonisation, which is facilitated by complement activation and which results in phagocytosis

Antibodies induced by conserved pneumococcal antigens common to all capsular types, i.e. the cell wall polysaccharides, different surface proteins and pneumolysin, are not serotype-specific. The onset of antibody production against proteins requires activation of B-cells by T-cells (102) and the process has been shown to induce immunologic memory (103). The protein antibodies interfere with the function of their target antigens, for example, by preventing adhesin-mediated binding of pneumococci to the host cells (49,50) or reducing the cytotoxic effects of pneumolysin (104).

Epidemiological studies have shown that even infants are capable of producing antibodies against the pneumococcal polysaccharides and proteins (105-112). The antibody concentrations in serum and saliva increase during the first 2 years of life but remain mostly below the level measured in adults (106-109). Previous culture-confirmed pneumococcal contacts, either carriage or AOM, have been associated with increased concentrations of antibodies against some pneumococcal capsular polysaccharide and protein antigens in children (106-114). In addition, polysaccharide antibodies from children who have previous culture-confirmed contacts with homologous serotypes have shown higher capacity to opsonise pneumococci for phagocytosis as compared to antibodies from children without such contacts (115). Increases in antibody concentrations have been measured between the acute and convalescent phases of culture-confirmed pneumococcal AOM, although this seems to occur only in a minority of events and vary by age, antigen and previous pneumococcal contacts (116-120). In adults, significant increases in polysaccharide antibody concentrations against some pneumococcal serotypes and proteins were observed, if either the individual or a family member carried the serotype during a 10-month follow-up (121).The observed kinetics of the development of antibodies in children has varied remarkably between different serotypes and by different protein antigens (106,107,109,122). It is not easy to interpret how much of this variation is due to real biological differences in the responses to these antigens and how much could be explained by other reasons, such as undetected previous encounters with pneumococci or other unrecognised factors affecting the antibody dynamics.

2.3.2 The protective effect of naturally acquired immunity against pneumococcal carriage and acute otitis media

The protective role of anti-capsular polysaccharide antibodies against the invasive forms of pneumococcal disease is well established (123). The protective role of naturally acquired polysaccharide antibodies, i.e. antibodies induced by encounters with pneumococci in carriage or disease against subsequent pneumococcal carriage and mucosal infections is less well understood. Repeated episodes of carriage and AOM due to the same serotype are not unusual (124,125) and pneumococcal AOM may occur even in the presence of serotype-specific antibodies (105).

In experimental colonisation studies among adult humans, susceptibility to colonisation could not be predicted by the pre-inoculation level of serum IgG against the homologous capsular polysaccharide (126,127). In contrast, an observational study among Israeli toddlers found a lower risk of colonisation with serotypes 6A, 14, and 23F after previous exposure to the same serotype, and for serotypes 14 and 23F the protection was associated with increased serotype-specific antibody concentration (114). Previous carriage of selected serotypes resulted in delayed re-acquisition of the homologous serotypes in a refugee camp in Thailand (128) and the same was observed for serotype 14 among Gambian infants (129). In a follow-up study among UK adults, a higher level of anticapsular IgG for serotype 14 at the beginning of a 10-month follow-up was associated with reduced odds of carriage of this serotype during the follow-up (121). In a study in US families in the 1970s, pre-existing serotype-specific antibodies did not protect from the acquisition of a homotypic strain but seemed to decrease the duration of carriage (113). Concerning AOM, lower pre-existing concentrations of antibodies to pneumococcal capsular antigens have been associated with later proneness to otitis (130) and lower acute phase concentrations have been observed in children with pneumococcal AOM than in children with non-pneumococcal AOM (118).

Development of anti-capsular polysaccharide antibodies have long been considered as the basis for immunity increasing with age against invasive pneumococcal diseases (123). However, other mechanisms must also be involved in the development of protection, as the decline in the incidence of invasive pneumococcal diseases by age is similar across different serotypes despite large variation in the exposure and carriage of different serotypes and their immunogenicity (131). The decline in disease incidence also starts at an earlier age than would be expected based solely on the rate of increase in polysaccharide-specific antibody levels. Thus, antibodies against antigens common to all

pneumococcal serotypes or adapted cellular immune mechanisms could be alternative mechanisms for protection.

Antibodies against the cell wall polysaccharides, common to all serotypes, did not prevent pneumococcal carriage from proceeding to disease or the worsening of disease in a study among young adults (132). Studies of the protective role of pre-existing antibodies against pneumococcal proteins in preventing pneumococcal carriage have resulted in inconclusive findings. Of the two studies in which volunteer adults were challenged intranasally with pneumococci, one suggested protection against pneumococcal carriage by pre-existing anti-protein antibodies (126), whereas the other did not (127). Studies among UK children aged 2–12 years and among young infants in The Gambia showed associations between low concentrations of antibodies against pneumococcal protein antigens and pneumococcal carriage (122,133). In contrast, in studies among Finnish children, naturally acquired protein antibodies in serum and saliva were not protective against carriage (120,134-137). Instead, Finnish studies have provided suggested evidence that naturally acquired serum and salivary antibodies against selected pneumococcal proteins correlate with protection against pneumococcal AOM during the second year of life (120,134,135,137). A higher antibody concentration against pneumococcal surface adhesin A, but not against pneumolysin, during the acute phase of AOM has also been associated with a lower risk of pneumococcal involvement (118,138). Similarly, a higher antibody concentration against pneumococcal surface protein A during the acute phase of invasive disease has been associated with a lower risk of pneumococcal involvement (139). After an intra-nasal challenge with pneumococcal serotype 6B, adult volunteers who became carriers developed higher IgG levels against pneumococcal proteins than non-carriers (127). When pooled sera from the carriers was inoculated into mice, the mice were protected against invasive disease after inoculation of lethal doses of a heterologous serotype 2, indicating protection due to antibodies independent of serotype (127). Likewise, previous pneumococcal carriage has been associated with serotype-independent protection against subsequent acquisition in a follow-up study among Bangladeshi infants (140).

The role of cellular immunity as another mechanism of serotype-independent protection against pneumococcal carriage was evidenced by the mouse models of Malley et al. (141). The authors found that colonisation with pneumococci induced similar protection against re-colonisation by both homologous and heterologous serotypes and that protection was independent of both anticapsular and non-capsular antibodies. Furthermore, protection was completely abrogated in mice

that congenitally or experimentally lacked TD4 positive T-cells. The fact that protection was long-lived argued for adapted cellular immunity instead of innate immune mechanisms (141). The role of T cells, either alone or together with serum and mucosal antibodies, is currently undergoing intensive research in animal and human studies (97,98,142-146).

2.4 Transmission and carriage of *Streptococcus pneumoniae*

S. pneumoniae is transmitted between human hosts by respiratory secretions, either as airborne droplets or through contaminated hands or fomites (147). If pneumococcal adherence to the mucosa is not prevented by the host defence mechanisms, pneumococci settle on the mucosa of the human nose, nasopharynx or oropharynx. Pneumococcal carriage remains usually asymptomatic, the bacteria staying on the mucosal surface without causing any harm until the bacteria are cleared. Only a few carriage episodes proceed to disease.

2.4.1 Sampling sites and methods for identification of pneumococcal carriers

Our knowledge of the dynamics of pneumococcal carriage depends on adequate identification of the carrier state. Pneumococci can be sampled from the human nasal cavity, nasopharynx or oropharynx.

Nasal or nasopharyngeal specimens have proved superior to those obtained from the oropharynx or tonsils in children, if the swab sampling technique has been used (148-154).The sensitivity of nasopharyngeal sampling was >90% in 7 of 9 reviewed studies in which sampling from both nasopharynx and oropharynx was used as the diagnostic standard (9). Strains from the nasopharynx have been shown to reflect those causing otitis media better than oropharyngeal strains (155). Usually the nasopharyngeal sample is obtained through the transnasal route, but sometimes the sample from the nasopharynx has been obtained through the transoral route by curving a flexible swab upwards into the nasopharynx (154,156). However, sampling the nasopharynx via the transnasal route has proved more sensitive in children (154).

Nasal or nasopharyngeal aspirate samples or nasal wash samples are also used in identifying children carrying pneumococci. In a study comparing different sampling

sites and methods among children with respiratory tract infection, the sensitivities of nasopharyngeal swab samples and nasopharyngeal aspirates were very similar, both performing significantly better than swab samples obtained from the posterior wall of the oropharynx (148). In a Kenyan study, the nasopharyngeal swab culture was positive in 85% of children with a positive nasal wash culture (157). A disadvantage of the aspiration and wash techniques is that these methods may be less tolerated and require more collaboration. Furthermore, aspiration without wash can only be used if nasal secretion is present. Similar pneumococcal yields have been observed in studies comparing nasal swabs and nasopharyngeal aspirates (148,158) or nasal swabs and nasopharyngeal swabs (148,159). However, the presence of respiratory infection may have affected the findings in some of these studies. Even cultures from paper tissues with visible secretions after blowing or wiping the nose have revealed good sensitivity (159,160)

Nasopharyngeal sampling has been used to identify pneumococcal carriers also among older children and adults. During the last decade, however, it has become obvious that simultaneous nasopharyngeal and oropharyngeal samples are required for optimal detection of carriage in adults (150,161-165). In some studies of adults and adolescents, swab samples obtained from the posterior pharyngeal wall or nasopharynx through the mouth have yielded pneumococci even more often than samples obtained through the nose (24,163,164,166). In a study among Dutch adults, pneumococci were initially found more often from nasopharyngeal samples obtained via the transnasal route as compared to the transoral route. However, the yield was significantly better when using the trans-oral route if initially culture-negative but PCR-positive samples were carefully re-cultured after enrichment (24). A re-examination of initially negative transnasal samples resulted in only very few positive results, which means that repeated sampling (157) was not the main reason for the additional yield gained from using both sampling routes.

The optimal composition of the sampling swab is not well known. Cotton swabs may include properties inhibitory to pneumococci (9,10). The performance of calcium-alginate, Dacron and rayon tipped swabs has varied in studies using conventional culture methods and no clear preference has been determined (167,168). Calcium alginate may inhibit some PCR-based assays and, like rayon, contains nucleic acids that interfere with the interpretation (9). Recently, high pneumococcal yields have been obtained with flocked nylon swabs (168).

A WHO Working Group published recommendations for a standard method for the detection of upper respiratory pneumococcal carriage in children initially in 2003 to advance the comparability across different studies (10). In the majority of

carriage studies, samples have been obtained from the nasopharynx as recommended, but variable swab types and swab transport media have been used (169). The WHO Working Group has updated their recommendations in 2013 (9). Deep nasopharyngeal sampling with the one-swab approach is recommended for children, whereas both nasopharyngeal and oropharyngeal sampling is recommended for adults. The use of any alternative methods and all deviations in the procedure should be reported to help with interpreting the results. Calcium alginate, rayon, Dacron or nylon materials are suitable for culture-based studies. For molecular analyses, synthetic materials such as nylon or Dacron are preferred. Skim milk-tryptone-glucose-glycerin is the recommended medium for transport and storage of the samples. Culture remains the standard for detection of pneumococci and the use of selective plates enriched with gentamicin is recommended. As a new recommendation, one of each morphologically distinct colony should be stored along with information of its order of prevalence. This allows for the possibility to return to all alpha-haemolytic colonies if the first colony turns out to be non-pneumococcal or if information about multiple colonisation will later be needed. As the optimal method for detection of multiple serotypes has not been identified, the recommendation encourages storing the original sample for future assessment. Culture may be complemented with non-culture techniques, of which the current recommendation favours the detection of DNA from major pneumococcal autolysin genes by real-time PCR (9). The core method for serotyping of pure pneumococcal isolates is the Quellung test. Latex agglutination may also be used, but evidence is insufficient to give recommendations for other methods.

If species other than *S. pneumoniae* should be detected as well, sampling methods should be revisited and complemented. For example, current data suggest that both naso- and oropharyngeal swabs may be needed for the optimal recovery of *H. influenzae* (148,150,152).

When interpreting findings of carriage studies, it must be borne in mind that the sensitivity of pneumococcal detection by culture is not perfect (24,170). For example, antimicrobial treatment may reduce the bacterial load below the detection level or bacteria organised in biofilms may be undetectable with conventional culture (22,23).

2.4.2   Exposure to pneumococci and transmission

The major reservoir of pneumococcal strains in the population is upper respiratory tract carriage. Asymptomatic carriage is the source for the vast majority of transmissions. Transmission occurs most likely when individuals are in close contact with one another. Since young children have the highest frequency of pneumococcal carriage, they form the most important source for pneumococcal transmission. For this reason, most transmission studies have been carried out in families with young children or in day care facilities.

The importance of intra-familial spread of pneumococci was first recognised already several decades ago (113,149,171). Family studies continue to be crucial in learning about the dynamics of pneumococcal transmission and colonisation. In a study among Bangladeshi families, exposure within families to selected target serotypes signified more than a ten-fold increase in the rate of acquisition in other family members (172). Large families, crowded living conditions (171,173,174) and respiratory infections (113,175) favour pneumococcal spread within families.

Young children are particularly susceptible to acquiring *S. pneumoniae*. The risk for acquisition is especially high if the young child is exposed by a carrying family member (140,149,173,175-180). In a subgroup of the FinOM Cohort Study, simultaneous carriage of the same serogroup by another family member presented a clearly higher risk of carriage at age 6 months to 2 years than with day care attendance (177). However, adults are less susceptible to acquiring *S. pneumoniae*, even when exposed at home (173,178,181,182). Adults with children in the family tend to carry pneumococci more often than adults living without children (183,184), but the parents still carry much less often than their children (33,128,150,180,181,183-188).

Even if different family members carry *S. pneumoniae* simultaneously, the strains are not always the same, especially in populations in which the frequency of carriage is high and the turnover of strains is rapid (181,184,185,187). In general, however, the same serotype seems to be a good indicator for pneumococcal transmission. In 17 of 18 pairs of children and their parents with concurrent carriage of the same serotype in Japan, the molecular typing by PFGE showed an identical or at least a related pattern (189). Similarly, the nasopharyngeal serotype and PFGE type were identical between siblings in 12 of 13 clusters of concurrent or closely related AOM events experienced in 11 families (190).

Children acquire *S. pneumoniae* effectively also from the community, thus introducing new strains into the family (173,176,182,191). Day care attendees

usually show higher prevalence of pneumococcal carriage than unselected healthy children. Thus, day care centres provide an effective environment for transmission of pneumococci (181,182). In Israel, carriage in infants and toddlers cared for at home was associated with previous carriage of similar strains in the day care centre of their older siblings, but not in other day care centres in the area, the strains being identified by serotyping, antibiogram, and PFGE (192). In a study of pneumococcal transmission among Finnish day care attendees and their family members, about 80% of new serotypes detected in the family were estimated to be attributable to the day care attendee (182). In this model, 65% of acquisitions in day care attendees were estimated to originate from fellow day care attendees, 25% from the community, and only 10% from family members.

There may be considerable differences in carriage prevalence between individual day care centres or primary schools even in the same area (193-198). In a comparative analysis of pneumococcal transmission in Portuguese and Finnish day-care centres, the higher prevalence of carriage in the Portuguese setting was assigned to the younger age of attendees, to a higher within-room transmission and to the larger day care centre size (199).

Clustering of strains within day care centres and schools has been demonstrated in several studies (181,200-204). Although the overall serotype distributions in different day care centres are often similar, more detailed pneumococcal typing with molecular methods may yield different patterns for different day care centres and even for different rooms within a facility (205). When studied longitudinally, pneumococcal microepidemics have been identified within facilities (182,206,207). Especially strains resistant to antimicrobials are able to circulate for months or become endemic in a day care centre (208). Sometimes observed repeated or prolonged epidemics of a serotype may actually represent several distinct epidemics of different strains expressing the same serotype, if the strain has not been studied in detail with molecular methods (206).

The staff of the day care centres usually have surprisingly low frequency of pneumococcal carriage, similar to that of other adults (197,209-212). However, exceptions with high carriage in staff have been reported, especially at the time of introduction of a new strain in the facility (33). In an Israeli study, pneumococcal strains identified by serotype, resistance patterns and PFGE did not spread effectively from day care children to adults, despite close contacts in a kibbutz community (181). Adults introduce new strains into the family less often than children because adults carry pneumococci less frequently. However, once

colonised, adults may effectively transmit the bacterium to susceptible family members (173,178).

2.4.3　Acquisition of pneumococcal carriage

Worldwide, practically all children acquire *S. pneumoniae* at least once by the age of 2 years (125,129,175,188,213,214). New-born infants can be colonised by the bacterium from the first days of life (125,175,179,185).

In developing countries and populations with high risk of pneumococcal carriage and disease, the first episode of colonisation occurs early in life (128,175,179,185,215,216). First pneumococcal carriage was detected in all children in studies conducted in Papua New Guinea (185,215) by the age of 3 months (mean 17 days) and in all Australian indigenous children by the age of 4–5 months (median 58 days) (216). First pneumococcal carriage has been recognised by the age of 1-2 months in at least half of the children followed from birth or early infancy in studies in The Gambia, Kenya, India, Bangladesh and among Burmese refugees (128,129,140,175,217).

In the industrialised countries, initial colonisation with *S. pneumoniae* occurs later. About 50% of Swedish children, swabbed four times at 2, 6, 10 and 18 months of age, had acquired pneumococci at least once (218). In a US birth cohort swabbed at 1–2-month intervals, the proportion was 38% at six months and 54% at one year of age (219). The mean age at first detected acquisition was about six months. These figures are likely to be underestimates, since duration of carriage may be short and the recognition of acquisition depends on the frequency of the sampling. This was evidenced in a Costa Rican study in which the cumulative acquisition rate during the first year of life was 36% if the children were swabbed at 1, 3, 6 and 12 months of age, but 72% if the children were swabbed weekly (186). As many as 38% of children followed from birth with biweekly sampling in Oxfordshire, UK, had acquired *S. pneumoniae* already by the age of 12 weeks (220), this proportion being still far smaller than in the high risk populations.

After first acquisition, new strains are acquired and even the same strain may be acquired again after a period of absence. In a birth cohort study in the USA, up to six different serotypes were sequentially acquired within the two first years of life (125). In a Swedish day care centre, children acquired up to seven different serotypes during a 2-year follow-up with monthly sampling (209).

It is not straightforward to infer whether sequential findings of the same serotype represent continuous carriage or whether the serotype has been cleared and re-acquired. Re-appearance of a previously carried serotype may occur even after several months of negative findings (113,128,206,209,221,222). An intervening negative culture may not be absolutely reliable, especially during antimicrobial treatment and if the time spacing between the two positive findings is short. In longitudinal data sets, variable algorithms have been used to differentiate continuous carriage and re-acquisition. Depending on the sampling frequency, re-acquisition has been defined as the re-appearance of a serotype or strain if more than a specified time (30 days to 14 weeks) has elapsed between two positive findings, often allowing one or even two negative findings between the positive ones, at least during antimicrobial treatment (113,125,185,209,213,222-224). The estimation of incidence rates of pneumococcal acquisition requires a longitudinal study with frequent sampling, and yet the exact times of acquisition between two samples with different status remain unknown, complicating the analysis. Different statistical models have been useful in estimating acquisition rates (128,172,175,182,191,225-227). Acquisition rates are higher in preschool children than in older children or adults (128,172,182,228). An increase in the acquisition rate with age during the first months or years of life has been suggested (191,229), but this pattern has not been consistent (225,230). Differences in acquisition rates across serotypes have been observed in several studies (125,172,175,213,225,228,230,231).

2.4.4   Clearance of pneumococcal carriage

The times and rates of pneumococcal clearance can be estimated in longitudinal studies with repeated sampling, although the exact timing remains largely unknown. Of note, the estimates of duration of carriage are not fully comparable across studies due to methodological differences, especially different definitions of acquisition and clearance of carriage. For example, studies that estimate serotype-specific durations of carriage by modelling the serotype-specific clearance rates often differentiate the overall disappearance of the serotype from its clearance in the absence of competition by other serotypes (immune clearance) (225)((228,232).

The observed duration of carriage has ranged from a few days up to 17 months (125). The observed distribution of duration tends to be skewed. Many short carriage episodes and a few long carriages are usually observed.

(129,183,207,209,222,233). Without frequent sampling, even long carriage episodes may be detected in one sample only and short episodes have a lower probability to be detected than long ones.

The clearance rates increase with age, so that the average duration of carriage shortens (128,173,176,191,199,229,230,233). In the US study by Gray et al., the estimated average duration of pneumococcal carriage decreased steadily from 4–6 months at the age of three months to 2–4 months at the age of 15 months, and remained constant thereafter up to the age of 24 months (229). In a Swedish study, the mean duration was about 2.5 months in children aged less than one year, whereas it was 1.5 months and one month in children aged 1–2 and 3–4 years, respectively (233). In children aged less than two years, the mean duration was about two months in Finland and France (191,206,225). Valuable data with frequent samplings from The Gambia (129,176) and Kenya (225) confirm the decreasing duration with age, the duration being similar or somewhat longer than observed in industrialised countries.

As compared to children aged less than five years, older children and adults have been found to carry pneumococci for shorter times, on average for one month or less in Finland, Sweden, the UK and in a refugee camp in Thailand, and for about two months in The Gambia (128,176,182,228,233). The effect of serotype on duration of carriage has been difficult to ascertain, since serotypes carried most frequently and for the longest time are also acquired at the youngest age (125,234). A number of studies have shown clear differences across serotypes in duration of carriage (129,213,230,231), especially in the youngest children (225,228,233). In the South Swedish Pneumococcal Intervention Program, it was confirmed that children aged less than four years carried serotypes belonging to serogroups 6 and 23 for longer time than those belonging to serogroups 9 and 14 in each of the narrower age groups (233). In Kenyan infants, the point estimates for time to immune clearance varied between 28 and 124 days for different serotypes (225). In a cohort study among UK families, the average duration of carriage for serotype 6B was 4 months as compared to less than two months for other serotypes in children aged less than five years (228). However, in older individuals, the average duration of carriage for serotype 6B was only 11 days (228).

2.4.5 Prevalence of pneumococcal carriage

The frequency of pneumococcal carriage in a population is usually characterised by the point prevalence of carriage, or sometimes as the proportion of positive findings out of the total number of samples in studies with repeated samples. The prevalence of carriage depends on the acquisition and clearance rates of pneumococcal carriage in the population.

The prevalence of pneumococcal carriage increases during the first months of life, as infants become exposed and start acquiring pneumococcal strains after birth (129,186,229,235-237).In several populations the prevalence levels off or peaks already during the first year of life, (128,129,164,174,214,238-245) whereas in others the prevalence continues to increase at least through the second and sometimes the third year of life (73,184,236,244,246). After the peak prevalence, carriage starts to decrease slowly but usually remains relatively prevalent up to about 3 or 7 years of age (73,184,207,214,244,247,248), after which it decreases more quickly (73,79,149,164,177,183,187,188,241,249-252).

In high risk populations with a large burden of pneumococcal disease, the turnover and acquisition of new strains is rapid and the prevalence of pneumococcal carriage is high. Extremely high prevalence levels (~90%) have been reported among African children in The Gambia and Ethiopia and among HIV positive children in Tanzania (129,249,253). Similarly, very high pneumococcal prevalence has been observed among highland children in Papua New Guinea and Australian indigenous children (215,254). High levels of pneumococcal carriage of 60–80% have also been observed in several other African and Asian countries (164,181,217,238,241,243,255,256), among the indigenous peoples in North and South America (180,246), among the Bedouin population in Israel (257), in refugee camps in Hong Kong and Thailand (128,239) and in an urban slum community in Brazil (247).

The turnover of pneumococcal strains is slower in industrialised countries and middle- and high-income populations, and the average carriage prevalence in healthy children under school age usually settles at a lower level, at about 20–50% (153,186,188,197,198,218,248,258-262). In studies conducted among ill children and in day care centres the prevalence tends to be higher and high point prevalences (70–90%) have been observed occasionally also in industrialised countries in day care centres or orphanages, especially in young children, (205,206,224,263). In contrast, low carriage levels (3–20%) have also been reported, but in these studies at least some participating children have been older

than five years of age, children with even mild respiratory symptoms have been excluded, the samples have been obtained via oral route, and/or the samples have been cultured on unselected plates (156,193,195,239).

Adults carry *S. pneumoniae* less frequently than children. Across a wide range of studies, the prevalence of pneumococcal carriage has been 10% or less (79,149,174,181,186,188,214,250). Higher levels of about 15–20% have been reported in some studies in day care centres and military service (33,162). Among high risk populations even adults carry pneumococci frequently, and the prevalence has been as high as ~50–60% among Gambian villagers (249) and ~20-40% in some other African countries and among Australian and South-American indigenous peoples (180,241). Sampling only the nasopharynx is likely to underestimate the proportion of adult carriers. The prevalence increased from 19% to 35% among parents of young children in the Netherlands after careful examination of the oropharyngeal swab samples in addition to the nasopharyngeal ones (24) and when nasal aspiration was used as a third detection method in hospital employees with common cold in the USA, the prevalence was as high as 45% (163). Even with both nasopharyngeal and oropharyngeal sampling, the carriage prevalence remains clearly lower in adults as compared to children in the same population or family (150,164). It is not well known how much of the low carriage frequency in adults is attributable to immunological resistance against acquisition, or is due to low sensitivity to detect carriage of short duration.

The prevalence of carriage in older children and adolescents is between those found in children and adults (73,79,149,164,177,181,188,214,241,249,250). There is only sparse knowledge about pneumococcal carriage among the elderly, the other major risk group for invasive pneumococcal disease. However, carriage seems to be rare in this age group (184,264,265), except among some institutionalised populations (183,266).

2.4.6   Carriage of different pneumococcal serotypes

The diversity of carried serotypes is wide. For example, in a cross-sectional survey among Gambian children and adults, the 2428 carriage isolates comprised 74 of the more than 90 known serotypes (249).

In unvaccinated children, the most frequently carried serotypes have belonged to serogroups 6, 19, 23 and often also 14 with only little temporal and geographical variation   (73,125,164,214,220,240,261,267,268).   These   frequently   carried

serogroups account for the majority of first acquisitions (125,129,207,213) and re-acquisitions (125). These serogroups tend to have higher acquisition rates and lower clearance rates compared to most other serotypes (125,175,213,225,228). The diversity of carried serotypes increases with age (175,263).

Several reasons for the relative fitness of the most frequently carried serotypes have been suggested. The serotype has been shown to affect adherence to human respiratory epithelial cells in vitro (269). Although a thinner capsule is associated with attachment and colonisation, a thicker capsule helps to escape clearance by mucus and surface phagocytosis. A novel hypothesis suggests that a simple structure of the capsule requires less energy consumption and leads to better growth in a nutrient-restricted environment and to better fitness of the serotype (270,271). The serotype-specific antibodies seem to have a role in the regulation of acquisition and/or clearance of carriage. Immunogenicity seems to vary between serotypes (114,121) and it has been suggested that high immunogenicity is correlated with lower acquisition rates (231).

More than one pneumococcal serotype can be carried at the same time. As many as one third of nasal samples collected from children in Papua New Guinea yielded more than one distinct pneumococcal serotype, and up to four serotypes in one sample (215). Altogether 17% of positive samples from Kenyan infants yielded multiple serotypes (157). In most studies, the proportion of positive samples that have yielded multiple serotypes has been up to 12% (29,125,164,181,187,188,205,220,254,272,273). However, these numbers may be underestimates since colonies representing a minority serotype may be sparse and indistinguishable from the majority of colonies, and in most studies only a few pneumococcal colonies have been picked from the culture plate for serotyping. Kaltoft et al. performed direct serotyping from an enrichment serum broth culture by a capsular reaction test and found multiple serotypes from 8-12% and 38% of positive samples obtained from children and staff in study day care centres, respectively (33). In a study among Burmese refugee infants, multiple serotypes were detected in 11% of samples positive for pneumococci by picking two or more morphologically different colonies for serotyping, while the proportion was 43% when a sweep of colonies were serotyped by latex agglutination and it was 49% with a molecular-serotyping microarray (274).

Pneumococcal serotypes compete with each other in the nasopharynx. Competition may concern limited nutrients and sites for adherence on the host cells. Carriage of a resident strain has been shown to inhibit colonisation by a challenge strain in a mouse model (275). Pneumococcal strains have been shown to

produce small antimicrobial peptides called bacteriocins, which inhibit the growth of other pneumococcal strains without causing harm for the producer strain in vitro and decrease colonisation by a competing strain in experimental animal models (276). Lower acquisition rates for new pneumococcal strains in the presence of another strain have been estimated by statistical modelling, based on longitudinal carriage data from epidemiological studies, whereas competition through enhanced clearance has not been evident (172,225,227,228). The most fit and most frequently carried serotypes (belonging to serogroups 6, 19, 23) with the highest acquisition and lowest spontaneous clearance rates are also less susceptible to competition by other strains than most other serotypes (225,228). However, even the most competitive serotypes do not out-compete other serotypes and the diversity of carried serotypes is maintained. One suggested theory is that carriage induces serotype-specific immunity against the carried serotype itself, and this immunity opens niches for the less fit serotypes not carried earlier in life (87,277-279).

For the prevention of pneumococcal diseases with serotype-specific vaccines the competition between pneumococcal serotypes poses an important challenge. In the clinical trials with pneumococcal conjugate vaccines, it was soon observed that while vaccination reduces carriage due to the vaccine serotypes, it increases carriage due to other serotypes (280-282). It was first speculated that the explanation for this phenomenon could be the more sensitive detection of pre-existent minor serotypes, detectable after the elimination of the major ones, so-called 'unmasking'. However, it was soon understood that the increase in carriage with the non-vaccine serotypes was true and due to the disappearance of the competing vaccine types, a phenomenon termed 'replacement' (28,283). Accordingly, clear increases have been observed in the incidence of pneumococcal diseases due to the non-vaccine-serotypes, first in the clinical trials and later also in clinical surveys during ongoing vaccination programmes (284-286).

2.5   Clinical manifestations of pneumococcal infection in children

The pneumococcus causes a wide variety of diseases. These range from relatively mild but common mucosal infections, such as otitis media, sinusitis and non-bacteraemic pneumonia, to rare but serious and even life-threatening invasive diseases like bacteraemia and meningitis. The incidence of pneumococcal diseases

is highest in both extremes of life and these diseases constitute an enormous burden throughout the world.

2.5.1   Otitis media

Otitis media is a continuum of inflammatory conditions of the middle ear, accompanied by the collection of effused fluid. The healthy middle ear is an air-filled cavity within the temporal bone, lined with a mucosal membrane and separated from the external ear with the tympanic membrane. It is essential for hearing as it conducts sound to the inner ear through the ear ossicles. The middle ear is connected to the nasopharynx by the Eustachian tube, which balances the pressure between the two locations.

The acute form of otitis media, AOM, is characterised by an acute symptomatic inflammation of the middle ear accompanied by MEF (287). AOM is mostly, if not always, caused by a microbial infection and with sensitive methods pathogenic bacteria and/or viruses are detected in the MEF in a majority of AOM cases (288,289). Otitis media with effusion (OME) is an inflammation of the middle ear, associated with collection of effusion without symptoms or signs of acute infection (287). OME often follows AOM due to the slow resolution of inflammation and MEF. OME can also be associated with disturbances in the function of the Eustachian tube and, in turn, predispose to acute and recurrent infections (290-292). In prolonged OME, the MEF becomes viscous because of increased mucus production, leading to 'glue ear'. Chronic forms of otitis media may cause perforation of the tympanic membrane with or without chronic discharge, adhesion of the tympanic membrane to the bony parts of the tympanic cavity, erosion of the bones in the middle ear or development of cholesteatoma.

The development of AOM occurs in the following pattern: A predisposing event, usually a viral upper respiratory infection, causes inflammatory changes and congestion of the respiratory mucosa. The impaired function of the Eustachian tube leads to negative pressure in the middle ear, accumulation and trapping of secretions and ascending of pathogens from the nasopharynx into the middle ear cavity (287,290). Inflammation also disrupts the function of the mucociliary system, important in preventing bacteria from ascending into the middle ear and in clearing them from it (293). Furthermore, inflammation enhances bacterial adherence and invasion into the cells (57). As a consequence, viruses and bacteria effectively proliferate in the secretions in the middle ear and the infection proceeds

to disease (287). It is likely that the host inflammatory response to infection in the middle ear is responsible for most clinical symptoms and signs of AOM (294).

The criteria for the clinical diagnosis of AOM and its differentiation from OME have been debated in recent decades and various criteria have been used in both clinical practice and clinical studies (295). The diagnosis of AOM has mainly been based on signs suggesting inflammation of the tympanic membrane and presence of fluid in the middle ear as observed in pneumatic otoscopy, together with recent onset of signs or symptoms of local or systemic illness (296,297). Otoscopic signs suggesting presence of MEF behind an intact tympanic membrane include its opacity, yellow or greyish colour, bulging and impaired mobility (297). Sometimes the MEF can be seen directly as a visible air-fluid level behind the tympanic membrane, or occasionally in the ear canal after spontaneous perforation of the membrane (297,298). Tympanometry or reflectometry can be used as an aid in the assessment of ear-drum mobility (299-301). The symptoms of AOM and upper respiratory tract infection are essentially the same, and AOM cannot be distinguished from viral respiratory infection on the basis of symptoms, especially in young children (302-304). Also ear-related symptoms (ear pain, tugging or rubbing of the ear), although relatively often associated with AOM (296,303,305) can also be due for example to teething or transient alterations of the middle ear pressure. The recently updated US recommendation sets more stringent criteria for the clinical diagnosis of AOM and the differentiation between AOM and OME. This recommendation emphasises bulging as a specific sign of acute inflammation of the tympanic membrane (306,307). While increasing the specificity in recognising cases with truly acute bacterial infection, with the highest probability to benefit from antimicrobial treatment, these criteria may be insufficient in recognising the whole spectrum of AOM (306,308).

The standard method for the etiological diagnosis of AOM is culturing live pathogens from the MEF obtained by myringotomy, tympanocentesis or drainage through a perforation. *S. pneumoniae* has long been the most important bacterial cause of AOM. The proportion of AOM cases with pneumococcal aetiology has ranged from 20 to 50% in studies across different populations (237,288,309-319). After the introduction of large scale pneumococcal conjugate vaccination the relative proportion of AOM caused by pneumococci, i.e. the serotypes included in the vaccine initially decreased and accordingly, the relative proportion of AOM caused by other etiologic bacteria increased. The increase was true especially for non-typable *H. influenzae*, which has rivalled pneumococcus as the other major otopathogen (312,320). However, several years after vaccine introduction, the

pneumococcal serotype replacement has led to an increase in the incidence of AOM caused by serotypes not included in the pneumococcal vaccines, and thus the relative proportion of pneumococcal AOM has increased again (321). The third bacterium commonly causing AOM is *M. catarrhalis* (316). *Streptococcus pyogenes* is nowadays a rare otitis pathogen (284,288,314). Viruses are detected in a small proportion of MEF samples, if only culture methods are used (322). However, with culture combined with antigen detection or especially with the sensitive PCR-based methods, the proportion of viral presence in MEF samples from children with AOM can reach up to more than 70% (288,323). In a majority of these cases, viruses are found together with bacteria, but sometimes viruses are the only detected pathogens (288,322). *S. pneumoniae* tends to cause more severe AOM than the two other major otopathogens, non-typable *H. influenzae* and *M. catarrhalis.* Although the aetiology of AOM cannot usually be determined by its clinical picture (315), fever and earache, bulging or spontaneously perforated eardrum and myringitis have been associated with pneumococcal AOM (324-327). The spontaneous recovery rate has been shown to be lower (20%) for pneumococcal AOM than for AOM caused by *H. influenzae* (50%) (328). Higher leucocyte counts and higher concentrations of specific interleukins as markers of vigorous inflammation have been found in the blood of MEF in children with pneumococcal AOM, as compared to those with AOM caused by *H. influenzae* or *M. catarrhalis* (329-331). In contrast, the relative proportion of *H. influenzae* tends to be higher in recurrent AOM and OME and in older children (332-334). AOM caused by mixed bacteria (335) and the presence of viruses in the MEF concomitantly with bacteria have been associated with persistence and recurrence of otitis media and poor responsiveness to antimicrobial treatment (336).

Pneumococcal serotypes that most frequently cause AOM are largely the same as the most frequently carried ones, at least in unvaccinated children. The most frequent pneumococcal serotypes causing AOM serotypes in unvaccinated young children have thus belonged to serogroups 6, 14, 19 and 23 (124,125,284,337). The prominence of these serogroups decreases with age (124,337). When pneumococcal isolates recovered from MEF samples obtained during AOM were compared with those from age matched nasopharyngeal samples obtained at visits scheduled by age in the FinOM Cohort Study, small differences were found between some serotypes but not between genotypes in their relative ability to cause AOM, and there was no significant difference in the genotypic diversities of the AOM and carriage strains (338). In another Finnish study, likewise no major

differences in the clinical presentation of AOM across different serotypes were found (339).

2.5.2 Sinusitis and bronchitis

Pneumococci may spread from the nasopharynx and nose into the paranasal sinuses during upper respiratory infection and cause sinusitis, especially in older children and adults (340). Upper respiratory infection often spreads to the paranasal sinuses also in young children, but trapping of secretions is rare and the disease often resolves without antimicrobial treatment (341).

Acute bronchitis is considered to have a viral aetiology. However, *S. pneumoniae* and *H. influenzae* are involved in the chronic forms of bronchitis, chronic suppurative lung disease and bronchiectasis (342). In the pre-antibiotic era, these disease entities were common also in children as sequelae of respiratory infections, and they still remain a problem in developing countries and among disadvantaged indigenous groups (343). In industrialised countries these conditions are mostly associated with cystic fibrosis, immunodeficiency, tarcheomalacia and neuromuscular conditions (344).

2.5.3 Pneumonia

Pneumonia is an acute infection of the lung tissue, filling the alveoli with exudation and interfering with breathing and gas exchange. Bacterial pneumonia develops mostly as a result of aspiration of the bacteria from the nasopharynx or inhalation of the bacteria directly in air-borne droplets (345). The clinical diagnosis of pneumonia is based on the symptoms (cough, fever, pain or difficulty breathing, or gastrointestinal symptoms (346,347) and clinical signs (crackles or decreased breath sounds detected by auscultation of the lungs and signs of difficult breathing (346,348,349). A WHO working group has published guidelines for standard interpretation of chest radiographs in verifying the diagnosis of pneumonia in children, especially for purposes of epidemiological studies and vaccine trials (350).

The etiological diagnosis of pneumonia is difficult. If pneumonia remains restricted to the mucosa, there are no reliable tools for its etiologic diagnosis. Good quality sputum samples (351) are difficult to obtain without contamination by the upper respiratory flora. Classical pneumococcal pneumonia is considered to present with lobar or segmental consolidation in chest radiography and to respond

promptly to penicillin (352,353), but these characteristics are not reliable (346,347). If the bacteria spread to the blood and cause bacteraemic pneumonia, they may be detectable with blood culture (347,354).

S. pneumoniae is recognised as the most common cause of pneumonia in unvaccinated children all over the world, together with *H. influenzae* type b (355,356). The proportion of pneumococcal involvement in pneumonia has been estimated to be 20% or more, depending on diagnostic methods, severity of disease and population studied (347,355,357,358). Viruses are frequently detected in association with childhood pneumonia, either as the only causative agents or as counterparts in mixed bacterial-viral infections (347,357,359). Especially the influenza virus is known to predispose to pneumococcal pneumonia (360) and influenza-associated pneumococcal pneumonia tends to be especially severe (61).

2.5.4   Pneumococcal invasive disease

Invasive pneumococcal disease is an infection caused by pneumococci in a normally sterile body site. The bacteria can spread into the blood during pneumonia or locally from the nasopharynx to cause bacteraemia or they may spread through the blood-brain barrier to cause meningitis (69,340). Meningitis and other intracranial infections can be sequelae of AOM if the infection spreads from the middle ear via venous channels, through the inner ear, or through infected bone (292). Pneumococci can also gain entrance into the host through lesions caused by trauma or surgical procedures. Occasionally pneumococci cause cellulitis, osteomyelitis, pericarditis, pleuritis or arthritis. Because of strong inflammation, invasive pneumococcal diseases are always potentially life-threatening.

