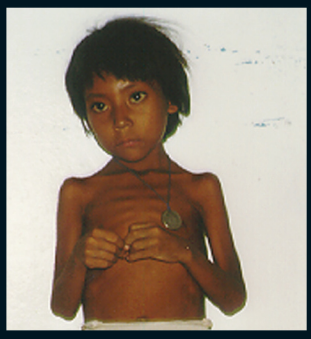
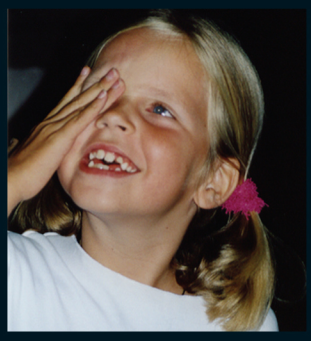





Host-pathogen interaction during *Streptococcus pneumoniae* colonization and infection


Debby Bogaert


HOST-PATHOGEN INTERACTION DURING *STREPTOCOCCUS PNEUMONIAE* COLONIZATION AND INFECTION

Cover: Tonny Moonen, Marcel Sluijter & Debby Bogaert

ISBN: 90-77595-49-X

Printed by: Optima Grafische Communicatie Rotterdam

HOST-PATHOGEN INTERACTION DURING *STREPTOCOCCUS PNEUMONIAE* COLONIZATION AND INFECTION

INTERACTIE TUSSEN GASTHEER EN PATHOGEEN
TIJDENS COLONISATIE EN INFECTIE
MET *STREPTOCOCCUS PNEUMONIAE*

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 2 juni 2004 om 11.45 uur

door

Debby Bogaert

geboren te Goes

Promotiecommissie

Promotor:	Prof.dr. R. de Groot
Overige leden:	Prof.dr. A. Finn
	Prof.dr. A.F. van Belkum
	Prof.dr. H. Hooijkaas
Copromotor:	Dr. P.W.M. Hermans

The research represented in this thesis was financially supported by:
Sophia foundation for medical research, The Netherlands (grant 268)
Dutch science foundation (grant SGO-Inf. 005)

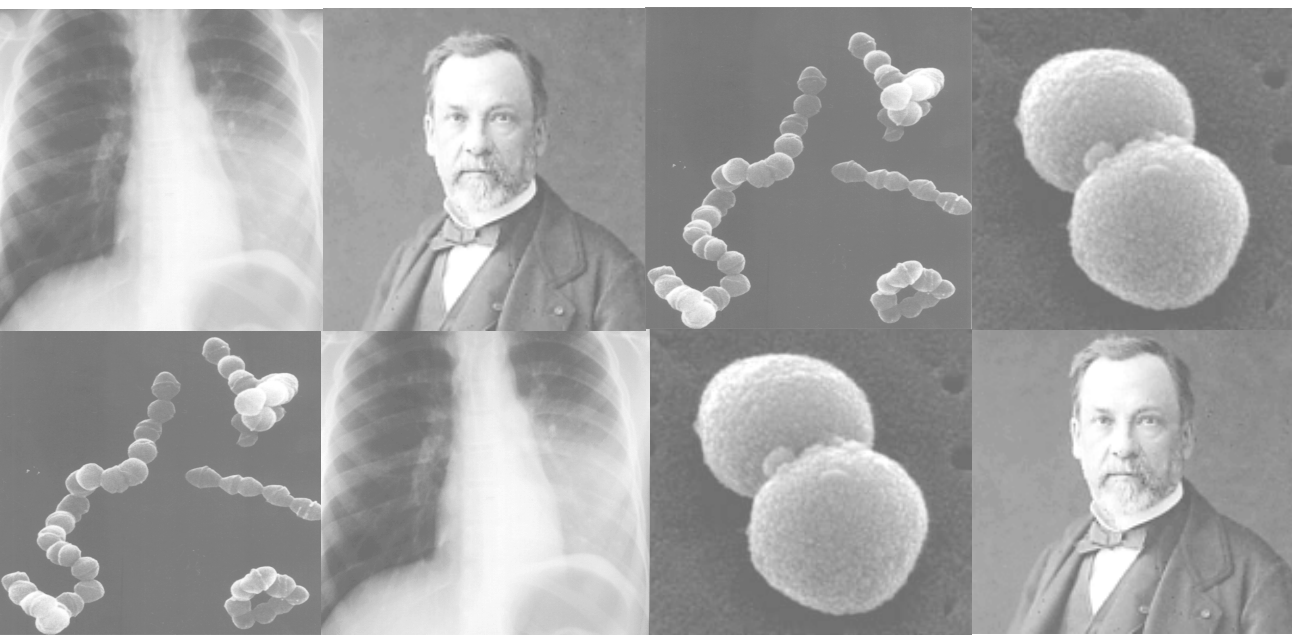
“Luctor et Emergo”

Voor Tonny

Contents

Chapter 1

General introduction



Streptococcus pneumoniae was discovered by Sternberg and Pasteur in 1880. It took another six years to discover that this microorganism, called the pneumococcus, was the actual cause of bacterial pneumonia [1]. Subsequently, this bacterium has been shown to provoke an impressive number of diseases, which can be roughly classified in respiratory and invasive. Respiratory diseases by *S. pneumoniae* are sinusitis, otitis media, bronchitis and pneumonia, of which the latter one may be complicated by septicemia [2]. Disseminated invasive infections caused by the pneumococcus include sepsis, meningitis, endocarditis and arthritis [2]. Morbidity and mortality are high both in the developing and the developed world: annually 3 million people die worldwide of pneumococcal infections. Approximately 1 million are children under the age of five years [3]. Risk groups for pneumococcal disease are children younger than 2 years, elderly people and immunocompromised patients [2]. In children, the increased risk for pneumococcal infections is mainly due to a relatively immature immune response to type II T-cell independent (TI-2) antigens such as capsular polysaccharides [4]. In the elderly, the ability of both the innate as well as the adaptive immunity to respond to pneumococcal infection are thought to decline [5]. In addition, higher rates of pneumococcal invasive diseases have been observed among populations such as Alaskan Natives, American Indians and African Americans [6]. Patient groups who are at risk for particular variants of pneumococcal diseases are well defined. For example functional asplenia as in sickle cell disease as well as anatomic asplenia are serious risk factors for pneumococcal sepsis. This is due to the absence or dysfunction of the spleen, which is involved in systemic clearance of *S. pneumoniae* [2]. In addition, complement deficiency and lower levels of circulating antibodies are thought to contribute to the increased susceptibility to pneumococcal infections in patients with sickle cell disease and other haemoglobinopathies [6]. A relatively new risk group for pneumococcal meningitis are children with a cochlear implantation [7]. Disease in these patients is thought to occur by the presence of a continuum between the outer ear and the inner skull. In general, patients with cerebrospinal fluid leakage, immunodeficiencies, chronic cardiovascular and pulmonary disease, HIV infections and diabetes mellitus are considered at risk for pneumococcal invasive disease [2,6].

Nasopharyngeal carriage of *S. pneumoniae* is common. The majority of infants become colonized at least once early in life. Pneumococcal colonization is mostly asymptomatic and not followed by disease. However, in situations of disturbance of the host-pathogen balance, for example through viral infection, malnutrition or local damage of the mucosa, asymptomatic colonization can proceed to (invasive) disease (Figure 1) [8-10]. In addition, healthy individuals with asymptomatic pneumococcal carriage may spread this pathogen within the community. Especially crowding of individuals as occurs in hospitals, day-care centers and jails has been shown to enhance the horizontal spread of pneumococci [11-13].

Because the highest incidence in pneumococcal colonization and the highest crowding index is found in young children, this risk group is believed to be the most important vector for horizontal dissemination of pneumococcal strains within the community [14]. The nasopharynx is also believed to be the main reservoir and thus source of acquisition, exchange and spread of antibiotic resistance among pneumococcal isolates [15]. Penicillin- and multidrug-resistant pneumococci have emerged in several countries over the last decades. In some of these countries, up to 60% of the pneumococci are resistant to one or more antibiotics [16-18]. A significant proportion of pneumococcal resistance is the result of worldwide spread of a limited number of multidrug-resistant clones [19-22].

Until recently, intervention strategies by means of vaccination failed in the major risk group of young children. Recently, a 7-valent conjugate vaccine has shown to be highly efficacious against pneumococcal invasive disease caused by the predominant serotypes in young children. Moreover, the vaccine partially protects against diseases such as pneumonia and otitis media [23-26]. Unfortunately, the coverage of the conjugate vaccine is limited to 7 of the more than 90 known pneumococcal serotypes, which may result in replacement of vaccine serotypes by non-vaccine serotypes after vaccination. Several studies have shown that this replacement phenomenon occurs at the nasopharyngeal level [27]. Whether this observation will also occur with respect to disease remains unclear.

In conclusion, nasopharyngeal colonization is of crucial importance in the pathogenesis of infection with, resistance to, spread of and intervention against *S. pneumoniae* and, therefore, important to study in more detail. Though many studies have investigated overall pneumococcal colonization and serotype distributions in young children, few of these have extended the age group until adulthood. Therefore no data are available on the natural dynamics of pneumococcal colonization and serotype distribution with increasing age. In addition, few studies have been performed on the molecular epidemiology of pneumococci with respect to colonization and infection, although these are extremely important to generate knowledge about pneumococci, especially in the context of vaccination. Moreover, only a limited number of studies have investigated the effect of conjugate vaccination on colonization. Therefore, in this thesis we asked ourselves the following questions: a) what is the prevalence and which are the determinants of nasopharyngeal colonization with *S. pneumoniae* and other pathogenic bacteria in children, b) is nasopharyngeal colonization with *S. pneumoniae* during health and (mucosal) infection a static or dynamic process, and c) how efficiently do antibiotic-resistant pneumococci spread within the population and which events are involved in the acquisition and spread of resistance and resistance genes in pneumococci? In addition we asked d) what the effect of pneumococcal vaccination is on colonization and infection with *S. pneumoniae* and e) what the response of the host immune system is and what type of response occurs with respect to pneumococcal colonization,

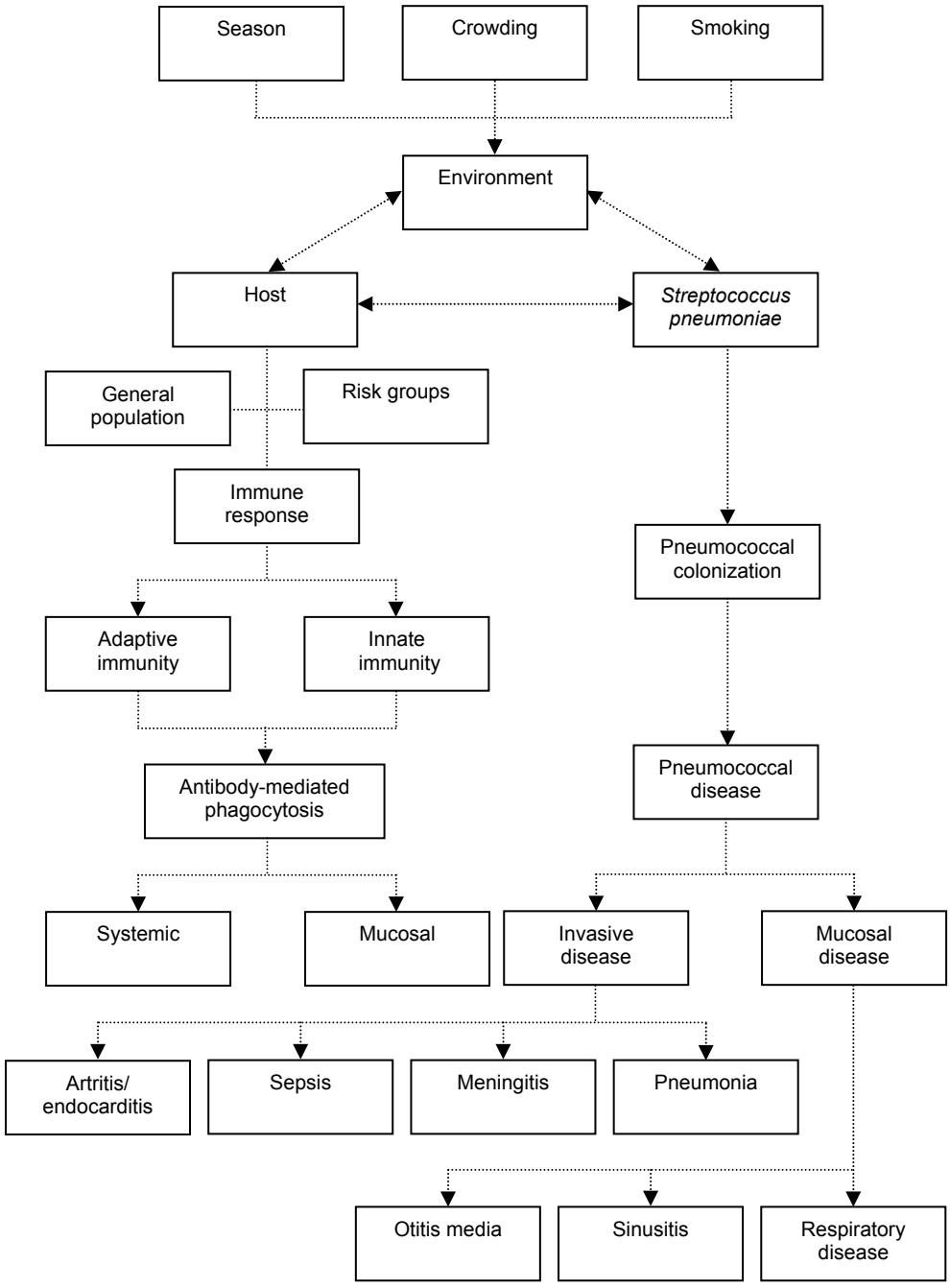


Figure 1.

Interaction between the environment, the host immune system and the different routes of infection by *S. pneumoniae*

infection and vaccination.

In **Chapter 2**, we discuss the mechanism of pneumococcal colonization and the importance of colonization for pneumococcal disease. In **Chapter 3**, the epidemiology and determinants for pneumococcal colonization and the interaction with other nosocomial pathogens are described. In **Chapter 4**, we specifically focus on the molecular behavior of drug-resistant pneumococci in Greece and The Netherlands. In **Chapter 5**, the molecular dynamics of colonization and infection with *S. pneumoniae*, especially the acquisition and spread of resistance is investigated. In **Chapter 6**, an update on the current vaccination strategies is provided, followed by the description of the impact of pneumococcal conjugate vaccination on colonization and infection with *Streptococcus pneumoniae* in **Chapter 7**. Finally, in **Chapter 8** our most recent studies regarding the host immune response to pneumococcal infection and vaccination are presented.

REFERENCES

1. 1. Austrian R. The pneumococcus at the millennium: not down, not out. *J Infect Dis* 1999;179 Suppl 2:S338-41.
2. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 1997;46(RR-8):1-24.
3. Jaffar S, Leach A, Hall AJ, et al. Preparation for a pneumococcal vaccine trial in The Gambia: individual or community randomisation? *Vaccine* 1999;18(7-8):633-40.
4. Goldblatt D. Immunisation and the maturation of infant immune responses. *Dev Biol Stand* 1998;95:125-32.
5. Meyer KC. The role of immunity in susceptibility to respiratory infection in the aging lung. *Respir Physiol* 2001;128(1):23-31.
6. Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2000;49(RR-9):1-35.
7. Pneumococcal vaccination for cochlear implant recipients. *MMWR Morb Mortal Wkly Rep* 2002;51(41):931.
8. Hament JM, Kimpen JL, Fleer A, Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. *FEMS Immunol Med Microbiol* 1999;26(3-4):189-95.
9. Mulholland K. Strategies for the control of pneumococcal diseases. *Vaccine* 1999;17 Suppl 1:S79-84.
10. Plotkowski MC, Puchelle E, Beck G, Jacquot J, Hannoun C. Adherence of type I *Streptococcus pneumoniae* to tracheal epithelium of mice infected with influenza A/PR8 virus. *Am Rev Respir Dis* 1986;134(5):1040-4.
11. Kellner JD, Ford-Jones EL. *Streptococcus pneumoniae* carriage in children attending 59 Canadian child care centers. Toronto Child Care Centre Study Group. *Arch Pediatr Adolesc Med* 1999;153(5):495-502.
12. Nuorti JP, Butler JC, Crutcher JM, et al. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. *N Engl J Med* 1998;338(26):1861-8.
13. Principi N, Marchisio P, Schito GC, Mannelli S. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. *Pediatr Infect Dis J* 1999;18(6):517-23.
14. Leiberman A, Dagan R, Leibovitz E, Yagupsky P, Fliss DM. The bacteriology of the nasopharynx in childhood. *Int J Pediatr Otorhinolaryngol* 1999;49 Suppl 1:S151-3.
15. Sa-Leao R, Tomasz A, Sanches IS, et al. Carriage of internationally spread clones of *Streptococcus pneumoniae* with unusual drug resistance patterns in children attending day care centers in lisbon, portugal. *J Infect Dis* 2000;182(4):1153-60.
16. Lee HJ, Park JY, Jang SH, Kim JH, Kim EC, Choi KW. High incidence of resistance to multiple antimicrobials in clinical isolates of *Streptococcus pneumoniae* from a university hospital in Korea. *Clin Infect Dis* 1995;20(4):826-35.
17. Fairchok MP, Ashton WS, Fischer GW. Carriage of penicillin-resistant pneumococci in a military population in Washington, DC: risk factors and correlation with clinical isolates. *Clin Infect Dis* 1996;22(6):966-72.
18. Baquero F, Garcia-Rodriguez JA, Garcia de Lomas J, Aguilar L. Antimicrobial resistance of 1,113 *Streptococcus pneumoniae* isolates from patients with respiratory tract infections in Spain: results of a 1-year (1996-1997) multicenter surveillance study. The Spanish Surveillance Group for Respiratory Pathogens. *Antimicrob Agents Chemother* 1999;43(2):357-9.
19. Corso A, Severina EP, Petruk VF, Mauriz YR, Tomasz A. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. *Microb Drug Resist* 1998;4(4):325-37.
20. Hermans PW, Sluijter M, Dejsirilert S, et al. Molecular epidemiology of drug-resistant pneumococci: toward an

international approach. *Microb Drug Resist* 1997;3(3):243-51.
21. Sibold C, Wang J, Henrichsen J, Hakenbeck R. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. *Infect Immun* 1992;60(10):4119-26.
22. Tomasz A, Corso A, Severina EP, et al. Molecular epidemiologic characterization of penicillin-resistant *Streptococcus pneumoniae* invasive pediatric isolates recovered in six Latin-American countries: an overview. PAHO/Rockefeller University Workshop. Pan American Health Organization. *Microb Drug Resist* 1998;4(3):195-207.
23. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000;19(3):187-95.
24. Black SB, Shinefield HR, Ling S, et al. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. *Pediatr Infect Dis J* 2002;21(9):810-5.
25. Fireman B, Black SB, Shinefield HR, Lee J, Lewis E, Ray P. Impact of the pneumococcal conjugate vaccine on otitis media. *Pediatr Infect Dis J* 2003;22(1):10-6.
26. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a Pneumococcal Conjugate Vaccine against Acute Otitis Media. *N Engl J Med* 2001;344(6):403-409.
27. Mbelle N, Huebner RE, Wasas AD, Kimura A, Chang I, Klugman KP. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis* 1999;180(4):1171-6.

Chapter 2

Streptococcus pneumoniae colonisation: the key to pneumococcal disease

D. Bogaert, R. de Groot and P.W.M. Hermans

Lancet Infect Dis 2004; 4(3):144-154

ABSTRACT

Streptococcus pneumoniae is an important pathogen causing invasive diseases such as sepsis, meningitis and pneumonia. The burden of disease is highest in the youngest and oldest parts of the population in both developed and developing countries. The therapy of pneumococcal infections is complicated by the worldwide emergence of resistance to penicillin and other antibiotics. Especially in children pneumococcal disease is preceded by asymptomatic colonisation.

The current 7-valent conjugate vaccine is highly efficacious against invasive disease caused by the vaccine type strains. However, the vaccine coverage is limited and replacement by non-vaccine serotypes resulting in disease is a serious threat for the near future. Therefore, the search for new vaccine candidates that elicit protection against a broader range of pneumococcal strains is still an important field of investigation. To this respect, several surface-associated protein vaccines are currently studied. Another important matter of debate is whether to prevent pneumococcal disease by eradication of nasopharyngeal colonisation, or whether to aim for the prevention of bacterial invasion leaving colonisation relatively unaffected and hence, preventing the occurrence of replacement colonisation and disease.

To illustrate the importance of pneumococcal colonisation in relation to pneumococcal disease and prevention of disease we will discuss in detail the mechanism and epidemiology of colonisation, the complexity of inter- and intra-species relations, and the consequences of the different preventive strategies for pneumococcal colonisation.

INTRODUCTION

Streptococcus pneumoniae is a common cause of invasive disease and respiratory tract infections in developing as well as developed countries. Risk groups for pneumococcal infections such as meningitis, sepsis and pneumonia include young children, the elderly and patients with immunodeficiencies [1]. Annually, 1 million children under the age of 5 years die due to pneumonia and invasive diseases. In the United States, the yearly incidence of fatal pneumococcal infection is 40.000 deaths [2]. Community-acquired pneumococcal meningitis has a very high case-fatality rate (20% and 50% in developed and developing countries, respectively). Depending on the age, 30%-60% of the survivors develop long-term sequelae including hearing-loss, neurological deficits and neuropsychological impairment [3].

Protection against pneumococcal infections is mediated by opsonin-dependent phagocytosis. This antibody-initiated complement-dependent opsonisation, which activates the classical complement pathway, is considered to be the major immune mechanism protecting the host against infections with pneumococci [4]. The mechanism of clearance depends on the interaction of type-specific antibodies (IgA, IgM, IgG), complement and neutrophils or phagocytic cells from lung, liver and spleen. The absence of the spleen or cirrhosis of the liver predisposes for severe pneumococal infection. Congenital deficiencies in immunoglobulin or complement are also associated with predisposition to pneumococcal infection [5]. *S. pneumoniae* is part of the commensal flora of the upper respiratory tract. Together with *Moraxella cattarrhalis, Haemophilus influenzae, Neisseria meningitidis*, *Staphylococcus aureus* and various haemolytic streptococci, they colonise the nasopharyngeal niche. Though colonisation with pneumococci is mostly asymptomatic, it can progress to respiratory or even systemic disease (Figure 1). Importantly, pneumococcal disease will not occur without being preceded by nasopharyngeal colonisation with the homologous strain [6,7]. In addition, pneumococcal carriage is believed to be an important source of horizontal spread of this pathogen within the community. Especially, crowding like in hospitals, day-care centres and jails has shown to enhance horizontal spread of pneumococcal strains [8-16]. Because the highest incidence in pneumococcal colonisation and the highest crowding index is found in young children, this risk group is believed to be the most important vector for horizontal dissemination of pneumococcal strains within the community [17]. Therefore, part of the prevention strategies for pneumococcal disease focus on prevention of nasopharyngeal colonisation, especially in children.

Because of the key role for nasopharyngeal colonisation in pneumococcal disease and pneumococcal spread, we will focus in this review on the different aspects of nasopharyngeal colonisation in children. In order to gain insight into the route of pneumococcal disease we will discuss the current knowledge on the mechanism of

colonisation, the epidemiology and determinants of pneumococcal carriage, and the actual status of prevention of colonisation by means of vaccination.

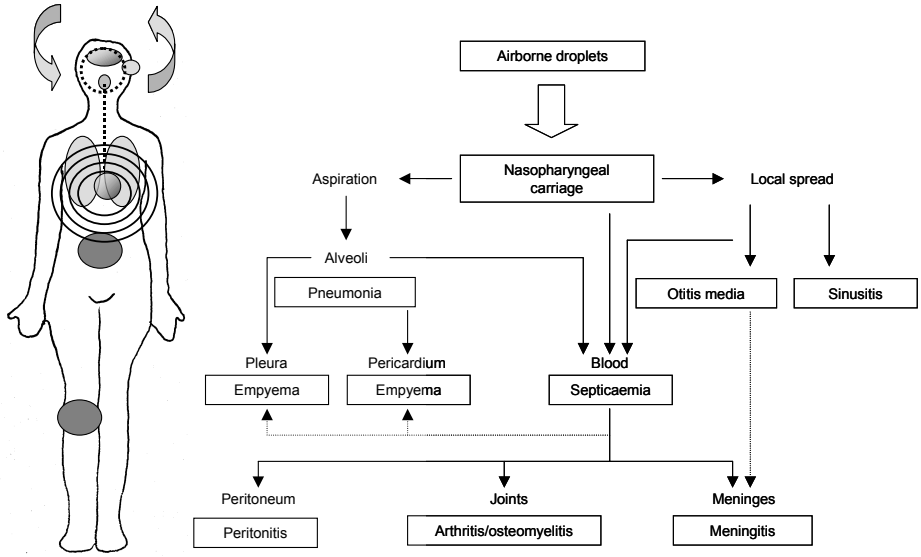


Figure 1. Pathogenic route for *S. pneumoniae* infection (adapted from Obaro et al., J. Med. Microbiol. 2002;51: 98-104). In dark and light grey the organs infected through the hematogenic and airborn route, respectively, are depicted.

SEARCH STRATEGY AND SELECTION CRITERIA

PubMed searches and references from relevant articles and recent conferences were used for this paper. Search terms were “Streptococcus pneumoniae and (colonization or carriage)”, “Streptococcus pneumoniae and children”, “Streptococcus pneumoniae and (vaccin*)”, “streptococ* and protein and vaccin*”, “streptococcus and (interference or interaction or competition)”, “Streptococcus pneumoniae and (protection or immun*)”. Only English language papers were reviewed.

DYNAMICS OF NASOPHARYNGEAL COLONISATION

The upper respiratory tract is the ecological niche for many bacterial species. In children, the nasopharyngeal flora becomes established during the first months of life [18,19]. A broad variety of microorganisms including *S. pneumoniae, H. influenzae* and *M. catarrhalis* may

colonise the nasopharyngeal niche. It is estimated that all humans are colonised with these pathogens at least once during life. In most cases, this concerns asymptomatic carriage, but occasionally colonisation is followed by disease [20,21]. Colonisation is commonly followed by horizontal dissemination of these pathogens to individuals in the direct environment leading to spread within the community [22-24]. The reported rates of bacterial acquisition and carriage depend on age, geographical area, genetic background and socio-economic conditions [11,24-27]. The local host immune response plays an important regulatory role in the trafficking of pathogens in the upper respiratory tract [28]. A poor mucosal immune response may lead to persistent and recurrent colonisation and consequently infection, whereas a brisk local immune response to the pathogen will eliminate colonisation and prevent re-colonisation [29,30]. In general, mucosal immunity matures earlier than systemic immunity, and is detected from the age of 6 months [29]. IgG as well as secretory IgA antibodies directed against capsular polysaccharides and surface-associated proteins have been observed in saliva of children in response to colonisation with *S. pneumoniae* [31,32].

Nasopharyngeal colonisation is a dynamical process regarding the turnover of colonising species and serotypes. Moreover, interspecies competition is believed to be present and to interfere with the composition of the nasopharyngeal flora. First of all, the balance between the resident flora and transient invaders is important. To this purpose, the resident flora including α-haemolytic streptococci have shown to inhibit *S. pneumoniae, H. influenzae*, *S. aureus* as well as *M. catarrhalis* [23,29,33,34]. The importance of this inhibitory role has been shown by Ghaffar et al., who found a competitive balance between α-haemolytic streptococci and *S. pneumoniae* and *H. influenzae*, which could be altered by antibiotics. Faden et al. reported a negative correlation between viridans streptococci and *S. pneumoniae, H. influenzae* and *M. catarrhalis* with the last three becoming predominant during upper respiratory tract infections [23,35].

In addition, a competitive relation between the different pathogenic species exists. In vitro studies of Pericone et al. have shown a significant correlation between *N. meningitidis* and *S. pneumoniae*. *S. pneumoniae* growth did increase in the presence of meningococci, a process probably mediated by meningococcal catalase. However, meningococcal growth was decreased in the presence of pneumococci or pneumococcal culture supernatant. The authors attributed the latter effect to the presence of pneumococcal peroxide [36]. This inhibitory effect of *S. pneumoniae* was also observed in co-cultures with *H. influenzae* and *M. catarrhalis*. Moreover, *S. pneumoniae* may interfere with the growth of *S. aureus,* which has also been assigned to pneumococcal hydrogen peroxide [37,38]. Bogaert et al. showed in a cross-sectional carriage study among 3200 children that this interspecies competition between *S. aureus* and *S. pneumoniae* significantly contributes to the age-related dynamics of nasopharyngeal colonisation in children [39], which was recently confirmed by data of

Regev-Yochay et al. [40]. Bogaert et al. found parallel to the age-related decline of pneumococcal colonisation, caused by the maturation of the immune system, a simultaneous increase in *S. aureus* ranging from 10% in the first years of life to a maximum carriage rate of 50% at the age of ten years was found. In addition to these ecological interactions, the composition of the nasopharyngeal niche was influenced by environmental factors such as crowding and smoking [39]. Finally, limited evidence is available for the competition between the different pneumococcal serotypes. For example, Lipsitch et al. have used a mouse model of intranasal carriage of pneumococci to test whether competition occurs between different pneumococcal strains. They have found reduced levels of colonization with a serotype 6B strain when challenged intranasally with a serotype 23F pneumococcus. This inhibitory effect could be overcome by increasing the dose of the challenge strain [41]. We have to realise, that interference in this complex pattern of interaction and inhibition by means of vaccination may have serious and unpredictable consequences for the composition of the entire nasopharyngeal population.

MECHANISM OF COLONISATION

The pneumococcal outer surface is covered by a polysaccharide capsule. Capsular polysaccharides are highly heterogeneous, and thus far, almost 100 different capsular serotypes have been described [5]. The polysaccharide capsule is the most important virulence factor of the pneumococcus since it protects the bacteria from phagocytosis [42]. Reduced expression results in greater access of antibodies and complement to the pneumococcal surface [43], and hence, increased clearance by the immune system. Capsular polysaccharides are highly immunogenic and antibodies against these polysaccharides protect against infection with the homologous serotype by induction of opsonophagocytosis. The antigenicity of the capsule is type-specific, however, cross-reaction can occur because of shared polysaccharide epitopes [5].

The layer underneath the capsule, the cell wall, consists of polysaccharides and teichoic acid and serves as an anchor for cell wall associated surface proteins (CBPs). The cell wall is responsible for the intense inflammatory reaction that accompanies pneumococcal infection as it stimulates the influx of inflammatory cells and activates the complement cascade, and cytokine production [44]. The cell wall is believed to be protected from the host response by the surrounding polysaccharide capsule.

Colonisation of *S. pneumoniae* requires adherence to the epithelial lining of the respiratory tract. Asymptomatic colonisation involves pneumococcal binding to cell-surface carbohydrates (GlcNAc) on non-inflamed resting epithelium. Adherence to these sugars is mediated by CBPs, like pneumococcal surface adhesion A (PsaA) (Figure 2). In addition,

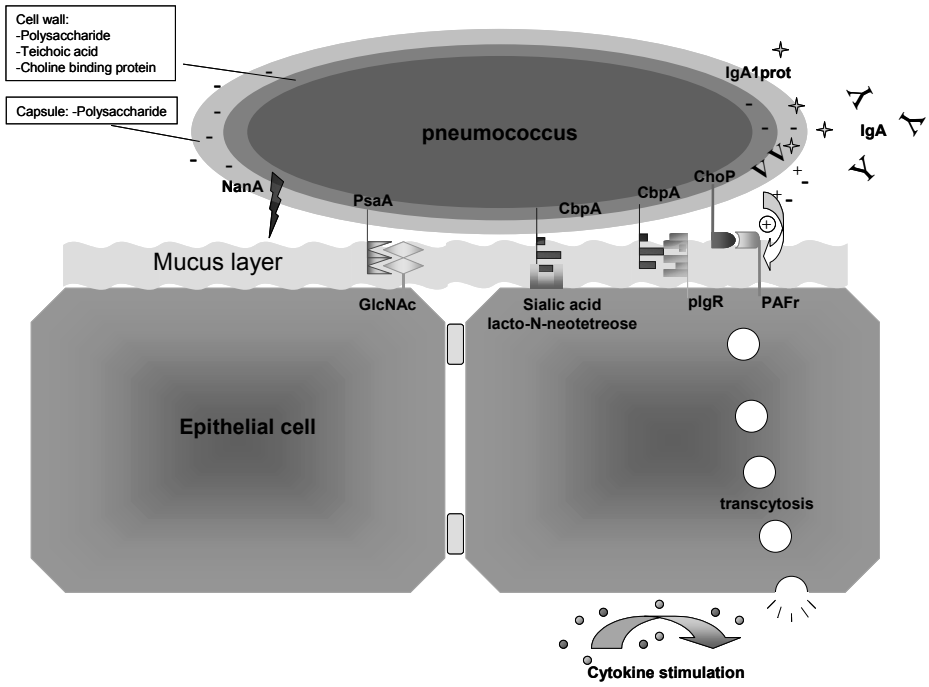


Figure 2. Interaction between *S. pneumoniae* and epithelial cells. Neuraminidase (NanA) decreases the viscosity of the mucus and exposes the N-acetyl-glycosamine (GlcNAc) receptors on the epithelial cells, which are able to interact with pneumococcal surface associated proteins like PsaA. In response to cytokine stimulation, host epithelial cells upregulate the platelet activating receptors (PAFr). The pneumococcus has increased affinity via its cell-wall phosphocholine (ChoP) for PAFr. Moreover, a second choline binding protein, CbpA, displays increased affinity for immobilised sialic acid and lacto-N-neotreatose, and binds directly to the polymeric Ig receptor (pIgR), which enhances migration through the mucosal barrier (transcytosis). Pneumococcal IgA1 protease cleaves opsonizing IgA, which results in a change (neutralisation) of surface charge and increases the physical proximity of ChoP to the PAF receptor.

CPBs contribute to the hydrophobic and electrostatic surface characteristics of pneumococci and may facilitate adherence to host cells partially through non-specific, physiochemical interactions [45]. In general, colonisation is not followed by symptomatic disease. Conversion of asymptomatic colonisation to invasive disease requires the local generation of inflammatory factors like interleukin-1 and tumor necrosis factor, as seen in the presence of viral infections [46]. This inflammatory cascade changes the type and number of receptors on target epithelial and endothelial cells. Pneumococcal cell wall choline (ChoP) shows increased affinity for one of these upregulated receptors, the platelet activating factor (PAF) receptor. Binding to the PAF receptor induces internalisation of pneumococci and promotes the transcellular migration through respiratory epithelium as well as vascular endothelium,

resulting in invasion of living bacteria (Figure 2) [47,48]. In addition, one of the CBPs, choline binding protein A (CbpA) shows increased affinity for immobilised sialic acid and lacto-N-neotetraose on cytokine-activated human cells [49]. Moreover, CbpA directly interacts with the polymeric Ig receptor (pIgR) which enhances migration through the mucosal barrier [50]. How the pneumococcus escapes endocytosis-mediated killing remains unclear [46,51]. The function of IgA1 protease has recently been unravelled by Weiser et al. They showed an enhanced adherence of pneumococci to lung epithelial cells in the presence of human IgA. This effect is thought to occur due to the cleavage of opsonising IgA by IgA1 protease, which results in change of surface charge and increased physical proximity of ChoP to the PAF receptor [52,53]. In addition, CbpA binds to the secretory component of IgA and interacts with the complement pathway, thus interfering with the human immune-response [50,54]. Another pneumococcal enzyme neuraminidase (NanA), enhances colonisation by cleaving N-acetylneuraminic acid from mucin decreasing the viscosity of the mucus. NanA also cleaves glycolipids, glycoproteins and oligosaccharides, and thus is thought to be responsible for exposing the N-acetyl-glycosamine receptors on the host epithelial cells [55]. In this way, the neuraminidase activity of viruses like influenza and parainfluenza viruses may contribute to the increased adherence of pneumococci observed during viral infections [56]. Variability in the composition, expression or exposure of surface-associated proteins may explain differences in colonisation and invasion capacities between strains. The complexity of this process is underlined by previous studies of Weiser et al., in which they identified a reversible phenotypic variation within pneumococcal strains and its role in host interaction. Transparent phase variants show enhanced adherence compared to opaque variants. This phenotypic variation is associated with the lower expression of capsule polysaccharides, and higher expression of certain cell surface proteins and carbohydrate containing cell wall structures [57-59].

With increasing knowledge about the mechanisms of colonisation, the surface-associated proteins have become of major interest as potential vaccine candidates. Although surface-associated proteins like pneumolysin and PspA have shown to elicit protection against systemic diseases, PsaA and CbpA have proven to be promising candidates for prevention of colonisation [50,60]. Theoretically, enhanced protection against colonisation and infection with *S. pneumoniae* may be expected when a combination of proteins with distinct roles in bacterial virulence is used.

PNEUMOCOCCAL COLONISATION IN CHILDREN

Nasopharyngeal colonisation of *S. pneumoniae* in children is primarily age dependent. Recently, the age-dependent carriage rate was investigated in a large cohort of healthy

children ranging from 1 to 19 years of age. The peak-incidence of pneumococcal colonisation was 55% at the age of 3 years. This was followed by a steady decline until a stable prevalence was observed of 8% after the age of 10 years [39]. Although most other colonisation studies have not extended the age of children until adulthood, the few studies that did study this show a comparable decline [61-63]. In contrast, in the first year of life the nasopharyngeal niche will become colonised. Therefore, pneumococcal carriage shows an increase before the age of two years (Table 1)[73,82]. For example, in a Finnish study the incidence of nasopharyngeal carriage in children 2 to 24 months of age increased from 13% under 6 months of age to 43% in children older than 19 months of age [67]. This percentage increased during respiratory infections to 22-45%, supporting the theory of enhanced adherence during (viral) infections.

In the healthy population, risk factors also seem to determine the incidence of pneumococcal carriage. Independent determinants for nasopharyngeal colonisation are ethnicity, crowding, environmental and socio-economic factors. Socio-economic and environmental risk factors include family size, specifically the number of older siblings, income, smoking (passive and active) and recent antibiotic use [11,17,29,64,84]. Crowding is a major factor in colonisation and in spread of pneumococcal strains. In young children, especially day-care visits are correlated to significant increased colonisation rates (Table 1) [24,39,75,82-87]. In a day-care study in The Netherlands a 1.6-fold increased risk of nasopharyngeal colonisation of pneumococci in day-care centre attendees was found compared to children who were taken care for at home [24]. In addition, this study has shown increased genetic clustering among pneumococcal isolates, which is in line with previous reports [87-89]. This supports the hypothesis of increased horizontal spread of specific pneumococcal strains among day-care centre attendees [24]. In agreement with these findings, Raymond et al. reported a colonisation rate of up to 82% in infants living in an orphanage [80]. Furthermore, close relatedness between the pneumococcal isolates was found in this study, suggesting frequent horizontal spread.

According to ethnicity, risk-groups for pneumococcal colonisation as well as invasive disease are African American, native American (Apache and Navajo) and Alaskan native populations [90]. The risk for invasive pneumococcal diseases in children 24-35 months of age is 64.7 cases per 100.000, whereas African American citizens in the USA have a risk incidence of 116.4 per 100.000 and native Americans 73-227 cases per 100.000 [90]. For example, the risk for invasive disease in American native population is increased to such a degree that the Advisory Committee for Immunization Practices (ACIP; USA) has recommended pneumococcal vaccination for this population in all age groups [1]. Also for

Table 1. Pneumococcal colonisation and serotype-distribution studies. Country, age, size of the study population, risk group, carriage rate and potential coverage of the pneumococcal conjugate vaccine are depicted.

Investigator	Year	Country	Number of children	Age	risk group	Type of culture	Carriage (%)	Coverage 7-valent conjugate vaccine (%)
Coles[64]	1998-99	India	464	2-6 months	Healthy	transnasal	64-70*	50†
Syrogiannopoulos[65]	1997-99	Greece	2448	2-23 months	Healthy	transnasal	34	65
Jebaraj[66]	1994-95	India	100	6-18 months	Healthy	transnasal	40	46**
Syrjanen[67]	1994-95	Finland	329	2-24 months	Healthy	transnasal	13-43*	53
Soewignjo[68]	1997	Indonesia	484	0-25 months	Healthy	transnasal	48	
Bogaert[24]	1999	The Netherlands	535	3-36 months	Healthy	transnasal	37	56
Rusen[69]	1990	Kenya	26	0-2 years	Healthy	transnasal	22	59‡
Chiou[70]	2905	Taiwan	2905	0-7 years	Healthy	transnasal	21	-
Polack[71]	1997	USA	85	0-14 years	Healthy	transnasal	19	-
Parry[72]	1996	Vietnam	911	1-16 years	Healthy	transnasal	44	70**
Bogaert[39]	2002	The Netherlands	3200	1-19 years	Healthy	transnasal	50-8*	42
Faden[7]	1995	Buffalo	306	6 months	Healthy	unknown	23	-
Vives[73]	1988-92	Costa Rica	440	1-12 months	Healthy	unknown	3-19*	-
Berkovitch[74]	(2002)	Israel	1000	1-24 months	Healthy	throat	2	-
Marchisio[75]	1998-00	Italy	55	6-84 months	Healthy	throat	24	-
Marchisio[76]	2000	Italy	2799	0-7 years	Healthy	throat	9	63
Principi[11]	1996	Italy	1723	1-7 years	Healthy	throat	4	-
Bakir[26]	2000	Turkey	1382	0-10 years	Healthy	throat	8	-
Muhlemann[61]	1998-99	Switzerland	2769	0-16 years	RTI	transnasal	48-39*	49-65
Syrjanen[67]	1994-95	Finland	329	2-24 months	URTI	transnasal	22-45*	68
Rusen[69]	1990	Kenya	26	0-2 years	URTI	transnasal	29	59‡
Desjirilert[77]	1992-94	Thailand	1783	0-5 years	URTI	transnasal	35	-
Syrjanen[67]	1994-95	Finland	329	2-24 months	AOM	transnasal	45-56*	68
Dagan[78]	1994-96	Israel	120	3-36 months	AOM	transnasal	63	61
Veenhoven[79]	1998-02	The Netherlands	383	1-7 years	rAOM	transnasal	55	55
Raymond[80]	1996	France	71	0-24 months	Orphanage	transnasal	58	85
Leibovitch[81]	1996	Romania	162	1-38 months	Orphanage	transnasal	50	98**
Rusen[69]	1990	Kenya	26	0-2 years	HIV	transnasal	20	59‡
Rusen[69]	1990	Kenya	26	0-2 years	HIV+ URTI	transnasal	86	59‡
Leibovitch[81]	1996	Romania	40	3-9 years	HIV	transnasal	30	98**
Polack[71]	1997	USA	85	0-14 years	HIV	transnasal	20	-

Daw[62]	1994-95	USA	312	0-18 years	SCD	transnasal	21-11*	56
Norris[63]	1994-95	USA	278	1-19 years	SCD	transnasal/throat	32-5*	79
Bogaert[24]	1999	Holland	535	3-36 months	DCC	transnasal	58	59
Lee[82]	1998-99	Asia	4963	0-5 years	DCC/OPD	transnasal	11-43	65**
Chiu[83]	1999-00	Hong Kong	1978	2-6 years	DCC	transnasal	39	-
Petrosillo[84]	1999	Italy	610	2-65 months	DCC	unknown	15	57
Vives[73]	1988-92	Costa Rica	280	2-5 years	DCC	unknown	39	-
Marchisio[75]	1998-00	Italy	85	6-84 months	rAOM	throat	29	-
Marchisio[75]	1998-00	Italy	113	6-84 months	cOME	throat	35	-
Fujimore[85]	1994	Japan	43	2-12 years	cOME	throat	23	-

*with increasing age. **including cross-reactive serotypes. †coverage for 9-valent conjugate vaccine. ‡Average for all isolates of the study. URTI: upper respiratory tract infection. RTI: respiratory tract infection. AOM: acute otitis media. rAOM: recurrent acute otitis media. cOME: chronic otitis media with effusion. HIV: human immunodeficiency virus. SCD: sickle cell disease. OPD: out-patient department.

children attending day-care centres the risk of pneumococcal infection is so high that immunisation with a 7-valent pneumococcal conjugate vaccine (Prevnar, Wyeth, USA) covering the most prevalent serotypes 4, 6B, 9V, 14, 18C, 19F and 23F is advised. Pneumococcal colonisation, especially colonisation with antibiotic-resistant pneumococci is also increased as a result of recent antibiotic treatment [35,84]. It is commonly assumed that the selection of antibiotic-resistant pneumococci at the nasopharynx causes the spread of resistant pneumococcal strains within the comunity [78]. Consequently, several multidrug-resistant clones have already spread throughout the world [91,92].

Not all risk groups for pneumococcal diseases show increased prevalences of colonisation compared to the general population. For example children with HIV and sickle cell disease have similar colonisation rates compared to healthy children (Table 1) [71,93]. Their predisposition is presumably caused by the underlying immune disorder: instead of a defect or augmented challenge of the primary defence mechanism against pneumococcal invasion, an immune disorder is present related to an impaired response to or clearance mechanism for pneumococci after invasion has occurred. For children with HIV/AIDS, CD4 positive T-cells, necessary for an appropriate anti-polysaccharide response, are decreased, whereas for children with sickle cell disease the splenic function, involved in direct phagocytosis and initiation of the anti-polysaccharide response, is impaired. However, the primary mucosal barrier including the mucosal immune response is still intact in these patients [93,94].

Figure 3. A nasopharyngeal swab is taken from a 10-year old girl during a large cohort study in Rotterdam, The Netherlands (September 2002). The nasopharynx is approached via the nasal route: the swab is passed gently back from one nostril along the floor of the nasal cavity until it touches the posterior wall of the nasopharynx. After gentle rubbing or twisting for 1-2 seconds the swab is withdrawn. The swab is stored in stuart transport medium and plated within 6 hours onto gentamycin blood agar plates.

In Table 1 recent colonisation studies in different areas of the world are summarised. Though variable colonisation rates have been observed, the colonisation rates are higher during respiratory tract infections and otitis media and in risk groups like day-care centre attendees compared to healthy populations. An additional difference is the tendency towards lower colonisation rates when nasopharyngeal samples were obtained via the oropharynx compared with the transnasal approach, though this was more obvious in healthy

children than in risk groups. For future research, we believe the transnasal route for approaching the nasopharynx will be preferable (Figure 3).

SEROTYPE DISTRIBUTION AMONG PNEUMOCOCCAL ISOLATES

The serotype distribution among nasopharyngeal carriage isolates shows slightly variable results depending on the country, age group and type of cohort. First of all, Europe and the United States show comparable serotype distributions with minor differences regarding several serotypes. For example, in The Netherlands serotype 19F (19%), 6B (16%), 6A (13%), 9V (7%) and 23F (7%) are most frequently found among children under 3 years of age [24]. Comparable results originate from Greece where the most predominant serotypes among children younger than 2 years of age are 6B, 19F, 23F, 14 and 18C [65], and from Finland where the serotypes 6B (16%), 23F (14%), 19F (14%) and 6A (9%) are most prevalent [67]. In the United States, the serotypes 6B, 14, 19F and 23F are also highly prevalent [95]. In Asia, similar serotypes and serogroups have been found among nasopharyngeal isolates in healthy children. For example, in India the most common serogroups are 6, 14, 19 and 15 [64,66] and in Vietnam the serogroups 19, 23, 14, 6 and 18 [72]. The serogroup distribution in Indonesia is slightly different, with the most common serogroups being 6 (25%) and 23 (21%) followed by 15 (8%), 33 (8%), 19 (6%), 12 (5%) and 3 (4%) [68]. In Kenya, serotype 13 was besides the serotypes 15, 14, 6B and 19F most predominantly present [69]. In South Africa a similar distribution was found with the exception of serotype 13 which was not found at all [96].

No major differences were found in the serotype distribution among children with risk factors such as day care centre attendance and upper respiratory tract infections compared to healthy children [24,67,75].

In contrast, a major variable is the age group investigated. In general, the incidence of vaccine serotypes is declining with age [97]. In a recent study in The Netherlands, nasopharyngeal carriage of vaccine-type strains generally declined from 30% at the age of one year to 3% at the age of eight years, after which a stable prevalence was observed until the age of 19 years. In contrast, non-conjugate vaccine serotypes, especially serotype 3, 8, 10, 11 and 15 show an initial increase till the age of 7-10 years after which a delayed decline compared to the vaccine serotypes was observed [39].

In general the serotype distribution among nasopharyngeal isolates from different parts of the world is rather similar. This is also reflected by the potential conjugate vaccine coverage (Table 1). As shown by Lloyd-Evans et al., invasive disease originates from nasopharyngeal colonisation with the homologous serotype [98]. Therefore, the serotype distribution of colonisation isolates should be representative for invasive disease, antibiotic resistance profiles and potential vaccine coverage. Alternatively, certain serotypes and genotypes

seem to cause invasive diseases more often when corrected for prevalence of nasopharyngeal colonisation [98]. This is recently shown by Brueggemann et al. who found serotype and clone-specific differences in invasive disease potential with an increased capacity to cause disease for specific serotype 14 and 18C clones [99]. In contrast, the most commonly carried serotypes 6B, 19F and 23F are least invasive, whereas certain non-vaccine serotypes (8, 38, 33F) are infrequent colonisers but appear to be more invasive. This counts in particular for the serotypes 5, 7F and 1 [100,101]. This knowledge is extremely important in view of the replacement of colonising strains observed after conjugate vaccination. Therefore, surveillance of pneumococcal invasive disease and colonisation isolates remains a necessity in those countries where large-scale pneumococcal vaccination is initiated.

CURRENT VACCINE STRATEGIES

The ACIP has recommended vaccination against pneumococcal infections for several risk groups. Although the 23-valent vaccine, with a theoretical coverage of 85-90% of the circulating strains, has shown to be immunogenic in adults and children > 5 years of age, children < 2 years of age have a severely impaired antibody response upon polysaccharide vaccination [94,102-104]. Therefore, the recommendations of the ACIP in 1997 excluded the major risk group of children under 2 years of age. The remaining groups were immunocompetent children above 2 years of age at increased risk for illness and death associated with pneumococcal disease because of chronic cardiac and pulmonary diseases, persons older than 2 years with functional or anatomic asplenia and immunocompromised patients above 2 years of age [1]. Fortunately, the new generation of conjugate vaccines is highly immunogenic in children under 2 years of age. Moreover, these vaccines elicit immunological memory [105]. In several large recent studies, a 7-valent conjugate vaccine had an almost 100% efficacy against invasive diseases caused by the serotypes included [106,107]. The new vaccines contain polysaccharides of 7-11 pneumococcal serotypes conjugated to a carrier protein inducing a T-cell-dependent immune response which is already present in humans from birth on. The ACIP therefore changed the childhood recommendations for pneumococcal vaccination in 2000. Vaccination with the 7-valent conjugate vaccine Prevnar (Wyeth, USA) for all children under two years of age and in children 2-5 years of age at increased risk of pneumococcal disease is advised. In the latter case, conjugate vaccination is followed by a polysaccharide booster because this has been shown to improve pneumococcal antibody titers in this age group [108]. The conjugate vaccine is highly effective against invasive disease caused by vaccine serotype strains. The efficacy against mucosal diseases such as pneumonia and otitis media is much lower and more difficult to measure

because culture based proof of diagnosis are often missing [106,107,109,110]. Moreover, several investigators have shown a significant reduction in nasopharyngeal carriage of vaccine type pneumococci as a result of conjugate vaccination [96,97,111-113]. In addition to individual protection, diminished colonisation is thought to elicit protection against pneumococcal colonisation and disease in the vaccinated community, i.e. herd immunity. Dagan and co-workers for example have shown a decreased colonisation rate in siblings of day-care centre attendees who were vaccinated with a 9-valent conjugate vaccine [114]. Moreover, penicillin and multidrug resistance is common among pneumococcal strains, especially among the conjugate vaccine serotypes. Therefore, it has been suggested that conjugate vaccination will also reduce resistance among pneumococcal strains in vaccinated individuals as well as the open community as a result of herd immunity [90]. Recently, Dagan et al. have proven a significant reduction in penicillin- and multidrug resistance among carriage strains as a result of vaccination with a nine-valent conjugate vaccine [115].

Unfortunately, the 7-11 valent vaccines cover a limited number of serotypes. Protection is also dependent on the geographic area with a potential coverage of the 7-valent conjugate vaccine for invasive strains of over 85% for the USA, 60-70% for Europe and around 55% for Asia [90], although a large proportion of these differences might be explained by variation in blood-culture practices [116]. In addition to the limited coverage of these conjugate vaccines, another long-term risk needs to be considered. Because of the limited coverage of circulating pneumococcal strains by the conjugate-vaccine, the remaining non-vaccine serotype strains will actually benefit from this selective immunological pressure. A phenomenon called replacement may occur, causing a shift in serotype strains circulating in the population and, consequently, in disease. Since the start of large-scale vaccination trials, replacement has been observed in subjects colonised with pneumococci as well as in patients with acute otitis media [79,96,110]. So far, the effect of this phenomenon on invasive diseases remains unclear. However, though not yet significant, the first alarming findings have been reported on partial replacement of invasive strains with non-vaccine serotypes in vivo [117]. In addition, Brueggemann et al. have shown a high invasive capacity for certain non-vaccine serotypes, which may also imply that replacement of carriage will lead to replacement of disease [99]. Thus, close monitoring of serotype distribution among invasive as well as colonisation strains remains of major importance. In Table 2, the seven studies that investigated the impact of conjugate vaccination on nasopharyngeal colonisation are summarised. In two studies, no significant effect was observed on the overall pneumococcal colonisation nor on vaccine type carriage [95,111]. In the remaining studies, a positive effect of vaccination was found on colonisation of vaccine serotype pneumococci. However, replacement of these strains with non-vaccine serotypes reduced the effect on overall pneumococcal colonisation.