The standard diagnosis of invasive pneumococcal disease is based on culture of pneumococci from normally sterile body sites. This diagnosis is specific but insensitive and thus underestimates the incidence of pneumococcal disease, especially if preceding antimicrobial treatment has been given. In an Italian study, for example, only 34% of patients with clinical suspicion of invasive pneumococcal disease with positive pneumococcal PCR-tests from normally sterile body sites yielded pneumococci in culture (361). Using data from vaccine trials for epidemiological purposes, the difference in the disease incidence between vaccinated and unvaccinated individuals can be attributed to serotypes included in the vaccine (355,362,363).

A relatively small number of serotypes are responsible for the majority of invasive pneumococcal disease in children (364). There are differences across age groups and geographical areas in the most frequent serotypes causing invasive disease (364). In addition to true epidemiological differences, diagnostic practises may affect the observed serotype distribution, mild diseases being under-diagnosed with strict sampling policies (365). In unvaccinated populations, both the most frequently carried serotypes (6, 14, 19 and 23) and some rarely carried serotypes (1, 4, 5, and 7) with a special invasive capacity often cause invasive diseases (213,215,267,366). The capsular type is important to the ability of pneumococci to cause invasive disease (366). Nevertheless, distinct genetic clones showing the same serotype may have different abilities to cause invasive disease. (267,367). Within serotypes, the genetic diversity is lower among clones causing invasive diseases than among those carried (367,368).

2.5.5   Burden of pneumococcal diseases

The most common pneumococcal disease is otitis media. According to a recent review on the global burden of otitis media, the incidence of AOM is highest in Sub-Saharan Africa, Oceania and South Asia and lowest in East Asia, Central and Eastern Europe and Southern Latin America (369). Early onset, persistence and frequent sequelae of ear infections are very common also in many indigenous populations, e.g. among the native populations in Australia, North America and Greenland (370,371). The incidence is highest in children aged less than five years and half of the estimated more than 700 million cases per year occur in this age group (369).

AOM is also the most common cause of children's visits to outpatient clinics and antimicrobial treatment in developed countries (372-374). Up to 70-80% of children experience AOM by the age of 2–3 years (375,376). A rising trend of AOM occurrence was observed in both countries in the last decades of the 20th century (377,378). In Finland, with about 5 million inhabitants, an estimated 500 000 cases of otitis media occur annually, and in the USA, pneumococci alone are responsible for 5 to 7 million AOM cases each year (379,380).

Apart from causing more severe symptoms, pneumococcal AOM leads to serious bacterial complications more often than AOM caused by *H. influenzae* and *M. catarrhalis* (292,381). Such complications include mastoiditis and related infections (periostitis, retroauricular abscess), and intracranial infections like

meningitis, brain abscesses and lateral sinus thrombosis, all of which are life threatening and may be associated with permanent neurological damage (382). Inner ear problems, e.g. sensorineural hearing loss or vestibular dysfunction may also occur, although rarely (383). Instead, conductive hearing loss associated with long-lasting bilateral MEF is a common consequence of recurrent AOM and OME and chronic forms of otitis media often lead to permanent hearing loss. The burden of the sequelae and complications of AOM is especially high in the developing and low-income countries with limited health care resources (369).

Studies of the effects of hearing loss on speech, language skills or educational achievements have produced contradictory conclusions (382,384-386). In industrialised countries with adequate treatment possibilities the impact is mostly considered to be relatively minor (382,387-391).relatively minor (382,388-390). However, the harm may be considerable for children with co-existing conditions associated with delayed development, like Down's syndrome (389). In children in developing countries and among indigenous populations with high prevalence of chronic ear problems, hearing loss is common and has been found to lead to learning problems and disability (392,393). The unfavourable impact of recurrent AOM on the quality of life of the child and its caregivers has been recognised (394).

Since ear infections are very common, they form a considerable economic burden. In addition to the direct costs due to physician visits, antimicrobial treatment, surgical treatment to prevent recurrent cases and treatment of complications and sequelae, considerable indirect costs incur from parental absence from work, travel, and care arrangements for the sick child (379,395,396). High AOM morbidity and frequent antimicrobial use has led to increased resistance of bacterial strains against antibiotics, which poses a serious global problem (397-399).

The overall average annual incidence of invasive pneumococcal diseases in children aged less than two years was 44 per 100 000 before the implementation of pneumococcal infant vaccination in Europe (400). The corresponding incidence was 188 per 100 000 in the USA (401) and even higher in the developing countries and in some high risk populations (402-405). Pneumococcal pneumonia and invasive disease are rarer than ear infections, but pneumonia and invasive diseases usually require hospitalisation and the severity of these disease entities highlights their importance. Although the case fatality ratio for invasive pneumococcal diseases is lower for children than for adults (404,406), it has been estimated that almost one million children aged less than five years die because of pneumococcal infections each year (355). Most deaths occur in developing countries in Africa and

Asia, pneumonia being the most common cause (407). In Finland, on average only one child dies annually of pneumococcal diseases. Meningitis, although rare, has a very high case fatality ratio (355) and causes invalidising sequelae even in industrialised countries (408).

2.5.6   Treatment of acute otitis media

AOM, pneumonia and invasive diseases are mostly treated with antimicrobial agents. Since etiologic diagnosis is rarely available, treatment is usually empirical and relies on epidemiological data on the causative bacteria and their antimicrobial susceptibility in the population. For decades, pneumococci have been highly susceptible to penicillin, but recently the frequent use of wide-spectrum antibiotics has led to increasing antimicrobial resistance. Therefore, the need to reduce unnecessary antibiotic use, to improve prevention strategies and to find tools for monitoring and predicting the epidemiology of pneumococcal diseases has become evident.

A number of studies conducted in the 1980s and 1990s revealed that most AOM episodes resolve without antibiotic treatment (409-412). Thereafter, a new treatment strategy for AOM, 'an observation option' or 'watchful waiting' (413,414) was introduced for selected uncomplicated cases, which means delaying the decision on antibiotic treatment for a few days, with symptomatic treatment and appropriate follow-up. The option is included in the guideline recommendations to primary care clinicians, published by the American Academy of Pediatrics (306,307) and implemented in many other countries including Finland (415).

Surgical drainage of the middle ear by tympanocentesis or myringotomy with or without aspiration was an important treatment for AOM before the antibiotic era. The role of the procedure as a valuable adjunct to antimicrobial treatment was still stressed by otologists in the Finnish consensus conference 1985 (416,417). Later studies showed that the procedure does not offer any additional benefit over antimicrobial treatment (418-420) and it is currently mostly restricted to children with serious disease or an underlying condition, intensive pain or special need of etiologic diagnosis. However, the role of tympanocentesis and myringotomy in the diagnosis and treatment of AOM has been occasionally revisited (421). Pichicero et al. showed that recurrent AOM and tympanostomy could be prevented if tympanocentesis was performed in event of failure of the primary treatment and

antimicrobial treatment was selected according to the etiologic bacterium and its antimicrobial susceptibility (422).

2.5.7   Prevention strategies for pneumococcal diseases

Vaccination is the most effective way to prevent pneumococcal invasive diseases and efforts have been paid to investigate its potential in reducing the burden of pneumococcal otitis media. A 23-valent polysaccharide vaccine has been available since 1983. It has some effect against bacteraemic disease in adults, but children younger than two years of age show minimal immune responses against most polysaccharide antigens. The efficacy of the polysaccharide vaccine against mucosal infections like otitis media is poor (423). When the polysaccharide antigens are conjugated to carrier proteins, however, these antigens become immunogenic also in young children and induce immunologic memory.

The first conjugate vaccine was licensed in 2000. The vaccine was primarily targeted to prevent invasive diseases, and it included antigens against seven serotypes (4, 6B, 9V, 14, 18C 19F and 23F). This vaccine covered >80% of serotypes causing pneumococcal invasive disease in children in the USA (365,424). The corresponding coverage was 60–70% in Europe before the vaccination era, with some additional coverage if serotypes cross-reacting with the vaccine serotypes are also considered (364,400,424), while coverage was only about 50% or less of serotypes causing invasive diseases in children in Asia and Africa, areas with the highest burden of pneumococcal diseases (364,424). In studies among children in the USA and Finland, the efficacy of the 7-valent conjugate vaccine was 97% against invasive pneumococcal diseases (284,425). The next step in the vaccine development was to add serotypes 1 and 5, which are common causes of invasive disease in many developing countries. In studies in South Africa and The Gambia, an experimental, never licensed 9-valent vaccine showed 72-83% efficacy against paediatric invasive pneumococcal diseases and 17-37% efficacy against all-cause radiologically confirmed pneumonia (426,427). A 10-valent vaccine including an additional serotype 7F prevented 100% of vaccine-type invasive disease in Finnish children (428).

In a Finnish vaccine trial, the efficacy of the 7-valent vaccine was 57% against AOM attacks caused by the vaccine serotypes (284). In Finland and the USA the vaccine prevented only 6-8% of all AOM cases (284,425,429), but protection against frequent episodes of otitis media and tympanostomy tube placement was

better, at 20–40% (425,429-431). In the 10-valent vaccine, the conjugated carrier protein originates from *H. influenzae*. The vaccine prevented 67% of vaccine-type AOM in Panama (432), and supplemented with serotype 3 it prevented 34% of all-cause clinical episodes of AOM in a trial conducted in the Czech Republic (433). A 13-valent vaccine with additional antigens against serotypes 6A and 19A, also relative common otopathogens, especially in unvaccinated populations, was licensed in 2009 on the basis of immunogenicity studies. The use of 10- and 13-valent vaccines has now practically replaced the use of the 7-valent vaccine.

In clinical trials of the 7- to 10-valent conjugate vaccines the relative reduction in the risk of pneumococcal carriage due to serotypes included in the vaccine has been about 50–60% as based either on carriage prevalence ratios or odds ratios (282,434-436). The vaccine prevents new acquisitions rather than affecting carriage established before vaccination (434,436). Rinta-Kokko et al. presented a method for evaluating the vaccine efficacy against acquisition of carriage using prevalence data (437). The authors applied their approach to data from five published trials and found that the vaccine efficacy against acquisition of vaccine-type carriage varied between 44% and 65% and the overall average efficacy against pneumococcal carriage acquisition seemed to be about 50% (437). Vaccination has been shown to also decrease the density of vaccine-type carriage (434). The protection against carriage lasts for several years (280). Most vaccine trials have demonstrated almost complete serotype replacement in carriage after vaccination (282,434,436,438).

By the end of 2013, pneumococcal conjugate vaccines had been introduced into childhood vaccination programs in 103 countries, and the global coverage of infant immunisation was estimated to be 25% (439). Following vaccine introduction, the incidence of invasive vaccine-type disease has declined remarkably, not only in the vaccine target population but also in other age groups. This indicates herd immunity, i.e. indirect protection through reduced carriage and transmission of the vaccine serotypes by vaccinated children (400,401). Although the direct effects in the target population for vaccination are clearly beneficial in most countries, the benefit of the indirect effect has been questioned because the other type of indirect effect, i.e. replacement disease in the non-target population has been relatively high. There is heterogeneity between countries in the occurrence and timing of replacement in disease, (285,440) and in some countries, especially in Europe, the net benefit in the non-target population has been questioned. Reasons for the variation in patterns of replacement may be the different and changing blood culture practices, with only the most severe cases being captured with more

stringent policies (440). Secular changes in serotype distributions and vaccination coverage may also have an effect (285,440).

One solution to the problem of replacement is to increase the serotype coverage of pneumococcal vaccines. A 15-valent vaccine is already under clinical testing (Clinicatrials.gov NCT01215175), but considering the wide variety of carried serotypes, the replacement phenomenon may remain troublesome. Nurhonen and Auranen introduced a model for predicting the optimal serotype composition in a vaccine to gain the best possible net benefit in the population. The model is based on assumptions about the good effectiveness of vaccination in reducing carriage of the vaccine serotypes and on their replacement with the non-vaccine serotypes. In addition, knowledge of pre-vaccination age- and serotype-specific carriage prevalences and probabilities of invasive disease per carriage episode ("case-to-carrier ratios") is needed (441). As another approach for overcoming the problem with replacement, several proteins conserved across all serotypes have been tested as vaccine antigens, some of which have reached clinical trial phase in humans (442,443).

General strategies for preventing childhood respiratory infections including pneumococcal otitis media comprise of favouring small day care groups with improved hygiene routines (444,445), avoiding use of a pacifier at the otitis-prone age (444,446) and using xylitol chewing gum (447). Anti-adhesive oligosaccharides(448), probiotics (449) and alpha-haemolytic streptococci with bacteriocin-like activity (71) have proved promising in inhibiting growth of otopathogens in vitro and in preclinical studies (528), but their ability to prevent ear infections in clinical studies has been controversial or disappointing (450-453). Recurrent attacks of otitis media and prolonged OME can be prevented by the insertion of ventilation tubes, with or without adenoidectomy (454). According to current recommendations the benefits of prophylactic antimicrobial treatment should be carefully weighed against potential harms (306). Influenza vaccination is an effective way to reduce otitis media and other secondary bacterial infections during the epidemic (455), and vaccines against other respiratory viruses are under development.

2.6 Pneumococcal carriage, viral upper respiratory infection and otitis media

Pneumococcal carriage with frequent turnover of a large number of different strains is common in healthy children. At the same time, carriage is a pre-requisite for pneumococcal diseases, including AOM. Both pneumococcal carriage and otitis media are associated with upper respiratory tract infection, generally considered to be a viral disease, and all three share several common predisposing factors. Although much is known about the dynamics of pneumococcal carriage, respiratory infections and development of otitis media, the interplay between these conditions is far from fully understood.

2.6.1 Pneumococcal carriage and respiratory infection

Acute upper respiratory tract infection is most often caused by rhinoviruses. Other major respiratory viruses are respiratory syncytial virus, influenza virus, parainfluenza virus, adenovirus, coronavirus, enterovirus, bocavirus and metapneumovirus, most of which can be divided into subtypes. Combining different detection methods including PCR to search for a wide panel of viruses, the presence of viral particles can be confirmed in virtually all nasopharyngeal specimens from young children during symptoms of respiratory infection (76,456,457). Accordingly, symptoms of respiratory tract infection are frequently considered as markers of a clinically significant viral infection (458). Serial respiratory infections are mostly not caused by the same viruses, as detection of the same virus strain ≥2 weeks apart was unusual in a prospective follow-up study among US infants (459). Upper respiratory tract infection is very common in children except during the first few months of life. Preschool children suffer on average about five episodes per year (460-462).

Viral infection of the upper respiratory tract has a substantial impact on pneumococcal transmission and acquisition. Symptoms of upper respiratory infection, coughing, sneezing and increased nasal secretion offer effective means for the spread of bacteria by droplets or through contamination of hands or fomites. In a study of intra-familial transmission of pneumococci and its relation to respiratory infection, the inferred donors of pneumococci to other family members tended to have respiratory symptoms more often in the two weeks before transmission events than at other times (113). Viral co-infection has been

associated with increased pneumococcal load in the nasal cavity of children (463) as well as in the nasopharynx of ferrets and infant mice (464,465). In the mouse and ferret models, pneumococcal transmission was more intense if both the donor animals, colonised with pneumococci, and the contact animals were experimentally infected with an influenza virus (464,465). In ferrets, influenza infection increased especially the susceptibility of the contact animals to pneumococcal acquisition and these could acquire pneumococci over longer distances than animals not infected with influenza (464).

Viral infection increases bacterial adherence to the epithelial cells and enhances translocation of bacteria through the epithelial barrier (61). In several in vitro studies, enhanced binding of *S. pneumoniae* to the human epithelial cells occurred if the cells were infected with influenza virus, rhinovirus or a specific adenovirus type as compared to uninfected cells (466,467).In a study among adult volunteers pneumococci, adhered in increased numbers to cells from individuals experimentally infected with influenza virus (468).

Knowledge on the mechanisms by which viral respiratory infection contributes to bacterial adherence has increased considerably during the last two decades. Viral infection up-regulates receptor molecules responsible for pneumococcal attachment (56,61,469). The neuraminidases of the influenza virus, together with the pneumococcal neuraminidases, probably cleave sialic acids from the cell surface molecules (467). It has also been suggested that fibrin and fibrinogen deposits during the regenerative processes after viral infection or viral glycoproteins act as mediators for bacterial binding (61). Viral infection also leads to impairment of the ciliary function, thus slowing down the clearing of bacteria from the mucosa (61,470), and it impairs the function of the polymorphonuclear leucocytes (471).

Experimental studies in animals have shown that viral infection predisposes to the establishment of pneumococcal carriage in vitro (464,472). When chinchillas were challenged intranasally first with an influenza virus and subsequently with pneumococci seven days later, the frequency of nasopharyngeal pneumococcal carriage was higher when compared to animals inoculated with pneumococci without preceding viral infection (472). No difference was seen in chinchillas infected with the adenovirus.

In a large number of epidemiological studies from different parts of the world, the prevalence of pneumococcal nasopharyngeal carriage in children has been 1.2 to almost 3 times more likely during acute respiratory symptoms or AOM than during health (79,113,151,153,175,180,242,259,473,474). In a follow-up study among US children during the first three years of life, significantly higher

nasopharyngeal isolation rates of *S. pneumoniae* were observed at physician visits due to upper respiratory tract infection or otitis media as compared to routine healthy visits (473). In an outpatient clinic in Mexico, the frequency of pneumococcal carriers in patients with upper respiratory tract infection, 33% of which had otitis media, was greater than in patients without upper respiratory tract infection (33% *vs.* 21%)(242). In Uruguay, 42% of children seeking ambulatory medical care for acute respiratory infection carried *S. pneumoniae* in their nasopharynx whereas only 15% of healthy children were carriers, and the frequency of heavy or moderate oropharyngeal carriage was higher in hospitalised children with pneumonia than in healthy controls (153). In Pakistan, children fulfilling criteria of acute lower respiratory tract infection carried *S. pneumoniae* significantly more often than healthy children attending immunisation clinics (64% *vs.* 52%, respectively) (474). Also preceding respiratory infection within one month has been identified as a risk factor for pneumococcal carriage (475).

There are also clear associations between pneumococcal carriage and clinical AOM. In Greenland, children with concurrent AOM carried *S. pneumoniae* significantly more often than their age-matched controls (48% *vs.* 29%) (476). In US children followed up to 3 years of age, otitis-prone children carried pneumococci significantly more often than non-otitis-prone children during respiratory infection or AOM (473). In another US study, children were at low risk for AOM during respiratory infection if they did not harbour pathogenic bacteria in their nasopharynx (477). In an Australian study, nasopharyngeal load of *S. pneumoniae* was higher in children with suppurative otitis media than in children with healthy ears (478).

Pneumococcal carriage has been associated with the frequency of AOM also in many longitudinal studies (216,219,240,479). In a one-year follow-up of healthy children receiving immunisations and medical care in a study clinic in the USA, AOM was diagnosed more often during visits at which pneumococci were carried than during those at which it was not isolated (240). In Swedish children followed with three nasopharyngeal samplings during the first year of life, the occurrence of AOM was significantly associated with nasopharyngeal carriage of pneumococci at age 8 to 12 months but not earlier (479). Among Australian Indigenous children followed from birth at 2 to 4 week intervals for up to 15 months, the early onset and frequency of otitis media was better explained by early colonisation with pathogenic bacteria, especially *S. pneumoniae*, *H. influenzae* or mixed colonisation, than by age (216). In an intensive follow up of US children during the first year of life the frequency of pneumococcal carriage was correlated with the occurrence of

AOM and OME, but in this study early detection of pneumococcal carriage (at <3 months of age) did not increase the risk of the first otitis media episode (219). Pneumococcal carriage during the first year of life also did not affect the risk of otitis media during the second year of life in a cohort of children in the Netherlands (480).

There are also studies in which no difference has been observed in the frequency of pneumococcal carriage between respiratory infection or AOM and health (150,207,215,217,222,237,481,482). In a study in Papua New Guinea, almost all children carried pneumococci even during health with little differences according to the health status (215). However, children carried serotypes defined as 'invasive' more often during acute lower respiratory tract infection than during health. Similarly, in studies in Pakistan and in Uruguay, children with respiratory infections carried more frequently serotypes that caused invasive pneumococcal disease in the same area, as compared to healthy children (153,474). This association may mainly apply to children with severe or lower respiratory infection, since children suffering from mild respiratory infections or AOM mostly carry similar serotypes than healthy children (242,483,484).

2.6.2 Respiratory infection and acute otitis media

Both viral and pneumococcal structures induce production of cytokines and other inflammatory mediators (57,62,64,485,486). The development of inflammation has an important role when asymptomatic pneumococcal carriage progresses to AOM. Inflammatory markers have been found in MEF from children with AOM (487), and higher concentrations in the nasopharynx have predicted development of AOM during upper respiratory infection (488). In a mouse model, replication of the influenza virus and presence of haemagglutinin were associated with middle ear inflammation, which in turn induced growth of pneumococci in the middle ear cavity (489).

Viral infection and the subsequent inflammation cause cytotoxic cell damage (61). The inflammation induces vasodilatation, increased vascular permeability, secretive activity and congestion of the mucosa, which leads to dysfunction of the Eustachian tube and accumulation of fluid into the middle ear. This has been demonstrated by abnormal tympanometric findings in animals experimentally infected with influenza virus (471). In a clinical study among schoolchildren, the middle ear pressure decreased transiently during 66% of upper respiratory

infections not leading to AOM, mostly within seven days after the onset of symptoms (490). In adults, experimental viral infection caused abnormally negative middle ear pressure even without development of symptoms of respiratory infection (491). Furthermore, viral infection, especially influenza infection, causes impairment of the leucocyte function as shown by animal experiments (471) and also observed in humans (492). According to mouse models and in vitro studies, influenza infection contributes to bacterial growth via the formation of neutrophil extracellular traps (493,494) and by inducing release of bacteria from the biofilm (88).

Experimental studies have shown a clear synergistic effect between the influenza virus and pneumococci in development of otitis media. In a study of Giebink et al., otitis media developed in 67% of chinchillas inoculated intranasally concurrently with pneumococci and influenza virus, versus 21% and 4% when the animals were inoculated with *S. pneumoniae* or influenza virus only (495). In another experiment, the middle ear pressure in chinchillas declined and reached a nadir on the seventh day after intranasal inoculation with influenza virus, while polymorphonuclear leucocyte dysfunction was seen 4–6 days after inoculation, whereas neither occurred after inoculation with pneumococci alone. In this study, the highest incidence of pneumococcal otitis media occurred when pneumococci were inoculated just before the time of influenza-induced leucocyte dysfunction and negative middle ear pressure (471). In a third study, in which chinchillas were inoculated intranasally first with influenza virus and then with pneumococci seven days later, the otoscopic signs of tympanic membrane inflammation peaked eight days after the pneumococcal inoculation (496). Influenza virus also increased the incidence and severity of otitis in an experimental model with sequential inoculation first with viruses and *S. pneumoniae* seven days later (472).

Epidemiological studies have documented clear temporal associations between seasonal laboratory-documented epidemics of respiratory viruses and the occurrence of AOM, viral epidemics peaking either shortly before or concurrently with the peak occurrence of AOM (223,497-499). The clinical association between viral infection and AOM is evident as well: more than 90% of children with AOM have symptoms of upper respiratory tract infection at the time of diagnosis (296,303,324). Rhinovirus is the virus most frequently found in children during upper respiratory infection and AOM and also when asymptomatic (288,456,456,457,500,501), but during rhinovirus infection the risk of AOM has not been consistently higher than during viral infection in general (461,497,502).

About 20-50% of the upper respiratory infection episodes in children lead to AOM (458,500,503). In follow-up studies, most AOM cases have been diagnosed within two weeks after the onset of symptoms of upper respiratory infection, the average and peak occurrence falling between days 2 and 6 after symptom onset (458,477,501,504). Upper respiratory infections are associated also with increased risk of OME (461), and detection of viruses in the nasopharynx of even asymptomatic children has been associated with development of OME (500,505).

Numerous clinical studies show that children with respiratory syncytial virus infection have a special risk for development of AOM (223,461,499,505). Further, of influenza infections of children aged less than three years 40% were complicated by AOM in a Finnish follow-up study (506). There is more variation in the observed propensity of other specific virus infections to lead to AOM (76,223,461,497,499,502,505,507,508). However, as the most common virus causing respiratory infections, rhinovirus is the most important virus associated with AOM. High loads of viruses in the nasopharynx have inconsistently been associated with the risk of AOM (502).

2.6.3   Pneumococcal carriage and pneumococcal acute otitis media

The frequent occurrence of pneumococci in the nasopharynx during culture-confirmed pneumococcal AOM in children has been almost a uniform finding (125,155,262,509-513). This means that the sensitivity of nasopharyngeal culture in predicting pneumococcal aetiology of concurrent AOM is high. The sensitivity was 90% based on aggregated data of 4791 paired nasopharyngeal and MEF samples available in six published studies (514). Generally, if pneumococci have been isolated concurrently both from the nasopharynx and from the MEF, the serotypes have been homologous in more than 90% of cases (124,125,510,513,515). If characterised beyond the serotype level, a vast majority of paired isolates from the nasopharynx and the MEF showed the same resistance patterns (513) or indistinguishable genetic patterns in a PFGE analysis (516).

The high sensitivity of nasopharyngeal culture means that the value of a negative nasopharyngeal finding in excluding pneumococcal aetiology of concurrent AOM (negative predictive value) is high. However, pneumococcal carriage is common both in healthy children and in children with non-pneumococcal AOM, which results in low specificity and a modest value of a positive nasopharyngeal finding to predict pneumococcal aetiology of concurrent

AOM (positive predictive value) (262,510,512). The positive predictive value based on combined data from six published AOM studies was 50% (514).

Temporal associations between pneumococcal carriage, respiratory infection and development of acute otitis media

Understanding the development of carriage and disease requires approaching them as dynamic longitudinal processes in time. Only a few follow-up studies have assessed the temporal relationship between acquisition and carriage of *S. pneumoniae*, respiratory infection and the development of otitis media.

Brimblecombe et al. observed a close temporal relationship between acute upper respiratory infection and acquisition of pneumococcal carriage in a follow-up study with fortnightly visits to UK families in the 1950s (171). Based on thorough monitoring of respiratory symptoms and collection of nasal and throat swab samples, carriage was observed to increase 1-2 days after the start of the symptoms of acute coryza and children carried *S. pneumoniae* twice as often when they had nasal discharge as compared to when they did not (171).

Sleeman et al. followed a cohort of UK children from birth up to 24 weeks of age collecting nasopharyngeal swab samples 2–4 weeks apart to study the relationship between pneumococcal carriage and physician visits for infection (220). Pneumococcal acquisition, defined as the first detection of a new serotype, was significantly associated with visits for infection potentially caused by pneumococci, but not with visits because of other infections, whereas no association was found between established pneumococcal carriage (subsequent to acquisition) and visits for infection.

Grijalva et al. conducted a study among Peruvian Andean children aged less than three years, during the introduction of pneumococcal conjugate vaccines in the population in 2009–2010 (517,518). If acute respiratory infection was present during weekly household visits, nasal swabs were collected for identification of respiratory viruses. Nasopharyngeal swab samples were obtained at monthly visits for detecting acquisitions of new pneumococcal strains by culture and PCR-based methods. The risk of pneumococcal acquisition was about two-fold after exposure to influenza or parainfluenza virus and increased also after viral co-infections, whereas exposure to viruses in general symptoms of respiratory infection irrespective of the viral finding did not increase the risk of pneumococcal acquisition. The increase in the acquisition risk was only observed in children who already carried a pneumococcal strain. The pattern was relatively constant when stratified by vaccination status (518).

Gray et al. conducted a prospective follow-up among 82 Alabaman children aged less than two years (125,229). Nasopharyngeal swab samples were collected at 1 to 3 month intervals as well as during visits due to respiratory infections, and MEF samples were obtained if AOM was diagnosed. The authors observed that pneumococcal AOM developed mostly within one month after acquisition of the causative serotype (125,229). In contrast, in the study in Papua New Guinea, the authors observed an association between the frequency of serotypes detected from children admitted to hospital because of pneumonia and frequency of serotypes carried in a nearby area, the association being similar for new acquisitions and prevalent carriage (231).

Henderson et al. conducted a longitudinal study among children in a day care centre in North Carolina to assess the relative importance of viral respiratory infection and nasopharyngeal colonisation with *S. pneumoniae* in development and occurrence of AOM (223). The health status of the children was reviewed by nurses five days a week and nasal wash and throat swab samples for bacterial and viral detection were collected every two weeks and at onset of respiratory illness. A higher incidence of otitis media was associated with concurrent or preceding (within 14 days) detection of respiratory syncytial virus, adenovirus and influenza viruses, with only a modest increase if *S. pneumoniae* was also present. The presence of *S. pneumoniae* or *H. influenzae* had a more pronounced effect on the risk of otitis in the presence of such viruses which alone posed a small risk of otitis media, such as rhinovirus, enterovirus and parainfluenza virus. If no viruses were detected, the incidence of otitis media was only marginally associated with pneumococcal carriage during the preceding two weeks and pneumococcal acquisitions measured as first detections of individual serotypes did not increase the risk of otitis media as compared to established carriage (223). Overall, the frequency of otitis media was correlated with early or recurrent infection with respiratory syncytial virus, adenovirus and influenza viruses but not with early or frequent colonisation with *S. pneumoniae*.

2.7 Pneumococcal carriage and pneumococcal invasive disease

Children very often harbour *S. pneumoniae* in their nasopharynx also during invasive culture-confirmed pneumococcal disease. In studies conducted in Pakistan, Papua New Guinea and The Gambia, pneumococci were found in 90–96% of nasal or

nasopharyngeal samples in children with pneumococcal invasive diseases and in 77–99% of these cases the serotypes found in the nasopharynx and the normally sterile body site were homologous (187,474,519). In the study in Papua New Guinea, 94% of the blood serotypes were also found from the nasopharynx if they belonged to the frequently carried serogroups 6, 14, 19, 23, or to a large group of serotypes that were rarely carried and rarely caused invasive pneumococcal disease in the study area (519). By contrast, only 50% of serotypes found in the blood were concurrently harboured in the nasopharynx if they were labelled 'invasive', i.e. belonged to a group of serotypes frequently causing invasive diseases but less frequently found in carriage in the study area.

2.8   Other determinants of pneumococcal carriage, upper respiratory infection, acute otitis media and pneumococcal disease

Studies from different parts of the world document an extraordinary wide range of pneumococcal carriage prevalence (3.5-90%) in children (156,249,520). This variation is likely due to differences across the study populations with respect of age, current and underlying health status and treatment, genetic background as well as socio-economic living conditions and cultural habits. In addition, different sampling and laboratory methods may contribute to the differences. Factors affecting the occurrence of upper respiratory infections and otitis media are mostly the same as those associated with pneumococcal carriage (521) and also pneumococcal invasive diseases share similar risk factors. However, there are also differences, and it is not well known whether these differences are due to shortcomings in the methodology in investigating the associations, or whether there are mechanisms by which some factors specifically affect the progress of carriage to disease.

The incidence of respiratory tract infections, AOM and invasive pneumococcal diseases starts to increase after protection by maternal antibodies has waned by 6 months of age (110,111,462), especially in developed populations (112). Pneumococcal carriage often starts to increase already during the first months of life. The increase of pneumococcal carriage frequency may continue during the second and third years of life, after which it remains at a relatively high level up to school age, whereas the incidence of respiratory infections, AOM and invasive diseases usually peaks earlier at about one year of age or even earlier

(131,164,223,375,376,462,522-524). The maturation of immunological mechanisms, increasing acquired immunity to microbes as well as anatomical changes in the respiratory tract and Eustachian tube are the likely factors contributing to decreasing susceptibility of children to these infections by age.

Both viral and pneumococcal infections are common in children, and both are transmitted in close contacts. Thus, spending time with other children such as day care mates or siblings is the most indisputable risk factor for pneumococcal carriage (175,197,246,261,479), respiratory tract infections (525,526), AOM (444,527-532) and invasive pneumococcal disease (533-535). Only few studies have failed to demonstrate the impact of day care (243,480) or siblings (243,536) on carriage, AOM and pneumococcal invasive disease. Some authors have been able to distinguish differences in the risk by the degree of exposure to other children, such as the size of day care group (212,444,537,538), time spent in the day care (206,444,539) and the number of siblings (239), while others have not (125,205,218,540). Some but not all studies have associated the increased risk specifically with young age of siblings (184,195,540), with their day-care attendance (192,218,537), with their pneumococcal carriage, respiratory symptoms or AOM (177,246,375,529) or with sharing a sleeping room with siblings (375).

Viral respiratory infections, AOM, pneumonia and invasive pneumococcal diseases show clear seasonality with peaks in winter (223,497,499,541).Alternatively, a bimodal pattern with peaks in the spring and autumn has been observed (376,460,498,523,524,529). Seasonal trends have been associated with cold or ambient temperatures, humidity and darkness (529,541) or also with dry seasons in Africa (164). Additional explanations may be increased transmission associated with people spending more time inside in close contact with each other during the cold, dark or rainy seasons (209), or with periodical changes in the susceptibility of the population to various infections (541). Seasonality in pneumococcal carriage is less clear and several studies have failed to show any seasonal trends (183,215,217,237,248,542,543). A trend to more prevalent carriage in the winter or in association with cold weather or rainy seasons has been reported in a number of studies (153,171,175,179,207,209,222,544), but occasionally even higher prevalence of carriage in summer has been reported(250). In a follow-up study among US families, clear peaks were observed in carriage but the peaks did not occur at the same time during different years and no consistent seasonal trend was seen (542). In two other US studies, the pneumococcal carriage prevalence fluctuated mildly throughout the year, while the acquisition peaked in midwinter (229). In a Finnish study, the seasonal trend was clear for AOM caused

by *H. influenzae* and *M. catarrhalis* but weaker for pneumococcal AOM, which occurred frequently also in the summer (324). The authors of two Swedish studies explained the observed seasonality in pneumococcal carriage in day care centres by the influence of major holidays (209,212).

Males are over-represented among patients with many infectious diseases (545). In adults, the higher risk has been partly attributed to behavioural patterns like smoking and alcohol use (266,546). Nevertheless, male sex has remained a risk factor even when adjusting for these habits (266), and sex steroids are hypothesised to affect immunity and susceptibility to infections (545). Likewise, invasive pneumococcal infections in children tend to be more common in boys than girls (405,523,524). Furthermore, many authors have found a significant association between the risk of otitis media and male gender (375,377,522,530,532) whereas others have not (319,444,547-549). Instead, the frequency of pneumococcal carriage has been similar in boys and girls in most studies (156,174,180,207,218,235,243,248,249). Only in a few studies has the frequency of carriage been higher in either boys or girls, and often in narrow age groups only (217,222,246,252).

Many chronic diseases and conditions impair the host defence mechanisms, predisposing to pneumococcal invasive infections, pneumonia and AOM. Anatomical or functional defects in the lungs, liver, kidneys, nervous system or middle ear and adjacent organs, metabolic and neoplastic diseases or their treatment predispose to these infections (287,550). Globally, the most important of immunological deficiencies predisposing to pneumococcal infections are those caused by human immunodeficiency virus (HIV) infection (551), hereditary sickle-cell disease (552) and various causes of anatomical or functional splenic dysfunction. The effect of these conditions on pneumococcal carriage is not as clear, and the treatment often interferes with interpretation (553), but at least immunological disorders are suggested to affect the carriage (554). In two Kenyan studies, the prevalence of pneumococcal carriage was higher in HIV infected than in healthy children (238), especially during respiratory infection (238,555), and in a South African study even HIV exposed although uninfected children carried pneumococci more often than HIV unexposed children (165). Obvious immunodeficiencies are rarely diagnosed in children suffering from frequent repeated episodes of respiratory infections and AOM, but minor or transient deviations in their immunological system may occur (130,556,557). Severe atopy, allergy, asthma and gastrointestinal reflux probably predispose to respiratory infections (558) but their impact on prolonged and recurrent middle ear problems

or carriage has remained controversial and associations with pneumococcal carriage have been found only occasionally (181).

In developed countries, differences in socio-economic conditions are relatively small and the roles of income, education and employment of parents, type of housing, living conditions and other markers of socio-economic status on carriage of pneumococci, respiratory infections, AOM and invasive pneumococcal diseases in children have remained controversial (235,375,529,530,532,534,547). However, crowding has been identified as a risk factor for these infections in several studies also in developed countries (171,177,209,533,539), although not in all (195,222). Furthermore, when investigated in detail, a diet rich in added sugar increased the frequency of pneumococcal carriage, whereas a diet rich in fruit and berries and low in sugar decreased the risk of AOM in Finnish children (559). It is likely that the high occurrence of pneumococcal carriage and infectious diseases in developing countries and high risk populations in developed countries is largely attributable to socio-economic factors (185,535,560,561), malnutrition, vitamin A deficiency (180), poor housing or hygiene, smoky firewood, and crowding (175,215,239,562). Poor access to health care may worsen these problems (563).

Genetic and environmental factors affecting the risk of infection are difficult to differentiate from each other. Indigenous populations with high risk of pneumococcal infections and otitis media do not share common genetic heritage, although according to a recent theory, their non-European background could refer to a genetic background for their higher susceptibility to pneumococcal infections against which people with a European background could be genetically less susceptible (564). However, the socio-economic status and cultural lifestyles of indigenous people often differ from those of the non-indigenous populations, and this difference complicates the interpretation of epidemiological data. Furthermore, the declining rate of invasive pneumococcal diseases in Navajo children aged less than 3 years from 1989 to 1996 speaks for environmental factors affecting the risk in this population (402). Genetic factors may predispose to infectious diseases through inherited anatomical structure (565), specific hereditary diseases (552), or immunological aberrations (557). Data from the Netherlands suggest that genetic variation of innate immune responses plays a key role both in the defence against middle ear disease in childhood and in responsiveness to pneumococcal vaccination (566). Studies in the USA have shown no differences in the risk of otitis media between black and white Americans (319). However, in twin/triplet and adoption studies, a genetic component has been found in susceptibility to otitis

media (567) and modern genetic studies are expected to identify genes that influence the risk of otitis media (568).

Tobacco smoke contains chemicals that cause inflammation of the respiratory tract mucosa and harm its function. Adherence of bacteria to human cells has been shown to be more effective in smokers than non-smokers (569,570). Active smoking has been identified as a very strong risk factor for invasive pneumococcal disease in adults (266). In children, the real quantity of exposure to smoke is difficult to measure and it may be associated with socio-economic factors. Thus, the role of passive smoking is clearly recognised in many studies as a risk factor for pneumococcal carriage (128,217,239,252), otitis media (530,532,537,547), and pneumococcal invasive disease (534,535), but remains undetected in others (235,529,571). In Alaska, the presence in the household of at least one person chewing tobacco rather than the presence of a smoker was an independent risk factor, indicating that the effect of parental smoking may be at least partly due to increased susceptibility of the smoking parents to infections and subsequent transmission of infection to their children (572). Indoor air pollution due to combustion of biofuels is regarded as a risk factor for pneumococcal carriage and acute respiratory infections in developing countries.

There are also factors that decrease the susceptibility of children to pneumococcal carriage and infection, including otitis media. Human milk has properties that protect against bacterial attachment in the human respiratory epithelial cells (573). The milk secretory immunoglobulin A antibodies inhibit microbes on the mucosa, while antimicrobial molecules such as lactoferrin and oligosaccharides may resemble the receptors for pneumococcal attachment (573,574). It has also been suggested that breast milk has a unique capacity to stimulate the immune system of the infant after which it might respond better to infections (575). Breast-feeding is often associated with other potential factors affecting bacterial carriage or disease, e.g. age, nutritional and hygienic quality of alternative food, time spent in day care, education of the parents or other socio-economic factors. Hence, it appears plausible that such confounding could explain some of the controversial findings about the protective effect of breast-feeding against pneumococcal carriage (156,576), AOM (375,479,480,529,532,537,547,576), and invasive pneumococcal diseases (533,534).

Xylitol inhibits the growth of *S. pneumoniae* (577). It has been found to reduce pneumococcal adherence to human epithelial cells in vitro in a Finnish study (578), but not in a Spanish study. (579). Xylitol decreased the occurrence of AOM in Finnish day care children when used regularly five times a day (447). In animal

studies, xylitol did not decrease nasopharyngeal carriage of pneumococci in rats (580) but activated neutrophils and increased the survival time of rats during experimental pneumococcal sepsis (581). Also probiotics decrease pneumococcal adhesion to human epithelial cells in vitro (449). The ingestion of probiotics reduces pneumococcal carriage in adults (582), and a beneficial effect in preventing respiratory infections and otitis media in children has been suggested (453), although in a randomised, controlled trial no effect was seen against either otitis media or pneumococcal carriage (452). Of other child care habits, use of a pacifier has been identified as a risk factor for otitis media (547), whereas sucking of the thumb or use of nursing bottle have not (583).

The use of antimicrobial agents to treat pneumococcal diseases and AOM also affects pneumococcal carriage. With very few exceptions, lower carriage rates have been reported in studies that compare children with recent antimicrobial treatment in the last month to children without treatment (184,221,246,484,584,585). In other studies, the carriage rate had clearly decreased a few days or 2–3 weeks after the end of the antimicrobial treatment, after which it increased again, rebounded to the initial level or even surpassed it about one or two months after the treatment (221). A trend towards a protective effect of antibiotics for as long as two months has occasionally been suggested (586).

There appears to be an association between clearance of the carried strain and its susceptibility to the antimicrobial agent: carriage of susceptible strains is reduced whereas carriage of non-susceptible strains remains at the same level (484,584,587), resulting in an increase in the proportion of non-susceptible strains. Consequently, antimicrobial treatment selects resistant strains, and recent or frequent antimicrobial use has proven to be a risk factor for carriage of non-susceptible strains both at the individual (193) and population levels (397-399). In an Israeli study, a resistant strain in the nasopharynx replaced the initially susceptible strain in the middle ear within a few days after the start of antimicrobial treatment of AOM (588), which is a good example of the close relationship and dynamics between the bacterial flora in the nasopharynx and the middle ear.

3 AIMS OF THE STUDY

The aims of this thesis were to investigate the relationship between respiratory infection, pneumococcal carriage and pneumococcal AOM.

The specific objectives were:

1. To evaluate the course of nasopharyngeal carriage of *S. pneumoniae* during health and during respiratory infection with and without acute otitis media in children from the age of 2 months to 24 months (I).

2. To evaluate the value of nasopharyngeal culture in predicting the pneumococcal aetiology of acute otitis media (II).

3. To evaluate the dynamic interplay between acquisition of pneumococcal carriage, respiratory infection and development of pneumococcal acute otitis media (III and IV).

4 MATERIALS AND METHODS

4.1 Finnish Otitis Media (FinOM) Cohort Study

The material for this thesis was derived from the Finnish Otitis Media (FinOM) Cohort Study, in which 329 children were prospectively followed from 2 months to 24 months of age. The FinOM Cohort Study was conducted by the National Institute for Health and Welfare (THL, the former National Public Health Institute, KTL) in collaboration with the Public Health Centre of Tampere City and the Tampere University Hospital (TAUH).

The aims of the FinOM Cohort Study were to assess the natural course of nasopharyngeal carriage of *S. pneumoniae* and to collect data on the epidemiology of pneumococcal carriage and pneumococcal AOM prior to The Finnish Otitis Media (FinOM) Vaccine Trial, which addressed the efficacy of two 7-valent pneumococcal conjugate vaccines in the prevention of culture-confirmed AOM (284,589).

4.1.1 Study population and facilities

Infants born in the Hervanta area of Tampere, Finland, were invited to participate in the FinOM Cohort Study, the enrolment lasting from April 1994 to August 1995. Families with a newborn baby were informed about the study during their first routine visit to the local child health centre. All healthy infants born or residing in the area were eligible to participate in the study if they were aged 2 months +/- 2 weeks, if they had no prior immunisation with a pneumococcal vaccine, and if their mothers were able to communicate fluently in Finnish. During the study, the participating children were vaccinated following the Finnish national immunisation programme at the time. The programme did not include any pneumococcal vaccine.

The follow-up started in April 1994 and continued through July 1997. A special study clinic with one or two study physicians, including the author of the thesis, and several study nurses was established close to the local child health centre. The

study physicians were specially trained to diagnose and treat AOM and obtain MEF samples and all of the personnel were trained to interview the parents and obtain nasopharyngeal and blood samples. The services at the study clinic were available for the study children from 8 am to 3 pm during week days, and from 9 am to 12 noon at all other times.

The study data were collected on paper case-report forms by the study staff and double-recorded manually into an electronic form by the personnel at THL (KTL) in Helsinki.

4.1.2   Study visits.

The study design comprised ten visits scheduled by age. In addition, the parents of the children were asked to contact the study clinic if the parents suspected AOM according to predefined symptoms and signs. After diagnosis of AOM, a control visit was arranged to check-up of the healing of the AOM. After a physical examination, the type of visit was reassessed according to the health status of the child. Sometimes more than one visit types could be carried out concurrently, depending on the health status of the child and the time windows of the visits. Finally, two types of visits were defined, i.e. *age-based visits* (n=3026) at which the child was healthy or had only mild respiratory symptoms, and *sick visits* (n=2122) with respiratory symptoms needing medical aid, suspected or diagnosed AOM, fever, or (occasionally) viral exanthema or gastrointestinal symptoms together with respiratory symptoms or AOM. Figure 1 illustrates the flow of the data from the clinical study visits to the final visit types.

Age-based visits

The children were examined in ten visits at the study clinic scheduled at 2, 3, 4, 5, 6, 9, 12, 15, 18 and 24 months of age of the child, allowing a time window of +/- 2 weeks at 2–15 months and +/- 4 weeks at 18 and 24 months of age. At each scheduled visit, the study physician performed a physical examination, including pneumatic otoscopy. If the child was healthy or had only minor respiratory infection, the visit was carried out as an age-based visit (n=2811). If the child was diagnosed to have AOM, febrile infection (Trec $\geq$38°C), viral exanthema or acute gastroenteritis at the scheduled visit, the age-base visit was not carried out but a new age-based visit was arranged later within the time window if the child had passed the acute phase. Further, if the time window of the age-based visit

overlapped with the time-window of a visit scheduled to check up on the healing of AOM and the child was healthy or had only minor respiratory infection, the two visits were combined and carried out as a second type of an age-based visit (n=215, see below).

The study physician or the study nurse gathered background information including family structure and education of the parents at the first age-based visit of the child. At each age-based visit, the study staff carried out an extensive interview using a structured questionnaire about potential risk factors for pneumococcal carriage or AOM, including feeding patterns, day care attendance and indoor smoking in the family. Use of antimicrobial agents during the 28 days preceding the visit was recorded. The child's visits to other than the study physicians were registered according to a patient diary, and medical records were requested and evaluated for confirmation of AOM events diagnosed outside the study clinic

Sick visits

The parents were asked to bring the child to the study clinic if the child had respiratory infection or fever and the parents suspected AOM due to the presence of at least one of the following symptoms: earache, tugging or rubbing of the ear, ear discharge, irritability, poor sleeping, poor eating, vomiting or diarrhoea. The parents were also advised to bring the child to the clinic if respiratory symptoms continued for more than five days without improving. These visits due to parental request because of suspicion of AOM were recorded as a **spontaneous sick visit**. At these spontaneous sick visits, the study physician recorded the history of symptoms and current antimicrobial treatment in a structured way and performed a thorough physical examination, including pneumatic otoscopy and tympanometry. The spontaneous sick visits (n=1897) were *the first of the three types of sick visits.*

If the child was diagnosed as having AOM, febrile infection ($\geq$38°C), viral exanthema or symptoms of respiratory and gastrointestinal symptoms at a scheduled visit, the visit was carried out as *a second type of sick visits* (n=106). At these visits, all study procedures were identical with those carried out at the spontaneous sick visits.
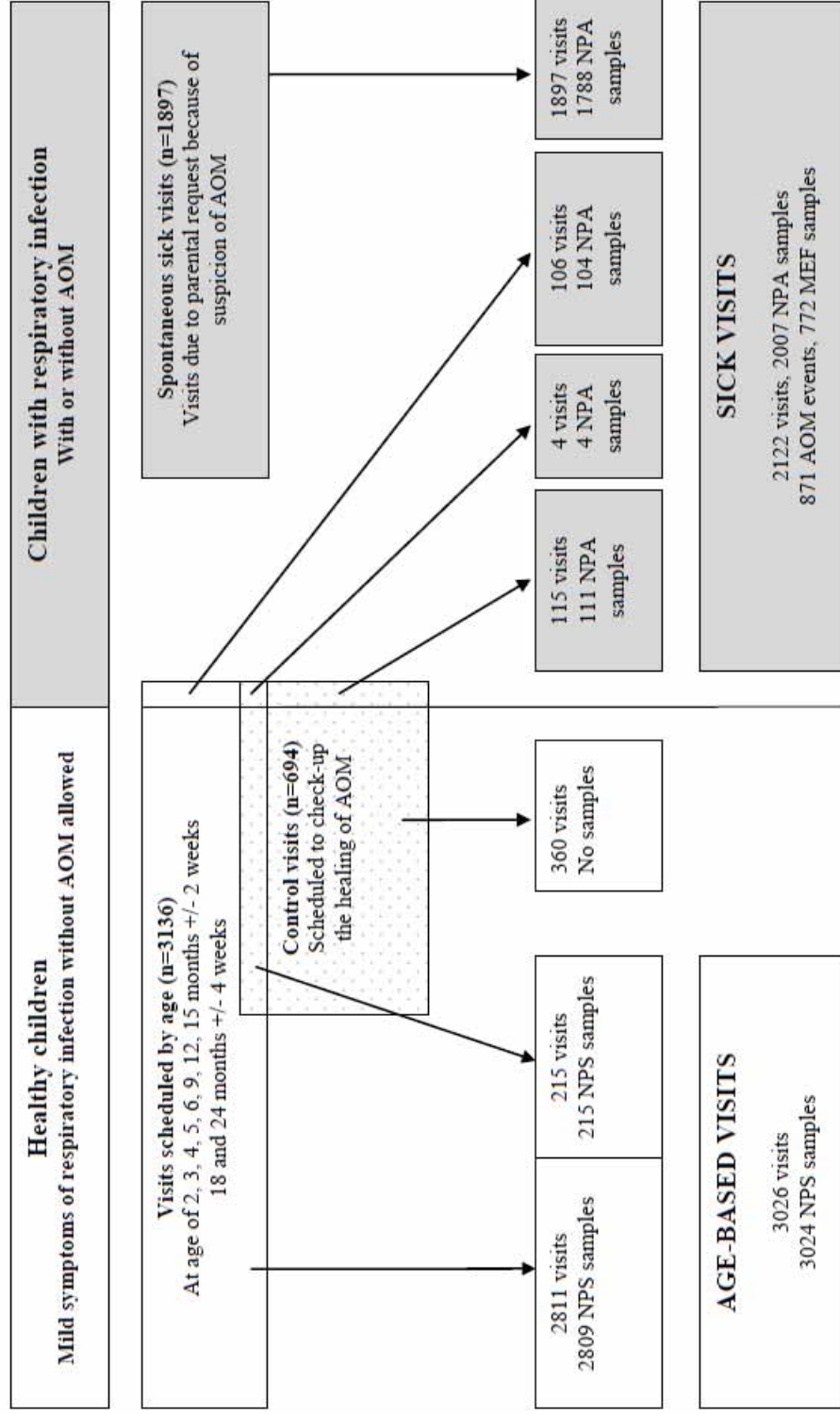
Figure 1. Visits in the FinOM Cohort Study.

NPS, nasopharyngeal swab; NPA nasopharyngeal aspirate; MEF, middle ear fluid

If the study physician diagnosed AOM at any study visit, a control visit (n=694) was scheduled at 4 weeks (+/-7days) after the diagnosis to check up on the healing of the AOM. If AOM, febrile infection (Trec ≥38°C), viral exanthema or gastrointestinal symptoms (together with even mild respiratory symptoms) were diagnosed at the control visit, the visit was classified as *a third type of sick visits* (n=119; 115+4). At these visits, the interview focused on the healing of the previous AOM and not on the current symptoms, while the physical examination and other study procedures were identical with those at the spontaneous sick visits.

If the child was healthy or had only minor respiratory infection at the control visit, no clinical samples were obtained, and the visit was not included in the material of this thesis (n=360), unless the control visit fell within the time window of an age-based visit, in which case these two visits were combined and carried out as the second type of an age-based visits (n=215; see above).

4.1.3   Diagnosis of acute otitis media

The criteria for diagnosing AOM were based on the statement of the Finnish consensus conference on the approach of the treatment of AOM in 1985 (344). The criteria included symptoms or signs of acute infection together with a visually abnormal tympanic membrane suggesting fluid in the middle ear cavity in pneumatic otoscopy. For diagnosis, at least one of the following signs or symptoms was required: symptoms of respiratory infection, fever, earache, tugging or rubbing of the ear, irritability, restless sleep, loss of appetite or other gastrointestinal symptoms. The otoscopic criteria for AOM diagnosis were cloudiness, bulging or decreased mobility of the tympanic membrane, visible air-fluid level in the middle ear or discharge through a spontaneous perforation or ventilation tube.

In the pneumatic otoscopy, soft speculums of appropriate size were used (Welch Allyn 20200 otoscope, Welch Allyn, Skaneateles falls, NY, USA). Tympanometry (GSI 38 Autotymp, Grason Stadler, Milford, NH, USA) was used as a diagnostic aid (299). Also an examination microscope (OPMI 99FC, Carl Zeiss, Oberkochen, Germany) was available for verification of the diagnosis, though it was used routinely only during the first few months of the study.

4.1.4   Clinical samples

Nasopharyngeal swab samples

At each age-based visit, a nasopharyngeal swab (NPS) sample was obtained using a sterile swab with flexible aluminium shaft and a dry calcium alginate tip (Galgiswab® Type 1, Spectrum Laboratories, INC., Dallas, Texas). The child's head was tipped backwards and immobilised. The shaft of the swab was first bent in the peel pouch to follow the arch of the nasopharynx, and then the swab was inserted into the nostril, passed into the nasopharynx to a distance equal to that from the child's nose to the tip of the ear and maintained for five seconds. The sample was stroked immediately with the swab on the culture plates.

Nasopharyngeal aspirates

At each sick visit, a nasopharyngeal aspirate (NPA) was obtained with a sterile paediatric mucus extractor (UNO sterile EtO, UnoPlast A/S, Hundested, Denmark). The catheter was guided to a depth of 4–8 cm in the nasopharynx through a nostril and drawn back while applying a gentle suction with an electric suction device. If the amount of the sample was less than 0.5 ml, the sample was diluted with 0.5 ml phosphate-buffered saline (PBS) (from October 9, 1995, 1.0 ml PBS). The sample was stroked immediately on the culture plates with a 10 µl disposable loop for semi quantitative culture.

Middle ear fluid samples

Whenever AOM was diagnosed, myringotomy with aspiration was performed. After mechanical cleaning of the ear canal, the tympanic membrane was anaesthetised with Bonain's solution (equal amounts of cocaine hydrochloride, menthol, and phenol), maintained for 20 minutes or with a touch with 70% liquid phenol. A small incision was made in the anteroinferior part of the tympanic membrane. A middle ear fluid (MEF) sample was aspirated with an electric suction apparatus into a sterile suction tip. The aspirate was immediately flushed into a tube with 1.0 ml of PBS and stroked on the culture plates with a 10 µl disposable loop for a semi quantitative culture.

4.1.5   Bacteriological methods

The NPS, NPA and MEF samples were cultured on selective sheep blood agar plates containing 5 µg/ml gentamicin and on enriched chocolate agar plates. The plates were incubated in 5% CO2 at 36–37°C in the study clinic, generally overnight, and transported to the bacteriological laboratory in Oulu. The plates inoculated on Fridays and Saturdays were incubated in the study clinic until Sunday. In the laboratory, the plates were incubated further if needed to reach a total incubation time of at least 48 hours.

After at least 48 hours of incubation, the numbers of colonies on the one of the two plates with more abundant growth were counted in the bacteriological laboratory. Colony counts ≥100 per plate were labelled as abundant growth. To identify *S. pneumoniae*, four different alpha-haemolytic colonies were tested for optochin sensitivity. If the optochin test was negative but colony morphology was suggestive of *S. pneumoniae*, the bile solubility test was used. Pneumococcal isolates were frozen in skim milk and later serotyped by counterimmunoelectrophoresis and, for serogroups 7 and 14, by latex agglutination using antiserum pools and serotype/serogroup specific antisera. The Quellung test was used when necessary to confirm an uncertain result. The isolates belonging to serogroups 6, 9, 18, 19 and 23 were further serotyped using pneumococcal factor antisera purchased from Statens Serum Institut, Copenhagen, Denmark. No particular methods were used to search for multiple serotypes. In this thesis, both the individual serotypes and serogroups that were not divided into individual subtypes are referred to as serotypes for convenience, unless the serogroup level is specifically considered.

H. influenzae was identified from the NPA and MEF samples. The colonies suggestive of *H. influenzae* on enriched chocolate agar plates were screened using the satellite test. Satellite test positive strains were further identified by their requirement for X and V growth factors (A/S Rosco, Taastrup, Denmark) on Mueller-Hinton agar plates. The 'abundant growth' of *H. influenzae* was quantified as that of *S. pneumoniae*. Other relevant bacteria, such as *S. pyogenes*, *S. aureus* and gram negative rods were identified from MEF samples using the standard operation procedures of the laboratory.

4.1.6   Treatment of acute otitis media

The treatment of AOM was based on the recommendations of the Finnish consensus conference, adjusted according to the knowledge of local resistance of

bacteria to antimicrobial agents. The first-line drugs used at the study clinic were V-penicillin or amoxicillin. Second line drugs included sulfonamides with trimethoprim, amoxicillin-clavulanate or second generation cephalosporins. The second line drugs were used if the child was suspected to be allergic against the first line drugs or if the bacterium in the MEF was not susceptible to the first-line drug used. Second-line drug were also used if AOM did not respond to the primary drug, if AOM recurred within one month, or if the treatment had failed several times. In case of spontaneous perforation or a discharging ventilation tube, topical antimicrobial agents were used as an additional treatment, or sometimes alone in recurrent cases. Symptomatic treatment was used according to the judgment of the study physician. All prescriptions of antimicrobial agents, whether systemic or topical, were recorded and the use of prescribed antibiotics was asked at the age-based as well as the control visits.

The children were referred to the otologists in Tampere University Hospital for assessment of the need of ventilation tubes and/or adenoidectomy if the child had had MEF continuously for two months, if the child had had three AOM events within six months or if the child had had four to five AOM events within one year.

4.1.7   Ethical consideration

Written consent was obtained from the parent or guardian after describing the study procedures and discussion before the child was enrolled in the study. The study protocol was approved by the Ethics Committees of THL (KTL), Department of Social and Health Care of Tampere City and Tampere University Hospital, Tampere, Finland.

4.2   Definitions of pneumococcal carriage, respiratory infection and pneumococcal acute otitis media

In the four studies comprising this thesis, *pneumococcal carriage (Pnc carriage)* means the process of harbouring *S. pneumoniae* in the nasopharynx, irrespective of whether the bacterium is associated with disease or not. Pnc carriage starts with the acquisition of a pneumococcal strain in the nasopharynx and ends with the clearance of the strain from the nasopharynx. At discrete time points, the presence of Pnc carriage is observed as a nasopharyngeal culture positive for *S.*

pneumoniae. The acquisition and clearance of carriage, which cannot be directly detected, are defined as changes in the individual's serotype-specific carriage status. The methods applied to set or estimate the change points in the carriage status are defined in detail in the descriptions of the data subsets for Studies III and IV. Two pneumococcal isolates were considered to represent the same strain if they expressed the same serotype and were temporally or spatially associated, e.g. cultured sequentially from the nasopharynx, or found concurrently both in the nasopharynx and MEF.

In the studies of this thesis ***respiratory infection*** with or without AOM refers to the symptoms of an infection in the respiratory tract, likely caused by one or several sequential viral infections. At separate time points, the presence of respiratory infection was defined as presence of symptoms of respiratory tract infection in a child presenting at sick visits in the study clinic. In Studies III and IV, respiratory infection is approached as a longitudinal process, observed at one or more sequential sick visits. The methods for separating sequential episodes of respiratory infections and identifying the start of a new respiratory infection are defined in detail in the descriptions of the data subsets for Studies III and IV.

In this thesis, ***pneumococcal acute otitis media (Pnc AOM)*** is defined as a clinical AOM event with at least one MEF culture positive for *S. pneumoniae*. The bacterium or pneumococcal serotype in the MEF sample was recorded once if it was isolated from both ears.

4.3   Data subsets and designs of Studies I-IV

4.3.1   Natural course of nasopharyngeal pneumococcal carriage (Study I)

To describe the natural course of Pnc carriage during health and respiratory infection, the culture results from all available NPS (n=3024), NPA (n=2007) and MEF (n=772) samples were included in the analysis. The proportion of samples positive for *S. pneumoniae* was analysed by age, sex, season and treatment with antimicrobial agents within the preceding 28 days. For comparisons between carriage during health and respiratory infection, the NPS and NPA samples were grouped according to the age they were obtained, i.e. at 1–6, 7–12, 13–18 and 19–24 months of age. Within each age group, the proportion of samples positive for *S. pneumoniae* was then calculated in several clinical categories: age-based visits, sick

visits with or without clinical AOM, sick visits with non-pneumococcal AOM, and sick visits with a diagnosis of Pnc AOM. In Study I, all age-based and sick visits were included.

Study I presents, in a descriptive way, the natural course of nasopharyngeal carriage in a heterogeneous cohort of basically healthy children, who have variable proneness to infections, including AOM. The Kaplan-Meier method and the χ^2 test were used to compare demographic and background factors between children who discontinued or completed the study and to compare the cumulative proportions of carriers among boys and girls. SPSS for Windows® software, release 8.0.1 (SPSS Inc., Chicago, Illinois) was used for computation.

4.3.2   The value of nasopharyngeal culture in predicting the aetiology of acute otitis media (Study II)

Study II assessed the value of the nasopharyngeal culture of *S. pneumoniae* in the prediction of *S. pneumoniae* presence in the MEF sample at the time of clinical AOM diagnosis. For comparison, the analysis was repeated for *H. influenzae*. The analyses were based on AOM events with the MEF sample and a concurrent available NPA sample (n=761). We excluded events at which the child had ongoing oral antimicrobial treatment (n=104) or history of preceding AOM diagnosis in the 14 days (n=71) prior to the analyses, since antimicrobial treatment was found to affect differently the occurrence of *S. pneumoniae* and *H. influenzae* in the NPA and MEF samples. This left 586 AOM events of 200 children available in the analysis. The quantity of colonies was determined for all NPA samples positive for *S. pneumoniae* and for all but one NPA sample positive for *H. influenzae*.

A positive culture for *S. pneumoniae* or *H. influenzae* from the MEF sample was defined as the standard method for the aetiologic diagnosis of AOM. Accordingly, the sensitivity of the nasopharyngeal culture was calculated as the probability for *S. pneumoniae* or *H. influenzae* to be present in the NPA if the bacterium was found in the MEF. The specificity was calculated as the probability of the bacterium to be absent from the NPA if it was not found in the MEF. The positive predictive value (PPV) refers to the probability of the MEF to be positive for the bacterium if it was found in the NPA. The negative predictive value (NPV) means the probability of the MEF to be negative for the bacterium if it was not found in the NPA. To control for a possible intra-child correlation between measurements from the same child, 95% confidence intervals for sensitivity, specificity, PPV and NPV were

estimated by logistic regression using generalised estimating equations with an exchangeable correlation structure. The effects of age, sex, season and the number of previous AOM events on sensitivity, specificity and the predictive values were assessed by multivariate logistic regression.

4.3.3 Pneumococcal acute otitis media in relation to preceding pneumococcal carriage (Study III)

To investigate the association between pneumococcal acquisition and the development of Pnc AOM, each child's follow-up was divided into six non-overlapping *observation periods*. The periods started at age-based visits with NPS sampling at 3, 6, 9, 12, 15 and 18 months of age and ended at the next among these age-based NPS samplings or after exactly 3 months if the next NPS sample was not obtained but the child was still in clinical follow-up. A total of 1771 such observation periods were identified.

For Study III, only spontaneous sick visits were considered (Figure 1). These visits were considered to represent an acute start or worsening of respiratory symptoms suggestive for AOM. To avoid repeated measures from a single episode of infection, only visits at least 30 days apart were used. Of these visits, the first after the start of the observation period was included in the analysis. This visit was referred to as *a focus sick visit.* If the NPA sample or, in the case of AOM, the MEF sample was not available at the visit, the whole observation period was excluded. This led to the exclusion of a total of 56 observation periods (3% of all), thus leaving 1715 observation periods and 774 focus sick visits for 318 children in the analysis.

The occurrence, timing and clinical features of the focus sick visits were compared between those children who carried *S. pneumoniae* at the start of the observation period (initial carriers) and those who did not (initial non-carriers). The relationship between the serotype-specific carriage status at the start of the period and occurrence of Pnc AOM caused by that serotype at the focus sick visit was assessed, allowing concurrent carriage of two serotypes and including these as two separate observation periods in the serotype specific analysis.

The analyses of Study III were largely based on focus sick visits with Pnc carriage, referred to as *NPA positive focus sick visits*. Using conditional logistic regression, the relative risk of Pnc AOM at the NPA positive focus sick visits was estimated for the initial carriers and non-carriers of the homologous serotype.

Since the child could have focus sick visits during several observation periods, a frailty variable was included to account for any heterogeneity across children in their proneness to Pnc AOM. The temporal association between the initial carriage status and the timing of the focus sick visit was investigated in a descriptive way, comparing the visits with and without Pnc AOM. In addition, the preceding carriage status of the serotype harboured at the focus sick visit was assessed retrospectively in these two types of visits. A conditional logistic regression approach was applied in the R software, version 1.9.0 (Foundation for Statistical Computing, Vienna, Austria, 2004). Other computations were performed with SPSS for Windows® software, release 8.0.1 (SPSS Inc., Chicago, Illinois).

4.3.4 Dynamics of pneumococcal carriage acquisition, respiratory infection and pneumococcal acute otitis media (Study IV)

In Study IV, the acquisition of Pnc carriage, respiratory infection and the development of Pnc AOM were approached in more detail as longitudinal processes. The subset of data in Study IV comprised of all age-based visits and all spontaneous sick visits (Figure 1).

Episodes of carriage and non-carriage were constructed using all NPS samples (n=3024) obtained at the age-based visits and all NPA samples obtained at spontaneous sick visit (n=1778). After excluding 17 samples (9 NPS, 8 NPA) with no serotype information available and 731 samples (685 NPS and 46 NPA samples) obtained at least 62 days apart from any other sample, 4064 nasopharyngeal samples were available. Episodes of carriage (n=585) were defined as a series of consecutive samples yielding the same serotype. Episodes of non-carriage (n=828) were defined as a series of consecutive negative samples. The onset and end of each episode were defined as the midpoints between two samples with heterologous findings. If no sample was available less than 62 days before the first observation of an episode, the onset was considered unknown. A negative nasopharyngeal finding was considered falsely negative and therefore imputed with a positive finding if the child had antimicrobial treatment and the previous and subsequent samples were of the same serotype. In addition, because nasopharyngeal carriage is considered a prerequisite for Pnc AOM, a missing or negative nasopharyngeal finding for the serotype detected in the MEF sample was imputed with a positive finding. The MEF serotype was retained as the carriage serotype if more than one serotype was carried simultaneously. In the absence of

Pnc AOM, the newer serotype of multiple ones was retained, or a random one if more than one new serotype was found concurrently. All children were considered non-carriers at birth.

Sick episodes (n=1146) were defined to start by spontaneous sick visits, if at least 30 days had elapsed since the previous spontaneous sick visit of the child. Since the respiratory infections may be overlapping and the fluctuations of the respiratory symptoms of the child were not carefully monitored, the onset of a new sick episode was used to represent the start of a new respiratory infection, although in most cases the symptoms had started or worsened earlier. A sick episode was defined to end 30 days after the last spontaneous sick visit of the sick episode, or 30 days before the next sick episode onset, whichever occurred earlier.

An exploratory analysis of pneumococcal acquisition hazard was first performed using data only from children with at least one sick episode. Based on this subset of the data, a non-parametric estimate of the (cumulative) hazard was used to identify three strata according to sick episode onsets: *health* (i.e. more than 30 days before the sick episode onset), *pre-sickness* (i.e. within 30 days before any sick episode onset), and *sickness* (i.e. during a sick episode).

As a sensitivity analysis, the exploratory analysis was repeated after defining the carriage and non-carriage episodes using only samples obtained only 45 days apart, allowing more precise estimation of carriage acquisition but reducing the amount of data in the analysis.

Unadjusted estimates of the acquisition hazards were calculated as the numbers of acquisitions per at-risk time, separately for carriage and non-carriage episodes. The hazards of pneumococcal acquisition and clearance were further estimated in the three strata using a Markov transition model and all age-based and sick visit samples (description in the Appendix of the Original Communication IV). In the Markov model, the 31 possible states included 'non-carrier' or 'carrier' of any of the 30 different serotypes in the data, same hazards of acquisition and clearance were assumed for each of the serotypes and the hazards were stratified into two age classes (<12 and ≥12 months). A 50% reduction in the acquisition hazard was assumed if the child already carried pneumococci as compared to a non-carrying child of the same age (182).

Risk episodes (n=411) were defined as overlapping periods of carriage and sick episodes, for which both the onset time of the carriage episode and the timing of the first Pnc AOM event were known (i.e. the MEF sample was not missing for any AOM before the first Pnc AOM, if any, during the risk episode).

The hazard of the first Pnc AOM during the risk episodes was estimated. In an exploratory analysis, the risk episodes were stratified according to the temporal relationship between onset of the episode of carriage and onset of the sick episode so that the Pnc AOM hazard within each stratum was as constant as possible. Three strata were recognized and the risk episodes were defined according to the underlying episode of carriage starting *long before sickness*, *around sickness onset* and *later during sickness*. A Poisson regression model was used to estimate the Pnc AOM hazards in the three strata of risk episodes, with adjustment for age group (1–5, 6–11, 12–17, 18–24 months of age at the risk episode onset), previous homologous carriage (yes/no), and previous homologous Pnc AOM (yes/no). To remove the potential impact of repeated sick episodes during the same carriage episode before the onset of the risk episode, the analysis was repeated involving only the first sick episodes during each carriage episode (n=335). Another sensitivity analysis included also those overlapping periods of episodes of carriage and sick episodes for which the exact acquisition time of carriage was not known, but the carriage had been first detected more than 30 days before the sick episode onset. This produced 28 additional risk episodes with known aetiology of the first potential Pnc AOM and the occurrence of previous sick episodes during the carriage.
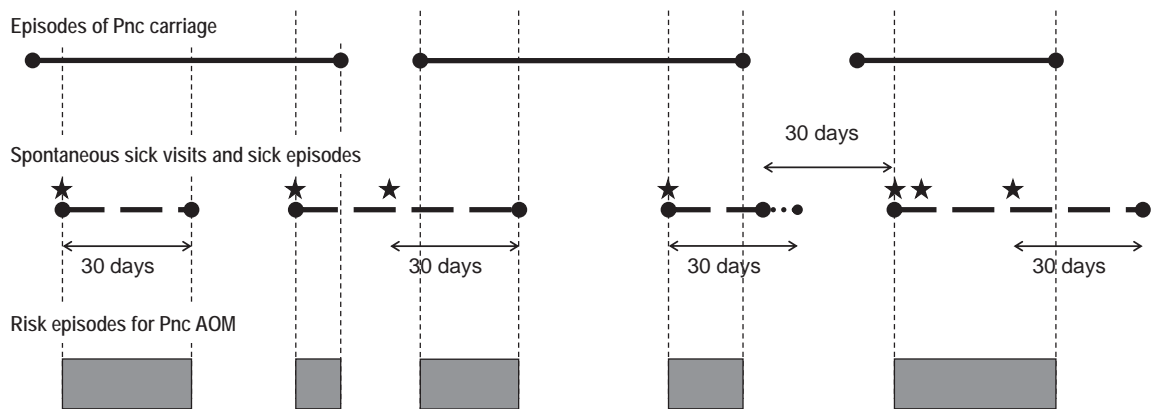

Figure 2. Risk episodes for pneumococcal acute otitis media.

Overlapping episodes of pneumococcal carriage (Pnc carriage) and sick episodes were defined for assessing the hazard of pneumococcal acute otitis media (Pnc AOM). Solid lines, episodes of Pnc carriage; stars, spontaneous sick visit; dashed lines, sick episodes; bars, risk episodes for assessing the hazard of Pnc AOM.

5 RESULTS

5.1 Characteristics of the study children and the follow-up

A total of 329 children were enrolled, representing 53% of all children of eligible age and registered in the child health centres in the study area during the enrolment period. Of the 329 study children, 158 (48%) were boys, 163 (50%) had older siblings living in the household and 122 (37%) attended care during the follow-up. Of the enrolled 329 children, 167 (51%) were breastfed for at least 24 weeks and 135 (41%) were exclusively breastfed for at least 12 weeks. Altogether 225 children (68%) had a mother or father with higher education, i.e. senior high school, college or academic degree. Families of 12 (4%) children reported smoking indoors in the home during the follow-up. Of the 329 participating children, 316 (96%), 309 (94%), 284 (86%) and 281 (85%) were followed until at least 7, 13, 19 and 24 months of age, respectively. The reasons for discontinuation were moving out of the study area (40% of those who discontinued), withdrawal of consent (33%), loss to follow-up (23%), and unknown (4%). Girls discontinued more often than boys (19% $vs.$ 10%; χ^2, p=0.03) and children without older siblings discontinued more frequently than children with older siblings (19% $vs.$ 10%; χ^2, p=0.03). Otherwise there were no significant differences between those who discontinued and those who were followed up to 24 months of age. The total follow-up time of the 329 children from the first visit to the last visit or discontinuation was 565 person-years.

Figure 1 illustrates the numbers of clinical study visits. At the age-based visits, 3024 NPS samples were obtained, the total number decreasing from 328 to 260 from the first to the tenth scheduled visit. All ten scheduled NPS samples were obtained from 212 (64%) of the 329 children and at least 8 samples were available from 91% of the children.

Altogether 2122 sick visits were carried out, 1897 (89%) of them were spontaneous sick visits and 225 were initially scheduled by age or for check-up of the healing of AOM (Figure 1). Of all 329 children, 288 (88%) presented at least once at a sick visit during the follow-up (91% of boys and 85% of girls; χ^2, p=0.12). The maximum number of sick visits per child was 32 (median, 5). Study

physicians diagnosed AOM at 871 sick visits (at 41% of all sick visits), and in 772 (89%) of these, the diagnosis was verified by collection of a MEF sample from one or both ears. AOM was diagnosed at least once in 215 (65%) study children, one or two times in 88 (27%), 3–5 times in 75 (23%) and more than 5 times in 52 (16%) of the 329 children, maximally 17 times in one child (median, three). These numbers comprise 86% of all AOM cases recorded to have occurred among the study children during the follow-up.

5.2 Natural course of nasopharyngeal pneumococcal carriage (I)

5.2.1 Cumulative incidence of the first detection of carriage

A vast majority of the 329 children encountered *S. pneumoniae* during the follow-up. Of the 281 children who remained in the follow-up until 24 months of age, 244 (87%) were observed to harbour *S. pneumoniae* in the nasopharynx at least once, either at age-based or sick visits (Figure 3). The cumulative proportions of the children who were still in follow-up and who were known to have carried *S. pneumoniae* at least once were 34%, 56% and 80% at 6, 12 and 18 months of age, respectively. No difference was observed in the cumulative incidence of the first detection of carriage between boys and girls followed for 24 months (90% in boys and 83% in girls; χ^2 p=0.097).
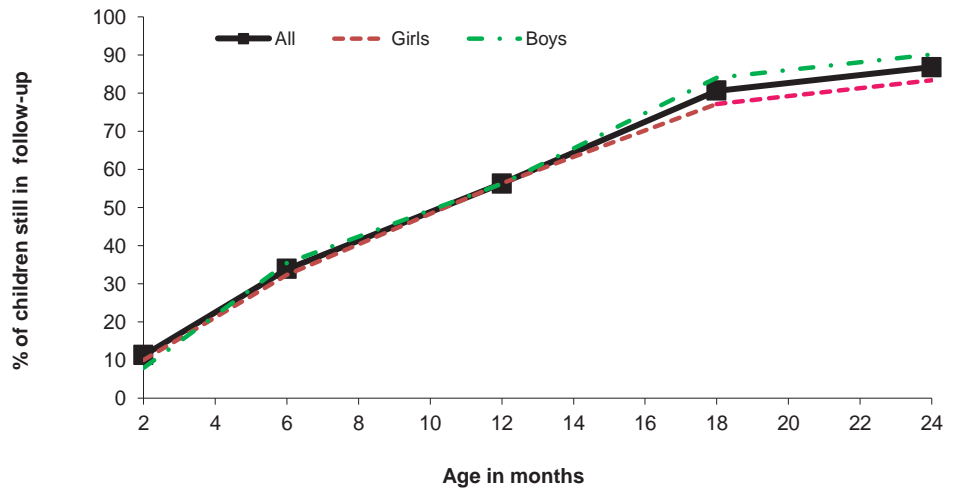
Children in follow-up	329	316	309	284	281
Carriers	37	107	174	229	244

Figure 3. Cumulative proportion of the first detection of nasopharyngeal carriage of *S. pneumoniae* by age and sex.

Carriers, children with at least one nasopharyngeal sample positive for *S. pneumoniae* during the follow-up.

5.2.2 Nasopharyngeal carriage during health, respiratory infection and acute otitis media

The total proportion of NPS samples positive for *S. pneumoniae* obtained at the age-based visits during the study follow-up was 21% (649/3024). When adjusted for age, the average proportion of NPS samples positive for *S. pneumoniae* was 27% (29% in boys and 26% in girls). At sick visits, the proportion of all NPA samples positive for *S. pneumoniae* was 41% (826/2007) and was almost twice as high at the sick visits as compared to the age-based visits (42% in boys and 40% in girls).

In all, 201 Pnc AOM events were diagnosed, *S. pneumoniae* causing 26% of the 772 AOM events with at least one MEF sample available. Of the 761 AOM events at which both NPA and at least one MEF sample were available, pneumococci caused 199 (26%), the percentages being 26%, 25%, 31% and 22% in four age groups of 1-6, 7-12, 13-18 and 19-24 months, respectively.

The percentages of samples positive for pneumococci, obtained at the age-based visits and different types of sick visits at 1–6, 7–12, 13–18 and 19–24 months of age are presented in Table 1. When assessed by each month of age, clear trends

in the pneumococcal carriage frequency were observed by age and health status, as illustrated in Figure 4.

Table 1. Nasopharyngeal samples positive for *S. pneumoniae* by age group and clinical category.