Table 2. Conjugate vaccination studies investigating the effect of vaccination on colonisation rate, serotype distribution and replacement.

Investigator	Year	Country	Age (mths)	Number of children	Follow-up (mths)	Risk group	Vaccine	Vaccination Schedule†	Carriage (%)‡		Carriage (%) vaccine types¥		Replacement
									VG	CG	VG	CG	
Lakshman [111]	2000-01	UK	2	607	24-60	-	7-valent	3x CV + PV	25/43	27/41	10/30	14/32	Not relevant
Veenhoven [118]	1998-99	The Nether-lands	12-72	383	26	rAOM	7-valent	1-2x CV + PV	55	55	50	25	Yes
Edwards [119]		USA	2	260	10	-	9-valent	3x CV	41	40	48	60	Yes
O'Brien [120]	1998-99	USA	7-12	577	11	American Indians	7-valent	3x CV	63	65	24	36	Yes
Mbelle [96]	1997	South Africa	2	500	9	-	9-valent	3x CV	54	61	18	36	Yes
Dagan [97]	1996-97	Israel	12-35	262	24	DCC	9-valent	2x CV	~65	~70	13	21	Yes
Dagan [112]	(1997)	Israel	2	75	11	-	4-valent	3 x CV + PV**	44-52	52	5-12	30	No
Yeh [95]	1995	USA	2	81	13	-	7-valent*	4x CV	47	53	27	28	Not relevant
Dagan [113]	1994	Israel	12-18	263	12	DCC	7-valent	2x CV	43	57	11	25	No

*7-valent pneumococcal vaccine conjugated to outer membrane protein of *Neisseria meningitidis*
**Efficacy data do not include the effect of the polysaccharide booster
† CV, conjugate vaccine; PV, polysaccharide vaccine
‡ VG, vaccine group; CG, control group
¥ Depending on the season

NEW VACCINE STRATEGIES

New vaccine strategies focus on the use of pneumococcal surface-associated proteins. This approach has several potential advantages. First of all, the production of protein vaccines is expected to be cheaper thus within reach of developing countries. Secondly, a protein-based vaccine is expected to elicit protection in all age groups, including children under 2 years of age. Finally, in case highly conserved proteins or protein epitopes will be used as vaccine components, broad and serotype-independent protection can be expected. However, the level and type of protection will be influenced by the function of the protein(s) included in the vaccine. We will illustrate this by discussing the most promising protein vaccine candidates.

Pneumococcal surface protein A (PspA) belongs to the family of structurally related choline-binding surface proteins and is able to interfere with complement fixation by blocking recruitment of the alternative pathway through reduction of the amount of C3b, deposited on the pneumococci, thereby reducing the effectiveness of the complement receptor mediated pathways of clearance [121-124]. This is particularly important when bacterial invasion has occurred and suggests a significant role for PspA in the maintenance of invasive pneumococcal disease. Active immunisation data with PspA in animal models yields a protective effect against invasive infections and to a lesser extent against mucosal disease and nasopharyngeal carriage [125-128]. The first phase I vaccination trial with a single recombinant PspA variant in humans showed induction of cross-reactive antibodies to heterologous PspA molecules [129], which were found to protect mice challenged intraperitoneally with pneumococci [130].

Another candidate is pneumococcal surface adhesin A (PsaA), a member of the family of metal binding lipoproteins, part of an ABC transporter complex thought to be involved in the transport of manganese into pneumococci [131,132]. This protein is mainly involved in asymptomatic colonisation [46]. The first immunisation studies with PsaA have shown significant protection against colonisation but limited to modest protection against invasive infections [60,133-135]. Recently, Seo et al. have shown that oral vaccination of mice with PsaA encapsulated in microalginate microspheres elicits significant protection against colonisation, pneumonia as well as septicaemia when challenged orally [136]. These data suggest that vaccination with PsaA elicits primary protection against colonisation with secondary protection against invasive disease. However, clinical studies on the correlation between anti-PsaA antibodies and the risk of pneumococcal AOM have shown contradictory results. Rapola et al. have shown an association between a higher anti-PsaA antibody titer and lower risk of pneumococcal AOM, but only in children older then 9 months of age, whereas in children younger than 9 months of age the risk was increased with higher anti-PsaA concentration [137,138]. These data suggest a basic difference between age-groups with

respect to protectivity of anti-PsaA antibodies, and perhaps to the origin of the antibody response. A higher anti-PsaA antibody titer might be associated with increased pneumococcal contacts in the past, i.e. through colonisation as well as through infection. Consequently, this might explain the correlation with the underlying increased susceptibility to pneumococcal AOM rather than a decreased risk of infections.

Pneumolysin is a protein that also contains a choline-binding domain, and is suggested to interfere with host immunity and inflammatory responses by a variety of functions, including complement fixation and inhibition of phagocyte function. It also inhibits ciliary activity in the bronchus and is thus important in pathogenesis of pulmonary infection [139]. Knock-out mutagenesis of genes encoding pneumolysin has suggested its role in virulence, in colonisation as well as in infection [140-142]. Several investigators described the protective properties of pneumolysin against challenge with pneumococci in mice, albeit only against invasive diseases [143,144]. The combination of PspA and pneumolysin yields complementary protection to invasive diseases in animal models [60,126]. Additionally, the combination of PsaA and PspA has been shown to prevent colonisation and otitis media in animal models [126,132]. Hence, depending on the target, a different combination of vaccine components may be used. The optimal combination of proteins for vaccination remains to be determined.

Alternative routes of vaccination are also explored. Several recent studies suggest that administration of a vaccine via the oral or nasal route is as effective as systemic application [125,128,136]. In addition, a recent study by Lynch et al. has shown that intranasal administration of a conjugate vaccine plus interleukin-12 not only elicits protection against invasive disease but, in contrast to intramuscular administration, also induces protection against nasal carriage [145]. The latter effect occurs through the induction of significant mucosal IgA responses. A mucosal route of administration is highly preferable because it is less invasive and the number of vaccines already administered intramuscularly to children, as part of community vaccination programs is high. Moreover, in contrast to pneumococcal conjugate vaccines and polysaccharide vaccines, protection is also expected in children with HIV/AIDS, even during progression of disease, because of the intact mucosal immune response in these patients [136].

DISCUSSION

Nasopharyngeal colonisation provides an important key to the burden of pneumococcal disease and its prevention. Nasopharyngeal colonisation is not only obligatory for invasive disease, it also provides the basis for horizontal spread of the species.

Although the major goal of all vaccine strategies is to reduce the burden of pneumococcal disease, it implicates also the prevention of pneumococcal colonisation. Opinions about

reduction in colonisation differ from 'secondary aim' to 'fortunate side effect'. However, the importance of this essential link in pathogenesis has seldom received full attention.

The natural route of infection with *S. pneumoniae* starts with colonisation, which may progress to (invasive) disease by crossing of natural immunological barriers. Therefore, it seems rational to aim for prevention of colonisation thus eliciting protection against (invasive) diseases. Moreover, by preventing nasopharyngeal colonisation of *S. pneumoniae*, horizontal spread of pneumococcal strains may also diminish thus enhancing herd immunity [2,114,146]. This supports the use of polysaccharide-based vaccines such as the 23-valent polysaccharide vaccine and the 7-valent conjugate vaccine, or future protein-based vaccines comprising surface-exposed proteins involved in colonisation and adherence such as PsaA, CbpA and neuraminidase. An alternative to vaccination could be the use of anti-attachment agents such as receptor analogues or agents like xylitol, N-acetylcysteine or the recently found S-carboxymethylcysteine [147]. Unfortunately, none of these agents results in a complete eradication of pneumococcal colonisation. However, the same counts for vaccination: by prevention of colonisation without complete eradication of pneumococcal carriage, the immunological pressure will skew selection of non-covered serotypes or genotypes. Moreover, by clearing the nasopharyngeal niche for pneumococci, replacement with other species may occur. This is supported by findings of Veenhoven et al. who observed that pneumococcal conjugate vaccination resulted in fewer middle ear fluid cultures with vaccine serotype pneumococci, but in a 3-fold increase in *S. aureus* positive cultures [79]. Moreover, we have recently demonstrated an intra-individual competition between *S. aureus* and *S. pneumoniae* in healthy children between 4 and 9 years of age [39]. Similarly, competition between *S. pneumoniae* and species like *H. influenzae, M. catarrhalis* and *N. meningitidis* has also been shown in vitro. A possible solution for this problem might be to aim strictly for prevention of invasive disease and leave nasopharyngeal colonisation unhampered, although mucosal disease then remains unprevented. This is not possible with the currently available vaccines, but might be an option for future protein-based vaccines including disease-related proteins such as pneumococcal surface protein A, pneumolysin, the Pht family and autolysin [60,148,149]. A second option might be to consider the different levels of protective antibodies necessary for systemic and mucosal protection. It has been suggested that for mucosal protection against *S. pneumoniae* colonisation and infection, higher levels of immune-protective antibodies are needed compared to antibody levels for systemic infection [90]. Although highly speculative, one might consider adjusting the conjugate vaccines to such an extent that antibody levels are induced that are adequate enough to prevent invasive pneumococcal disease but insufficient to eradicate pneumococcal colonisation. However, such an approach would require individual monitoring and, hence, will not be achievable in case of large-scale vaccination.

In conclusion, although pneumococcal colonisation is mostly asymptomatic, it is the first step in the pathogenic route of pneumococci towards invasive disease. Moreover, it plays a crucial role in the prevention of pneumococcal infections and horizontal spread of virulent strains. Finally, a natural balance between pneumococci and co-colonising bacterial species exists, which may influence the outcome of vaccination strategies. These facts underline the key role for pneumococcal colonisation in pathogenesis and prevention of pneumococcal infections, which justifies extensive consideration in the decision-making regarding mass vaccination and future vaccine strategies.

CONFLICT OF INTEREST

The authors have no conflicts of interest with respect to the content of the paper.

REFERENCES

1. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 1997;46(RR-8):1-24.
2. Obaro S, Adegbola R. The pneumococcus: carriage, disease and conjugate vaccines. *J Med Microbiol* 2002;51(2):98-104.
3. Koedel U, Scheld WM, Pfister HW. Pathogenesis and pathophysiology of pneumococcal meningitis. *Lancet Infect Dis* 2002;2(12):721-36.
4. Paton JC, Andrew PW, Boulnois GJ, Mitchell TJ. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. *Annu Rev Microbiol* 1993;47:89-115.
5. Bruyn GA, Zegers BJ, van Furth R. Mechanisms of host defense against infection with *Streptococcus pneumoniae*. *Clin Infect Dis* 1992;14(1):251-62.
6. Gray BM, Converse GM, 3rd, Dillon HC, Jr. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* 1980;142(6):923-33.
7. Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D, Tung Y. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. *J Infect Dis* 1997;175(6):1440-5.
8. Hoge CW, Reichler MR, Dominguez EA, et al. An epidemic of pneumococcal disease in an overcrowded, inadequately ventilated jail. *N Engl J Med* 1994;331(10):643-8.
9. Kristinsson KG. Epidemiology of penicillin resistant pneumococci in Iceland. *Microb Drug Resist* 1995;1(2):121-5.
10. Munoz R, Coffey TJ, Daniels M, et al. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. *J Infect Dis* 1991;164(2):302-6.
11. Principi N, Marchisio P, Schito GC, Mannelli S. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. *Pediatr Infect Dis J* 1999;18(6):517-23.
12. de Galan BE, van Tilburg PM, Sluijter M, et al. Hospital-related outbreak of infection with multidrug-resistant *Streptococcus pneumoniae* in The Netherlands. *J Hosp Infect* 1999;42(3):185-92.
13. Mandigers CM, Diepersloot RJ, Dessens M, Mol SJ, van Klingeren B. A hospital outbreak of penicillin-resistant pneumococci in The Netherlands. *Eur Respir J* 1994;7(9):1635-9.
14. Millar MR, Brown NM, Tobin GW, Murphy PJ, Windsor AC, Speller DC. Outbreak of infection with penicillin-resistant *Streptococcus pneumoniae* in a hospital for the elderly. *J Hosp Infect* 1994;27(2):99-104.
15. Reichler MR, Rakovsky J, Slacikova M, et al. Spread of multidrug-resistant *Streptococcus pneumoniae* among hospitalized children in Slovakia. *J Infect Dis* 1996;173(2):374-9.
16. Shi ZY, Enright MC, Wilkinson P, Griffiths D, Spratt BG. Identification of three major clones of multiply antibiotic-resistant *Streptococcus pneumoniae* in Taiwanese hospitals by multilocus sequence typing. *J Clin Microbiol* 1998;36(12):3514-9.
17. Leiberman A, Dagan R, Leibovitz E, Yagupsky P, Fliss DM. The bacteriology of the nasopharynx in childhood. *Int J Pediatr Otorhinolaryngol* 1999;49 Suppl 1:S151-3.
18. Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D, Tung Y. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. *J Infect Dis* 1997;175(6):1440-5.
19. Faden H, Duffy L, Williams A, Krystofik DA, Wolf J. Epidemiology of nasopharyngeal colonization with nontypeable *Haemophilus influenzae* in the first two years of life. *Acta Otolaryngol Suppl* 1996;523:128-9.
20. Kyaw MH, Christie P, Jones IG, Campbell H. The changing epidemiology of bacterial meningitis and invasive non-

meningitic bacterial disease in scotland during the period 1983-99. *Scand J Infect Dis* 2002;34(4):289-98.
21. Nouwen JL, van Belkum A, Verbrugh HA. Determinants of *Staphylococcus aureus* nasal carriage. *Neth J Med* 2001;59(3):126-33.
22. Givon-Lavi N, Fraser D, Porat N, Dagan R. Spread of *Streptococcus pneumoniae* and antibiotic-resistant *S. pneumoniae* from day-care center attendees to their younger siblings. *J Infect Dis* 2002;186(11):1608-14.
23. Faden H, Stanievich J, Brodsky L, Bernstein J, Ogra PL. Changes in nasopharyngeal flora during otitis media of childhood. *Pediatr Infect Dis J* 1990;9(9):623-6.
24. Bogaert D, Engelen MN, Timmers-Reker AJ, et al. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. *J Clin Microbiol* 2001;39(9):3316-20.
25. El Ahmer OR, Essery SD, Saadi AT, et al. The effect of cigarette smoke on adherence of respiratory pathogens to buccal epithelial cells. *FEMS Immunol Med Microbiol* 1999;23(1):27-36.
26. Bakir M, Yagci A, Ulger N, Akbenlioglu C, Ilki A, Soyletir G. Asymtomatic carriage of *Neisseria meningitidis* and *Neisseria lactamica* in relation to *Streptococcus pneumoniae* and *Haemophilus influenzae* colonization in healthy children: apropos of 1400 children sampled. *Eur J Epidemiol* 2001;17(11):1015-8.
27. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 1997;10(3):505-20.
28. Garcia-Rodriguez JA, Fresnadillo Martinez MJ. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *J Antimicrob Chemother* 2002;50 Suppl C:59-74.
29. Ghaffar F, Friedland IR, McCracken GH, Jr. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. *Pediatr Infect Dis J* 1999;18(7):638-46.
30. Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. Nasopharyngeal colonization with nontypeable *Haemophilus influenzae* and recurrent otitis media. Tonawanda/Williamsville Pediatrics. *J Infect Dis* 1994;170(4):862-6.
31. Simell B, Korkeila M, Pursiainen H, Kilpi TM, Kayhty H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin a, pneumolysin, and pneumococcal surface protein a in children. *J Infect Dis* 2001;183(6):887-96.
32. Simell B, Kilpi TM, Kayhty H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal capsular polysaccharides in children. *J Infect Dis* 2002;186(8):1106-14.
33. Uehara Y, Kikuchi K, Nakamura T, et al. Inhibition of methicillin-resistant *Staphylococcus aureus* colonization of oral cavities in newborns by viridans group streptococci. *Clin Infect Dis* 2001;32(10):1399-407.
34. Tano K, Grahn-Hakansson E, Holm SE, Hellstrom S. Inhibition of OM pathogens by alpha-hemolytic streptococci from healthy children, children with SOM and children with rAOM. *Int J Pediatr Otorhinolaryngol* 2000;56(3):185-90.
35. Ghaffar F, Muniz LS, Katz K, et al. Effects of large dosages of amoxicillin/clavulanate or azithromycin on nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, nonpneumococcal alpha-hemolytic streptococci, and *Staphylococcus aureus* in children with acute otitis media. *Clin Infect Dis* 2002;34(10):1301-9.
36. Pericone CD, Overweg K, Hermans PW, Weiser JN. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. *Infect Immun* 2000;68(7):3990-7.
37. Mc Leod JW, Gordon J. Production of hydrogen peroxide by bacteria. *Biochemistry Journal* 1922;16:499-506.
38. Dahiya RS, Speck ML. Hydrogen peroxide formation by lactobacilli and its effect on Staphylococcus aureus. *J Dairy Sci* 1968;51(10):1068-72.
39. Bogaert D, Koppen S, Boelens H, et al. Epidemiology and determinants of nasopharyngeal carriage of bacterial pathogens in healthy Dutch children. Program and abstracts of the 21st Annual Meeting of the European Society for Paediatric Infectious Diseases, Giardini Naxos, April 9-11, 2003. ESPID 2003.
40. Regev-Yochay G, Dagan R, Raz M, et al. Is nasopharyngeal carriage of *Streptococcus pneumoniae* protective against carriage of *Staphylococcus aureus*? 43rd ICAAC 2003, Chicago, Illinois, USA. Abstract G-2048, page 302.
41. Lipsitch M, Dykes JK, Johnson SE, et al. Competition among *Streptococcus pneumoniae* for intranasal colonization in a mouse model. *Vaccine* 2000;18(25):2895-901.
42. Watson DA, Musher DM. A brief history of the pneumococcus in biomedical research. *Semin Respir Infect* 1999;14(3):198-208.
43. Magee AD, Yother J. Requirement for Capsule in Colonization by *Streptococcus pneumoniae*. *Infect Immun* 2001;69(6):3755-61.
44. Bruyn GA, van Furth R. Pneumococcal polysaccharide vaccines: indications, efficacy and recommendations. *Eur J Clin Microbiol Infect Dis* 1991;10(11):897-910.
45. Swiatlo E, Champlin FR, Holman SC, Wilson WW, Watt JM. Contribution of choline-binding proteins to cell surface properties of *Streptococcus pneumoniae*. *Infect Immun* 2002;70(1):412-5.
46. Tuomanen EI. The biology of pneumococcal infection. *Pediatr Res* 1997;42(3):253-8.
47. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature* 1995;377(6548):435-8.
48. McCullers JA, Rehg JE. Lethal synergism between influenza virus and *Streptococcus pneumoniae*: characterization of a mouse model and the role of platelet-activating factor receptor. *J Infect Dis* 2002;186(3):341-50.
49. Rosenow C, Ryan P, Weiser JN, et al. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol Microbiol* 1997;25(5):819-29.
50. Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, Hollingshead SK, Briles DE. Role of pneumococcal

surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. *Infect Immun* 2002;70(5):2526-34.
51. Tuomanen EI. Pathogenesis of pneumococcal inflammation: otitis media. *Vaccine* 2000;19 Suppl 1:S38-40.
52. Ring A, Weiser JN, Tuomanen EI. Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway. *J Clin Invest* 1998;102(2):347-60.
53. Weiser JN, Bae D, Fasching C, Scamurra RW, Ratner AJ, Janoff EN. Antibody-enhanced pneumococcal adherence requires IgA1 protease. *Proc Natl Acad Sci U S A* 2003;100(7):4215-20.
54. Hammerschmidt S, Talay SR, Brandtzaeg P, Chhatwal GS. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Mol Microbiol* 1997;25(6):1113-24.
55. Tong HH, Blue LE, James MA, DeMaria TF. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase- deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infect Immun* 2000;68(2):921-4.
56. McCullers JA, Tuomanen EI. Molecular pathogenesis of pneumococcal pneumonia. *Front Biosci* 2001;6:D877-89.
57. Weiser JN, Markiewicz Z, Tuomanen EI, Wani JH. Relationship between phase variation in colony morphology, intrastrain variation in cell wall physiology, and nasopharyngeal colonization by *Streptococcus pneumoniae*. *Infect Immun* 1996;64(6):2240-5.
58. Weiser JN, Kapoor M. Effect of intrastrain variation in the amount of capsular polysaccharide on genetic transformation of *Streptococcus pneumoniae*: implications for virulence studies of encapsulated strains. *Infect Immun* 1999;67(7):3690-2.
59. Kim JO, Weiser JN. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J Infect Dis* 1998;177(2):368-77.
60. Briles DE, Hollingshead S, Brooks-Walter A, et al. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. *Vaccine* 2000;18(16):1707-11.
61. Muhlemann K, Matter HC, Tauber MG, Bodmer T. Nationwide surveillance of nasopharyngeal *Streptococcus pneumoniae* isolates from children with respiratory infection, Switzerland, 1998-1999. *J Infect Dis* 2003;187(4):589-96.
62. Daw NC, Wilimas JA, Wang WC, et al. Nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae* in children with sickle cell disease. *Pediatrics* 1997;99(4):E7.
63. Norris CF, Mahannah SR, Smith-Whitley K, Ohene-Frempong K, McGowan KL. Pneumococcal colonization in children with sickle cell disease. *J Pediatr* 1996;129(6):821-7.
64. Coles CL, Kanungo R, Rahmathullah L, et al. Pneumococcal nasopharyngeal colonization in young South Indian infants. *Pediatr Infect Dis J* 2001;20(3):289-95.
65. Syrogiannopoulos GA, Grivea IN, Davies TA, Katopodis GD, Appelbaum PC, Beratis NG. Antimicrobial use and colonization with erythromycin-resistant *Streptococcus pneumoniae* in greece during the first 2 years of life. *Clin Infect Dis* 2000;31(4):887-93.
66. Jebaraj R, Cherian T, Raghupathy P, et al. Nasopharyngeal colonization of infants in southern India with *Streptococcus pneumoniae*. *Epidemiol Infect* 1999;123(3):383-8.
67. Syrjanen RK, Kilpi TM, Kaijalainen TH, Herva EE, Takala AK. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish children younger than 2 years old. *J Infect Dis* 2001;184(4):451-9.
68. Soewignjo S, Gessner BD, Sutanto A, et al. *Streptococcus pneumoniae* nasopharyngeal carriage prevalence, serotype distribution, and resistance patterns among children on Lombok Island, Indonesia. *Clin Infect Dis* 2001;32(7):1039-43.
69. Rusen ID, Fraser-Roberts L, Slaney L, et al. Nasopharyngeal pneumococcal colonization among Kenyan children: antibiotic resistance, strain types and associations with human immunodeficiency virus type 1 infection. *Pediatr Infect Dis J* 1997;16(7):656-62.
70. Chiou CC, Liu YC, Huang TS, et al. Extremely high prevalence of nasopharyngeal carriage of penicillin- resistant *Streptococcus pneumoniae* among children in Kaohsiung, Taiwan. *J Clin Microbiol* 1998;36(7):1933-7.
71. Polack FP, Flayhart DC, Zahurak ML, Dick JD, Willoughby RE. Colonization by Streptococcus penumoniae in human immunodeficiency virus-infected children [In Process Citation]. *Pediatr Infect Dis J* 2000;19(7):608-12.
72. Parry CM, Diep TS, Wain J, et al. Nasal carriage in Vietnamese children of *Streptococcus pneumoniae* resistant to multiple antimicrobial agents. *Antimicrob Agents Chemother* 2000;44(3):484-8.
73. Vives M, Garcia ME, Saenz P, et al. Nasopharyngeal colonization in Costa Rican children during the first year of life. *Pediatr Infect Dis J* 1997;16(9):852-8.
74. Berkovitch M, Bulkowstein M, Zhovtis D, et al. Colonization rate of bacteria in the throat of healthy infants. *Int J Pediatr Otorhinolaryngol* 2002;63(1):19-24.
75. Marchisio P, Claut L, Rognoni A, et al. Differences in nasopharyngeal bacterial flora in children with nonsevere recurrent acute otitis media and chronic otitis media with effusion: implications for management. *Pediatr Infect Dis J* 2003;22(3):262-8.
76. Marchisio P, Esposito S, Schito GC, Marchese A, Cavagna R, Principi N. Nasopharyngeal carriage of *Streptococcus pneumoniae* in healthy children: implications for the use of heptavalent pneumococcal conjugate vaccine. *Emerg Infect Dis* 2002;8(5):479-84.
77. Dejsirilert S, Overweg K, Sluijter M, et al. Nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae* among children with acute respiratory tract infections in Thailand: a molecular epidemiological survey. *J Clin Microbiol* 1999;37(6):1832-8.
78. Dagan R, Leibovitz E, Greenberg D, Yagupsky P, Fliss DM, Leiberman A. Dynamics of pneumococcal nasopharyngeal colonization during the first days of antibiotic treatment in pediatric patients. *Pediatr Infect Dis J* 1998;17(10):880-5.

79. Veenhoven R, Bogaert D, Uiterwaal C, et al. Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. *Lancet* 2003;361:2189-95.
80. Raymond J, Le Thomas I, Moulin F, Commeau A, Gendrel D, Berche P. Sequential colonization by *Streptococcus pneumoniae* of healthy children living in an orphanage. *J Infect Dis* 2000;181(6):1983-8.
81. Leibovitz E, Dragomir C, Sfartz S, et al. Nasopharyngeal carriage of multidrug-resistant *Streptococcus pneumoniae* in institutionalized HIV-infected and HIV-negative children in northeastern Romania. *Int J Infect Dis* 1999;3(4):211-5.
82. Lee HJ, Park JY, Jang SH, Kim JH, Kim EC, Choi KW. High incidence of resistance to multiple antimicrobials in clinical isolates of *Streptococcus pneumoniae* from a university hospital in Korea. *Clin Infect Dis* 1995;20(4):826-35.
83. Chiu SS, Ho PL, Chow FK, Yuen KY, Lau YL. Nasopharyngeal carriage of antimicrobial-resistant *Streptococcus pneumoniae* among young children attending 79 kindergartens and day care centers in Hong Kong. *Antimicrob Agents Chemother* 2001;45(10):2765-70.
84. Petrosillo N, Pantosti A, Bordi E, et al. Prevalence, determinants, and molecular epidemiology of *Streptococcus pneumoniae* isolates colonizing the nasopharynx of healthy children in Rome. *Eur J Clin Microbiol Infect Dis* 2002;21(3):181-8.
85. Fujimori I, Hisamatsu K, Kikushima K, Goto R, Murakami Y, Yamada T. The nasopharyngeal bacterial flora in children with otitis media with effusion [see comments]. *Eur Arch Otorhinolaryngol* 1996;253(4-5):260-3.
86. Dunais B, Pradier C, Carsenti H, et al. Influence of child care on nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae*. *Pediatr Infect Dis J* 2003;22(7):589-92.
87. Yagupsky P, Porat N, Fraser D, et al. Acquisition, carriage, and transmission of pneumococci with decreased antibiotic susceptibility in young children attending a day care facility in southern Israel. *J Infect Dis* 1998;177(4):1003-12.
88. Givon-Lavi N, Dagan R, Fraser D, Yagupsky P, Porat N. Marked differences in pneumococcal carriage and resistance patterns between day care centers located within a small area. *Clin Infect Dis* 1999;29(5):1274-80.
89. Sa-Leao R, Tomasz A, Sanches IS, et al. Carriage of internationally spread clones of *Streptococcus pneumoniae* with unusual drug resistance patterns in children attending day care centers in lisbon, portugal. *J Infect Dis* 2000;182(4):1153-60.
90. Pelton SI, Dagan R, Gaines BM, et al. Pneumococcal conjugate vaccines: proceedings from an Interactive Symposium at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy. *Vaccine* 2003;21(15):1562-71.
91. Davies T, Goering RV, Lovgren M, Talbot JA, Jacobs MR, Appelbaum PC. Molecular epidemiological survey of penicillin-resistant *Streptococcus pneumoniae* from Asia, Europe, and North America. *Diagn Microbiol Infect Dis* 1999;34(1):7-12.
92. Sibold C, Wang J, Henrichsen J, Hakenbeck R. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. *Infect Immun* 1992;60(10):4119-26.
93. Overturf GD. American Academy of Pediatrics. Committee on Infectious Diseases. Technical report: prevention of pneumococcal infections, including the use of pneumococcal conjugate and polysaccharide vaccines and antibiotic prophylaxis. *Pediatrics* 2000;106(2 Pt 1):367-76.
94. Kroon FP, van Dissel JT, Ravensbergen E, Nibbering PH, van Furth R. Antibodies against pneumococcal polysaccharides after vaccination in HIV-infected individuals: 5-year follow-up of antibody concentrations. *Vaccine* 1999;18(5-6):524-30.
95. Yeh SH, Zangwill KM, Lee H, et al. Heptavalent pneumococcal vaccine conjugated to outer membrane protein of *Neisseria meningitidis* serogroup b and nasopharyngeal carriage of *Streptococcus pneumoniae* in infants. *Vaccine* 2003;21(19-20):2627-31.
96. Mbelle N, Huebner RE, Wasas AD, Kimura A, Chang I, Klugman KP. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis* 1999;180(4):1171-6.
97. Dagan R, Givon-Lavi N, Zamir O, et al. Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J Infect Dis* 2002;185(7):927-36.
98. Lloyd-Evans N, O'Dempsey TJ, Baldeh I, et al. Nasopharyngeal carriage of pneumococci in Gambian children and in their families. *Pediatr Infect Dis J* 1996;15(10):866-71.
99. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* 2003;187(9):1424-32.
100. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* 2000;30(1):100-21.
101. Porat N, Trefler R, Dagan R. Persistence of two invasive *Streptococcus pneumoniae* clones of serotypes 1 and 5 in comparison to that of multiple clones of serotypes 6B and 23F among children in southern Israel. *J Clin Microbiol* 2001;39(5):1827-32.
102. Koskela M, Leinonen M, Haiva VM, Timonen M, Makela PH. First and second dose antibody responses to pneumococcal polysaccharide vaccine in infants. *Pediatr Infect Dis* 1986;5(1):45-50.
103. Leinonen M, Sakkinen A, Kalliokoski R, Luotonen J, Timonen M, Makela PH. Antibody response to 14-valent pneumococcal capsular polysaccharide vaccine in pre-school age children. *Pediatr Infect Dis* 1986;5(1):39-44.
104. O'Brien KL, Steinhoff MC, Edwards K, Keyserling H, Thoms ML, Madore D. Immunologic priming of young children by pneumococcal glycoprotein conjugate, but not polysaccharide, vaccines. *Pediatr Infect Dis J* 1996;15(5):425-30.

105. Peeters CC, Tenbergen-Meekes AM, Haagmans B, et al. Pneumococcal conjugate vaccines. *Immunol Lett* 1991;30(2):267-74.
106. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000;19(3):187-95.
107. Black SB, Shinefield HR, Ling S, et al. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. *Pediatr Infect Dis J* 2002;21(9):810-5.
108. Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2000;49(RR-9):1-35.
109. Fireman B, Black SB, Shinefield HR, Lee J, Lewis E, Ray P. Impact of the pneumococcal conjugate vaccine on otitis media. *Pediatr Infect Dis J* 2003;22(1):10-6.
110. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a Pneumococcal Conjugate Vaccine against Acute Otitis Media. *N Engl J Med* 2001;344(6):403-409.
111. Lakshman R, Murdoch C, Race G, Burkinshaw R, Shaw L, Finn A. Pneumococcal nasopharyngeal carriage in children following heptavalent pneumococcal conjugate vaccination in infancy. *Arch Dis Child* 2003;88(3):211-4.
112. Dagan R, Muallem M, Melamed R, Leroy O, Yagupsky P. Reduction of pneumococcal nasopharyngeal carriage in early infancy after immunization with tetravalent pneumococcal vaccines conjugated to either tetanus toxoid or diphtheria toxoid. *Pediatr Infect Dis J* 1997;16(11):1060-4.
113. Dagan R, Melamed R, Muallem M, et al. Reduction of nasopharyngeal carriage of pneumococci during the second year of life by a heptavalent conjugate pneumococcal vaccine. *J Infect Dis* 1996;174(6):1271-8.
114. Givon-Lavi N, Fraser D, Dagan R. Vaccination of day-care center attendees reduces carriage of *Streptococcus pneumoniae* among their younger siblings. *Pediatr Infect Dis J* 2003;22(6):524-32.
115. Dagan R, Givon-Lavi N, Zamir O, Fraser D. Effect of a nonavalent conjugate vaccine on carriage of antibiotic-resistant *Streptococcus pneumoniae* in day-care centers. *Pediatr Infect Dis J* 2003;22(6):532-40.
116. Hausdorff WP, Siber G, Paradiso PR. Geographical differences in invasive pneumococcal disease rates and serotype frequency in young children. *Lancet* 2001;357(9260):950-2.
117. Hsu K, Pelton D, Heisey-Grove S, Hashemi J, Klein J, Health aMotMDoP. Conjugate vaccine era serotype-specific surveillance for invasive pneumococcal disease in massachusetts children:Program and abstracts of the 21st Annual Meeting of the European Society for Paediatric Infectious Diseases, Giardini Naxos, April 9-11, 2003. 2003.
118. Veenhoven R. Impact of combined pneumococcal conjugate and polysaccharide vaccination on nasopharyngeal carriage in children with recurrent acute otitis media: Program and abstracts of the 3rd International Symposium on Pneumococci and Pneumococcal Diseases, Anchorage, May 5-8, 2002.
119. Edwards K, Wandling G, Palmer P, Decker M. Carriage of pneumococci among infants immunized with a 9-valent pneumococcal conjugate vaccine at 2, 4, and 6 months of age. *Clin Infect Dis* 1999;29:966.
120. O'Brien K, Bronsdon M, Carlone G, Facklam R, Schwartz B, Reid R. Effect of a 7-valent pneumococcal conjugate vaccine on nasopharyngeal carriage among navajo and white mountain apache infants. Proceedings of the 19th Annual Meeting of the European Society for Paediatric Infectious Diseases, Istanbul, Turkey, 26-28 March 2001. p.22 2001.
121. Yother J, Handsome GL, Briles DE. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the pspA gene. *J Bacteriol* 1992;174(2):610-8.
122. Yother J, Briles DE. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. *J Bacteriol* 1992;174(2):601-9.
123. Tu AH, Fulgham RL, McCrory MA, Briles DE, Szalai AJ. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. *Infect Immun* 1999;67(9):4720-4.
124. Neeleman C, Geelen SP, Aerts PC, et al. Resistance to both complement activation and phagocytosis in type 3 pneumococci is mediated by the binding of complement regulatory protein factor H. *Infect Immun* 1999;67(9):4517-24.
125. Wu HY, Nahm MH, Guo Y, Russell MW, Briles DE. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. *J Infect Dis* 1997;175(4):839-46.
126. Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 2000;68(5):3028-33.
127. Briles DE, King JD, Gray MA, McDaniel LS, Swiatlo E, Benton KA. PspA, a protection-eliciting pneumococcal protein: immunogenicity of isolated native PspA in mice. *Vaccine* 1996;14(9):858-67.
128. Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infect Immun* 2001;69(11):6718-24.
129. Nabors GS, Braun PA, Herrmann DJ, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine* 2000;18(17):1743-54.
130. Briles DE, Hollingshead SK, King J, et al. Immunization of Humans with Recombinant Pneumococcal Surface Protein A (rPspA) Elicits Antibodies That Passively Protect Mice from Fatal Infection with *Streptococcus pneumoniae* Bearing Heterologous PspA. *J Infect Dis* 2000;182(6):1694-1701.
131. Dintilhac A, Alloing G, Granadel C, Claverys JP. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal

permeases. *Mol Microbiol* 1997;25(4):727-39.
132. Briles DE, Ades E, Paton JC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect Immun* 2000;68(2):796-800.
133. Johnson SE, Dykes JK, Jue DL, Sampson JS, Carlone GM, Ades EW. Inhibition of pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin a. *J Infect Dis* 2002;185(4):489-96.
134. Gor DO, Ding X, Li Q, Schreiber JR, Dubinsky M, Greenspan NS. Enhanced immunogenicity of pneumococcal surface adhesin A by genetic fusion to cytokines and evaluation of protective immunity in mice. *Infect Immun* 2002;70(10):5589-95.
135. Romero-Steiner S, Pilishvili T, Sampson JS, et al. Inhibition of Pneumococcal Adherence to Human Nasopharyngeal Epithelial Cells by Anti-PsaA Antibodies. *Clin Diagn Lab Immunol* 2003;10(2):246-51.
136. Seo JY, Seong SY, Ahn BY, Kwon IC, Chung H, Jeong SY. Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin a encapsulated in microspheres. *Infect Immun* 2002;70(3):1143-9.
137. Rapola S, Kilpi T, Lahdenkari M, Takala AK, Makela PH, Kayhty H. Do antibodies to pneumococcal surface adhesin a prevent pneumococcal involvement in acute otitis media? *J Infect Dis* 2001;184(5):577-81.
138. Rapola S, Jantti V, Eerola M, Makela PH, Kayhty H, Kilpi T. Anti-PsaA and the risk of pneumococcal AOM and carriage. *Vaccine* 2003;21(25-26):3608-13.
139. Hirst RA, Sikand KS, Rutman A, Mitchell TJ, Andrew PW, O'Callaghan C. Relative roles of pneumolysin and hydrogen peroxide from *Streptococcus pneumoniae* in inhibition of ependymal ciliary beat frequency. *Infect Immun* 2000;68(3):1557-62.
140. Berry AM, Yother J, Briles DE, Hansman D, Paton JC. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. *Infect Immun* 1989;57(7):2037-42.
141. Kadioglu A, Taylor S, Iannelli F, Pozzi G, Mitchell TJ, Andrew PW. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect Immun* 2002;70(6):2886-90.
142. Wellmer A, Zysk G, Gerber J, et al. Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis. *Infect Immun* 2002;70(11):6504-8.
143. Lock RA, Paton JC, Hansman D. Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*. *Microb Pathog* 1988;5(6):461-7.
144. Paton JC, Lock RA, Hansman DJ. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. *Infect Immun* 1983;40(2):548-52.
145. Lynch JM, Briles DE, Metzger DW. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. *Infect Immun* 2003;71(8):4780-8.
146. Dagan R, Fraser D. Conjugate pneumococcal vaccine and antibiotic-resistant *Streptococcus pneumoniae*: herd immunity and reduction of otitis morbidity. *Pediatr Infect Dis J* 2000;19(5 Suppl):S79-87; discussion S88.
147. Cakan G, Turkoz M, Turan T, Ahmed K, Nagatake T. S-carboxymethylcysteine inhibits the attachment of *Streptococcus pneumoniae* to human pharyngeal epithelial cells. *Microb Pathog* 2003;34(6):261-5.
148. Adamou JE, Heinrichs JH, Erwin AL, et al. Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infect Immun* 2001;69(2):949-58.
149. Berry AM, Lock RA, Hansman D, Paton JC. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. *Infect Immun* 1989;57(8):2324-30.

Chapter 3

Epidemiology of bacterial colonization in healthy children and COPD patients

Chapter 3.1

Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study

D. Bogaert, M.N. Engelen, A.J.M. Timmers-Reker, K.P. Elzenaar, P.G.H. Peerbooms, R.A. Coutinho, R. de Groot and P.W.M. Hermans

J Clin Microbiol 2001; 39(9): 3316-20

ABSTRACT

In 1999, Engelen and coworkers investigated colonization in Amsterdam among 259 children attending 16 day-care centers (DCCs) and among 276 children who did not attend day-care centers (NDCCs). A 1.6- to 3.4-fold increased risk for nasopharyngeal colonization was observed in children attending DCCs compared with NDCC children, while no difference in antibiotic resistance was found between groups. The serotype and genotype distributions of 305 nasopharyngeal *Streptococcus pneumoniae* isolates of the latter study were investigated. The predominant serotypes in both the DCC and the NDCC groups included 19F (19 and 18%, respectively), 6B (14 and 16%, respectively), 6A (13 and 7%, respectively), 23F (9 and 7%, respectively), and 9V (7 and 7%, respectively). The theoretical vaccine coverage of the 7-valent conjugate vaccine was 59% for the DCC children and 56% for the NDCC group. Genetic analysis of the pneumococcal isolates revealed 75% clustering among pneumococci isolated from DCC attendees versus 50% among the NDCC children. The average pneumococcal cluster size in the DCC group was 3.8 and 4.6 isolates for two respective sample dates (range, 2 to 13 isolates per cluster), while the average cluster size for the NDCC group was 3.0 (range, 2 to 6 isolates per cluster). Similar to observations made in other countries, these results indicate a higher risk for horizontal spread of pneumococci in Dutch DCCs than in the general population. This study emphasizes the importance of molecular epidemiological monitoring before, during, and after implementation of pneumococcal conjugate vaccination in national vaccination programs for children.

INTRODUCTION

Streptococcus pneumoniae is worldwide one of the major causes of severe infections such as meningitis, septicemia, and respiratory tract infections. In addition, *S. pneumoniae* is, together with *Moraxella catarrhalis* and *Haemophilus influenzae*, a dominant pathogen in middle ear infections and sinusitis. Risk groups for pneumococcal infections are young children under the age of 2 years, elderly people, and immunocompromised patients (1).

Pneumococci are often part of the nasopharyngeal flora; probably all humans are colonized with this organism at least once early in life. The risk of pneumococcal colonization is high, especially under conditions with crowding, such as day-care centers (DCCs), nursing homes, hospitals, and jails (16, 22, 23). A strong relation between carriage and middle ear infections has been found, but the association between colonization and invasive disease has not been confirmed (11, 31).

The emergence of penicillin- and multidrug-resistant pneumococci has been observed in various countries over the last decade. In some countries and populations, up to 60% of the pneumococcal isolates are resistant to one or more antibiotics (3, 12, 17). A significant proportion of pneumococcal resistance is the result of the worldwide spread of a limited number of multidrug-resistant clones (4, 14, 30, 35). Carriage of organisms with decreased antibiotic susceptibility is associated with young age, female sex, winter season, and exposure to antimicrobial drugs during the previous month (37).

Children attending DCCs have several risk factors for carriage, i.e., young age, crowding, and frequent usage of antimicrobial agents. Furthermore, it is believed that DCCs may be a global reservoir for multidrug-resistant pneumococci (27). Therefore, prevention of carriage and infection with *S. pneumoniae* in these risk groups will become an important tool in the battle against (antibiotic-resistant) *S. pneumoniae*.

Prevention of infections caused by *S. pneumoniae* and of spread of this pathogen is an important goal of an effective vaccine. Therefore, new vaccines have been developed that are also immunogenic in risk groups, such as young children, the elderly, and immunocompromised patients. Results with these conjugate vaccines, containing polysaccharides from up to 11 different serotypes conjugated to a protein carrier (tetanus-diphtheria toxoid-Hib protein or meningococcal outer membrane protein), are promising (6, 9, 21, 28, 29). Recently, one of these vaccines, the 7-valent pneumococcal conjugate vaccine from Wyett Lederle, has been approved by the Department of Health and Human Services in the United States and the European Agency for the Evaluation of Medicine for Europe.

The introduction of the conjugate vaccines underscores the need for detailed and long-term epidemiological surveillance of *S. pneumoniae* in the target groups in order to calculate the theoretical vaccine coverage and to evaluate the (long-term) effects of large-scale introduction of this vaccine in the general population by using serological and molecular techniques. So far, no data are available on the molecular epidemiology of *S. pneumoniae* carriage in young children in The Netherlands.

In 1999, a study was performed in Amsterdam, The Netherlands, among 259 children attending 16 DCCs and 276 children who did not attend day-care centers (NDCC). We investigated nasopharyngeal carriage rates and susceptibility to antibiotics of the nasopharyngeal flora. Carriage rates for *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae* of 37, 48, and 11%, respectively, were observed in the NDCC group. Increased risks of 1.6, 1.7, and 3.4 for carriage of *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae*, respectively, were observed among DCC attendees. Finally, DCC attendees were ill more frequently and used more antibiotics than the controls. Similar to earlier surveillance data from The Netherlands (20), only 2% of the pneumococcal isolates showed reduced susceptibility to erythromycin and no penicillin resistance was found (P. Peerbooms, M. Engelen, A. van Belkum, and R. Coutinho, 11th Eur. Cong. Clin. Microbiol. Infect. Dis., abstr. 8, 2001). These results are in contrast to data from previous DCC studies in other countries, where antibiotic resistance among pneumococcal isolates was high, associated with previous antibiotic consumption, and correlated to increased spread of drug-resistant pneumococci among DCC attendees (2, 5, 24, 33, 37). Because drug resistance among pneumococci is negligible in our study group, we hypothesized that crowding, which is also a risk factor for nasopharyngeal carriage, is playing an important role in facilitating the transmission of bacteria among children in DCCs. To obtain insight in the transmission of pneumococci in children, the molecular epidemiology of the pneumococcal isolates collected from both the general population and DCCs was investigated by serotyping and genotyping.

MATERIALS AND METHODS

Bacterial sampling. *S. pneumoniae* strains were isolated from the nasopharynges of 259 children, aged 3 to 36 months, attending 16 DCCs in Amsterdam, The Netherlands, from January to March 1999. All children were sampled twice, with a time interval of 4 weeks. In the same period, an additional 276 children from three well-baby clinics in Amsterdam, aged 3 to 36 months, who did not attend DCCs were evaluated for *S. pneumoniae* carriage (P. Peerbooms et al., 11th Eur. Cong. Clin. Microbiol. Infect. Dis., 2001). Nasopharyngeal

samples were obtained with a dacron pernasal swab (Medical Wire & Equipment Co., Wiltshire, England). The swabs were transported in Amies transport medium to the Microbiology Laboratory of the Municipal Health Service (Amsterdam, The Netherlands), immediately plated on 5% sheep blood agar plates, and grown overnight at 36°C in a CO2-enriched atmosphere. *S. pneumoniae* isolates were identified according to standard microbiological procedures (18). Molecular analyses were performed on all the pneumococcal isolates that were available for use, i.e., 115 and 129 strains collected from the 16 DCCs on the two occasions, respectively, and 61 strains collected from the NDCC children.

Serotyping. Pneumococci were serotyped by the capsular quellung method (Quellung reaction) and observed microscopically using commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

RFEL. Typing of the 305 pneumococcal strains by restriction fragment end labeling (RFEL) analysis was performed as described by Van Steenbergen et al. (36) and as adapted by Hermans et al. (15). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for variable lengths of time at room temperature to ECL Hyperfilms (Amersham, Little Chalfont, United Kingdom).

***Computer-assisted analysis of RFEL banding patterns*.** The RFEL types were analyzed using the Windows version of the Gelcompar software (version 4; Applied Maths, Kortrijk, Belgium) after imaging the RFEL autoradiograms using the Image Master DTS (Pharmacia Biotech, Uppsala, Sweden). For this purpose, DNA fragments in the molecular weight range of 160 to 400 bp were explored. The DNA banding patterns were normalized using pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the banding patterns was performed by unweighted pair group method using arithmetic averages (25) and the Jaccard similarity coefficient applied to peaks (32). Computer-assisted analysis and methods and algorithms used in this study were carried out according to the instructions of the manufacturer of Gelcompar. A tolerance of 1.2% in band position was applied during comparison of the DNA patterns. For evaluation of the genetic relatedness of the isolates, we used the following definitions: score of 1, strains of the particular RFEL type are considered 100% identical by RFEL analysis; score of 2, the RFEL cluster represents a group of RFEL types that differs in only one band (>95% genetic

relatedness); score of 3, the RFEL lineage represents a group of RFEL types that differs in less than four bands (>85% genetic relatedness).

The genotypes of the pneumococcal isolates were also compared with an international collection of pneumococcal strains representing about 320 distinct RFEL types originating from 17 different countries in America, Europe, Africa, and Asia (M. Sluijter, unpublished observations), including the 16 international clones described by the Pneumococcal Epidemiology Network (http://www.wits.ac.za/pmen/pmen.htm).

Statistical analysis. For statistical analysis of the results, the Fisher exact test was used.

Table 1. Contribution of vaccine serotypes to carriage of *S. pneumoniae* in Dutch infants

Serotype	% of children with the serotype	
	DCC [a]	NDCC
19F	19	18
6B	14	16
9V	7	7
23F	9	7
14	7	5
18	1	3
4	0	0
6A	13	7
19A	4	2
23A+23B	4	5
7-valent conjugate vaccine (4, 6B, 9V, 14, 18C, 19F, and 23F)	59	57
7-valent conjugate vaccine (including 6A)	72	64
11-valent conjugate vaccine (7-valent + 1, 3, 5 and 7F)	62	62
11-valent conjugate vaccine (including 6A)	75	70

[a] Average percentages were calculated from the two sampling dates.

RESULTS

We investigated the serotypes of 244 DCC isolates and 61 NDCC isolates (Table 1). The predominant serotypes in both the DCC and NDCC groups were the serotypes 19F (19 and 18%, respectively), 6B (14 and 16%, respectively), 6A (13 and 7%, respectively), 23F (9 and 7%, respectively), 9V (7 and 7%, respectively), and 14 (7 and 5%, respectively). The

serotypes 19F, 6B, 23F, 9V, and 14 are covered by the 7-valent pneumococcal conjugate vaccine. The other two serotypes covered by the 7-valent conjugate vaccine are serotypes 4 and 18C. No children were colonized with vaccine type 4, and only 1 to 3% of the children were colonized with vaccine serotype 18. The theoretical vaccine coverages of the 7-valent conjugate vaccine are 59% for the DCC group and 57% for the NDCC group. The theoretical vaccine coverage of the 11-valent conjugate vaccine, in which the additional capsular types 1, 3, 5, and 7F are included, is 62% for both the DCC and NDCC groups.

Genotyping of the 305 pneumococcal isolates from this study was performed by RFEL analysis. The 61 isolates from the NDCC children showed 50 different genotypes; 50% of the strains represented 10 distinct genetic clusters. Cluster sizes in the NDCC group ranged from two to six strains per cluster with an average cluster size of 3.0. The DCC group displayed 66 and 75 different genotypes at the first and second sampling dates, respectively. Seventy-five percent of the strains from both the first and the second sampling dates represented 24 and 20 clusters, respectively. This percentage differed significantly from the 50% genetic clustering observed in the NDCC group ($P < 0.01$). Cluster sizes within the individual sampling data ranged from 2 to 13 strains, with an average cluster size of 3.8 and 4.6 for the two sampling dates, respectively (Fig. 1). The majority of the clusters in both the DCC group (29 of 44 clusters) and the NDCC group (8 of 10 clusters) displayed a serotype covered by the 7-valent conjugate vaccine, including the cross-protective serotype 6A (Fig. 1). We also investigated carriage turnover in the DCC group, defined as the percentage of children with positive pneumococcal samples at both sampling dates that changed genotypes within the 4-week interval between sampling dates. A total of 209 of 259 children in the DCC group were carrying a pneumococcus on the nasopharynx at least once. Of the 259 children, 107 were carrying pneumococci on the nasopharynx at both sampling dates. The isolates from 69 children who were identified as being colonized by *S. pneumoniae* at both sampling dates were available for molecular analysis. Fourty-four of these 69 children had changed genotypes between the two sampling dates, i.e., a carriage turnover of 64%.