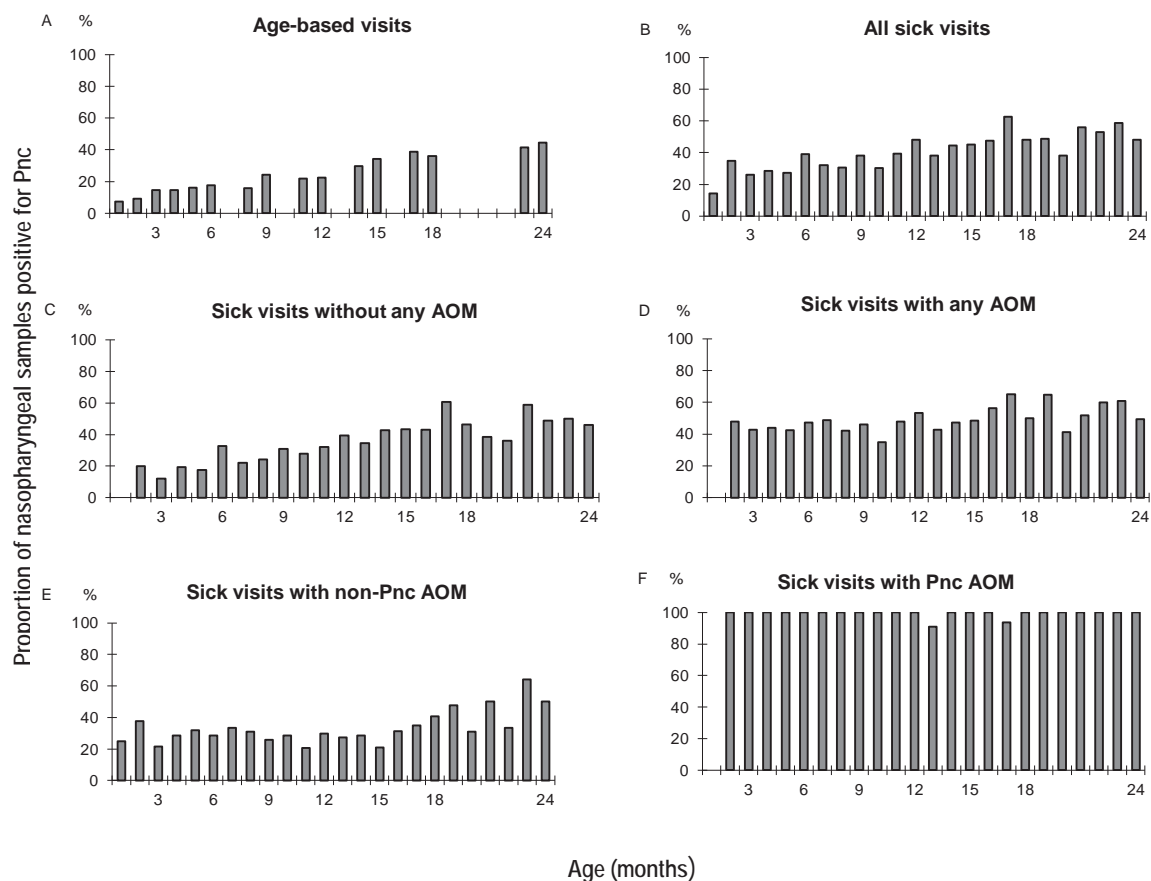| Clinical category	Age group									
	1-6 months		7-12 months		13-18 months		19-24 months		1-24 months	
	%	n/N[1]	%	n/N	%	n/N	%	n/N	%	n/N
All visits	17	341/1989	29	369/1276	41	456/1118	48	309/648	29	1475/5031
Age-based visits	13	215/1594	21	130/617	35	191/553	43	113/260	21	649/3024
All sick visits	32	126/395	36	239/659	47	265/565	51	196/388	41	826/2007
No AOM[2]	22	50/230	29	106/371	45	151/339	46	100/218	35	407/1158
Any AOM[3]	46	76/165	46	133/288	50	114/226	56	96/170	49	419/849[2]
Non-Pnc AOM[4]	30	33/109	28	55/195	30	42/138	46	55/120	33	185/562
Pnc AOM[5]	100	38/38	100	65/65	97	60/62	100	34/34	99	197/199

The numbers are based on nasopharyngeal samples obtained at all age-based and sick visits, aggregated into four age groups. [1]Samples positive for *S. pneumoniae* of all samples; [2]samples obtained at sick visits at which no clinical acute otitis media (AOM) was diagnosed; [3]samples obtained at sick visits at which any clinical AOM was diagnosed, irrespective of aetiology; [4]samples obtained at sick visits at which AOM not caused by *S. pneumoniae* (non-Pnc AOM) was diagnosed; [5]samples obtained at sick visits at which pneumococcal AOM (Pnc AOM) was diagnosed. The aetiology was known for 761 of 849 AOM events.

The prevalence of Pnc carriage at age-based visits increased slowly and almost linearly with age up to 24 months of age (Figure 4A). The samples obtained at sick visits (Figure 4B) showed a similar trend. The carriage frequency was higher at sick visits than at age-based visits at all ages, especially in the youngest age. There was no systematic difference in the carriage frequency by age between boys and girls, either at age-based visits or at sick visits

At sick visits with no AOM, carriage increased in a similar way by age, and the proportion of samples positive for pneumococci was not much lower than in those obtained at any sick visit (Figure 4C). Of the 1158 NPA samples obtained at sick visits without any AOM, 407 (35%) were positive for pneumococci. If any AOM irrespective of aetiology was diagnosed at the sick visit, 419 of the 849 samples (49%) grew pneumococci (Figure 4D). During AOM there was very little if any trend by age, and the carriage frequency was relatively high in all age groups, including the youngest one. The higher carriage frequency at sick visits as compared to age-based visits was most obvious in the youngest age group during AOM.

When examining the nasopharyngeal carriage during AOM by AOM aetiology, we found that most of the excess carriage frequency during AOM was clearly due to Pnc AOM. During non-pneumococcal AOM, the carriage was as low as at sick visits without any AOM (Figure 4E), the overall percentage of positive findings being 33% (185/565). Instead, during Pnc AOM, 197/199 (99%) of nasopharyngeal samples grew pneumococci (Figure 4F).

Figure 4. Nasopharyngeal samples positive for *S. pneumoniae* by age month and clinical category.

The bars present the proportions of nasopharyngeal cultures positive for *S. pneumoniae* (Pnc) in samples obtained at the age-based visits and at the sick visits with and without acute otitis media, by age month. A, samples obtained at age-based visits; B, samples obtained at all sick visits; C, samples obtained at sick visits at which no clinical acute otitis media (AOM) was diagnosed; D, samples obtained at sick visits at which any clinical AOM irrespective of aetiology was diagnosed; E, samples obtained at sick visits at which AOM not caused by *S. pneumoniae* (non-Pnc AOM) was diagnosed; E, samples obtained at sick visits at which pneumococcal AOM (Pnc AOM) was diagnosed. The aetiology was known for 761 of 849 AOM events.

5.2.3 Nasopharyngeal carriage by history of previous sick visits with respiratory infection

The increase of Pnc carriage with age observed in all NPS samples obtained during the age-based visits was not evident if only those 1344 NPS samples were considered which were obtained before any sick visit of the child, including those that occurred outside the study clinic (Figure 5). Although there was an increase in the carriage prevalence during the first 9 months, prevalence then levelled off and remained at 16–23%.
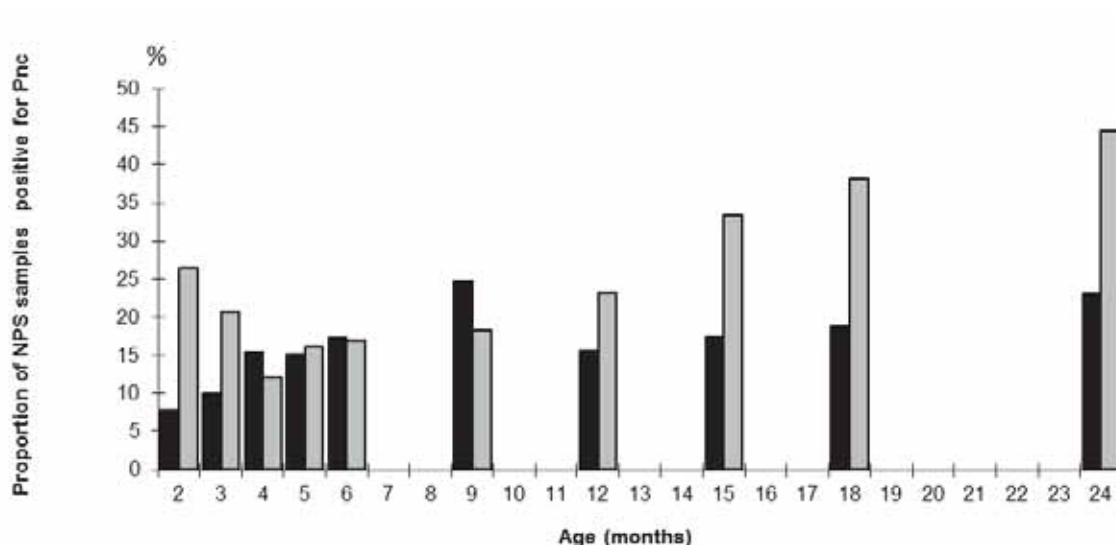

Figure 5. Nasopharyngeal carriage of *S. pneumoniae* by age and history of respiratory infections.
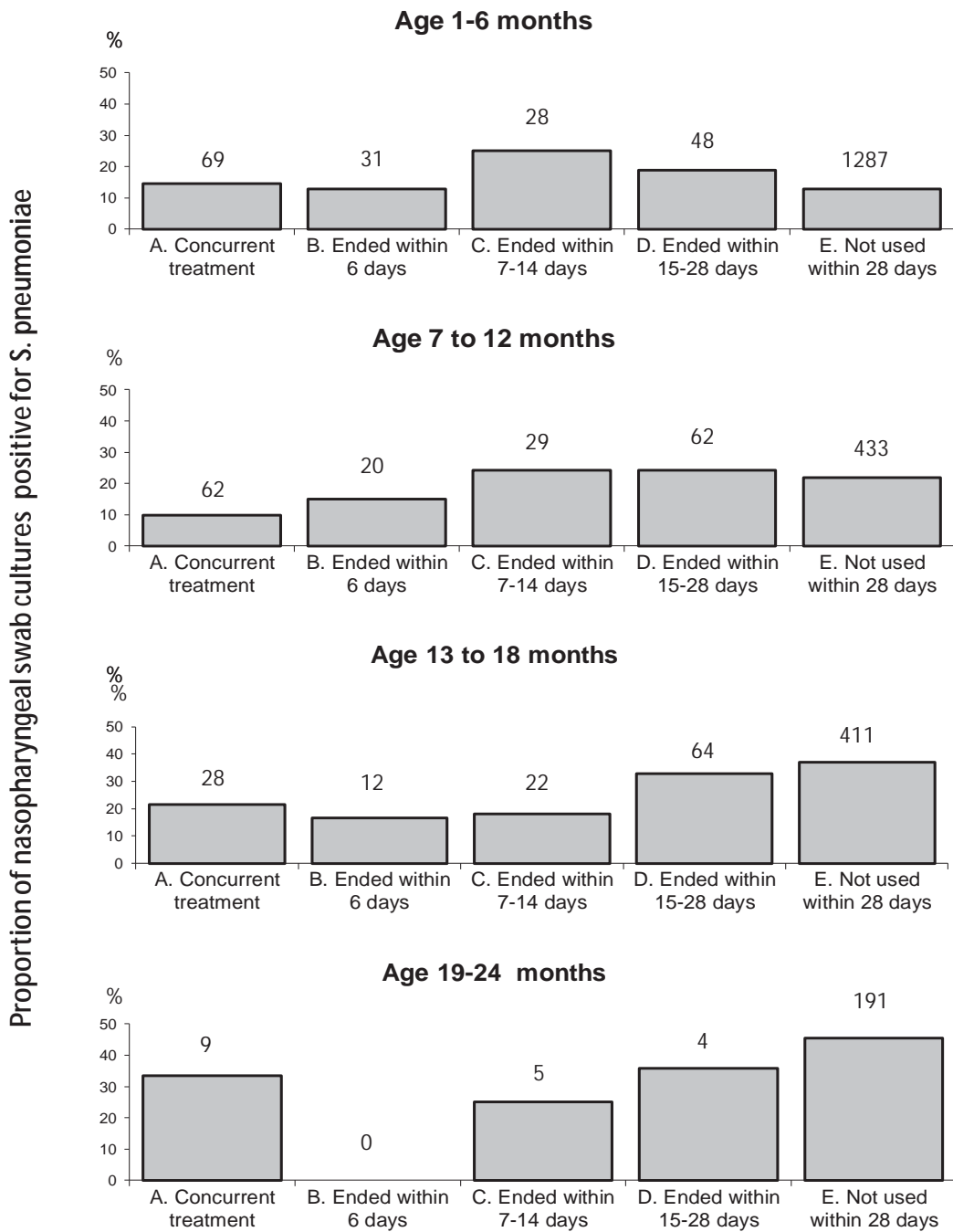
The bars show the proportions of cultures positive for *S. pneumoniae* (Pnc) in nasopharyngeal swab samples obtained at age-based visits, by preceding history of respiratory infections needing medical consultations. Grey bars, at least one sick visit carried out at the study clinic or any ambulatory visit to other physicians because of respiratory infection or AOM before the sampling date. Black bars, no sick visits at the study clinic and no ambulatory visits to other physicians because of respiratory infection or AOM before the sampling date. The ambulatory visits to other physicians because of respiratory infections were recorded according to the diary cards and interviews during the study visits and uncertain cases and AOM diagnoses were verified from medical records. For definitions of age-based and sick visits, see text.

5.2.4 Effect of antimicrobial treatment on pneumococcal carriage

The children had ongoing oral antimicrobial treatment at 6% of the age-based visits and 11% of the sick visits. For 0.4% and 3%, of the visits, respectively, it was not known whether the child received antimicrobials.

Figure 6 illustrates the effect of antimicrobial treatment on Pnc carriage. According to information derived from the diaries and interviews collected at the study visits, the child had a history of antimicrobial treatment within the previous 28 days at 18% (532/3024) of the age-based visits (in 6%, the history was not known, or only topical antibiotics had been used).

In children aged seven months or older, antimicrobial treatment was associated with lower carriage prevalence, but only temporarily. In children who had received antimicrobial treatment within one week prior to the sampling, the proportion of samples positive for *S. pneumoniae* was half that in samples obtained during visits with history of no antimicrobial treatment within the previous 28 days. If 1-2 weeks had elapsed since the end of treatment, the proportion of positive samples was higher, and if more than two weeks had elapsed, it reached 80–100% of the level in samples obtained in the absence of treatment within 28 days. In the youngest children aged less than seven months, previous antimicrobial treatment was not at all associated with reduced carriage.

Figure 6. Effect of antimicrobial treatment on nasopharyngeal carriage of *S. pneumoniae* by age group and time since the end of the treatment.

The bars represent the proportions of cultures positive for *S. pneumoniae* in nasopharyngeal swab samples obtained at the age-based visits (for definition of age-based visits, see text). A, antimicrobial treatment ongoing at the day of the sampling; B, antimicrobial treatment ended 1-6 days before the sampling; C, antimicrobial treatment ended 7-14 days before the sampling; D, antimicrobial treatment ended 15-28 days before the sampling; E, antimicrobial treatment not used within 28 days before the sampling. The numbers of samples are indicated above the bars.

5.2.5   Seasonality of pneumococcal carriage

A   **B**
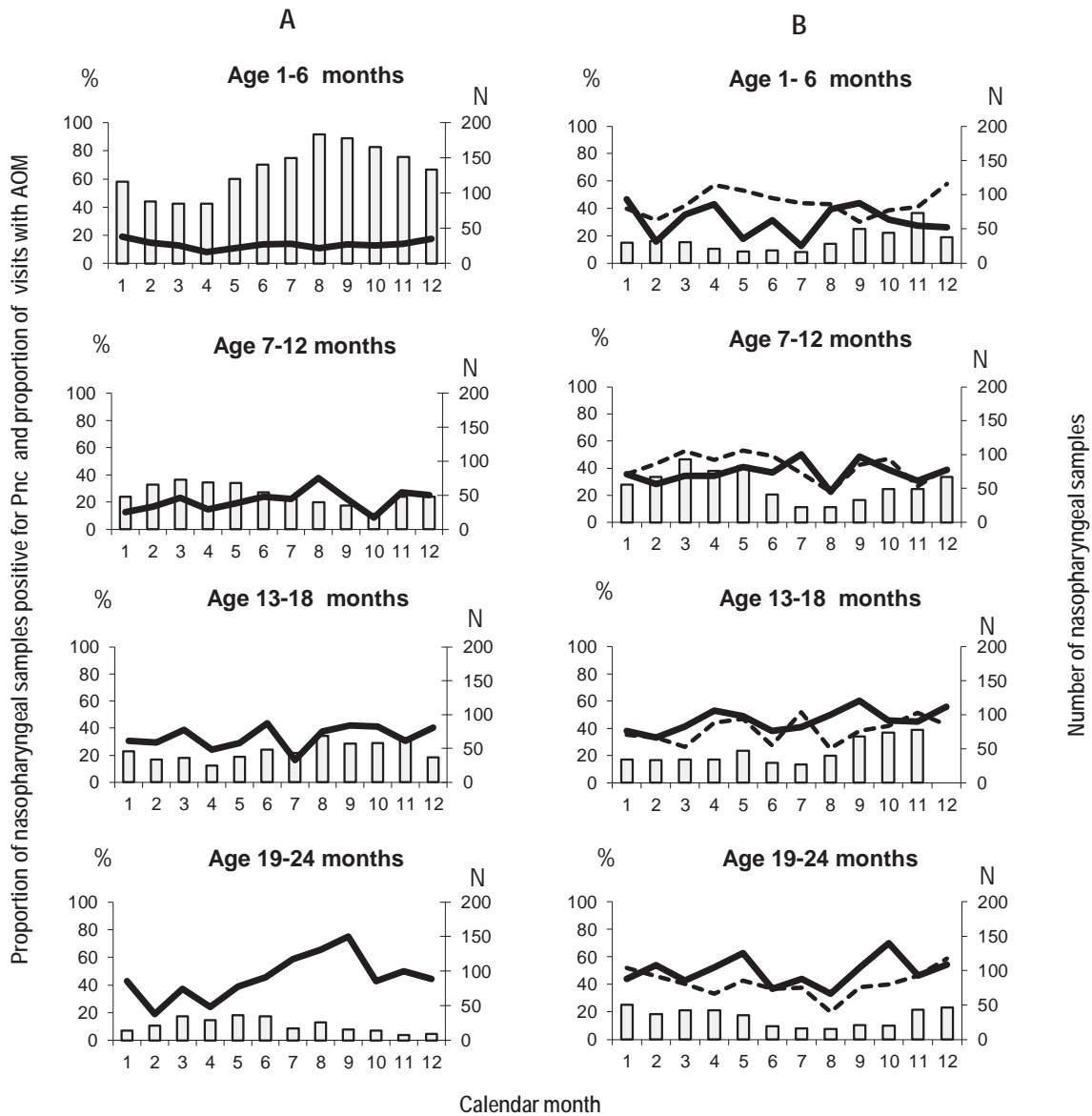
Figure 7.   Seasonality of pneumococcal carriage of *S. pneumoniae*.

The figure presents the proportion of nasopharyngeal samples positive for *S. pneumoniae* (Pnc) by age, health status and calendar month from January to December. Panel A, nasopharyngeal swab samples (NPS) obtained at age-based visits; panel B, nasopharyngeal aspirates (NPA) obtained at sick visits. Solid lines, proportion of samples positive for pneumococci; bars, numbers of nasopharyngeal samples; dashed lines in panel B, proportion of sick visits with AOM diagnosis of all sick visits.

No obvious seasonal trend was observed in Pnc carriage. The proportion of all NPS samples positive for *S. pneumoniae* fluctuated between 14% and 25% throughout the year. After stratifying the samples by age group, no systematic seasonality was seen either (Figure 7). The number of sick visits per month was

clearly smaller in the summer from June to August than during the rest of the year, but only a faint decrease in the proportion of NPA samples positive for *S. pneumoniae* was seen during the summer months.

5.2.6 Serotypes carried during health and during respiratory infection

Of the 267 children who carried *S. pneumoniae* at least once, 37% carried only one serotype during the follow-up. Two different serotypes were carried during the follow-up by 31% and 3–6 serotypes by 45% of these children. In one child, 7 different serotypes were found.

Altogether 1530 pneumococcal isolates were found in the 1475 nasopharyngeal samples (649 NPS samples and 826 NPA samples) positive for *S. pneumoniae* (Table 2). Of these isolates, 1457 represented 30 different serotypes/groups, 55 were non-encapsulated (rough), 16 were non-typable and 2 were lost before serotyping. Maximally a child carried a single serotype on 19 occasions. Table 2 lists the numbers of the most frequently isolated serotypes in three categories, i.e. in all NPS samples obtained at age-based visits, in NPA samples obtained during sick visits without any AOM and in NPA samples obtained during sick visits with AOM, and in irrespective of aetiology. The distribution of serotypes in in the MEF samples is shown for comparison. From the MEF samples, 205 pneumococci were isolated and all could be serotyped. Serotypes 6A, 6B, 19F and 23F were the most frequently isolated serotypes in all clinical categories in varying order, followed by 11 and 35 in the NPS samples and 14 and 11 in the MEF samples. During Pnc AOM (n=201), two NPA samples were not available, two were negative, one could not be serotyped and one comprised a serotype not found in the MEF sample. During all other Pnc AOM events, the serotype in the MEF was concurrently found in the NPA. An additional serotype, not found in the other site was found from three MEF samples and from eight NPA samples.

Table 2. Distribution of serotypes of *S. pneumoniae* in nasopharyngeal and middle ear fluid samples

	Isolates by sample type and clinical category							
	Nasopharyngeal samples[1]						MEF[2] samples	
	Age-based visits[3]		Sick visits, no AOM[4]		Sick visits, any AOM[5]		Sick visits, Pnc AOM[2]	
Serotype	n	%	n	%	n	%	N	%
6B	106	16	60	14	47	11	18	9
23F	93	14	87	21	88	20	40	20
19F	93	14	74	17	91	21	51	25
6A	61	9	36	9	44	10	23	11
11	57	8	35	8	24	6	11	5
35	35	5	5	1	9	2	1	<1
14	28	4	14	3	25	6	15	7
15	26	4	15	4	15	3	4	2
9N	18	3	8	2	9	2	3	1
19A	17	3	12	3	10	2	6	23
7	17	3	2	<1	2	<1	1	<1
9V	15	2	12	3	9	2	5	2
4	13	2	5	1	5	1	2	1
18C	10	1	1	<1	10	2	6	3
22	9	1	10	2	7	2	1	<1
3	8	1	6	1	6	1	3	1
Others[6]	30	4	21	5	17	4	9	4
Nonencapsulated	28	4	14	3	13	3	6	3
Nontypable	8	1	6	1	2	<1	0	0
Not typed	1	<1	0	0	1	<1	0	0
Total	673	100	423	100	434	100	205	100

[1]Nasopharyngeal samples positive for *S. pneumoniae* (n=1475; 1530 isolates); [2]middle ear fluid samples obtained during pneumococcal acute otitis media (Pnc AOM; n=201, 205 isolates); [3]nasopharyngeal swab (NPS) samples obtained at age-based visits (n=649; 673 isolates); [4] nasopharyngeal aspirate (NPA) samples obtained at sick visits without any clinical diagnosis of AOM (n=407; 423 isolates); [5]NPA samples obtained at sick visits with any clinical diagnosis of acute otitis media irrespective of aetiology (n=419; 434 isolates); [6]other serotypes: 8, 10, 16, 18B, 20, 21, 23A, 25, 28, 31, 33, 34, 38 and 42. In 54 nasopharyngeal samples from 45 children and in 4 MEF samples from 4 children, two (and in one NPA sample, three) serotypes were isolated at the same time. The serotype was included only once, if isolated in MEF samples from both ears.

5.3 The value of nasopharyngeal culture in predicting the aetiology of acute otitis media (II)

5.3.1 Presence of *S. pneumoniae* and *H. influenzae* in nasopharyngeal culture according to the aetiology of acute otitis media

The value of nasopharyngeal culture in predicting the presence of these two bacteria in the MEF was studied using all available pairs of NPA and MEF samples (N 586) obtained during AOM in the absence of concurrent oral antimicrobial treatment and more than two weeks after any previous AOM diagnosis. In total, 200 children provided at least one AOM event (1-11, median, 2).

S. pneumoniae grew in 27% (160/586) and *H. influenzae* grew in 22% (126/586) of the MEF samples obtained during AOM. Table 3 shows the occurrence of pneumococci or *H. influenzae* concurrently in the nasopharynx and in the MEF during AOM.

Table 3. Occurrence of *S. pneumoniae* and *H. influenzae* in the nasopharynx and the same bacterium in the middle ear fluid during acute otitis media.

	Culture of the bacterium from the NPA[1] sample	Culture of the bacterium from the MEF[2] sample		
		Positive n	Negative n	Total N
S. pneumoniae	Positive	159[3]	157	316
	Negative	1	269	270
	Total	160	426	586
H. influenzae	Positive	97	54	151
	Negative	29	406	435
	Total	126	460	586

[1]NPA, nasopharyngeal aspirate; [2]MEF, middle ear fluid sample. [3]In 158 of these 159 events, the pneumococci from both MEF and NPA were serotyped and the same serotype was found in 157 of the 158 events. The bacterium isolated from MEF samples from both ears were regarded as one finding.

5.3.2 Predictive value of nasopharyngeal culture of *S. pneumoniae* and *H. influenzae* in predicting their presence in the MEF during acute otitis media

Using the results from Table 3, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of nasopharyngeal culture to predict the aetiology of AOM were calculated and are presented in Table 4. For *S. pneumoniae*, the sensitivity (i.e. detecting Pnc AOM with positive nasopharyngeal culture) was very high, 99% (159/160). Accordingly, the NPV (i.e. ruling out Pnc AOM in presence of negative nasopharyngeal culture) was also very high, >99% (269/270). Instead, the specificity of pneumococcal nasopharyngeal culture was relatively low 63% (269/426), as pneumococci were often carried also during non-pneumococcal AOM. Moreover, PPV was low (50%), as only half of the pneumococcal carriers had Pnc AOM. The sensitivity and NPV remained close to 100% irrespective of age, sex, season or the number of previous AOM events. The PPV seemed to decrease towards two years of age.

In predicting AOM caused by *H. influenzae* (Hi AOM) by nasopharyngeal culture of *H. influenzae*, the sensitivity (77%) and NPV (93%) were lower than those for *S. pneumoniae*, whereas the specificity (88%) and PPV (64%) were higher (Table 4).

Table 4. Nasopharyngeal culture of *S. pneumoniae* and *H. influenzae* as a predictor for isolation of the same bacterium from a middle ear fluid sample obtained concurrently during acute otitis media.

	Sensitivity	Specificity	PPV[1]	NPV[2]
S. pneumoniae	99	63	50	>99
n/N	159/169	269/426	159/316	269/270
95% CI[3]	95-100	57-68	43-56	97-100
H. influenzae	77	88	64	93
n/N	97/126	406/460	97/151	406/435
95% CI	69-83	85-91	56-71	90-95

[1]Positive predictive value; [2]negative predictive value; [3]95% confidence interval, estimated by logistic regression using generalised estimating equations with an exchangeable correlation structure to control for a possible intra-child correlation between measurements from the same child. The same bacterium isolated from both ears was regarded as one finding.

5.3.3 Quantity of pneumococcal growth in nasopharyngeal culture as a predictor of pneumococcal acute otitis media

The occurrence of both Pnc AOM and Hi AOM was associated with the quantity of the bacterial colonies in the NPA. As seen in Table 5, *S. pneumoniae* grew more often from the MEF if the same bacterium grew abundantly from the NPA. Similarly, there was a clear association between a high density of *H. influenzae* in the NPA culture and a positive culture in the MEF, although the probability of Hi AOM was relatively high even with low numbers of colonies in the NPA sample (Table 5).

Table 5. Quantity of growth of *S. pneumoniae* and *H. influenzae* in the nasopharynx and occurrence of the same bacterium in the middle ear fluid during acute otitis media.

	Culture of the bacterium from the NPA[1] sample	Culture of the bacterium from the MEF[2] sample		
		Positive n (%)	Negative n	Total N
S. pneumoniae	Abundant growth[3]	153 (55)	123	276
	Sparse growth[4]	6 (15)	34	40
	No growth	1 (<1)	269	270
	Total	160 (27)	426	586
H. influenzae	Abundant growth	83 (71)	34	117
	Sparse growth	14 (42)	19	33
	No growth	29 (7)	406	435
	Total	126 (22)	459	585[5]

[1]NPA, nasopharyngeal aspirate; [2]MEF, middle ear fluid; [3]abundant growth, ≥ 100 colonies per plate after a 10 μl-loopful plated; [4]sparse growth, 1-99 colonies per plate after a 10 μl-loopful plated; [5]for one NPA sample the quantity of growth was not known for *H. influenzae*. If the same bacterium was isolated from both ears, the event was included only once, with the most abundant growth.

Comparison between Tables 4 and 6 reveals that using abundant growth in the NPA as a positive finding and sparse or no growth as a negative finding, the specificity and PPV increased whereas the sensitivity and NPV decreased for both *S. pneumoniae* and *H. influenzae*.

Table 6. Abundant nasopharyngeal growth of *S. pneumoniae* and *H. influenzae* as a predictor for isolation of the same bacterium from a middle ear fluid sample obtained concurrently during acute otitis media.

	Sensitivity	Specificity	PPV[1]	NPV[2]
Abundant growth[3] of *S. pneumoniae* n/N	96 153/160	71 303/426	55 153/276	98 303/310
Abundant growth of *H. influenzae* n/N	66 83/126	93 425/459	71 83/117	91 425/468

[1]Positive predictive value; [2]negative predictive value; [3]positive nasopharyngeal finding, $\geq$100 bacterial colonies per plate after a 10 µl-loopful plated; negative nasopharyngeal finding, no growth or sparse growth (1-99 colonies per plate after a 10 µl-loopful plated). If the same bacterium was isolated from both ears, the event was included only once, with the most abundant growth.

5.3.4 Combinations of *S. pneumoniae* and *H. influenzae* in the nasopharyngeal samples in predicting their presence in middle ear fluid

There was a clearly inverse association between the presence of *H. influenzae* in the NPA and Pnc AOM while the inverse association between the occurrence of *S. pneumoniae* in the NPA and Hi AOM was less obvious. Table 7 shows the frequency of *S. pneumoniae* and *H. influenzae* findings in the MEF by different combinations of *S. pneumoniae* and *H. influenzae* in the NPA sample. The PPV for *S. pneumoniae* was 58% (140/241) if it grew in the NPA without *H. influenzae*. If *H. influenzae* grew without *S. pneumoniae* in the NPA, the probability of Hi AOM was 66% (50/76). Both *S. pneumoniae* and *H. influenzae* grew in the NPA in 75 (13%) cases. Of these, *S. pneumoniae* alone grew in 12 (16%) of the MEF samples, *H. influenzae* alone grew in 40 (53%) of the MEF samples and both *S. pneumoniae* and *H. influenzae* grew in 7 (9%) of the MEF samples.

Table 7. Occurrence of *S. pneumoniae* and *H. influenzae* in the middle ear fluid of children with acute otitis media by combinations of *S. pneumoniae* and *H. influenzae* in the concurrently obtained nasopharyngeal aspirate.

Combination of *S. pneumoniae* and *H. influenzae* in the NPA[1]		*S. pneumoniae* in the MEF[2]		*H. influenzae* in the MEF		All AOM[3] events
S. pneumoniae	*H. influenzae*	n	%	n	%	N
Positive	Negative	140[4]	58	17[4]	7	241
Positive	Positive	19[5]	25	47[5]	63	75
Negative	Positive	0	0	50	66	76
Negative	Negative	1	< 1	12	6	194
Any combination		160	27	126	22	586

[1]Nasopharyngeal aspirate; [2]middle ear fluid; [3]acute otitis media. [4]Includes five events with both *S. pneumoniae* and *H. influenzae*; [5]includes seven events with both *S. pneumoniae* and *H. influenzae*. The same bacterium isolated from both ears was regarded as one finding.

5.4 Pneumococcal acute otitis media in relation to preceding pneumococcal carriage (III)

5.4.1 The observation periods

Altogether 318 children provided 1715 observation periods (1–6 per child, median, 6). The overall proportion of carriers of *S. pneumoniae* was 23% (397/1715) at the age-based visits (at 3, 6, 9, 12, 15 and 18 months of age) that started the six 3-month observation periods (initial carriers). The proportion of initial carriers increased from 12% at three months of age to 38% at 18 months of age.

The duration of individual observation periods varied because a time window of +/- 2 or +/- 4 weeks was allowed for the age-based visits, but by definition, the periods never overlapped for an individual child. The median duration of the six observation periods varied from 89 days to 93 days (mean 88-95 days) without any systematic trend by age. The median and mean durations were similar (91-92 days) for observation periods starting with and without Pnc carriage, periods with and without a *focus sick visit* (spontaneous sick visit fulfilling the inclusion criteria for this study, see definition), periods with and without AOM diagnosed at the focus sick visit, periods with and without *S. pneumoniae* in the NPA at the focus sick visit, and periods with and without Pnc AOM at the focus sick visit. This is important for assessing the timing of the *NPA positive focus sick visits* with and without Pnc AOM in relation to the age-based visits starting the observation periods.

The 318 children came to 774 focus sick visits (1–6 per child, median, 3). The children carried *S. pneumoniae* at 316 (41%) of the 774 focus sick visits. Any AOM was diagnosed at 224 (29%) of these 774 visits and of the 224 AOM events, 70 (31%) were caused by *S. pneumoniae*. During Pnc AOM, the MEF always yielded only one serotype and the same serotype was also always found concurrently in the nasopharynx.

Table 8 describes the observation periods of the initial carriers and initial non-carriers. There was little difference in the occurrence of the focus sick visits and the presence of AOM at the focus sick visits. By contrast, Pnc carriage at the focus sick visits was clearly associated with the initial carriage status. The initial carriers harboured pneumococci in their nasopharynx at the focus sick visits about three times as often as initial non-carriers (87% *vs.* 26%).

Table 8. Observation periods for investigating the association between the development of pneumococcal acute otitis media and preceding pneumococcal carriage.

Focus sick visits	Observation periods					
	Initial carriers[1] 23% (n=397)		Initial non-carriers[2] 77% (n=1318)		All observation periods (N=1715)	
	%	n/N	%	n/N	%	n/N
Occurrence of focus sick visit[3]	47	185/397	45	589/1318	45	774/1715
Any AOM at the focus sick visit[4]	32	60/185	28	164/589	29	224/774
NPA positive focus sick visit[5]	87	161/185	26	155/589	41	316/774
Pnc AOM at the focus sick visit[6]	12	22/185	8	48/589	9	70/774
Non-Pnc AOM at the focus sick visit[7]	21	38/185	20	116/589	20	154/774

[1]Children who carried and [2]children who did not carry *S. pneumoniae* at the age-based visit starting the 3-month observation period: [3]for definition of a focus sick visit, see text; [4]clinical acute otitis media (AOM) irrespective of aetiology diagnosed at the focus sick visit; [5]nasopharyngeal aspirate positive for *S. pneumoniae* at the focus sick visit; [6]Pnc AOM and [7]non-pneumococcal AOM diagnosed at the focus sick visit. Concurrent carriage of two serotypes at the focus sick visit was included as two separate observation periods in this analysis.

Nevertheless, initial carriers had Pnc AOM at the focus sick visits only 1.5 times as often as the initial non-carriers (12% *vs.* 8%). The median time from the start of the observation period to the focus sick visits was 38 days (42 days and 37 days during the periods of initial carriers and non-carriers, respectively). The median duration of symptoms leading to the sick visit was 4 days in both initial carriers and non-carriers.

5.4.2 Association of pneumococcal acute otitis media with serotype-specific carriage history

The analysis of the development of Pnc AOM according to carriage history in Study III was based on those 316 sick visits at which the child carried *S. pneumoniae*. At 12 of these sick visits, the child carried two (or once, three) serotypes simultaneously, giving a total of 329 pneumococcal serotypes. Of the 329 serotypes, 197 (60%) were not present at the start of the period and the serotype-specific carriage was therefore termed 'newly acquired'. In the remaining 132 (40%) cases the serotype carried at the sick visit was present already at the period onset and carriage of that serotype was termed 'established'. Of the 197 newly acquired serotypes, 159 (81%) had been acquired after a negative finding (in initial non-carriers) while in 38 (19%) cases the serotype carried (by initial carriers) had changed.

Of the events of newly acquired and established carriage at the sick visit, 28% (55/197) and 11% (15/132) were associated with concurrent Pnc AOM caused by that serotype, respectively. According to the logistic regression model, the OR for occurrence of Pnc AOM was 3.8 (95% CI 1.4-10.0) if the child had a newly acquired carriage as compared to established carriage. Of the newly acquired serotypes that were seen after a negative NPS sample at the onset of the observation period, 30% (48 of 159) were associated with Pnc AOM, whereas if the serotype had been acquired after initial carriage of another serotype (i.e. the serotype had changed), the proportion was 18% (7 of 38). Of the 70 Pnc AOM events, 55 (79%) were caused by a serotype not present at the start of the observation period.

Newly acquired Pnc carriage was not associated with the risk of development of AOM per se, e.g. as a marker for a high infection pressure, since acquisition of Pnc carriage was not associated with non-pneumococcal AOM at a focus sick visit with Pnc carriage. Of those children who at the focus sick visit carried at least one newly acquired pneumococcal serotype, 13% had non-pneumococcal AOM, whereas the proportion was 19% for those who at the focus sick visit carried only established serotypes.

The propensity of newly acquired and established carriage to lead to Pnc AOM showed clearly different age trends. The probability of established carriage to cause Pnc AOM at the focus visit decreased steadily with age. As seen in Table 9, during the first observation period starting at 3 months of age, 33% (3/9) of established carriage cases caused Pnc AOM, whereas during the last period starting at 18

months of age none of the 25 cases of established carriage did so. Instead, the propensity of a newly acquired carriage to lead to Pnc AOM peaked at 9 to 12 months of age.

Table 9. Occurrence of pneumococcal AOM at focus sick visits with pneumococcal carriage, according to the presence of the same serotype at start of a 3-month observation period.

NPA positive focus sick visits[2]	Observation periods[1]											
	From 3 to 6 months		From 6 to 9 months		From 9 to 12 months		From 12 to 15 months		From 15 to 18 months		From 18 to 21 months	
	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N
Established carriage[3]	33	3/9	19	5/27	13	3/24	14	3/22	4	1/25	0	0/25
Newly acquired carriage[4]	18	4/22	21	5/24	42	15/36	27	10/37	25	10/40	29	11/38
Any carriage	23	7/31	20	10/51	30	18/60	22	13/59	17	11/65	17	11/63

[1]Six 3-month observation periods starting at age-based visits; [2]focus sick visits (see definition in text) with *S. pneumoniae* cultured from the nasopharyngeal aspirate (NPA); [3]the serotype carried at the focus sick visit present in the nasopharynx already at the age-based visit starting the observation period; [4]the serotype carried at the focus sick visit acquired after the start of the observation period.

A rapid development of Pnc AOM after acquisition of a pneumococcal serotype was indirectly suggested by the difference in timing of the NPA positive focus sick visit with and without Pnc AOM (Figure 8). The median time elapsed from the start of the observation period to the time the parents brought the child to a focus sick visit (i.e. because of suspicion of AOM) was shorter if Pnc AOM was diagnosed at the visit than if it was not, although in both cases the child carried pneumococci at the visit (Figure 6). This difference was especially informative in the case of focus visits with newly acquired carriage. The median time of 29 days between the period onsets and the focus sick visits with Pnc AOM means, by definition, that at least half of these Pnc AOM events had developed 29 days or less after acquisition. In the case of established carriage, the corresponding median time from period onset to Pnc AOM at the focus sick visit was only 15 days, indicating that also a majority of these Pnc AOM events could have developed relatively soon after acquisition. The median time from period onsets to the NPA positive focus sick visits was shorter in the case of established carriage than in the case of newly acquired carriage, probably because, the established carriage had been acquired already before the start of the observation period (if not cleared and re-acquired). The early timing of focus sick visits with AOM was again specific to Pnc AOM.
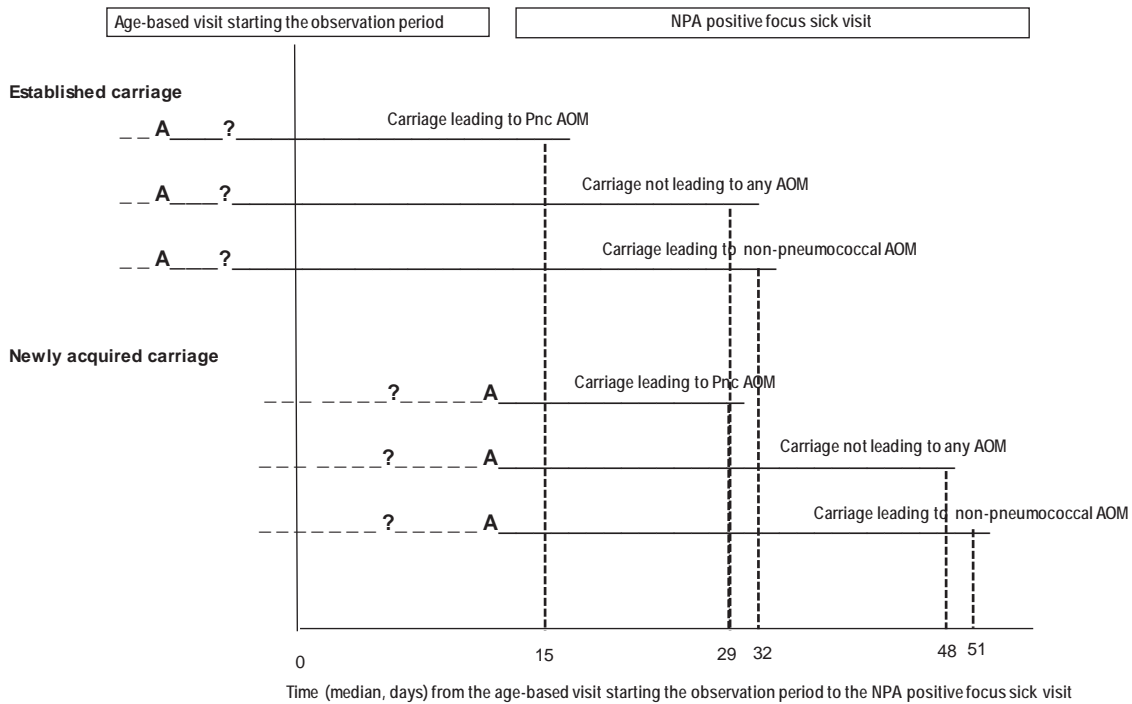
Figure 8. Temporal association between the age-based visits starting the observation periods and the NPA positive focus sick visits with different outcomes.

Dashed horizontal lines, time before pneumococcal acquisition; A, pneumococcal acquisition event; solid horizontal lines, pneumococcal carriage; dashed vertical lines, average (median) time from the start of the observation periods to the NPA positive focus sick visits. The question marks indicate the unknown time between the start of the observation period and pneumococcal acquisition which occurs either before (established carriage) or after (newly acquired carriage) the start of the observation period.

The rapid development of Pnc AOM after pneumococcal acquisition was further demonstrated in a retrospective analysis of carriage preceding the sick visit (Figure 9). The serotypes causing Pnc AOM were found at 21% (15/70) of the age-based visits starting the observation periods, and the frequency was only 14% (8/56) at visits 2 to 15 weeks before the Pnc AOM. Instead, the preceding carriage of the serotypes found at the NPA positive focus sick visits without Pnc AOM was constantly high before the sick visit, the average proportion being 45% (117/259).
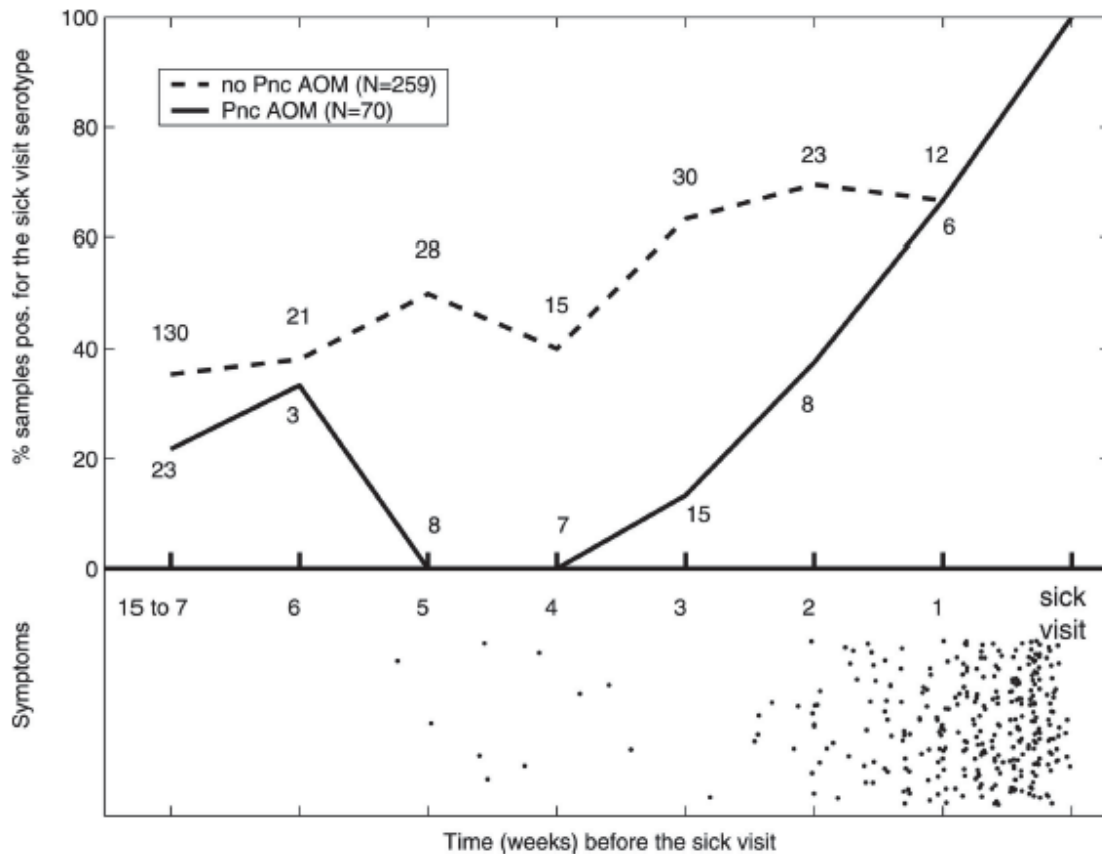
Figure 9. Preceding carriage of pneumococcal serotypes carried at the focus sick visit with and without pneumococcal acute otitis media

Solid line, pneumococcal acute otitis media (Pnc AOM, n=70); dashed line, no Pnc AOM (n=259); Note that during the last week before the focus sick visit the dashed line is hidden behind the solid line. The preceding carriage was assessed at the six age-based visits starting the observation periods (numbers of samples in the figure). Dots indicate the onset times of symptoms leading to the focus sick visit. Figure with permission from PIDJ September 2005–Volume 24–Number 9, pp 801–806). Wolters Kluwer Health Lippincott Williams & Wilkins©.

5.5 Dynamics of pneumococcal acquisition and pneumococcal acute otitis media in temporal relationship to respiratory infection (IV)

The proportion of pneumococcal carriers in Study IV was 41% at the 1788 spontaneous sick visits and 21% at 3024 age-based visits. The difference in the frequency of carriage between the spontaneous sick visit and age-based visits was clear in children aged less than six months (25% *vs.* 12%) but only moderate in children at least 18 months of age (51% *vs.* 40%). Figure 10 demonstrates the

definition of the 585 episodes of carriage in 228 children (1–9 per child, median, 2). The 585 *episodes of carriage* and the 828 *episodes of non-carriage* comprised 11% and 33% of the total follow-up time (from birth to the last nasopharyngeal sampling) of 607 years in 327 children. In the episode-based analysis, the time of acquisition could be determined for 404 episodes of carriage. The 1146 *sick episodes* comprised 17% of the total follow-up time in the 329 children and 19% of the total follow up in those 286 children who had at least one sick episode.
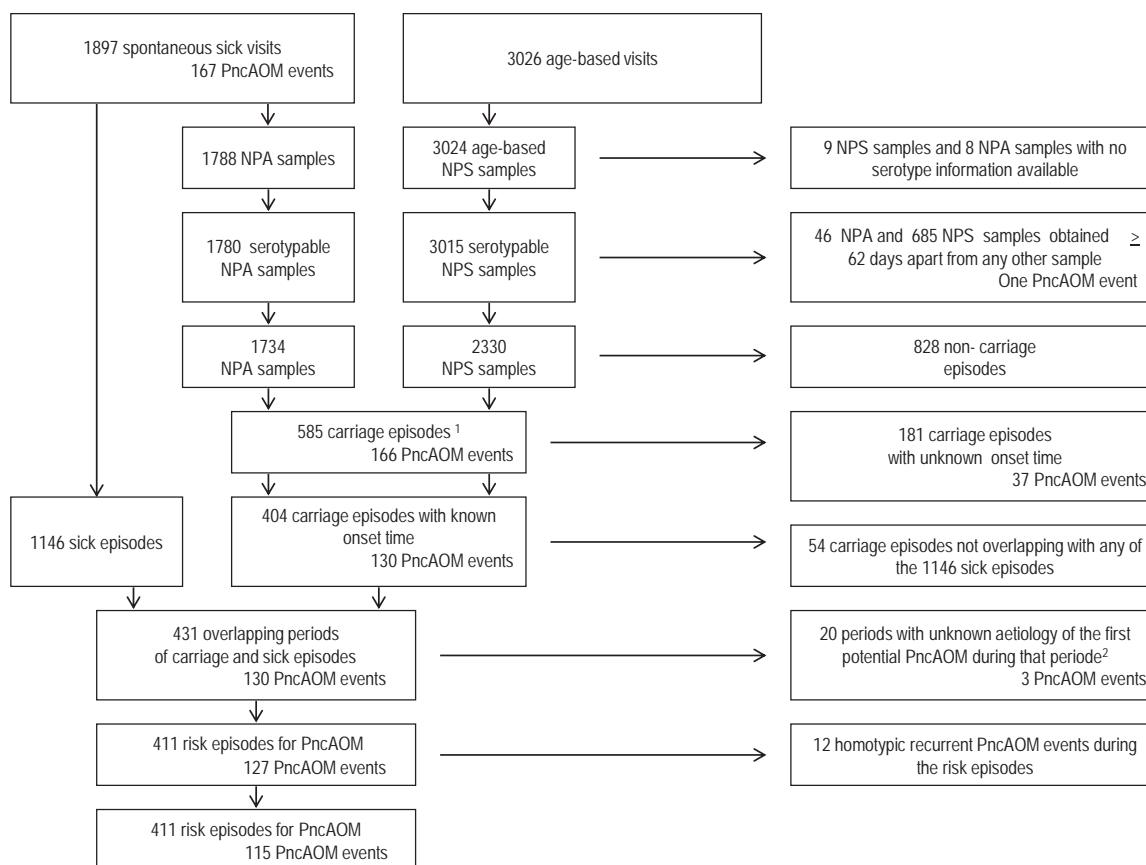
Figure 10. Defining episodes of carriage and non-carriage, sick episodes and risk episodes for pneumococcal acute otitis media.

NPA, nasopharyngeal aspirate; NPS nasopharyngeal swab; PncAOM, pneumococcal acute otitis media. [1]Six NPS and seven NPA samples negative for pneumococci were imputed with positive carriage because the child was on antibiotic medication and the previous and subsequent samples were of the same serotype. In addition, a negative NPA (two), missing NPA (two), or NPA with a different serotype from that in the middle ear fluid (MEF; one) were imputed with the serotype isolated from the concurrent positive MEF sample. In the 52 cases where two (or in one case, three) serotypes were identified concurrently, the serotype not present in the previous sample less than 62 days apart was chosen 35 times, the serotype in MEF was chosen twice, and in 15 cases the serotype was chosen randomly. [2]If there were any AOM event with missing MEF sample before the first Pnc AOM (if any) during the risk period, the aetiology of the first potential Pnc AOM remained unknown. Figure adapted from Original Communication IV, with permission of all authors.

5.5.1   Acquisition of carriage in relation to respiratory infection

Tables 10 and 11 present the 404 observed acquisition events and person-times during the 585 episodes of carriage and the 828 episodes of non-carriage stratified by age group (0–5, 6–11, 12–17, 18–24 months at the episode onset) and current carriage status of the child (Table 10, non-carriers; Table 11, carriers). For this episode-based analysis, only episodes with known onset were used. The follow-up time was further stratified by the proximity to the sick episode onset, based on an exploratory analysis which revealed a clear change point in the overall acquisition hazard about one month before the sick episode onset.

The overall unadjusted acquisition hazard in the episode-based analysis was clearly highest in non-carrying children during *pre-sickness'* (i.e. during 30 days preceding the sick episode onset) and continued to be high during 'sickness' (i.e. during the sick episode), the acquisition hazards being more than 5-times and 3-times higher as compared to *'health'* (i.e. more than 30 days before sick episode onset). The acquisition hazards were lower in children who already carried pneumococci than in children, who did not, except during sickness. During health, the acquisition rate was very low in both carrying and non-carrying children.

Table 10.   Episode-based crude estimates of hazard of pneumococcal acquisition in non-carriers stratified by age group and proximity to sick episode onset.

|	Acquisitions in relation to sick episode onset					
	Health[1]		Pre-sickness[2]		Sickness[3]	
Age[4] (months)	Aquisition hazard[5]	Acquisitions/ person-time[6]	Aquisition hazard	Acquisitions/ person-time	Aquisition hazard	Acquisitions/ person-time
0-5	0.05	65/1206.0	0.19	47/248.5	0.08	18/218.3
6-11	0.02	1/52.2	0.30	39/130.0	0.14	25/180.1
12-17	0.03	1/39.3	0.45	43/94.6	0.28	33/116.8
18-24	0.00	0/10.2	0.31	11/35.5	0.24	14/58.6
Total	0.05	67/1307.5	0.28	140/508.5	0.16	90/573.8

The at-risk time for pneumococcal acquisition during episodes of non-carriage with known onset time is divided into three strata: [1] *health*, >30 days before any sick episode onset; [2] *pre-sickness*, within 30 days before any sick episode onset; and [3]*sickness*, during a sick episode. [4]Age group at the beginning of the episode of non-carriage. [5]A crude estimate of pneumococcal acquisition hazard is calculated as acquisitions [6]per person-time in months. Table adapted from Original Communication IV, with permission of all authors.

Table 11. Episode-based crude estimates of hazard of pneumococcal acquisition in carriers stratified by age group and proximity to sick episode onset.

	Acquisitions in relation to sick episode onset					
	Health[1]		Pre-sickness[2]		Sickness[3]	
Age[4] (months)	Aquisition hazard[5]	Acquisitions/ person-time[6]	Aquisition hazard	Acquisitions/ person-time	Aquisition hazard	Acquisitions/ person-time
0-5	0.03	4/145.1	0.13	9/69.3	0.17	9/52.7
6-11	0.00	0/20.5	0.19	14/71.6	0.16	13/83.3
12-17	0.04	1/25.3	0.16	14/87.8	0.17	18/106.9
18-24	0.00	0/8.4	0.27	12/45.9	0.16	13/79.0
Total	0.03	5/199.3	0.18	49/274.6	0.16	53/321.8

The at-risk time for pneumococcal acquisition during episodes of carriage with known onset time is divided into three strata: [1] *health*, >30 days before any sick episode onset; [2] *pre-sickness*, within 30 days before any sick episode onset; and [3]*sickness*, during a sick episode. [4]Age group at the beginning of the episode of carriage. [5]A crude estimate of pneumococcal acquisition hazard is calculated as acquisitions [6]per person-time in months. Table adapted from Original Communication IV, with permission of all authors.

Due to the less frequent age-based sampling in children aged more than 6 months, however, most of the episode-based person-time of risk for acquisition during health was accrued from children aged less than six months. Using a Markov transition model and data from all age-based and sick visit samples, the acquisition rate in healthy non-carrying children was significantly higher in the age group ≥12 months than in the age group <12 months (0.18 *vs.* 0.08 per month, respectively). Instead, the hazards of clearing carriage were similar in the two age groups, corresponding to median carriage durations of 1.9 months with overlapping 90% PIs for the medians (1.7–2.2 and 1.7–2.1, respectively).

The acquisition hazard during the pre-sickness period was 3.5 times higher as compared to health (hazard ratio (HR) 90% PI 2.9-4.1). The hazards continued to be elevated during sickness (HR 2.2, 90% PI 1.9-2.7). The model predictions about the Pnc carriage prevalence at the age-based and sick visits agreed relatively well with the prevalence figures observed from the data (Figure 11, panels A and B), although there was a slight tendency to overestimation in the age-based samples.

The model was also used to predict the age-specific prevalence of carriage assuming the absence of respiratory infections (i.e. sick episodes) and this was compared to the observed prevalence in samples obtained from children who had not had sick visits during the follow-up before the sampling. Both predicted and

observed age-specific prevalence was lower as compared to that based on all age-based samples (Figure 11, panel C).
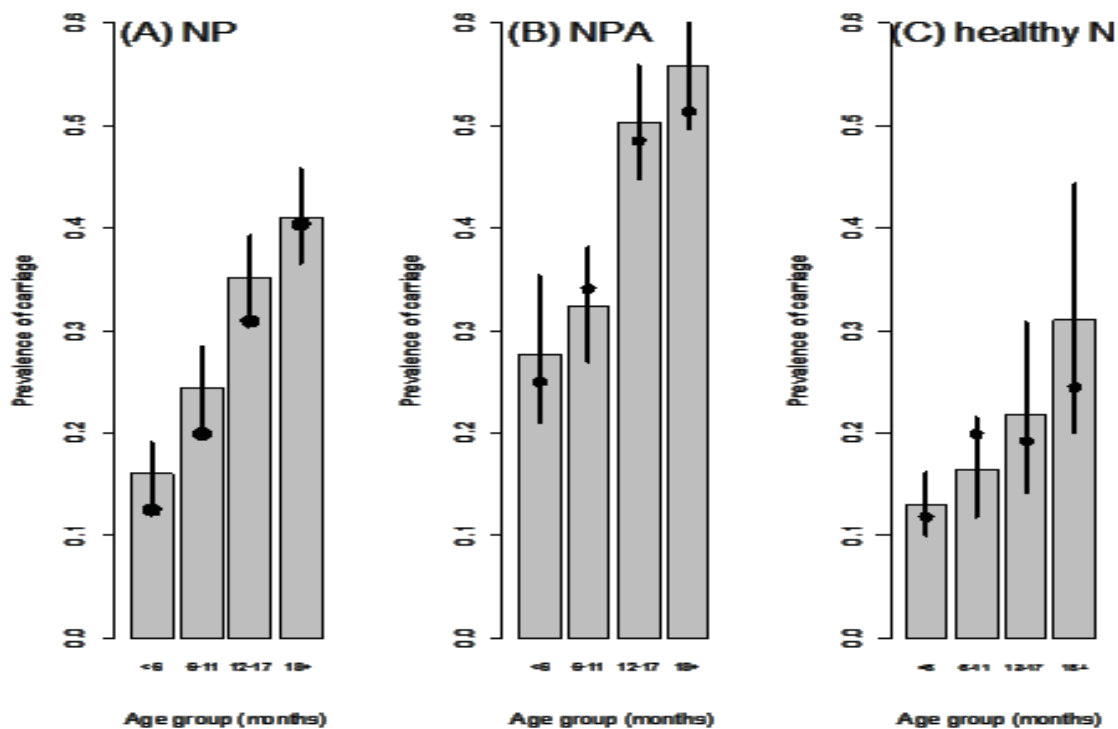
Figure 11. The prevalence of pneumococcal carriage predicted by Markov transition model, by age group.

The Markov model used data of all nasopharyngeal samples obtained during age-based visits and spontaneous sick visits. The frequency of pneumococcal carriage observed directly from the samples is shown for comparison. Panel A, nasopharyngeal swab (NPS) samples obtained at age-based visits; panel B, nasopharyngeal aspirates (NPA) obtained during spontaneous sick visits; panel C, age-based NPS samples collected more than 30 days prior to the first spontaneous sick visit of the child. The bar heights indicate the predicted prevalence values, with vertical lines showing the 90% predictive intervals. The black dots indicate the actual observed values. Figure from Original Communication IV, with permission of all authors.

5.5.2   Pneumococcal acute otitis media in relation to pneumococcal acquisition and respiratory infection

Of the 585 episodes of carriage, 491 overlapped with 541 of 1146 sick episodes. One episode of carriage overlapped with 1-5 sick episodes, and one sick episode overlapped with 1-4 episodes of carriage. This produced a total of 603 overlapping periods with 166 Pnc AOM events. Figure 10 shows the flow chart for the definition of episodes of carriage and *risk episodes* for Pnc AOM. Considering only

the 404 carriage episodes with known onset, 431 overlapping periods with 130 Pnc AOM events were available. After excluding 20 overlapping periods with MEF samples missing for the first potential Pnc AOM during that period (three Pnc AOM events) and 12 repeated homologous Pnc AOM events during the same risk episode (always only once and in different children), 411 risk episodes and 115 Pnc AOM events remained in the analysis.

Altogether, 91 (58%) of boys and 73 (46%) of girls had at least one risk episode (1-9; median, 2). These 164 children had more often older siblings living in the household (59%) as compared to those not providing risk episodes (41%).

Pnc AOM was diagnosed in 80 children during 28% (115/411) of all risk episodes, with little variation by age group (27%, 29%, 30%, and 24% in children aged 1-5, 6-11, 12-17, and 18-24 months at the risk episode onset, respectively). Of the 115 Pnc AOM events, 79% (91/115) occurred within 30 days after carriage acquisition.

In the explorative analysis, the 411 risk episodes were stratified according to temporal proximity of the acquisition of the underlying carriage to the current sick episode onset, so that the Pnc AOM hazard appeared constant within each stratum. As illustrated in Figure 12, the Pnc AOM hazard during the risk episode was considerably higher if carriage had started '*around sickness onset'*, i.e. within 30 days before or within seven days after the sick episode onset (Figure 12, blue curve, marked with a star), as compared to carriage which had started '*later during sickness'*, i.e. later than seven days after the sick episode onset (Figure 12, red curve, marked with a triangle) or to carriage which had started '*long before sickness'*, i.e. more than 30 days before the sick episode onset (Figure 12, black curve, marked with a sphere).
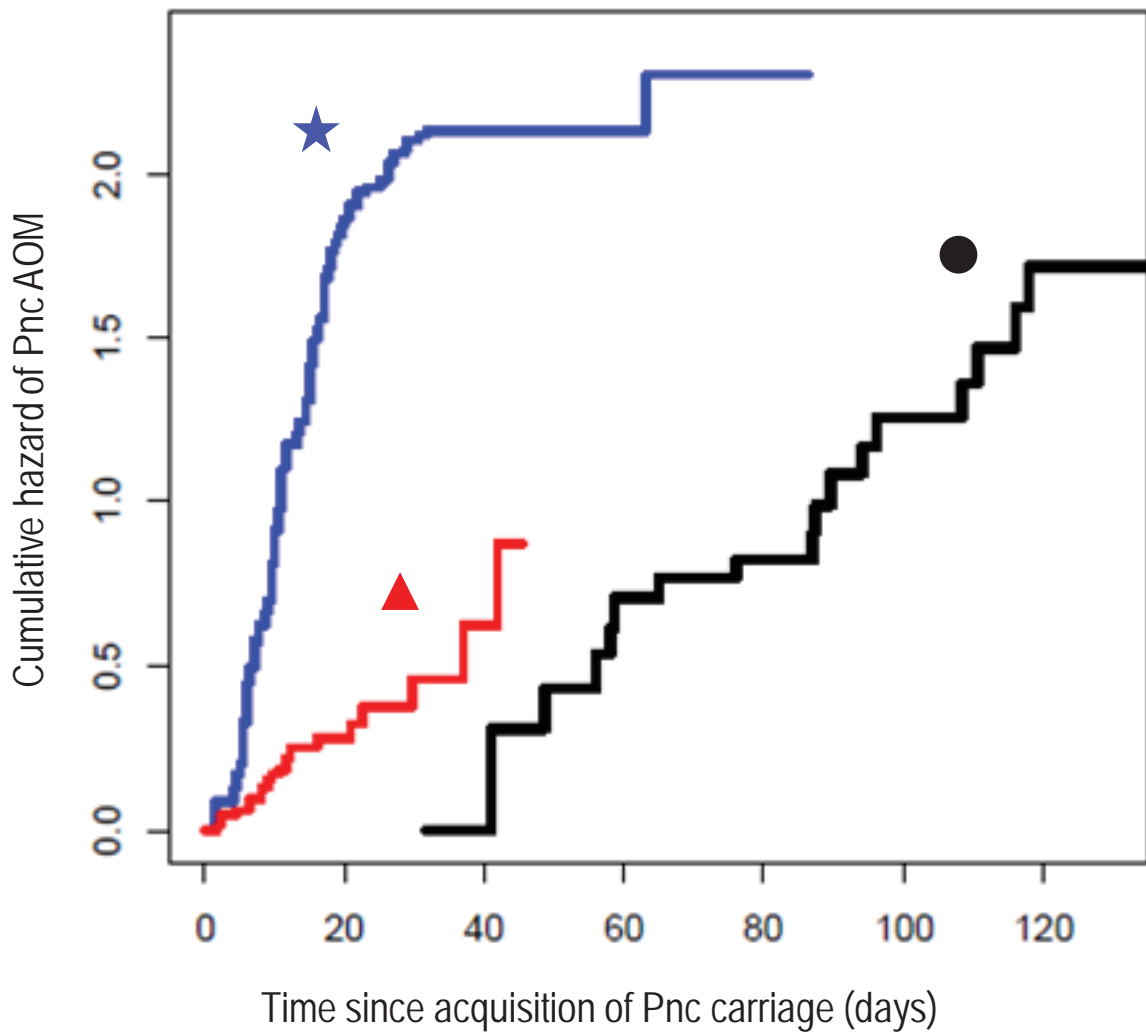
Figure 12. Cumulative hazard rate of pneumococcal otitis media by day since acquisition of pneumococcal carriage.

The hazard is presented in three strata, defined by the time of acquisition in relation to the sick episode onset. Pnc AOM, pneumococcal acute otitis media; black curve (with sphere), *long before sickness*, i.e. more than 30 days before the sick episode onset; blue curve (with star), *around sickness onset*, i.e. within 30 days before or within seven days after the sick episode onset; red curve (with triangle), *later during sickness,* i.e. during the sick episode more than seven days after its onset. The slopes of the curves represent the hazards of Pnc AOM. Note, that if the carriage had started more than 30 days before the sick episode onset, the risk episode could only start 30 days or more after acquisition. Figure from Original Communication IV, with permission of all authors.

Of note, the hazard during carriage that had started around sickness onset remained high only for the first 30 days after acquisition. Thereafter, the hazard dropped to a very low level, only 3 of 70 Pnc AOM events in this stratum occurring more than 30 days after carriage acquisition, as indicated by the change in the slope of the blue curve. Therefore, the at-risk time only up to 30 days after acquisition with 67 Pnc AOM events was included from this stratum in the further analyses, so that 112 Pnc AOM events (instead of 115) were included in the Poisson regression model (Table 12).

During those risk episodes for which the underlying carriage episode had started around sickness onset (n=204), 67 Pnc AOM events occurred during 39.1 months at risk, corresponding to a crude estimate of the Pnc AOM hazard of 1.71 per month (90% PI 1.40-2.09; Table 12). During risk episodes for which carriage started later during the sick episode (n=111), the crude Pnc AOM hazard was 0.50 per month (26 Pnc AOM events during 52.5 months at risk, 90% PI 0.36-0.68; Table 12). During risk episodes for which carriage had started more than 30 days before the sick episode onset (n=96), the crude Pnc AOM hazard was 0.49 per month (19 Pnc AOM events during 38.7 months at risk, 90% PI 0.34-0.72; Table 12).

According to the Poisson regression model with adjustment for age group, previous homologous carriage and previous homologous Pnc AOM, the hazard of Pnc AOM was significantly higher for carriage acquired around sickness onset as compared to carriage acquired later during sickness (HR 3.6, 90% PI 2.4-5.2). The relative hazard was even larger when compared to carriage acquired long before sickness (HR 4.5, 90% PI 2.5-6.8). The proportion of Pnc AOM events that occurred during carriage acquired around sickness onset comprised 60% (67/112) of all Pnc AOM events although the total duration of risk episodes in this stratum (57 months) comprised only 25% of the total follow-up time of 230 months during the risk episodes. There was a non-significant trend towards a lower Pnc AOM hazard in the case of previous homologous carriage (HR 0.68, 90% PI 0.42-1.03), but previous homologous AOM was not associated with the Pnc AOM hazard.

Risk episodes for which the underlying carriage had started around the sickness onset or later during sickness were necessarily (i.e. by definition) the first risk episodes during that carriage episode. In contrast, this was not the case for risk episodes for which the underlying carriage had started long before sickness, and in 76 of 96 of these risk episodes the child had actually had at least one earlier sick episode (1-4) during the same carriage episode. The unadjusted Pnc AOM hazard during these repeated risk episodes during the same carriage episode was notably

low (0.38 per month), although 60 of these risk episodes were observed in children at an infection-prone age of 6 to 18 months (median time from the carriage acquisition to the risk episode onset, 85 days). Instead, the hazard was higher (1.00 per month) during the 20 first risk episodes for which the carriage had also started long before the risk episode onset but the child had not had any preceding sick episodes during the same carriage episode, and which all were observed in children less than 12 months of age (median time from the carriage acquisition to the risk episode onset, 66 days).

The Poisson model was next used to compare Pnc AOM hazards only between risk episodes without any previous sick episodes during the same carriage episode in the three strata (n=335). The relative Pnc AOM hazard during carriage acquired around sickness onset was now only about two times higher than the hazard during carriage acquired long before sickness, with 90% credible interval for the relative hazard including zero (HR 2.1, 90% PI 0.9-4.1). Instead, the hazard remained high as compared to the hazard during carriage acquired later during sickness (HR 3.8, 90% PI 2.5-5.4). Previous homologous carriage now significantly decreased the Pnc AOM hazard (HR 0.57, 90% PI 0.30-0.94) but previous homologous Pnc AOM was not associated with the Pnc AOM hazard.

There were 34 additional overlapping periods of carriage and sick episodes, for which the episode of carriage had been first detected more than 30 days before the sick episode onset although the exact acquisition time could not be defined in the episode-based analysis. For 28 of these additional risk episodes, information was available on the aetiology of the first potential Pnc AOM event and of the presence (n=15) or absence (n=13) of previous sick episodes overlapping with the same carriage episode. When the analysis of Pnc AOM during the first risk episodes of carriage was repeated while including these 13 additional risk episodes, the Pnc AOM hazard was again significantly higher if carriage had been acquired around sickness onset than if carriage had been acquired more than one month before sickness onset (HR 1.9, 90% CI 1.1-3.4). The augmented data including the additional 28 risk episodes are summarised in Table 12.

Table 12. Hazard of acute otitis media caused by S. pneumoniae by timing of the carriage acquisition in relation to the onset of respiratory infection, stratified by age group.

| | At least one previous sick episode during the carriage episode[1] | | | No previous sick episodes during the carriage episode[2] | | | | | | | | |
| | First detection of carriage long before sickness[3] (n=91) | | | First detection of carriage long before sickness[3] (n=33) | | | Acquisition of carriage around sickness onset[4] (n=204) | | | Acquisition of carriage later during sickness[5] (n=111) | | |
Age group[6] (months)	Pnc AOM hazard	PncAOM/ person-time	90% PI	Pnc AOM hazard	PncAOM/ person-time	90% PI	PncAOM hazard	PncAOM/ person-time	90% PI	PncAOM hazard	PncAOM/ person-time	90% PI
1-5	0.0	0/2.8	NA	1.5	4/2.6	0.7-1.7	1.1	7/6.3	0.6-2.1	0.3	1/3.2	0.1-1.6
6-11	0.5	8/15.2	0.3-0.9	0.6	5/7.9	0.3-1.3	1.8	19/10.6	1.2-2.6	0.5	6/13.3	0.2-0.9
12-17	0.1	2/16.6	0.0-0.4	0.5	1/1.9	0.1-2.7	2.2	28/13.0	1.6-2.9	0.6	13/22.9	0.4-0.9
18-24	0.8	3/3.7	0.3-2.1	0.0	0/0.3	NA	1.4	13/9.2	0.9-2.2	0.5	6/13.1	0.2-0.9
Total	**0.3**	**13/38.3**	**0.2-0.5**	**0.8**	**10/12.8**	**0.5-1.3**	**1.7[7]**	**67/39.1**	**1.4-2.1**	**0.5**	**26/52.5**	**0.4-0.7**

The hazard of pneumococcal acute otitis media (Pnc AOM) was assessed during overlapping episodes of carriage and sick episodes (for definition of risk episodes, see text). In this table, also overlapping time without known onset of the underlying carriage episode was considered, if the carriage was first detected > 30 days before the sick episode onset. The at-risk time was first divided according to whether [1]the underlying carriage had overlapped with at least one sick episode also earlier, or whether [2]the the sick episode was the first one during the same episode of carriage. The at-risk time was further divided into three strata according to whether the underlying carriage had started or was first detected [3]long before sickness (i.e. more than 30 days before the sick episode onset), [4]around sickness onset (i.e. within 30 days before or within seven days after the sick episode onset) or [5]later during sickness (i.e. during the sick episode more than seven days after its onset). [6]The at-risk time was further groped by age at the start of the risk episode. [7]During carriage acquired around sickness onset, only Pnc AOM events occurring during the first 30 days of carriage (67 of 70) were included, since thereafter, only three Pnc AOM events occurred during a person time of 34.7 months, corresponding to a very low Pnc AOM hazard of 0.09 per month. The hazard of Pnc AOM was calculated as Pnc AOM events per at-risk person-time in months. For further details, see text.

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6 DISCUSSION

All four studies constituting this thesis are based on material collected in the Finnish Otitis Media (FinOM) Cohort Study, conducted in 1994–1997 before licensure of the pneumococcal conjugate vaccines. In the FinOM Cohort Study, an extensive amount of information on nasopharyngeal carriage of *S. pneumoniae* during health and respiratory infection as well as on the occurrence and aetiology of AOM was collected. The study thus provided exceptional data for examining the dynamics between Pnc carriage and the development of Pnc AOM before the era of pneumococcal conjugate vaccines.

6.1 Study population and follow-up

Practically all eligible families living in the study area (Hervanta) received information about the study at their local child health care centres. These municipal health care facilities are frequented by 99% of Finnish families with young children, regardless of socio-economic status. Thus about half of the children in the birth cohort in the study area participated in the FinOM Cohort Study.

The study children were fairly representative of the Finnish child population aged 2 to 24 months. The mean number of children in the participating families was 1.8, corresponding to the average child count in Finland at the time (590). Of the study children, 63% never attended day care during the follow-up. This proportion is comparative to the proportion (64%) of Finnish children cared at home during the second to third years of life (591). Of the study children and the Finnish children in general, 51% were breastfed for at least 24 weeks and the proportion of those exclusively breastfed for at least 12 weeks was 41% and 45%, respectively (592). Of the parents, 56% had an educational attainment of senior high school or higher. The corresponding proportion was only 34% in the Finnish population >15 years of age in 1997, which, however, includes the elderly with a lower average educational attainment (593). Children of immigrant families were usually not eligible for the study, as the child's mother was required to be able to

communicate adequately in Finnish. However, in 1995, only 3% of Finnish residents had been born outside the country (594).

The study clinic was established especially to collect data for the FinOM Cohort Study. In the clinic, full-time and trained study physicians and nurses were available for the study children whenever the children needed medical care for suspicion of AOM. The study clinic was located in the centre of the study area and access to the clinic was kept easy to enhance the capture of potential AOM cases.

The compliance of the study participants to follow-up, visits and sampling was very good. Only 15% of children discontinued, the main reason being moving out of the area. Of the scheduled age-based visits, 92% were carried out, and at only two of these visits was the NPS sample not obtained. According to the diaries and interviews, 81% of ambulatory visits due to respiratory infection or AOM took place at the study clinic, and at 95% of these sick visits, an NPA sample was obtained.

The FinOM Cohort Study was conducted between April 1994 and July 1997. No pneumococcal conjugate vaccines were licensed at the time, and none of the study participants had received any pneumococcal vaccine. Between December 1995 and April 1997, about 37% of children living in the Tampere area, including Hervanta, were vaccinated at 6 to 13 weeks of age with either of two 7-valent pneumococcal conjugate vaccines investigated in the FinOM Vaccine Trial (284,589). Therefore, it has been speculated whether there could have been herd effects on Pnc carriage among the FinOM Cohort Study children. No significant reduction in carriage of the vaccine serotypes, however, was observed at the age of 12 months in the vaccinated children in the trial (595). A moderate reduction was observed in the vaccinated children at the age of 18 months, i.e. starting from February 1997, by which time more than 80% of the FinOM Cohort children had already finished their follow-up. Thus, it is unlikely that there were significant herd effects on Pnc carriage or disease in the FinOM Cohort Study children.

6.2   Design of FinOM Cohort Study and the sub-studies

Young children are prone to frequent respiratory infections. The presence of mild respiratory symptoms was therefore allowed at the age-based visits for feasibility reasons. In contrast, the parents were instructed to bring the child to a sick visit specifically when they suspected AOM on the basis of predefined symptoms, including symptoms of respiratory infection or fever. This strategy seemed to be

successful in distinguishing the conditions of health and respiratory infection, as Pnc carriage was clearly more prevalent at the sick visits than at the age-based visits. This observation is in concordance with a wide range of previous studies, in which the carriage frequency has been higher during respiratory infection as compared to health (151,238,240,242,259,473,474). Furthermore, AOM was diagnosed in 36% of the spontaneous sick visits, in 15% of the control visits scheduled to check-up healing of AOM and in only 3% of the visits initially scheduled by age. Since visits with AOM or fever were defined as sick visits, it is thus fairly appropriate to consider the child as relatively healthy at the time of the age-based visits and 'having respiratory infection' at the time of the sick visits, especially the spontaneous ones.

The longitudinal design of the FinOM Cohort Study offered an excellent possibility to examine the natural course of Pnc carriage. At the same time, however, the design posed many challenges for the analysis. Children with frequent sick visits were probably more infection-prone than children with few sick visits, either because of a higher infection pressure in the environment or because of an individual frailty. This infection-proneness could also affect the frequency of Pnc carriage at the sick visits. However, because there is always heterogeneity between children in their proneness to infection, this type of selection was not a major problem in Study I, in which the aim was to describe the natural course of Pnc carriage in an unselected child population. In studies II–IV, different measures were taken to diminish the impact of repeated infections and over-representation of events from infection-prone children. In Study II, AOM events with a preceding AOM event in the two weeks prior were excluded to diminish the proportion of recurrent AOM events. Moreover, in calculating the confidence intervals for the sensitivity, specificity, PPV and NPV of nasopharyngeal culture in predicting the aetiology of AOM, the possibility of correlation between the measurements from the same child was controlled. In Study III, only spontaneous sick visits due to parental request 30 days apart were included in order to avoid repeated measurements of the same infection period, thus diminishing over-representation of data from infection-prone children with frequent visits. In Study IV, the onset of a new respiratory infection was defined even stricter, by requiring 30 days between the last spontaneous sick visit and the start of a new episode. Moreover, the assessment of Pnc AOM hazard in Study IV was restricted to episodes during immediate risk for Pnc AOM. Comparisons to healthy children were thus not relevant.

In addition to the above designs, control of statistical uncertainty was required because of the possibility of intra-child correlation between measurements from the same child. In Studies II and III, the statistical models included a frailty variable to account for possible heterogeneity across children in their proneness to Pnc AOM. Inclusion of a frailty variable in these analyses did not essentially change the results, which indicates that individual proneness is not likely to be a significant confounder. In Study IV, previous homologous carriage and AOM of the child were included as covariates in the Poisson regression model to estimate the Pnc AOM hazard.

Age-based visits were scheduled at intervals of one month between the ages of 2 and 6 months, every three months between the ages of 6 and 18 months, and the interval between the last two scheduled visits was as long as six months. The uneven schedule of age-based visits was another challenge in the design of the FinOM Cohort Study. The youngest age group was thus over-represented among the NPS samples, whereas the frequency of sick visits peaked at the infection-prone age between 6 and 18 months. This potential source of confounding had to be carefully considered in interpreting the data and in designing the sub-studies. Stratification of samples into age groups and including age as a covariate in the statistical analyses were used to control for confounding. In Study III, only age-based visit 3-months apart were allowed to start a new observation period to ensure comparability; in estimating the acquisition hazard rates in Study IV, the Markov analysis used all data instead of being dependent on the definition of episodes of carriage.