Comparison of the RFEL data with the 16 international clones described by the Pneumococcal Epidemiology Network demonstrated that six isolates (10%) of the NDCC group were identical to the clones Taiwan19F-14 (1 isolate), France9V-3 (3 isolates), Slovakia14-10 (1 isolate), and Tennessee23F-4 (1 isolate), whereas 25 of the isolates from the DCC group (10%) belonged to the clones France9V-3 (15 isolates), Slovakia14-10 (6 isolates), and Tennessee23F-4 (4 isolates). All but one of the isolates were susceptible to the antibiotics penicillin, tetracycline, erythromycin, and cotrimoxazole, in contrast to the

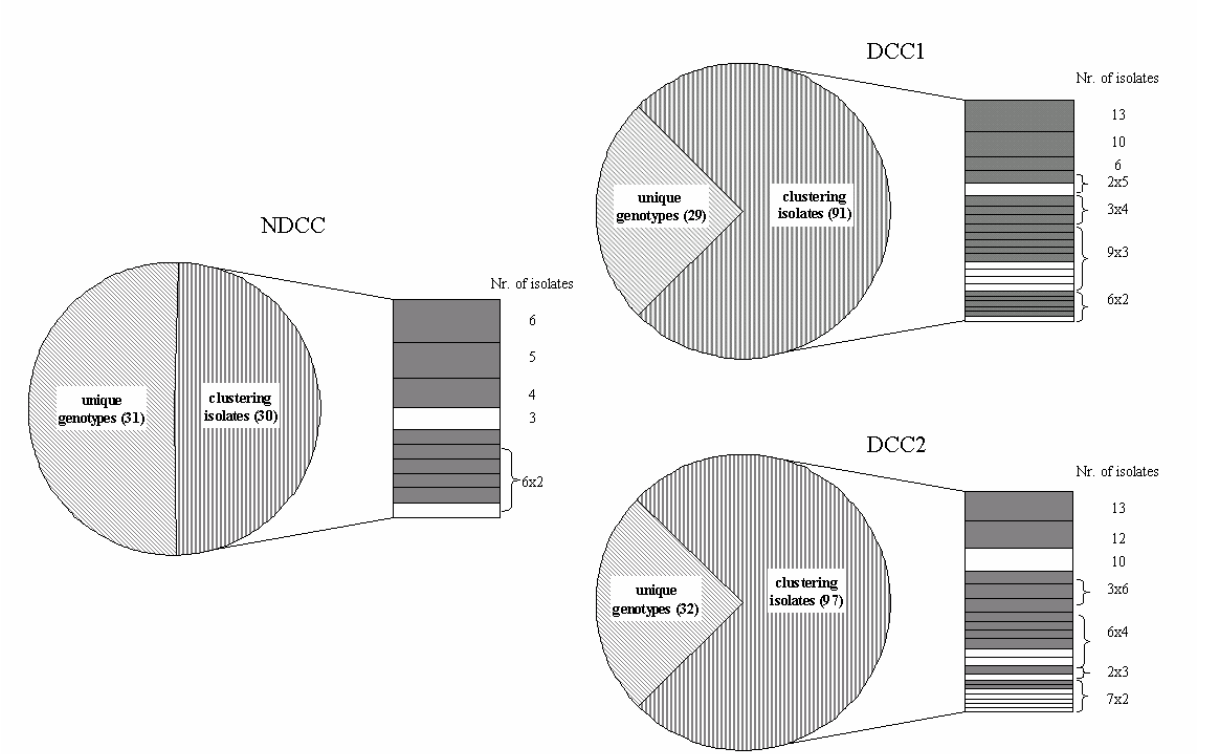


Figure 1. Number and distribution of pneumococcal isolates with unique genotypes or genetically clustered isolates from both the NDCC children and the DCC attendees. The clustered isolates are further grouped into separate clusters. Cluster sizes are depicted on the right; clusters representing conjugate-vaccine serotypes are depicted in gray, and nonvaccine serotypes are depicted in white. DCC1 and DCC2 refer to the two different sampling dates.

antibiotic resistance patterns of their genetically homologous clones. Only the isolate identical to clone Taiwan19F-14 was resistant to erythromycin (Peerbooms et al., 11th Eur. Cong. Clin. Microbiol. Infect. Dis., 2001).

DISCUSSION

In The Netherlands, the prevalence of pneumococcal colonization in the pediatric population was found to be 58% in DCC attendees versus 37% in the NDCC group, i.e., a 1.6-fold higher risk of pneumococcal colonization in DCC attendees compared to age-matched NDCC children (Peerbooms et al., 11th Eur. Cong. Clin. Microbiol. Infect. Dis., 2001). Known risk factors for carriage of *S. pneumoniae* are young age, crowding, and antibiotic usage. Since only 2% of the pneumococcal isolates were resistant to erythromycin and no resistance to the antibiotics penicillin, cotrimoxazole, or tetracycline was found, crowding is presumed to be the most important contributor to the difference in carriage seen in this study. We hypothesize that crowding facilitates horizontal transfer of bacteria from one child to another. Therefore, we investigated the molecular epidemiology of 305 pneumococcal isolates from this study. Both the DCC group and the NDCC group showed a serotype distribution comparable to what is found in many other countries, such as Israel, Finland, Canada, and South Africa (6, 10, 16, 19, 34). The most predominant serotypes were 19F (19 and 18% in DCC and NDCC attendees, respectively), 6B (14 and 16%, respectively), 6A (13 and 7%, respectively), 23F (9 and 7%, respectively), and 9V (7 and 7%, respectively). This serotype distribution implicates a theoretical coverage of 57 to 59% by the 7-valent conjugate vaccine. The additive value of an 11-valent conjugate vaccine is only 3 to 5% (62% total coverage for both study groups). An additive cross-protective effect is expected at least for serotype 6A (26), which increases the theoretical coverage of the 7-valent conjugate vaccine to 64% for the NDCC group and to 72% for the DCC group. It is unknown whether a cross-protective effect can be expected for the serotypes 19A, 23A, and 23B (13, 26, 38). With respect to the theoretical vaccine coverage, the long-term effect of large-scale implementation of the conjugate vaccine for the Dutch pediatric population remains unknown. Eskola et al. have found a similar serotype distribution before vaccination with the 7-valent pneumococcal conjugate vaccine and a shift towards nonvaccine serotypes causing middle ear infection after vaccination (10). Such a shift in distribution after conjugate vaccination was also observed among nasopharyngeal carriage isolates (7, 8, 19; S. K.

Obaro, R. A. Adegbola, W. A. Banya, and B. M. Greenwood, Letter, Lancet 348:271-272, 1996). Therefore, it is concluded that a shift in distribution towards nonvaccine serotypes will reduce the efficacy of conjugate vaccination with respect to carriage and disease.

In the DCC group, 75% of the pneumococci represented genetic clusters, in contrast to 50% in the NDCC group. These molecular epidemiological data suggest augmented spread of pneumococci among DCC attendees compared to the NDCC group. In addition, the average cluster size for the first and second sampling dates in the DCC group were 3.8 and 4.6, respectively, with a range of 2 to 13 isolates per cluster, compared to 3.0 for the control group with a range of 2 to 6 isolates per cluster (Fig. 1). These data show larger clusters in the DCC group, which supports the hypothesis that pneumococci are spread more frequently by horizontal transfer between DCC attendees than among NDCC attendees. A carriage turnover of 64% in the DCC children with two positive pneumococcal isolates at both sampling dates was observed. At present, no carriage turnover data are available for the NDCC population. Whether the genotype shift in the DCC population is due to recolonization of the nasopharyngeal niche by new genotypes or whether it is due to unmasking of genotypes which were already present but not detected as a result of the abundant presence of other genotypes needs to be further investigated.

The RFEL patterns of both the DCC group and the NDCC group were compared with the 16 international (multidrug-resistant) clones described by the Pneumococcal Epidemiology Network. Twenty-five isolates (10%) in the DCC group were homologous to 3 of the reference clones (100% identical), whereas 6 isolates (10%) in the NDCC group matched with 4 of these clones. In contrast to the multidrug-resistant reference clones, all isolates but one were fully susceptible to penicillin, erythromycin, and cotrimoxazole. The resistant isolate, identical to the clone Taiwan19F-14, had reduced susceptibility to erythromycin only, whereas the reference clone was resistant to erythromycin, penicillin, and tetracycline. These results suggest that these Dutch isolates represent members of the ancestor lineages of the resistant reference clones. The overall absence of resistant pneumococcal strains in The Netherlands may be explained by the restricted use of antibiotics in general compared to that in many other countries.

In conclusion, an increased frequency of horizontal spread of *S. pneumoniae* strains was shown in DCCs. At least 56% of the nasopharyngeal pneumococcal isolates would be theoretically covered by a 7-valent conjugate vaccine. Furthermore, the majority of the horizontal spreading genotypes (70 to 80%) express capsular types that are covered by the conjugate vaccine. These data indicate that implementation of the pneumococcal conjugate vaccine in the near future in Dutch infants, and especially in risk groups like DCC attendees,

should be considered. Importantly, to investigate the long-term efficacy of the vaccine against pneumococcal infections, detailed molecular epidemiological monitoring of pneumococcal colonization and infection is required.

ACKNOWLEDGMENTS

We thank M. Sluijter for technical support. This study was supported by the Sophia Foundation for Medical Research (grant 268) Rotterdam, The Netherlands, and NOW (grant SGO-Inf.005), The Hague, The Netherlands.

REFERENCES

1. Anonymous. 1997. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). Morb. Mortal. Wkly. Rep. 46(RR-8):1-24.
2. Arnold, K. E., R. J. Leggiadro, R. F. Breiman, H. B. Lipman, B. Schwartz, M. A. Appleton, K. O. Cleveland, H. C. Szeto, B. C. Hill, F. C. Tenover, J. A. Elliott, and R. R. Facklam. 1996. Risk factors for carriage of drug-resistant *Streptococcus pneumoniae* among children in Memphis, Tennessee. J. Pediatr. 128:757-764.
3. Baquero, F., J. A. Garcia-Rodriguez, J. Garcia de Lomas, and L. Aguilar. 1999. Antimicrobial resistance of 1,113 *Streptococcus pneumoniae* isolates from patients with respiratory tract infections in Spain: results of a 1-year (1996-1997) multicenter surveillance study. The Spanish Surveillance Group for Respiratory Pathogens. Antimicrob. Agents Chemother. 43:357-359.
4. Corso, A., E. P. Severina, V. F. Petruk, Y. R. Mauriz, and A. Tomasz. 1998. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. Microb. Drug Resist. 4:325-337.
5. Dagan, R., E. Leibovitz, D. Greenberg, P. Yagupsky, D. M. Fliss, and A. Leiberman. 1998. Dynamics of pneumococcal nasopharyngeal colonization during the first days of antibiotic treatment in pediatric patients. Pediatr. Infect. Dis. J. 17:880-885.
6. Dagan, R., R. Melamed, M. Muallem, L. Piglansky, D. Greenberg, O. Abramson, P. M. Mendelman, N. Bohidar, and P. Yagupsky. 1996. Reduction of nasopharyngeal carriage of pneumococci during the second year of life by a heptavalent conjugate pneumococcal vaccine. J. Infect. Dis. 174:1271-1278.
7. Dagan, R., R. Melamed, O. Zamir, and O. Leroy. 1997. Safety and immunogenicity of tetravalent pneumococcal vaccines containing 6B, 14, 19F and 23F polysaccharides conjugated to either tetanus toxoid or diphtheria toxoid in young infants and their boosterability by native polysaccharide antigens. Pediatr. Infect. Dis. J. 16:1053-1059.
8. Dagan, R., M. Muallem, R. Melamed, O. Leroy, and P. Yagupsky. 1997. Reduction of pneumococcal nasopharyngeal carriage in early infancy after immunization with tetravalent pneumococcal vaccines conjugated to either tetanus toxoid or diphtheria toxoid. Pediatr. Infect. Dis. J. 16:1060-1064.
9. Eskola, J. 2000. Immunogenicity of pneumococcal conjugate vaccines. Pediatr. Infect. Dis. J. 19:388-393.
10. Eskola, J., T. Kilpi, A. Palmu, J. Jokinen, J. Haapakoski, E. Herva, A. Takala, H. Kayhty, P. Karma, R. Kohberger, G. Siber, P. H. Makela, S. Lockhart, and M. Eerola. 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N. Engl. J. Med. 344:403-409.
11. Faden, H., L. Duffy, R. Wasielewski, J. Wolf, D. Krystofik, and Y. Tung. 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J. Infect. Dis. 175:1440-1445.
12. Fairchok, M. P., W. S. Ashton, and G. W. Fischer. 1996. Carriage of penicillin-resistant pneumococci in a military population in Washington, D.C.: risk factors and correlation with clinical isolates. Clin. Infect. Dis. 22:966-972.
13. Giebink, G. S., J. D. Meier, M. K. Quartey, C. L. Liebeler, and C. T. Le. 1996. Immunogenicity and efficacy of *Streptococcus pneumoniae* polysaccharide-protein conjugate vaccines against homologous and heterologous serotypes in the chinchilla otitis media model. J. Infect. Dis. 173:119-127.
14. Hermans, P. W., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. Goessens, and R. de Groot. 1997. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. Microb. Drug Resist. 3:243-251.
15. Hermans, P. W., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 33:1606-1612.
16. Kellner, J. D., and E. L. Ford-Jones. 1999. *Streptococcus pneumoniae* carriage in children attending 59 Canadian child care centers. Toronto Child Care Centre Study Group. Arch. Pediatr. Adolesc. Med. 153:495-502.

17. Lee, H. J., J. Y. Park, S. H. Jang, J. H. Kim, E. C. Kim, and K. W. Choi. 1995. High incidence of resistance to multiple antimicrobials in clinical isolates of *Streptococcus pneumoniae* from a university hospital in Korea. Clin. Infect. Dis. 20:826-835.
18. Lenette, E. H., A. Balows, W. J. Hausler, Jr., and H. J. Shadomy. 1985. Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
19. Mbelle, N., R. E. Huebner, A. D. Wasas, A. Kimura, I. Chang, and K. P. Klugman. 1999. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. J. Infect. Dis. 180:1171-1176.
20. Neelingde, A. J. D. 2001. Resistentie tegen antibiotica bij pneumokokken in Nederland en Europa. National Institute for Public Health and the Environment, Bilthoven, The Netherlands.
21. Nieminen, T., H. Kayhty, O. Leroy, and J. Eskola. 1999. Pneumococcal conjugate vaccination in toddlers: mucosal antibody response measured as circulating antibody-secreting cells and as salivary antibodies. Pediatr. Infect. Dis. J. 18:764-772.
22. Nuorti, J. P., J. C. Butler, J. M. Crutcher, R. Guevara, D. Welch, P. Holder, and J. A. Elliott. 1998. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. N. Engl. J. Med. 338:1861-1868.
23. Principi, N., P. Marchisio, G. C. Schito, and S. Mannelli. 1999. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. Pediatr. Infect. Dis. J. 18:517-523.
24. Reichler, M. R., A. A. Allphin, R. F. Breiman, J. R. Schreiber, J. E. Arnold, L. K. McDougal, R. R. Facklam, B. Boxerbaum, D. May, R. O. Walton, et al. 1992. The spread of multiply resistant *Streptococcus pneumoniae* at a day care center in Ohio. J. Infect. Dis. 166:1346-1353.
25. Romesburg, H. 1990. Cluster analysis for researchers, p. 9-28. Krieger, Malabar, Fla.
26. Saeland, E., H. Jakobsen, G. Ingolfsdottir, S. T. Sigurdardottir, and I. Jonsdottir. 2001. Serum samples from infants vaccinated with a pneumococcal conjugate vaccine, PncT, protect mice against invasive infection caused by *Streptococcus pneumoniae* serotypes 6A and 6B. J. Infect. Dis. 183:253-260.
27. Sa-Leao, R., A. Tomasz, I. S. Sanches, A. Brito-Avo, S. E. Vilhelmsson, K. G. Kristinsson, and H. de Lencastre. 2000. Carriage of internationally spread clones of *Streptococcus pneumoniae* with unusual drug resistance patterns in children attending day care centers in Lisbon, Portugal. J. Infect. Dis. 182:1153-1160.
28. Shinefield, H. R., and S. Black. 2000. Efficacy of pneumococcal conjugate vaccines in large scale field trials. Pediatr. Infect. Dis. J. 19:394-397.
29. Shinefield, H. R., S. Black, P. Ray, I. Chang, N. Lewis, B. Fireman, J. Hackell, P. R. Paradiso, G. Siber, R. Kohberger, D. V. Madore, F. J. Malinowski, A. Kimura, C. Le, I. Landaw, J. Aguilar, and J. Hansen. 1999. Safety and immunogenicity of heptavalent pneumococcal CRM197 conjugate vaccine in infants and toddlers. Pediatr. Infect. Dis. J. 18:757-763.
30. Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck. 1992. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. Infect. Immun. 60:4119-4126.
31. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. Goessens, A. van Belkum, and P. W. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. J. Clin. Microbiol. 36:2248-2253.
32. Sneath, P. 1973. Numerical taxonomy. Freeman, San Francisco, Calif.
33. Syrogiannopoulos, G. A., I. N. Grivea, N. G. Beratis, A. E. Spiliopoulou, E. L. Fasola, S. Bajaksouzian, P. C. Appelbaum, and M. R. Jacobs. 1997. Resistance patterns of *Streptococcus pneumoniae* from carriers attending day-care centers in southwestern Greece. Clin. Infect. Dis. 25:188-194.
34. Takala, A. K., J. Vuopio-Varkila, E. Tarkka, M. Leinonen, and J. M. Musser. 1996. Subtyping of common pediatric pneumococcal serotypes from invasive disease and pharyngeal carriage in Finland. J. Infect. Dis. 173:128-135.
35. Tomasz, A., A. Corso, E. P. Severina, G. Echaniz-Aviles, M. C. Brandileone, T. Camou, E. Castaneda, O. Figueroa, A. Rossi, and J. L. Di Fabio. 1998. Molecular epidemiologic characterization of penicillin-resistant *Streptococcus pneumoniae* invasive pediatric isolates recovered in six Latin-American countries: an overview. PAHO/Rockefeller University Workshop. Pan American Health Organization. Microb. Drug Resist. 4:195-207.
36. van Steenbergen, T. J., S. D. Colloms, P. W. Hermans, J. de Graaff, and R. H. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc. Natl. Acad. Sci. USA 92:5572-5576.
37. Yagupsky, P., N. Porat, D. Fraser, F. Prajgrod, M. Merires, L. McGee, K. P. Klugman, and R. Dagan. 1998. Acquisition, carriage, and transmission of pneumococci with decreased antibiotic susceptibility in young children attending a day care facility in southern Israel. J. Infect. Dis. 177:1003-1012.
38. Yu, X., B. Gray, S. Chang, J. I. Ward, K. M. Edwards, and M. H. Nahm. 1999. Immunity to cross-reactive serotypes induced by pneumococcal conjugate vaccines in infants. J. Infect. Dis. 180:1569-1576.

Chapter 3.2

Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children

D. Bogaert, A. van Belkum, M. Sluijter, A. Luijendijk, R. de Groot, H.C. Rümke, H.A. Verbrugh, and P.W.M. Hermans

Lancet; in press

ABSTRACT

A trial with a 7-valent pneumococcal-conjugate vaccine in children with recurrent acute otitis media showed a shift in pneumococcal colonisation towards non-vaccine serotypes and an increase in *Staphylococcus aureus*-related acute otitis media after vaccination.

We investigated prevalence and determinants of nasopharyngeal carriage of *S. pneumoniae* and *S. aureus* in 3198 healthy children aged 1-19 years.

Nasopharyngeal carriage of *S. pneumoniae* was detected in 598 children (19%), and was affected by age (peak incidence at 3 years) and day-care attendance (odds ratio [OR] 2.14, 95% CI: 1.44-3.18). *S. aureus* carriage was affected by age (peak incidence at 10 years) and male sex (OR 1.46, 95% CI: 1.25-1.70), family size (≥ 5 members, OR: 1.17, 95% CI: 1.00-1.37) and passive smoking (OR: 1.22, 95% CI: 1.04-1.42), whereas active smoking was inversely related to *S. aureus* carriage (OR: 0.75, 95% CI: 0.53-1.04). Serotyping showed 42% vaccine type and 58% non-vaccine type pneumococci. We noted a negative correlation for co-colonisation of *S. aureus* and vaccine-type pneumococci (OR 0.68, 95% CI: 0.48-0.94), but not for *S. aureus* and non-vaccine serotypes.

These findings suggest the presence of a natural competition between vaccine-type pneumococci and *S. aureus*, which might explain the increase in *S. aureus*-related otitis media after vaccination.

INTRODUCTION

Streptococcus pneumoniae causes morbidity and mortality in young children, elderly people, and immunodeficient patients [1], but asymptomatic carriage of pneumococci is also common, especially in children. Children are thought to be an important vector for community-wide spread of this micro-organism, and prevention of pneumococcal carriage may therefore contribute to reduction in the prevalence of infections [2].

Pneumococcal conjugate vaccination protects young children against invasive diseases with *S. pneumoniae* [3,4], but does not adequately prevent mucosal infections and colonisation, and, moreover, replacement with non-vaccine serotypes occurs [5]. A study of pneumococcal vaccination in children with a history of recurrent acute otitis media showed an increased incidence of *Staphylococcus aureus*-related acute otitis media after vaccination [5]. We postulated that the natural balance of microbial species in the nasopharynx might be altered by vaccination. To study age-related dynamics of pneumococcal and *S. aureus* carriage, we measured pneumococcal and staphylococcal colonisation in 3198 healthy children in The Netherlands.

MATERIALS AND METHODS

We enrolled 3198 children with no previous pneumococcal vaccination, aged between 12 months and 19 years, who were participating in a national meningococcal vaccination campaign [6]. We obtained signed informed consent from the parent, child or both, and recorded demographic data with a standardised questionnaire. The study was approved by the Medical Ethics Review Board of the Erasmus MC.

A team of 10 specifically trained research nurses and medical doctors obtained one nasopharyngeal swab per child. The flexible swab (Copan Italia, Brescia, Italy) was inserted into the anterior nares, gently rubbed on the posterior nasopharyngeal wall, removed, and stored in Amies transport medium at room temperature. Swabs were transported to the medical microbiology laboratory and plated within 6 hours of sampling, first on a gentamicin blood agar plate, then submerged in phenyl mannitol broth, to isolate *S. pneumoniae* and *S. aureus*, respectively. We identified bacteria by use of standard methods [7], and serotyped pneumococci by the capsular swelling method (Quellung reaction) with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark), according to the instructions of the manufacturer.

We analysed data by SPSS version 11.0. We did a univariate regression analysis on the variables age, family size (0: < 5 persons, 1: ≥ 5 persons), passive and active smoking, antibiotic use within the last 7 days, and proxy measures of social contact (at least 3 hours per week of sport activities, youth club, sports club, discotheque visits; at least 3 days per

week day-care attendance). Colonisation with *S. aureus* or *S. pneumoniae* were dependent variables. We considered all variables with univariate associations as potential determinants of colonisation, and confirmed independence by multivariate logistic regression analysis by use of the stepwise backward Wald method for variables with a p-value of < 0.10. To assess co-colonisation we cross-tabulated *S. aureus* and *S. pneumoniae* carriage, and *S. aureus* and *S. pneumoniae* vaccine and non-vaccine serotype carriage. We did bivariate logistic regression analysis with *S. aureus* as dependent variable and *S. pneumoniae* vaccine serotype and non-vaccine serotype carriage as covariates. Because carriage of vaccine serotype pneumococci was negatively associated with *S. aureus* carriage with p<0.01, we did multivariate logistic regression analysis adjusting for the independent determinants of colonisation. We estimated reliability of the final analysis with the Hosmer & Lemeshow test [8]. 103 children had missing values, so we assessed data for 3085 out of 3198 children (97.4%).

RESULTS AND DISCUSSION

The 3198 children included in our study were a balanced representation of all age groups (Figure). 1670 (52%) were male, and the mean family size was 4.3 members with a mean of 2.4 children per family. 45% of the households contained at least one smoker, and 275 children above 12 years of age (21%) were active smokers. 1317 (42%) of all children above 4 years of age practised a sporting activity, 909 (29%) attended a youth society or sports club, and 311(21%) of all children older than 10 years attended discotheques for more then 3 hours per week. 262 children younger than 4 years (56%) were at a day-care centre for at least 3 days per week.

We cultured 3198 swabs for *S. pneumoniae;* 3097 swabs were also cultured for *S. aureus*. We isolated *S. pneumoniae* from 598 children (19%) with a peak incidence during the first three years of life, followed by a gradual decline until reaching a stable colonisation rate after the age of 10 years (Figure). 250 of the pneumococcal isolates (41.6%) expressed capsular serotypes included in the 7-valent conjugate vaccine. The serotypes most frequently found were 6B (69, 11.5%), 19F (68, 11.3%), 23F (53, 8.8%), 6A (40, 6.7%), 3 (36, 6.0%) and 11 (31, 5.2%). The conjugate vaccine serotypes showed a peak incidence at age 1 year followed by a steady decline till the age of 8 years. The non-vaccine type pneumococci showed initially a slight increase with a peak incidence of 27% at the age of 4 years, followed by a decrease in incidence (Figure). *S. aureus* was isolated from 1117 children (36%) showing an age-related parabolic distribution with a peak incidence at age 10 years (Figure).

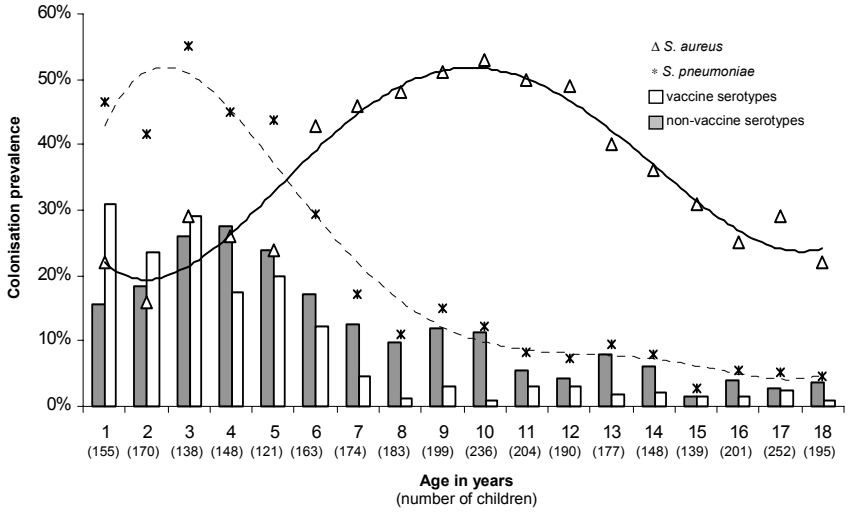


Figure 1. Age-related prevalence of *S. aureus, S. pneumoniae* and vaccine type (VT) and non-vaccine type (non-VT) pneumococci. Trend lines are shown [polynomial function with 5 degrees (quintic function)].

Age, passive smoking, active smoking, 3 days or more per week day-care attendance, family-size, 3 hours or more per week of sports, youth club visits, or discotheque attendance correlated with pneumococcal carriage. Age and day-care (odds ratio [OR] 2.14, 95% CI 1.44-3.18) were found to be independent determinants of pneumococcal carriage by multivariate logistic regression analysis. The variables found to correlate with *S. aureus* colonisation by means of univariate analysis were age, number of children in the family, family-size, passive smoking, active smoking, sports, youth club and discotheque visits. Multivariate logistic regression identified age, male sex (OR 1.46, 95% CI 1.25-1.70), family-size (OR 1.17, 95% CI 1.00-1.42), active smoking (OR 0.75, 95% CI 0.53-1.04) and passive smoking (OR 1.2, 95% CI 1.04-1.42) as independent determinants of *S. aureus* colonisation. Other variables that have previously been shown to be determinants of pneumococcal carriage [9], such as passive smoking at home, large households, and crowding among teenagers, did not correlate with pneumococcal carriage in our study.

187 (6%) children had cultures positive for *S. aureus* and *S. pneumoniae*. We noted a negative correlation for co-colonisation of vaccine type pneumococci and *S. aureus* (23.2% co-colonisation), but not for non-vaccine serotypes (37.0%) compared with cultures negative for pneumococci (37.2%) (Table 1). This was supported by bivariate logistic regression analysis, which showed an odds ratio of 0.51 (95% CI 0.38-0.70) for co-colonisation of

Table 1. Cross-tabulation for colonisation with *S. pneumoniae* and *S. aureus*

		S. pneumoniae colonisation			
		No	Non-vaccine serotypes	Vaccine serotypes	Total
S. aureus	No	1570	219	191	1980
Colonisation	Yes	930	129	58	1117
	Total	2500	348	249	3097

Chi-square: 19.63, df: 2, p-value: < 0.001

Table 2. Odds Ratios of colonisation with *Staphylococcus aureus* for co-colonisation with *Streptococcus pneumoniae* and other independent risk factors (n = 3085)

	Odds Ratio	95% CI	p-value
Age (years)	1.45	1.34-1.54	< 0.0001
Age*Age[‡]	0.98	0.98-0.99	<0.0001
Gender (male versus female)	1.46	1.25-1.70	<0.0001
Family size (<5 versus ≥5 members)	1.17	1.00-1.37	0.049
Passive smoking (yes versus no)	1.22	1.04-1.42	0.013
Active smoking (yes versus no)	0.75	0.53-1.04	0.083
Pneumococcal vaccine serotypes (present versus absent)	0.68	0.48-0.94	0.020
Pneumococcal non-vaccine serotypes (present versus absent)	1.04	0.81-1.34	0.741

[‡] Non-linear effect modification suggested by significance of the square of age

vaccine serotype pneumococci and *S. aureus.* When adjusting for age, sex, family-size, and active and passive smoking in a multivariate logistic regression model, this difference was still significant for vaccine-type pneumococci but not for non-vaccine serotypes (Table 2). Because a non-linear effect modification of age was suggested by the significant correlation of *S. aureus* and the square of age, we did a subsidiary analysis for children younger than five years (data not shown). Again, we noted a negative correlation for co-colonisation of vaccine-type pneumococci and *S. aureus* (OR 0.61, 95% CI 0.38-0.98), and no correlation with non-vaccine serotypes. For the remaining determinants, the subsidiary analysis was too small to show significance.

More research is needed to clarify the molecular basis of these serotype-related interactions between *S. pneumoniae* and *S. aureus*, and to ascertain the potential effect of pneumococcal conjugate vaccination on *S. aureus* carriage and *S. aureus*-related disease.

ACKNOWLEDGMENTS

We are grateful to Onno de Zwart (Department of Infectious Diseases, Municipal Health Services, Rotterdam, The Netherlands) for facilitating our study. Furthermore, we thank Rianne Oostenbrink (ErasmusMC, Rotterdam, The Netherlands) for statistical advise. In addition, we thank the medical students, PhD students and laboratory personnel of Erasmus MC Rotterdam who contributed to the study. Finally, we are grateful to all children and their parents who participated in this study.

REFERENCES

1. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 1997;46(RR-8):1-24.
2. Leiberman A, Dagan R, Leibovitz E, Yagupsky P, Fliss DM. The bacteriology of the nasopharynx in childhood. *Int J Pediatr Otorhinolaryngol* 1999;49 Suppl 1:S151-3.
3. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000;19(3):187-95.
4. Whitney CG, Farley MM, Hadler J, et al. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* 2003;348(18):1737-46.
5. Veenhoven R, Bogaert D, Uiterwaal C, et al. Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. *Lancet* 2003;361:2189-95.
6. de Greeff SC, de Melker HE, Spanjaard L, van den Hof S, Dankert J. The first effect of the national vaccination campaign against meningococcal-C disease: a rapid and sharp decrease in the number of patients. *Ned Tijdschr Geneeskd* 2003;147(23):1132-5.
7. Lenette E, Balows A, Hauser Jr. W, Shadomy H. Manual of Clinical Microbiology. Washington, 1985.
8. Hosmer DW, Lemeshow S e. Applied logistic regression. New York: John Wiley & Sons. Inc, 1989.
9. Ghaffar F, Friedland IR, McCracken GH, Jr. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. *Pediatr Infect Dis J* 1999;18(7):638-46.

Chapter 3.3

Epidemiology of nasopharyngeal carriage of *Neisseria meningitidis* in healthy Dutch children

D. Bogaert, S. Koppen, H. Boelens, M. Sluijter, A. Luijendijk, H.C. Rümke, P.W.M. Hermans, A. van Belkum, R. de Groot and H.A. Verbrugh

Submitted

ABSTRACT

Background. In Western countries *Neisseria meningitidis* is the most important cause of sepsis and meningitis in children. An increase in meningococcal sepsis and meningitis caused by group C meningococci in The Netherlands prompted a nation-wide campaign in the summer of 2002 to vaccinate all children between 12 months and 19 years of age against group C meningococcal disease.

Methods. We investigated the prevalence and determinants of nasopharyngeal carriage of *N. meningitidis* in healthy children participating in the meningococcal vaccination campaign in Rotterdam, The Netherlands. A nasopharyngeal swab and a short questionnaire were obtained from 3,198 children between 12 months and 19 years of age.

Results. We observed meningococcal carriage in 46 children (1.5%). Subtyping of the meningococcal strains showed 8 group B isolates, 9 group C isolates, 24 type xyzw 135 isolates and 8 others. Peak incidences were seen in the first year of life (3.2% carriage) and after the age of 15 years (3.7% carriage). Independent determinants of *N. meningitidis* carriage included age, pneumococcal carriage (OR 4.1) and regular visits of youth clubs and dancings (OR 2.2 and 4.3, respectively).

Conclusions. Nasopharyngeal carriage of *N. meningitidis* was low during the national meningococcal vaccination campaign in the summer of 2002 in The Netherlands. Age, crowding and pneumococcal carriage showed to be determinants of meningococcal colonization in healthy Dutch children.

INTRODUCTION

Asymptomatic nasopharyngeal colonization with bacterial pathogens is common in children and in adults, and may predispose to the development of infections (1, 2). Furthermore, the upper respiratory tract is considered to be the primary reservoir from which community-wide spread of these microorganisms occurs (3-5). Detailed information about asymptomatic carriage of pathogenic bacteria in the population can provide insight into the nature of infections caused by these bacteria, which may ultimately help to prevent or control outbreaks. One of the pathogens of major interest is *Neisseria meningitidis.* This pathogen is an important cause of invasive diseases including meningitis and septicemia (6). *N. meningitidis* resides in the posterior nasopharynx. Morbidity and mortality is high despite the availability of antibiotic treatment. Known determinants for *N. meningitidis* colonization are winter season, large households, day-care visits, visits to discotheques, youth clubs and sport clubs and *Streptococcus pneumoniae* carriage (7-9). Unfortunately, most studies investigating nasopharyngeal carriage have limitations due to restricted age- and patient groups, small numbers of participants and the range of pathogens investigated.

Because of a nation-wide increase in invasive meningococcal disease caused by group C meningococci in the first months of 2002 (10), all Dutch children between 12 months and 19 years of age were offered immunization during a national vaccination campaign in the summer of 2002. The vaccination coverage nationwide was 80% (Municipal Health Services, personal communication). This opportunity was exploited to screen over 3,000 healthy children in Rotterdam for nasopharyngeal carriage of *N. meningitidis, S. pneumoniae* and *Staphylococcus aureus* and to obtain information regarding age distribution, and social and environmental factors related to nasopharyngeal carriage of the three pathogens. Prevalences and determinants of *S. pneumoniae* and *S. aureus* colonization have been described previously (11). In the present paper, we will focus on the prevalence and determinants of *N. meningitidis* carriage in this Dutch cohort of healthy children.

MATERIALS AND METHODS

Study population. In total, 3198 children between 12 months and 19 years of age visiting the national meningococcal vaccination campaign in Rotterdam were enrolled in this study. All children were residents of Rotterdam who were vaccinated in July (age 12 months to 5 years and 15 to 19 years) and September (age 6 to 15 years) of the year 2002. Signed informed consent was obtained from the parent accompanying the participating child under the age of 16 years and directly from the child above the age of 16 years.

Study design. When children and their parents agreed to participate in this study, demographic data were recorded for each child through a standardized questionnaire that was completed by the parent or the child under the supervision of an instructed interviewer. Questions addressed month and year of birth, zip code, family size, frequent sport activities (at least 3 hours per week), crowding (at least 3 hours per week attendance of youth and sport clubs, discotheques and at least 3 times per week day-care visits), active and passive smoking and recent antibiotic use (within last 7 days). Finally, a nasopharyngeal swab was obtained by a skilled research nurse or medical doctor. The study was approved by the Medical Ethics Review Board of the Erasmus MC, Rotterdam, The Netherlands.

Cultures. The nasopharyngeal samples were obtained with rayon tipped dacron pernasal swabs (Copan Italia, Brescia, Italy) and transported in Amies transport medium to the medical microbiology laboratory of the Erasmus MC, samples were plated within 6 hours of sampling on Thayer-Martin medium for isolation of *N. meningitidis*. Bacteriological determination was performed according to standard procedures (12). In case morphologically different colonies were observed within one sample, multiple colonies were stored for further analysis.

Data analysis. Statistical analysis was performed by SPSS version 11.0 for Windows. To evaluate determinants of colonization, we performed univariate regression analysis on the variables age, family size (0: < 5 persons, 1: ≥ 5 persons), a minimum of 3 hours per week sport activities, a minimum of 3 hours per week attendance of youth clubs, a minimum of 3 hours per week attendance of sport clubs, a minimum of 3 hours per week discotheque visits, a minimum of 3 days per week day-care visits, passive and active smoking, recent antibiotic use (within the last 7 days) and co-colonization with *S. pneumoniae* and *S. aureus*. Colonization with *N. meningitidis* was used as dependent variable. The variables were coded 1 when present and 0 when absent. All variables with univariate associations (p-value < 0.10), were considered potential determinants of colonization. To identify independent markers, the variables were further analyzed by multivariate logistic regression analysis using the stepwise backward Wald method (including the variables with a p-value of <0.10). As cases with missing values were rejected from multivariate analyses, the analysis was based on the data of 2,850 out of 3,198 children (89.2%) participating in the study.

RESULTS

In total, 3,198 children participated in the study. We retrieved questionnaires and nasopharyngeal swabs of all 3,198 children, whereas 3,098 of the swabs were cultured for *N. meningitidis*. Full sets of data and clinical materials were obtained for 2850 children.

Demography. The 3,198 children formed a balanced representation of all age groups varying in size from 121 to 199 children per year of age. Fifty-two percent of all children were male. The average family size was 4.3 persons with on average 2.4 children per family. In 45% of the households at least one person smoked regularly. Twenty-one percent of all children above 12 years of age were active smokers. Overall, 42% of all children above 4 years of age were actively involved in physical sports (≥ 3 hours per week), 29% attended a youth society or sports club for 3 hours or more per week and 21% of all children above 10 years of age visited discotheques for more than 3 hours per week. Of all children under 4 years of age, 56% visited a day-care center for at least 3 days a week.

Bacterial carriage and susceptibility. Nasopharyngeal carriage of *N. meningitidis* was observed in 46 out of 3098 children (1.5%). Eight and nine children carried meningococci group B and group C, respectively. Twenty-four children were carriers of serotype xyzw 135 meningococci. Eight children were carrier of other groups of meningococci. Two children were carriers of two types of meningococci; one child had a group B strain and a nontypeable strain and the second child had a group B and group C meningococcal strain. Meningococci were most frequently isolated during the second year of life with a peak incidence of 3.2%. Thereafter, the incidence dropped to 0% after the fifth year of life with a second peak incidence after the age of 14 years (Figure 1). Sixteen out of the seventeen group B and group C meningococcal strains were isolated in children older than 14 years of age. The remaining group C meningococcal isolate was retrieved from a one year old child.

Determinants of meningococcal carriage. To evaluate potential determinants of nasopharyngeal carriage of *N. meningitidis,* we calculated the Odds Ratios by means of univariate logistic regression followed by multivariate logistic regression analysis. The initial correlation of active smoking and *N. meningitidis* carriage showed to be an indirect effect of age in the multivariate logistic regression model and was excluded from the model. Finally, we identified age, age*age, pneumococcal carriage (OR 4.1, 95% CI: 1.87-9.01), youth club visits for more than 3 hours a week (OR 2.3, 95% CI: 0.99-4.63) and discotheque visits for more than 3 hours a week (OR 4.4, 95% CI: 1.54-12.17) as significant determinants for *N. meningitidis* carriage (Table 1). Carriage of both vaccine serotype pneumococci (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) and non-vaccine serotypes significantly correlated with meningococcal carriage with odds ratios of 3.5 (95% CI: 1.19-10.40) and 4.4 (95% CI: 1.90-10.27), respectively.

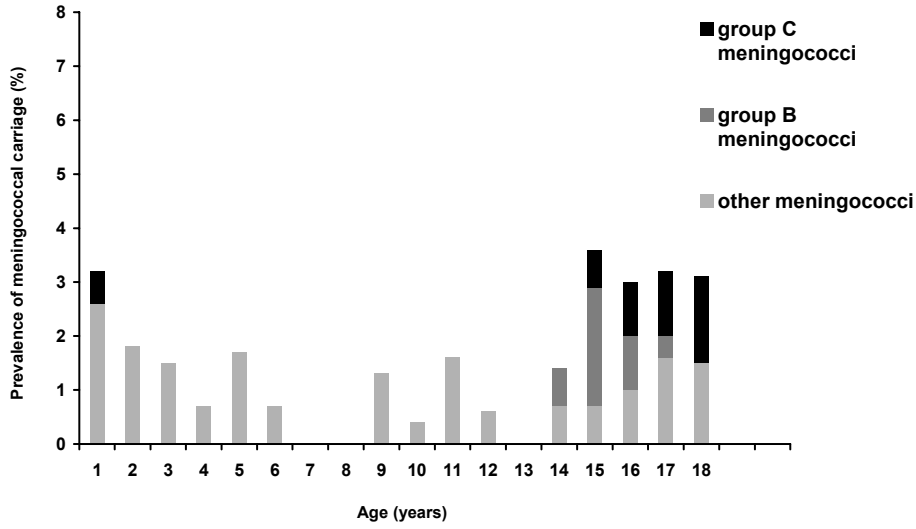


Figure 1. Age-related prevalence of nasopharyngeal colonization with *N. meningitidis.*

Table 1. Odds Ratios for the independent risk factors of colonization with *Neisseria meningitidis* (n = 2850)

	Odds Ratio	95% CI	p-value
Age (years)	0.69	0.53-0.91	0.008
Age*Age	1.02	1.01-1.03	0.007‡
Youth club visits (>3 hours/week versus < 3 hours/week)	2.32	0.99-4.63	0.054
Discotheque visits (>3 hours/week versus < 3 hours/week)	4.36	1.54-12.17	0.005
Pneumococcal vaccine serotype carriage (present versus absent)	3.51	1.19-10.40	0.023
Pneumococcal non-vaccine serotype carriage (present versus absent)	4.42	1.90-10.27	0.001

‡ Non-linear effect modification suggested by the significance of the square of age

DISCUSSION

In 2002, the Netherlands Reference Laboratory for Bacterial Meningitis noticed an increase in invasive diseases caused by meningococci group C (10). Therefore, a large-scale vaccination campaign was undertaken to prevent group C meningococcal disease in all

children up to the age of 19 years. This opportunity was used to evaluate asymptomatic colonization with *N. meningitidis* in this large open cohort of healthy children.

Surprisingly, the incidence of *N. meningitidis* colonization was low, ranging between 0 and 4% depending on the age. In the light of the recent increase in meningococcal disease, a higher incidence was expected, comparable to previously described incidences of up to 26% (8, 9). One might argue that this is caused by differences in the method of sampling, i.e. nasopharyngeal instead of oropharyngeal cultures. At present, there is still uncertainty about the most optimal method of meningococcal sampling (7, 13-16). Furthermore, high colonization rates of more than 20% have mostly been reported in certain risk groups including students and military recruits. These risk groups were not included in our study. However, our highest incidence in group B and group C meningococcal carriage was also observed in the children older then 15 years of age, which is in line with these findings (15, 17). More importantly, the time of sampling, i.e. the summer, might explain the relatively low incidence in carriage. Most often, a peak in meningococcal colonization and infection is observed in autumn and winter when the crowding factor increases. For example, Neal et al. have shown a significant increase in pharyngeal colonization among university students during the first week of term starting with 6.9% on day 1 to 23.1% on day 4 (18). The seasonal variation also counts for invasive meningococcal diseases in Dutch children, where the highest incidence is found from January until April (10). With respect to the outbreak of meningococcal disease, also a rapid decline in meningococcal disease was observed after April of the year 2002. Also in line with the findings of the Netherlands Reference Laboratory for Bacterial Meningitis is the age-related distribution of colonization and infection. Both follow a similar pattern with peak incidences of colonization and infection during the second year of life, followed by a decrease with the lowest incidence at the age of 7 to 8 years, after which a small peak in incidence at the age of 11 years is observed and a major increase after the age of 14 years (according to the statistics of the Netherlands Reference Laboratory for Bacterial Meningitis: incidence of serogroup C meningococcal disease 2001).

We investigated determinants of meningococcal colonization. After multivariate analysis we found colonization with pneumococci, age and crowding in case of teenagers (frequent youth club and discotheque visits) to correlate positively with meningococcal colonization. Although these determinants were described previously, other parameters such as smoking, large households and elementary school attendance showed no significant correlation with meningococcal carriage in our study. However, in contrast to our study these studies performed univariate regression. Our results are in agreement with Dominguez et al. who found the same determinants of meningococcal carriage after multivariate logistic regression analysis (16).

We also investigated co-colonization of *S. pneumoniae* and *S. aureus* as potential determinants of meningococcal colonization. Although we did not find a correlation between *S. aureus* and meningococcal carriage, we found a positive correlation between *S. pneumoniae* and *N. meningitidis* colonization. This finding is in accordance with a previous clinical study performed by Bakir et al. (7). Moreover, these data are supported by findings of Pericone et al. who have shown an enhanced growth of *S. pneumoniae* in co-culture with *N. meningitidis* mediated by catalase produced by the meningococcus. (19). In a previous analysis of nasopharyngeal carriage with *S. pneumoniae* and *S. aureus* in the same cohort of children, we observed a negative correlation between *S. aureus* and *S. pneumoniae* carriage, however, for vaccine serotype pneumococci only (11). Therefore, we re-analyzed the data for meningococcal and conjugate vaccine serotype and non-vaccine serotype carriage. A negative correlation was found with both groups of pneumococci, indicating that this correlation is independent from the pneumococcal serotype.

Our data suggest a complex pattern of interactions between the different species colonizing the nasopharynx. Whether this interaction affects vaccination against one of these species is still unknown. For mass vaccination with the meningococcal C conjugate vaccine the first efficacy data are available. These data show a reduction in group C meningococcal disease of 66% after 12 months and no significant rise in other meningococcal serogroups yet (20). A possible effect on colonization with other species still has to be investigated.

In conclusion, we performed a large meningococcal colonization study among children between 1 and 19 years of age at the time of the National meningococcal group C vaccination campaign in The Netherlands in the summer of 2002. We observed a relatively low incidence of meningococcal carriage with peak incidences in the first year of life and after the age of 15 years. Determinants of meningococcal carriage were age, crowding, especially in teenagers, and pneumococcal carriage.

ACKNOWLEDGMENTS

We are grateful to Onno de Zwart, MPH, head of the Department of Infectious Diseases of the Municipal Health Services for facilitating this study. Furthermore, we thank Wim Hop for statistical advise. We thank Baxter BV, The Netherlands, for sponsoring gifts for the children. We are also thankful to the medical students, PhD students and laboratory personnel of the Erasmus MC in Rotterdam, The Netherlands, for their assistance. Finally, we thank all children and their parents who participated in this study.

REFERENCES

1. Riordan T, Cartwright K, Andrews N, et al. Acquisition and carriage of meningococci in marine commando recruits. Epidemiol Infect 1998;121(3):495-505.
2. Sharon N, Ofek I. Safe as mother's milk: carbohydrates as future anti-adhesion drugs for bacterial diseases. Glycoconj J 2000;17(7-9):659-64.
3. Faden H, Stanievich J, Brodsky L, et al. Changes in nasopharyngeal flora during otitis media of childhood. Pediatr Infect Dis J 1990;9(9):623-6.
4. Bogaert D, Engelen MN, Timmers-Reker AJ, et al. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J Clin Microbiol 2001;39(9):3316-20.
5. van Deuren M, Brandtzaeg P, van der Meer JW. Update on meningococcal disease with emphasis on pathogenesis and clinical management. Clin Microbiol Rev 2000;13(1):144-66, table of contents.
6. Kyaw MH, Christie P, Jones IG, Campbell H. The changing epidemiology of bacterial meningitis and invasive non-meningitic bacterial disease in scotland during the period 1983-99. Scand J Infect Dis 2002;34(4):289-98.
7. Bakir M, Yagci A, Ulger N, et al. Asymtomatic carriage of *Neisseria meningitidis* and *Neisseria lactamica* in relation to *Streptococcus pneumoniae* and Haemophilus influenzae colonization in healthy children: apropos of 1400 children sampled. Eur J Epidemiol 2001;17(11):1015-8.
8. Conyn-van Spaendonck MA, Reintjes R, Spanjaard L, et al. Meningococcal carriage in relation to an outbreak of invasive disease due to Neisseria meningitidis serogroup C in the Netherlands. J Infect 1999;39(1):42-8.
9. Reintjes R, Conyn-van Spaendonck MA. Carriage of meningococci in contacts of patients with meningococcal disease. Age and other risk factors need to be taken into account. Bmj 1999;318(7184):665-6.
10. de Greeff SC, de Melker HE, Spanjaard L, et al. [The first effect of the national vaccination campaign against meningococcal-C disease: a rapid and sharp decrease in the number of patients]. Ned Tijdschr Geneeskd 2003;147(23):1132-5.
11. Bogaert D, van Belkum A, Sluijter M, et al. Natural competition between *Streptococcus pneumoniae* and *Staphylococcus aureus* during colonisation in healthy children. Lancet: accepted 2003.
12. Lenette E, Balows A, Hauser Jr. W, Shadomy H. Manual of Clinical Microbiology. Washington; 1985.
13. Cartwright KA, Stuart JM, Jones DM, Noah ND. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect 1987;99(3):591-601.
14. Sim RJ, Harrison MM, Moxon ER, Tang CM. Underestimation of meningococci in tonsillar tissue by nasopharyngeal swabbing. Lancet 2000;356(9242):1653-4.
15. Jordens JZ, Williams JN, Jones GR, Heckels JE. Detection of meningococcal carriage by culture and PCR of throat swabs and mouth gargles. J Clin Microbiol 2002;40(1):75-9.
16. Dominguez A, Cardenosa N, Izquierdo C, et al. Prevalence of *Neisseria meningitidis* carriers in the school population of Catalonia, Spain. Epidemiol Infect 2001;127(3):425-33.
17. Block C, Gdalevich M, Buber R, et al. Factors associated with pharyngeal carriage of *Neisseria meningitidis* among Israel Defense Force personnel at the end of their compulsory service. Epidemiol Infect 1999;122(1):51-7.
18. Neal KR, Nguyen-Van-Tam JS, Jeffrey N, Slack RC, et al. Changing carriage rate of Neisseria meningitidis among university students during the first week of term: cross sectional study. Bmj 2000;320(7238):846-9.
19. Pericone CD, Overweg K, Hermans PW, Weiser JN. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect Immun 2000;68(7):3990-7.
20. Maiden MC, Stuart JM. Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination. Lancet 2002;359(9320):1829-31.

Chapter 3.4

Bacterial colonization, inhaled corticosteroids and risk of exacerbations in COPD in the COPE study

P. van der Valk, D. Bogaert , E. Monninkhof, J. van der Palen, P.W.M. Hermans, G. Zielhuis, C. van Herwaarden and R. Hendrix

Submitted

ABSTRACT

The aim of this study was to investigate the effect of bacterial colonization during stable disease on time to first exacerbation in patients who either discontinued or continued fluticasone propionate (FP).

In a randomized controlled trial, 244 COPD patients either continued using FP or received placebo for 6 months, after 4 months of treatment with FP. Sputum samples were collected at randomization and during exacerbations. We calculated the time to development of an exacerbation in colonized compared to non-colonized patients in relation to ICS use.

At randomization, we collected sputum samples of 209 patients, of which 36% were colonized with potentially pathogenic microorganisms. We did not find effect modification by FP status (FP or placebo), but effect modification by FEV_1 was present. The adjusted hazard ratio of a first exacerbation in colonized compared to non-colonized patients was 1.29 (95% CI 0.76-2.20) and 1.01(95% CI 0.56; 1.81) in patients with a $FEV_1 \geq 50\%$, and $FEV_1 < 50\%$, respectively.

Bacterial colonization in moderately severe COPD patients is not an independent risk factor for the development of acute exacerbations. There is no interaction between colonization and discontinuation of ICS on time to first exacerbation.

INTRODUCTION

The clinical course of chronic obstructive pulmonary disease (COPD) is one of gradual progressive impairment, which despite optimal pharmacological treatment may lead to substantial functional limitations and eventually to respiratory failure (1,2). Morbidity and mortality among patients with COPD are for the most part related to acute exacerbations of COPD (3,4) which occur on average one to three times a year (5). The clinical effects of inhaled corticosteroids (ICS) on exacerbations in COPD observed in recent studies were a more rapid onset and higher recurrence-risk of exacerbations after discontinuation of ICS (6), and a decreased frequency (7) or severity (8) of exacerbations. ICS affect airway inflammation in COPD, which may explain the decrease in exacerbations (9). However, many issues related to exacerbations remain unclear or controversial including the role of microorganisms in the airways of the COPD patient. (10-12). A significant proportion of patients with COPD have lower airway bacterial colonization with a spectrum of pathogens including *non- typeable Haemophilus influenzae, Streptococcus pneumoniae, and Moraxella catarrhalis* (13,14). Simple bronchial colonization or overgrowth of potentially pathogenic microorganisms (PPM) may promote bronchial inflammation (15) hence promoting bronchial injury and consequently, by higher sputum levels of inflammatory mediators, exacerbations (16,17). Recent longitudinal studies compared for the first time the role of bacterial colonization COPD exacerbations and in stable state (18-20). These have clearly demonstrated that especially the acquisition of a new bacterial strain promoted the development of a bacteriological proven exacerbation (19). Since ICS affect airway inflammation in COPD, discontinuation of these corticosteroids in colonized patients might have profound effects on the acquisition of new bacterial strains and so promoting exacerbations.

The aim of this single center prospective study was to investigate the effect of bacterial colonization during stable disease on time to first exacerbation in patients who either discontinued or continued the inhaled steroid fluticasone propionate (FP) in a randomized double blind fashion.