Since the estimated average duration of Pnc carriage is about two months in children aged less than two years and even longer in the youngest ones (129,185,191,206,228,233), it is likely that the majority of carriage episodes in the very young were captured. In contrast, episodes of carriage were more likely missed in children aged more than six months, especially if the child had only a few sick visits to provide additional samples. The possibility of missing carriage episodes was a challenge in Studies III and IV, which addressed temporal associations of carriage acquisition and development of Pnc AOM. Without the possibility for very frequent sampling, it is impossible to know when Pnc carriage actually starts and when it has cleared. In Study III, carriage and subsequent occurrence of Pnc AOM were assessed at systematically defined and comparative pairs of age-based and sick visits. In the episode-based analysis in Study IV, the less frequent sampling resulted in a lack of sufficiently detailed information on episodes of carriage and non-carriage in older children during health. However, carriage frequency was

similar in samples used to define carriage episodes and in samples obtained at least 62 days apart from any other sample, when stratified by age and visit type. Thus, the detected carriage episodes probably represented relatively well those that remained undetected. Furthermore, the Markov transition model used all data, including the samples excluded from the episode based analysis in estimating the acquisition hazards. Nevertheless, the estimates of individual acquisition and Pnc AOM hazards in Study IV should be interpreted with some caution, while the relative hazards are likely to be relatively reliable (596).

Antimicrobial treatment decreases bacterial growth and interferes temporarily with its detection, as was evident also in the data comprising this thesis. Data on the history of preceding treatment were only available for the age-based visits. Data on ongoing treatment were available for almost all age-based and sick visits with samples available, the treatment having been ongoing at 6% and 11% of these visits, respectively. In Study II, the analysis was based only on samples obtained in the absence of antimicrobial treatment and recent AOM events (within 14 days), which probably reduced the likelihood of recent antimicrobial treatment. The predictive ability of nasopharyngeal culture of *S. pneumoniae* was similar when AOM cases with ongoing antimicrobial treatment or repeated AOM events were also included (597), but these cases were excluded from the analysis in Study II to improve the comparability between *S. pneumoniae* and *H. influenzae*. To avoid data reduction, the analysis in Study III was based on all data, after confirming that the conclusions were essentially unchanged when the analysis was repeated using only samples obtained in the absence of antimicrobial treatment. In defining episodes of carriage in Study IV, any one sample negative for pneumococci in the presence of antimicrobial treatment was considered positive if the preceding and next samples within two months were positive for the same serotype.

6.3   Detection of events and episodes of acute otitis media

According to the collected diaries and interviews at the age-based visits and after verifying relevant diagnoses from medical records, 81% of all visits due to acute infection and 86% of visits at which AOM was diagnosed took place in the study clinic. These high percentages indicate that the study quite likely succeeded in capturing a vast majority of AOM events occurring in the study children during the follow-up. This assertion is further supported by the observed incidence of AOM in the FinOM Cohort Study (332), which is comparative to or slightly higher than

the AOM incidences observed in three other studies conducted in Finland during the period 1978–1995 (376,377,522).

Diagnosing AOM is not easy, especially in young children who cannot complain or locate specific ear-related symptoms verbally. The disease is often misdiagnosed, which may be a problem in studies based on routine clinical praxis (295,598). In the FinOM Cohort Study special attention was paid to increasing the accuracy of the otologic diagnosis. Optimal diagnostic equipment and sufficient time were available for cleaning the cerumen from the external auditory canal and for examining the appearance and movement of the tympanic membrane. Adequate assistance for restraining the child was always available and the study physicians were thoroughly trained in diagnostic skills. Routine use of tympanometry reduced the subjectivity of the diagnosis. In addition, in 89% of the AOM events the presence of MEF was confirmed by myringotomy.

The design using myringotomy was justified by the statement of the Finnish consensus conference on the approach of the treatment of AOM in 1985. The otologists emphasised the value of drainage of the middle ear in the prevention of prolongation and complications of otitis media (417). However, new studies arguing against the effect of the procedure had been published and especially paediatricians and general practitioners had started to avoid the procedure in primary uncomplicated cases. The design necessitating the routine use of the procedure may have decreased the participating in the study, which, however, was still very high. Moreover, given the similarity of the background factors of the study children and Finnish children in general, it is not likely that any systematic selection bias would have arisen. The high compliance and the low percentage of those (9% of all children) who discontinued for reasons beyond moving out of the study area indicate that the study procedures were tolerated reasonably well.

The easy access to the study clinic and regular ear examinations at scheduled visits may have led to inclusion of relatively mild AOM cases. Moreover, as the FinOM criteria for AOM comprised signs of MEF with at least one symptom of acute infection, but without specific signs of acute inflammation of the tympanic membrane, some cases of residual MEF after previous AOM or OME with concurrent symptoms of infection not related to the ear condition may have been included. Although differences in the pathogenesis between AOM, recurrent AOM and OME are likely (92,292), these conditions form a continuum of diseases that are difficult to distinguish also in clinical praxis. Furthermore, pneumococci are involved in all three clinical entities and pneumococcal conjugate vaccines seem to reduce tympanostomy tube placements due to prolonged or recurrent forms of

otitis media even more than the acute form of otitis media (425,429-431). Thus, all three disease entities are relevant in the scope of prevention of otitis media with pneumococcal vaccines.

The difference between acute and prolonged otitis media is more important when examining the dynamics of AOM development and its relation to Pnc carriage and respiratory infection. Therefore, only spontaneous sick visits were considered in Studies III and IV, and repeated events during the same infection episode were excluded. To increase the likelihood to correctly identify new episodes of acute respiratory infection in Study IV, a new sick episode was defined to start only if more than 30 days had elapsed without the child showing up at the clinic for a spontaneous sick visit.

6.4   Clinical samples and bacteriological methods

At the age-based visits, NPS samples were collected from the deep nasopharynx with calcium-alginate tipped swabs, as recommended in the standard method for detecting upper respiratory carriage of *S. pneumoniae* by the WHO Working Group 2003 (10). The only difference to the recommendation was that the shaft of the swab was bent in the peel pouch to follow the arch of the child's nasopharynx before entering the swab into the nostril. This method allowed entering the swab very deeply into the nasopharynx but may have resulted in less tight contact of the swab tip with the mucosa and therefore to less sensitive detection of bacteria. This method has not been reported in other carriage studies and its impact is thus difficult to assess.

At the sick visits, nasopharyngeal aspirates (NPA) with a paediatric mucus extractor were used, since NPA was considered better for detection of viruses (599). The sampling techniques were specifically compared in a preceding study of children with acute respiratory infection, and the agreement between NPS and NPA sampling in detecting *S. pneumoniae* was excellent (148). Therefore, it is not likely that the higher frequency of pneumococcal recovery during respiratory infection as compared to health was due to the different sampling methods. It has been a constant finding across many studies worldwide that carriage frequency is higher during respiratory infection as compared to health, even when the same sampling technique has been used during health and respiratory infection (242,473). However, it cannot be ruled out that respiratory infection with increased discharge from nasal mucosa may enhance detection of Pnc carriage. Furthermore,

in the preceding study, NPA was found to perform significantly better than nasopharyngeal swabbing in the detection of *H. influenzae* (148), so using NPA probably improved the comparability of microbial findings in Study II.

The NPS, NPA and MEF samples were cultured on enriched chocolate agar plates and sheep blood agar plates including 5% gentamicin and thus selective for *S. pneumoniae*. The FinOM Cohort Study was primarily designed to examine the epidemiology of *S. pneumoniae*. Culture on selective agars has proven more sensitive than standard agars for detecting otitis pathogens, especially in the nasopharynx where the pathogens are easily overgrown by other bacteria (8,125). However, neither of the plates was selective for *H. influenzae*, which may have reduced the detection sensitivity for this pathogen, thus complicating the comparison between the two pathogens in Study II.

All samples were analysed in the same bacteriological in-house laboratory by a small number of trained laboratory technicians, assuring uniform quality of the analyses. The serotyping methods used in the study, counterimmunoelectrophoresis and latex agglutination have been shown to perform as well as the Quellung test (25). Uncertain results were confirmed by means of the Quellung test.

If the child carried the same serotype at two temporally related visits, the serotypes were considered to represent the same pneumococcal strain without further identification. However, a portion of the NPS and MEF isolates were analysed with MLST in a sub-study comparing the ability of serotypes and clones to cause AOM or invasive pneumococcal disease (338,367). The vast majority of temporally associated isolates with the same serotype in the same child were found to have the same sequence type. Therefore, the use of serotype as an indicator for continuous or repeated carriage should be relatively reliable. The assumption of the equivalence of serotype and strain has also been made in most of carriage and transmission studies conducted in the pre-vaccination era. However, the relevance of this assumption should be monitored closely during large scale pneumococcal vaccinations, which may exert selective immunological pressure on the pneumococcal population.

6.5   Basic features of pneumococcal carriage

The overall age-adjusted Pnc carriage prevalence based on the age-based visits was 27%. This figure is comparable to prevalence estimates in studies of unvaccinated

children aged less than two years in Sweden, the USA and Costa Rica in the 1990s (186,218,248,473). Importantly, these studies were not conducted in day care centres or medical settings treating respiratory infections. The prevalence was higher than in Chinese children in Hong Kong (239) and Japanese children (666), but lower than in more recent reports from the UK, the Netherlands, Israel and Sweden (73,181,188,220,235,236,261). As expected, the prevalence of Pnc carriage in the FinOM Cohort Study was clearly lower than in populations with special risk for pneumococcal infections in low-income circumstances or in tropical environment (214-217,238,249,255).

At 12 months of age, 56% of the children had encountered *S. pneumoniae* at least once, and at 24 months of age the proportion was 87%. This figure suggests that most, if not all, children in the FinOM Cohort Study harboured pneumococci at some time during the follow-up, considering that some carriage episodes have presumably remained unobserved (186). Missed carriage episodes are probable, although additional sampling at sick visits completed the relatively infrequent age-based sampling in children aged more than six months and increased the cumulative proportion of carriers at 24 months of age by about 10 percentage points. The pattern in the cumulative incidence of first acquisition is concordant with previous data about the slow accumulation with age of the first pneumococcal encounter in developed countries (10,125,186,218-220), in contrast to populations with high risk of Pnc carriage and diseases (129,215,216). However, practically all children in any population carry *S. pneumoniae* at least once during the first two years of life (125,128,175,186,213-216,600).

The prevalence of carriage at the age-based visits increased up to 24 months of age, which is a common pattern in many studies (73,184,229,236,246). In other studies, pneumococcal prevalence has peaked or levelled off earlier (128,129,164,174,214,238-243). The latter pattern is common especially in countries where the strain turnover is fast and carriage prevalence settles at a very high level. One could speculate that in these populations pneumococci occupy their ecological niche already at very young age and/or an intensive stimulation of the immunological system leads to an earlier equilibrium between the infection pressure and carriage. Occasionally the prevalence of carriage has been found to settle at a low level (about 10% or less) at early age, e.g. in studies among exclusively healthy Chinese children in Hong Kong (239). Interestingly, the prevalence of carriage in the current study was relatively stable between 9 and 12 months of age, increasing again thereafter. A similar plateau during the second half of the first year of life and a subsequent rise can be perceived from data in a small

number of other studies conducted in the USA, Israel and Costa Rica (186,229,236). In the US study, the prevalence of serotypes 6 and 23 continued to increase during the second year of life, whereas the prevalence of serotypes 14 and 19 decreased after 18 months of age (229). Furthermore, in the current study, the prevalence of carriage increased only a little, if at all, after nine months of age, if the child had not had sick visits before the age-based sampling, suggesting some kind of change in the dynamics of carriage development between 6 and 12 months of age.

Interestingly, higher concentrations of anti-PsaA antibodies were associated with a smaller proportion of pneumococcal aetiology of AOM in the FinOM Cohort Study children aged more than nine months if the child concurrently harboured pneumococci in the nasopharynx and if the AOM was the child's first clinical AOM (138). This finding suggests that there might be an improvement in the immunological competence during the second half of the first year of life. However, this pattern remains largely unresolved, since higher concentrations of anti-PsaA antibodies did not provide protection against subsequent carriage in these children and, although there was a slight trend towards decreasing risk of Pnc AOM in the presence of high anti-PsaA antibodies after the age of 12 months, it remained statistically non-significant (138).

There was no systematic difference in Pnc carriage between boys and girls, which is consistent with most other studies (156,180,207,218,235,237,243,249,536). The lack of a systematic sex difference in Pnc carriage is interesting, since boys are known to be at higher risk for invasive pneumococcal diseases in Finland as well as elsewhere (405,523,524). Moreover, many studies have shown higher incidences of AOM in boys (375,522,530,532). Likewise, the incidence of Pnc AOM in the FinOM Cohort Study was higher in boys than in girls, although the difference was smaller than for *H. influenzae* (332). It seems that the higher risk of pneumococcal diseases among boys cannot be explained directly with the propensity to Pnc carriage, but there must be other mechanisms in the process of development of carriage to disease (545).

Pnc carriage at the age-based visits showed no clear seasonality. A small trend towards a bipolar seasonality in carriage frequency was seen in the two oldest age groups during respiratory infection, which is in concordance with the increased acquisition observed in association with respiratory infection. A similar bipolar seasonality was observed also in the incidence of respiratory infections (498) and AOM in the FinOM Cohort Study (332), as well as in the occurrence of AOM (529) and invasive pneumococcal diseases (601) in Finnish children in general.

Concordantly with the weak seasonality in Pnc carriage, the seasonality in the incidence of Pnc AOM was less prominent than seasonality in AOM caused by *H. influenzae* or *M. catarrhalis* (332). It is not clear why different carriage studies present conflicting results about the seasonality of Pnc carriage. Certainly, the climate varies considerably across different countries, but there may also be other reasons for monthly variation than those associated with weather. Although periodical trends have usually been associated with changes in weather conditions (541), in Kenya, the monthly variation in carriage was not associated with temperature, humidity or rainfall (238). The monthly variation in carriage observed in two Swedish studies among day care attendees was attributed to main vacations by the authors (209,212). In a US study, peaks and lows were observed in Pnc carriage, without regular seasonal patterns across different years (542). Gray et al. described an interesting detail in the seasonality of Pnc carriage in children: acquisition peaked in midwinter, while the prevalence fluctuated mildly throughout the year (229). One could speculate that if carriage acquisition is associated with viral respiratory infections with bipolar seasonality, the relatively long duration of carriage in children levels off the carriage prevalence between the epidemic peaks. The observation that acquisition of Pnc carriage and AOM present a similar seasonality, while prevalent carriage does not follow a similar pattern supports the link between carriage acquisition and development of AOM. This was suggested by the findings in Studies III and IV. Strong local seasonal epidemics of other nasopharyngeal microbes could also offer an explanation for variable observations of seasonality in carriage.

This thesis focused on the interplay between respiratory infection, Pnc carriage and Pnc AOM. Given the limited number of children, this approach did not allow for extensive adjustment for their background characteristics. Contacts with other children, siblings and day care mates are known to be major risk factors for infectious diseases. Furthermore, the importance of Pnc carriage of the family members on Pnc carriage among the FinOM Cohort Study children has been shown by other authors (177,191). However, since most of the impact of child contacts is likely to be due to viral and bacterial transmissions, relatively little effect could be expected beyond occurrence of respiratory infections and Pnc carriage. Nutritional and socio-economic differences are not likely to have a major impact on carriage of otitis morbidity in Finland and the parents rarely reported smoking indoors in the vicinity of the child. The Finnish population is relatively homogeneous genetically and only a few immigrants were included because of the language criterion. Adjusting for repeated measurements from the same child in

Studies II and III diminished over-representation of samples from children who may have had special risk factors for carriage and infection.

6.6   Pneumococcal carriage during respiratory infection and acute otitis media

S. pneumoniae detected at the age-based visits was considered to represent Pnc carriage during health. Although the study children were not required to be completely free of mild respiratory symptoms for feasibility reasons, the children underwent a physical examination with otoscopy at each visit, and if fever or AOM was recognised, the visit was recorded as a sick visit. In that case, NPA instead of NPS was obtained and the healthy visit was carried forward in time.

The overall proportion of positive sick visit samples was 41%, to be compared to the age-weighted average of 27% in the age-based samples. The higher frequency of Pnc carriage in association with respiratory infections or AOM has been an almost universal pattern (79,151,153,242,252,259,473,474). However, there are a number of studies in which no difference has been observed in carriage frequency between respiratory infection and health (150,207,215,217,222,237,481,482). A possible reason is that the classification of young children as healthy or symptomatic is not straightforward because mild respiratory infections are very common and minor symptoms may not be easy to recognize. In addition, antimicrobial treatment is frequently used in association with respiratory infections, and the opposite effects of infection and treatment may interfere with the interpretation of results. Some of these studies were also based on oropharyngeal rather than nasopharyngeal samples (222,481) and others included children aged up to 9–16 years (207,222,481,482), which could have affected the findings. In particular, differences in carriage frequency during respiratory infection and health may be smaller in the throat than in the nose, as reported in a family study by Masters (151). The difference between infection and health also diminished with age and was not at all seen in throat swabs from adults (151). However, the sampling site or older age do not likely explain entirely the lack of observed effects of respiratory infection on carriage, since in a study among Israeli children pneumococci were found more often during respiratory infection than during health with both nasopharyngeal and oropharyngeal swab samples (150), while in a Brazilian study, upper respiratory infection was clearly associated with an increased

risk of carriage in adolescents aged 10–19 years (252). In studies in India and Papua New Guinea, the level of carriage was very high even during health, which may explain the lack of any additional increases during respiratory infection (215,217).

The increasing prevalence of Pnc carriage with age was evident at both the age-based and sick visits. When sick visits were stratified by AOM occurrence, the age trend remained at visits with no AOM, but was hardly seen at visits with AOM. This difference is partly explained by the fact that practically all children carried pneumococci during Pnc AOM irrespective of age. In children aged less than 6 months, however, the carriage frequency tended to be relatively high also during non-pneumococcal AOM, although in all age groups combined, the carriage frequency during non-pneumococcal AOM was similar to that during sick visits with no AOM. This could be due to confounding through over-representation of AOM cases in the oldest children within this age group, since respiratory infections and AOM are rare during the first months of life and increase towards six months of age. However, further stratification by each month of age revealed relatively high carriage frequency during non-pneumococcal AOM and also during sick visits with no AOM as compared to health at each age band.

The frequency of Pnc carriage during respiratory infection and AOM as compared to health was especially high in the youngest age group. A possible explanation for this finding is that children presenting at sick visits already during the first months of life had been either more exposed to both viral and bacterial infections or were individually more prone to catch infections in general. In other words, Pnc carriage could be a marker of proneness to infection, especially in the youngest children, while older children with more contacts to other children carry pneumococci even without special proneness. Whatever the reason, these results highlight the importance of considering both age and the health status of the participants when carriage levels in different studies and populations are compared.

Although Pnc carriage, respiratory infections, and Pnc AOM all generally show clear age patterns, there were interesting differences between these entities in the FinOM Cohort Study. First, although the prevalence of carriage continued to increase throughout the second year of life, the incidence of the respiratory infections and Pnc AOM peaked at about 12 months of age and decreased thereafter (332,498). Nevertheless, the proportion of Pnc AOM events of all AOM events showed little if any variation by age. Interestingly, when examined by risk time, i.e. the child both carrying pneumococci and having respiratory infection, the age pattern almost disappeared. The age distribution in the occurrence of Pnc AOM with the peak at about one year of age thus reflected the incidence of

respiratory infections, not the prevalence of carriage. Likewise, the seasonality of AOM was similar to that of respiratory infections, whereas there was little if any seasonality in Pnc carriage

In agreement with numerous previous studies, the prevalence of carriage in the FinOM Cohort children decreased temporarily during or soon after antimicrobial treatment (221,483,484,584,586). When analysed in detail, the effect was largest during and one week after the end of the treatment, and became diluted thereafter, the carriage frequency reaching the age-group specific level at 15 to 28 days after the end of treatment, and even earlier in the age group 7 to 13 months. In children aged less than seven months antimicrobial treatment did not lower the carriage frequency below the overall level. One reason could be the especially high carriage frequency during AOM in the youngest children, as compared to health, counteracting the effect of antimicrobial treatment.

6.7 Nasopharyngeal carriage as a predictor of the aetiology of acute otitis media

The higher prevalence of Pnc carriage during AOM than during respiratory infection without AOM was mainly attributable to Pnc AOM. In particular, during Pnc AOM the child practically always harboured pneumococci in the nasopharynx, whereas during non-pneumococcal AOM, the overall proportion of positive samples was almost similar to that at sick visits without AOM. The high occurrence of *S. pneumoniae* in the nasopharynx during Pnc AOM means that the sensitivity of nasopharyngeal culture for detecting the pneumococcal aetiology of concurrent AOM was almost 100%. Accordingly, the negative predictive value of a nasopharyngeal sampling in ruling out Pnc AOM was excellent. By contrast, the specificity and the positive predictive value of the nasopharyngeal finding in predicting the presence of pneumococci in the MEF were only moderate. The high sensitivity and NPV of Pnc carriage in predicting Pnc AOM have been constantly documented by numerous studies (155,262). In the current study, however, the sensitivity and NPV were extraordinarily high as compared, for example, to the sensitivity and NPV derived from six AOM studies in a systematic review of the literature (514). One reason for the high sensitivity of nasopharyngeal carriage in predicting AOM aetiology may be high sensitivity for detecting Pnc carriage. In the FinOM Cohort Study, efforts were paid to obtaining truly deep nasopharyngeal samples and selective plates were used to diminish growth of other bacteria so as

to increase detection of pneumococci (9). Luotonen et al. used nasal rather than nasopharyngeal samples, studied children aged 2 to 6 years and cultured pneumococci on unselective plates, reaching a sensitivity of 79% in predicting the pneumococcal aetiology of concurrent AOM (510). The older age of the study children may explain the lower sensitivity, since although nasal culture has shown a good agreement with nasopharyngeal culture, especially in young children (148) it might not be as sensitive in older children (602). It is difficult to detect all existing potentially pathogenic bacteria in nasopharyngeal samples with ample normal flora, whereas the pathogens are more easily found from the normally sterile middle ear. Using selective plates to avoid overgrowth of other bacteria, even sparse nasopharyngeal growth can be detected, which increases the sensitivity of predicting AOM aetiology.

A higher sensitivity and NPV of carriage of *S. pneumoniae* than carriage of *H. influenzae* in the prediction of the respective presence of the pathogens in the MEF has not been observed consistently in previous studies (262,510,512). In a study combining previously published data, the sensitivity was lower (76% *vs.* 90%) but the NPV was higher (80% *vs.* 70%) for *H. influenzae* than for *S. pneumoniae* (514). In our study, the use of selective plates for *S. pneumoniae* but not for *H. influenzae* could have led to better sensitivity to detect pneumococci in the nasopharynx and therefore to better sensitivity to predict Pnc AOM by Pnc carriage. Furthermore, nasopharyngeal sampling is likely to detect Pnc carriage more sensitively than carriage of *H. influenzae*, since even young children carry *H. influenzae* often also in the oropharynx without nasopharyngeal involvement (148,150,152). Therefore, nasopharyngeal sampling and even aspirate may detect *H. influenzae* less sensitively than *S. pneumoniae*. However, it is not clear whether additional oropharyngeal sampling would have been beneficial in predicting Hi AOM, since a Swedish study suggested that oropharyngeal culture unlike nasopharyngeal culture has no value in assessing the aetiology of otitis media (155), possibly because the Eustachian tube is located in the nasopharynx.

As *S. pneumoniae* was a common finding in the nasopharynx even during non-pneumococcal AOM, the specificity and PPV in predicting Pnc AOM were relatively low. The vast majority of positive NPA samples yielded abundant growth and there was a clear association between the quantity of pneumococcal growth in the NPA and the presence of *S. pneumoniae* in the MEF. However, using abundant growth increased PPV only slightly, since there were only a few samples with non-abundant pneumococcal growth. The association of high nasopharyngeal bacterial

load with bacterial presence in the middle ear and also with otitis media irrespective of aetiology has been reported in Australian and US studies (262,478,509)

The specificity and PPV of nasopharyngeal culture of *H. influenzae* in predicting Hi AOM were higher than those for *S. pneumoniae*, while the sensitivity and NPV were lower. The PPV of Pnc carriage was clearly lower if *H. influenzae* was also present in the nasopharynx. Instead, the presence of pneumococci in the nasopharynx hardly affected the PPV for *H. influenzae*. It is difficult to interpret whether this asymmetry is explained with lower sensitivity for detecting *H. influenzae* carriage or whether there were true interactions between the two bacteria in causing AOM. Inverse associations between the concurrent presence of *S. pneumoniae* in the nasopharynx and *H. influenzae* in MEF and vice versa have been observed also in other studies but the associations have been of similar magnitude for both bacteria (509,510) or prediction of Hi AOM has occasionally been even poorer if pneumococci have been carried concurrently with *H. influenzae* (155). However, research in children vaccinated with the 7-valent conjugate vaccine suggests that although *S. pneumoniae* and *H. influenzae* are relatively frequently carried concurrently, they compete in the development of AOM, *H. influenzae* performing better than most serotypes of pneumococci (603).

In general, evidence on both positive and negative interactions between *S. pneumoniae* and *H. influenzae* has been presented (20,76-78). Any interaction between the two bacteria may be strain-specific (84) and affected by other bacteria (75), viruses (72), host defence mechanisms (83,84,86,92) and biofilm formation (93). Animal and in vitro observations of decreased density of pneumococci and increased density of *H. influenzae* during concurrent colonisation with the two bacteria, as compared to single bacterium colonisation, provide further evidence for the hypothesis of the higher propensity of *H. influenzae* to cause AOM during simultaneous colonisation (83,84). Furthermore, *H. influenzae* stimulates innate and pneumococcal-specific immunity, which favours the success of *H. influenzae* during co-colonisation of the two bacteria (83,84). *S. pneumoniae* in turn is known to inhibit survival of *H. influenzae* by producing hydrogen peroxide and neuraminidase (81,85).

Numerous studies addressing the ability of nasopharyngeal bacterial culture of pneumococci and *H. influenzae* to predict AOM aetiology have been conducted, in the hope that it would be helpful in clinical praxis (155,262,509-513). However, although the high NPV of nasopharyngeal culture has been acknowledged, the overall performance of the procedure has been disappointing, since the PPV remains low. The PPV of 50% in our study doubled the overall probability of Pnc

AOM (26%), but it cannot be considered optimal for clinical decisions. Schwartz et al. suggested reappraising nasopharyngeal culture in predicting AOM aetiology (509). They found that a single bacterium comprising at least 25–50% of the total number of colonies in the nasopharynx during AOM was the most valuable predictor of the aetiology of suppurative otitis caused by that bacterium. Similarly, the PPVs in our study were highest for both *S. pneumoniae* and *H. influenzae* if abundant nasopharyngeal growth of one bacterium was found in the absence of the other.

As a future consideration, adding the clinical picture to the quantified multibacterial nasopharyngeal finding could probably further increase the specificity and PPV in predicting pneumococcal AOM. Palmu et al. evaluated the association between clinical symptoms and signs or their combinations and the bacterial aetiology of AOM and constructed an algorithm that doubled the probability of pneumococcal aetiology of AOM as compared to the overall prevalence (324). The problem with increasing specificity and PPV is that it increases the false negative findings, i.e. decreases sensitivity. It has also to be considered that PPV and NPV are dependent on the prevalence of the disease in the population, in this case Pnc AOM, which is usually not known, since aetiological samples are not obtained in uncomplicated cases.

The usefulness of nasopharyngeal culture in choosing treatment for AOM is compromised by the fact that the culture takes 1–2 days to yield a result. Instead, if the 'observation option' is chosen, the culture result would probably be available at a second visit, potentially with antimicrobial susceptibility data. A rapid pneumococcal antigen detection test would provide the result during the first consultation, but it does not allow quantification of bacteria. Furthermore, there are currently no rapid antigen tests adapted to nasopharyngeal samples available for other most relevant otopathogens to allow an evaluation of the concurrent presence of multiple bacteria. High NPV, due to high sensitivity has clinical value even without detection of other pathogens, because exclusion of the potentially most severe Pnc AOM could ease choosing the 'observation option' and thus reduce antimicrobial overuse.

Despite problems in clinical work, an algorithm for predicting pneumococcal aetiology of AOM with high specificity would be useful in defining outcomes for research purposes, for example in clinical trials of pneumococcal vaccines. The time needed to analyse nasopharyngeal samples is not a problem in a study setting. The low sensitivity inevitably associated with high specificity is not a major problem in a cohort design, except for the large number of participants needed to

detect a sufficient number of outcome events, and thus high cost. The high work load and cost could be tolerable if the nasopharyngeal samples could additionally be used to answer other study questions. For example, the samples could be used for epidemiological surveillance to assess serotype distributions and antimicrobial resistance in the population. The distribution of serotypes in the NPA samples obtained during clinical AOM resembled slightly more the distribution in the MEF samples than did the distribution in isolates from the NPS samples obtained at the age-based visits. This difference may be at least partly due to repeated and uneven sampling, some serotypes being carried for longer time or at younger age and thus more frequently detected by repeated age-based sampling, rather than being real differences across serotypes in their biological propensity to cause AOM. However, as demonstrated by our Studies III and IV, Pnc AOM is not often caused by prolonged carriage. Thus, the distribution of nasopharyngeal serotypes detected during AOM may reflect the MEF serotypes better than the distribution of serotypes detected during health. The same was also suggested by a study conducted in the USA to monitor the distribution of serotypes in the nasopharynx and MEF during AOM 8 to 10 years after the introduction of pneumococcal conjugate vaccination (321). Indeed, there were some, though slight differences across serotypes in their ability to cause AOM when random nasopharyngeal samples were compared with random age-matched MEF samples in a study using data from the FinOM Cohort Study (412). A difference between carried serotypes in their capacity to cause AOM has also been shown in an Israeli study (604). Thus, better markers of pneumococcal serotypes causing AOM would be needed to evaluate the effects of serotype-specific pneumococcal vaccination against AOM.

The possibilities to evaluate the distribution of serotypes causing AOM would be even better if the specificity of predicting the pneumococcal aetiology of AOM could be increased, e.g. by using multibacterial and quantitative detection of nasopharyngeal bacteria together with the clinical picture. During Pnc AOM, the aetiologic serotype is almost always present in the nasopharynx, as demonstrated by our study as well as numerous other studies. This possibility is important because MEF samples are not currently used in clinical praxis and thus are seldom ethically and practically justifiable for research purposes.

Frequent scheduled nasopharyngeal sampling could still provide an additional possibility to increase the specificity in predicting the pneumococcal aetiology of AOM for research purposes. As shown in Studies III and IV, the strain carried during AOM has the highest probability to be present in the MEF if it has been acquired recently, i.e. the serotype harboured in the nasopharynx during the AOM

has not been present in a preceding nasopharyngeal sample. Thus, scheduled nasopharyngeal samples obtained frequently enough could provide additional information to increase the specificity in predicting the pneumococcal aetiology of AOM for research purposes.

6.8 Dynamics of pneumococcal carriage acquisition, respiratory infection and development of acute otitis media

The longitudinal nature of the FinOM Cohort Study offered a possibility to examine the temporal association of Pnc carriage acquisition and development of Pnc AOM. We found that Pnc AOM tends to develop soon after acquisition of a new serotype. This phenomenon was explained by the distinct increase in the rate of carriage acquisition in association with the onset of a new episode of respiratory infection, and the fact that carriage acquired around the acute phase of respiratory infection was most prone to proceed to otitis media.

The close association of pneumococcal acquisition with the onset of a new respiratory infection appears biologically plausible. Respiratory infections are mostly caused by viruses, and there is ample evidence from both in vitro and experimental studies that viral infection enhances bacterial adhesion and invasion to the host cells (466,467,469), thus contributing to carriage acquisition (464,472,605). Viral infection is also a well-known contributor in the development of AOM. It is likely that the viral and pneumococcal virulence mechanisms act in a synergistic way in the development of inflammation and disease (51,467). It seems that the risk of acquiring a new pneumococcal strain is highest at the onset of respiratory infection and these newly acquired pneumococci are prone to rapidly proceed to AOM. Since only a minority of respiratory infections lead to pneumococcal acquisition (477) and only a minority of new pneumococcal acquisitions lead to AOM, other factors such as the virulence of the pneumococcal strain and host immunity and other host defence mechanisms of the child must be involved in the dynamic interplay between pneumococci, viruses and the individual.

A large majority of Pnc AOM events were due to strains acquired recently, within the last month. The sensitivity analysis in Study IV showed that the inferred timing of increased acquisition before the respiratory infection onset depends on the rule used to define the carriage episodes. In the sensitivity analysis, only those samples obtained less than 45 days apart were included in defining carriage

episodes (as opposed to the 62 days in the base-case model). The period of increased acquisition before the sickness was then estimated to be shorter and the Pnc AOM hazard during newly acquired carriage was even higher as compared to carriage acquired long before sickness or later during sickness. Thus, the time of increased acquisition is likely to start later than one month before the respiratory infection onset, presumably rather at the time or soon after the beginning of the respiratory infection. Indeed, in Study III, the frequency of the preceding carriage of the serotype causing Pnc AOM was found to increase most within the two weeks before the AOM diagnosis. As symptoms had often lasted for several days before the child was brought to the visit and since viral shedding had probably started several days before the start of symptoms (606), it is quite possible that pneumococcal acquisition had occurred at the time of viral infection onset or even later. This assumption is in accordance with an animal study in which influenza virus and pneumococci were inoculated sequentially with varying intervals. In that study, the highest incidence of Pnc AOM was observed when pneumococci were inoculated just before the time of influenza-induced leucocyte dysfunction and negative middle ear pressure, both occurring within one week after inoculation of the influenza viruses (578). In another animal study, in which the animals were inoculated with influenza virus and then with pneumococci seven days later, the otoscopic signs of tympanic membrane inflammation peaked eight days after the inoculation of pneumococci (496).

Pnc carriage acquired more than a month before new respiratory infection was less prone to lead to Pnc AOM than carriage acquired around the respiratory infection onset. The Pnc AOM hazard was not as low if the child had not had previous respiratory infection during the same carriage episode as compared to purely asymptomatic carriage. Unfortunately, the majority of long asymptomatic carriage episodes were captured at less than six months of age when age-based sampling was most frequent, whereas long carriage episodes with intervening respiratory infections were mostly captured at 6 to 18 months of age. Thus, it was not possible to identify whether it was the older age or history of respiratory infection during the same episode of carriage that affected the Pnc AOM hazard more. It is possible that children who carry pneumococci already at a young age may have a special propensity to developing Pnc AOM during respiratory infection even after relatively long carriage, either because of more intense exposure to infections or because of some individual frailty which could not be recognised in these studies. In contrast, children aged more than six months may have host defence mechanisms effective enough to prevent development of otitis media after

establishment of carriage, even at an infection-prone age. It is also possible that continuing carriage itself stimulates immunological response which decreases the hazard of Pnc AOM. Based on tis data is impossible to say, whether this protection process was accelerated by an intervening viral infection or whether the slightly longer average carriage duration before the risk episode onset decreased the Pnc AOM hazard in children with repeated respiratory infections during the same carriage episode. Interestingly, having at least one previous carriage episode due to the same serotype was associated with decreased Pnc AOM hazard after re-acquisition at all ages. Similarly, when the occurrence of the first Pnc AOM event during the whole carriage episode was considered in the FinOM Cohort Study children, repeated acquisitions of the same serotype were associated with decreased occurrence of Pnc AOM, whereas previous carriage of different serotypes did not have any effect on the occurrence (607). This is in accordance with a US study among children aged less than two years, in which serotype-specific antibody responses to carriage acquisition or Pnc AOM tended to be greatest after repeated exposure (105). Thus, our findings may indicate a protective role of preceding homologous carriage against Pnc AOM, rarely demonstrated in children despite the broad, although fragmentary body of knowledge on the development and function of natural immunity against pneumococcal infections.

The hazard of Pnc AOM was relatively low and the events accumulated slowly if pneumococcal acquisition had occurred later than during the first week since the first visit due to a new respiratory infection. This lower hazard is concordant with findings from an experimental chinchilla study, in which the incidence of pneumococcal otitis media was lower if pneumococci were inoculated 12 days after inoculation of influenza virus, as compared to the incidence observed if pneumococci were inoculated four days after influenza inoculation, i.e. at the time when the virus-induced leucocyte dysfunction and a concurrent virus-induced decrease in the middle ear pressure were most prominent (471). In our study, the yield of the virus may have been short-lived after the symptom onset (495) and it is thus possible that there were few, if any, active viruses left to interact with newly acquired pneumococci in the development of AOM later during the sick episode.

Interestingly, although the Pnc AOM hazard was relatively low if the underlying carriage had been acquired during the respiratory infection after its acute phase, the hazard of pneumococcal acquisition remained high throughout the episode of respiratory infection. Thus, after the acute phase of respiratory infection, the continuing or healing viral infection still seems to enhance acquisition of Pnc carriage, but for some reason, at this phase the viral infection seems to have lost

much of its ability to promote progression of Pnc carriage to AOM. This raises the question of whether there are mechanisms that prevent AOM without preventing carriage acquisition and, if so, whether these mechanisms could have implications on planning the prevention of pneumococcal diseases.

6.9 The role of pneumococcal carriage studies in the prevention of pneumococcal diseases with conjugate vaccines

Pneumococcal conjugate vaccines prevent carriage as well as mucosal infections like AOM caused by the serotypes included in the vaccine, unlike a vaccine that only includes purified polysaccharides. In two vaccine trials, pneumococcal conjugate vaccines reduced the incidence of AOM caused by the vaccine serotypes already before the booster dose, whereas the reduction of vaccine-serotype carriage was less pronounced and observed only after the booster dose (595,608). In two other trials, the reduction in carriage became apparent when the child grew older even in the absence of a booster dose (282,609). It has been hypothesised that primary vaccine doses could reduce Pnc AOM by reducing the bacterial load in the nasopharynx without reducing the frequency of carriage (434,608). The reduction in carriage frequency could then follow later, after a booster dose, owing to enhanced immunity with increasing age and previous encounters with the serotypes.

The temporal association between pneumococcal acquisition and Pnc AOM might offer another explanation for the earlier effect of vaccination on Pnc AOM than carriage. Vaccination is believed to affect acquisition of carriage rather than established, prevalent carriage (434,436). Thus, if vaccination prevents new acquisitions, it prevents also Pnc AOM events which would have promptly followed these acquisitions. Instead, if vaccination does not affect established carriage, the prevalence in the vaccinated population is maintained until the carriage episodes have ended. Thus, the effect of vaccination on carriage may be delayed, especially in young children who on average carry pneumococci for a longer time than older children.

Pnc AOM is much milder than pneumococcal invasive infection, but because Pnc AOM is very common and usually treated with antimicrobials, its prevention has a significant public health impact. Although the effect of the 10-valent pneumococcal conjugate vaccine was only 8% in preventing antimicrobial purchases in a cluster-randomised trial in Finland, vaccination would lead to more

than 12 000 fewer antimicrobial purchases per year for children aged less than two years (610).

The most important goal for pneumococcal vaccination is invasive disease. The seriousness of invasive disease highlights the need to investigate the dynamics of pneumococcal acquisition, carriage and development of invasive diseases. Invasive pneumococcal diseases in children are often caused by serotypes that are frequently acquired and carried, but invasive diseases are also relatively often caused by serotypes that are rarely found in the nasopharynx, probably due to their rapid clearance from the nasopharynx. As a report of the work of a large consortium of researchers of pneumococcal epidemiology (PneumoCarr), Simell et al. emphasised the important role of vaccine efficacy against carriage acquisition in protecting the individual against mucosal pneumococcal diseases (611). Furthermore, the differences in the epidemiology and vaccine efficacy by age and differences in the case-to–carrier ratios across different serotypes in development of mucosal and invasive diseases are important. These facts highlight the need for reliable data on the incidence of both Pnc carriage and pneumococcal disease in different populations so as to assess the effectiveness of vaccination with conjugate vaccines (612). Nevertheless, vaccination also prevents the progress of (breakthrough) carriage to invasive disease and its worsening (611), so that other mechanisms than those preventing carriage or reducing nasopharyngeal load of pneumococci are also likely to be involved.

7   SUMMARY AND CONCLUSIONS

The high global burden of pneumococcal diseases, the emerging resistance of pneumococcal strains against antimicrobial treatment and the availability of pneumococcal conjugate vaccines emphasise the importance of the investigation of pneumococcal infections. Nasopharyngeal carriage of pneumococci is common in children and it comprises the main source of transmission of pneumococcal strains in the population. Carriage is a prerequisite for development of pneumococcal otitis media. The Finnish Otitis Media (FinOM) Cohort Study, a prospective follow-up of children during the first two years of life, offered a unique possibility to assess the natural course of pneumococcal carriage as well as the dynamic relationship between pneumococcal acquisition and carriage, respiratory infection and pneumococcal acute otitis media (Pnc AOM).

The following conclusions were drawn on the basis of the studies in this thesis:

Young children carry *S. pneumoniae* more frequently during respiratory infection than during health.

The hazard of pneumococcal acquisition of is higher in association with the onset of a respiratory infection than during health and the increased hazard continues several weeks after the onset of the respiratory infection.

A pneumococcal strain acquired around the onset of a respiratory infection is especially prone to cause AOM. The majority of Pnc AOM events occur within one month after the acquisition of a new strain.

The prevalence of pneumococcal carriage increases with age during the first two years of age. However, in the absence of respiratory infections, pneumococcal carriage increases only moderately with age.

If the child carries pneumococci during respiratory infection, age has little if any effect on the occurrence of Pnc AOM. The age-dependency in the incidence of

Pnc AOM mainly reflects the age-dependence in the incidence of respiratory infections.

Antimicrobial treatment decreases pneumococcal carriage frequency temporarily, but sex and season have little effect.

If pneumococci are not found from the nasopharynx during AOM, the pneumococcal etiology of AOM is very unlikely. A positive nasopharyngeal finding increases the possibility of pneumococcal aetiology of AOM but pneumococcal carriage is common also during non-pneumococcal AOM.

Nasopharyngeal carriage during AOM could be used as information in predicting the aetiology of AOM for research purposes. The negative nasopharyngeal finding could be used for ruling out Pnc AOM in selected cases also in clinical practice.

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10 ORIGINAL COMMUNICATIONS

Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Finnish Children Younger Than 2 Years Old

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To describe the natural course of nasopharyngeal carriage of *Streptococcus pneumoniae* and its relationship to acute otitis media (AOM), 329 Finnish children were followed from ages 2 to 24 months. In total, 3024 nasopharyngeal (NP) swabs (obtained at 10 scheduled healthy visits) and 2007 NP aspirates (obtained during respiratory infections) were cultured. Carriage during health increased gradually (9%–43%) with age. Within 4 age intervals, carriage was lower during health (13%–43%) than during respiratory infection without AOM (22%–45%). Higher proportions of positive samples were found during AOM (45%–56%), in particular during pneumococcal AOM (97%–100%). Antimicrobial treatment reduced carriage only temporarily. The most frequent NP serotypes were 6B, 6A, 11, 19F, and 23F. Both age and health status were important determinants of NP carriage of *S. pneumoniae* and these features should be considered carefully during analysis of carriage rates.

Streptococcus pneumoniae causes a wide variety of infections worldwide, from invasive diseases with considerable mortality to relatively benign and very common mucosal infections, such as acute otitis media (AOM). It is also a common component of the nasopharyngeal (NP) flora in healthy persons. The increasing occurrence of penicillin-resistant strains challenges the treatment and prevention strategies for pneumococcal diseases and emphasizes the need for knowledge about factors that affect *S. pneumoniae* carriage and its progression to disease.

Most children acquire *S. pneumoniae* in their nasopharynx during the first few years of life [1–6]. Colonization is very rapid in high-risk populations, such as infants in Papua New Guinea, who are colonized by the age of 3 months (the pattern is similar among Australian aboriginal infants) [3, 4]. In industrialized countries such as Sweden and the United States, about half of children are colonized with *S. pneumoniae* at least once by age

1 year [7, 8]. The reasons for the differences between populations are not fully understood. The prevalence of NP carriage increases during the first months of life [6, 7, 9, 10] and starts to decrease after age 3–5 years [2, 11–13]. The wide range of pneumococcal carriage (5%–89%) in different parts of the world [14, 15] reflects variations in study populations with respect to age, ethnicity, socioeconomic living conditions, and differences in sampling and isolation techniques. The health conditions at the time of sampling may also vary. The terms "carriage" or "colonization" apply to both healthy persons and to subjects with symptoms of acute infection that is possibly or definitely caused by *S. pneumoniae.*

Many studies have shown that recovery of *S. pneumoniae* from the nasopharynx or oropharynx is more likely during respiratory infection than during health [16–20] but others failed to find such a connection [21–23]. Most often, the carried serotypes are the same as those that cause most cases of AOM and other pneumococcal diseases in children. As a rule, *S. pneumoniae* of the causative serotype is found in the nasopharynx or nasal cavity during pneumococcal AOM [1, 24, 25]. Antimicrobial treatment may interfere with isolation rates, and lower isolation rates have been observed during and soon after antibiotic treatment, compared with pretreatment rates, but antimicrobial treatment does not eradicate the bacteria from the nasopharynx [23, 26–29]. New strains appear after treatment [1, 26, 28], but the long-term effect of antimicrobials on carriage rates is poorly documented. In the Finnish Otitis Media (FinOM) Cohort Study, we followed an unselected group of 329 infants from ages 2 to 24 months, during health at 10 scheduled visits and during respiratory infection and AOM, to evaluate the natural course of pneumococcal carriage and its relationship to respiratory infection with and without AOM.

Materials and Methods

Study population and facilities. In total, 329 healthy infants born in the Hervanta area, Tampere, Finland, were consecutively enrolled in the FinOM cohort study between April 1994 and August 1995 and were followed-up prospectively from ages 2 through 24 months. Families with newborns were informed of the study during their first routine visit to their local well-baby clinic. All infants born or residing in the area were eligible to participate in the study if they were 2 months ± 2 weeks old and had no prior immunization with a pneumococcal vaccine and if their mothers could communicate fluently in Finnish. During the study, the children were vaccinated in accordance with the Finnish schedule, which does not include pneumococcal vaccine.

Follow-up began in April 1994 and continued through July 1997. A study clinic with 1 or 2 doctors and 1–3 nurses was established near the local well-baby clinic. Study personnel were specially trained to interview parents, obtain samples, and diagnose and treat AOM. Study clinic services were available for the study children from 8 AM to 3 PM on work days and for 3 h on Saturdays, Sundays, and national holidays.

Scheduled visits and NP swabs (NPS). The children were examined at age-scheduled healthy visits at ages 2, 3, 4, 5, 6, 9, 12, and 15 months (±2 weeks) and at age 18 months (±4 weeks). A close-out visit was done at age 24 months (±4 weeks). The background information gathered at entry included family structure, parents' education, history of family allergies, and history of otitis media (OM) of siblings. At each scheduled visit, the study staff questioned parents about potential risk factors for carriage or AOM, including feeding patterns, allergies, day care attendance of the study child and siblings, and passive smoking. Responses were recorded on structured case report forms. Parents were also asked about antimicrobial drug usage during the 28 days preceding the visit. A physical examination, including pneumatic otoscopy, was performed by the study doctor. If the child was diagnosed with AOM, febrile infection, viral exanthem, or acute gastroenteritis, the visit was recorded as a "sick visit" and the healthy visit was carried forward within the time window, if possible.

An NPS specimen was obtained at each healthy visit with a sterile swab with a flexible aluminum shaft and a dry calcium alginate tip (Galgiswab; Spectrum Laboratories). The child's head was tipped backwards and was immobilized. The bent swab was inserted into the nostril, was passed into the nasopharynx to a distance equal to that from the child's nose to the tip of the ear, and was maintained for 5 s. The sample was cultured immediately for *S. pneumoniae.*

Sick visits and NP aspirates (NPAs). If symptoms of acute infection suggested AOM, the parents were asked to bring the child to the study clinic. The event was recorded as a sick visit. A patient history was taken, and the study doctor performed a physical examination, including pneumatic otoscopy and tympanometry. Whenever AOM (see definition below) was suspected, myringotomy was performed to confirm the diagnosis, and a middle ear fluid sample (MEF) was obtained for etiologic diagnosis. Resolution of each AOM was followed at a check-up visit 4 weeks after the diagnosis. If AOM, febrile infection (≥38°C), viral exanthem, or acute gastroenteritis was diagnosed at the check-up visit or at the close-out visit, the visit was classified as a sick visit.

An NPA was obtained at each sick visit with a sterile pediatric mucus extractor (UNO sterile EtO; UnoPlast). The catheter was guided to a depth of 4–8 cm in the nasopharynx through a nostril and was drawn back while a gentle suction was applied with an electric suction device. The sample was diluted with 0.5 mL of PBS, if needed, and was cultured immediately with a 10-μL loop for *S. pneumoniae.* The child's visits to other than study doctors were registered if shown in a patient diary, and medical records were requested to confirm AOM diagnoses.

Bacteriologic methods. NPS, NPA, and MEF samples were cultured on enriched chocolate agar plates and selective sheep blood agar plates containing 5 μg/mL gentamicin. The plates were incubated in 5% CO_2 at 36°C–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 α-hemolytic 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. 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. The susceptibility of pneumococcal strains to penicillin was tested by the agar dilution method, with breakpoints ≤0.06 μg/mL (susceptible), 0.125–1.0 μg/mL (intermediately resistant), and ≥2 μg/mL (highly resistant).

Definitions. The carrier state refers to harboring *S. pneumoniae* in the nasopharynx either at a healthy visit or during respiratory infection—even during pneumococcal AOM. AOM was visually diagnosed as an abnormal tympanic membrane in pneumatic otoscopy (by color, position, and mobility), suggesting effusion in the middle ear cavity, concomitantly with ≥1 of the following signs or symptoms of acute infection: fever, earache, tugging of the ear, irritability, acute gastrointestinal symptoms, or other symptoms of respiratory infection. Tympanometry was used as a diagnostic aid [30]. We defined pneumococcal AOM as an AOM with ≥1 MEF sample with positive culture for *S. pneumoniae.* Because age proved to be an important determinant of prevalence rates, the NPS and NPA samples were grouped according to the child's age at sampling in 4 intervals: ≤6, 7–12, 13–18, and ≥19 months.

Statistical analysis. The results describe the natural situation in a heterogeneous cohort where some children are healthy and some have repeated infections, including AOM. We used the Kaplan-Meier method and χ^2 test to compare the similarity of children who discontinued and children who completed the study. We used SPSS for Windows software (release 8.0.1; SPSS) for computation.

Results

Population and samples. In all, 329 children were enrolled in the study, representing 53% of the children registered in the well-baby clinics in the study area during the enrollment period. Table 1 lists the background characteristics of the enrolled chil-

Table 1.   Characteristics of the 329 study children.

Characteristic	No. (%) of children
Sex	
Boys	158 (48)
Girls	171 (52)
Feeding patterns	
Any breast-feeding	
No	4 (1)
≥12 Weeks	232 (71)
≥24 Weeks	167 (51)
Not known	18 (5)
Exclusive breast-feeding	
No	28 (9)
≥12 Weeks	135 (41)
Not known	3 (1)
Day care attendance[a]	
Home care only	207 (63)
Age at entrance	
≤6 Months	4 (1)
≤12 Months	35 (11)
≤18 Months	85 (26)
≤24 Months	122 (37)
Older siblings[b]	
Yes	163 (50)
No	166 (50)
Education of mother or father[c]	
Low	103 (31)
High	225 (68)
Not known	1 (<1)
Smoking indoors in home[d]	
Yes	12 (4)
No	292 (89)
Unknown	25 (8)

[a] Any type of day care ≥4 h/week.
[b] Living in household at the time of enrollment.
[c] Highest education of either mother or father: low, comprehensive school, lower high school, or vocational education; high, senior high school, college, or academic degree.
[d] At any time during follow-up.

dren. From 4 families, 2 children (twins from 3 families and siblings from 1 family) participated in the study. Compliance of the families was high: 316 (96%), 309 (94%), and 284 (86%) of the children were followed until ages 7, 13, and 19 months, respectively, and 281 (85%) completed the entire follow-up. The main reason for dropping from the study was moving from the residential area (40% of those who discontinued). The children who were lost to follow-up were similar to those who were followed-up for 24 months, in regard to duration of breast-feeding, age when first attending day care, parents' education, occurrence of first NP *S. pneumoniae,* and occurrence of first AOM, when compared by the Kaplan-Meier method. However, girls were lost to follow-up more often than boys (18.7% vs. 10.1%; $P = .027$, χ^2), and children without older siblings living in the household were lost to follow-up more frequently than children with older siblings (18.7% vs. 10.4%; $P = .034$, χ^2).

Of the scheduled 3290 healthy visits, 3026 (92%) took place. During these visits, 3024 NPS samples were obtained. All 10 samples were obtained from 212 (64%) children. The history of antimicrobial treatment within 28 days before the swabbing was known for 2993 (99%) of the NPS samples. There was no

treatment in 2322 (77%) cases. Oral treatment was given in 532 (18%) cases. In 168 cases, the treatment was ongoing at the time of sampling, in 68 it ended 1–7 days before sampling, and in 296, it ended 7–28 days before the sampling. Only topical antibiotics were used in 139 cases (5%).

Of all children, 288 (88%) were seen at a sick visit in the study clinic at least once during the study period. In all, there were 2122 sick visits during which 2007 (95% of the planned) NPA samples were obtained. One NPA sample each was obtained from 30 children, 2–10 NPA samples each were obtained from 196 children, and 11–31 NPA samples each were obtained from 61 children. In all, 81% of all known visits due to acute infections took place at the study clinic.

Study doctors diagnosed AOM at 871 sick visits (41% of all sick visits); in 89% of these, the diagnosis was verified by collection of a MEF sample from 1 or both ears. During the entire follow-up period, clinical AOM was diagnosed at least once in 215 (65%) study children, once or twice in 88 (27%) children, 3–5 times in 75 (23%) children, >5 times in 52 (16%) children, and 17 times in 1 child. These numbers comprise 86% of all cases of AOM known to have occurred among the study children during the follow-up. Only AOMs diagnosed in the study clinic are considered here.

Children were ≤6 months old at 395 (20%) sick visits during which NPA was obtained, 7–12 months old at 659 (33%) such visits, 13–18 months old at 565 (28%) such visits, and ≥19 months old at 388 (19%) such visits. The corresponding percentages of AOM events were almost the same: 167 (19%), 293 (34%), 234 (27%), and 177 (20%) for the 4 age groups, respectively.

NP carriage at healthy visits, by age and sex.   Of the 3024 NPS samples obtained, 649 (21%) were positive for *S. pneumoniae* (figure 1). The prevalence of pneumococcal carriage clearly increased with age; the age-specific rates were 9% at 2 months, 17% at 6 months, 22% at 12 months, 37% at 18 months, and 43% at 24 months. The age-weighted average proportion of positive NPS samples for the whole follow-up period was 27%. The increasing trend was seen in both sexes; the age-weighted average mean of positive samples was 29% for boys and 26% for girls. There were no systematic differences in age-specific prevalence between boys and girls. The number of colonies was >20 (84%) in 545 and 1–20 (16%) in 104 positive cultures.

NP carriage of S. pneumoniae *during health and respiratory infection with and without AOM.*   At sick visits, 2007 NPA samples were obtained, and 826 (41%) were positive for *S. pneumoniae.* At visits without concurrent diagnosis of AOM, the proportion of positive NPA samples was 35% (407 of 1158); at visits with concurrent AOM, the proportion was 49% (419 of 849).

The percentages of positive samples obtained within the 4 age intervals were compared among different clinical categories (figure 2A). The proportion of positive NPS samples obtained at healthy visits increased from 13% during the first age interval to 43% during the last age interval. In samples obtained at sick
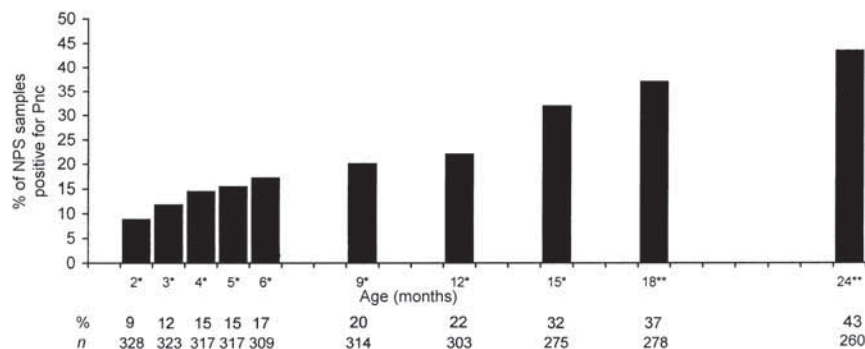
Figure 1. Age-specific prevalence of *Streptococcus pneumoniae* (Pnc) carriage in 329 children, detected in cultures of 3024 nasopharyngeal swab (NPS) samples obtained during 10 scheduled healthy visits at ages 2–6, 9, 12, and 15 months and at ages 18 and 24 months *±2 Weeks; **±4 weeks.

visits without concurrent AOM, the proportions of positive NPA samples were somewhat higher (22%–46%) and increased with age. At sick visits with concurrent AOM, the child's age had little, if any, effect on the carriage rate: within the 4 age categories, *S. pneumoniae* was found in 46%, 46%, 50%, and 56% of samples, respectively. All percentages were higher than those observed within the respective age groups at healthy visits

or at sick visits without AOM. This difference was most prominent during the first year of life.

We then examined NPA samples obtained at 761 visits during which AOM was diagnosed and for which ≥1 MEF sample was available for culture (figure 2*B*). *S. pneumoniae* was isolated from MEF in 26% of the AOM events: 26%, 25%, 31%, and 22%, respectively, by age interval. Throughout the age range,
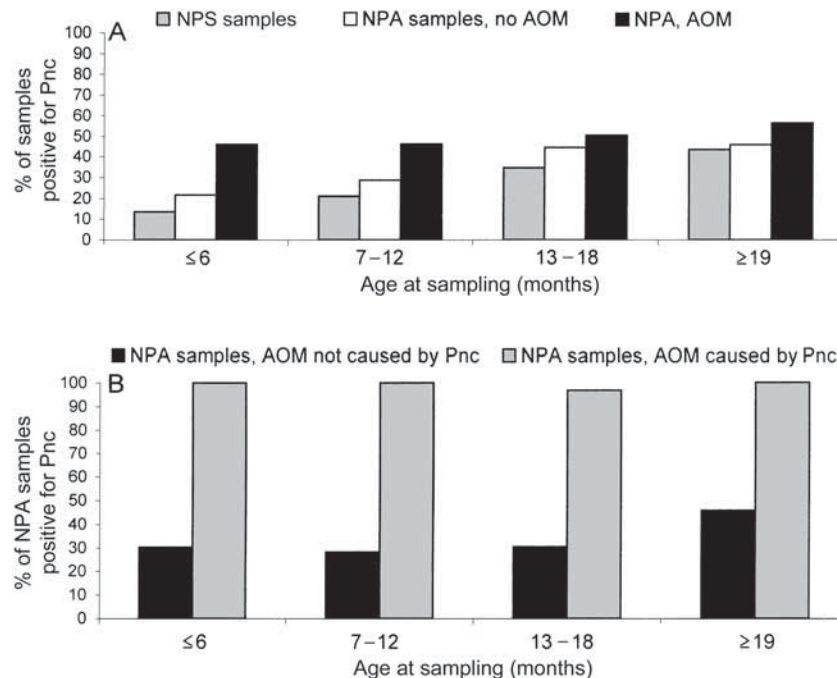
Figure 2. Proportions of 5031 nasopharyngeal (NP) cultures positive for *Streptococcus pneumoniae* (Pnc) during health or respiratory infection with or without acute otitis media (AOM). Samples were obtained during 4 age intervals from children followed from ages 2 to 24 months. *A,* NP swab (NPS) samples obtained during scheduled healthy visits (*n* = 3024, *gray bars*) and NP aspirates (NPA) obtained during respiratory infections without (*n* = 1158, *white bars*) or with (*n* = 849, *black bars*) concurrent AOM. *B,* NPA samples obtained during concurrent AOM without (*n* = 562, *black bars*) or with (*n* = 199, *gray bars*) pneumococcal etiology.

the NPA samples obtained during pneumococcal AOM usually were positive for *S. pneumoniae* (97%–100%), whereas the proportion of positive NPA samples obtained during AOM with no evidence of pneumococcal etiology was about the same as in samples obtained at visits without concurrent AOM.
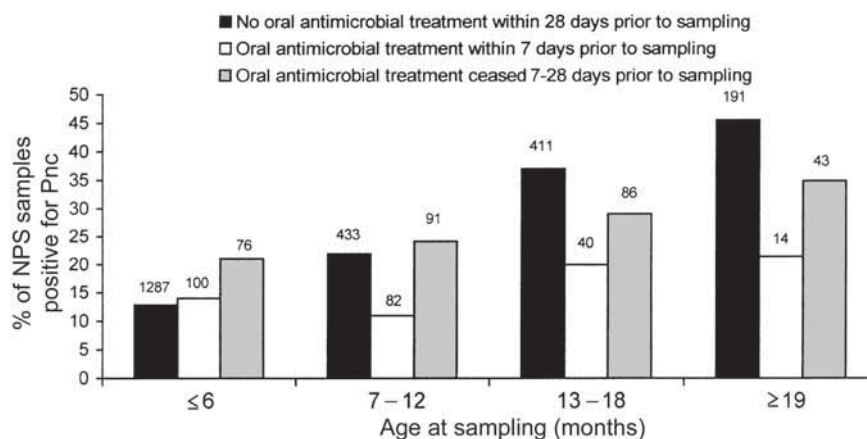
NP carriage at healthy visits in relation to history of antimicrobial medication.   The effect of antimicrobial treatment was studied for 2854 (94%) of the 3024 NPS samples for which there were data of an oral antimicrobial treatment or of no treatment within 28 days before swabbing. The most frequently used oral antimicrobials were amoxicillin (40%), trimethoprim-sulfadiazine or trimethoprim-sulfamethoxazole (27%), amoxicillin-clavulanate (10%), and penicillin V (8%). *S. pneumoniae* grew in 499 (21%) of 2322 samples obtained at visits without and in 112 (21%) of 532 samples obtained at visits with a history of an oral antibiotic treatment within 28 days. However, differences were seen when the data were examined in more detail. Figure 3 shows the proportions of positive NPS samples according to whether oral antibiotics had not been used within 28 days or whether they had been used within 7 days or whether the use had ended 7–28 days before sampling. Up to age 6 months, the percentages of positive NPS samples were the same in the first 2 groups and were higher if the child had received antibiotics 7–28 days before sampling. At later ages, the proportion of positive samples was lower (11%–21%) if antibiotic treatment was recent (within 7 days) but increased (24%–35%) if ≥1 week had elapsed since the end of medication.

Season.   The percentages of NPS and NPA samples positive for *S. pneumoniae* by calendar month are shown in figure 4. The occurrence of *S. pneumoniae* in NPS samples obtained during healthy visits fluctuated throughout the year (14%–25%), without any clear seasonal trend, as did the percentages of positive NPA samples obtained during sick visits, only at a

higher level (33%–52%). However, there was no obvious seasonal trend, in contrast to the numbers of sick visits, which varied seasonally. The average number of sick visits was 106/month in the summer months (June to August) and 200/month the rest of the year.

Cumulative colonization by S. pneumoniae.   Of the 329 children, 267 (81%) carried *S. pneumoniae* at least once (NPS or NPA) during the follow-up period. By the ages of 2, 6, 12, 18, and 24 months, 37 (11%), 107 (34%), 174 (56%), 228 (80%), and 244 (87%) of the children still being followed had carried *S. pneumoniae* at least once (figure 5). Of the 281 children with full follow-up, we detected 219 carriers (78%) during healthy visits and 207 (74%) during sick visits. For the 212 children with all 10 NPS samples, the individual number of positive samples at healthy visits varied from 1 (for 49 children) to 8 (for 3 children; median, 2).

Serotypes.   The distribution of serogroups or serotypes of *S. pneumoniae* isolated in NPS and NPA samples is shown in table 2. Of the 1530 isolates, 1456 belonged to 30 different serotypes, 17 were nontypeable, 55 were rough, and 2 were lost before typing. In NPS samples, the 6 most frequently isolated serotypes were 6B, 23F, 19F, 6A, 11, and 35, which accounted for 66% of all NPS isolates. In NPA samples, 23F, 19F, 6B, 6A, 11, and 14 were the most frequent (73% of all NPA isolates). Serotypes 19F, 23F, 6B, 6A, 11, and 14 were most often acquired first (as detected in either NPS or NPA samples) and comprised 62% of the first acquisitions. A serotype included in the only currently available pneumococcal conjugate vaccine (7-valent, with types 4, 6B, 9V, 14, 18C, 19F, and 23F) was identified in 58% of the isolates overall (53% of NPS and 62% of NPA isolates). Together with the cross-reactive types 6A, 9N, 18B, 19A, and 23A, the vaccine-related types comprised 68% of NPS and 76% of NPA isolates.

Figure 3.   Nasopharyngeal (NP) carriage of *Streptococcus pneumoniae* (Pnc) in relation to oral antimicrobial medication within 28 days before sampling. NP swab (NPS) samples obtained at 2854 scheduled healthy visits during 4 age intervals: ≤6 months; 7–12 months; 13–18 months; and ≥19 months (±4 weeks). Results are shown by antibiotic usage before sampling. Total nos. of samples are shown above the bars.
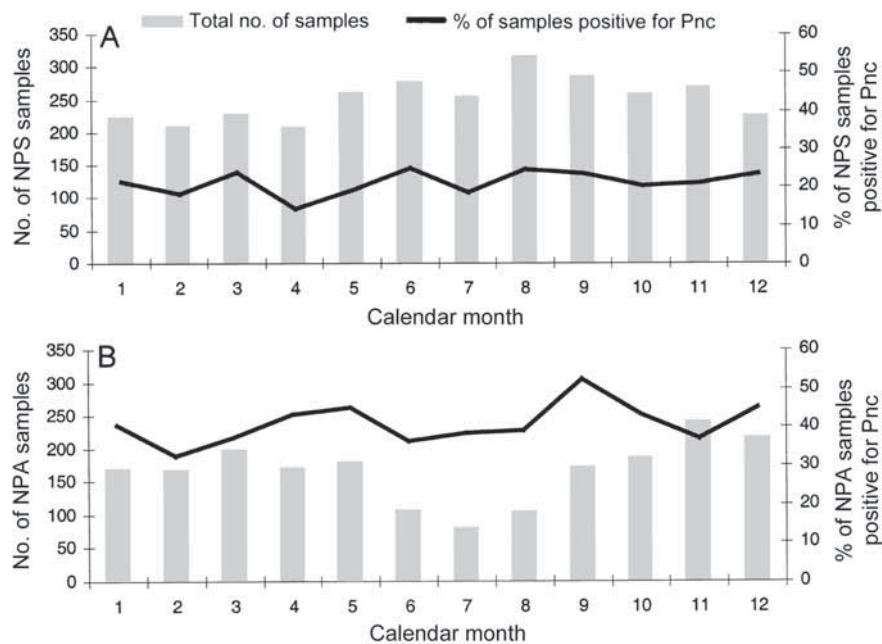
Figure 4. Proportion of nasopharyngeal (NP) samples positive for *Streptococcus pneumoniae* (Pnc) by calendar month, January–December (1–12, respectively). *A,* NP swab (NPS) samples obtained during health. *B,* NP aspirate (NPA) samples obtained during respiratory infection. *Bars,* Total samples; *lines,* percentage of samples positive for *S. pneumoniae.*

In all, 168 children (63% of the 267 carriers) acquired >1 identified serotype during the follow-up. Two, 3, and 4–6 serotypes were carried by 84, 38, and 45 children, respectively, and 1 child carried his seventh serotype at age 22 months. A single serotype was found in 6 consecutive NPS samples in 2 children (6B in both cases).

In 24 (3.7%) NPS samples and 29 (3.5%) NPA samples that were positive for *S. pneumoniae,* 2 different serotypes were isolated at the same time, and, in 1 NPA sample, 3 different serotypes (3, 6B, and 23F) were found. Multiple serotypes were recovered from 45 children. In all, 21 different serotypes were responsible for multiple pneumococcal colonization, but again, 23F, 6B, 19F, 14, 6A, and 11 were the most common, in this order, accounting for 68% of the multiple isolations.

Susceptibility of NP strains to penicillin. Resistance of the pneumococcal isolates to penicillin was rare. Eight (1.2%) of 649 NPS isolates and 32 (3.9%) of 826 NPA isolates had reduced susceptibility to penicillin. Only 2 NPS isolates and 2 NPA isolates were highly resistant (MIC, ≥ 2 μg/mL); others showed intermediate resistance (MIC, 0.125–1.0 μg/mL). Of the 267 carriers (NPS or NPA), only 12 (4.5%) at least once carried *S. pneumoniae* with reduced susceptibility to penicillin.

Discussion

Most children in this closely followed cohort carried *S. pneumoniae* at least once in their nasopharynx during the first 2 years of life. The carriage prevalence during health increased with age. The isolation rates were higher during respiratory infection without AOM than during health, again with an increasing trend with age. During AOM, the rates were high, regardless of age, and, during pneumococcal AOM, practically every child carried *S. pneumoniae* (also in the nasopharynx). Antimicrobial treatment reduced the carriage rates only temporarily. The serotypes included in the 7-valent conjugate vac-
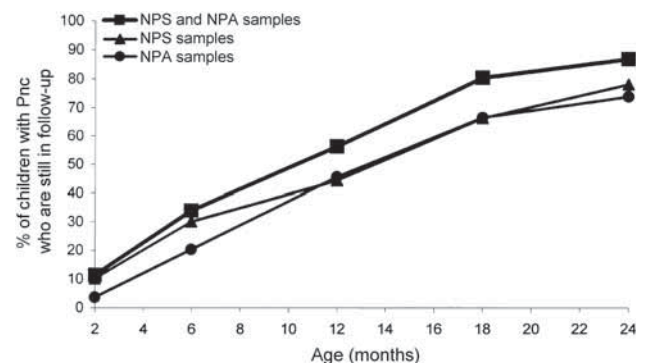
Figure 5. Cumulative rates of first acquisition of *Streptococcus pneumoniae* (Pnc) of children still in follow-up by age. Nasopharyngeal (NP) swab (NPS) samples were obtained at scheduled healthy visits at 2–6, 9, 12, and 15 months (± 2 weeks) and at 18 and 24 months (± 4 weeks); second NP aspirate (NPA) samples were obtained at sick visits during respiratory infections with or without acute otitis media.

Table 2. Distribution of *Streptococcus pneumoniae* serogroups or serotypes in 1475 nasopharyngeal (NP) samples obtained from 267 children during health (NP swab [NPS]) and during respiratory infection (NP aspirate [NPA]) with or without acute otitis media.

Serogroup or serotype	Isolate, by sample type					
	NPS (*n* = 673)		NPA (*n* = 857)		All (*n* = 1530)	
	No. (%)	Cumulative percentage	No. (%)	Cumulative percentage	No. (%)	Cumulative percentage
6B	106 (15.8)	15.8	107 (12.5)	12.5	213 (13.9)	13.9
23F	93 (13.8)	29.6	175 (20.4)	32.9	268 (17.5)	31.4
19F	93 (13.8)	43.4	165 (19.3)	52.2	258 (16.9)	48.3
6A	61 (9.1)	52.5	79 (9.2)	61.4	140 (9.2)	57.5
11	57 (8.5)	60.9	59 (6.9)	68.3	116 (7.6)	65.0
14	28 (4.2)	65.1	39 (4.6)	72.8	67 (4.4)	69.4
15	26 (3.9)	68.9	30 (3.5)	76.3	56 (3.7)	73.1
35	35 (5.2)	74.1	14 (1.6)	77.9	49 (3.2)	76.3
Others	137 (20.4)	94.5	152 (17.7)	95.7	289 (18.9)	95.2
R	28 (4.2)	98.7	27 (3.2)	98.8	55 (3.6)	98.8
NT	8 (1.2)	99.9	9 (1.1)	99.9	17 (1.1)	99.9
Not typed	1 (.1)	100.0	1 (.1)	100.0	2 (.1)	100.0
Total	673 (100.0)		857 (100.0)		1530 (100.0)	

NOTE. In 54 samples from 45 children, ⩾1 serotype was isolated at the same time. NT, nontypeable; not typed, not typed for another reason; R, rough.

cine used in recent efficacy trials in California [31] and Finland [32] and licensed in 2000 comprised 53% of the pneumococcal strains isolated from the nasopharynx during health and 62% of strains isolated during respiratory infection.

Although 87% of the study children carried *S. pneumoniae* at least once during the study, the children acquired their strains relatively slowly, and the overall carriage rate resembled that in the United States [16] and Sweden [7, 11]. The age-weighted mean proportion of positive NPS samples was 27%, a value near the prevalence (21%) among unselected healthy children sampled once at age 0–2 years in a Swedish study in 1975–1976 [11]. The cumulative acquisition rates were very near those reported by Faden et al. [8] (38% at 6 months and 54% at 12 months).

The prevalence of carriage at scheduled healthy visits (NPS) increased steadily with age from 9% at 2 months to 43% at 24 months. Dagan et al. [9] reported similar gradually increasing carriage rates from ages 2 to 24 months (from 26% to 62%) in Israeli children. In some other studies, increasing isolation rates leveled off at a younger age [5, 7, 10, 20]. In the present study, the health status of the study children seemed to have an important association with the carriage rates and also modified the association between carriage rates and age. The increase with age was most clear in the prevalence of carriage in healthy children, less clear in the NPA samples taken during respiratory infection without concurrent AOM, and absent in NPA samples taken during AOM (figure 2).

The NPA samples obtained during sick visits due to respiratory infection with or without AOM grew *S. pneumoniae* almost twice as often (41%) as the NPS samples obtained at healthy visits (21%). The percentage was even higher (49%) in the subgroup of NPA samples associated with concurrent AOM. This result is in agreement with that of Faden et al. [16], who reported significantly higher NP isolation rates during vis-

its with upper respiratory infection or OM than during routine healthy visits in a follow-up study of US children <3 years old. Higher isolation rates in samples obtained at visits with signs of unspecific respiratory infection also were reported in an earlier study among British families [17] and in studies in Uruguay, Mexico, and Pakistan [19, 20, 33]. However, several studies failed to find any differences in carriage rates between healthy and ill children [21–23, 34]. In these studies, the study subjects were older than those in our study; in 2 studies, throat swabs rather than NP swabs were used, which could affect the results.

In the present study, the difference in isolation rates between healthy and ill children depended on age. The difference was most prominent during the first year of life and almost disappeared by age 2 years, when the rates were also high during health. Furthermore, the excess of carriage strains recovered during AOM seemed to be related to the concurrent occurrence of *S. pneumoniae* in MEF, since nonpneumococcal AOM was not associated with higher carriage than was respiratory infection without AOM. This conclusion is also consistent with the finding that the seasonal variation was less prominent in pneumococcal AOM than in AOM associated with *Haemophilus influenzae* or *Moraxella catarrhalis* in this same cohort [35].

One might argue that the difference in pneumococcal recovery observed in our study during health and respiratory infection was due to the different sampling methods (NPS or NPA). We consider this to be unlikely because the techniques were specifically compared in a previous study of children with acute respiratory infection, and the agreement between the NPS and the NPA techniques was excellent [36].

The antimicrobial treatment of the children was assumed to affect the results, but the overall proportion of samples positive for *S. pneumoniae* was similar (21%), whether or not the child had received oral antimicrobial treatment within 28 days. This

finding parallels the observation of Dagan et al. [9] among children 2–13 months old, which showed no association between carriage rate and use of antibiotics during the preceding month. In the present study, the isolation rates were lower if the therapy was ongoing or had ended within 7 days, but if 1–4 weeks had elapsed since the end of the medication, the rates increased again or, during the first year of life, were even higher than without antimicrobial treatment (figure 3). This is in accordance with findings of others that a more recent antimicrobial treatment reduced carriage rates [23, 26–28]. Leach et al. [28] found lower pneumococcal isolation rates (29%) 2–3 weeks after a single dose of azithromycin administered to Australian aboriginal children with trachoma than before treatment (68%), but, at the 2-month follow-up, the rate had rebounded to a higher level (78%) than before treatment. Thus, the lower carriage rates after use of antimicrobials seem to be of very short duration.

The most frequently isolated NP serogroups or serotypes in young children in industrialized countries are 6, 14, 19, and 23 [1, 12, 34]. These are related to most OM and invasive infections caused by *S. pneumoniae* in children in these areas [24, 29, 35, 37–39]. These serogroups or serotypes were among the most common in our present study, but group 11 also was common in both NPS and NPA samples. As expected, serogroups or serotypes 4, 7, and 18, which cause serious diseases but are less frequently recovered as carriage strains [37–39], were relatively rare among our NP isolates. In addition, type 3, a common cause of AOM in several reports [24, 29, 40], was uncommon in the present series. Type 3 is found frequently in the naso- or oropharynx in older children or adults rather than among infants and young children [15, 21, 37]. The frequency of multiple serotypes was low (3.7% of positive samples) in this study, which is in accordance with other studies in which a few colonies (3–5) were serotyped [1, 41]. Higher frequencies of multiple serotypes were detected by serotyping 50 colonies in Papua New Guinea (29%) [42], but such serotyping is impractical and expensive [41]. More sensitive methods would be needed to assess multiple carriage.

In conclusion, we found a high pneumococcal colonization rate (49%) in the presence of AOM throughout the first 2 years of life, in contrast to the colonization rates during health, which increased with age from 9% at 2 months to 43% at 2 years. The first practical conclusion from these findings is that both age and health status should be considered carefully when carriage rates in young children are compared and interpreted. The reasons for these findings and the implications for pneumococcal disease will be of interest for further study. Numerous additional data collected in the FinOM Cohort Study currently are being analyzed to further characterize the factors that affect the development of pneumococcal carriage and disease.

Acknowledgments

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The Value of Nasopharyngeal Culture in Predicting the Etiology of Acute Otitis Media in Children Less Than Two Years of Age

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Background: In selecting treatment of acute otitis media (AOM), knowledge of its etiology would be valuable. We revisited the possibility to use the nasopharyngeal culture of *Streptococcus pneumoniae* (Pnc) and *Haemophilus influenzae* (Hi) for predicting their presence in the middle ear fluid (MEF) during AOM.

Methods: The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of bacterial culture of the nasopharyngeal aspirate (NPA) in predicting the presence of the same pathogen in the MEF were assessed during AOM events among children followed from 2 to 24 months of age.

Results: The data comprised 586 AOM events. For Pnc, the sensitivity and NPV were high, 99% (95% confidence interval = 95–100%) and >99% (97–100%), respectively. The specificity and PPV were relatively low, 63% (57–68%) and 50% (43–56%). For Hi, the sensitivity and the NPV were lower (77%, 69–83% and 93%, 90–95%) than for Pnc, but the specificity and the PPV were higher (88%, 85–91% and 64%, 56–71%). The quantity of Pnc and Hi in the NPA was clearly related to their presence in the MEF. If both Pnc and Hi were found in the nasopharynx, Hi was more likely cultured from MEF.

Conclusion: Together with clinical and epidemiologic features of AOM, the nasopharyngeal culture can be helpful in selecting specific antimicrobial therapy.