PATIENTS AND METHODS

Patients. From May 1999 through March 2000, patients were recruited from the outpatient pulmonary clinic of the Medisch Spectrum Twente: an 1150 bed teaching hospital, in Enschede, The Netherlands. To be eligible for the study the patients had to meet the following criteria: 1) a clinical diagnosis of stable COPD, as defined by American Thoracic Society criteria (21); 2) no history of asthma; 3) no exacerbation in the month prior to enrollment; 4) current or former smoker; 5) age between 40-75 years; 6) baseline pre-

bronchodilator FEV_1 25- 80% of predicted; 7) pre-bronchodilator ratio FEV_1 inspiratory vital capacity(IVC) value of 60% or less; 8) reversibility of FEV_1 post inhalation of 80 µg of ipratropium bromide via metered dose inhalator with Aerochamber 12% of predicted value or less (22); 9) Total Lung Capacity (TLC) greater than the TLC predicted minus 1.64*SD; 10) no maintenance treatment of oral steroids or antibiotics; 11) no medical condition with low survival or serious psychiatric morbidity (e.g. cardiac insufficiency, alcoholism); 12) absence of any other active lung disease (e.g. sarcoidosis); and 13) use of medication such as nasal corticosteroids, theophyllines, chronic use of acetylcysteine and all other bronchodilators was allowed.

The hospital's medical ethical committee approved this study. All patients provided written informed consent.

Trial design. This study was designed as a randomized, double blind, parallel-group single center study that comprised of 4 months run-in, followed by 6 months of active treatment or placebo (6). In the run-in phase all patients were prescribed the inhaled corticosteroid, fluticasone propionate (FP) via Diskus/Accuhaler 500 µg twice daily, and bronchodilators to optimize lung function. After 4 months patients were randomly assigned to continue FP 500 µg twice daily or to receive placebo for 6 months. Randomization was performed in blocks of 6 by computer-generated allocation. Sputum samples were collected at randomization at 4 month and at exacerbation. Only patients producing sputum at randomization were included in this analysis. Follow-up visits were scheduled only when the patient was in a stable condition at 4, 7 and 10 months post baseline. At the regular follow-up visits data were collected, spirometry was performed post bronchodilation, and sputum was collected for analysis. At any time patients experienced any worsening of their respiratory symptoms they were instructed to contact the study personnel by telephone. They were subsequently invited to attend the outpatients department within 12 hours for spirometry measurements, sputum collection and consultation by one of the study physicians**.**

Exacerbations were defined as worsening of respiratory symptoms that required treatment with a short course of oral corticosteroids or antibiotics as judged by the study physician. The type of an exacerbation was expressed by the Anthonisen scores type I, II, III (23). An exacerbation was scored Type 0 as the exacerbation was treated with a short course of oral corticosteroids or antibiotics, but did not fulfill the Anthonisen scores. A short course of oral corticosteroids was defined as 30 mg prednisolone for a period of 10 days. First choice of antibiotics was amoxicillin/clavulanic acid 625 mg four times daily for a period of 10 days. Second choice was doxycycline 100 mg daily for a period of 10 days. In order to differentiate between an ongoing and a new or recurrent exacerbations all patients' clinical and pharmacy records were collected and analyzed. In order to avoid the impact of treatment

with antibiotics on lower airway colonization, we only analyzed the time to the first exacerbation of COPD.

Sputum Samples. The laboratory technicians processing the sputum samples were unaware of the clinical condition of the patients. Spontaneously expectorated sputa were collected in sterile vials and processed in the laboratory within 4 hours. Sputa were homogenized by incubation at 37^{o} C for 15 minutes with an equal volume of 0,1 percent dithiothreitol. Appropriate dilutions of homogenized sputum samples were placed on blood, chocolate, CLED and saboroud agar plates. Bacterial and mycological isolation and identification was performed with the use of standard techniques (24). *Streptococcus pneumoniae*, *Haemophilus influenzae* or *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Aspergillus and Candida species* and other gram-negative rods were considered to be potentially pathogenic microorganisms (PPM). Other bacterial species were classified as normal flora.

Bacterial colonization was defined as the demonstration of bacteria in cultures of lower respiratory tract sample in a clinically stable COPD patient without symptoms attributable to infection (16). A bacterial infection was defined by the abundant presence of one or more potentially pathogenic microorganisms in relation to the normal microbiological flora in sputum in the presence of more than 10^5 per ml.

Statistical Analysis. We calculated that 192 patients (96 per treatment group) were required to detect a hazard ratio of a first exacerbation of 1.50 (colonized compared to non colonized) with 80% power and a two-sided 0.05 α-level test (25). Baseline characteristics are reported as means $\pm$ SD or as percentages stratified by colonization at baseline yes or no. The effect of colonization on time to first exacerbation (the primary outcome) was assessed using multivariate Cox proportional hazard analyses. We adjusted for potential confounding variables, including age, sex, smoking status, number of exacerbations in preceding year and FEV_1% predicted. We forced all of these variables in the final model(s) because they have been demonstrated to be associated with exacerbations or risk of bacterial colonization. The presence of effect modification by ICS (FP or placebo) and FEV_1 (continuous) was investigated by adding the product terms to the multivariate Cox proportional hazard model. If the p-value of the product term was lower than 0.1, further analyses were stratified by subgroups of the tested variable. For the stratified analysis, FEV_1 was dichotomized into patients with a relatively low (FEV_1< 50%) and high FEV_1 ($FEV_1 \geq$ 50%) according to the GOLD criteria (1). Furthermore, we analyzed time to a first Anthonisen type 0, I, II and III exacerbations for colonization (yes or no) in the parsimonious model. All statistical analyses were performed using SPSS version 10 (26).

RESULTS

Of 509 eligible patients, 269 were enrolled as described in an earlier publication (6) (see Figure 1). After 4 months of treatment with fluticasone propionate (FP), 244 COPD patients were randomized to either continue using FP (FP-group) or to receive placebo for 6 months (placebo group). At randomization, sputum samples could be collected in 209 patients, 106 in the FP and 103 in the placebo group. The mean age (± SD) of the participants was 64.0 ± 7.2 years. During the double blind phase, two patients were withdrawn from the study; one in the FP group due to cancer and the other in the placebo group due to a cerebrovascular accident. Table 1 shows the baseline characteristics of the study population stratified by colonization (yes or no) in stable state.

Table 1. Baseline characteristics of the patients with sputum samples at randomization

	Colonized	**Non colonized**
Number of patients	76	133
Mean age in years (SD)	65 (7)	64 (8)
Male (%)	86	87
Mean number of exacerbations in preceding year (sd)	1.7 (1.8)	1.2 (1.6)
Smoking status (%)		
Ex-smokers	67	74
Current smokers	33	26
Placebo (%)	54	47
Lung function post bronchodilation (SD)		
FEV_1 in liters	1.6 (0.5)	1.8 (0.6)
FEV_1 % predicted of normal	54 (15)	58 (15)

The excluded patients without sputum sample at baseline (n=35) experienced slightly fewer exacerbations in the preceding year (0.9 ± 0.9), contained fewer current smokers (20%) and more women (30%) compared to the study population with a sputum sample at baseline.

At randomization, 76 (36%) of the 209 patients were colonized. *Haemophilus influenzae* was observed in 50 patients, *Streptococcus pneumoniae* in 22 and *Moraxella catarrhalis* in 19 patients. In the colonized group, 47 (62%) of the patients developed at least one exacerbation compared to 67 (50%) in the non-colonized group. At exacerbation predominantly new PPM were isolated compared to stable state COPD.

The crude hazard ratio of a first exacerbation in colonized patients compared to non-colonized was 1.30 (95% CI 0.89-1.89). Adding the product term (ICS* colonization) to the multivariate model, including age, sex, FEV_1, number of exacerbations in preceding year and

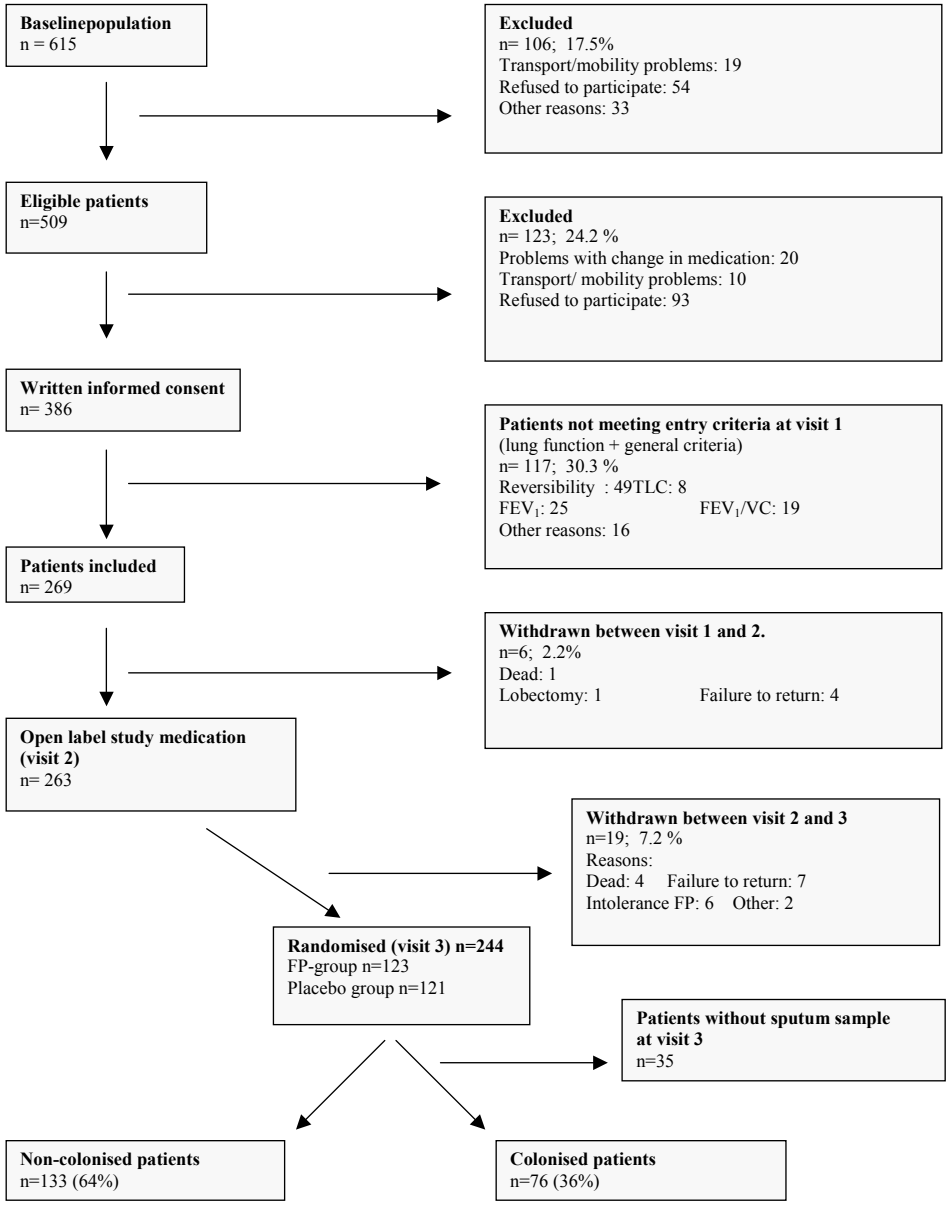


Figure 1. Flow diagram of subject progress through the study.

smoking status, did not indicate effect modification by discontinuation of ICS (p=0.85). However, adding the product term (FEV_1 * colonization) to this model, suggests effect modification by FEV_1 (p=0.038). We therefore stratified the analysis by patients with a relatively low (FEV_1< 50%) and high FEV_1 ($FEV_1 \geq 50\%$) according to the GOLD criteria (1). The adjusted hazard ratio of a first exacerbation in colonized compared to non-colonized patients was 1.01 (95% CI 0.56-1.81) in patients with a low FEV_1 and 1.29 (95% CI 0.76-2.20) in the patients with a high FEV_1 (see Table 3).

Analysis for the different types of exacerbation revealed that colonization increased the risk of a first Anthonisen type I exacerbation in the low FEV_1 group: adjusted HR 4.42 (95% CI 1.20-16.25) (see Table 4). No hazard ratio's for the risk of a first type III could be calculated because of an insufficient number of cases. The type of the exacerbations differed significantly between colonized and non-colonized patients. Colonized patients experienced predominantly type I exacerbations: 53% (18/34) compared to 25% (13/52) in the non-colonized patients. In the non-colonized patients, a type II exacerbation occurred most often (39% 20/52) (see Table 2).

Table 2. Distribution of exacerbation types according to Anthonisen criteria in the colonised and non colonised patients

	Type 0	**Type 1**	**Type 2**	**Type 3**
Colonized n(%)	1 (3%)	18 (53%)	8 (23%)	7 (21%)
Non colonized n(%)	7 (13%)	13 (25%)	20 (39%)	12 (23%)

Table 3. Adjusted hazard ratios of the first exacerbation for colonisation and other risk factors stratified by FEV_1

	FEV_1 < 50% HR (95% CI)	$FEV_1 \geq 50\%$ HR (95% CI)
Colonization at randomization (yes versus no)	1.01 (0.56; 1.81)	1.29 (0.76; 2.20)
Treatment (placebo versus FP))	2.14 (1.14; 4.03)	1.35 (0.81; 2.26)
Smoking status at baseline (yes versus no)	0.32 (0.15; 0.68)	0.70 (0.37; 1.29)
FEV_1% predicted	1.00 (0.96; 1.04)	0.98 (0.96; 1.01)
Gender (male versus female)	1.61 (0.51; 5.08)	0.65 (0.36; 1.19)
Age (years)	0.97 (0.93; 1.01)	0.99 (0.95; 1.02)
Number of exacerbations in preceding year	1.30 (1.11; 1.52)	1.45 (1.25; 1.69)

Table 4. Adjusted hazard ratios for colonisation (yes/no) of the first Anthonisen type I and II exacerbation stratified by FEV_1 *

Outcome	FEV_1 < 50% HR (95% CI)	FEV_1 ≥ 50% HR (95% CI)
Colonization and risk of type I exacerbation	4.42 (1.20-16.25)	1.23 (0.42-3.61)
Colonization and risk of type II exacerbation	0.75 (0.13- 4.28)	0.89 (0.32-2.47)

* No hazard ratio's for the risk of a first type III could be calculated because of an insufficient number of cases

DISCUSSION

This study revealed three important new findings. First, in contrast to our hypothesis no interaction between colonization and discontinuation of ICS on the time to first exacerbation was found. Second, different from recent literature, bacterial colonization in moderately severe COPD patients was not shown to be an independent risk factor for the development of acute exacerbations. Third, as already indicated in an earlier publication (6) patients with a FEV_1< 50% had a prominent increased risk of exacerbation following withdrawal of ICS, while such increased risk was not found in patients with a FEV_1 of 50% or higher.

This is the first study longitudinally investigating the effects of bacterial colonization on well-documented exacerbations in moderately severe COPD in relation to discontinuation of ICS. The lack of effect of bacterial colonization on the development of exacerbations is in contrast to the findings of the Sethi study and a study of the East London Cohort (19,20), which both reported that bacterial colonization in stable state modulated frequency of COPD exacerbations. Both studies suggest that colonization is a risk factor for exacerbations, but they did not report the results corrected for potential confounders indicating the severity, like FEV_1, smoking, and age. Since, we assumed FEV_1 is not an intermediate between colonization and exacerbation, we considered FEV_1 as a potential confounder or effect modifier. Although, in our study unadjusted Cox regression analysis showed a trend of increased risk in colonized patients, after correction for potential confounding factors no increased risk was found. The suggestion that an outgrow of colonizing bacteria, prompts an exacerbation (20) is disputed by the study of Sethi et al. (19) indicating that the increased exacerbations risk in colonized patients is caused by newly acquired bacteria. In the latter study, the relative risk of an exacerbation for colonized patients in stable state was 1.44, and rose to a maximum of 2.15 if a new strain of a bacterial pathogen was isolated. Similarly, we found in our study that most bacteria at exacerbations were different compared to those seen in stable state.

Since, patients with bacterial colonization in the low FEV_1 group experienced predominantly Anthonisen type I exacerbations and hence have a high chance to be treated with antibiotics

at exacerbation (23), new studies on the effects of frequent use of antibiotics in this subgroup of COPD patients are warranted.

The increased risk of exacerbations following withdrawal of ICS was already reported in our recent publication (6) and is in accordance with other limited evidence available (27,28). There is still debate whether or not ICS are beneficial in airway inflammation in COPD (29,30)following reports of conflicting results on the effects of ICS on markers of inflammation (31-34). In a recent study, the inhaled corticosteroid FP (9) showed beneficial effects on airway inflammation in bronchial biopsies in COPD, which may explain the decrease in exacerbations seen in long-term studies with FP. We are not aware of a study investigating the interaction of ICS with lower airway bacterial colonization. We found that bacterial colonization is not a risk factor for a more rapid onset of exacerbations in patients who discontinue ICS.

Although, we did not study colonization and infections with viruses and atypical bacteria, some non-differential sampling errors might have occurred and minimal oropharyngeal contamination of the sputum samples cannot be excluded, we feel confident about the following conclusions: Bacterial colonization in moderately severe COPD is not an independent risk factor for new bacterial exacerbations and colonized patients do not benefit more from the preventive effects of ICS in reducing the frequency and severity of exacerbations than non-colonized patients.

ACKNOWLEDGEMENTS

We gratefully acknowledge the members of the department of Pulmonary Medicine of the Medisch Spectrum Twente hospital, Enschede, especially the technicians of the lung function department, the data managers Betty Rinsma and Petra Meerlo and the technicians of the Regional Laboratory of Public Health, Enschede.

REFERENCES

1. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-1276.
2. Wouters EFM. Pulmonary rehabilitation in chronic obstructive pumonary disease. *Eur Respir Rev* 1999;9:189-192.
3. Seneff MG, Wagner DP, Wagner RP, Zimmerman JE, Knaus WA. Hospital and 1-year survival of patients admitted to intensive care units with acute exacerbation of chronic obstructive pulmonary disease. *JAMA* 1995;274:1852-1857.
4. Sin DD, Tu JV. Inhaled corticosteroids and the risk of mortality and readmission in elderly patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:580-584.
5. Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ, Wedzicha JA. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:1608-1613.
6. van der Valk P, Monninkhof E, van der Palen J, Zielhuis G, van Herwaarden C. Effect of Discontinuation of Inhaled Corticosteroids in Patients with Chronic Obstructive Pulmonary Disease: The COPE Study. *Am J Respir Crit Care Med* 2002;166:1358-1363.

7. Burge PS, Calverley PM, Jones PW, Spencer S, Anderson JA, Maslen TK. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: the ISOLDE trial. *BMJ* 2000;320:1297-1303.
8. Paggiaro PL, Dahle R, Bakran I, Frith L, Hollingworth K, Efthimiou J. Multicentre randomised placebo-controlled trial of inhaled fluticasone propionate in patients with chronic obstructive pulmonary disease. International COPD Study Group. *Lancet* 1998;351:773-780.
9. Hattotuwa KL, Gizycki MJ, Ansari TW, Jeffery PK, Barnes NC. The effects of inhaled fluticasone on airway inflammation in chronic obstructive pulmonary disease: a double-blind, placebo-controlled biopsy study. *Am J Respir Crit Care Med* 2002;1592-1596.
10. Ewig S. Legionella spp.in acute exacerbations of chronic obstructive pulmonary disease: what is the evidence? *Eur Respir J* 2002;19:387-389.
11. Miravitlles M. Exacerbations of chronic obstructive pulmonary disease: when are bacteria important? *Eur Respir J Suppl* 2002;36:9s-19s.
12. Sohy C, Pilette C, NMS, Sibille Y. Acute exacerbation of chronic obstructive pulmonary disease and antibiotics: what studies are still needed? *Eur Respir J* 2002;19:966-975.
13. Monso E, Ruiz J, Rosell A, Manterola J, Fiz J, Morera J, Ausina V. Bacterial infection in chronic obstructive pulmonary disease. A study of stable and exacerbated outpatients using the protected specimen brush. *Am J Respir Crit Care Med* 1995;152:1316-1320.
14. Cabello H, Torres A, Celis R, El Ebiary M, Puig dlB, Xaubet A, Gonzalez J, Agusti C, Soler N. Bacterial colonization of distal airways in healthy subjects and chronic lung disease: a bronchoscopic study. *Eur Respir J* 1997;10:1137-1144.
15. Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *Am J Med* 2000;109:288-295.
16. Ewig S, Rodriguez-Roisin R, Torres A. 2002. Indications for and choice of antibiotics in COPD. In T. Similowski, Whitelaw.W., and J. Derenne, editors Clinical management of Chronic Obstructive Pulmonary Disease. Marcel Dekker Inc, New York. 201-220.
17. Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax* 2000;55:629-30.
18. Sethi S, Muscarella K, Evans N, Klingman KL, Grant BJ, Murphy TF. Airway inflammation and etiology of acute exacerbations of chronic bronchitis. *Chest* 2000;118:1557-1565.
19. Sethi S, Evans N, Grant BJ, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *N Engl J Med* 2002;347:465-471.
20. Patel IS, Seemungal TA, Wilks M, Lloyd-Owen SJ, Donaldson GC, Wedzicha JA. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. *Thorax* 2002;57:759-764.
21. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152:s77-s120.
22. Brand PL, Quanjer PH, Postma DS, Kerstjens HA, Koeter GH, Dekhuijzen PN, Sluiter HJ. Interpretation of bronchodilator response in patients with obstructive airways disease. The Dutch Chronic Non-Specific Lung Disease (CNSLD) Study Group. *Thorax* 1992;47:429-436.
23. Anthonisen NR, Manfreda J, Warren CPW, Hershfield ES, Harding GKM, Nelson NA. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. *Ann Intern Med* 1987;106:196-204.
24. Isenberg, HD. 1992. Clinical microbiology procedures handbook Washington DC.
25. Piantadosi P. 1997. Sample Size and Power. In S. Paintadosi, editor Clinical Trials John Wiley & Sons, Inc., New York. 148-185.
26. SPSS for Windows, release 10. 2001; SPSS Inc, Chicago IL, USA.
27. Jarad NA, Wedzicha JA, Burge PS, Calverley PM. An observational study of inhaled corticosteroid withdrawal in stable chronic obstructive pulmonary disease. ISOLDE Study Group. *Respir Med* 1999;93:161-166.
28. O'Brien A, Russo-Magno P, Karki A, Hiranniramol S, Hardin M, Kaszuba M, Sherman C, Rounds S. Effects of withdrawal of inhaled steroids in men with severe irreversible airflow obstruction. *Am J Respir Crit Care Med* 2001;164:365-371.
29. Calverley PM. Inhaled corticosteroids are beneficial in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:341-342.
30. Barnes PJ. Inhaled corticosteroids are not beneficial in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:342-344.
31. Hill A, Gompertz S, Stockley R. Factors influencing airway inflammation in chronic obstructive pulmonary disease. *Thorax* 2000;55:970-977.
32. Llewellyn-Jones CG, Harris TA, Stockley RA. Effect of fluticasone propionate on sputum of patients with chronic bronchitis and emphysema. *Am J Respir Crit Care Med* 1996;153:616-621.
33. Confalonieri M, Mainardi E, Della PR, Bernorio S, Gandola L, Beghe B, Spanevello A. Inhaled corticosteroids reduce neutrophilic bronchial inflammation in patients with chronic obstructive pulmonary disease. *Thorax* 1998;53:583-585.
34. Keatings VM, Jatakanon A, Worsdell YM, Barnes PJ. Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* 1997;155:542-548.

Chapter 4

Molecular epidemiology of drug-resistant *Streptococcus pneumoniae*

Chapter 4.1

Molecular epidemiology of penicillin-nonsusceptible *Streptococcus pneumoniae* among children in Greece

D. Bogaert, G.A. Syrogiannopoulos, I.N. Grivea, R. de Groot,
N.G. Beratis and P.W.M. Hermans

J Clin Microbiol 2000; 38(12): 4361-4366

ABSTRACT

A total of 145 penicillin-nonsusceptible *Streptococcus pneumoniae* strains were isolated from young carriers in Greece and analyzed by antibiotic susceptibility testing, serotyping, restriction fragment end labeling (RFEL), and penicillin-binding protein (PBP) genotyping. The serotypes 23A and 23F (54%), 19A and 19F (25%), 9V (5%), 15A, 15B, and 15C (4%), 6A and 6B (4%), and 21 (4%) were most prevalent in this collection. Fifty-three distinct RFEL types were identified. Sixteen different RFEL clusters, harboring 2 to 32 strains each, accounted for 82% of all strains. Eight of these genetic clusters representing 60% of the strains were previously identified in other countries. A predominant lineage of 66 strains (46%) harboring five RFEL types and the serotypes 19F and 23F was closely related to the pandemic clone Spain23F-1 (genetic relatedness of 85%). Another lineage, representing 11 strains, showed close genetic relatedness to the pandemic clone France9V-3. Another lineage of 8 serotype 21 strains was Greece specific since the RFEL types were not observed in an international collection of 193 genotypes from 16 different countries. Characterization of the PBP genes *pbp1a*, *pbp2b*, and *pbp2x* revealed 20 distinct PBP genotypes of which PBP type 1-1-1, initially observed in the pandemic clones 23F and 9V, was predominantly present in 11 RFEL types in this Greek collection of penicillin-nonsusceptible strains (55%). Sixteen PBP types covering 52 strains (36%) were Greece specific. This study underlines the strong contribution of penicillin-resistant international clones to the prevalence and spread of penicillin-nonsusceptible pneumococci among young children in Greece.

INTRODUCTION

Streptococcus pneumoniae is worldwide a common cause of invasive diseases such as meningitis, bacteremia, and pneumonia and of upper respiratory tract infections (1). Pneumococci are often part of the normal nasopharyngeal flora. Especially young children and elderly people are at risk of becoming colonized with *S. pneumoniae*. The colonization risk increases where crowding occurs, e.g., in day care centers, hospitals, and nursing homes (23, 30, 31). Although a positive correlation between colonization with pneumococci and acute otitis media has been found (12, 36), a relation between pneumococcal carriage and invasive disease has not yet been proven.

Since the late 1970s and 1980s, antibiotic resistance among pneumococci has become an emerging problem. Several (multi)drug-resistant clones have rapidly spread throughout various European countries, North and Latin America, and Asia (2, 18, 20). Some of these clones were initially discovered in Spain, where so far the prevalence of penicillin resistance has reached levels of up to 60% (4). The most striking example is the spread of the pandemic multidrug-resistant clone 6B from Spain to Iceland in the late 1980s, where until 1988 no drug resistance among *S. pneumoniae* isolates had been reported. From 1989 to 1992 penicillin resistance rose steeply from 2.3 to 17% (39). In 1993, the resistance level among pneumococcal isolates in Iceland reached 20%, of which the vast majority was associated with the serogroups 6, 19, and 23 (25). In 1991, Munoz et al. have reported the intercontinental spread of a multidrug resistant *S. pneumoniae* clone of serotype 23F from Spain to the United States (29). Since then this clone, recently designated pandemic clone Spain23F-1 (http://www.wits.ac.za/pmen/pmen.htm) has rapidly spread throughout the United States (27). Finally, Gasc et al. have described in 1995 the spread of a penicillin-resistant pneumococcal clone of serotype 9V (pandemic clone France9V-3) from Spain to France (14). Throughout the years these clones have been identified in many countries in different parts of the world (10, 20, 42). In addition, novel drug-resistant clones have been reported in France, former Czechoslovakia, Spain, Hungary, Japan, South Africa, the United States, Chile, England, etc. which tend to spread in a nationwide manner (6, 13, 16, 17, 19, 35, 37, 45). The risk for colonization with and spread of antibiotic-resistant strains is related to younger-age children, occurrence of refractory middle ear infections, previous antibiotic consumption, and day care attendance (9, 28, 32).

In Greece, the emergence of antibiotic resistance among pneumococcal isolates was recognized in the mid 1990s (41). During the period December 1995 through February 1996, 53% of the pneumococci isolated from healthy carriers attending day care centers appeared to be resistant to one or more antibiotics, while 29% of these isolates were penicillin nonsusceptible (41). In a recent study in which 2,448 infants and toddlers were screened during a 2-year period (1997 to 1999) for pneumococcal carriage, 16% of the pneumococci

demonstrated reduced susceptibility to penicillin (40; G. Syrogiannopoulos, I. Grivea, G. Katopodis, and N. Beratis, unpublished data). The aim of the current study was to identify the molecular epidemiological nature of the penicillin-nonsusceptible pneumococci isolated in Greece. For this purpose, molecular analysis was performed on penicillin-nonsusceptible isolates of the two Greek studies. A total of 145 (multi)resistant pneumococcal isolates collected from both studies were characterized by drug susceptibility testing, serotyping, restriction fragment end labeling (RFEL), and penicillin-binding protein (PBP) genotyping.

MATERIALS AND METHODS

Bacteriology. Penicillin-nonsusceptible *S. pneumoniae* strains were isolated from the nasopharynx of 338 children attending seven day care centers in the city of Patras, Southwestern Greece, from December 1995 to February 1996 and from 2,448 children under the age of 2 years visiting health care centers in Southern and Central Greece from February 1997 to February 1999. Bacteriological diagnosis and susceptibility testing were carried out at the Laboratory of the Division of Pediatric Infectious Disease of the University of Patras, Patras, Greece. The bacteriological methods and serotyping have been described previously (40, 41). Molecular analysis was performed on 92% of the penicillin-nonsusceptible isolates, i.e., 34 strains collected from seven day care centers in Patras and 111 strains collected from 12 different provinces in Central and Southern Greece. In addition, the Greek isolates were compared with an international collection of pneumococcal strains representing 193 distinct RFEL types originating from 16 different countries in America, Europe, Africa, and Asia (M. Sluijter, unpublished observations), in which the international clones pandemic clone Spain23F-1, pandemic clone France9V-3, and pandemic clone Spain6B-2 are present (http://www.wits.ac.za/pmen/pmen.htm).

RFEL analysis. Typing of the 145 pneumococcal strains by RFEL analysis was performed as described by van Steenbergen et al. (44) and as adapted by Hermans et al. (22). Briefly, purified pneumococcal DNA was digested by the restriction enzyme EcoRI. The DNA restriction fragments were end labeled at 72°C with [-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for various times at room temperature to ECL Hyperfilms (Amersham, Bucks, United Kingdom).

BOX PCR fingerprinting. Typing of the 145 pneumococcal strains by BOX PCR fingerprinting was performed as described by van Belkum et al. (43). Briefly, 50 ng of pneumococcal DNA was amplified by PCR (4 min at 94°C [predenaturation]; 40 cycles of 1

min at 94°C, 1 min at 60°C, and 2 min at 74°C; and 2 min at 74°C [extension]), using primer BOX-A (5'-ATACTCTTCGAAAATCTCTTCAAAC), which was designed from the primary structure of the pneumococcal BOX repeat motif. The amplified products were separated on a 1.5% agarose gel. Gels were stained with ethidium bromide, and the banding patterns were evaluated visually.

PBP genotyping. Genetic polymorphism of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* of the penicillin-nonsusceptible isolates was investigated by restriction fragment length polymorphism (RFLP) analysis as described previously (20). Briefly, we amplified the genes by PCR. The primers used to amplify the genes *pbp1a*, *pbp2b*, and *pbp2x* were described previously (7, 11). The amplification products (5 μl) were digested by restriction endonuclease HinfI and separated by agarose gel electrophoresis. Gels were scanned and analyzed by the Geldoc 2000 system (Bio-Rad). The different PBP genotypes received a three-number code (e.g., 6-12-34) referring to the RFLP patterns of the genes *pbp1a* (6), *pbp2b* (11), and *pbp2x* (33), respectively.

Computer-assisted analysis of the DNA banding patterns. The RFEL types were analyzed using the Windows version of the Gelcompar software version 4 (Applied Maths, Kortrijk, Belgium) after imaging of the RFEL autoradiograms using the Image master DTS (Pharmacia Biotech, Uppsala, Sweden). To this end, the DNA fragments in the molecular size range of 160 to 400 bp were explored. The DNA banding patterns were normalized using pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the banding patterns was performed by unweighted-pair-group method using arithmetic averages (34) and using the Jaccard similarity coefficient applied to peaks (38). Computer-assisted analysis and methods and algorithms used in this study were carried out according to the instructions of the manufacturer of Gelcompar. A tolerance of 1.2% in band position was applied during comparison of the DNA patterns. For evaluation of the genetic relatedness of the isolates, we used the following definitions: (i) strains of particular RFEL type are 100% identical by RFEL analysis; (ii) a RFEL cluster represents a group of RFEL types that differs in only one band (ca. >95% genetic relatedness); and (iii) an RFEL lineage represents a group of RFEL types that differs in <4 bands (ca. >85% genetic relatedness).

Statistical analysis. For statistical analysis of the results, we used the Fisher exact test.

RESULTS

A total of 145 penicillin-nonsusceptible pneumococcal strains were analyzed using serotyping, RFEL, and PBP genotyping. Serotyping revealed 12 different serotypes, namely, 23F (52%); 23A (2%); 19F (15%); 19A (10%); 9V (5%); 15A, 15B, and 15C (4%); 6A (2%); 6B (1%); 21 (4%); 14 (2%); 22 (1%); and 33F (1%) (40, 41; Syrogiannopoulos et al.,

unpublished). RFEL analysis divided the strains into 53 distinct RFEL genotypes (Fig. 1). Sixteen genetic clusters were observed in this collection of strains, representing 82% of the strains and varying in size from 2 to 32 strains. The genetic relatedness within these clusters was confirmed by BOX PCR (43) (data not shown). Five of the sixteen clusters contained two serotypes, while one of the clusters harbored three different serotypes. We compared the 53 Greek RFEL types with our international library in which 193 RFEL genotypes of pneumococci from 16 different countries are present (M. Sluijter, unpublished observations). Six of the clusters representing 60% of the isolates were previously seen. To analyze the genetic heterogeneity in the penicillin resistance genes, we performed PBP genotyping. Twenty distinct PBP genotypes were observed (Table 1). PBP genotype 1-1-1, initially observed in the pandemic clones 23F and 9V, was most predominantly observed in this Greek collection; 81 strains representing 13 distinct RFEL types shared this penicillin resistance genotype.

Clusters IX and X were the most predominantly observed clusters and consisted of 31 (21%) and 32 strains (22%), respectively (Fig. 1, Table 1). Both clusters belonged to one predominant lineage of 66 genetically related strains, representing five RFEL types and harboring the serotypes 23F and 19F. This lineage was closely related to the pandemic clone Spain23F-1 (genetic relatedness of 90%) which is widely spread all over the world (8, 26, 42). All strains belonging to this lineage showed resistance to penicillin, chloramphenicol, tetracycline, and sulfamethoxazole-trimethoprim. In addition, cluster I was also resistant to erythromycin and clindamycin. Similar to the characteristics of the pandemic clone Spain23F-1, this lineage invariably demonstrated PBP genotype 1-1-1.

The second lineage representing cluster VII contained 10 strains of serotypes 19F (Fig. 1, Table 1). Cluster VII was observed previously in The Netherlands, Thailand, and Vietnam and showed reduced susceptibility to penicillin, tetracycline, erythromycin and, in most cases, to sulfamethoxazole-trimethoprim. This cluster had another common feature since the genetic analysis of *pbp1a* by PBP genotyping of the vast majority of the strains was not applicable. Consequently, the PBP genotype of the majority of these strains was 0-3-30. Interestingly, this observation is in agreement with the genetically related strains present in the international data library.

The third lineage representing 12 strains and the clusters III, IV, and V displayed serotype 19A (Fig. 1, Table 1). These strains did not cluster with isolates from the international library and their PBP genotype did not match with any of the PBP genotypes present in the international library. All strains displayed the *pbp1a* and *pbp2b* genotype 2-2 that matched with the majority of the penicillin-susceptible pneumococci analyzed so far (Sluijter, unpublished). We observed alterations in the PBP profile of *pbp2x*, resulting in the

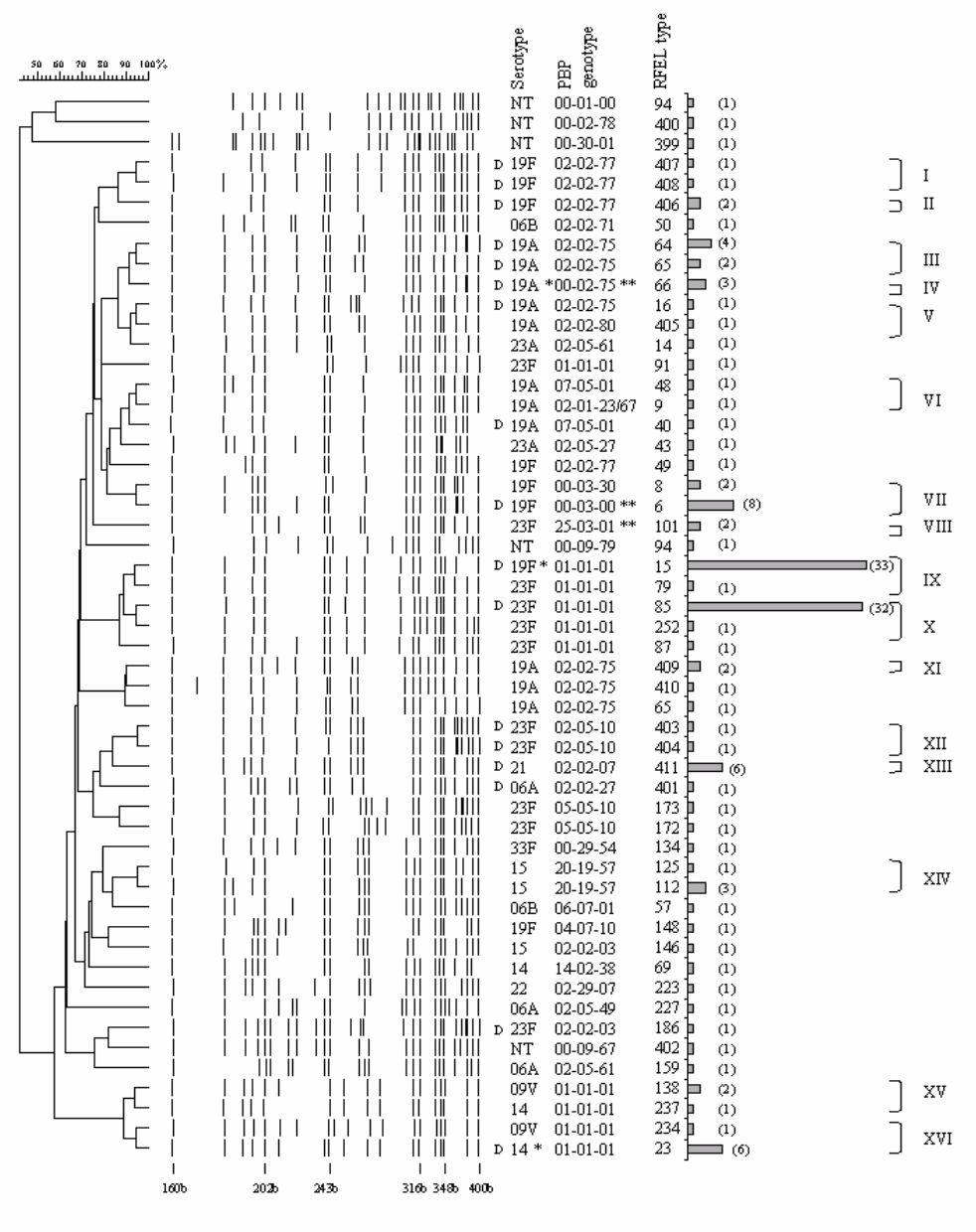


Figure 1. Dendrogram of the 53 RFEL types observed among 145 penicillin-nonsusceptible pneumococcal isolates from the nasopharynxes of Greek children. Molecular sizes of reference bands are indicated in bases (b). Serotypes, PBP types, RFEL types, bars representing the number of isolates per RFEL type, clusters and cluster codes are also shown. NT, nontypeable; D, RFEL types observed at least once in day care centers. Code "00" refers to untypeable PBP genotypes. Major serotypes (*) and PBP types (**) within RFEL types are indicated (for details, see Table 1).

genotypes 2-2-75 and 2-2-80. These PBP genotypes invariably corresponded to intermediate resistance to penicillin only.

The fourth lineage represented the clusters XV (two strains) and XVI (nine strains) and harbored the serotypes 9V, 14, and 23A (Fig. 1, Table 1). These clusters corresponded to the pandemic clone France9V-3. All strains showed the PBP genotype 1-1-1 and were invariably resistant to penicillin, sulfamethoxazole-trimethoprim and, in some strains, to chloramphenicol and tetracycline.

The fifth lineage represented the clusters XIII (serotype 21; six strains) and XII (serogroup 23F; two strains) (Fig. 1, Table 1). These clusters were Greece specific since they were not present in the international library. Interestingly, these clusters consisted of strains that were isolated in day care centers only. Cluster XIII was mostly observed in a single day care center, whereas cluster XII was exclusively observed in another day care center (Fig. 1). Cluster XIII displayed PBP type 2-2-76 corresponding to low penicillin resistance. Cluster XII showed a different PBP genotype 2-5-10 with alterations in *pbp2b* and *pbp2x*. None of the latter two PBP genotypes were present in the international library.

Both study groups were divided in day care center attendees and non-day care attendees to analyze the contribution of day care center attendance on clustering of pneumococcal strains. We observed that 86% of the strains from the day care center group containing 45 pneumococcal isolates belonged to a cluster. Within the non-day care center group representing 100 pneumococcal isolates, 80% of the strains demonstrated genetic clustering. There was no statistical difference between the two groups. We reanalyzed our data for the largest day care center in Patras, where 21 strains were isolated. We observed that 19 of these 21 strains (90%) clustered within four clusters. Although this percentage demonstrated a higher degree of genetic clustering in children attending day care centers, this difference was statistically not significant.

DISCUSSION

Serotyping of the penicillin-nonsusceptible isolates revealed that 79% of the Greek strains belonged to serotypes 23F, 23A, 19F, and 19A (40, 41; Syrogiannopoulos et al., unpublished). These data correspond to earlier findings in other European countries and in the Americas in which these serogroups also significantly contributed to the prevalence of penicillin-resistant *S. pneumoniae* among young carriers (2, 3, 15, 23, 33, 42).

RFEL genotyping of the 145 penicillin-nonsusceptible isolates showed that 82% of the strains matched within genetic clusters. These data suggest that penicillin-resistant pneumococci rapidly spread among children. This is in line with earlier findings by Hermans et al., who demonstrated that the degree of genetic clustering of penicillin-resistant strains in

the Netherlands and Thailand is 70 and 74%, respectively, whereas the degree of genetic clustering among Dutch penicillin-susceptible isolates is 32% (21). Similar data have been reported in various other parts of the world, including Europe, the United States, and South America (8, 20, 26, 42).

The present study clearly demonstrates the significant contribution of the pandemic clone Spain23F-1 to the prevalence and spread of penicillin-nonsusceptible pneumococci among young children in Greece. This is in agreement with studies in other countries in which the predominance of this pandemic clone has also been observed (8, 26, 42). In addition, in the latter studies a second predominant clone, the pandemic clone France9V-3, was highly contributive to the penicillin-resistant pneumococcal population. This clone is also present in our Greek collection, but it does not play a predominant role.

Serogroup 19 represented the major serotype among five lineages. The lineages of cluster VI and cluster VII represented mainly multidrug-resistant isolates and were closely related to isolates found in The Netherlands, Thailand, and Vietnam. Multilocus sequence typing (MLST) has demonstrated that cluster VII also matches with multidrug-resistant serotype 19F isolates from Taiwan (B. Spratt, personal communication). The three other lineages, representing clusters I and II, clusters III, IV, and V, and cluster XI, respectively, showed intermediate resistance to penicillin only and displayed new PBP genotypes with alterations in *pbp2x* only. MLST has demonstrated that clusters III, IV, and V match with a drug-susceptible serotype 19A invasive isolate from the United Kingdom. In general, this study showed a relation between accumulation of alterations in the three PBP genes and level of penicillin resistance; high resistance levels were often associated with changes in the DNA banding patterns of all three PBP genes (18). In addition, there was also a correlation between multidrug resistance and cumulative alterations in all three PBP genes. This phenomenon is in agreement with the previous observations of Hermans et al. and is hypothesized to be the effect of frequent horizontal cotransfer of resistance genes other than PBP genes in pneumococci with high-level penicillin resistance (20).

Only one lineage, representing two RFEL clusters of serotype 21 and 23F, respectively, was found to be Greece specific since they were not present in the international data library. In addition, this lineage was found only in three day care centers. This is strongly suggestive for the dissemination of pneumococcal clones among day care center attendees within and between day care centers. MLST typing has recently demonstrated that this RFEL cluster of serotype 21 matches a drug-susceptible invasive isolate of serotype 21 in the United Kingdom (B. Spratt, personal communication).

In conclusion, our observations demonstrate a high degree of genetic clustering among penicillin-nonsusceptible, often multidrug-resistant pneumococci in young children in

Table 1. Clusters, serotypes, PBP genotypes and resistance patterns of the 145 penicillin-resistant pneumococci isolated from the nasopharynx of young infants in Greece.

Cluster [a]	No. of strains/no. of RFEL types	Serotype(s) (no. of strains)	PBP type [b] (nr. of strains)	Resistance Pattern [c]
I	(2/2)	19F	2-2-77	P
II	(2/1)	19F		
III	(6/2)	19A/F	2-2-75	P
IV	(3/1)	19A (2)	2-2-75	P
		9V (1)	1-1-1	
V	(2/2)	19A	2-2-75	P
	(1/1)	23A	2-5-61	PCTS
VI	(2/2)	19A	7-5-1	P(TE)S
			2-1-23	
	(1/1)	19A	7-5-1	PCTS
	(1/1)	23A	2-5-27	
VII	(10/2)	19F	1(0)-3-30(0)	PTE(S)
VIII	(2/1)	23F	2-2-71	PCT
			25-3-1	PS
IX	(32/2)	23F	1-1-1	PCTEC^{L}S
X	(33/2)	23F (32)		PCTS
		19F (1)		
	(1/1)	23F		PCTES
XI	(2/1)	19A	2-2-75	PS
	(2/2)	19A		
XII	(6/1)	21 (5)	2-2-76	P
XIII	(2/2)	23F	2-5-10	
	(2/2)	23F	5-5-10	P
XIV	(4/2)	15	2-19-57 (2)	P
			20-19-57 (2)	
XV	(8/2)	9V/ 14/ 23A	1-1-1	P(CT)S
XVI	(3/2)	23A/ 14		PS

[a] Genetic clustering according to RFEL typing exclusively matched with BOX PCR fingerprinting.

[b] Code “(0)” refers to untypeable PBP genotypes.

[b] The antibiotic resistance pattern was evaluated for penicillin (P), erythromycin (E), chloramphenicol (C), tetracycline (T), clindamycin (C^{L}) and sulfamethoxazole-trimethoprim (S).

Greece, mainly caused by the spread of a restricted number of penicillin-resistant *S. pneumoniae* clones. Limiting antibiotic prescription and promoting compliance would probably contribute to the control of this problem. A better alternative to prevent the spread of multidrug-resistant clones in the near future, however, is large-scale vaccination using pneumococcal conjugate vaccines. Although the initial results of the conjugate vaccination trials look promising (5, 24; R. Dagan, N. Givon, P. Yagupsky, et al., Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. G552, 1998), the epidemiological consequences of such strategies need to be monitored in detail.

REFERENCES

1. Anonymous. 1997. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). Morb. Mortal. Wkly. Rep. 46:1-24.
2. Appelbaum, P. C., C. Gladkova, W. Hryniewicz, et al. 1996. Carriage of antibiotic-resistant *Streptococcus pneumoniae* by children in eastern and central Europea multicenter study with use of standardized methods. Clin. Infect. Dis. 23:712-717.
3. Arnold, K. E., R. J. Leggiadro, R. F. Breiman, et al. 1996. Risk factors for carriage of drug-resistant *Streptococcus pneumoniae* among children in Memphis, Tennessee. J. Pediatr. 128:757-764.
4. Baquero, F., J. A. Garcia-Rodriguez, J. Garcia de Lomas, and L. Aguilar. 1999. Antimicrobial resistance of 1,113 *Streptococcus pneumoniae* isolates from patients with respiratory tract infections in Spain: results of a 1-year (1996-1997) multicenter surveillance study. The Spanish Surveillance Group for Respiratory Pathogens. Antimicrob. Agents Chemother. 43:357-359.
5. Black, S., H. Shinefield, B. Fireman, et al. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr. Infect. Dis. J. 19:187-195.
6. Coffey, TJ, S Berron, . Daniels, et al. 1996. Multiply antibiotic-resistant *Streptococcus pneumoniae* recovered from Spanish hospitals (1988-1994): novel major clones of serotypes 14, 19F and 15F. Microbiology 142:2747-2757.
7. Coffey, T. J., C. G. Dowson, M. Daniels, J. Zhou, C. Martin, B. G. Spratt, and J. M. Musser. 1991. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. Mol Microbiol. 5:2255-2260.
8. Corso, A., E. P. Severina, V. F. Petruk, Y. R. Mauriz, and A. Tomasz. 1998. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. Microb. Drug Resist. 4:325-337.
9. Dagan, R., E. Leibovitz, D. Greenberg, P. Yagupsky, D. M. Fliss, and A. Leiberman. 1998. Dynamics of pneumococcal nasopharyngeal colonization during the first days of antibiotic treatment in pediatric patients. Pediatr. Infect. Dis. J. 17:880-885.
10. Davies, T., R. V. Goering, M. Lovgren, J. A. Talbot, M. R. Jacobs, and P. C. Appelbaum. 1999. Molecular epidemiological survey of penicillin-resistant *Streptococcus pneumoniae* from Asia, Europe, and North America. Diagn. Microbiol. Infect. Dis. 34:7-12.
11. Dowson, C. G., A. Hutchison, and B. G. Spratt. 1989. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. Mol. Microbiol. 3:95-102.
12. Faden, H., L. Duffy, R. Wasielewski, J. Wolf, D. Krystofik, and Y. Tung. 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J. Infect. Dis. 175:1440-1445.
13. Figueiredo, A. M., R. Austrian, P. Urbaskova, L. A. Teixeira, and A. Tomasz. 1995. Novel penicillin-resistant clones of *Streptococcus pneumoniae* in the Czech Republic and in Slovakia. Microb. Drug Resist. 1:71-78.
14. Gasc, A. M., P. Geslin, and A. M. Sicard. 1995. Relatedness of penicillin-resistant *Streptococcus pneumoniae* serogroup 9 strains from France and Spain. Microbiology 141(Pt. 3):623-627.
15. Ghaffar, F., I. R. Friedland, and G. H. McCracken, Jr. 1999. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. Pediatr. Infect. Dis J. 18:638-646.
16. Gherardi, G., J. S. Inostrozo, M. O'Ryan, V. Prado, S. Prieto, C. Arellano, R. R. Facklam, and B. Beall. 1999. Genotypic survey of recent beta-lactam-resistant pneumococcal nasopharyngeal isolates from asymptomatic children in Chile. J. Clin. Microbiol. 37:3725-3730.
17. Gherardi, G., C. G. Whitney, R. R. Facklam, and B. Beall. 2000. Major related sets of antibiotic-resistant pneumococci in the United States as determined by pulsed-field gel electrophoresis and *pbp1a-pbp2b-pbp2x-dhf* restriction profiles. J. Infect. Dis. 181:216-229.
18. Hakenbeck, R. 1999. β-Lactam-resistant *Streptococcus pneumoniae*: epidemiology and evolutionary mechanism.

Chemotherapy 45:83-94.
19. Hall, L. M., R. A. Whiley, B. Duke, R. C. George, and A. Efstratiou. 1996. Genetic relatedness within and between serotypes of *Streptococcus pneumoniae* from the United Kingdom: analysis of multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and antimicrobial resistance patterns. J. Clin. Microbiol. 34:853-859.
20. Hermans, P. W., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. Goessens, and R. de Groot. 1997. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. Microb. Drug Resist. 3:243-251.
21. Hermans, P. W., M. Sluijter, K. Elzenaar, A. van Veen, J. J. Schonkeren, F. M. Nooren, W. J. van Leeuwen, A. J. de Neeling, B. van Klingeren, H. A. Verbrugh, and R. de Groot. 1997. Penicillin-resistant *Streptococcus pneumoniae* in the Netherlands: results of a 1-year molecular epidemiologic survey. J. Infect. Dis. 175:1413-1422.
22. Hermans, P. W., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 33:1606-1612.
23. Kellner, J. D., and E. L. Ford-Jones. 1999. *Streptococcus pneumoniae* carriage in children attending 59 Canadian child care centers. Toronto Child Care Centre Study Group. Arch. Pediatr. Adolesc. Med. 153:495-502.
24. Korkeila, M., H. Lehtonen, H. Ahman, O. Leroy, J. Eskola, and H. Kayhty. 2000. Salivary anti-capsular antibodies in infants and children immunised with *Streptococcus pneumoniae* capsular polysaccharides conjugated to diphtheria or tetanus toxoid. Vaccine 18:1218-1226.
25. Kristinsson, KG1995. Epidemiology of penicillin resistant pneumococci in Iceland. Microb. Drug Resist. 1:121-125.
26. Marchese, A., M. Ramirez, G. C. Schito, and A. Tomasz. 1998. Molecular epidemiology of penicillin-resistant *Streptococcus pneumoniae* isolates recovered in Italy from 1993 to 1996. J. Clin. Microbiol. 36:2944-2949.
27. McDougal, L. K., R. Facklam, M. Reeves, S. Hunter, J. M. Swenson, B. C. Hill, and F. C. Tenover. 1992. Analysis of multiply antimicrobial-resistant isolates of *Streptococcus pneumoniae* from the United States. Antimicrob. Agents Chemother. 36:2176-2184.
28. Melander, E., S. Molstad, K. Persson, H. B. Hansson, M. Soderstrom, and K. Ekdahl. 1998. Previous antibiotic consumption and other risk factors for carriage of penicillin-resistant *Streptococcus pneumoniae* in children. Eur. J. Clin. Microbiol. Infect. Dis. 17:834-838.
29. Muñoz, R., T. J. Coffey, M. Daniels, et al. 1991. Intercontinental spread of a multiresistant clone of genotype 23F *Streptococcus pneumoniae*. J. Infect. Dis. 164:302-306.
30. Nuorti, J. P., J. C. Butler, J. M. Crutcher, R. Guevara, D. Welch, P. Holder, and J. A. Elliott. 1998. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. N. Engl. J. Med. 338:1861-1868.
31. Principi, N., P. Marchisio, G. C. Schito, and S. Mannelli. 1999. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. Pediatr. Infect. Dis. J. 18:517-523.
32. Reichler, M. R., A. A. Allphin, R. F. Breiman, J. R. Schreiber, J. E. Arnold, L. K. McDougal, R. R. Facklam, B. Boxerbaum, D. May, R. O. Walton, et al. 1992. The spread of multiply resistant *Streptococcus pneumoniae* at a day care center in Ohio. J. Infect. Dis. 166:1346-1353.
33. Reichler, M. R., J. Rakovsky, A. Sobotova, M. et al. 1995. Multiple antimicrobial resistance of pneumococci in children with otitis media, bacteremia, and meningitis in Slovakia. J. Infect. Dis. 171:1491-1496.
34. Romesburg, H. 1990. Cluster analysis for researchers, p. 9-28. Krieger, Malabar, Fla.
35. Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck. 1992. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. Infect. Immun. 60:4119-4126.
36. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. Goessens, A. van Belkum, and P. W. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. J. Clin. Microbiol. 36:2248-2253.
37. Smith, A. M., and K. P. Klugman. 1997. Three predominant clones identified within penicillin-resistant South African isolates of *Streptococcus pneumoniae*. Microb. Drug Resist. 3:385-389.
38. Sneath, P. 1973. Numerical taxonomy, p. 131-132. W. H. Freeman, San Francisco, Calif.
39. Soares, S., KG Kristinsson, JM Musser, and A Tomasz. 1993. Evidence for the introduction of a multiresistant clone of serotype 6B *Streptococcus pneumoniae* from Spain to Iceland in the late 1980s. J Infect Dis 168:158-163.
40. Syrogiannopoulos, G., I. Grivea, G. Katopodis, G. P, M. Jacobs, and N. Beratis. 2000. Carriage of antibiotic-resistant *Streptococcus pneumoniae* in Greek infants and toddlers. Eur. J. Clin. Microbiol. Infect. Dis. 19:288-293.
41. Syrogiannopoulos, G. A., I. N. Grivea, N. G. Beratis, A. E. Spiliopoulou, E. L. Fasola, S. Bajaksouzian, P. C. Appelbaum, and M. R. Jacobs. 1997. Resistance patterns of *Streptococcus pneumoniae* from carriers attending day-care centers in southwestern Greece. Clin. Infect. Dis. 25:188-194.
42. Tomasz, A., A. Corso, E. P. Severina, G. Echaniz-Aviles, M. C. Brandileone, T. Camou, E. Castaneda, O. Figueroa, A. Rossi, and J. L. Di Fabio. 1998. Molecular epidemiologic characterization of penicillin-resistant *Streptococcus pneumoniae* invasive pediatric isolates recovered in six Latin-American countries: an overview. PAHO/Rockefeller University Workshop. Pan American Health Organization. Microb. Drug Resist. 4:195-207.
43. van Belkum, A., M. Sluijuter, R. de Groot, H. Verbrugh, and P. W. Hermans. 1996. Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 34:1176-1179.
44. van Steenbergen, T. J., S. D. Colloms, P. W. Hermans, J. de Graaff, and R. H. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc. Natl. Acad. Sci. USA 92:5572-5576.
45. Yoshida, R., Y. Hirakata, M. Kaku, K. Tomono, S. Maesaki, Y. Yamada, S. Kamihira, M. R. Jacobs, P. C. Appelbaum, and S. Kohno. 1999. Genetic analysis of serotype 23F *Streptococcus pneumoniae* isolates from several countries by penicillin-binding protein gene fingerprinting and pulsed-field gel electrophoresis. Chemotherapy 45:158-165.

Chapter 4.2

Molecular epidemiology of penicillin-susceptible, multidrug-resistant serotype 6B pneumococci isolated from children in Greece

G.A. Syrogiannopoulos, D. Bogaert, I.N. Grivea, N.G. Beratis,
R. de Groot and P.W.M. Hermans

J Clin Microbiol 2001; 39(2): 581-5

ABSTRACT

Since January 1996, and over a 3-year time span, a significant spread of serotype 6B multidrug-resistant (MDR) pneumococci, susceptible to penicillin and resistant to erythromycin, clindamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole, was noted in young carriers living in central and southern Greece. Using restriction fragment end labeling and penicillin binding protein (PBP) genotyping, we studied 41 serotype 6B penicillin-susceptible MDR pneumococci isolated during two independent studies in Greece. Forty (98%) of these 41 isolates were strongly related, representing a single lineage (genetic relatedness, 91%). The Greek isolates were closely related (genetic relatedness, ~91%) to the penicillin-resistant MDR clone of serotype 6B that spread from Spain to Iceland in the late 1980s. Moreover, the Greek group of isolates was genetically distinct (genetic relatedness < 83%) from other penicillin-susceptible or -resistant serotype 6B strains from various parts of the world. All serotype 6B penicillin-susceptible MDR isolates displayed a penicillin-susceptible PBP 1A-2B-2X genotype. Our findings suggest that the penicillin-susceptible MDR 6B clone that was found in Greece between the years 1996 and 1999 represents the ancestor of the pandemic penicillin-resistant MDR clone 6B.

INTRODUCTION

Streptococcus pneumoniae is a common cause of invasive diseases, such as meningitis and bacteremia, and of respiratory tract infections (10, 15). *S. pneumoniae* isolates that are resistant to penicillin and/or to non-β-lactam agents have been reported with increasing frequency worldwide (15). This global increase in antibiotic-resistant and especially in multidrug-resistant (MDR) pneumococci appears, in part, to result from the spread of individual highly resistant pneumococcal clones (1, 4, 5, 12-14). A penicillin-resistant MDR clone of serotype 6B was initially isolated from Spain (14) and then spread to Iceland (20). This clone has been designated the Spain^{6B}-2 clone (11). Additional clones of antibiotic-resistant serotype 6B pneumococci have been found in Europe, North America, and South Africa (2, 4, 6, 7, 17, 19); it is not clear whether they share a common ancestor or emerged independently.

Recently, penicillin-susceptible MDR serotype 6B pneumococci, resistant to chloramphenicol, tetracycline, erythromycin, clindamycin, and trimethoprim-sulfamethoxazole (SXT) were isolated from young carriers in Greece. Specifically, this notable serotype 6B resistance pattern was described for the first time in the report of a study that was performed between December 1995 and February 1996 in the city of Patras, southwestern Greece (21). Following the initial isolation of pneumococci with this phenotype, between the years 1996 and 1999, isolates with the same characteristics were recovered from young carriers living in various Greek areas (21, 22). In addition, similar serotype 6B penicillin-susceptible MDR pneumococci were isolated from young carriers in Italy and Israel in 1997 to 1998 (23).

The present study was undertaken to investigate, by using restriction fragment end labeling (RFEL) analysis and penicillin-binding protein (PBP) genotyping, the molecular characteristics of the Greek serotype 6B penicillin-susceptible MDR pneumococci and their genetic relatedness with other serotype 6B pneumococcal isolates, both penicillin-susceptible and penicillin-resistant strains, isolated in various parts of the world.

MATERIALS AND METHODS

Study population. Penicillin-susceptible MDR pneumococci were isolated from nasopharyngeal cultures obtained from children during two independent studies in Greece (21, 22). The first study was performed with 338 children attending seven day-care centers in the city of Patras, southwestern Greece, during the 2-month period from 19 December 1995 to 15 February 1996. In this study, 20 penicillin-susceptible MDR pneumococcal isolates were recovered from 132 carriers. The second study, the 'Hellenic Antibiotic-Resistant Respiratory Pathogens' (HARP) Study, was conducted from 10 February 1997 to 10 February 1999. Nasopharyngeal cultures for *S. pneumoniae* from 2,448 children younger

than 24 months old living in central and southern Greece were performed. Ninety-five (3.9%) of the 2,448 children attended day-care centers. In the HARP Study, screening of the children revealed 46 penicillin-susceptible MDR pneumococci.

Bacteriological procedures. Isolation, identification, and susceptibility testing of the Greek *S. pneumoniae* isolates were performed at the Laboratory of the Division of Pediatric Infectious Disease of the University of Patras as described previously (22). Penicillin and erythromycin MICs for the Greek isolates were determined by the E-test method (AB Biodisk, Solna, Sweden). Susceptibility to clindamycin, chloramphenicol, tetracycline, and SXT was determined by the disk-diffusion method (16). Multidrug resistance was defined as resistance to at least three classes of antimicrobial agents. Pneumococci were serotyped at the Centre National de Référence du Pneumocoque, Créteil, France, by latex agglutination using specific antisera prepared by the investigators at the Centre or at the Laboratory of the Division of Pediatric Infectious Disease of the University of Patras by the capsular swelling method using commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

International collection of serotype 6B S. pneumoniae strains. Twenty-five serotype 6B penicillin-susceptible or -resistant pneumococci, isolated from various parts of the world, were compared to 41 randomly selected Greek penicillin-susceptible MDR isolates of serotype 6B (Table 1). The international collection of serotype 6B pneumococci included strains from Thailand (*n* = 6), the United States (*n* = 5), The Netherlands (*n* = 4), Iceland (*n* = 3), Poland (*n* = 3), Spain (*n* = 1), South Africa (*n* = 1), Finland (*n* = 1), and Cuba (*n* = 1) (7).

RFEL typing. Pneumococcal strain typing by RFEL was done as described by van Steenbergen et al. (24) and adapted by Hermans et al. (8, 9). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for various times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, United Kingdom).

PBP genotyping. Genetic polymorphism of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* of the penicillin-resistant isolates was investigated by restriction fragment length polymorphism (RFLP) analysis as described previously (9). The different PBP genotypes received a three-number code (i.e., 6-12-34), referring to the RFLP patterns of the genes *pbp1a* (6), *pbp2b* (12), and *pbp2x* (34), respectively (18).

Computer-assisted analysis of DNA band patterns. RFEL autoradiographs were converted to images (Image Master DTS; Pharmacia Biotech, Uppsala, Sweden) and

analyzed by computer (Windows version Gelcompar software, version 4; Applied Mathematics, Kortrijk, Belgium). DNA fragments were analyzed as described previously (9). For evaluation of the genetic relatedness of the isolates, we used the following definitions: (i) strains of a particular RFEL type are 100% identical by RFEL analysis; (ii) an RFEL cluster represents a group of RFEL types that differ in only one band (>95% [approximate] genetic relatedness); and (iii) an RFEL lineage represents a group of RFEL types that differ in fewer than four bands (>85% [approximate] genetic relatedness).

RESULTS

Epidemiology of the Greek penicillin-susceptible MDR pneumococci. Of the 66 penicillin-susceptible MDR pneumococci isolated during the two studies in Greece, 79% displayed capsular type 6B. These serotype 6B isolates were invariably susceptible to penicillin and resistant to erythromycin and clindamycin, while 98, 94, and 92% of them were resistant to tetracycline, chloramphenicol, and SXT, respectively.

RFEL analysis. DNA fingerprinting of 41 penicillin-susceptible MDR 6B pneumococci from Greece and an international group of 25 serotype 6B pneumococcal strains revealed 34 distinct types (Fig. 1 and Table 1). The 41 randomly selected Greek isolates represented a total of nine RFEL types. Forty (98%) of the 41 isolates were strongly related, representing a single lineage (genetic relatedness, 91%). RFEL analysis showed six clusters of strains.

Only one strain had a unique type that was genetically unrelated to those of the other isolates (genetic relatedness, < 80%). There was no correlation between the slightly different resistance patterns and the distinct genotypes (Table 1). The group of Greek serotype 6B isolates was closely related (genetic relatedness, ~91%) to the penicillin-resistant MDR Spain^{6B}-2 clone that spread from Spain to Iceland in the late 1980s, as represented by the isolates IC 08, IC 55, and IC 213. The group of Greek serotype 6B isolates was genetically distinct (genetic relatedness < 83%) from the other 22 serotype 6B pneumococci isolated from various parts of the world.

PBP genotyping. All Greek serotype 6B isolates harbored PBP types which were clearly distinct from those of the penicillin-resistant isolates. They invariably displayed a penicillin-susceptible PBP 1A-2B-2X genotype, namely, 2-2-71 (Table 1).

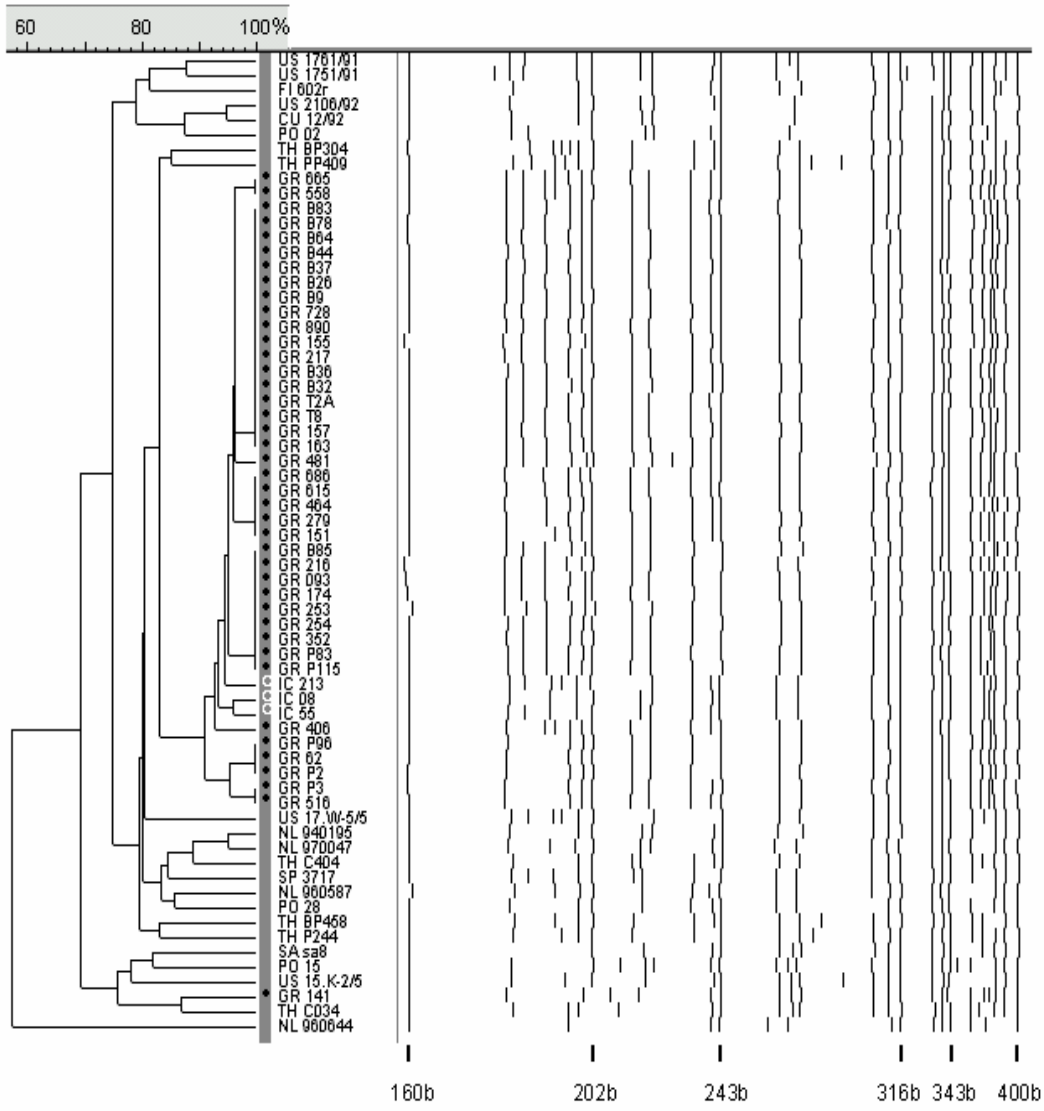


Figure 1. Dendrogram depicting genetic distances and overall relatedness of 6B pneumococcal isolates on the basis of RFLP data. Numbers of base pairs of the single-stranded DNA are indicated below the banding patterns. See Table 1 for the characteristics of the isolates.

DISCUSSION

The molecular and epidemiological factors that control the emergence of antibiotic resistance, increase in resistance levels, and the geographic spread of antibiotic-resistant pneumococci are not fully understood. The present study investigated the emergence and spread of antibiotic resistance in *S. pneumoniae* strains of serotype 6B.

In Greece, serotype 6B is currently the leading capsular type among the penicillin-susceptible, non-β-lactam-resistant pneumococci isolated from young carriers, representing 46% of these isolates (references 21 and 22 and our unpublished data). Seventy-three percent of these serotype 6B pneumococci were resistant to four or five classes of non-β-lactam agents. The penicillin-susceptible pneumococci of serotype 6B that are resistant to several non-β-lactams have to be considered potentially epidemic, since between the years 1996 and 1999, they were recovered from a significant number of carriers living in various areas of Greece. These pneumococci were isolated from children both attending day-care centers and cared for at home.

The relative importance of the clonal spread of strains and horizontal spread of resistance genes depends on the extent of recombination between chromosomal genes in nature. We used the combination of a method that indexes the overall genetic relatedness of isolates and one that can assess whether the isolates carry the same penicillin resistance determinants (altered PBP genes) to distinguish the clonal spread of strains from the horizontal spread of resistance genes.

RFEL fingerprinting showed that the Greek serotype 6B penicillin-susceptible MDR pneumococcal isolates indicate the spread of a single clone. Another molecular technique, BOX PCR fingerprinting, was also tested (data not shown), but it did not increase the discriminatory power. All Greek isolates displayed an identical penicillin-susceptible PBP 1A-2B-2X genotype, namely, 2-2-71. This PBP type has been previously described for penicillin-susceptible pneumococci after application of the same molecular analysis (18).

A comparison of the RFEL patterns of the Greek serotype 6B isolates with those of serotype 6B pneumococci recovered from diverse geographic locales has brought to light a notable finding: the close relatedness between the Greek and Icelandic serotype 6B isolates.

Since the Greek penicillin-susceptible MDR serotype 6B isolates are closely related to the penicillin-resistant MDR serotype 6B pneumococci that spread from Spain to Iceland in the late 1980s, these results suggest that the penicillin-susceptible MDR 6B clone that was found throughout Greece represents the ancestor of one of the three most virulent, internationally disseminated, penicillin-resistant MDR clones, the Spain^{6B}-2 clone (11, 14).

Another molecular technique, multilocus sequence typing (3), has demonstrated that the Greek penicillin-susceptible MDR serotype 6B pneumococcal isolate GR 890, representative of the RFEL cluster of isolates that match their Spanish ancestor 6B, is genetically related to

Table 1. Source as well as genotypic and phenotypic properties of serotype 6B penicillin-susceptible and penicillin-resistant pneumococcal isolates [a]

Strain(s)	Country	Clinical origin	RFEL type	PBP genotype	Penicillin MIC (µg/ml)	Resistance pattern
US 1761/91	United States	MEF	102	16-01-01	1	PEN-ERY-STX
US 1751/91	United States	MEF	221	NA-3-30	1	PEN-ERY-STX
FI 602r	Finland	NA	107	03-02-01	1	PEN-ERY-TET-STX
US 2106/92	United States	Sputum	3	02-01-04	1	PEN-ERY-STX
CU 12/92	Cuba	NP	256	02-02-40	0.03	STX
PO 02	Poland	NA	55	02-05-51	1	PEN
TH BP304	Thailand	NP	220	01-03-13	2	PEN-ERY-TET-STX
TH PP409	Thailand	NP	214	01-06-13	2	PEN-ERY-TET-STX
GR 665, GR 558	Greece	NP	253	02-02-71	0.016	ERY-CLI-TET-CHL-STX
GR B83, GR B78, GR B64, GR B44, GR B37, GR B26, GR B9, GR 728, GR 890, GR 155, GR T8 GR B36, GR B32, GR T2A, GR 157, GR 163	Greece	NP	244	02-02-71	≤ 0.016	ERY-CLI-TET-CHL-STX
GR 217	Greece	NP	244	02-02-71	0.016	ERY-CLI-TET-CHL
GR 481	Greece	NP	254	02-02-71	0.016	ERY-CLI-TET-CHL-STX
GR 686, GR 615, GR 464, GR 151	Greece	NP	249	02-02-71	≤ 0.016	ERY-CLI-TET-CHL-STX
GR 279	Greece	NP	249	02-02-71	0.016	ERY-CLI-TET-CHL
GR B85, GR 216, GR 093, GR 174, GR 253, GR 254, GR 352, GR P83, GR 115	Greece	NP	245	02-02-71	≤ 0.016	ERY-CLI-TET-CHL-STX
IC 213	Iceland	NA	288	01-03-13	2	PEN-ERY-CLI-CHL-STX
IC 08	Iceland	NA	187	01-03-13	1	PEN-ERY-CLI-TET-CHL-STX
IC 55	Iceland	NA	188	01-03-13	0.75	PEN-ERY-CLI-TET-CHL-STX
GR 406	Greece	NP	251	02-02-71	0.016	ERY-CLI-TET-CHL-STX
GR P96, GR 62, GR P2	Greece	NP	246	02-02-71	0.016	ERY-CLI-TET-CHL-STX
GR P3, GR 516	Greece	NP	248	02-02-71	0.016	ERY-CLI-TET-CHL-STX
US 17.W-5/5	United States	NP	218	01-16-47	0.5	PEN-STX
NL 940195	The Netherlands	Blood	255	02-02-02	NA	NA
NL 970047	The Netherlands	Blood	51	04-26-01	0.5	PEN-STX
TH C404	Thailand	NP	203	01-10-13	2	PEN-ERY-TET-STX
SP 3717	Spain	Blood	199	01-03-13	1	PEN-STX
NL 960587	The Netherlands	NP	211	01-03-13	NA	PEN-ERY-TET-STX
PO 28	Poland	NA	96	02-02-55	< 0.1	TET-ERY-STX
TH BP458	Thailand	NP	206	01-10-13	1	PEN-ERY-TET-STX
TH P244	Thailand	NP	215	01-06-13	2	PEN-ERY-TET-STX
SA sa8	South Africa	NA	138	NA-02-27	2	PEN-STX
PO 15	Poland	Blood	156	02-02-09	0.12	PEN-TET-STX
US 15.K-2/5	United States	NP	133	02-02-03	0.5	PEN-TET
GR 141	Greece	NP	247	02-02-71	0.016	ERY-CLI-TET-CHL-STX
TH C034	Thailand	NP	140	12-09-01	0.5	PEN-TET-STX
NL 960644	The Netherlands	NP	97	NA-29-70	2	PEN-ERY-STX

[a] MEF, middle ear fluid; NA, not available; NP, nasopharyngeal culture; PEN, penicillin; ERY, erythromycin; TET, tetracycline; CLI, clindamycin; CHL: chloramphenicol; STX, trimethoprim-sulfamethoxazole.

the Spain^{6B}-2 clone but is not identical (five of seven loci are identical). In addition, isolate GR 890 is closely related to a Finnish penicillin-resistant MDR serotype 6B strain (six of seven loci are identical). The Finnish strain is related to the Spain^{6B}-2 clone, with five of seven loci being identical (B. G. Spratt, personal communication).

Our findings suggest that the penicillin-susceptible MDR 6B clone that was found in Greece represents the ancestor of the penicillin-resistant MDR Spanish-Icelandic clone. An alternative hypothesis to explain these findings is that a Spanish-Icelandic strain restored penicillin susceptibility by mutations and that this genetic event gave rise to the Greek isolates. However, restoration of penicillin susceptibility through reacquisition of penicillin-susceptible PBP genes is a process that, in a natural setting, has not been observed until now.

The clinical implication of the significant spread of this penicillin-susceptible, MDR 6B clone in Greece is important, as it limits the available options for the therapy of pneumococcal infection, particularly in penicillin-allergic patients. In addition, caution should be directed to the fact that penicillin-susceptible MDR 6B strains are not identified when the susceptibility testing is performed by screening only for penicillin susceptibility status. Clinical microbiologists and infectious disease specialists have to be aware that, apart from widely spread penicillin-resistant pneumococci, other MDR, penicillin-susceptible pneumococcal strains circulate widely as well.

REFERENCES

1. Doit, C., B. Picard, C. Loukil, P. Geslin, and E. Bingen. 2000. Molecular epidemiology survey of penicillin-susceptible and -resistant *Streptococcus pneumoniae* recovered from patients with meningitis in France. J. Infect. Dis. 181:1971-1978.
2. Enright, M. C., A. Fenoll, D. Griffiths, and B. G. Spratt. 1999. The three major Spanish clones of penicillin-resistant *Streptococcus pneumoniae* are the most common clones recovered in recent cases of meningitis in Spain. J. Clin. Microbiol. 37:3210-3216.
3. Enright, M. C., and B. G. Spratt. 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 144:3049-3060.
4. Fenoll, A., I. Jado, D. Vicioso, A. Perez, and J. Casal. 1998. Evolution of *Streptococcus pneumoniae* serotypes and antibiotic resistance in Spain: update (1990 to 1996). J. Clin. Microbiol. 36:3447-3454.
5. Gherardi, G., C. G. Whitney, R. R. Facklam, and B. Beall. 2000. Major related sets of antibiotic-resistant pneumococci in the United States as determined by pulsed-field gel electrophoresis and *pbp1a-pbp2b-pbp2x-dhf* restriction profiles. J. Infect. Dis. 181:216-229.
6. Harakeh, H., G. S. Bosley, J. A. Keihlbauc, and B. S. Fields. 1994. Heterogeneity of rRNA gene restriction patterns of multiresistant serotype 6B *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 32:3046-3048.
7. Hermans, P. W., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. Goessens, and R. de Groot. 1997. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. Microb. Drug Resist. 3:243-251.
8. Hermans, P. W., M. Sluijter, K. Elzenaar, A. van Veen, J. J. Schonkeren, F. M. Nooren, W. J. van Leeuwen, A. J. de Neeling, B. van Klingeren, H. A. Verbrugh, and R. de Groot. 1997. Penicillin-resistant *Streptococcus pneumoniae* in The Netherlands: results of a 1-year molecular epidemiologic survey. J. Infect. Dis. 175:1413-1422.
9. Hermans, P. W. M., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 33:1606-1612.
10. Klein, J. O. 1981. The epidemiology of pneumococcal disease in infants and children. Rev. Infect. Dis. 3:246-253.

11. Klugman, K. 1998. Pneumococcal Molecular Epidemiology Network. ASM News 64:371.
12. McDougal, L. K., R. Facklam, M. Reeves, S. Hunter, J. M. Swenson, B. C. Hill, and F. C. Tenover. 1992. Analysis of multiply antimicrobial-resistant isolates of *Streptococcus pneumoniae* from the United States. Antimicrob. Agents Chemother. 36:2176-2184.
13. Muñoz, R., T. R. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J. M. Musser, B. G. Spratt, and A. Tomasz. 1991. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. J. Infect. Dis. 164:302-306.
14. Muñóz, R., J. M. Musser, M. Crain, D. E. Briles, A. Marton, A. J. Parkinson, U. Sorensen, and A. Tomasz. 1992. Geographic distribution of penicillin resistant clones of *Streptococcus pneumoniae*: characterization by penicillin binding protein (PBP) profile, surface protein A typing and multilocus enzyme analysis. Clin. Infect. Dis. 15:112-118.
15. Musher, D. M. 2000. *Streptococcus pneumoniae*, p. 2128-2144. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases, 5th ed. Churchill Livingstone, New York, N.Y.
16. National Committee for Clinical Laboratory Standards. 1997. Performance standards for antimicrobial disk susceptibility tests, 6th ed. Approved standard M2-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
17. Rudolph, K. M., M. J. Crain, A. J. Parkinson, and M. C. Roberts. 1999. Characterization of a multidrug-resistant clone of invasive *Streptococcus pneumoniae* serotype 6B in Alaska using pulsed-field gel electrophoresis and PspA serotyping. J. Infect. Dis. 180:1577-1583.
18. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. Goessens, A. van Belkum, and P. W. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. J. Clin. Microbiol. 36:2248-2253.
19. Smith, A. M., and K. P. Klugman. 1997. Three predominant clones identified within penicillin-resistant South African isolates of *Streptococcus pneumoniae*. Microb. Drug Resist. 3:385-389.
20. Soares, S., K. G. Kristinsson, J. M. Musser, and A. Tomasz. 1993. Evidence for the introduction of a multiresistant clone of serotype 6B *Streptococcus pneumoniae* from Spain to Iceland in the late 1980s. J. Infect. Dis. 168:158-163.
21. Syrogiannopoulos, G. A., I. N. Grivea, N. G. Beratis, A. E. Spiliopoulou, E. L. Fasola, S. Bajaksouzian, P. C. Appelbaum, and M. R. Jacobs. 1997. Resistance patterns of *Streptococcus pneumoniae* from carriers attending day-care centers in southwestern Greece. Clin. Infect. Dis. 25:188-194.
22. Syrogiannopoulos, G. A., I. N. Grivea, T. A. Davies, G. D. Katopodis, P. C. Appelbaum, and N. G. Beratis. 2000. Antimicrobial use and colonization with erythromycin-resistant *Streptococcus pneumoniae* in Greece during the first 2 years of life. Clin. Infect. Dis. 31:887-893.
23. Syrogiannopoulos, G. A., F. Ronchetti, R. Dagan, I. Grivea, M. P. Ronchetti, N. Porat, T. A. Davies, R. Ronchetti, P. C. Appelbaum, and M. R. Jacobs. 2000. Mediterranean clone of penicillin-susceptible, multidrug-resistant serotype 6B *Streptococcus pneumoniae* in Greece, Italy and Israel. Int. J. Antimicrob. Agents, 16:219-224.
24. van Steenbergen, T. J. M., S. D. Colloms, P. W. M. Hermans, J. de Graaff, and R. H. A. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc. Natl. Acad. Sci. USA 92:5572-5576.

Chapter 4.3

Molecular epidemiology of penicillin-susceptible, non-beta-lactam resistant pneumococci isolated from Greek children

D. Bogaert, P.W.M. Hermans, I.N. Grivea, G.S. Katopodis, T.J. Mitchell, M. Sluijter, R. de Groot, N.G. Beratis and G.A. Syrogiannopoulos

J Clin Microbiol 2003; 41(12): 5633-9

ABSTRACT

A total of 128 *Streptococcus pneumoniae* isolates that were susceptible to penicillin but resistant to non-β-lactam agents were isolated from young carriers in Greece and analyzed by antibiotic susceptibility testing, serotyping, restriction fragment end labeling (RFEL), and antibiotic resistance genotyping. The serotypes 6A/B (49%), 14 (14%), 19A/F (11%), 11A (9%), 23A/F (4%), 15B/C (2%), and 21 (2%) were most prevalent in this collection. Of the isolates, 65% were erythromycin resistant, while the remaining isolates were tetracycline and/or trimethoprim-sulfamethoxazole resistant. Fifty-nine distinct RFEL types were identified. Twenty different RFEL clusters, harboring 2 to 19 strains each, accounted for 76% of all strains. Confirmatory multilocus sequence typing analysis of the genetic clusters showed the presence of three international clones (Tennessee^{23F}-4, England^{14}-9, and Greece^{6B}-22) representing 30% of the isolates. The *erm*(B) gene was present in 70% of the erythromycin-resistant isolates, whereas 18 and 8% contained the *mef*(A) and *mef*(E) genes, respectively. The pneumococci representing *erm*(B), *erm*(A), and *mef* genes belonged to distinct genetic clusters. In total, 45% of all isolates were tetracycline resistant. Ninety-six percent of these isolates contained the *tet*(M) gene. In conclusion, penicillin-susceptible pneumococci resistant to non-β-lactams are a genetically heterogeneous group displaying a variety of genotypes, resistance markers, and serotypes. This suggests that multiple genetic events lead to non-β-lactam-resistant pneumococci in Greece. Importantly, most of these genotypes are capable of disseminating within the community.

INTRODUCTION

Streptococcus pneumoniae is a common cause of invasive diseases, such as meningitis and bacteremia, and of respiratory tract infections (5). *S. pneumoniae* isolates that are resistant to penicillin and/or non-β-lactam agents have been frequently reported (6, 12, 35). Resistance of *S. pneumoniae* to erythromycin and the other macrolides is increasing in many parts of the world (15, 17). Strains resistant to erythromycin are also resistant to azithromycin, clarithromycin, and roxithromycin (37). This global increase in antibiotic-resistant and especially multidrug-resistant pneumococci is the result of the spread of various highly resistant pneumococcal clones (7, 25).

In Greece, the emergence of antibiotic resistance among pneumococcal isolates was recognized in the mid-1990s (30). Recently, pneumococcal isolates susceptible to penicillin and resistant to chloramphenicol, tetracycline, erythromycin, clindamycin, and trimethoprim-sulfamethoxazole (SXT) have been isolated from young Greek carriers. During the period from December 1995 to February 1996, 24% of the pneumococci isolated from healthy carriers attending day care centers were demonstrated to be penicillin-susceptible, non-β-lactam-resistant isolates (29). In a recent study in which 2,448 children younger than 2 years old living in various areas in Greece were screened during a 2-year period (1997 to 1999) for pneumococcal carriage, 15% of the pneumococci appeared to have reduced susceptibility to non-β-lactam agents (31).

The present study was undertaken to investigate the molecular epidemiological characteristics of the Greek pneumococci susceptible to penicillin but resistant to erythromycin and/or other non-β-lactam agents. Furthermore, penicillin, erythromycin, and tetracycline resistance determinants were studied at a molecular level.

MATERIALS AND METHODS

Bacterial isolates. We studied a collection of 128 *S. pneumoniae* isolates susceptible to penicillin but resistant to erythromycin and/or other non-β-lactam agents; the isolates were recovered from nasopharyngeal cultures obtained from children during two independent studies in Greece (3, 30, 31). The first study was performed in 338 children attending seven day care centers in the city of Patras in southwestern Greece during the 2-month period from December 1995 to February 1996. In this study, 30 penicillin-susceptible pneumococci resistant to one or more non-β-lactam agents were recovered from 132 carriers. Of these 30 *S. pneumoniae* isolates, 26 were available for further analysis in the present investigation. The second study, the ‘Hellenic Antibiotic-Resistant Respiratory Pathogens’ (HARP) study,

was conducted from February 1997 to February 1999. Nasopharyngeal cultures for *S. pneumoniae* were performed in 2,448 children younger than 2 years old living in central and southern Greece. Ninety-five (3.9%) of the 2,448 children attended day care centers. In the HARP study, screening of the children revealed 119 pneumococci which were penicillin susceptible but resistant to non-β-lactam agents. Of these 119 pneumococci, 102 were available for further analysis. Thirteen of these pneumococci were isolated from children attending day care. Only two of these children attended the same day care center.

Bacteriological procedures. Isolation, identification, and susceptibility testing of the Greek *S. pneumoniae* isolates were performed by applying standard methods as described previously (30, 31). Penicillin and erythromycin MICs for the Greek isolates were determined by the E-test method (AB Biodisk, Solna, Sweden). Susceptibility to clindamycin, chloramphenicol, tetracycline, and SXT was determined by the disk diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (21). Multidrug resistance was defined as resistance to three or more classes of antimicrobial agents. Pneumococci were serotyped by the capsular swelling method or the latex agglutination technique (30, 33).

Penicillin-binding protein (PBP) genotyping. Genetic polymorphism of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* of the pneumococcal isolates was investigated by restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified genes, as described previously (13).

Detection and analysis of the erm*(B) *and mef genes. To detect the presence of *erm*(B) within the pneumococcal isolates, we used the protocol of Sutcliffe et al. (28). In summary, we amplified the genes by PCR and analyzed the amplified DNA products by agarose gel electrophoresis. The presence of the *mef* gene was also detected by PCR (28). In order to discriminate between *mef*(A) and *mef*(E), PCR-RFLP analysis was performed by the method of Del Grosso et al. (8). The amplicon was digested using *Bam*HI and *Dra*I. The *mef*(A) gene contains a single *Bam*HI site, which is absent in the *mef*(E) gene. Digestion of *mef*(A) and *mef*(E) with *Dra*I yields two and three fragments, respectively.

Detection of the tet*(M) *and tet*(O) *genes. In order to discriminate between *tet*(M) and *tet*(O), a PCR-RFLP analysis was performed as described previously (2). In summary, a PCR mix was made of 25-μl reaction buffer containing 0.5 U of thermostable DNA polymerase, diluted in the buffer supplied by the manufacturer (Integro, Leuvenheim, The Netherlands), 0.2 mM (each) deoxynucleoside triphosphate, 1.5 mM MgCl2, 10 pmol of each primer, and 10 to 50 ng of pneumococcal DNA. Amplification cycling in a programmable thermal controller (PTC-100; MJ Research, Watertown, Mass.) consisted of

the following steps: predenaturation for 1 min at 94°C, followed by 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. Amplification was finished after 3 min at 72°C. A 1.5% agarose gel in 0.045 M Tris-borate buffer with 0.001 M EDTA (0.5x TBE buffer) containing 0.1 μg of ethidium bromide per ml was used to visualize the PCR products.

RFEL typing. Pneumococcal strain typing by restriction fragment end labeling (RFEL) was performed by the method of van Steenbergen et al. (36) as adapted by Hermans et al. (13). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [α-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, United Kingdom).

Computer-assisted analysis of DNA band patterns. RFEL autoradiographs were converted to images (Image Master DTS; Pharmacia Biotech, Uppsala, Sweden) and analyzed with a computer (Windows version Gelcompar software version 4; Applied Math, Kortrijk, Belgium). DNA fragments were analyzed as described previously (26). For evaluation of the genetic relatedness of the isolates, we used the following definitions: (i) isolates of a particular RFEL type are 100% identical by RFEL analysis; (ii) an RFEL cluster represents a group of RFEL types that differ in only one band (approximately >95% genetic relatedness); (iii) an RFEL lineage represents a group of RFEL types that differ in less than four bands (approximately >85% genetic relatedness).

International comparison. The Greek genotypes were compared with an international collection of pneumococcal isolates representing 751 distinct RFEL types originating from 17 different countries in America, Europe, Africa, and Asia (2, 12; M. Sluijter, unpublished observations). The international collection includes the first 16 international pandemic clones described by the Pneumococcal Molecular Epidemiological Network in 2000 (http://www.pneumo.com/physician/pmen_clone_collection.asp) (18).

MLST. The genotypes of all clusters were verified by multilocus sequence typing (MLST) analysis. For this purpose, a fully automated method for MLST was used as described previously (14), and one or two isolates per cluster were analyzed. The MLST types were compared with the global database at www.mlst.net.

RESULTS

The serotypes 6A/B (49%), 14 (14%), 19A/F (11%), 11A (9%), 23A/F (4%), 15B/C (2%), and 21 (2%) were most prevalent in the collection of 128 Greek pneumococcal isolates. All isolates were invariably susceptible to penicillin, while 84 (65%), 78 (60%), 69 (54%), 63 (49%), and 62 (48%) isolates were resistant to erythromycin, SXT, tetracycline, chloramphenicol, and clindamycin, respectively.

The isolates were classified as penicillin-susceptible erythromycin-resistant (65%) and penicillin-susceptible erythromycin-susceptible (35%) isolates. The latter group was represented by tetracycline- and/or SXT-resistant pneumococci. The 84 penicillin-susceptible erythromycin-resistant pneumococci displayed capsular types 6B (56%), 14 (18%), 19F (13%), 11A (11%), 10A (1.2%), and 15C (1.2%). Of these erythromycin-resistant isolates, 59 (70%), 15 (18%), and 7 (8.3%) isolates carried the *erm*(B), *mef*(A), and *mef*(E) erythromycin resistance determinants, respectively. In four erythromycin-resistant isolates belonging to a serotype 11A clone, an *erm*(A) gene was previously detected (32). Furthermore, 62 (74%), 57 (68%), 46 (55%), and 42 (50%) of the 84 penicillin-susceptible erythromycin-resistant pneumococci, were also resistant to clindamycin, tetracycline, SXT, and chloramphenicol, respectively. Clindamycin resistance was always identified in combination with erythromycin resistance. All 62 clindamycin- and erythromycin-resistant isolates carried an *erm* resistance gene. Of the 54 erythromycin-resistant isolates that were resistant to tetracycline, 51 carried the *tet*(M) resistance gene. The three remaining isolates were negative for both the *tet*(M) and *tet*(O) genes.

The 44 penicillin-susceptible erythromycin-susceptible isolates belonged to serotypes 6B (29%), 23F (11%), 14 (6.7%), 21 (6.7%), 1 (4.4%), 6A (4.4%), 15B (4.4%), 18C (4.4%), 19A (4.4%), 8 (2.2%), 10A (2.2%), 11A (2.2%), 16F (2.2%), 19F (2.2%), 20 (2.2%), and 24F (2.2%) and nontypeable serotypes (4.4%).

Sulfamethoxazole resistance was identified in 34 of the 44 penicillin-susceptible erythromycin-susceptible isolates (78%), whereas tetracycline resistance was identified in 12 (27%) isolates. All but one carried the *tet*(M) resistance gene. All isolates were resistant to a single agent except for one isolate that was resistant to tetracycline and chloramphenicol and two isolates that were resistant to tetracycline and SXT.

Fourteen distinct PBP genotypes were observed. Of the 128 isolates, 76 (59%), 24 (19%), and 4 (3.1%) displayed a known penicillin-susceptible PBP 1A-2B-2X genotype, being 2-2-71, 2-2-3, and 2-2-2, respectively. The remaining isolates displayed alterations in *pbp2x* (11 distinct types), *pbp2b* (2 types), and *pbp1a* (1 type).

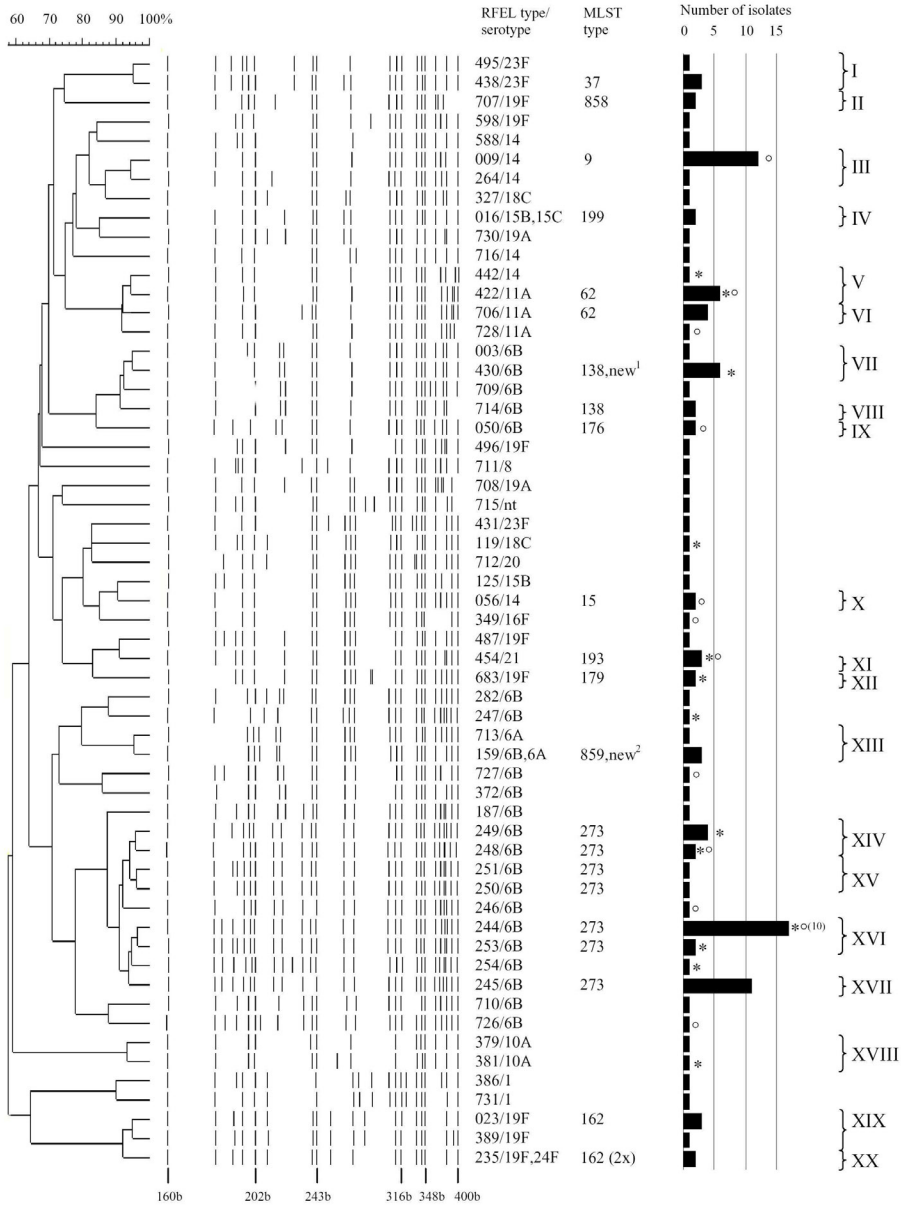


Figure 1. Dendrogram of the 59 RFEL types observed among the 128 Greek *S. pneumoniae* nasopharyngeal isolates. The molecular sizes of reference bands (in bases [b]), serotypes, PBP types, RFEL types, MLST types, number of isolates per RFEL type, clusters, and cluster codes are depicted. One or two strains per RFEL clusters were analyzed by MLST. Day care center isolates from the 1995 to 1996 study (asterisks) and day care center isolates from the 1997 to 1999 study (degree symbols) are indicated. If more than one day care center isolate belongs to a specific genotype, the total number is displayed in parentheses. Two new strains with new MLST types (new1 and new2) are indicated. These two strains have been submitted to the MLST database. nt, nontypeable.

RFEL analysis divided the 128 penicillin-susceptible isolates, which were resistant to one or more non-β-lactam agents into 59 distinct RFEL genotypes (Fig. 1). Ninety-nine isolates belonged to 20 genetic clusters, representing 77% of the isolates and varying in size from 2 to 19 isolates (Table 1). The average cluster size was 5.0 isolates. Five of the 20 clusters contained two serotypes, while the remaining clusters contained only one serotype each. The 20 clusters belonged to 11 lineages. The three largest clusters were cluster III, XVI, and XVII. Cluster III consisted of 13 (10%) isolates and was identical to the pandemic clone England^{14}-9 which was confirmed by MLST analysis (Fig. 1 and Table 1). This cluster belonged to a single predominant lineage of 14 genetically related isolates, representing three RFEL genotypes and harboring the serotypes 11A, 14, and 18C. The serotype 14 pneumococci carried the *mef*(A) erythromycin resistance determinant and had a low to moderate level of resistance to erythromycin. The remaining pneumococci were resistant to SXT.

Clusters XVI (19 isolates) and XVII (11 isolates) belonged, together with cluster XIV (6 isolates) and XV (2 isolates), to one predominant lineage, representing 10 RFEL types and all harboring the serotype 6B. Most isolates were resistant to erythromycin, clindamycin, tetracycline, chloramphenicol, and SXT. These pneumococci carried the *erm*(B) erythromycin resistance determinant and had a high level of resistance to erythromycin. The latter clusters were identical to the pandemic clone Greece^{6B}-22 and closely related to the penicillin-resistant MDR clone Spain^{6B}-2 that has spread from Spain to Iceland in the late 1980s (29).

Clusters I (three isolates) and XIX (three isolates) represented genotypes identical to the pandemic clone Tennessee^{23F}-4 and closely related to the pandemic clone Spain^{9V}-3 (six of seven alleles) as described by the Pneumococcal Molecular Epidemiology Network. The genetic relatedness of the observed MLST profiles of the 20 RFEL clusters is depicted in Fig. 2.

The 39 isolates recovered from children attending day care centers displayed 21 different genotypes; 10 isolates displayed unique genotypes, whereas the remaining 29 isolates displayed 13 genotypes belonging to 11 clusters. Cluster XVI contained nine isolates from one day care center, whereas cluster XVII contained three isolates from a second single day care center. The remaining clustering day care center isolates originated from different day care centers (Fig. 1).

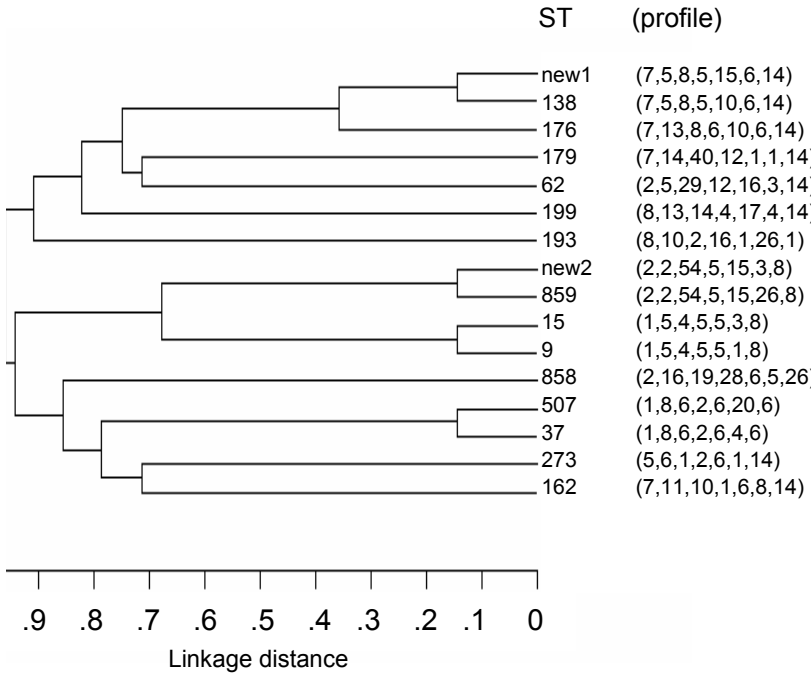


Figure 2. Genetic relatedness of the 15 MLST sequence types (ST) observed within the 20 RFEL clusters.

DISCUSSION

We evaluated 128 *S. pneumoniae* isolates that were susceptible to penicillin but resistant to non-β-lactam agents from young carriers in Greece by antibiotic susceptibility testing, serotyping, RFEL, and antibiotic resistance genotyping. In general, the isolates could be divided into two groups: one group consisting of 84 erythromycin-resistant isolates, while the second group of 44 isolates were erythromycin-susceptible. Multidrug resistance, i.e., resistance to three or more different classes of antibiotics, was seen predominantly within the first group of isolates, whereas the erythromycin-susceptible isolates predominantly displayed monodrug resistance to tetracycline or sulfamethoxazole. Furthermore, clustering of isolates, which is the result of horizontal spread of the isolates within the community, was higher among multidrug-resistant genotypes. This is in line with previous findings where multidrug-resistant clones have shown to spread from country to country (3, 4, 7, 20, 22, 25, 27, 35). This has led to the classification of pandemic clones by the Pneumococcal Molecular Epidemiological Network (18). Although the majority of these pandemic clones are

Table 1. Molecular and phenotypic characteristics of the 129 Greek pneumococcal isolates. Number (nr) of genotypes, size, serotypes, resistance patterns and genotypes are presented.

RFEL cluster	Number of isolates\ RFEL types	Serotypes (nr of strains)	Resistance pattern* (nr of strains)	PBP type (nr of strains)	E/T genotype (nr of strains)
I	4\2	23F(4)	S	2-2-3	
II	2\1	19F(2)	TECLS	2-2-3	*erm*(B)*/ tet*(M)
			ECLS		*erm*(B)
III	13/2	14(13)	E(13)	2-2-71	*mef*(A)
IV	2\1	15B	S	2-2-44	
		15C	ECL	2-2-3	*erm*(B)
V	7\2	11A(6) 14(1)	TECL(4) S(2) E(1)	2-2-3(6) 2-2-71(1)	*erm*(B) *(*4),*tet*(M)(4*)* *mef*(E) (1)
VI	4\1	11A (4)	TECL	2-2-3	*erm*(A)*, tet*(M)
VII	7\2	6B(7)	S(6)	2-2-71	*erm*(B) (1),*tet*(M)(1)
			E(1)		*mef*(E)
VIII	2\1	6B (2)	S(2)	2-2-71	
IX	2\1	6B (2)	CTECLS	2-2-71	unknown
			S		
X	2\1	14(2)	S(2)	2-2-71	
XI	3\1	21	S(3)	2-2-7	
				2-2-98	
XII	2\1	19F(2)	TECL	2-2-98	*erm*(B) (2)
					tet(M) (2)
XIII	4\2	6B (2)	T(3), TS(1)	2-5-22(2)	*tet*(M) (4)
		6A (2)		2-5-99(1)	
				2-2-71(1)	
XIV	6\2	6B (6)	CTECLS(5)	2-2-71	*erm*(B) (6)
			CTECL		*tet*(M) (6)
XV	2\2	6B (2)	CTECLS	2-2-71	*erm*(B) (2)
			CTECL		*tet*(M) (2)
XVI	19\2	6B (19)	CTECLS (17)	2-2-71 (18)	*erm*(B) (19)
			CTECL (1)	0-0-0 (1)	*tet*(M) (18)
			TECLS (1)		
XVII	11\1	6B (11)	CTECLS (11)	2-2-71 (10)	*erm*(B) (11)
				0-0-0 (1)	*tet*(M) (11)
XVIII	2/2	10A	TECL(1) S	2-2-71 2-2-2	*erm*(B)(1), *tet*(M)(1)
XIX	4\2	19F (4)	E	2-2-2 (4)	*mef*(A) (1)
					mef(E) (3)
XX	2\1	19F (1)	S	2-2-2 (1)	
		24F (1)		2-2-3 (1)	

* C, chloramphenicol; T, tetracyclin; E, erythromycin; C, clindamycin; S, trimethoprim-sulfamethoxazole

penicillin-resistant, our study as well as previous studies have shown that this is not a prerequisite for clonal spread.