Key Words: nasopharyngeal culture, predictive value, etiology, acute otitis media

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Acute otitis media (AOM) forms a considerable disease burden for children in developed countries. *Streptococcus pneumoniae* (Pnc) and *Haemophilus influenzae* (Hi) are the most important pathogens causing acute otitis media (AOM) followed by *Moraxella catarrhalis* (Mc) and, decreasingly, group A beta-hemolytic streptococci.[1–3] Detection of pathogens in the middle ear fluid (MEF) by culture is the "gold standard" for etiologic diagnosis of AOM. Tympanocentesis and myringotomy are, however, invasive procedures that are at present rarely considered indicated in uncomplicated cases. Many studies evaluating the possibility to use nasopharyngeal culture as a predictor of the bacterial etiology of otitis have shown that if Pnc or Hi are not found in the nasopharynx during AOM, it is unlikely that they would be found in the MEF.[4–10] Some of the studies have acknowledged the value of nasopharyngeal culture in prediction of the etiology of AOM,[4,5] whereas others have considered it too nonspecific.

The nasopharyngeal sample takes up to 48 hours to culture and may thus not be practical and cost-effective in the routine treatment of all AOM cases in the clinical practice, because antimicrobial agents for empiric treatment of AOM are available. However, increasing resistance of the otitis pathogens to the commonly used antimicrobials prompts a reevaluation of the treatment strategies and stresses the need to restrict the use of antibiotics in general and of those with a wide spectrum of activity in particular. Because uncomplicated AOM often resolves without antimicrobial treatment, an option for "watchful waiting" for 48 to 72 hours without initial antimicrobial treatment has been recommended for selected uncomplicated cases pending assurance of appropriate follow up.[11] Should the child appear for a second visit because of persistent or aggravating symptoms, the culture result of the nasopharyngeal sample taken at the first visit would by then be available and provide useful guidance for selection of treatment or, in borderline cases, further waiting without an antimicrobial.

For example, there is increasing evidence that Pnc AOM is likely to have a more severe clinical picture and more suppurative complications, whereas Hi is associated with recurrent AOM.[3,12–19] Although clearly recognized as a pathogen, Mc tends to be associated with a milder clinical picture and is therefore of less importance in this context.[12] Recently, studies suggest that the nasopharyngeal culture at diagnosis may be a valuable predictor for the outcome of the AOM although the etiology is not known.[20,21]

In the current study, based on 586 AOM cases among 329 children followed from 2 to 24 months of age, we revisited the possibility to use the nasopharyngeal culture of Pnc and Hi for prediction of their presence in the MEF during AOM.

MATERIALS AND METHODS

Study Population and Clinical Samples

The study population consisted of 329 healthy children enrolled in the Finnish Otitis Media (FinOM) Cohort Study and followed prospectively in 1994–1997 from 2 to 24 months of age in a special study clinic established for assessing the epidemiology of AOM.[3,22] AOM was diagnosed during visits requested by the parents because of predefined symptoms suggesting AOM, at the follow-up visits scheduled for 3 to 5 weeks after the initial diagnosis of AOM and occasionally at age-scheduled visits that were part of the study design. During these visits, AOM was diagnosed by the following criteria: a visually abnormal tympanic membrane in pneumatic otoscopy (in regard to color, position and mobility) suggesting effusion in the middle ear cavity concomitantly with at least one of the following signs and/or symptoms of acute local or general infection: fever, earache, tugging of the ear, irritability, acute gastrointestinal symptoms or other symptoms of respiratory infection. Each occasion in which AOM was diagnosed was defined as an AOM event. Current use of antimicrobials and any AOM recently diagnosed in another medical setting than the study clinic were asked from the parents. AOM events treated outside the clinic were verified from medical case records.

Whenever AOM was diagnosed, a sample of MEF and a nasopharyngeal aspirate (NPA) were obtained. In bilateral cases, the MEF sample was obtained from both ears. The MEF sample was aspirated after myringotomy or through perforation or discharging ventilation tube. If the amount of the NPA sample was less than 0.5 mL, phosphate-buffered saline was added to reach an amount of at least 0.5 mL. All samples were cultured immediately with a 10-μL loop on chocolate agar and on blood agar with 5% gentamicin and incubated in 5% CO_2 at 36° to 37°C in the study clinic overnight. Thereafter, they were sent to the National Reference Laboratory for Pneumococcus of the National Public Health Institute (KTL) in Oulu for identification of relevant pathogens and for serotyping of Pnc with standard methods as described earlier.[3,22] After 48 hours' incubation, the numbers of colonies on the plate with more growth were counted. Colony counts >100/plate were labeled as "abundant growth."

Calculating Predictive Values and Statistical Analyses. Positive culture for Pnc or Hi from a MEF sample was defined as the gold standard for the etiologic diagnosis of the AOM. In this analysis, 2 MEF samples obtained at the same visit were regarded as one sample and any Pnc or Hi found in either or both of these 2 samples was treated as a single Pnc or Hi finding in a single sample. The sensitivity, specificity and positive and negative predictive values (PPV, NPV) of the nasopharyngeal culture of Pnc and Hi in predicting the homologous etiology of AOM were calculated as conditional probabilities from 2 × 2 tables with positive and negative findings in NPA and MEF samples. To control possible intrachild correlation between the measurements from the same child, the 95% confidence intervals were determined by logistic regression using generalized estimating equations with exchangeable correlation structure. The effects of age, sex, season and the number of previous AOM on sensitivity, specificity and the predictive values were assessed by multivariate logistic regression. There was no significant intrachild correlation between the outcomes. The quantity of nasopharyngeal growth and occurrence of homologous AOM were compared with χ^2 test.

Ethical Review. Written informed consent was obtained from the parents or guardians. The study protocol was approved by the Ethics Committees of the Finnish National Public Health Institute, Department of Social and Health Care of Tampere City and Tampere University Hospital.

RESULTS

During the study, AOM was diagnosed 871 times at the study clinic. Both NPA and at least one MEF sample were available for 761 AOM events. Altogether, 104 events at which antimicrobial treatment was ongoing (or not known) were excluded from the analysis as were 71 events that occurred within 14 days after a previous AOM event (diagnosed in the study clinic or in another medical setting and as a rule treated with antibiotics). This left 586 events in 200 children available for the analysis. Of the 200 children, 60 (30%) had one AOM event that was evaluable in the current analysis, 70 (35%) had 2 or 3 events and 70 (35%) >3 events. Pnc grew from 316 (54%) of the NPA samples and from 160 (27%) of the MEF samples (Table 1). Hi grew from 151 (26%) of the NPA samples and from 126 (22%) of the MEF samples. The same pneumococcal serotype grew from both the NPA sample and the MEF sample in all but one of the 158 events in which Pnc was found in both of these samples and serotyped.

TABLE 1. *Streptococcus pneumoniae* (Pnc) and *Haemophilus influenzae* (Hi) Findings From 586 Middle Ear Fluid (MEF) Samples and Nasopharyngeal Aspirates (NPA) Obtained Concurrently From 200 Children During Acute Otitis Media

Bacterium Cultured From NPA	Homologous Bacterium Cultured From MEF		
	−	+	
Pnc			
−	269	1	270
+	157	159*	316
	426	160	586
Hi			
−	406	29	435
+	54	97	151
	460	126	586

Two MEF samples obtained at the same visit were regarded as one sample and Pnc or Hi found in either or both of these 2 samples was treated as a single Pnc or Hi finding of a single sample.

*In 158 of these 159 cases, the Pnc from both NPA and MEF samples were serotyped; the same serotype was found in 157 of these 158.

TABLE 2. Nasopharyngeal Culture of *Streptococcus pneumoniae* (Pnc) and *Haemophilus influenzae* (Hi) as a Predictor for Isolation of the Same Bacterium From a Middle Ear Fluid (MEF) Sample Obtained Concurrently During Acute Otitis Media (AOM)

The Bacterium	Prevalence			Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
	N	MEF Percent	NPA Percent				
Pnc	586	27	54	99 (95–100)	63 (57–68)	50 (43–56)	>99 (97–100)
Hi	586	22	26	77 (69–83)	88 (85–91)	64 (56–71)	93 (90–95)

Two MEF samples obtained at the same visit were regarded as one sample and Pnc or Hi found in either or both of these 2 samples was treated as a single finding of a single sample.

Sensitivity, the probability of presence of the bacterium in NPA, if found from MEF (%); specificity, the probability of absence of the bacterium from NPA, if not found from MEF (%); PPV, positive predictive value, the probability of presence of the bacterium in MEF, if found from NPA (%); NPV, negative predictive value, the probability of absence of the bacterium from MEF, if not found from NPA (%); CI, confidence interval (%).

Presence of Streptococcus pneumoniae *and* Haemophilus influenzae *in Nasopharyngeal Culture as a Predictor of Their Presence in Middle Ear Fluid.* The sensitivity, specificity, PPV and NPV of the nasopharyngeal culture of Pnc and Hi in predicting the homologous etiology of AOM are shown in Table 2. The sensitivity (99%) and NPV (>99%) for Pnc were very high, but the specificity (63%) and PPV (50%) were relatively low. For Hi, the sensitivity (77%) and NPV (93%) were lower, but the specificity (88%) and PPV (64%) were higher than those for Pnc.

The sensitivity and NPV for Pnc remained close to 100% irrespective of age, sex, season or the number of previous AOM events. Coinciding with a high prevalence of carriage but low frequency of Pnc AOM, the specificity and PPV decreased by age (odds ratio [OR] = 0.94 by each month of age, 95% confidence interval [CI] = 0.91–0.98), especially after 18 months of age. The PPV was higher in case of the second or third AOM event of the child as compared with the first event (OR = 1.86, 95% CI = 1.01–3.42), but this trend was not evident for more than 3 AOM events. For Hi, the PPV increased with age (OR = 1.08 by each month of age, 95% CI = 1.01–1.14) and it was higher in boys than in girls (OR = 3.11, 95% CI = 1.39–6.95), whereas the NPV tended to decrease during autumn and winter months and with increasing number of previous AOM events, all these categories being associated with a relatively high proportion of Hi in the MEF samples (data not shown).

Abundance of Bacterial Growth in the Nasopharyngeal Aspirate as a Predictor for Streptococcus pneumoniae *and* Haemophilus influenzae *Acute Otitis Media.* The majority of the 316 NPA cultures positive for Pnc (87%) contained ≥100 colonies in the 10-μL loopful plated, and Pnc grew from the MEF in 55% of these events (Fig. 1). The sensitivity, specificity, PPV and NPV for abundant growth of Pnc in predicting Pnc AOM were 96%, 71%, 55% and 98%, respectively.

For Hi, the association of a positive MEF culture with high number of bacteria in the NPA culture was also clear, although less pronounced (Fig. 2). An estimate of the number of Hi colonies was available for 150 of the 151 Hi-positive NPA samples. Of these, 78% contained ≥100 Hi colonies and Hi was found in the MEF in 71% of these cases. The sensitivity, specificity, PPV and NPV for abundant growth of Hi in predicting Hi AOM were 66%, 93%, 71% and 91%, respectively.

Effect of Concurrent Presence of the Other Bacterium in the Nasopharyngeal Aspirate. Pnc was found in the NPA without Hi in 241 AOM events. Of these, Pnc grew from the MEF in 140 cases, increasing the PPV of a positive Pnc finding to 58%. If both Pnc and Hi were found concurrently

FIGURE 1. Occurrence of *Streptococcus pneumoniae* (Pnc) in the middle ear fluid (MEF) sample by Pnc colony count (in 10-μL sample plated) in the concurrently obtained nasopharyngeal aspirate (NPA) (N = 586). The increase in occurrence of Pnc in the MEF by increasing nasopharyngeal growth was statistically significant (*P* < 0.001).
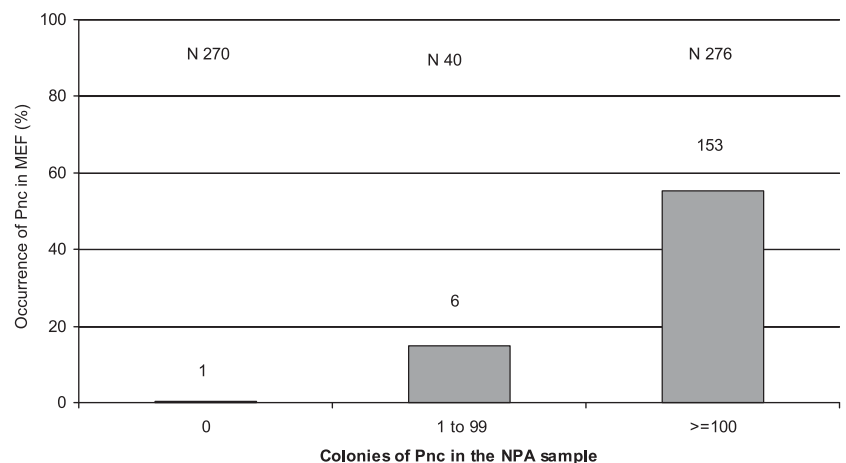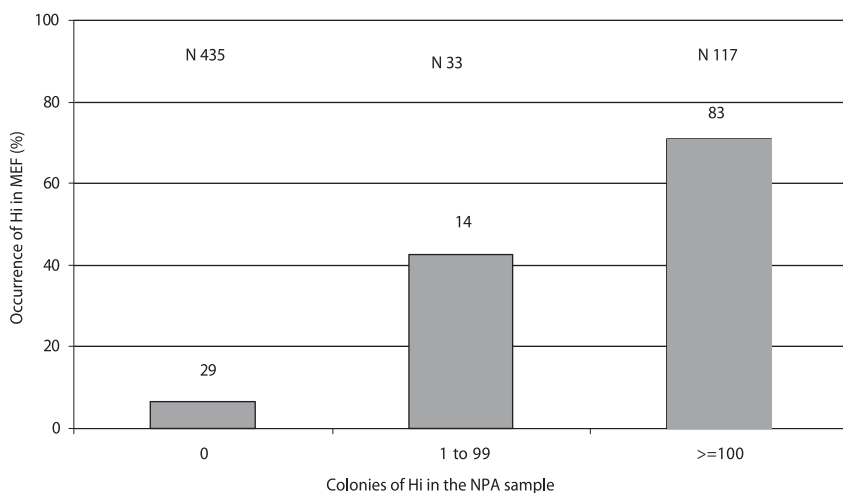
FIGURE 2. Occurrence of *Haemophilus influenzae* (Hi) in the middle ear fluid (MEF) sample by Hi colony count (in 10-μL sample plated) in the concurrently obtained nasopharyngeal aspirate (NPA) (N = 585). The increase in occurrence of Hi in the MEF by increasing nasopharyngeal growth was statistically significant ($P < 0.001$).

in the NPA, the probability of finding Pnc in the MEF was only 25% (19 of 75) and the probability of finding Hi was 63% (47 of 75). Hi grew from the MEF in 66% (50 of 76) of AOM cases in which Hi was found in the NPA without Pnc. In the 194 AOM cases, in which neither Pnc nor Hi was found in the NPA, Pnc grew from one MEF sample and Hi grew from 12 MEF samples.

DISCUSSION

The results of the present study show that nasopharyngeal culture can be useful in predicting the bacterial etiology of AOM. The study comprised a large sample of AOM cases among children in otitis-prone age, representing a wide range of clinical pictures in a primary care setting. Because the children with previous AOM within 2 weeks and those receiving antibiotics were excluded, the results are applicable only to patients with a new episode of AOM.

The study confirmed the previous knowledge that if the nasopharyngeal culture is negative for Pnc and/or Hi, these bacteria are unlikely to be present in the MEF.[5,7–10,23,24] Unlike in previous studies,[7,9] the sensitivity and NPV were higher for Pnc than for Hi. In the study by Luotonen and colleagues,[6] the sensitivities were lower for both Pnc (79%) and Hi (74%), which may be the result of their use of nonselective culture media[25] and nasal rather than nasopharyngeal sampling.[26] In the present study, the higher sensitivity for Pnc than for Hi may similarly be the result of the use of highly selective medium for Pnc but not for Hi. Another explanation might be that young children carry Pnc mainly in their nasopharynx, but they may carry Hi also in their oropharynx without nasopharyngeal involvement.[27,28] However, in young children with respiratory infection, nasopharyngeal aspiration has performed significantly better than nasopharyngeal swabbing in detection for Hi, and it has proved to be optimal for detection of both Pnc and Hi.[29]

Despite a relatively low specificity, a positive NPA culture for Pnc doubled the probability of Pnc AOM and a positive NPA culture for Hi tripled the probability of Hi AOM as compared with the prevalences without this knowledge. The colonization density was directly related to AOM: the more colonies in the NPA, the more likely the presence of the bacterium in the MEF. The specificity increased slightly (from 63% to 71% for Pnc and from 88% to 93% for Hi) when only abundant growth ($\geq$100 colonies in the 10-μL sample) was regarded as positive. This classification had little effect on the sensitivity for Pnc but more for Hi. Consistent with our findings, Schwartz and colleagues[4] found a high PPV for both Pnc (80%) and Hi (79%) when classifying only nasopharyngeal growth of any single pathogen covering >25% of total colonies as positive.

Because the proportion of NPA samples with less than 100 colonies was small, PPV increased only slightly (from 50% to 55% for Pnc and from 64% to 71% for Hi) if such samples were considered to be negative for the respective bacterium. Although the higher cutoff for positive NPA culture achieved with quantitation decreases the rate of false-positive prediction, it correspondingly increases the false-negative prediction. Furthermore, quantitative culture is not a routine laboratory procedure and is therefore not likely to increase the usefulness of nasopharyngeal culture.

Nasopharyngeal culture has not been considered as a useful predictor of AOM etiology in clinical practice, but in light of the results of the present study, we encourage a reappraisal of its usefulness in attempts to implement more judicial antimicrobial treatment policy for AOM. Together with clinical and epidemiologic features of the AOM,[3,8,12–15,18,30,31] the result could be an important aid for reassessment of need of treatment after "observation option" without initial antimicrobial treatment. Development of sensitive and specific rapid tests for recognition of nasopharyngeal otopathogens, eg, based on antigen detection, would solve the problem of time needed for bacterial culture.[32] Prediction of antimicrobial resistance is another interesting application of the nasopharyngeal culture, because Pnc in the NPA and MEF represent almost always the same serotype as shown in this study and, with high probability, the same clone.[6,10,24,33–35]

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Pneumococcal Acute Otitis Media in Relation to Pneumococcal Nasopharyngeal Carriage

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Background: Acute otitis media (AOM) is closely associated with viral upper respiratory tract infections, but the most common microbial agent found in the middle ear fluid during AOM is *Streptococcus pneumoniae* (Pnc). Pnc is also a common colonizer of the nasopharynx, and its prevalence is further increased during the viral infection. The aim of this study was to investigate the interplay between viral infection, pneumococcal acquisition and carriage in the development of Pnc AOM.

Methods: Pnc carriage was assessed in a longitudinal study of 329 infants at scheduled visits at 3, 6, 9, 12, 15 and 18 months of age (N = 1715). The clinical outcome of the first episode of respiratory infection ("sick visit," N = 774) in the following 3-month period was recorded. The occurrence and timing of Pnc AOM in relation to serotype specific carriage at the start of the observation period were assessed.

Results: The occurrence, timing and duration of symptoms of the sick visits or the frequency of overall AOM were not associated with preceding pneumococcal carriage. Pnc AOM was in each case associated with concurrent carriage and 3.8 times (95% confidence interval, 1.4–10.0) more often with carriage acquired after the start of the observation period than with carriage already present at the scheduled visit. In all, 79% (55 of 70) of Pnc AOM events were caused by a serotype acquired after the start of the period.

Conclusion: The majority of Pnc AOM events develop in association with newly acquired carriage of pneumococcus.

Key Words: *Streptococcus pneumoniae*, pneumococcal, acquisition, carriage, acute otitis media

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Acute otitis media (AOM) is closely associated with upper respiratory tract infections. More than 90% of children with AOM have symptoms of upper respiratory tract infection at the time of diagnosis,[1,2] and the occurrence of AOM

episodes peaks 3–4 days after onset of symptoms of respiratory infection.[3,4] Although viruses are detected in the nasopharynx in only 30–50% of AOM cases by viral culture and/or antigen detection methods, the use of more sensitive, polymerase chain reaction-based techniques has confirmed viral infection in as many as 86% of children with AOM.[5–8] There are several pathogenic mechanisms by which viral infection could contribute to AOM. Viral infection causes dysfunction of the Eustachian tube, which results in impairment of pressure equilibration between the nasopharynx and the middle ear cavity and access of nasopharyngeal secretions to the middle ear. Some viruses induce leukocyte dysfunction[9] or cause other changes in the hosts' immune defense that could increase the risk of secondary bacterial infection.[7,10] Viral infection of the upper respiratory tract also has a substantial impact on the nasopharyngeal bacterial flora.[11–13]

Nasopharyngeal carriage of potential otitis pathogens is another contributor to bacterial AOM. The first episode of AOM correlates with onset of carriage in both U.S. and Australian Aboriginal children.[14,15] Carriage of pneumococci starts during the first months of life and is highest in young children,[16–24] largely at the same age at which AOM is most common.[25] The prevalence of pneumococcal carriage is higher during respiratory infection and AOM than during health,[24,26–31] although contradictory data have been reported.[32] Furthermore, during pneumococcal AOM, pneumococci are practically always found in the middle ear and in the nasopharynx,[20,33–35] and the serotypes are usually the same in both sites.[20,36–38]

The interplay between *Streptococcus pneumoniae* (Pnc) carriage, respiratory infection and development of Pnc AOM is not entirely clear. Does respiratory infection enhance acquisition of carriage, or does respiratory infection increase the capacity of preexisting carriage to progress to AOM? In a prospective study with 82 Alabaman children younger than 2 years of age with carriage sampling at intervals of 1–3 months, Gray et al[20] concluded that most Pnc AOMs developed within 1 month after acquisition of carriage. We have not been able to find other relevant data on the temporal relationship of Pnc carriage and Pnc AOM. This prompted us to investigate the relationship between pneumococcal carriage, respiratory infection and Pnc AOM in a subset of the Finnish Otitis Media (FinOM) Cohort Study. We addressed the question by assessing the carriage status at scheduled visits in children at 3, 6, 9, 12, 15 and 18 months of age and examining the occurrence, timing and outcome of the first

respiratory infection in the following 3 months in terms of Pnc AOM and Pnc carriage.

MATERIALS AND METHODS

The FinOM Cohort Study. The present study comprises a subset of samples (see "Setting for the Present Analysis") from the FinOM Cohort Study, in which 329 children were enrolled for a prospective follow-up from 2 to 24 months of age. During the follow-up, the study children made 10 scheduled visits and additional ad hoc visits due to acute illness to the study clinic.[24] At the scheduled visits during which the child was considered healthy, a nasopharyngeal sample (NP) was obtained with a calcium alginate swab. The parents were encouraged to bring the child to the study clinic at any time if predefined symptoms of acute respiratory infection suggesting AOM occurred. At these visits, termed sick visits, a nasopharyngeal aspirate (NPA) was obtained and the length of symptoms leading to the visit was assessed by interviewing the parent(s) using a structured questionnaire. The criteria for the diagnosis of AOM included a visually abnormal tympanic membrane in pneumatic otoscopy, suggesting effusion in the middle ear cavity, concomitantly with signs and/or symptoms of acute infection. Whenever AOM was diagnosed, a sample of middle ear fluid (MEF) was aspirated after myringotomy or through a discharging ventilation tube or spontaneous perforation. In bilateral disease, both ears were tapped. Pneumococcal AOM was defined as an AOM with Pnc identified by culture from at least 1 ear. The NP, NPA and MEF samples were cultured for Pnc, and the isolates were serotyped as described elsewhere in detail.[24,39] Briefly the samples were cultured immediately on enriched chocolate agar plates and selective sheep blood agar plates containing 5 μg/mL gentamicin. The plates were incubated overnight in 5% CO_2 at 36–37°C in the study clinic and transported to the bacteriologic laboratory in Oulu, where the pneumococci were identified by standard methods. Four different colonies were serotyped by counterimmunoelectrophoresis and latex agglutination (for types 7 and 14) with the use of antisera from Statens Serum Institut, Copenhagen, Denmark. Written informed consent was obtained from the parents or guardians, and human experimentation guidelines of the National Public Health Institute were followed in the clinical research. The study protocol was approved by the Ethics Committees of the Finnish National Public Health Institute, Department of Social and Health Care of Tampere City and Tampere University Hospital.

Setting for the Present Analysis. To assess the relation of Pnc AOM to carriage we divided the follow-up time of all study children into 6 observation periods, each lasting for 3 months. The periods started by a scheduled visit with NP sampling at the age of 3, 6, 9, 12, 15 or 18 months and ended at the next of these scheduled NP samplings, or after exactly 3 months if the next scheduled visit did not take place. Within each observation period, we recorded only the first sick visit; however, to avoid repeated measures from a single episode of infection, these sick visits were excluded if <30 days had elapsed since the previous one (occurring in the previous observation period). If the NPA sample or, in case of AOM,

the MEF sample was not obtained at the sick visit, the observation period was not included.

Statistical Methods. To compare the frequency of Pnc AOM associated with carriages that were detected already at the scheduled visit and those that were acquired after the scheduled visit, their relative frequency was estimated by using a conditional logistic regression model. Because a child could have a sick visit during several observation periods, we included a frailty variable to account for possible heterogeneity across children in their proneness to Pnc AOM. A conditional logistic regression approach was applied in the R software, version 1.9.0 (R Foundation for Statistical Computing, Vienna, Austria, 2004).

RESULTS

Observation Periods. Altogether 1802 NP samples were obtained at the 6 defined ages from 324 of the 329 children enrolled in the FinOM Cohort Study, but in 31 cases the child discontinued the follow-up before the end of the observation period of 3 months. Of the remaining 1771 observation periods of 319 children, 56 periods were excluded, because the NPA sample or, in case of AOM, the MEF sample was not obtained at the sick visit, which left 1715 observation periods of 318 children available for the analysis.

Sick Visits by Carriage at Start of the Observation Periods. The point prevalence of Pnc carriage at the scheduled visits increased from 12% at 3 months to 38% at 18 months of age (mean, 23% throughout the study). Two serotypes were carried simultaneously in 15 (4%) of the positive NP samples. Antimicrobial treatment was ongoing at 109 (6%) scheduled visits (this information was available for >99% of visits).

A sick visit was recorded in a total of 774 of the 1715 observation periods. Of carriers and noncarriers at the scheduled visits, 47% (185 of 397) and 45% (589 of 1318), respectively, were seen at a sick visit during the subsequent period (Table 1). The median time from the start of the period to the sick visit was 42 and 37 days in carriers and noncarriers, respectively. Also the duration of symptoms was similar in both cases (median, 4 days). AOM was diagnosed at 32% (60 of 185) of the sick visits of carriers and at 28% (164 of 589) of the sick visits of noncarriers. Thus the occurrence, the timing and the clinical features of the sick visits were not associated with the preceding Pnc carriage status. This was true for all observation periods independent of age (data not shown). In contrast, Pnc carriage during the sick visit (41%, 316 of 774) was strongly associated with carriage at the start of the period: 87% (161 of 185) of the initial carriers but only 26% (155 of 589) of the noncarriers had Pnc in their nasopharynx at the sick visit. Pnc AOM was diagnosed at the sick visit in 6% (22 of 397) of initial carriers and in 4% (48 of 1318) of noncarriers. In the case of Pnc AOM, the MEF always contained only 1 serotype, and the same serotype was without exception also found concurrently in the nasopharynx.

Occurrence of Pnc AOM by Established and Newly Acquired Carriage. Because of the strong association of Pnc AOM with concurrent Pnc carriage, we next focused on the sick visits, at

TABLE 1. The First Sick Visits During the 6 Observation Periods of 3 Months (N = 1715), According to the Carriage Status of Pnc at the Scheduled Visits at Start of the Period

Characteristics of the Sick Visits	Pnc Carriage at the Scheduled Visit		
	Yes (N = 397)	No (N = 1318)	Total (N = 1715)
Sick visits, N (% of observation periods in the category)	185 (47)	589 (45)	774
Pnc carriage at the sick visit, N (% of the sick visits)	161 (87)	155 (26)	316
AOM* at the sick visit, N (% of the sick visits)	60 (32)	164 (28)	224
Pnc AOM at the sick visit, N (% of the observation periods in the category)	22 (6)	48 (4)	70
Time between start of the period and the sick visit (median, days)	42	37	
Duration of symptoms leading to the sick visit (median, days)	4	4	

*Haemophilus influenzae and *Moraxella catarrhalis* were found in at least 1 middle ear fluid during 39 and 53 of the 224 AOM cases, respectively.

which Pnc were found in the NPA and divided the cases according to whether or not the serotype was present already at the start of the period. Pnc were carried at 316 sick visits; at 12 visits 2 or 3 serotypes were carried simultaneously, giving a total of 329 Pnc isolates. In 132 of these cases, the serotype in the nasopharynx was already present at the start of the period, and the carriage was therefore termed "established carriage." In the remaining 197 cases, the serotype in the nasopharynx at the sick visit was not present at the start of the period and the carriage was therefore termed "newly acquired carriage." A total of 11% (15 of 132) of the established carriages and 28% (55 of 197) of the newly acquired carriages were associated with Pnc AOM. Thus the logistic regression model showed that Pnc AOM occurred 3.8 times (95% confidence interval, 1.4–10.0) more often if the child had a newly acquired carriage than if he or she had an established one. Altogether 79% (55 of 70) of Pnc AOM events were caused by a newly acquired serotype.

The occurrence of Pnc AOM associated with established carriage declined with age: during the first observation period (age 3–5 months), 3 of 9 established carriages had led to Pnc AOM, whereas none of the 25 established carriages had done so during the last period (age 18–20 months) (Fig. 1). This trend was not seen with newly acquired carriage; instead

FIGURE 1. Occurrence of Pnc AOM at the first sick visit during observation periods of 3 months according to carriage of Pnc at start of the period. – – – – indicates that the serotype present in the nasopharynx at the sick visit was already present at start of the period ("established carriage"); ——, the serotype present in the nasopharynx at the sick visit was acquired after start of the period ("newly acquired carriage").

the highest proportion of carriages that had led to Pnc AOM was seen at age 9–12 months. Moreover this pattern was specific for Pnc AOM, because the occurrence of nonpneumococcal AOM in children who carried Pnc at the sick visit showed no trend by history of pneumococcal carriage (data not shown).
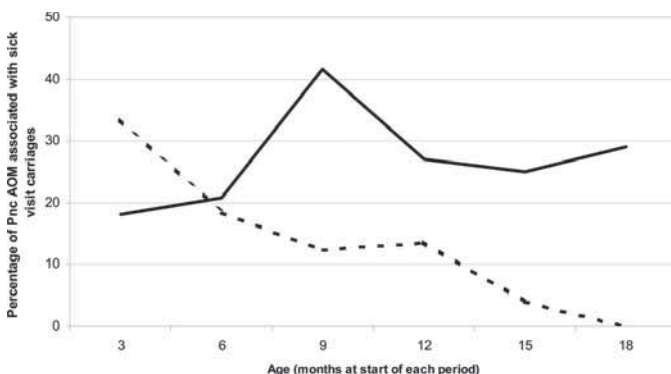
Temporal Association of Pnc AOM and Carriage. The 70 sick visits with Pnc AOM occurred earlier during the observation period than did the 259 sick visits at which Pnc were carried in the nasopharynx without diagnosis of Pnc AOM. This was true both for the visits with newly acquired carriage (time from start of the period; median, 29 versus 48 days in cases with or without Pnc AOM) and those with established carriage (15 versus 32 days, respectively) (Fig. 2). The early timing of sick visits with Pnc AOM compared with sick visits with only carriage clearly suggests that Pnc AOM was associated with recent acquisition of carriage. This early timing was specific for Pnc AOM; no difference in timing of sick visits with and without Pnc carriage or with and without overall AOM was observed, and in the Pnc carriers the sick visits with nonpneumococcal AOM did not time earlier than visits without any AOM.

We further conducted a retrospective examination of the serotype-specific carriage history of the 70 events with Pnc AOM and the 259 events at which Pnc were carried at the sick visits without Pnc AOM and found a clear-cut difference (Fig. 3). By definition, 100% of the children in both categories were Pnc carriers at the sick visit. Markedly few of the children in the Pnc AOM category were carriers before the visit (21%) as contrasted to the children in the without Pnc AOM category (45%). In both categories, there was a rapid increase of carriage in the final couple of weeks before the sick visits. This increase broadly coincided with the onset of symptoms of viral respiratory infection (Fig. 3).
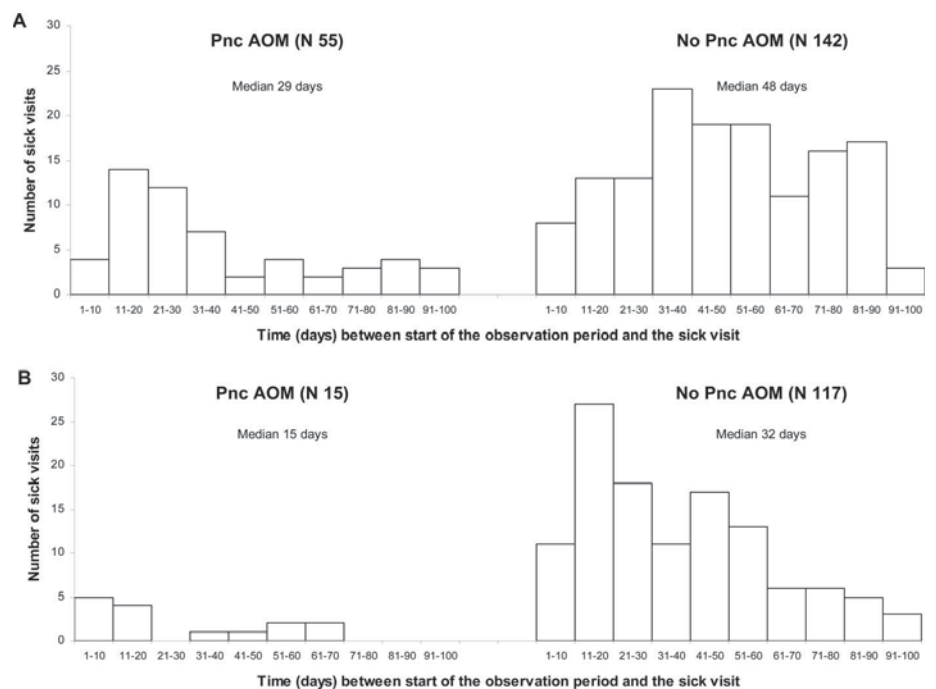
The duration of symptoms leading to the sick visit was similar whether Pnc AOM was diagnosed or not (median, 4 days). There was also little difference according to whether the children carried Pnc or not at the sick visit (median, 4 days) or whether the carried serotype had been acquired after start of the period (median, 5 days).

DISCUSSION

We studied the sequential relationship among Pnc carriage, respiratory infection and development of Pnc AOM

FIGURE 2. Timing between the start of the observation period and the sick visit because of respiratory symptoms at which Pnc was carried, shown separately for visits with and without Pnc AOM. **A** indicates newly acquired carriage (ie, the serotype acquired after the start of the observation period); **B**, established carriage (ie, the same serotype carried already at the start of the observation period).

and found that the occurrence and clinical features of the sick visits did not differ according to the preceding carriage status. In 79% of the 70 Pnc AOM events, the serotype found in MEF (and concurrently in the nasopharynx) was not present in the NP at start of the observation period but was acquired thereafter (newly acquired carriages). Carriage of serotypes acquired after start of the period were 3.8-fold more likely to proceed to Pnc AOM than carriage of serotypes present

FIGURE 3. Preceding carriage of pneumococcal serotypes carried at the sick visits with (——, N = 70) and without (– – – –, N = 259) Pnc AOM. The preceding carriage was assessed with nasopharyngeal swabs obtained at the scheduled visits starting the observation periods (numbers in the figure). Dots indicate the start of the symptoms leading to the sick visit.

already at start of the period, suggesting that Pnc AOM more easily develops after recent acquisition than after long carriage. This finding confirms, in a larger study material and in more precision, the data of Gray et al,[20] according to which 74% of 31 pneumococcal infections (mainly AOM) were caused by a serotype that was carried 1 month or less before the onset of acute illness. Our study focused on only part of the sick visits in the FinOM Cohort Study (ie, the first visits in each observation period of 3 months). This selection was made to reduce the confounding effect of repeated episodes and to better allow comparisons across all periods. This could have introduced a bias by minimizing the effect of an "otitis-prone" condition. However, repeating the essential analyses with the whole FinOM Cohort Study material did not change the conclusions (unpublished data).

We addressed the duration of carriage before AOM by focusing on the timing of the sick visit with Pnc carriage during each observation period. A temporal association between acquisition of carriage and development of Pnc AOM was evident because sick visits with Pnc AOM occurred on average earlier than sick visits without Pnc AOM. The presence or absence of nonpneumococcal AOM did not influence the finding. Although the precise date when the carriage had been acquired is not known, at least one-half of the Pnc AOM cases associated with newly acquired carriage must have occurred in <1 month after acquisition (the median time since the start of the period was 29 days). One-half of the Pnc AOM cases associated with a carriage acquired before the start of the period occurred within 15 days, indicating that many of them could in reality be the result of relatively recently acquired carriage. However, the data also show a minority of cases in which Pnc AOM seems to have developed in association with prolonged (present for >1 month) carriage, especially in the younger children. A similar subset
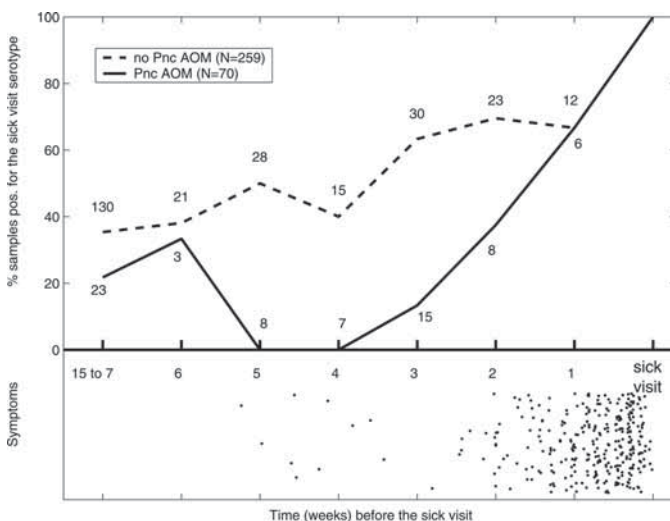
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of Pnc infection developing after prolonged carriage was reported by Gray et al.[20]

The short duration of carriage before Pnc AOM was further shown by the retrospective analysis of pneumococcal carriage before the sick visits: it was surprisingly low for several weeks preceding the sick visits with Pnc AOM (Fig. 3). Neither the serotype in MEF nor any other serotype was found in the 15 samples obtained 3–5 weeks before the Pnc AOM, indicating that absence of Pnc from the nasopharynx might be a risk factor for Pnc AOM. Antimicrobial treatment is not likely to confound this conclusion, because the results were very similar, when repeated after exclusion of the few samples obtained during antimicrobial treatment (data not shown).

The proportion of samples positive for Pnc was almost twice as high at the sick visits as at the scheduled visits, in accordance with data from the entire FinOM Cohort Study.[24] The rapid increase in carriage before the sick visit was here shown to broadly coincide with start of the symptoms of respiratory infection (Fig. 3), raising a question whether acquisition of Pnc caused the symptoms that lead to the sick visit, or whether the symptomatic mucosal reaction to viral infection facilitated acquisition of carriage. We thought that the duration of symptoms before the sick visit is likely to indicate their severity: the shorter the time, the more severe were the symptoms prompting a sick visit. What we found, however, was that the duration (and, by inference, the severity), of the symptoms before the sick visit did not correlate with the presence or new appearance of pneumococci in the nasopharynx. Thus the data did not support the hypothesis that the acquisition had caused the symptoms leading to the sick visit. Instead the data are consistent with the hypothesis that viral respiratory infection caused the symptoms and enhanced the acquisition of pneumococci. The primary role of a viral infection has also been shown by Brimblecombe et al,[26] who assessed the temporal relationship between onset of acute coryza and increase in Pnc carriage in a survey among British families. In that study, Pnc carriage only started increasing 1–2 days after the start of symptoms. This hypothesis is indirectly supported by experimental studies showing that viruses can increase the adherence of bacteria to human epithelial cells[11,40–41] and influenza A infection increases pneumococcal colonization both in chinchillas and in human volunteers.[12,13]

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