With emerging non-β-lactam resistance among pneumococci, the spread of penicillin-susceptible non-β-lactam-resistant pneumococci has become apparent (1, 24, 29, 34). This is underlined by our observation that, in addition to one large lineage of serotype 6B isolates that were mostly susceptible to penicillin and resistant to erythromycin, tetracycline, chloramphenicol, and sulfamethoxazole, 16 smaller clusters were found. In total, 30% of the isolates displayed a genotype identical to those of the pandemic clones Tennessee^{23F}-4, England^{14}-9, and Greece^{6B}-22 as reported by the Pneumococcal Molecular Epidemiological Network. These findings clearly indicate heterogeneity among the penicillin-susceptible, non-β-lactam-resistant isolates. Furthermore, these clusters represent not only the conjugate vaccine serotypes 6B, 14, 19F, and 23F, but also non-vaccine serotypes, such as 11A, 15B/C, and 21. This observation implicates that non-vaccine serotypes are also able to spread among children; hence, vaccination with pneumococcal conjugate vaccines is not a solution for the emergence of multidrug resistance among pneumococcal isolates.

In this study, 70% of the erythromycin-resistant isolates harbored the *erm*(B) gene, while 18 and 8% of the isolates contained *mef(*A) and *mef*(E) genes, respectively. These data differ from previous findings made by Reinert and coworkers, who observed an almost equal distribution of *erm*(B) (43%) and *mef*(E) (56%) genes among erythromycin-resistant isolates in Germany (23). However, our data are in line with a recent study performed in Italy and Vietnam where the majority of the strains also displayed the *erm*(B) gene (2, 19). In the four low-level erythromycin-resistant isolates displaying an identical RFEL genotype and serotype 11A, the *erm*(A) gene was previously identified (32).

Genetic analysis of tetracycline resistance genes *tet*(M) and *tet*(O) revealed that the *tet*(M) gene was exclusively observed in 91% of the Greek isolates. This is in line with the Vietnamese study where the *tet*(M) gene was also exclusively observed (2). In contrast to other studies, the remaining tetracycline-resistant isolates did not harbor the *tet*(O) gene (16). So far, no other tetracycline resistance determinants have been described; therefore, the underlying mechanism remains unclear. Also in line with the Vietnamese study is the isolation of *tet*(M)-containing but tetracycline-susceptible strains in Greece, suggesting the presence of a nonfunctional or unexpressed tetracycline resistance gene (2, 9). A similar phenomenon was seen for the PBP genes. Though the majority of the isolates displayed (known) susceptible genotypes, 10% of the isolates displayed alterations in one or two of the three major PBP genes, *pbp1a*, *pbp2b*, and *pbp2x*, which often implicates intermediate penicillin resistance (10). In our study, the observed alterations in *pbp1a*, *pbp2b*, and four of

the *pbp2x* alterations were identified previously in non-penicillin-susceptible pneumococci isolated in Thailand, the United States, and The Netherlands (11). Although these alterations have shown to be related to intermediate susceptibility, in all cases an additional alteration in one or two of the PBP genes was present (Sluijter, unpublished). These findings support the hypothesis that not all genetic alterations lead to amino acid substitutions or to substitutions which are relevant for penicillin resistance.

We hypothesize that the ongoing antibiotic pressure will continue the process of alteration and spread of resistance genes among pneumococci. Our study underlines that antibiotic resistance in any form and irrespective of its serotype is of benefit for *S. pneumoniae* with respect to survival and spread in the community. This implies that despite the introduction of new and effective pneumococcal conjugate vaccines, restrictive use of antibiotics will be of major importance.

REFERENCES

1. 1. 1997. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep 46:1-24.
2. Bell, J. M., J. D. Turnidge, and R. N. Jones. 2002. Antimicrobial resistance trends in community-acquired respiratory tract pathogens in the Western Pacific Region and South Africa: report from the SENTRY antimicrobial surveillance program, (1998-1999) including an in vitro evaluation of BMS284756. Int J Antimicrob Agents 19:125-32.
3. Bogaert, D., N. T. Ha, M. Sluijter, N. Lemmens, R. De Groot, and P. W. Hermans. 2002. Molecular epidemiology of pneumococcal carriage among children with upper respiratory tract infections in Hanoi, Vietnam. J Clin Microbiol 40:3903-8.
4. Bogaert, D., G. A. Syrogiannopoulos, I. N. Grivea, R. de Groot, N. G. Beratis, and P. W. Hermans. 2000. Molecular Epidemiology of Penicillin-Nonsusceptible *Streptococcus pneumoniae* among Children in Greece. J Clin Microbiol 38:4361-4366.
5. Castaneda, E., I. Penuela, M. C. Vela, and A. Tomasz. 1998. Penicillin-resistant *Streptococcus pneumoniae* in Colombia: presence of international epidemic clones. Colombian pneumococcal study group. Microb Drug Resist 4:233-9.
6. Corso, A., E. P. Severina, V. F. Petruk, Y. R. Mauriz, and A. Tomasz. 1998. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. Microb Drug Resist 4:325-37.
7. Davies, T., R. V. Goering, M. Lovgren, J. A. Talbot, M. R. Jacobs, and P. C. Appelbaum. 1999. Molecular epidemiological survey of penicillin-resistant *Streptococcus pneumoniae* from Asia, Europe, and North America. Diagn Microbiol Infect Dis 34:7-12.
8. Del Grosso, M., F. Iannelli, C. Messina, M. Santagati, N. Petrosillo, S. Stefani, G. Pozzi, and A. Pantosti. 2002. Macrolide efflux genes mef(A) and mef(E) are carried by different genetic elements in *Streptococcus pneumoniae*. J Clin Microbiol 40:774-8.
9. Doherty, N., K. Trzcinski, P. Pickerill, P. Zawadzki, and C. G. Dowson. 2000. Genetic diversity of the tet(M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. Antimicrob Agents Chemother 44:2979-84.
10. Hakenbeck, R., T. Grebe, Z. h. D, and J. B. Stock. 1999. beta-Lactam resistance in *Streptococcus pneumoniae*: penicillin-binding proteins and non-penicillin-binding proteins. Mol Microbiol 33:673-678.
11. Hermans, P. W., K. Overweg, M. Sluijter, and R. de Groot. 2000. Penicillin-resistant *Streptococcus pneumoniae*: An International Molecular Epidemiological Study, p. 457-566. *In* P. D. Alexander Tomasz (ed.), *Streptococcus pneumoniae*. Molecular Biology and Mechanisms of Disease. Mary Ann Liebert, Inc., New York.
12. Hermans, P. W., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. Goessens, and R. de Groot. 1997. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. Microb Drug Resist 3:243-51.
13. Hermans, P. W., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J Clin Microbiol 33:1606-12.
14. Jefferies, J., S. C. Clarke, M. A. Diggle, A. Smith, C. Dowson, and T. Mitchell. 2003. Automated pneumococcal

MLST using liquid-handling robotics and a capillary DNA sequencer. Mol Biotechnol 24:303-8.
15. Leclerq, S., J. S. Harms, G. M. Rosinha, V. Azevedo, and S. C. Oliveira. 2002. Induction of a th1-type of immune response but not protective immunity by intramuscular DNA immunisation with Brucella abortus GroEL heat-shock gene. J Med Microbiol 51:20-6.
16. Luna, V. A., D. B. Jernigan, A. Tice, J. D. Kellner, and M. C. Roberts. 2000. A novel multiresistant *Streptococcus pneumoniae* serogroup 19 clone from Washington State identified by pulsed-field gel electrophoresis and restriction fragment length patterns. J Clin Microbiol 38:1575-80.
17. Lynch, I. J., and F. J. Martinez. 2002. Clinical relevance of macrolide-resistant *Streptococcus pneumoniae* for community-acquired pneumonia. Clin Infect Dis 34 Suppl 1:S27-46.
18. McGee, L., L. McDougal, J. Zhou, B. G. Spratt, F. C. Tenover, R. George, R. Hakenbeck, W. Hryniewicz, J. C. Lefevre, A. Tomasz, and K. P. Klugman. 2001. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. J Clin Microbiol 39:2565-71.
19. Montanari, M. P., M. Mingoia, I. Cochetti, and P. E. Varaldo. 2003. Phenotypes and genotypes of erythromycin-resistant pneumococci in Italy. J Clin Microbiol 41:428-31.
20. Munoz, R., T. J. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J. M. Musser, B. G. Spratt, and et al. 1991. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. J Infect Dis 164:302-6.
21. NCCLS. 1999. Performance standards for antimicrobial susceptibility testing: ninth informational supplement, M 100-S9, 19 (1). NCCLS.
22. Overweg, K., P. W. Hermans, K. Trzcinski, M. Sluijter, R. de Groot, and W. Hryniewicz. 1999. Multidrug-resistant *Streptococcus pneumoniae* in Poland: identification of emerging clones. J Clin Microbiol 37:1739-45.
23. Reinert, R. R., A. Queck, A. Kaufhold, M. Kresken, and R. Lutticken. 1995. Antimicrobial resistance and type distribution of *Streptococcus pneumoniae* isolates causing systemic infections in Germany, 1992-1994. Clin Infect Dis 21:1398-401.
24. Ronchetti, M. P., R. Merolla, S. Bajaksouzian, G. Violo, R. Ronchetti, and M. R. Jacobs. 1998. Antimicrobial susceptibility of *Streptococcus pneumoniae* from children attending day-care centers in a central Italian city. Clin Microbiol Infect 4:622-626.
25. Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck. 1992. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. Infect Immun 60:4119-26.
26. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. Goessens, A. van Belkum, and P. W. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. J Clin Microbiol 36:2248-53.
27. Soares, S., K. G. Kristinsson, J. M. Musser, and A. Tomasz. 1993. Evidence for the introduction of a multiresistant clone of serotype 6B *Streptococcus pneumoniae* from Spain to Iceland in the late 1980s. J Infect Dis 168:158-63.
28. Sutcliffe, J., T. Grebe, A. Tait-Kamradt, and L. Wondrack. 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 40:2562-6.
29. Syrogiannopoulos, G. A., D. Bogaert, I. N. Grivea, N. G. Beratis, R. R. De Groot, and P. W. Hermans. 2001. Molecular epidemiology of penicillin-susceptible, multidrug-resistant serotype 6B pneumococci isolated from children in Greece. J Clin Microbiol 39:581-5.
30. Syrogiannopoulos, G. A., I. N. Grivea, N. G. Beratis, A. E. Spiliopoulou, E. L. Fasola, S. Bajaksouzian, P. C. Appelbaum, and M. R. Jacobs. 1997. Resistance patterns of *Streptococcus pneumoniae* from carriers attending day-care centers in southwestern Greece. Clin Infect Dis 25:188-94.
31. Syrogiannopoulos, G. A., I. N. Grivea, T. A. Davies, G. D. Katopodis, P. C. Appelbaum, and N. G. Beratis. 2000. Antimicrobial use and colonization with erythromycin-resistant *Streptococcus pneumoniae* in greece during the first 2 years of life [In Process Citation]. Clin Infect Dis 31:887-93.
32. Syrogiannopoulos, G. A., I. N. Grivea, A. Tait-Kamradt, G. D. Katopodis, N. G. Beratis, J. Sutcliffe, P. C. Appelbaum, and T. A. Davies. 2001. Identification of an erm(A) erythromycin resistance methylase gene in *Streptococcus pneumoniae* isolated in Greece. Antimicrob Agents Chemother 45:342-4.
33. Syrogiannopoulos, G. A., G. D. Katopodis, I. N. Grivea, and N. G. Beratis. 2002. Antimicrobial use and serotype distribution of nasopharyngeal *Streptococcus pneumoniae* isolates recovered from Greek children younger than 2 years old. Clin Infect Dis 35:1174-82.
34. Syrogiannopoulos, G. A., F. Ronchetti, R. Dagan, I. Grivea, M. P. Ronchetti, N. Porat, T. A. Davies, R. Ronchetti, P. C. Appelbaum, and M. R. Jacobs. 2000. Mediterranean clone of penicillin-susceptible, multidrug-resistant serotype 6B *Streptococcus pneumoniae* in greece, italy and israel. Int J Antimicrob Agents 16:219-24.
35. Tomasz, A., A. Corso, E. P. Severina, G. Echaniz-Aviles, M. C. Brandileone, T. Camou, E. Castaneda, O. Figueroa, A. Rossi, and J. L. Di Fabio. 1998. Molecular epidemiologic characterization of penicillin-resistant *Streptococcus pneumoniae* invasive pediatric isolates recovered in six Latin-American countries: an overview. PAHO/Rockefeller University Workshop. Pan American Health Organization. Microb Drug Resist 4:195-207.
36. van Steenbergen, T. J., S. D. Colloms, P. W. Hermans, J. de Graaff, and R. H. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc Natl Acad Sci U S A 92:5572-6.
37. Widdowson, C. A., and K. P. Klugman. 1998. Emergence of the M phenotype of erythromycin-resistant pneumococci in South Africa. Emerg Infect Dis 4:277-81.

Chapter 4.4

Molecular characteristics of penicillin-binding protein genes of penicillin-nonsusceptible *Streptococcus pneumoniae* isolated in The Netherlands

K. Overweg, D. Bogaert, M. Sluijter, R. de Groot and P.W.M. Hermans

Microb Drug Resist 2001; 7(4): 323-34

ABSTRACT

Recently, a nation-wide molecular epidemiologic survey of penicillin-nonsusceptible *Streptococcus pneumoniae* has been performed in The Netherlands. In the current study, we analyzed the *pbp* genes from these clinical isolates in detail. The pneumococcal strains were selected on the basis of differences in restriction fragment length polymorphism (RFLP) patterns of the complete genes *pbp1a, pbp2b* and *pbp2x*, representing 8, 7 and 10 distinct patterns, respectively. We characterized specific gene parts of *pbp1a* (nucleotide position 1741 to 2229), *pbp2b* (nucleotide position 1211 to 1714) and *pbp2x* (nucleotide position 1241 to 1786). Classification based upon sequence analysis of these *pbp* fragments correlated well with the classification according to RFLP analysis. Sequence analysis of *pbp2b* enables a refinement of the classification based on RFLP analysis. However, sequence analysis of *pbp1a* and *pbp2x* was less discriminatory compared to RFLP analysis. The mutations in the *pbp* sequences of the Dutch isolates invariably matched with the mutations described in *pbp* sequences of penicillin-nonsusceptible pneumococci isolated in other countries. This observation supports the hypothesis that multiple clones of penicillin-resistant pneumococci have been imported and spread in The Netherlands. Interestingly, novel combinations of mosaic structures were also identified indicating horizontal exchange of *pbp* gene parts among penicillin-nonsusceptible pneumococci.

INTRODUCTION

Streptococcus pneumoniae is a common cause of serious and life-threatening infections, such as pneumonia, bacteremia and of non-invasive infections such as otitis media and sinusitis. Ever since the beginning of the antibiotic era, penicillin has been the first choice drug against pneumococcal infections. However, in the last 20 years, increasing numbers of penicillin-nonsusceptible pneumococcal clinical isolates with rising MIC values have been isolated (11).

Penicillin resistance in *S. pneumoniae* has emerged through the development of altered penicillin-binding proteins (PBPs), which show a decreased affinity for penicillin and other β-lactam antibiotics. PBPs are membrane-bound D,D-peptidases, which catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. The β-lactam antibiotics interact with the PBPs and form a covalent penicilloyl complex via the active-site serine resulting in an inactivated enzyme. In penicillin-susceptible pneumococci, this interaction results in cellular lysis (4, 11). *S. pneumoniae* contains six PBPs: the high molecular weight proteins PBP1a, PBP1b, PBP2a, PBP2b and PBP2x, and the low molecular weight protein PBP3. In clinical isolates, altered PBP2b and PBP2x are the primary resistance determinants, i.e. they confer low-level resistance when introduced into sensitive strains, whereas alterations in PBP1a contribute to an increase in resistance levels (10, 13). The alterations in the PBPs are due to the formation of a mosaic structure of the genes, presumably as a result of horizontal gene transfer. The *pbp* genes of nonsusceptible isolates are composed of mosaic blocks that share identity with *pbp* genes from sensitive isolates and blocks that are identical to *pbp* genes from *Streptococcus mitis, Streptococcus sanguis* and *Streptococcus oralis* (23). It is therefore suggested that the mosaic *pbp* genes are the result of interspecies recombination. Some mosaic *pbp* genes are composed of a single block from viridans streptococci, whereas others are the result of complex multiple recombination events involving several donors (6). The presence of an enormous diversity of mosaic *pbp* genes suggests that resistance to penicillin has arisen on many independent occasions. These recombination events have resulted in multiple amino acid substitutions in the PBPs. Several amino acids are essential for the interaction of the PBPs with β-lactam antibiotics. Substitutions of these amino acids often result in a reduction of affinity and consequently in reduced penicillin susceptibility. Most mutations which confer resistance occur close to the motifs SXN or KT/SG, or close to the active-site serine which is part of the conserved STMK motif (11, 12).

We have recently investigated the epidemiologic characteristics of penicillin-nonsusceptible pneumococci in The Netherlands in a nation-wide survey (16). The penicillin-nonsusceptible

clinical isolates have been analyzed by restriction fragment length polymorphism (RFLP) analysis of the genes *pbp1a*, *pbp2b* and *pbp2x*. In this study, we sequenced gene parts of *pbp1a*, *pbp2b* and *pbp2x* representing distinct RFLP patterns. These data were used to investigate the correlation between *pbp* RFLP analysis and *pbp* sequence analysis, and to identify the molecular nature of pneumococcal penicillin resistance in The Netherlands.

MATERIALS AND METHODS

Bacterial strains, growth conditions and growth medium. Penicillin-nonsusceptible pneumococcal strains were isolated from patients in The Netherlands between March 1995 and March 1996. Their epidemiological characteristics have been described previously (16). The majority of the strains were isolates from patients with pneumococcal pneumonia. Clinical isolates representing distinct restriction fragment length polymorphism patterns of *pbp1a, pbp2b* and *pbp2x*, respectively, were used for nucleotide sequence analysis of their *pbp* genes. The pneumococcal strains reported in this study are listed in Fig. 1 to 3. The penicillin MICs of these strains ranged from 0.12 to 2 μg/ml. Bacteria were cultured at 37°C on Columbia agar supplemented with 5% sheep blood (Oxoid, Basingstoke, United Kingdom) in an atmosphere of increased CO_2. Bacterial colonies were inoculated in Todd Hewitt broth (Difco laboratories, Detroit, USA) supplemented with 0.5% Yeast Extract (Difco laboratories; THY-broth) and grown at 37°C.

PBP genotyping. Genetic polymorphisms of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* were investigated by restriction fragment length polymorphism (RFLP) analysis as described previously (16). Briefly, chromosomal DNA was extracted with cetyl trimethylammonium bromide (26). PCR amplification of the PBP-encoding genes was performed in 50 μl reactions containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 10 pmol of the individual primers, 0.5 U of DNA polymerase (Eurogentec, Liège, Belgium), and 10 ng of purified chromosomal DNA. Cycling was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, USA) and consisted of the following steps: predenaturation at 94°C for 1 min, 30 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C, and a final extension at 72°C for 3 min. The primers used to amplify the genes *pbp1a*, *pbp2b*, and *pbp2x* were described previously (see Table 1). The amplification products (5 μl) were digested with the restriction endonuclease *Hinf*I and separated by electrophoresis in 2.5% agarose gels. Gels were scanned and printed with the Geldoc 2000 system (Biorad, Veenendaal, The Netherlands). The different PBP genotypes are represented by a three-

number code (e.g., 06-14-43), referring to the RFLP patterns of the genes *pbp1a* (pattern 6), *pbp2b* (pattern 14), and *pbp2x* (pattern 43), respectively. The PBP RFLP codes used in this study refer to those described previously (16).

Nucleotide sequence analysis. Nucleotide sequencing was performed on parts of the penicillin-binding domain of *pbp1a*, of *pbp2b*, and of *pbp2x*, respectively, from the pneumococcal strains mentioned in Fig. 1 to 3. The primer pairs Pbp1a.fw/Pbp1a.rev, Pbp2b.fw/Pbp2b.rev and Pbp2x.fw/Pbp2x.rev were used to amplify nucleotides 1723 to 2350 of *pbp1a,* nucleotides 1199 to 1734 of *pbp2b*, and nucleotides 1217 to 1796 of *pbp2x*, respectively (Table 1). The numbering is based on published data for strain R6 [*pbp1a* (20), *pbp2b* (9), *pbp2x* (18)]. PCR-amplification was performed as described above in 100 µl reactions with 50 ng of purified pneumococcal chromosomal DNA. The PCR products were separated by electrophoresis in 1% agarose gels, cut from gel and purified with the QIAquick Gel Extraction kit (Qiagen, Westburg, Leusden, The Netherlands). Purified templates were sequenced with the Prism Ready Reaction Sequence Kit (Perkin Elmer, Roosendaal, The Netherlands) and 50 pmol of the primers M13.fw and M13.rev (Table 1).

Table 1. Primers used in this study. The underlined sequence parts of the primers Pbp1a.fw, Pbp1a.rev, Pbp2b.fw, Pbp2b.rev, Pbp2x.fw, Pbp2x.rev represent M13-based sequences.

primer name	primer sequence	reference
Pn1Aup	CGGCATTCGATTTGATTCGCTTCT	(5)
Pn1Adown	CTGAGAAGATGTCTTCTCAGG	(5)
Pn2Bup	GATCCTCTAAATGATTCTCAGGTGG	(8)
Pn2Bdown	CAATTAGCTTAGCAATAGGTGTTGG	(8)
Pn2Xup	CGTGGGACTATTTATGACCGAAATGG	(22)
Pn2Xdown	AATTCCAGCACTGATGGAAATAAACATATTA	(22)
Pbp1a.fw	TGTAAAACGACGGCCAGTCAAAGTCTCAAATCAGCAAG	this study
Pbp1a.rev	CAGGAAACAGCTATGACCGTTGTGTTACTTGAAATGGC	this study
Pbp2b.fw	TGTAAAACGACGGCCAGTCTGAAAAGTTATTTCAATTC	this study
Pbp2b.rev	CAGGAAACAGCTATGACCAAWCCAGTWGAYTCATCTGG	this study
Pbp2x.fw	TGTAAAACGACGGCCAGTAAAATGGGAGATGCTAC	this study
Pbp2x.rev	CAGGAAACAGCTATGACCTGGATACCTGAATAATG	this study
M13.fw	TGTAAAACGACGGCCAGT	this study
M13.rev	CAGGAAACAGCTATGACC	this study

Sequencing was performed on an Applied Biosystems Prism 377 (PE Applied Biosystems, Nieuwerkerk, The Netherlands). The nucleotide and deduced amino acid sequences were

compared to the published sequence of *pbp1a* (20)*, pbp2b* (9) and *pbp2x* (18) from the penicillin-susceptible strain R6 with BioEdit Sequence Alignment Editor (15). With the BLAST algorithm (1), the nucleotide sequences were analyzed for similarity to sequences deposited in the April 2000 version of the nucleotide database at the National Center for Biotechnology Information (Washinton D.C., USA).

Nucleotide sequence accession numbers. The *pbp1a* sequence data for the following clinical isolates were submitted to the EMBL/GenBank databases under the indicated accession numbers (acc. nos.): isolate 950225, AJ403974; isolate 960036, AJ403975; isolate 950710, AJ403976; isolate 950421, AJ403977; isolate 950423, AJ403978; isolate 950473, AJ403979; isolate 960027, AJ403980 and isolate 950035, AJ403981.

The *pbp2b* sequence data for the following clinical isolates have been submitted to the EMBL/GenBank databases under the indicated acc. nos.: isolate 950279, AJ278222; isolate 950961, AJ278223; isolate 950901, AJ278224; isolate 950138, AJ278225; isolate 960118, AJ278226; isolate 960035, AJ278227; isolate 960036, AJ278228; isolate 960030, AJ278229; isolate 954073, AJ278230 and isolate 960097, AJ278231.

The *pbp2x* sequence data for the following clinical isolates have been submitted to the EMBL/GenBank databases under the indicated acc. nos.: isolate 950181, AJ278232; isolate 950138, AJ278233; isolate 950423, AJ278234; isolate 960036, AJ278235; isolate 950454, AJ278236; isolate 950961, AJ278237; isolate 950637, AJ278238; isolate 950925, AJ278239, isolate 960097, AJ278240; and isolate 960165, AJ278241.

RESULTS

Sequence heterogeneity in pbp1a of penicillin-nonsusceptible pneumococcal isolates. We sequenced nucleotides 1741 to 2229 of *pbp1a* from 44 Dutch clinical pneumococcal isolates representing eight distinct *pbp1a* RFLP types. The *pbp1a* nucleotide sequences from strains representing identical *pbp1a* RFLP types were invariably identical (data not shown). A representative strain of each *pbp1a* RFLP type was chosen for sequence comparison. Fig. 1 provides a schematic illustration of the mosaic structures of *pbp1a*.

The nucleotide sequences and deduced amino acid sequences of *pbp1a* obtained from the RFLP type 1 and 10 strains were identical (Fig. 1). Nucleotides 1741 to 1789 differed from the penicillin-susceptible strain R6 by one synonymous substitution, nucleotides 1790 to 2039 were identical to *pbp1a* from *S. mitis* strain B6 (acc. no. AJ002290) (14), and nucleotides 2040 to 2229 differed from strain R6 by 11 synonymous substitutions and 11

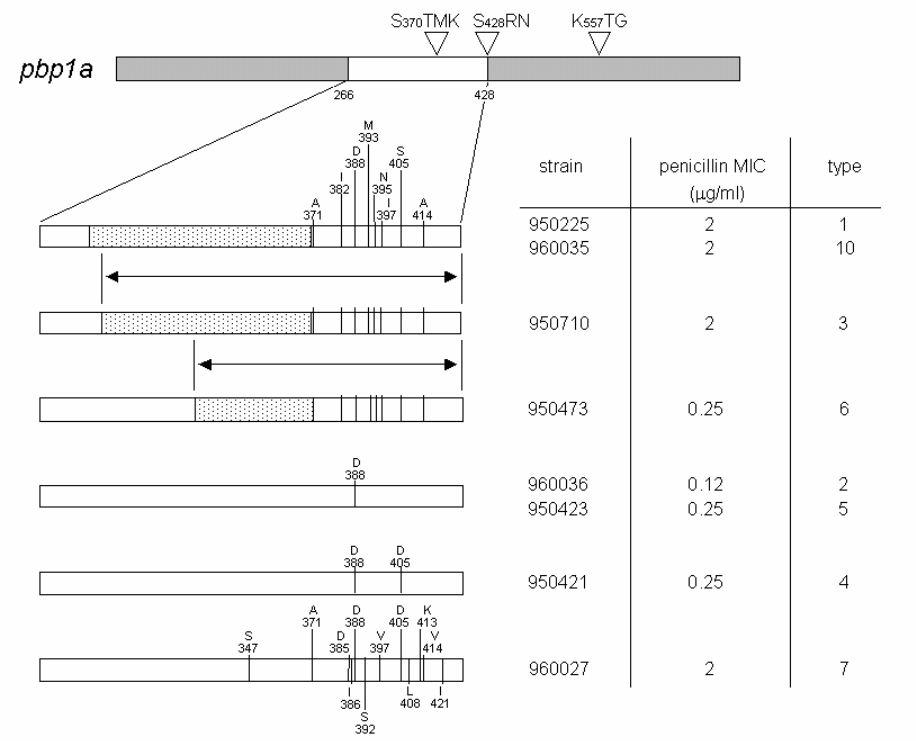


Figure 1. Schematic representation of *pbp1a*. The STMK-motif, SRN-motif and KTG-motif are depicted. The sequenced part is indicated by the open block. The mosaic structures in the deduced amino acid sequences of strains representing distinct RFLP types are shown below. Open blocks indicate DNA from susceptible pneumococcal strains and dotted blocks are homologous to *S. mitis* strain B6 DNA (14). Vertical lines indicate amino acid substitutions. Arrows indicate the identity between DNA sequences derived from genes representing distinct types. The pneumococcal strains, penicillin MICs, and *pbp1a* types are also listed.

nonsynonymous substitutions resulting in eight amino acid changes (Thr371Ala, Leu382Ile, Glu388Asp, Ile393Met, His395Asn, Glu397Ile, Asn405Ser, Gly414Ala) (Fig. 1). The *pbp1a* sequences of RFLP types 1 and 10 matched with the *pbp1a* sequence of *S. pneumoniae* strain D isolated in Spain (21) and *S. pneumoniae* strains #5/H31 and #17/246 isolated in Japan (3). Part of *pbp1a* of the RFLP type 3 strain (nucleotides 1880 to 2229) and part of *pbp1a* of the RFLP type 6 strain [nucleotides 1920 to 2229 except for five synonymous substitutions and one nonsynonymous substitution (Ala371Thr)] were identical to *pbp1a* of RFLP type 1 and 10 strains (Fig. 1). The *pbp1a* regions of the RFLP type 2 and 5 strains were identical and differed from strain R6 by two synonymous substitutions and a single

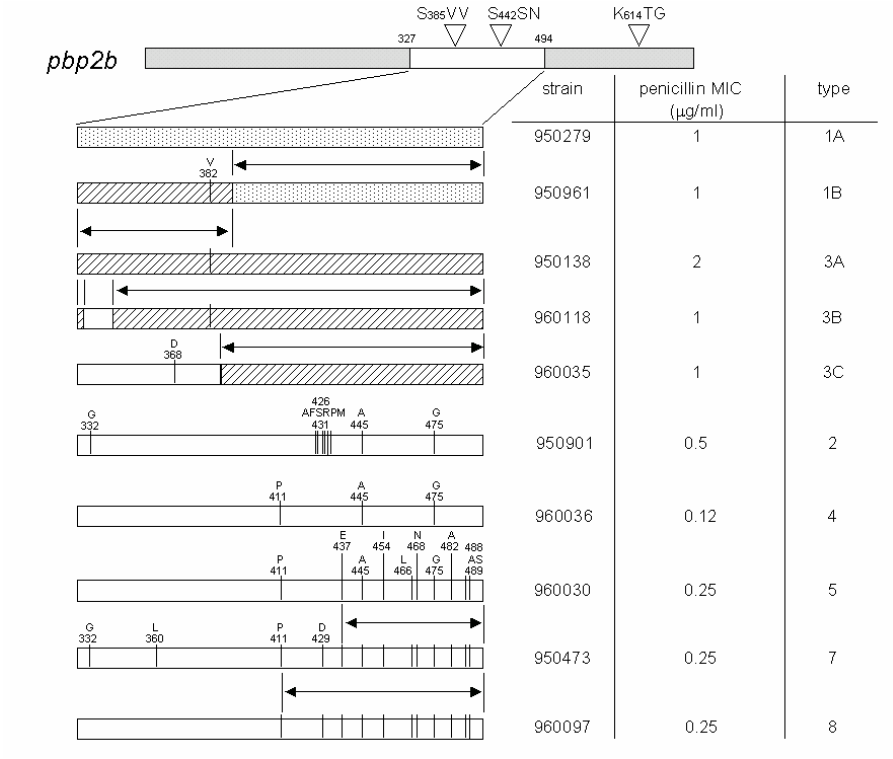


Figure 2. Schematic representation of *pbp2b*. The SVV-motif, SSN-motif and KTG-motif are depicted. The sequenced part is indicated by the open block. The mosaic structures in the deduced amino acid sequence of strains representing distinct RFLP types are shown below. Open blocks indicate DNA from susceptible pneumococcal strains, dotted blocks are homologous to *S. mitis* strain B6 (acc. no. AJ 002289) and *S. sanguis* strain 1907 DNA (acc. no. M32226), and striped blocks are homologous to penicillin-resistant *S. oralis* strain 5296 DNA (acc. no. M32228). Vertical lines indicate amino acid substitutions. Arrows indicate the identity between DNA sequences derived from genes representing distinct types. The pneumococcal strains, penicillin MICs, and *pbp2b* type are also listed.

nonsynonymous substitution (Glu388Asp) (Fig. 1). Interestingly, this substitution occurred in the deduced amino acid sequences from all isolates analyzed in this study. The *pbp1a* sequences of RFLP type 2 and 5 strains were identical to *S. pneumoniae* strain C, a penicillin-susceptible strain isolated in South Africa (21). The *pbp1a* sequence of the RFLP type 4 strain differed in seven additional synonymous substitutions and one additional nonsynonymous substitution (Asn405Asp) (Fig. 1). *pbp1a* of RFLP type 2, 4, and 5 strains were highly homologous to *pbp1a* of *S. pneumoniae* strain #23/HSB21 isolated in Japan (3). The *pbp1a* nucleotide and deduced amino acid sequence of the RFLP type 7 strain differed

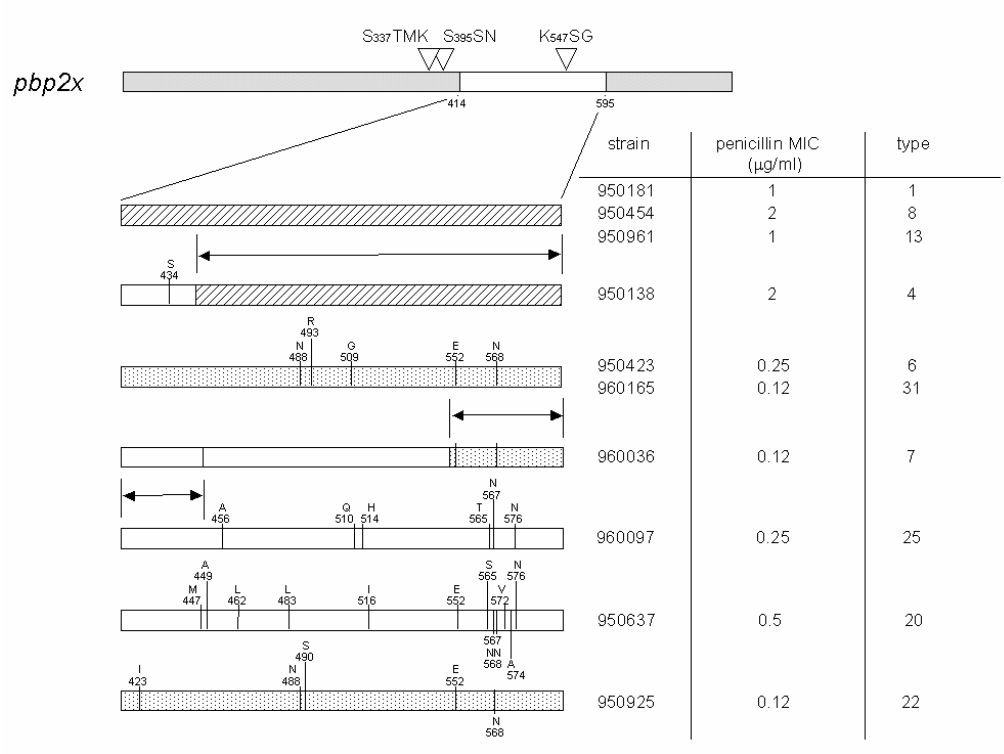


Figure 3. Schematic representation of *pbp2x*. The STMK-motif, SSN-motif and KSG-motif are depicted. The sequenced part is indicated by open block. The mosaic structures in the deduced amino acid sequences of strains representing distinct RFLP types are shown below. Open blocks indicate DNA from susceptible pneumococcal strains, dotted blocks are homologous to *S. mitis* strain 10712 DNA (acc. no. X78216), and striped blocks are homologous to *S. oralis* strain U5 DNA (acc. no. Y10536), *S. mitis* strains 476 (acc no. Y10534) and 197 (acc. no. Y10533) DNA. Vertical lines indicate amino acid substitutions. Arrows indicate the identity between the DNA sequences derived from genes representing distinct types. The pneumococcal strains, penicillin MICs, and *pbp2x* type are also listed.

significantly from the previous sequences, and differed from strain R6 by 52 synonymous substitutions and 13 nonsynonymous substitutions resulting in 12 amino acid changes (Ala347Ser, Thr371Ala, Gly385Asp, Val386Ile, Glu388Asp, Thr392Ser, Glu397Val, Asn405Asp, Val408Leu, Arg413Lys, Gly414Val, Leu421Ile) (Fig. 1). The partial *pbp1a* sequence of the RFLP type 7 was homologous to *S. pneumoniae* strain #14/Z42 isolated in Japan (3). Interestingly, the amino acid substitution Thr371Ala was found in all high-level penicillin-resistant strains.

Sequence heterogeneity in pbp2b of penicillin-nonsusceptible isolates. We sequenced nucleotides 1211 to 1714 of *pbp2b* from 57 Dutch clinical pneumococcal isolates representing seven distinct *pbp2b* RFLP types. The *pbp2b* sequences from strains representing identical *pbp2b* RFLP types were identical for RFLP types 2, 4 and 5, respectively (data not shown). In contrast, the *pbp2b* nucleotide sequences from strains representing RFLP type 1 could be divided into two sequence types designated type 1A and 1B. In addition, the *pbp2b* nucleotide sequences from strains representing RFLP type 3 could be divided into three sequence types designated type 3A, 3B and 3C. RFLP types 7 and 8 were represented by one strain each. A representative strain of each RFLP type and sequence-type, respectively, was chosen for sequence comparison (Fig. 2).

The *pbp2b* sequence of the RFLP type 1A strain was identical to *pbp2b* from the penicillin-resistant *S. sanguis* strain 1907 (acc. no. M32226), except for one synonymous substitution (Fig. 2). In addition, this *pbp2b* fragment was identical to *pbp2b* of *S. pneumoniae* strains SP1470 isolated in France (acc. no. AF210766), M15 isolated in the United Kingdom (acc. no. AJ243054), 577 isolated in the United Kingdom (acc. no. AJ243053) and 56762 isolated in South Africa (acc. no. U20080). *pbp2b* of the RFLP type 1B strain was in part identical to the *pbp2b* sequence of the RFLP type 3A strain (nucleotides 1211 to 1402) and in part to the *pbp2b* sequence of the RFLP type 1A strain (nucleotides 1403 to 1714) (Fig. 2). The *pbp2b* fragment of the RFLP type 3A strain was identical to *pbp2b* of *S. mitis* strain B6 (acc. no. AJ002289), except for one nonsynonymous mutation (Ile382Val) (Fig. 2). In addition, this *pbp2b* fragment was identical to penicillin-resistant *S. oralis* strain 5296 (acc. no. M32228), except for one synonymous mutation and one nonsynonymous mutation (Ile382Val). The *S. pneumoniae* strain 52328 isolated in South Africa (acc. no. U20073) also contained the same *pbp2b* sequence, except for two synonymous mutations. The *pbp2b* fragment of the pneumococcal RFLP type 3B strain was identical to that of the RFLP type 3A strain, with the exception of the fragment formed by nucleotides 1227 to 1279, which was identical to that in the penicillin-susceptible strain R6 (Fig. 2). *pbp2b* of the RFLP type 3C strain was in part homologous to the R6 sequence (nucleotides 1211 to 1391) differing in 6 synonymous mutations and one nonsynonymous mutation (Glu368Asp), and in part identical to *pbp2b* of RFLP type 3A and 3B strains, except for one synonymous mutation (Fig. 2). *pbp2b* of the RFLP type 2 strain contained 26 synonymous substitutions and 9 nonsynonymous (Glu332Gly, Gln426Ala, Ala427Phe, Tyr428Ser, Gly429Arg, Ser430Pro, Phe431Met, Thr445Ala, Glu475Gly) substitutions compared to the R6 sequence. The *pbp2b* fragment of this strain was identical to *S. pneumoniae* strains DN87/669 isolated in the United Kingdom. (acc. no. M25521) and 8249 isolated in South Africa (acc. no. M25520), except for one

synonymous substitution (7). The *pbp2b* fragment of the RFLP type 4 strain contained 22 synonymous substitutions and three nonsynonymous (Ser411Pro, Thr445Ala, Glu475Gly) substitutions compared to the R6 sequence and was not identical to any of the published sequences present in databases (Fig. 2). The *pbp2b* fragment of the RFLP type 5 strain differed in 49 synonymous mutations and 11 nonsynonymous mutations resulting in 10 amino acid changes (Ser411Pro, Gln437Glu, Thr445Ala, Leu454Ile, Gly466Leu, Ser468Asn, Glu475Gly, Gly482Ala, Thr488Ala, Ala489Ser) from *pbp2b* of strain R6 and was identical to *pbp2b* of *S. pneumoniae* strain SP 1513 isolated in France (acc. no. AF210762) (Fig. 2). Part of the *pbp2b* fragment of the RFLP type 5 strain (nucleotides 1460 to 1714) was homologous to *pbp2b* of *S. mitis* strain NCTC 11189 (acc. no. Z22183). In this fragment, we observed 11 synonymous mutations and 4 amino acid substitutions (Thr445Ala, Gly466Leu, Ser468Asn, Ala480Ser) compared to the *S. mitis* sequence, while 40 synonymous mutations and the 11 nonsynonymous mutations (Ser411Pro, Gln437Glu, Thr445Ala, Leu454Ile, Gly466Leu, Ser468Asn, Glu475Gly, Gly482Ala, Thr488Ala, Ala489Ser) were observed compared to the R6 sequence (Fig. 2). *pbp2b* of the RFLP type 7 and 8 strains were in part identical to *pbp2b* from the RFLP type 5 strain (Fig. 2). The *pbp2b* sequence of the RFLP type 7 strain differed in nucleotides 1211 to 1455 from the R6 sequence by eight synonymous substitutions and two nonsynonymous substitutions (Glu332Gly, Ile360Leu), was identical to *pbp2b* of the RFLP type 8 strain for nucleotides 1456 to 1714, and, except for two synonymous mutations, was identical to *pbp2b* of the RFLP type 5 strain for nucleotides 1528 to 1714 (Fig. 2). *pbp2b* of the RFLP type 8 strain was identical to *pbp2b* of R6 for nucleotides 1211 to 1455 and to *pbp2b* of the RFLP type 7 strain for nucleotides 1456 to 1714 (Fig. 2). The Thr445Ala substitution was found in all penicillin-nonsusceptible strains we investigated. In addition, the Glu475Gly substitution was found in all *pbp2b* sequence types of our strain collection. Six consecutive substitutions at residues 426 to 431 were found in *pbp2b* RFLP type 2 only.

Sequence heterogeneity in pbp2x of penicillin-nonsusceptible strains. We sequenced nucleotides 1241 to 1786 of *pbp2x* from 53 Dutch clinical pneumococcal isolates representing 10 distinct RFLP types. The *pbp2x* nucleotide sequences from strains representing identical *pbp2x* RFLP types were identical as far as RFLP type 1, 4, 6, 8, and 20 was concerned, except for two or less synonymous point mutations observed in RFLP type 1. The RFLP types 7, 13, 22, 25 and 31 were represented by single strains. A representative strain for each *pbp2x* RFLP type was chosen for sequence comparison (Fig. 3).

The *pbp2x* DNA fragments of the RFLP type 1, 8 and 13 strains were identical in nucleotide and deduced amino acid sequence (Fig. 3). This sequence type shared identity with *pbp2x* of *S. oralis* strain U5 (acc. no. Y10536), and *pbp2x* of *S. mitis* strains 476 (acc. no. Y10534) and 197 (acc. no. Y10533). In addition, the *pbp2x* sequence of RFLP type 1, 8 and 13 strains was identical to *pbp2x* of *S. pneumoniae* strain 577 isolated in the UNITED KINGDOM (19) and *S. pneumoniae* strains 34/H31 and 31/KU5 isolated in Japan (2). The *pbp2x* fragment from the RFLP type 4 strain was identical for the nucleotides 1319 to 1786 with *pbp2x* from RFLP type 1, 8 and 13 strains. The 5' part of the sequence (nucleotides 1211 to 1318) differed from *pbp2x* of strain R6 by 14 synonymous mutations and one nonsynonymous mutation (Ala434Ser) (Fig. 3). The *pbp2x* fragment of the RFLP type 6 strain was highly homologous to *pbp2x* of *S. mitis* strain 10712 (acc. no. X78216), and differed in 10 synonymous substitutions and 5 nonsynonymous substitutions (Asp488Asn, Lys493Arg, Ser509Gly, Gln552Glu, Tyr568Asn) (Fig. 3). The *pbp2x* fragment of the RFLP type 6 strain was identical to *pbp2x* of *S. pneumoniae* strain F1 isolated in France (acc. no. AJ238581), except for two synonymous substitutions. *pbp2x* of the RFLP type 31 strain was identical to *pbp2x* RFLP type 6 sequences, except for five synonymous substitutions (Fig. 3). The *pbp2x* sequence of the RFLP type 25 strain differed from *pbp2x* of the R6 sequence in 56 synonymous substitutions and 6 nonsynonymous substitutions (Val456Ala, Leu510Gln, Asn514His, Leu565Thr, Asp567Asn, Ser576Asn) (Fig. 3). The *pbp2x* sequence of the RFLP type 25 strain was identical to *pbp2x* of *S. pneumoniae* strain 53139/72 isolated in Papua New Guinea (19). *pbp2x* of the RFLP type 7 strain was in part homologous to *pbp2x* of the RFLP type 25 strain, in part to *pbp2x* of R6, and in part to *pbp2x* of the RFLP type 6 and 31 strains (Fig. 3): nucleotides 1241 to 1345 of *pbp2x* of the RFLP type 7 strain were identical to *pbp2x* of the RFLP type 25 strain except for two synonymous mutations, nucleotides 1346 to 1650 were identical to *pbp2x* of strain R6 except for two synonymous mutations, and nucleotides 1651 to 1786 were identical to *pbp2x* of strains representing RFLP types 6 except for two synonymous mutations. The *pbp2x* sequence of the RFLP type 20 strain differed from R6 in 60 synonymous substitutions and 12 nonsynonymous substitutions (Gln447Met, Ser449Ala, Ile462Leu, Ile483Leu, Val516Ile, Gln552Glu, Leu565Ser, Asp567Asn, Tyr568Asn, Ala572Val, Ser574Ala, Ser576Asn) (Fig. 3). The *pbp2x* RFLP type 20 sequence was identical to *pbp2x* of *S. pneumoniae* strains SP1258 isolated in France (acc. no. AF210756) and 669 isolated in the UNITED KINGDOM (acc. no. X65133) (19). The sequenced *pbp2x* fragment of the RFLP type 22 strain was homologous to *pbp2x* of *S. mitis* strain 10712 (acc. no. X78216) although 32 synonymous and 5 nonsynonymous substitutions (Val423Ile, Asp488Asn, Thr490Ser, Gln552Glu, Tyr568Asn) were present.

pbp2x of the RFLP type 22 strain was identical to *pbp2x* of *S. pneumoniae* strain SP1513 isolated in France (acc. no. AF210754), except for one nonsynonymous substitution (Asn567His).

DISCUSSION

Pneumococcal isolates express six PBPs. Many penicillin-nonsusceptible isolates are modified in PBP1a, PBP2b and PBP2x only (12). If one or more amino acids change in the conserved amino acid motifs SXXK (with active site serine), SXN and K(H)T(S)G or adjacent amino acids, penicillin and other β-lactam antibiotics are often unable to bind efficiently to PBPs, which results in resistance.

RFLP analysis has previously been performed to characterize *pbp1a*, *pbp2b* and *pbp2x* from penicillin-nonsusceptible strains isolated in The Netherlands that displayed a wide range of MIC levels (0.12 to 2 μg/ml) (16). In this study, we characterized parts of the DNA sequences of *pbp1a*, *pbp2b* and *pbp2x* of these clinical isolates. The classification based on sequence analysis of the *pbp* fragments correlated well with the classification according to *pbp* RFLP analysis. Sequence analysis of the *pbp2b* fragment (nucleotides 1211 to 1714) enables a refinement of the classification based on RFLP analysis of the complete *pbp2b* gene. The *pbp2b* RFLP types 1 and 3 were divided into sequence types 1A and 1B, and 3A, 3B and 3C, respectively. However, the classification of the *pbp2b* RFLP types 2, 4, 5, 7 and 8 was maintained after sequence analysis. We were unable to differentiate within the *pbp1a* RFLP types by *pbp1a* sequence analysis of nucleotides 1741 to 2229. Moreover, strains with *pbp1a* RFLP types 1 and 10, and strains with RFLP types 2 and 5 were classified to the same sequence type, respectively. In addition, we were not able to subdivide *pbp2x* RFLP types by sequence analysis of *pbp2x* nucleotides 1241 to 1786 and strains with *pbp2x* RFLP type 1, 8 and 13 were classified to the same sequence type.

Most of the *pbp* sequences characterized in this study matched with *pbp* sequences from strains isolated in other countries. Martin et al. described *pbp1a* sequence parts of eight resistant isolates (21) and Asahi et al. classified *pbp1a* and *pbp2x* sequences from clinical isolates into five sequence types (I to V) each (2, 3). All *pbp1a* sequence types identified in this study could be matched with *pbp1a* sequences described by both research groups. The *pbp2x* sequences of RFLP type 1, 8 and 13 strains were identical to *pbp2x* sequence types IV and V (2). In addition, the *pbp2x* sequences of the RFLP type 20 and 25 strains matched with sequences described by Laible et al. (19). Dowson and colleagues have divided *pbp2b* from penicillin-resistant isolates in two classes, A and B (7). *pbp2b* RFLP type 1A was similar to class B *pbp2b,* and *pbp2b* RFLP type 2 belongs to class A *pbp2b*. All other *pbp2b*

RFLP types described in this study did not match any of the classes A or B, pointing to the limitations of Dowson's classification.

Interestingly, penicillin-nonsusceptible strains with *pbp* sequences that were highly homologous to *pbp1a* sequences from susceptible strains were intermediately resistant. In contrast, penicillin-nonsusceptible strains with a high degree of heterogeneity in their *pbp* sequences compared to susceptible strains have often MIC levels of ≥1 μg/ml. The difference in penicillin susceptibility between strains belonging to the same sequence type may be due to amino acid alterations in non-sequenced parts of the penicillin-binding domain and in other *pbp* genes.

All prominent PBP1a amino acid substitutions in penicillin-nonsusceptible isolates described by Smith *et al*. (24) were also observed in this study. The amino acid change Asn405Asp was previously identified as being one of the prominent amino acid substitutions in *pbp1a* of resistant isolates (24). In addition, we confirmed that the Thr371Ala substitution in PBP1a was important for high-level resistance (3), since all high-level penicillin-resistant strains contained this substitution. The PBP1a amino acid substitution Glu388Asp, which has been identified in all penicillin-nonsusceptible isolates in this study, also occurs in susceptible strains. This substitution is probably not able to confer penicillin resistance (24). The amino acid substitutions found in PBP2b in strains of our collection confirmed the previously described amino acid substitutions in penicillin-resistant isolates (8, 25, 27). The Thr445Ala substitution was found in all penicillin-nonsusceptible strains adjacent to the Ser443-Ser-Asn motif, and its importance has previously been noted by Dowson and coworkers (8). The Asn residue of this motif has been proposed to form a hydrogen bond with the carbonyl group of the R1 side chain of penicillin, and the Thr445Ala substitution presumably disrupts this hydrogen bond (8). The significance of Glu475Gly has been noted by Smith and Klugman (25), and was found in all *pbp2b* sequence types of our strain collection. In addition, the six consecutive residues at nucleotide position 426 to 431 found in *pbp2b* RFLP type 2 were previously described (25). Since these residues do not occur in other sequence types, they are presumably not critical to resistance development. Most of the PBP2x amino acid substitutions found in this study were also described previously (2, 17, 19). The amino acid substitution Leu546Val proceeding the KSG motif found in PBP2x of RFLP type 4 and RFLP types 1, 8 and 13 strains is presumed to be involved in cefotaxime resistance (2). All strains with this amino acid substitution had a reduced susceptibility for cefotaxime (MIC range 0.5 to 1 μg/ml; unpublished observations), confirming the importance of the Leu546Val substitution for cefotaxime resistance. Although the amino acid substitution Gln552Glu has also been observed in cefotaxime resistant isolates, the impact on resistance is unknown

(17). Strains with the Gln552Glu substitution (RFLP types 6 and 31, RFLP type 7, RFLP type 20, and RFLP type 22) were all cefotaxime-susceptible (cefotaxime level: <0.016 to 0.032 μg/ml; unpublished observations), suggesting that the Gln552Glu does not play a role in cefotaxime resistance.

The *pbp* sequences identified in this study confirmed the acquisition of resistance by DNA transfer between non-pneumococcal species and pneumococci. Sequence blocks within *pbp1a*, *pbp2b* and *pbp2x* were identical to DNA from *S. mitis, S. oralis* and *S. sanguis*. Intra-species horizontal transfer events among different genetic lineages of pneumococci have probably occurred as well, and this is especially suggested between strains with *pbp1a* RFLP types 1, 3, 6 and 10 and between strains with *pbp2b* RFLP types 5, 7 and 8. A recurrence of the recombination with viridans streptococci may have also taken place giving rise to a smaller section of the mosaic and is highly suggestive for *pbp2b* of the RFLP type 1B and 3C strains and for *pbp2x* RFLP type 4 and 7.

In conclusion, mutations in *pbp* sequences of most Dutch isolates were similar to those described in *pbp* sequences of strains isolated in other countries. This supports the hypothesis that multiple clones of penicillin-resistant pneumococci have been introduced and subsequently spread in The Netherlands as reported by Hermans *et al.* (16). However, novel combinations of sequence blocks in *pbp1a*, *pbp2b* and *pbp2x* were also identified, and suggests horizontal transfer of DNA between *pbp* genes of penicillin-resistant pneumococci previously described and *pbp* genes of susceptible strains.

ACKNOWLEDGEMENTS

We thank Marjon Muller and Joris Schonkeren for excellent technical assistance. This work was financially supported by the Sophia Foundation for Medical Research, Rotterdam, The Netherlands (grants 183, 217 and 268) and by NWO (grant SGO-inf. 005).

REFERENCES

1. Altschul, S. F., G. W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 251:403-410.
2. Asahi, Y., Y. Takeuchi, and K. UbUnited Kingdomata. 1999. Diversity of substitutions within or adjacent to conserved amino acid motifs of penicillin-binding protein 2X in cephalosporin-resistant *Streptococcus pneumoniae* isolates. Antimicrob. Agents Chemother. 43:1252-1255.
3. Asahi, Y., and K. UbUnited Kingdomata. 1998. Association of a Thr-371 substitution in a conserved amino acid motif of penicillin-binding protein 1A with penicillin resistance of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 42:2267-2273.
4. Chambers, H. F. 1999. Penicillin-binding protein-mediated resistance in pneumococci and staphylococci. J. Infect. Dis. 179:S353-359.
5. Coffey, T. J., C. G. Dowson, M. Daniels, J. Zhou, C. Martin, B. G. Spratt, and J. M. Musser. 1991. Horizontal

transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. Mol. Microbiol. 5:2255-2260.
6. Dowson, C. G., V. Barcus, S. King, P. Pickerill, A. Whatmore, and M. Yeo. 1997. Horizontal gene transfer and the evolution of resistance and virulence determinants in *Streptococcus*. Soc. Appl. Bacteriol. Symp. Ser. 26:42S-51S.
7. Dowson, C. G., A. Hutchison, J. A. Brannigan, R. C. George, D. Hansman, J. Linares, A. Tomasz, J. M. Smith, and B. G. Spratt. 1989. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. U S A. 86:8842-8846.
8. Dowson, C. G., A. Hutchison, and B. G. Spratt. 1989. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. Mol. Microbiol. 3:95-102.
9. Dowson, C. G., A. Hutchison, and B. G. Spratt. 1989. Nucleotide sequence of the penicillin-binding protein 2B gene of *Streptococcus pneumoniae* strain R6. Nucleic Acids Res. 17:7518.
10. Grebe, T., and R. Hakenbeck. 1996. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of beta-lactam antibiotics. Antimicrob. Agents Chemother. 40:829-834.
11. Hakenbeck, R. 1999. Beta-lactam-resistant *Streptococcus pneumoniae*: epidemiology and evolutionary mechanism. Chemotherapy. 45:83-94.
12. Hakenbeck, R., and J. Coyette. 1998. Resistant penicillin-binding proteins. Cell Mol. Life Sci. 54:332-340.
13. Hakenbeck, R., T. Grebe, D. Zahner, and J. B. Stock. 1999. Beta-lactam resistance in *Streptococcus pneumoniae*: penicillin-binding proteins and non-penicillin-binding proteins. Mol. Microbiol. 33:673-678.
14. Hakenbeck, R., A. Konig, I. Kern, M. van der Linden, W. Keck, D. Billot-Klein, R. Legrand, B. Schoot, and L. Gutmann. 1998. Acquisition of five high-Mr penicillin-binding protein variants during transfer of high-level beta-lactam resistance from *Streptococcus mitis* to *Streptococcus pneumoniae*. J. Bacteriol. 180:1831-1840.
15. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis.
16. Hermans, P. W., M. Sluijter, K. Elzenaar, A. van Veen, J. J. Schonkeren, F. M. Nooren, W. J. van Leeuwen, A. J. de Neeling, B. van Klingeren, H. A. Verbrugh, and R. de Groot. 1997. Penicillin-resistant *Streptococcus pneumoniae* in the Netherlands: results of a 1-year molecular epidemiologic survey. J. Infect. Dis. 175:1413-1422.
17. Krauss, J., M. van der Linden, T. Grebe, and R. Hakenbeck. 1996. Penicillin-binding proteins 2x and 2b as primary PBP targets in *Streptococcus pneumoniae*. Microb. Drug Resist. 2:183-186.
18. Laible, G., R. Hakenbeck, M. A. Sicard, B. Joris, and J. M. Ghuysen. 1989. Nucleotide sequences of the pbpX genes encoding the penicillin-binding proteins 2x from *Streptococcus pneumoniae* R6 and a cefotaxime-resistant mutant, C506. Mol. Microbiol. 3:1337-1348.
19. Laible, G., B. G. Spratt, and R. Hakenbeck. 1991. Interspecies recombinational events during the evolution of altered PBP 2x genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Mol. Microbiol. 5:1993-2002.
20. Martin, C., T. Briese, and R. Hakenbeck. 1992. Nucleotide sequences of genes encoding penicillin-binding proteins from *Streptococcus pneumoniae* and *Streptococcus oralis* with high homology to *Escherichia coli* penicillin-binding proteins 1a and 1b. J. Bacteriol. 174:4517-4523.
21. Martin, C., C. Sibold, and R. Hakenbeck. 1992. Relatedness of penicillin-binding protein 1a genes from different clones of penicillin-resistant *Streptococcus pneumoniae* isolated in South Africa and Spain. EMBO J. 11:3831-3836.
22. Munoz, R., T. J. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J. M. Musser, B. G. Spratt, and A. Tomasz. 1991. Intercontinental spread of a multidrug-resistant clone of serotype 23F *Streptococcus pneumoniae*. J. Infect. Dis. 164:302-306.
23. Potgieter, E., and L. J. Chalkley. 1995. Relatedness among penicillin-binding protein 2b genes of *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus pneumoniae*. Microb. Drug Resist. 1:35-42.
24. Smith, A. M., and K. P. Klugman. 1998. Alterations in PBP 1A essential-for high-level penicillin resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 42:1329-1333.
25. Smith, A. M., and K. P. Klugman. 1995. Alterations in penicillin-binding protein 2B from penicillin-resistant wild-type strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 39:859-867.
26. van Soolingen, D., P. E. W. de Haas, P. W. M. Hermans, and J. D. A. van Embden. 1993. DNA fingerprinting of *Mycobacterium tuberculosis*. Methods Enzymol. 235:196-205.
27. Yamane, A., H. Nakano, Y. Asahi, K. UbUnited Kingdomata, and M. Konno. 1996. Directly repeated insertion of 9-nucleotide sequence detected in penicillin-binding protein 2B gene of penicillin-resistant *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 40:1257-1259.

Chapter 5

Molecular dynamics of *Streptococcus pneumoniae* during colonization and infection

Chapter 5.1

Molecular epidemiology of pneumococcal carriage among children with upper respiratory tract infections in Hanoi, Vietnam

D. Bogaert, N.T. Ha, M. Sluijter, N. Lemmens, R. de Groot, and P.W.M. Hermans

J Clin Microbiol 2002; 40(11): 3903-8

ABSTRACT

To investigate the molecular epidemiology of pneumococcal nasopharyngeal carriage in Hanoi, Vietnam, we studied 84 pneumococcal strains retrieved from children with upper respiratory tract infections. Serotypes 23F (32%), 19F (21%), 6B (13%), and 14 (10%) were found most often. A significant number of strains were antibiotic resistant. Fifty-two percent of the strains were (intermediate) resistant to penicillin, 87% were (intermediate) resistant to co-trimoxazole, 76% were resistant to tetracycline, 73% were resistant to erythromycin, and 39% were (intermediate) resistant to cefotaxime. Seventy-five percent were resistant to three or more classes of antibiotics. A high degree of genetic heterogeneity among the penicillin resistance genes was observed. In addition, the tetracycline resistance gene *tet*(M) and the erythromycin resistance gene *erm*(B) were predominantly observed among the isolates. Molecular analysis of the 84 isolates by restriction fragment end labeling (RFEL) revealed 35 distinct genotypes. Twelve of these genotypes represented a total of eight genetic clusters with 61 isolates (73%). The two largest clusters contained 24 and 12 isolates, and the isolates in those clusters were identical to the two internationally spreading multidrug-resistant clones Spain 23F-1 and Taiwan 19F-14, respectively. The remaining RFEL types were Vietnam specific, as they did not match the types in our reference collection of 193 distinct RFEL types from 16 countries. Furthermore, 57 of the 61 horizontally spreading isolates (93%) in the eight genetic clusters were covered by the seven-valent conjugate vaccine, whereas this vaccine covered only 43% of the isolates with unique genotypes. According to the serotype distribution of the nasopharyngeal pneumococcal isolates, this study suggests a high potential benefit of the seven-valent pneumococcal conjugate vaccine for children in Hanoi.

INTRODUCTION

Streptococcus pneumoniae is one of the major causes of respiratory tract infections and invasive diseases in children all over the world. At present, worldwide about 1 million children under 5 years of age annually die of pneumococcal disease (18). Pneumococci are often part of the nasopharyngeal flora; especially due to circumstances of crowding, as observed in day-care centers, nursing homes, and hospitals, the risk of being colonized with pneumococci is increased (3, 20, 27, 28). Usually, colonization is not followed by disease, as local barriers at the mucosal level of the respiratory tract and the human immune system are often protective. However, the balance between host and pathogen may be disturbed by highly pathogenic pneumococcal strains or by diminished host defense through viral infections, malnutrition, or immune deficiency (25).

Another problem is the growing (multi)drug resistance among pneumococcal isolates. The emergence of penicillin- and multidrug-resistant pneumococci has been observed in various countries over the last decade. In some countries and populations, up to 60% of the pneumococcal isolates are resistant to one or more antibiotics (1, 12, 22). A significant proportion of pneumococcal resistance is the result of the worldwide spread of a limited number of multidrug-resistant clones (6, 15, 35, 39).

Prevention of pneumococcal disease has become a major topic in the battle against pneumococcal disease. The prevailing 23-valent pneumococcal polysaccharide vaccines have been shown to be immunogenic in adults but not in the group most at risk of developing pneumococcal diseases, namely, young children (5). Recently, several conjugate vaccines have been developed which have proven to be effective in young children, especially against invasive diseases (2, 11). Unfortunately, these vaccines are protective against only a limited number of pneumococcal serotypes. Conjugate vaccines from up to 11 capsular serotypes have been developed, whereas over 90 serotypes exist. In order to evaluate the theoretical coverage of the vaccines and the effect of vaccination on future serotype distribution, we need to monitor in detail and on a large scale the molecular epidemiology of pneumococcal colonization and infection before and after the implementation of these vaccines.

So far, the coverage of the conjugate vaccine has been investigated in several parts of the world. For instance, approximately 75% of all pneumococcal central nervous system infections in children in Europe, the United States, and Canada are covered by the seven-valent conjugate vaccine. For China and Latin America, the rates are 50 and 48%, respectively (14). Several molecular epidemiological studies have been performed in Asian countries such as Thailand and Taiwan; however, in most studies the serotype distribution has not been investigated (8, 34). In South Korea the seven-valent conjugate vaccine covered 65% of the isolates retrieved in the period from 1991 to 1993 (22). Recently, the

Asian Network for Surveillance of Resistant Pathogens has studied nasal carriage of pneumococci in healthy children in Taiwan, South Korea, Sri Lanka, and Vietnam. The most common serogroups were 6, 23, 19, 14, and 15. Because no subtypes were determined, the exact vaccine coverage could not be calculated (23).

In this study, we investigated the molecular epidemiology of 84 pneumococcal carriage isolates of children attending several outpatient departments in Hanoi, Vietnam, with acute respiratory tract infections. The serotype distribution, vaccine coverage rates, resistance patterns, and genetic properties of these isolates will be discussed.

MATERIALS AND METHODS

Serotyping. Eighty-four *S. pneumoniae* strains were isolated from the nasopharynges of 410 children with upper respiratory tract infections visiting the outpatient departments of the Vietnam-Cuba Clinic and the Children's and Bach Mai Hospitals in Hanoi from September 1997 to December 1999. Bacteriological diagnosis was performed according to procedures published in the Manual of Clinical Microbiology (11a), and pneumococci were serotyped by the capsular swelling method (Quellung reaction) with capsular antisera (Statens Seruminstitut, Copenhagen, Denmark).

Susceptibility testing. The susceptibilities of the Vietnam strains to penicillin, co-trimoxazole, tetracycline, erythromycin, rifampin, vancomycin, ciprofloxacin, and cefotaxime were determined by the agar dilution method as described previously (13). To discriminate between susceptible and nonsusceptible strains, we used the antibiotic breakpoints according to the NCCLS guidelines (26).

Penicillin-binding protein (PBP) genotyping. The genetic polymorphisms of penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* of the pneumococcal isolates were investigated by restriction fragment length polymorphism (RFLP) analysis of the amplified genes, as described previously (4).

Detection and analysis of *erm*(B) and *mef* genes. To detect the presence of *erm*(B) in the pneumococcal isolates, we used the protocol described by Sutcliffe et al. (38). In summary, we amplified the genes by PCR and analyzed the amplified DNA products by agarose gel electrophoresis. The presence of the *mef* gene was also detected by PCR (38). In order to discriminate between *mef*(A) and *mef*(E), PCR-RFLP analysis was performed as described by Del Grosso et al. (9). The amplicon was restricted with *Bam*HI and *Dra*I. The *mef*(A) gene contains a single BamHI site, which is absent in the *mef*(E) gene. Restriction of *mef*(A) and *mef*(E) with DraI yields two and three fragments, respectively.

Detection of *tet*(M) and *tet*(O) genes. In order to discriminate between *tet*(M) and *tet*(O), PCR-RFLP analysis was performed. Primers tetOfw (5'-TGTCGGGTTGTCCATAGAG-3')

and tetOrev (5'-AAATTTACCAATAGCTGGC-3') were used to amplify *tet*(O). The primer sequences were based on the *tet*(O) gene (GenBank accession number Y07780; positions 1455 to 1474 and positions 1957 to 1976, respectively). Primers tetM-fw (5'-CCATTGGTTTATCTGTATCA-3') and tetM-rev (5'-CAGGTTCACCGGTAGTAACA-3') were used to amplify *tet*(M). The primer sequences were based on the *tet*(M) gene (GenBank accession number X90939; positions 3428 to 3447 and 3930 to 3949, respectively). The PCR mixture consisted of 25 μl of reaction buffer containing 0.5 U of thermostable DNA polymerase diluted in the buffer supplied by the manufacturer (Integro, Leuvenheim, The Netherlands), 0.2 mM each deoxynucleoside triphosphate, 1.5 mM MgCl2, 10 pmol of each primer, and 10 to 50 ng of pneumococcal DNA. Amplification cycling in a programmable thermal controller (PTC-100; MJ Research, Watertown, Mass.) consisted of the following steps: predenaturation for 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. Amplification was finished after 3 min at 72°C. A 1.5% agarose gel in 0.5x TBE (Tris-borate-EDTA) containing 0.1 μg of ethidium bromide per ml was used to visualize the PCR products.

RFEL analysis. The 84 pneumococcal isolates were subjected to DNA fingerprinting analysis. Genotyping of the pneumococcal strains by restriction fragment end labeling (RFEL) analysis was performed as described by Van Steenbergen et al. (40) and adapted by Hermans et al. (16). Briefly, purified pneumococcal DNA was digested with restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [-32P]dATP by using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for various lengths of time at room temperature to ECL Hyperfilms (Amersham, Little Chalfont, United Kingdom).

Computer-assisted analysis of RFEL banding patterns. The RFEL types were analyzed by using the Windows version of GelCompar software (version 4; Applied Maths, Kortrijk, Belgium) after the RFEL autoradiograms were imaged with an Image Master desk top scanner (Pharmacia Biotech, Uppsala, Sweden). To this end, the DNA fragments in the molecular size range of 160 to 400 bp were explored. The DNA banding patterns were normalized with the pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages (30) and by using the Jaccard similarity coefficient applied to peaks (37). Computer-assisted analysis was carried out, and the methods were performed and the algorithms were used according to the instructions of the manufacturer of GelCompar. A tolerance of 1.2% in band position was applied during comparison of the DNA

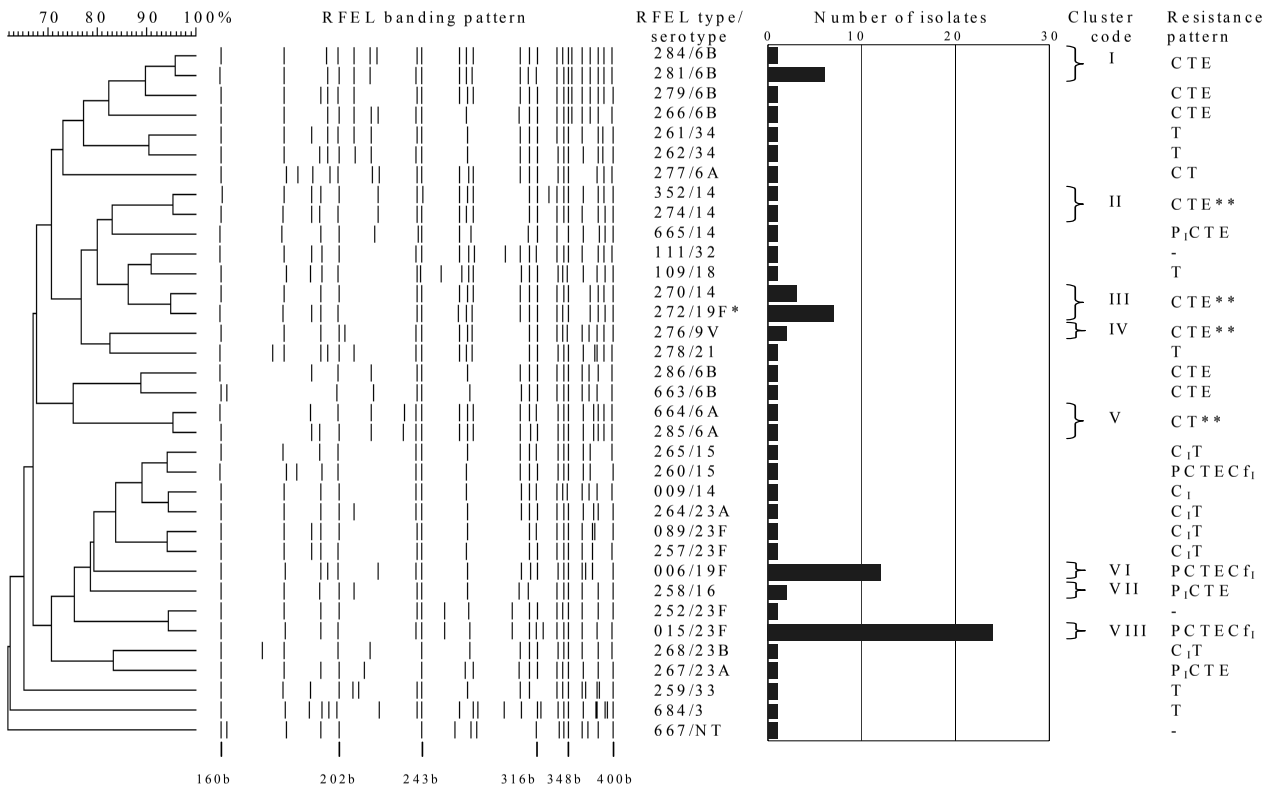


Figure 1. Dendrogram of the 38 RFEL types observed among the 84 strains isolated from the nasopharynx of Vietnamese children with an upper respiratory tract infection. Molecular sizes of reference bands in bases (b), RFEL banding patterns, RFEL types, serotypes, cluster codes and resistance patterns are depicted. penicillin, P; cotrimoxazole, C; tetracyclin, T; erytromycin, E; cefotaxim, Cf; and intermediate, I. Bars represent the number of isolates per RFEL type.

* Predominant serotypes within RFEL types.

**Major resistance patterns observed within clusters.

patterns. For evaluation of the genetic relatedness of the isolates, we used the following definitions: (i) strains of a particular RFEL type are 100% identical by RFEL analysis, (ii) an RFEL cluster represents a group of strains with RFEL types that differ by only one band (>95% genetic relatedness), and (iii) an RFEL lineage represents a group of strains with RFEL types that differ by less than four bands (>85% genetic relatedness).

International comparison. The Vietnamese genotypes were compared with the genotypes of an international collection of pneumococcal strains representing 193 distinct RFEL types originating from 15 different countries in Europe, Africa, and Asia and from the United States (M. Sluijter, unpublished observations, 2002), in which all international pandemic clones described by the pneumococcal epidemiological network are present (http://www.wits.ac.za/pmen/pmen.htm).

RESULTS

Eighty-four isolates retrieved from the nasopharynges of children under 5 years of age with acute respiratory tract infections were investigated by serotyping, susceptibility testing, and RFEL analysis. The most frequently observed serotypes were 23F (32% of strains), 19F (21%), 6B (13%), and 14 (10%). The rate of coverage of the seven-valent conjugate vaccine (covering serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) was 80%. The additive rate of coverage of the 11-valent conjugate vaccine (covering the additional serotypes 1, 3, 5, and 7F) was negligible: only one serotype 3 isolate was found, and isolates of the other three serotypes were absent. The most common serotypes not covered by the conjugate vaccine were 23A (2%), 6A (4%), and 15 (4%); one isolate could not be serotyped.

The rate of resistance to one or more antibiotics was 96%. The three isolates susceptible to all antibiotics were nonvaccine capsular types. The proportions of strains resistant to penicillin, co-trimoxazole, tetracycline, and erythromycin were 39, 71, 76, and 73%, respectively. None of the strains were resistant to rifampin, vancomycin, or ciprofloxacin. Intermediate resistance to penicillin, co-trimoxazole, and cefotaxime was found in 13, 16, and 39% of the strains, respectively. In total, 78% of the strains were multidrug resistant.

These multidrug-resistant strains were of serotypes 23F (24 strains), 19F (17 strains), 6B (12 strains), 14 (6 strains), 9V and 16 (2 strains each), and 15, 23A, and untypeable (1 strain each).

Molecular analysis of the 84 isolates revealed 35 different genotypes. Twelve of these genotypes represented eight genetic clusters with 61 isolates (72%) (Fig. 1). The largest cluster, cluster VIII, consisted of 24 isolates of serotype 23F and RFEL type 015. The second largest cluster was cluster VI, which consisted of 12 isolates of serotype 19F and RFEL type 006. The third and fourth largest clusters had 10 and 7 isolates, respectively, and

each cluster had two different genotypes. In contrast to all other clusters, cluster III, with 10 isolates, harbored isolates with two distinct capsular types, namely, serotypes 19F and 14. Cluster I, with seven isolates, consisted of serotype 6B strains only (Fig. 1).

We compared all Vietnamese RFEL types with the types in our international database representing 193 isolates from 16 different countries as well as those of all international pandemic clones described by the Pneumococcal Epidemiology Network (http://www.wits.ac.za/pmen/pmen.htm). Two of the genotypes, comprising the two largest clusters, clusters VIII and VI, were identical to international clones Spain 23F-1 and Taiwan 19F-14, respectively. The remaining 33 RFEL types were Vietnam specific, as these genotypes were not found in our present database.

PBP genotyping of the 84 pneumococcal isolates revealed 19 distinct types, and the cluster sizes ranged from 1 to 24 strains. Despite the observation that 61% of the strains were penicillin susceptible, only 15 isolates displayed wild-type PBP genotype 2-2-2, 2-2-3, or 2-2-71. Isolates in the major RFEL clusters, clusters VIII and VI, displayed PBP genotypes 1-1-1 and (26)-3-30, respectively. The *pbp1a* gene of the last PBP cluster could not be amplified from 5 of 12 strains. In addition, genetic analysis of tetracycline resistance genes *tet*(M) and *tet*(O) revealed that the *tet*(M) gene was exclusively observed in 77 of the 84 pneumococcal isolates (91%). Interestingly, 13 *tet*(M)-containing strains were fully susceptible to tetracycline. Finally, the erythromycin resistance gene *erm*(B) was present in 70 of the 84 pneumococcal isolates (83%). Seven of the *erm*(B)-containing strains also harbored the *mef*(E) gene. In the remaining 14 strains, *erm*(B), *mef*(A), and *mef*(E) were absent. Ten *erm*(B)-containing isolates and one *erm*(B)- and *mef*(E)-containing isolate were erythromycin susceptible (data not shown).

DISCUSSION

To determine the serotype distribution and theoretical rate of coverage of the new pneumococcal conjugate vaccines in Vietnam, we investigated 84 pneumococcal strains isolated from children under 5 years of age who visited several outpatient clinics in Hanoi with acute respiratory tract infections during the period from September 1997 to November 1999. Serotypes 23F (32%), 19F (21%), 6B (13%), and 14 (10%) were predominantly observed. The theoretical rate of coverage of the seven-valent conjugate vaccine was 80%. Since several studies have demonstrated a cross-reactive effect against serotype 6A, we assume a cumulative rate of coverage of the conjugate vaccine of 83% (7, 32). These data are comparable to those found in studies conducted in Europe and the United States, where rates of coverage of 60 to 80% are expected (17, 19, 21, 31, 33, 39).

A high rate of drug resistance was observed among the pneumococcal isolates. Ninety-six percent of the isolates were (intermediate) resistant to penicillin, co-trimoxazole, tetracycline, erythromycin, or cefotaxime. Seventy-eight percent of the isolates were resistant to three or more classes of antibiotics. This suggests a major problem with respect to treatment of pneumococcal infections with these antibiotics.

The epidemiological behavior of the pneumococcal strains isolated from the Vietnamese children was investigated by RFEL analysis. Among the 84 strains, 35 different genotypes were observed, of which 12 genotypes represented eight clusters with 61 strains. This indicates that 73% of the strains were recently transmitted. Ninety-three percent of the clustered isolates displayed capsular serotypes, which are covered by the seven-valent conjugate vaccine. The two largest clusters, clusters VI and VIII, containing 12 and 24 isolates, respectively, were identical to international clones Taiwan 19F-14 and Spain 23F-1, respectively. The remaining clusters, with 42% of the recently transmitted strains, and all strains with unique genotypes were considered to be Vietnam specific, as they were not previously observed for isolates from the 16 countries present in our database. These data indicate that strains of nationally and internationally spreading genotypes make a significant contribution to nasopharyngeal carriage and, consequently, respiratory tract infections among children in Hanoi. The four largest clusters, with 53 strains, i.e., 87% of the disseminating isolates, were multidrug-resistant. This indicates a significant transmission of multidrug-resistant pneumococci among children in Hanoi.

Because of the high percentage of resistance to penicillin, erythromycin, and tetracycline among the collection of isolates, we analyzed the corresponding resistance genes in detail. Since the degree of genetic heterogeneity of *pbp* genes in both penicillin-susceptible and penicillin-nonsusceptible isolates is high, a high degree of genetic plasticity irrespective of the penicillin resistance phenotype is suggested. This observation, which is in contrast to previous data, which showed that penicillin-susceptible pneumococci display a limited number of PBP genotypes (36), is in line with recent findings for pneumococcal isolates from Greece (4; Sluijter, unpublished). PBP genotype 1-1-1, which has been predominantly observed in various pneumococcal clones, including pandemic clones Spain 23F-1 and France 9V-3 (4), was also observed in Vietnamese cluster VIII, which represents clone Spain 23F-1. The second largest, Vietnam-specific cluster, cluster VI, primarily displayed PBP genotype 26-3-30.

Genetic analysis of tetracycline resistance genes *tet*(M) and *tet*(O) revealed the *tet*(M) gene in 91% of the Vietnamese isolates. This is in line with observations made by Luna and Roberts (24), who have demonstrated the presence of the *tet*(M) gene in 90% of the tetracycline-resistant strains. However, in the latter study, an additional 10% of the strains contained the *tet*(O) gene. Thirteen *tet*(M)-containing strains were fully susceptible to

tetracycline, suggesting the presence of a nonfunctional tetracycline resistance gene. A similar observation has previously been reported by Doherty et al. (10).

The erythromycin resistance gene *erm*(B) was present in 82% of the pneumococcal isolates, and 10% of the *erm*(B)-containing strains also harbored the *mef*(E) gene. This is in contrast to recent observations made by Reinert and coworkers (29), who have observed almost equal distributions of *erm*(B) (43%) and *mef*(E) (56%) genes among erythromycin-resistant isolates. Ten *erm*(B)-containing isolates and one *erm*(B)- and *mef*(E)-containing isolate were erythromycin susceptible. To our knowledge, this is the first study to describe the occurrence of nonfunctional erythromycin resistance genes in pneumococci.

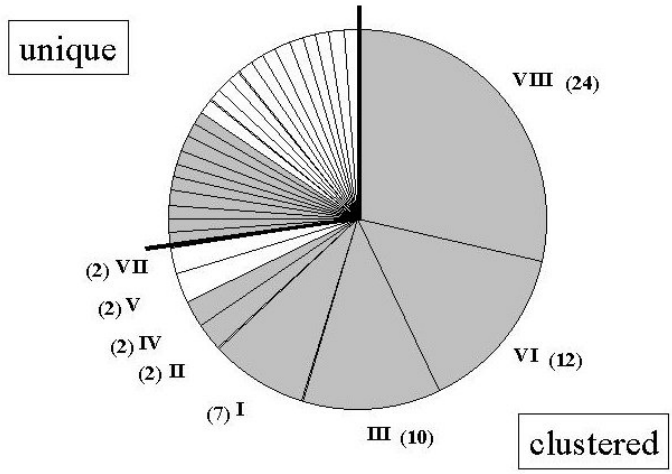


Figure 2. RFEL genotypes and theoretical coverage of the 7-valent conjugate vaccine. Vaccine serotypes are depicted in grey. Cluster codes and number of isolates per cluster (in brackets) are depicted.

When our data are compared with those from a recent study of Lee et al. (23), who have investigated pneumococcal carriage in healthy children in South Korea, we noticed slight differences in the serotype distributions. In both studies the three most common serogroups were 6, 23, and 19. However, the rate of carriage of serotype 23F was twice as high in the Vietnamese children with upper respiratory tract infections (30%) as in healthy carriers in South Korea (15%), whereas the rate of carriage of serogroup 6 was significantly higher in the healthy children in South Korea. In both studies, serogroup 23 isolates represented international clone Taiwan 23F. Assuming that the serotype distributions among children in Vietnam and South Korea are comparable, we hypothesize that clone Taiwan 23F is more often correlated with respiratory disease, whereas serogroup 6 is more frequently correlated with carriage. To strengthen this hypothesis, comparative epidemiological studies

demonstrating a significant correlation between pneumococcal carriage and respiratory disease are required.

In conclusion, our study demonstrated a high theoretical rate of coverage of the seven-valent conjugate vaccine against pneumococcal carriage strains isolated from children with acute respiratory tract infections in Hanoi. Moreover, the genotypes transmitted the most frequently were covered by the conjugate vaccine. Finally, 78% of the pneumococcal isolates are multidrug-resistant, and 92% of these multidrug-resistant isolates are theoretically covered by the seven-valent conjugate vaccine. Our data suggest that the children in Vietnam will benefit from implementation of vaccination with the pneumococcal conjugate vaccine.

REFERENCES

1. Baquero, F., J. A. Garcia-Rodriguez, J. Garcia de Lomas, and L. Aguilar. 1999. Antimicrobial resistance of 1,113 *Streptococcus pneumoniae* isolates from patients with respiratory tract infections in Spain: results of a 1-year (1996-1997) multicenter surveillance study. Antimicrob. Agents Chemother. 43:357-359.
2. Black, S., H. Shinefield, B. Fireman, E. Lewis, P. Ray, J. R. Hansen, L. Elvin, K. M. Ensor, J. Hackell, G. Siber, F. Malinoski, D. Madore, I. Chang, R. Kohberger, W. Watson, R. Austrian, K. Edwards, et al. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Pediatr. Infect. Dis. J. 19:187-195.
3. Bogaert, D., M. N. Engelen, A. J. Timmers-Reker, K. P. Elzenaar, P. G. Peerbooms, R. A. Coutinho, R. de Groot, and P. W. Hermans. 2001. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J. Clin. Microbiol. 39:3316-3320.
4. Bogaert, D., G. A. Syrogiannopoulos, I. N. Grivea, R. de Groot, N. G. Beratis, and P. W. Hermans. 2000. Molecular epidemiology of penicillin-nonsusceptible *Streptococcus pneumoniae* among children in Greece. J. Clin. Microbiol. 38:4361-4366.
5. Centers for Disease Control and Prevention. 1997. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). Morb. Mortal. Wkly. Rep. 46:1-24.
6. Corso, A., E. P. Severina, V. F. Petruk, Y. R. Mauriz, and A. Tomasz. 1998. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. Microb. Drug Resist. 4:325-337.
7. Dagan, R., N. Givon-Lavi, O. Zamir, M. Sikuler-Cohen, L. Guy, J. Janco, P. Yagupsky, and D. Fraser. 2002. Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J. Infect. Dis. 185:927-936.
8. Dejsirilert, S., K. Overweg, M. Sluijter, L. Saengsuk, M. Gratten, T. Ezaki, and P. W. Hermans. 1999. Nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae* among children with acute respiratory tract infections in Thailand: a molecular epidemiological survey. J. Clin. Microbiol. 37:1832-1838.
9. Del Grosso, M., F. Iannelli, C. Messina, M. Santagati, N. Petrosillo, S. Stefani, G. Pozzi, and A. Pantosti. 2002. Macrolide efflux genes *mef*(*A*) and *mef*(*E*) are carried by different genetic elements in *Streptococcus pneumoniae*. J. Clin. Microbiol. 40:774-778.
10. Doherty, N., K. Trzcinski, P. Pickerill, P. Zawadzki, and C. G. Dowson. 2000. Genetic diversity of the *tet*(*M*) gene in tetracycline-resistant clonal lineages of *S. pneumoniae*. Antimicrob. Agents Chemother. 44:2979-2984.
11. Eskola, J., T. Kilpi, A. Palmu, J. Jokinen, J. Haapakoski, E. Herva, A. Takala, H. Kayhty, P. Karma, R. Kohberger, G. Siber, P. H. Makela, S. Lockhart, and M. Eerola. 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N. Engl. J. Med. 344:403-409.

11a. Facklam, R. R., and J. A. Washington II. 1991. *Streptococcus* and related catalase-negative gram-positive cocci, p. 238-257. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrman, Henry D. Isenberg, and H. J. Shadomy, Manual of Clinical Microbiology, 5th ed. American Society for Microbiology, Washington, D.C.

12. Fairchok, M. P., W. S. Ashton, and G. W. Fischer. 1996. Carriage of penicillin-resistant pneumococci in a military population in Washington, DC: risk factors and correlation with clinical isolates. Clin. Infect. Dis. 22:966-972.
13. Goessens, W. H., N. Lemmens-den Toom, J. Hageman, P. W. Hermans, M. Sluijter, R. de Groot, and H. A. Verbrugh. 2000. Evaluation of the Vitek 2 system for susceptibility testing of *Streptococcus pneumoniae* isolates. Eur. J. Clin. Microbiol. Infect. Dis. 19:618-622.
14. Hausdorff, W. P., J. Bryant, C. Kloek, P. R. Paradiso, and G. R. Siber. 2000. The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II. Clin. Infect. Dis. 30:122-140.

15. Hermans, P. W., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. Goessens, and R. de Groot. 1997. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. Microb. Drug Resist. 3:243-251.
16. Hermans, P. W., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 33:1606-1612.
17. Huebner, R. E., A. D. Wasas, and K. P. Klugman. 2000. Trends in antimicrobial resistance and serotype distribution of blood and cerebrospinal fluid isolates of Streptococcus pneumoniae in South Africa, 1991-1998. Int. J. Infect. Dis. 4:214-218.
18. Jaffar, S., A. Leach, A. J. Hall, S. Obaro, K. P. McAdam, P. G. Smith, and B. M. Greenwood. 1999. Preparation for a pneumococcal vaccine trial in The Gambia: individual or community randomisation? Vaccine 18:633-640.
19. Jette, L. P., G. Delage, L. Ringuette, R. Allard, P. De Wals, F. Lamothe, and V. Loo. 2001. Surveillance of invasive *Streptococcus pneumoniae* infection in the province of Quebec, Canada, from 1996 to 1998: serotype distribution, antimicrobial susceptibility, and clinical characteristics. J. Clin. Microbiol. 39:733-737.
20. Kellner, J. D., E. L. Ford-Jones, et al. 1999. *Streptococcus pneumoniae* carriage in children attending 59 Canadian child care centers. Arch. Pediatr. Adolesc. Med. 153:495-502.
21. Kries, R., A. Siedler, H. J. Schmitt, and R. R. Reinert. 2000. Proportion of invasive pneumococcal infections in German children preventable by pneumococcal conjugate vaccines. Clin. Infect. Dis. 31:482-487.
22. Lee, H. J., J. Y. Park, S. H. Jang, J. H. Kim, E. C. Kim, and K. W. Choi. 1995. High incidence of resistance to multiple antimicrobials in clinical isolates of *Streptococcus pneumoniae* from a university hospital in Korea. Clin. Infect. Dis. 20:826-835.
23. Lee, N. Y., J. H. Song, S. Kim, K. R. Peck, K. M. Ahn, S. I. Lee, Y. Yang, J. Li, A. Chongthaleong, S. Tiengrim, N. Aswapokee, T. Y. Lin, J. L. Wu, C. H. Chiu, M. K. Lalitha, K. Thomas, T. Cherian, J. Perera, T. T. Yee, F. Jamal, U. C. Warsa, P. H. Van, C. C. Carlos, A. M. Shibl, M. R. Jacobs, and P. C. Appelbaum. 2001. Carriage of antibiotic-resistant pneumococci among Asian children: a multinational surveillance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP). Clin. Infect. Dis. 32:1463-1469.
24. Luna, V. A., and M. C. Roberts. 1998. The presence of the *tetO* gene in a variety of tetracycline-resistant *Streptococcus pneumoniae* serotypes from Washington State. J. Antimicrob. Chemother. 42:613-619.
25. Mulholland, K., O. Levine, H. Nohynek, and B. M. Greenwood. 1999. Evaluation of vaccines for the prevention of pneumonia in children in developing countries. Epidemiol. Rev. 21:43-55.
26. National Committee for Clinical Laboratory Standards. 1999. Performance standards for antimicrobial susceptibility testing: ninth informational supplement, M100-S9, 19 (1). National Committee for Clinical Laboratory Standards, Wayne, Pa.
27. Nuorti, J. P., J. C. Butler, J. M. Crutcher, R. Guevara, D. Welch, P. Holder, and J. A. Elliott. 1998. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. N. Engl. J. Med. 338:1861-1868.
28. Principi, N., P. Marchisio, G. C. Schito, S. Mannelli, et al. 1999. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Pediatr. Infect. Dis. J. 18:517-523.
29. Reinert, R. R., A. Al-Lahham, M. Lemperle, C. Tenholte, C. Briefs, S. Haupts, H. H. Gerards, and R. Lutticken. 2002. Emergence of macrolide and penicillin resistance among invasive pneumococcal isolates in Germany. J. Antimicrob. Chemother. 49:61-68.
30. Romesburg, H. 1990. Cluster analysis for researchers, p. 9-28.
31. Krieger, Malabar, Fla. Rudolph, K. M., A. J. Parkinson, A. L. Reasonover, L. R. Bulkow, D. J. Parks, and J. C. Butler. 2000. Serotype distribution and antimicrobial resistance patterns of invasive isolates of *Streptococcus pneumoniae*: Alaska, 1991-1998. J. Infect. Dis. 182:490-496.
32. Saeland, E., H. Jakobsen, G. Ingolfsdottir, S. T. Sigurdardottir, and I. Jonsdottir. 2001. Serum samples from infants vaccinated with a pneumococcal conjugate vaccine, PncT, potect mice against invasive infection caused by *Streptococcus pneumoniae* serotypes 6A and 6B. J. Infect. Dis. 183:253-260.
33. Saha, S. K., N. Rikitomi, M. Ruhulamin, H. Masaki, M. Hanif, M. Islam, K. Watanabe, K. Ahmed, K. Matsumoto, R. B. Sack, and T. Nagatake. 1999. Antimicrobial resistance and serotype distribution of *Streptococcus pneumoniae* strains causing childhood infections in Bangladesh, 1993 to 1997. J. Clin. Microbiol. 37:798-800.
34. Shi, Z. Y., M. C. Enright, P. Wilkinson, D. Griffiths, and B. G. Spratt. 1998. Identification of three major clones of multiply antibiotic-resistant *Streptococcus pneumoniae* in Taiwanese hospitals by multilocus sequence typing. J. Clin. Microbiol. 36:3514-3519.
35. Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck. 1992. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. Infect. Immun. 60:4119-4126.
36. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. Goessens, A. van Belkum, and P. W. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. J. Clin. Microbiol. 36:2248-2253.
37. Sneath, P. 1973. Numerical taxonomy, p. 131-132.
38. W. H. Freeman & Co., San Francisco, Calif. Sutcliffe, J., T. Grebe, A. Tait-Kamradt, and L. Wondrack. 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. 40:2562-2566.
39. Tomasz, A., A. Corso, E. P. Severina, et al. 1998. Molecular epidemiologic characterization of penicillin-resistant *Streptococcus pneumoniae* invasive pediatric isolates recovered in six Latin-American countries: an overview. Microb. Drug Resist. 4:195-207.
40. van Steenbergen, T. J., S. D. Colloms, P. W. Hermans, J. de Graaff, and R. H. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc. Natl. Acad. Sci. USA 92:5572-5576.

Chapter 5.2

Emergence of rifampicin-resistant *Streptococcus pneumoniae* as a result of antimicrobial therapy for penicillin-resistant strains

P.M. van Tilburg, D. Bogaert, M. Sluijter, A.R. Jansz, R. de Groot and P.W.M. Hermans

Clin Infect Dis 2001;33(8):e93-6

ABSTRACT

A multidrug-resistant strain of *Streptococcus pneumoniae* was isolated in The Netherlands during a nosocomial outbreak among 36 patients who mainly had chronic obstructive pulmonary disease. After the commencement of barrier nursing and short-term ceftriaxone-rifampin eradication therapy, the epidemic ceased. However, eradication therapy failed in 3 patients, and follow-up investigation of these patients showed the emergence of rifampin-resistant isolates.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) continues to be a common cause of serious and life-threatening infections, such as pneumonia, bacteremia, and meningitis, and of noninvasive infections, such as otitis media and sinusitis. Serious pneumococcal bacteremia is most prevalent in the extreme ages of life, resulting in illness in children in the first 2 years of life and in older patients (>70 years of age) [13]. An important risk factor for the acquisition and spread of drug-resistant pneumococci is chronic obstructive pulmonary disease (COPD) [3, 4]. Many studies have reported an increase in the prevalence of pneumococcal strains that are resistant to β-lactam antibiotics [5]. In addition, multidrug-resistant (MDR) strains have also been reported with increasing frequency. As a consequence, penicillin may no longer be the first-choice drug for treatment of pneumococcal infections in regions with a high prevalence of MDR strains. A variety of other antimicrobial agents can be used that have appropriate activity against penicillin-resistant pneumococci [5]. This includes rifampin, a semisynthetic derivative of the rifamycins that is primarily prescribed for the treatment of tuberculosis. Although not routinely used, it may be included in the treatment of penicillin-resistant pneumococcal infections in combination with the third-generation cephalosporins because of broad-spectrum activity, efficient (mucosal) tissue penetration, and a low side-effect profile [5-8]. Because of the possibility for rapid emergence of resistance, rifampin should never be prescribed as single-agent therapy [5, 7].

Rifampin resistance has arisen in several different species of bacteria because of alterations in the target of the antibiotic, the β-subunit of RNA polymerase encoded by *rpoB*. Rifampin acts by binding to the β-subunit of RNA polymerase, causing premature termination during DNA transcription, and point mutations, most of which map to 2 regions in the center of the *rpoB* gene and can lead to resistance to rifampin [8, 9]. Nucleotide sequence analysis of *rpoB* from rifampin-resistant and rifampin-susceptible isolates of *S. pneumoniae* has revealed that a defined set of point mutations within *rpoB* confers resistance to rifampin in *S. pneumoniae* in a similar manner as described elsewhere in resistant isolates of Mycobacterium tuberculosis, S. mitis, and Escherichia coli [8, 9]. In addition to the resistance to rifampin in *S. pneumoniae* caused by point mutation, interspecies gene transfer may also play a role in the evolution of rifampin resistance in *S. pneumoniae* [8]. Alternatives previously hinted at for organisms other than *S. pneumoniae* may also contribute to rifampin resistance. These include the modification in rifampin alteration in drug uptake or efflux mechanisms and changes in antibiotic permeability or metabolism [9].

In The Netherlands, a country with a low prevalence of pneumococcal resistance to antibiotics, several pneumococcal outbreaks have been reported in populations of patients with COPD [3, 4]. This study describes the emergence of primary rifampin resistance in 3 (of

which 2 were inadequately treated with rifampin monotherapy) patients with COPD in a Dutch hospital during therapeutic management of an outbreak of MDR pneumococci.

SETTING AND TREATMENT

In March 1997, isolates of restriction fragment end labeling (RFEL) type 19 pneumococci with reduced sensitivity to penicillin and other antibiotics were isolated from 36 patients admitted to the pulmonology ward of a 500-bed teaching hospital in Veldhoven, The Netherlands. This pneumococcal clone was unique for the current patient population, because it was not observed during the national survey of penicillin-resistant pneumococci in The Netherlands over the last 5 years. Retrospective research revealed nosocomial transmission among 36 COPD patients who had been hospitalized between July 1995 and August 1997. This outbreak was the continuation of the clonal spread of an MDR strain after interhospital transmission. In recognition of this outbreak, described elsewhere, an infection control team was set up in the hospital [3, 4].

Intervention therapy consisted of barrier nursing with separate rooms and cohort nursing. Antibiotic treatment before initiation of the treatment protocol followed susceptibility test results and always included a β-lactam antibiotic. After initiation of the protocol, a 7-day course of iv ceftriaxone 2 g once daily was prescribed. The treatment protocol was continued until 2 consecutive cultures were negative or until the patient was discharged from the hospital. The intervention therapy always included eradication therapy. Because colonization had been shown to contribute to further dissemination with drug-resistant pneumococci, eradication of carriage was sought. For this purpose, a 5-day course of rifampin (600 mg once daily) was added in the final stage of the patients' original antibiotic regimen as duotherapy. Rifampin was chosen as eradication therapy, because high levels can be obtained in respiratory secretions. In subsequent follow-up visits, nasopharyngeal swabs were taken for at least 1 year. Three patients did not respond to treatment, as described above.

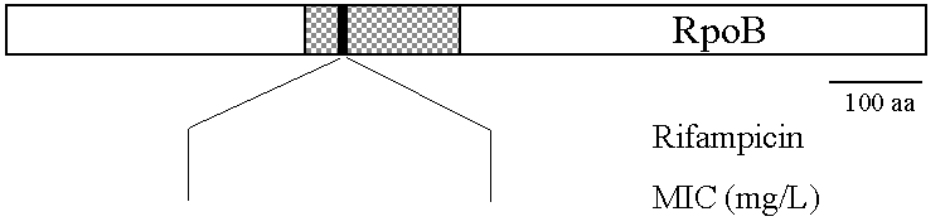
Microbiology. The DNA profiles of the isolates were examined by computerized analysis, after being confirmed as *S. pneumoniae* [4, 10]. A 617-bp internal region of the *rpoB* gene was amplified by PCR with primers RpoB2fw (5-GTACGTTTGGGACTTTCTCG-3, nucleotide position 10871106) and RpoB1rev (5-CATGCTGTTCGCAACGGCAAC-3, nucleotide position 16841703). Positioning of the primers is based on the *rpoB* gene sequence from the *S. pneumoniae* genome database of The Institute for Genomic Research (TIGR). Amplification was carried out in 100-mL volumes containing 75 mM Tris-HCl (pH 9.0), 20 mM (NH4)2SO4, 0.01% (w/v) Tween 20, 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, 50 pM of the individual primers, 0.5 U of *Taq* DNA polymerase (Goldstar,

Eurogentec), and 10 ng of purified chromosomal DNA. Cycling was performed in a PTC-100 Programmable Thermal Controller (MJ Research) and consisted of the following steps: predenaturation at 94°C for 1 min; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; and a final extension at 72°C for 3 min. Before nucleotide sequence analysis, the 617-bp amplicons were purified by sodium acetate precipitation. Each amplicon was sequenced by using 50 pM of the following primers: RpoB2fw, RpoB2rev (5-TTGTTCAAGTGTCCATAAG-3, position 14271446), RpoB1fw (5-ACTCACTATGGTCGTTATGTG-3, position 13541373), and RpoB1rev. DNA sequencing reactions were carried out with 300500 ng of DNA with the Thermo Sequenase dye terminator cycle sequencing premix kit (Pharmacia Amersham). The DNA sequencing procedure was performed according to the manufacturer's recommendations. Samples were analyzed with the Applied Biosystems Prism 377 (Pharmacia Amersham).

Transformation experiments were performed with *S. pneumoniae* Rx1 cells, as described by Lacks [11]. The cells were incubated for 2.5 h at 37°C with 0.5 mg of amplified *rpoB* DNA (617 bp) from rifampin-resistant pneumococcal isolates and 300 ng of synthetic competence stimulating factor 1 (CSP-1; kindly provided by D. Morrisson) before plating on Todd-Hewitt agar plates (Difco) containing 0.5% yeast extract (Difco) and 2 g/L rifampin (Yamanouchi Pharma). The susceptibility of the pneumococcal transformants for rifampin was determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards [12].

Patient 1. Patient 1 was admitted to the pulmonary department in November 1997 for exacerbation of COPD. Screening had revealed MDR rifampin-susceptible strains of *S. pneumoniae*, the first of which was isolated in March 1997. This patient received an antibiotic treatment followed by administrations of rifampin 600 mg for 5 days. In contrast to the protocol, rifampin was given in monotherapy. In December 1997, an MDR strain of *S. pneumoniae* with a rifampin resistance (MIC, 8 mg/L) was isolated from the nasopharynx of this patient. Although amoxicillin and rifamycin were given in duotherapy, rifampin-resistant isolates were collected on 5 different occasions.

Patient 2. Patient 2 was admitted to the hospital in September 1997 with exacerbation of COPD and constitutional eczema. MDR *S. pneumoniae* was isolated. She received a course of penicillin, followed by cotrimoxazole and rifampin for 5 days. During follow-up in the outpatient clinic in October 1997, an MDR pneumococcal isolate with an additional resistance for rifampin (MIC, >32 mg/L) was detected. A new rifampin eradication course for 5 days was prescribed in monotherapy. In December 1997, a rifampin-resistant strain of MDR *S. pneumoniae* (MIC, 8 mg/L) was detected. A combination course of an increased dose of penicillin and ceftriaxone iv was prescribed, resulting in the eradication of the rifampin-resistant strain in January 1998.




```
Rx1  **FGSSQFMDQHNPLSELSHKRRL****

1A   **FGSSQFMDQHNPLSELSHKRRL**** 0.032
1B   **FGSSQFMDQHNPLSELSNKRRL**** 8

2A   **FGSSQFMDQHNPLSELSHKRRL**** 0.023
2B   **FGSSQFMDQHNPLSELSDKRRL**** >32

3A   **FGSSQFMDQHNPLSELSHKRRL**** 0.047
3B   **FGPSQFMDQHNPLSELSHKRRL**** 4
```

Figure 1. Partial amino acid sequence of *Streptococcus pneumoniae rpoB* from rifampin-susceptible control strain Rx1 and the strains isolated from patients 1, 2, and 3 before (A) and after (B) eradication therapy. Amino acid residues are numbered according to their position in the *rpoB* protein. The striped bar indicates the PCR-amplified and PCR-sequenced gene part. Rifampin MIC values of the strains are also depicted.

Patient 3. Patient 3 had dehydration and obstipation. Microbiologic screening revealed an MDR, but rifampin-susceptible, strain of *S. pneumoniae* in both April and September 1997, without signs of COPD exacerbation. In contrast to the protocol, rifampin monotherapy was prescribed in an attempt to eradicate the pneumococcus. A subsequent specimen in November 1997 was negative for *S. pneumoniae*. In March 1998, a rifampin-resistant (MIC, 4 mg/L) MDR strain of *S. pneumoniae*, also highly resistant for tetracycline (MIC, >256 mg/L), emerged in the patient's sputum. In April 1998, the patient was readmitted for exacerbation of COPD. Antibiotic treatment was given without a second eradication regimen. After hospital discharge in May 1998, the MDR pneumococcus was isolated that year on several occasions.

Susceptibility testing of the rifampin-resistant pneumococcal MDR strains demonstrated a MIC for rifampin of 8, >32, and 4 mg/L for patients 1, 2, and 3, respectively (figure 1). Microbiologic analysis showed that all rifampin-resistant isolates belong to chromosomal genotype RFEL type 19 and serogroup 15. The penicillin-resistance genotype was PBP type

3-3-4. These findings match with the observations made during the initial outbreak [3], implying primary resistance of these *S. pneumoniae* isolates.

Mutations within *rpoB* showed a switch at position 526 from histidine to asparagine in patient 1, and in patient 2, from histidine to aspartic acid. Patient 3 had a point mutation at position 511 resulting in an amino acid substitution from serine to proline (figure 1). The amplified gene parts derived from the rifampin-resistant isolates were used to transform rifampin-susceptible pneumococcal strain Rx1. DNA transfer conferred rifampin resistance in the Rx1 transformants to MIC levels equal to the donor strains (data not shown). These observations indicate that the single point mutations in the *rpoB* genes of the COPD isolates were responsible for the rifampin-resistant phenotype.

DISCUSSION

COPD is a disease associated with frequent exacerbations, which are often accompanied by infections caused by *Haemophilus influenzae* or *S. pneumoniae*. As a consequence, antibiotic therapy is used widely in the treatment of exacerbations of COPD [3, 13]. Antimicrobial use is generally considered to be the driving force of bacterial resistance, resulting in the homing of MDR bacteria in this antibiotic "niche." Therefore, the prevalence of infections with MDR strains of pneumococci in this group of patients is often increased.

The combination of barrier nursing, a course of β-lactam antibiotics, and rifampin eradication therapy in the treatment protocol in Veldhoven was evidently effective. However, the MDR pneumococcus could not be eradicated in 3 patients. Microbiologic analysis confirmed that the studied isolates were of clonal origin and were identical to the initial outbreak strain. The mutations occurred in 2 different regions in the *rpoB* gene and displayed 3 distinct amino acid substitutions. We conclude that inadequate therapy caused by inaccurate prescription resulted in a primary *rpoB*-mediated rifampin resistance on 2 occasions. Obviously, the use of rifampin monotherapy in patients 1 and 3 contributed to the acquisition of rifampin resistance, and these patients did not respond to treatment [5, 7]. Patient 2 manifested primary resistance despite accurate therapy. We hypothesize that the pathological lung structure of these COPD patients has contributed to inadequacy of treatment by the treatment protocol. This study indicates the importance of accurate bacteriologic monitoring of patients with COPD who are colonized or infected with MDR pneumococci and suggests extension of the current eradication regimen with an additional (β-lactam) antibiotic.

REFERENCES

1. Caputo GM, Appelbaum PC, Liu HH. Infections due to penicillin-resistant pneumococci. Arch Intern Med 1993; 153:130110.
2. Musher DM. Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity, and treatment. Clin Infect Dis 1992; 14:8019.
3. De Galan BE, van Tilburg PMB, Sluijter M, et al. Hospital-related outbreak of infection with multidrug-resistant *Streptococcus pneumoniae* in The Netherlands. J Hosp Infect 1999; 42:18592.
4. Hermans PWM, Sluijter M, Elzenaar K, et al. Penicillin-resistant *Streptococcus pneumoniae* in The Netherlands: results of a 1-year molecular epidemiologic survey. J Infect Dis 1997; 175:141322.
5. Bradley JS, Scheld WM. The challenge of penicillin-resistant *Streptococcus pneumoniae* meningitis: current antibiotic therapy in the 1990s. Clin Infect Dis 1997; 24(Suppl 2):S21321. First citation in article | PubMed
6. Vesely JJ, Pien FD, Pien BC. Rifampin, a useful drug for nonmycobacterial infections. Pharmacotherapy 1998; 18:34557.
7. Friedland IR, McCracken GH Jr. Management of infections caused by antibiotic-resistant *Streptococcus pneumoniae*. N Engl J Med 1994; 331:37782.
8. Enright M, Zawadski P, Pickerill P, Dowson CG. Molecular evolution of rifampicin resistance in *Streptococcus pneumoniae*. Microb Drug Resist 1998; 4:6570.
9. Padayachee T, Klugman KP. Molecular basis of rifampicin resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 1999; 43:23615.
10. Hermans PWM, Sluijter M, Hoogenboezem T, Heersma H, van Belkum A, de Groot R. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J Clin Microbiol 1995; 33:160612.
11. Lacks S. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics 1966; 53:20735.
12. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 4th ed. Wayne, PA: National Committee for Clinical Laboratory Standards, 1997:M7A4.
13. Murphy TF, Sethi S, Klingman KL, Brueggemann AB, Doern GV. Simultaneous respiratory tract colonization by multiple strains of nontypeable Haemophilus influenzae in chronic obstructive pulmonary disease: implications for antibiotic therapy. J Infect Dis 1999; 180:4049.

Chapter 5.3

Molecular dynamics of pneumococcal colonization in healthy dutch children

D. Bogaert, M. Sluijter, N. Lemmens-den Toom, T.J. Mitchell, W.H.F. Goessens, S.C. Clarke, R. de Groot and P.W.M. Hermans

Submitted

ABSTRACT

Introduction. Streptococcus pneumoniae is a common cause of invasive diseases, such as sepsis and meningitis. Treatment is often complicated by multidrug resistance of this pathogen. Colonization with this species is common, particularly in young children. Recently, we have studied pneumococcal colonization among 3198 healthy children 1-19 years of age in The Netherlands. Overall, pneumococcal colonization was 19% with a peak-incidence of 55% at the age of 2 years. In addition, we have found an age-related serotype distribution among children, with a shift from vaccine to non-vaccine serotypes after the age of three years (Bogaert et al. Lancet: accepted).

Methods. In this study, we characterized the genetic background and resistance profiles of the 578 pneumococcal isolates from the latter study by means of chromosomal genotyping and susceptibility testing.

Results. In total, we observed 153 unique genotypes and 92 genetic clusters representing 425 isolates. In contrast to the observed age-related serotype distribution, the genetic background of the strains was not age-related. Comparison of the pneumococcal population to the international PMEN database of drug-resistant clones (PMEN) revealed close homology (>95%) with the international clones Spain^{9V}-3 (10 isolates), England ^{14}-9 (4 isolates), Tennessee^{23F}-4 (2 isolates), CSR^{14}-10 (1 isolate) and Sweden^{15A}-25 (1 isolate). In total 19% of all strains showed resistance to one or more antibiotics. Resistance to cotrimoxazole, tetracycline, erythromycin and penicillin was found in 12.9%, 5.6%, 5.0% and 2.7%, respectively. Multidrug resistance was found in 1.9% of all strains.

Conclusion. Pneumococcal colonization isolates from healthy Dutch children represent a heterogeneous, mostly susceptible genetic population with a high degree of genetic clustering, which suggests a high tendency to spread within the community.

INTRODUCTION

Streptococcus pneumoniae is worldwide one of the major pathogens causing invasive disease and respiratory tract infections. Risk groups for pneumococcal infections are young children, elderly and immunodeficient patients. Despite adequate antibiotic treatment, morbidity and mortality due to pneumococcal disease remains high (8). The increasing (multi) drug resistance among pneumococcal isolates hampers adequate treatment (1, 9, 19, 32).

Nasopharyngeal colonization with pneumococci is common; in general humans get colonized at least once early in life. Although colonization with pneumococci is mostly asymptomatic, it can progress to respiratory or even systemic disease as a result of a (temporary) defect in the mucosal barrier function, for example by a viral infection. Importantly, pneumococcal disease has to be preceded by nasopharyngeal colonization with the homologous strain (13, 14). Moreover, pneumococcal colonization causes horizontal spread of this pathogen within the community. For example crowding, which occurs in hospitals, day-care centers and jails, enhances horizontal spread of pneumococcal strains (17, 20, 23, 26, 29). Because the highest incidence in pneumococcal colonization and the highest crowding index is found in young children, this risk group is considered to be the most important vector for horizontal dissemination of pneumococcal strains within the community (21). New pneumococcal conjugate vaccines are highly effective against invasive diseases in young children (2). Furthermore, a protective effect against mucosal infections, such as (recurrent) otitis media, albeit limited, has been observed (2, 12). At nasopharyngeal level, however, replacement of vaccine-type pneumococci with non-vaccine serotypes as a result of vaccination has also been observed (10, 24, 35). In addition, replacement of mucosal disease, i.e. (recurrent) acute otitis media, has been found (12, 35). What effect this serotype replacement has on invasive diseases remains unclear, although it has clearly been demonstrated that several non-vaccine serotypes have high potential for causing invasive disease (7). Because few data are available on the age-related incidence and serotype distribution of *S. pneumoniae* among healthy children, a cross-sectional study has been performed in the summer of 2002 among 3200 healthy children aged 1-19 years in which the prevalence and determinants of pneumococcal carriage were studied (5). This study has shown a significant age-related colonization rate with a peak incidence at 3 years of age of 55% followed by a gradual decline till a stable colonization rate of 10% was reached after the age of 10 years. Moreover, a significant age-related serotype distribution was noticed with a primary peak of vaccine serotypes early in life, followed by a secondary peak with non-vaccine serotypes (5).

In this study, we investigated the molecular epidemiological dynamics and resistance profiles of the pneumococcal strains collected during the latter study in order to obtain

detailed insight in the occurrence and age-related distribution of pneumococcal genotypes and resistance profiles.

MATERIAL AND METHODS

Bacterial isolates. In total, 3198 healthy children, aged 1-19 years, participating in a national meningococcal vaccination campaign in Rotterdam were enrolled. All children were residents of Rotterdam and were vaccinated either in July (age 12 months to 5 years plus 15 to 19 years) or in September (age 6 to 15 years) of the year 2002. Signed informed consent was obtained from the parent accompanying the participating children under the age of 16 and directly from the children above the age of 16. Demographic data were collected as described previously (5). The study was approved by the Medical Ethics Review Board of Erasmus MC, Rotterdam, The Netherlands.

Cultures. Nasopharyngeal samples were obtained with rayon tipped dacron pernasal swabs (Copan Italia, Brescia, Italy) and transported in Amies transport medium to the medical microbiology laboratory and plated within 6 hours of sampling. The swabs were plated on gentamicin blood agar for the isolation of *S. pneumoniae.* Identification of *S. pneumoniae* isolates was performed by standard methods as described previously (22). Susceptibility testing was performed by the disc diffusion method. Resistance was defined by measuring the zone diameters for the respective antibiotics as defined by the NCCLS (27). Strains showing reduced susceptibility to oxacillin were additionally tested for penicillin and cefotaxime resistance by the E-test. Multidrug resistance was defined as resistance to $\geq$3 classes of antimicrobial agents.

Restriction fragment end labeling (RFEL) typing. Pneumococcal strain typing by RFEL was done as described by van Steenbergen et al. (33) and adapted by Hermans et al. (16). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72^{o}C with [α-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 *M* urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, NY), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, UK).

Computer-assisted analysis of DNA band patterns. RFEL autoradiographs were converted to images (Agfa Arcus II, Agfa Gevaert, Rijswijk, The Netherlands) and analyzed by computer (Windows version Bionumerics; Applied Maths, Sint-Martens-Latem, Belgium). DNA fragments were analyzed as described previously (31). For evaluation of the genetic relatedness of the isolates we used the following definitions: (1) isolates of a particular RFEL

type are 100% identical by RFEL analysis; (2) an RFEL cluster represents a group of RFEL types that differ in only one band (approximately >95% genetic relatedness).

International comparison. The genotypes were compared with an international collection of pneumococcal isolates representing 751 distinct RFEL types originating from 17 different countries in America, Europe, Africa and Asia (4, 15, 30), in which the first 26 international pandemic clones as described by the pneumococcal molecular epidemiological network (PMEN) in 2002 are present (25).

Multi locus sequence typing (MLST). The genotypes of the 24 largest clusters (n>4)were verified by MLST analysis, and one, two or three isolates per cluster were analyzed. For this purpose, a fully automated method for MLST was used as described previously (18). The MLST types were compared with the global PMEN database (http://www.pneumo.com/physician/pmen/pmen_history.asp).

Data-analysis. P-values for differences were calculated with the Chi-square test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, USA).

Table 1. Serotype distribution of the pneumococcal isolates.

	Total number of pneumococci (%)	
S. pneumoniae	601	(100 %)
Vaccine types	250	(41.6%)
Non-vaccine types	351	(58.4%)
Vaccine types		
Serotype 6B	69	(11.5%)
Serotype 19F	68	(11.3%)
Serotype 23F	53	(8.8%)
Serotype 14	23	(3.8%)
Serotype 18C	17	(2.8%)
Serotype 9V	14	(2.3%)
Serotype 4	6	(1.0%)
Non-vaccine types		
Serotype 6A	40	(6.7%)
Serotype 3	36	(6.0%)
Serotype 11	31	(5.2%)
Serotype 8	21	(3.5%)
Serotype 15	19	(3.2%)
Serotype 19A	19	(3.2%)
Serotype 10	18	(3.0%)
Serotype 23A	16	(2.5%)
Serotype 16	12	(2.0%)
Serotype 38	12	(2.0%)
Serotype 7	10	(1.7%)
Other serotypes	93	(15.4%)
Untypeable	31	(5.2%)

RESULTS

In total, 601 pneumococci were isolated from 3198 healthy children aged 1-19 years visiting the meningococcal vaccination campaign in the summer of 2002 in the city of Rotterdam, The Netherlands. The most prevalent serotypes found were the serotypes 6B, 19F, 23F, 6A, 3, 11 and 14 (Table 1). The age-related distribution of the most prevalent serotypes is depicted in Figure 1. The vaccine serotypes as well as the cross-protective serotypes invariably showed peak incidences at the age of 1-2 years, whereas the majority of the non-vaccine serotypes peaked at older age.

578 of the pneumococci were available for genetic analysis by means of RFEL genotyping (96%). We observed 337 genetically distinct genotypes of which 153 types were unique (found only once). The remaining 425 pneumococcal isolates (74%) comprised 92 genetic clusters. Of the RFEL clusters observed in this collection 5 showed close homology with RFEL profiles of the international PMEN clones. The PMEN genotype Spain^{9V}-3 was observed 10 times, England ^{14}-9 4 times, Tennessee^{23F}-4 2 times, CSR^{14}-10 1 time and Sweden^{15A}-25 1 time. In Figure 2 all genetic clusters are represented in a genetic dendrogram, including the serotype distribution, age distribution and number of isolates representing each cluster. In contrast to the age-related serotype distribution, no additional correlation between age and genetic profile was noted, except for Cluster I which showed an average age of 11 years compared to 6 years for the entire pneumococcal population.

We also investigated the clustering rate for the most common serotypes. In total, 55%, 72%, 56%, 48%, 67%, 67%, and 60% of the isolates representing the serotypes 6A, 6B, 14, 19F, 23F, 3 and 11 isolates, respectively, were part of one of the clusters. The remaining isolates represented unique genotypes. Statistical analysis of these data showed a significantly lower clustering rate for the serotypes 6A and 19F.

In Table 2 we depicted the 9 largest genetic clusters, representing more then 10 isolates each. The largest cluster, Cluster V, consisted of 45 serotype 6B (35), 6A (8) and 19F (2) strains, representing 5 genotypes. No homology was observed with any of the PMEN clones. The second and third largest clusters, i.e. Cluster VIII (18 strains) and Cluster I (17 strains) represented 2 and 3 genotypes, respectively and harbored mainly serotype 3. Only one major cluster, Cluster IX representing serotype 9V and 19F (13 strains), showed close homology (>95%) with an international clone, Spain^{9V}-3. MLST analysis showed that Cluster IX and Spain^{9V}-3 had 5 out of 7 alleles in common. In contrast to the original PMEN clone all Dutch isolates were fully susceptible to penicillin, tetracycline, erythromycin and cotrimoxazole. The remaining clusters, i.e. Cluster II (13 strains), Cluster III (10 strains), Cluster IV (13 strains), Cluster VI (10 strains) and Cluster VII (13 strains) represented predominantly the serotypes 14, 15, 23F, 23F, and 23F, respectively. Six of the 9 major clusters consisted of conjugate vaccine serotypes, whereas only three clusters

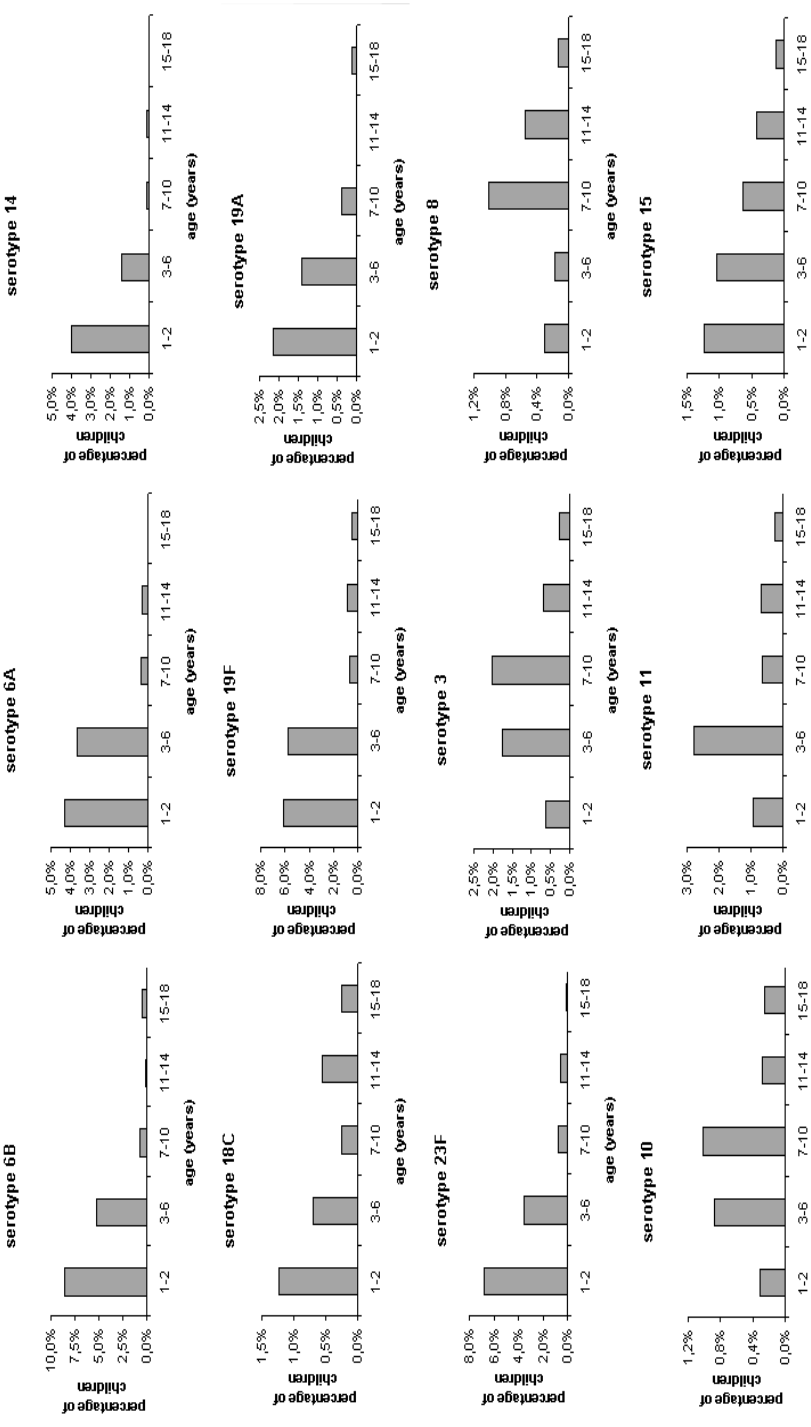


Figure 1. Age-related serotype-specific prevalence of pneumococcal strains during colonization. The prevalence is depicted as the percentage of the total number of children. Data are clustered for the ages 12-36 months, 3-6 years, 7-10 years, 11-14 years and 15-18 years.

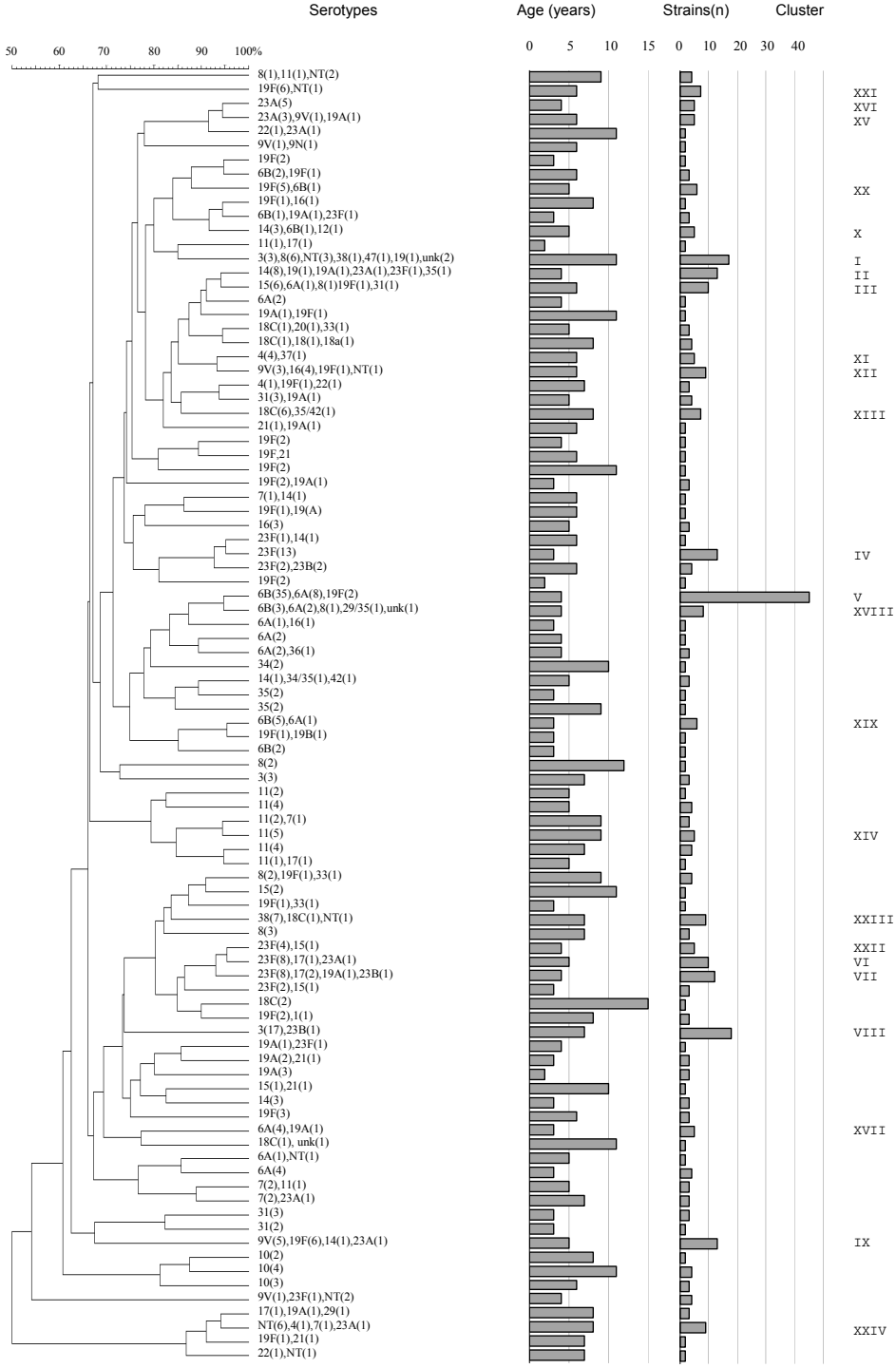

Serotypes
Age (years)
Strains(n)
Cluster
50 60 70 80 90 100%
0 5 10 15
0 10 20 30 40
8(1),11(1),NT(2)
19F(6),NT(1)
23A(5)
23A(3),9V(1),19A(1)
22(1),23A(1)
9V(1),9N(1)
19F(2)
6B(2),19F(1)
19F(5),6B(1)
19F(1),16(1)
6B(1),19A(1),23F(1)
14(3),6B(1),12(1)
11(1),17(1)
3(3),8(6),NT(3),38(1),47(1),19(1),unk(2)
14(8),19(1),19A(1),23A(1),23F(1),35(1)
15(6),6A(1),8(1)19F(1),31(1)
6A(2)
19A(1),19F(1)
18C(1),20(1),33(1)
18C(1),18(1),18a(1)
4(4),37(1)
9V(3),16(4),19F(1),NT(1)
4(1),19F(1),22(1)
31(3),19A(1)
18C(6),35/42(1)
21(1),19A(1)
19F(2)
19F,21
19F(2)
19F(2),19A(1)
7(1),14(1)
19F(1),19(A)
16(3)
23F(1),14(1)
23F(13)
23F(2),23B(2)
19F(2)
6B(35),6A(8),19F(2)
6B(3),6A(2),8(1),29/35(1),unk(1)
6A(1),16(1)
6A(2)
6A(2),36(1)
34(2)
14(1),34/35(1),42(1)
35(2)
35(2)
6B(5),6A(1)
19F(1),19B(1)
6B(2)
8(2)
3(3)
11(2)
11(4)
11(2),7(1)
11(5)
11(4)
11(1),17(1)
8(2),19F(1),33(1)
15(2)
19F(1),33(1)
38(7),18C(1),NT(1)
8(3)
23F(4),15(1)
23F(8),17(1),23A(1)
23F(8),17(2),19A(1),23B(1)
23F(2),15(1)
18C(2)
19F(2),1(1)
3(17),23B(1)
19A(1),23F(1)
19A(2),21(1)
19A(3)
15(1),21(1)
14(3)
19F(3)
6A(4),19A(1)
18C(1), unk(1)
6A(1),NT(1)
6A(4)
7(2),11(1)
7(2),23A(1)
31(3)
31(2)
9V(5),19F(6),14(1),23A(1)
10(2)
10(4)
10(3)
9V(1),23F(1),NT(2)
17(1),19A(1),29(1)
NT(6),4(1),7(1),23A(1)
19F(1),21(1)
22(1),NT(1)
XXI
XVI
XV
XX
X
I
II
III
XI
XII
XIII
IV
V
XVIII
XIX
XIV
XXIII
XXII
VI
VII
VIII
XVII
IX
XXIV

Table 2. Properties of the most predominant genetic clusters.

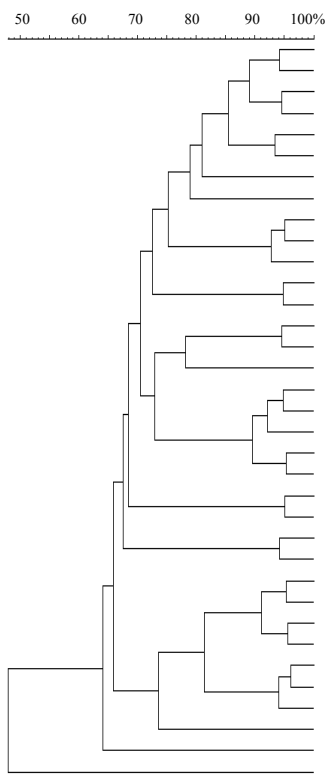
Cluster	Number of isolates	Serotypes	Number of genotypes	Average age (years)
I	17	3(3), 8(6), NT(3), others (6)	3	11
II	13	14(8), others (5)	4	4
III	10	15(6), others (4)	2	6
IV	13	23F(13)	4	3
V	45	6B(35), 6A(8), 19F(2)	5	4
VI	10	23F(8), others (2)	3	5
VII	12	23F(8), 17(2), others (2)	3	4
VIII	18	3(17), others (1)	2	7
IX	13	9V(5), 19F(6), others(2)	4	5

represented the non-vaccine serotypes 3 and 15.

We characterized the 24 largest clusters by MLST (Figure 3). This method confirmed the presence of an additional PMEN clone, namely England ^{14}-9. The remaining clusters showed ST types differing from the international multidrug-resitant clones, however most clusters matched ST types present in the international database.

Susceptibility testing was performed on 587 pneumococcal isolates for the following antimicrobial drugs: penicillin, erythromycin, tetracycline, cotrimoxazole, chloramphenicol, cefotaxime, ciprofloxacin and vancomycin. In total, 18.1% of the isolates showed resistance to one or more of the antimicrobial drugs investigated. Resistance to cotrimoxazole, erythromycin, and tetracycline was found in 12.9%, 5.0% and 5.6%, respectively. No resistance to vancomycin and ciprofloxacin was observed. In 13.6% of the isolates, resistance to a single drug was observed. In 2.6% of the isolates dual resistance was found and in 1.9% multidrug resistance. The resistance patterns observed are depicted in Table 3. High-level penicillin resistance was not observed. However, 2.7% of the pneumococcal isolates was intermediate susceptible to penicillin. In addition, the majority of these strains (11 out of 15 isolates) showed dual or multidrug resistance. In Table 4, the number of strains, resistance level and the average age of the children carrying these isolates is shown. No significant difference could be observed for the average age of the children carrying resistant isolates (5.4 years) versus children carrying susceptible isolates (5.9 years), nor for the individual resistance profiles.

Figure 2 (left). Dendrogram of the 92 genetic clusters observed by RFEL genotyping among the Dutch *S. pneumoniae* nasopharyngeal isolates. Serotypes with number of strains per serotype in brackets, bars representing average age of children carrying these isolates, number of isolates per RFEL cluster and cluster codes are depicted.



Cluster	Serotype	ST	*aroE*	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
II	14	124	7	5	1	8	14	11	14
X	14	9[a]	1	5	4	5	5	1	8
III	15	199	8	13	14	4	17	4	14
III	15	199	8	13	14	4	17	4	14
XII	16	414	1	5	27	5	1	28	1
XI	4	205	10	5	4	5	13	10	18
XIII	18C	113	7	2	1	1	10	1	21
IV	23F	36	1	8	4	1	1	4	6
I	8	447	29	33	19	1	36	22	31
I	8	447	29	33	19	1	36	22	31
I	3	447	29	33	19	1	36	22	31
XIV	11	408	2	5	6	12	16	3	14
XIV	11	62	2	5	29	12	16	3	14
XV	23A	42	1	8	9	9	6	4	6
XVI	23A	1040	1	8	9	9	6	26	6
XVII	6A	1141	2	13	9	6	6	19	14
V	6B	176	7	13	8	6	10	6	14
V	6A	1143	7	25	4	1	15	1	28
XVIII	6B	176	7	13	8	6	10	6	14
XIX	6B	176	7	13	8	6	10	6	14
V	6B	176	7	13	8	6	10	6	14
XX	19F	177	7	14	4	12	1	1	14
XX	19F	1045	7	14	4	12	1	14	14
XXI	19F	236	15	16	19	15	6	20	26
XXI	19F	236	15	16	19	15	6	20	26
VII	23F	440	7	5	1	1	13	31	14
VII	17	392	7	5	1	1	6	31	14
XXII	23F	440	7	5	1	1	13	31	14
VI	23F	440	7	5	1	1	13	31	14
XXIII	38	1140	7	43	41	18	13	49	6
XXIII	38	393	10	43	41	18	13	49	6
XXIII	38	393	10	43	41	18	13	49	6
VIII	3	180	7	15	2	10	6	1	22
IX[b]	9V	1142	29	11	10	1	6	8	14
XXIV	4	449	8	37	9	29	2	47	5

Figure 3. Dendrogram of 35 representative pneumococcal strains belonging to the 24 largest clusters. The MLST profiles of these strains are depicted.

[a] 100% homology with England ^{14}-9.

[b] 5/7 alleles homology with Spain^{9V}-3.

We also determined the contribution of vaccine serotypes to the total number of resistant strains. 60 out of 104 resistant strains depicted serotypes, which are covered by the 7-valent conjugate vaccine (58%) and 74 out of 104 resistant isolates (71%) were vaccine serotype isolates when the cross-protective serotypes 6A, 9N/L, 19A/B and 23A/B were included. In both cases, the contribution of vaccine serotypes to resistance is significantly higher compared to the contribution of vaccine serotypes to the total group of pneumococci. In case of dual resistance 10 out of 15 (67%) displayed vaccine serotypes and 11 out of 15 isolates (73%) when cross-protective serotypes were included. With respect to multidrug resistance 6 out of 11 (55%) strains represented vaccine serotypes and 7 out of 11 (64%) when cross-reactive serotypes were included. However, numbers were too small to show significant

differences compared to the contribution of vaccine serotypes to the susceptible pneumococcal population.

Table 3. Resistance profiles of the drug-resistant pneumococcal strains

Antibiotic*	Number of strains	Resistance rate		
P	4	0.5%		
T	13	2.2%		
E	6	0.9%		
S	57	9.7%	Mono resistance:	13.6%
PT	0	0.0%		
PS	3	0.5%		
TE	5	0.9%		
TS	4	0.5%		
SE	3	0.5%	Dual resistance:	2.6%
PTS	1	0.2%		
PTE	2	0.3%		
PES	0	0.0%		
TSE	2	0.3%		
PTSE	5	0.9%		
PSEC	1	0.2%	Multi resistance	1.9%
Total	106	18.1%		

* P, penicillin; T, tetracyclin; E, erythromycin; C; cotrimoxazole

Table 4. Susceptibility profiles of the pneumococcal isolates

	Percentage of Resistant strains	Percentage of intermediate Resistant strains	Total	Average age (years)
Penicillin		2.7%	2.7%	7.1
Tetracyclin	3.9%	1.7%	5.6%	5.9
Erythromycin	4.5%	0.6%	5.0%	5.1
Cotrimoxazole	7.5%	5.5%	12.9%	5.7
Chloramphenicol	0.2%		0.2%	18.0

DISCUSSION

We studied the molecular epidemiology of 578 pneumococcal isolates retrieved from healthy children varying from 12 months to 19 years of age in Rotterdam, The Netherlands. Genetic clustering was observed in 74% of the strains, which comprised 92 distinct genetic clusters. These data implicate that most pneumococci observed in this study have a high tendency to spread within the community. Moreover, because the majority of the strains is fully susceptible to the most common antibiotics, these data suggest that a high tendency to spread is per definition not related to (multi)drug resistance. Since the prevalence of

resistant pneumococci is very low (18.1%), we are unable to compare the tendency to spread within the community between resistant and fully susceptible strains.

In our cohort the largest genetic cluster comprised mainly serotype 6A and 6B isolates. These isolates were all fully susceptible to penicillin, tetracycline, erythromycin and cotrimoxazole. In addition, comparison of the genotypes of this cluster showed no close homology to any of the known serotype 6B clones from the PMEN database (25). Of the genotypes observed in this collection only 5 showed close homology with 5 distinct PMEN clones (25). These data indicate that the international drug-resistant clones do not significantly contribute to colonization and spread of pneumococci among children in The Netherlands. The data confirmed the observations of recent studies performed in The Netherlands among day-care center attendees and children suffering from recurrent acute otitis media (3, 6).

Recently, we have observed an age-related pneumococcal serotype distribution within our study population (5). Pneumococcal conjugate vaccine serotypes showed an initial peak incidence of 30% at the age of 1 years after which a decline is observed to an incidence of 2-3% after the age of 8 years. For non-vaccine serotypes an initial increase in incidence was observed to 27% at the age of 4 years, followed by a decline, which stabilizes after the age of 15 years at 4% (5). This was mainly caused by predominance of a limited number of predominant non-vaccine serotypes after the age of 3 years, i.e. serotype 3, 8, 10, and 11. Because children under 2 years of age who show the highest risk for pneumococcal infections carry mainly vaccine serotype strains, a difference in virulence between vaccine and non-vaccine serotypes might exist. We, therefore, investigated the age-related distribution of genotypes. In contrast to the predominant serotype 3 Cluster I with an average age of 11 years, no age-related genetic clustering was observed. This observation suggests that despite the observed shift in serotype with age, the chromosomal make-up and, corollarily, virulence of pneumococcal strains does not shift with increasing age.

The most frequently found serotypes in this study were 6A, 6B, 19F, 23F, 3 and 11. We questioned whether specific serotypes show a higher tendency to spread horizontally. The clustering rate of these serotype-specific isolates was on average 60-70%, whereas the total pneumococcal population showed 74% clustering. Surprisingly, the serotypes 6A and 19F showed a decreased tendency to spread in the community (55% and 48%, respectively), harboring more isolates representing unique genotypes. This is in line with previous findings among penicillin-susceptible isolates in The Netherlands (28). For the serotype 14 isolates a similar degree of clustering was found being 56%, although this was not significantly different because of the limited number of isolates.

When evaluating the 9 largest clusters each harboring 10 or more isolates, we observed that 6 of these clusters represented the conjugate vaccine serotypes 6B, 14 and 23F, whereas

the remaining three clusters consisted of the non-vaccine serotypes 3, 8 and 15. These data suggest that besides vaccine serotype pneumococci, also non-vaccine pneumococcal serotypes are able to spread at high frequency within the community. These findings are in accordance with several vaccination studies, where replacement of vaccine serotypes with non-vaccine pneumococcal serotype carriage was found at the nasopharynx after pneumococcal conjugate vaccination (10, 24, 35). The question remains, whether these non-vaccine serotype strains are as pathogenic as their vaccine-serotype counterparts. Several studies have already shown replacement from vaccine serotypes to non-vaccine serotype pneumococci causing mucosal disease (12, 34). With respect to invasive disease, replacement has not been observed yet. However, Brueggemann et al. have demonstrated epidemiological evidence for the high invasive capacity of certain non-vaccine serotypes (7).

Susceptibility testing showed in 18.1% of the strains resistance to at least one of the most commonly used antimicrobial drugs. Resistance to a single drug was observed most often (13.6%). Multidrug resistance was found in only 1.9% of the isolates. Most commonly, we observed resistance to cotrimoxazole (12.9%), which is a first line antibiotic used commonly among children in The Netherlands. Finally, we found antibiotic resistance to be associated with vaccine serotype pneumococci, which is in line with previous studies (4, 11, 36).

We conclude that drug resistance, with respect to pneumococcal carriage and disease, is still of minor concern in The Netherlands. However, a strict policy with respect to antibiotic prescription is still required.

In conclusion, pneumococcal colonization isolates from healthy Dutch children represent a heterogeneous, genetic population of mostly susceptible strains, which display a high tendency to spread horizontally irrespective of age of the colonized children.

REFERENCES

1. 1997. From the Centers for Disease Control and Prevention. Surveillance for penicillin-nonsusceptible *Streptococcus pneumoniae*--New York City, 1995. Jama 277:1585-6.
2. Black, S. B., H. R. Shinefield, S. Ling, J. Hansen, B. Fireman, D. Spring, J. Noyes, E. Lewis, P. Ray, J. Lee, and J. Hackell. 2002. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. Pediatr Infect Dis J 21:810-5.
3. Bogaert, D., M. N. Engelen, A. J. Timmers-Reker, K. P. Elzenaar, P. G. Peerbooms, R. A. Coutinho, R. de Groot, and P. W. Hermans. 2001. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J Clin Microbiol 39:3316-20.
4. Bogaert, D., N. T. Ha, M. Sluijter, N. Lemmens, R. De Groot, and P. W. Hermans. 2002. Molecular epidemiology of pneumococcal carriage among children with upper respiratory tract infections in Hanoi, Vietnam. J Clin Microbiol 40:3903-8.
5. Bogaert, D., A. van Belkum, M. Sluijter, A. Luijendijk, H. Rumke, R. de Groot, H. A. Verbrugh, and P. Hermans. 2003. Competition between *Streptococcus pneumoniae* and *Staphylococcus aureus* during colonisation is serotype-dependent. Lancet: accepted.
6. Bogaert, D., R. Veenhoven, M. Sluijter, and e. al. 2003. Molecular dynamics of pneumococcal colonization in response to pneumococcal conjugate vaccination in children with recurrent acute otitis media. Submitted.
7. Brueggemann, A. B., D. T. Griffiths, E. Meats, T. Peto, D. W. Crook, and B. G. Spratt. 2003. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis 187:1424-32.
8. Butler, J. C., E. D. Shapiro, and G. M. Carlone. 1999. Pneumococcal vaccines: history, current status, and future

directions. Am J Med 107:69S-76S.
9. Crook, D. W., and B. G. Spratt. 1998. Multiple antibiotic resistance in *Streptococcus pneumoniae*. Br Med Bull 54:595-610.
10. Dagan, R., N. Givon-Lavi, O. Zamir, M. Sikuler-Cohen, L. Guy, J. Janco, P. Yagupsky, and D. Fraser. 2002. Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J Infect Dis 185:927-36.
11. Dobay, O., F. Rozgonyi, E. Hajdu, E. Nagy, M. Knausz, and S. G. Amyes. 2003. Antibiotic susceptibility and serotypes of *Streptococcus pneumoniae* isolates from Hungary. J Antimicrob Chemother 51:887-93.
12. Eskola, J., T. Kilpi, A. Palmu, J. Jokinen, J. Haapakoski, E. Herva, A. Takala, H. Kayhty, P. Karma, R. Kohberger, G. Siber, P. H. Makela, S. Lockhart, and M. Eerola. 2001. Efficacy of a Pneumococcal Conjugate Vaccine against Acute Otitis Media. N Engl J Med 344:403-409.
13. Faden, H., J. Stanievich, L. Brodsky, J. Bernstein, and P. L. Ogra. 1990. Changes in nasopharyngeal flora during otitis media of childhood. Pediatr Infect Dis J 9:623-6.
14. Gray, B. M., G. M. Converse, 3rd, and H. C. Dillon, Jr. 1980. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. J Infect Dis 142:923-33.
15. Hermans, P. W., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. Goessens, and R. de Groot. 1997. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. Microb Drug Resist 3:243-51.
16. Hermans, P. W., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J Clin Microbiol 33:1606-12.
17. Hoge, C. W., M. R. Reichler, E. A. Dominguez, J. C. Bremer, T. D. Mastro, K. A. Hendricks, D. M. Musher, J. A. Elliott, R. R. Facklam, and R. F. Breiman. 1994. An epidemic of pneumococcal disease in an overcrowded, inadequately ventilated jail. N Engl J Med 331:643-8.
18. Jefferies, J., S. C. Clarke, M. A. Diggle, A. Smith, C. Dowson, and T. Mitchell. 2003. Automated pneumococcal MLST using liquid-handling robotics and a capillary DNA sequencer. Mol Biotechnol 24:303-8.
19. Klugman, K. P. 1996. Epidemiology, control and treatment of multiresistant pneumococci. Drugs 52:42-6.
20. Kristinsson, K. G. 1995. Epidemiology of penicillin resistant pneumococci in Iceland. Microb Drug Resist 1:121-5.
21. Leiberman, A., R. Dagan, E. Leibovitz, P. Yagupsky, and D. M. Fliss. 1999. The bacteriology of the nasopharynx in childhood. Int J Pediatr Otorhinolaryngol 49 Suppl 1:S151-3.
22. Lenette, E., A. Balows, W. Hauser Jr., and H. Shadomy. 1985. Manual of Clinical Microbiology, Washington.
23. Mandigers, C. M., R. J. Diepersloot, M. Dessens, S. J. Mol, and B. van Klingeren. 1994. A hospital outbreak of penicillin-resistant pneumococci in The Netherlands. Eur Respir J 7:1635-9.
24. Mbelle, N., R. E. Huebner, A. D. Wasas, A. Kimura, I. Chang, and K. P. Klugman. 1999. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. J Infect Dis 180:1171-6.
25. McGee, L., L. McDougal, J. Zhou, B. G. Spratt, F. C. Tenover, R. George, R. Hakenbeck, W. Hryniewicz, J. C. Lefevre, A. Tomasz, and K. P. Klugman. 2001. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. J Clin Microbiol 39:2565-71.
26. Munoz, R., T. J. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J. M. Musser, B. G. Spratt, and et al. 1991. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. J Infect Dis 164:302-6.
27. NCCLS. 2002. Performance standards for antimicrobial susceptibility testing: twenfth informational supplement, M 100-S12, 19 (1). NCCLS, Wayne, PA.
28. Overweg, K., D. Bogaert, M. Sluijter, J. Yother, J. Dankert, R. de Groot, and P. W. Hermans. 2000. Genetic Relatedness within Serotypes of Penicillin-Susceptible *Streptococcus pneumoniae* Isolates. J Clin Microbiol 38:4548-4553.
29. Principi, N., P. Marchisio, G. C. Schito, and S. Mannelli. 1999. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group [In Process Citation]. Pediatr Infect Dis J 18:517-23.
30. Sluijter, M. 2003. Unpublished observations.
31. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. Goessens, A. van Belkum, and P. W. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. J Clin Microbiol 36:2248-53.
32. Tomasz, A. 1997. Antibiotic resistance in *Streptococcus pneumoniae*. Clin Infect Dis 24 Suppl 1:S85-8.
33. van Steenbergen, T. J., S. D. Colloms, P. W. Hermans, J. de Graaff, and R. H. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc Natl Acad Sci U S A 92:5572-6.
34. Veenhoven, R. 2003. Impact of combined pneumococcal conjugate and polysaccharide vaccination on nasopharyngeal carriage in children with recurrent acute otitis media: Program and abstracts of the 3rd International Symposium on Pneumococci and Pneumococcal Diseases, Anchorage, May 5-8, 2002
35. Veenhoven, R., D. Bogaert, C. Uiterwaal, C. Brouwer, H. Kiezebrink, J. Bruin, P. Hermans, R. de Groot, W. Kuis, G. Rijkers, A. Schilder, and L. Sanders. 2003. Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. Lancet 361:2189-95.
36. Watanabe, H., N. Asoh, K. Hoshino, K. Watanabe, K. Oishi, W. Kositsakulchai, T. Sanchai, K. Kunsuikmengrai, S. Kahintapong, B. Khantawa, P. Tharavichitkul, T. Sirisanthana, and T. Nagatake. 2003. Antimicrobial susceptibility and serotype distribution of *Streptococcus pneumoniae* and molecular characterization of multidrug-resistant serotype 19F, 6B, and 23F Pneumococci in northern Thailand. J Clin Microbiol 41:4178-83.

Chapter 5.4

Genetic relatedness within serotypes of penicillin-susceptible *Streptococcus pneumoniae* isolates

K. Overweg, D. Bogaert, M. Sluijter, J. Yother, J. Dankert,
R. de Groot and P.W.M. Hermans

J Clin Microbiol 2000; 38(12):4548-4553

ABSTRACT

The molecular epidemiological characteristics of all Streptococcus pneumoniae strains isolated in a nationwide manner from patients with meningitis in The Netherlands in 1994 were investigated. Restriction fragment end labeling analysis demonstrated 52% genetic clustering among these penicillin-susceptible strains, a value substantially lower than the percentage of clustering among Dutch penicillin-nonsusceptible strains. Different serotypes were found within 8 of the 28 genetic clusters, suggesting that horizontal transfer of capsular genes is common among penicillin-susceptible strains. The degree of genetic clustering was much higher among serotype 3, 7F, 9V, and 14 isolates than among isolates of other serotypes, i.e., 6A, 6B, 18C, 19F, and 23F. We further studied the molecular epidemiological characteristics of pneumococci of serotype 3, which is considered the most virulent serotype and which is commonly associated with invasive disease in adults. Fifty epidemiologically unrelated penicillin-susceptible serotype 3 invasive isolates originating from the United States (n = 27), Thailand (n = 9), The Netherlands (n = 8), and Denmark (n = 6) were analyzed. The vast majority of the serotype 3 isolates (74%) belonged to two genetically distinct clades that were observed in the United States, Denmark, and The Netherlands. These data indicate that two serotype 3 clones have been independently disseminated in an international manner. Seven serotype 3 isolates were less than 85% genetically related to the other serotype 3 isolates. Our observations suggest that the latter isolates originated from horizontal transfer of the capsular type 3 gene locus to other pneumococcal genotypes. In conclusion, epidemiologically unrelated serotype 3 isolates were genetically more related than those of other serotypes. This observation suggests that serotype 3 has evolved only recently or has remained unchanged over long periods.

INTRODUCTION

Streptococcus pneumoniae continues to be a common cause of serious and life-threatening infections, such as pneumonia, bacteremia, and meningitis, in both adults and children (1). Pneumococci can be classified according to differences in capsular polysaccharide structure. As many as 90 different capsular types can be distinguished by serotyping (10). The distribution of serotypes varies in different populations and different geographic areas, and certain pneumococcal serotypes are known to be more virulent than others (24, 28). Pneumococcal serotype 3 isolates are considered to represent the most virulent serotype. These isolates are often responsible for invasive disease (17, 20), particularly in adults (15, 19). Bacteremia caused by this organism is considered to have the highest mortality rate compared to that caused by other serotypes (15, 20). To date, the frequency of penicillin resistance among serotype 3 isolates has remained low (16).

Serotyping as a tool for epidemiological studies has several disadvantages. *S. pneumoniae* is a naturally transformable species, and frequent exchange of capsular genes occurs (2-4, 13, 23). In addition, serotyping determines the variation in a single genetic locus, i.e., the *cps* locus. Therefore, several other typing methods have been developed to assist with the identification of relatedness between strains and their cellular structures. These methods include multilocus enzyme electrophoresis (9), penicillin-binding protein (PBP) profile analysis (21, 22), pneumococcal surface protein A typing (22), and DNA fingerprint methods, such as pulsed-field gel electrophoresis, multilocus sequence typing (MLST) (7), ribotyping, restriction fragment end labeling (RFEL) analysis, BOX PCR fingerprinting, and DNA fingerprinting of the PBP genes (14, 32). RFEL analysis provides a high degree of discriminatory power, and RFEL profiles are reproducible and suitable for computerized comparisons (14). In addition, RFEL analysis provides a DNA fingerprint that represents multiple loci in the pneumococcal genome. This technique is routinely used in our laboratory to generate a data library of pneumococcal DNA fingerprints. In this study, we investigated the molecular epidemiological characteristics of *S. pneumoniae* strains isolated in a nationwide manner from patients with meningitis in The Netherlands in 1994. The genetic relatedness within pneumococcal serotypes was determined. In addition, we studied the molecular epidemiological characteristics of epidemiologically unrelated serotype 3 pneumococci from four distinct countries. The isolates were characterized by serotyping, RFEL analysis, and PBP genotyping.

MATERIALS AND METHODS

Bacterial isolates. We studied a collection of *S. pneumoniae* strains (n = 153) isolated from Dutch patients suffering from meningitis in The Netherlands in 1994. These strains were

collected by the National Reference Center for Bacterial Meningitis in a nationwide manner and represent all pneumococcal meningitis isolates collected in a 1-year period. In addition, these strains were penicillin-susceptible and were presumed to be epidemiologically unrelated. In addition, 42 penicillin-susceptible invasive serotype 3 pneumococci were isolated from patients in the United States (n = 27), Thailand (n = 9), and Denmark (n = 6). The latter strains were also presumed to be epidemiologically unrelated, since they were isolated from various geographic regions within these countries and at different times ranging from 1960 to 1962 and from 1992 to 1998 (Table 1).

Serotyping. Pneumococci were serotyped on the basis of capsular swelling (Quellung reaction) observed microscopically after suspension in antisera prepared at Statens Seruminstitut, Copenhagen, Denmark (8).

Table 1. Geographic origins of and isolation dates for 50 penicillin-susceptible serotype 3 pneumococcal isolates.

Strain designation	State or country	Isolation date	RFEL type	(pbpla-pbp2b-pbp2x)
DK001	Denmark	1961	294	2-2-1971
DK002	Denmark	1961	294	2-2-1971
DK003	Denmark	1960	292	2-2-1971
DK004	Denmark	1991	167	2-2-1971
DK005	Denmark	1992	299	2-2-1971
DK006	Denmark	1962	289	2-2-1971
NL100	The Netherlands	1994	167	2-2-1971
NL101	The Netherlands	1994	167	2-2-1971
NL102	The Netherlands	1994	167	2-2-1971
NL103	The Netherlands	1994	393	2-2-1971
NL104	The Netherlands	1994	300	2-2-1971
NL106	The Netherlands	1994	291	2-2-1971
NL107	The Netherlands	1994	290	2-2-1971
NL108	The Netherlands	1994	294	2-2-1971
TH001	Thailand	1998	93	2-2-1971
TH002	Thailand	1998	96	9-2-1971
TH003	Thailand	1998	122	2-2-2003
TH004	Thailand	1998	122	2-2-2003
TH005	Thailand	1998	121	2-2-2003
TH006	Thailand	1998	123	2-2-2003
TH007	Thailand	1998	165	2-2-1971
TH008	Thailand	1998	105	2-2-2003
TH009	Thailand	1998	242	2-2-1971

US001	United States	1960	294	2-2-1971
US002	United States	1960	294	2-2-1971
US003	Alaska	1995	295	2-2-1971
US004	Colorado	1993	167	2-2-1971
US005	California	1992	167	2-2-1971
US006	Alaska	1993	167	2-2-1971
US007	Alaska	1993	167	2-2-1971
US008	Alaska	1993	167	2-2-1971
US009	Wisconsin	1992	167	2-2-1971
US010	Pennsylvania	1995	167	2-2-1971
US011	Pennsylvania	1995	167	2-2-1971
US012	Maryland	1995	167	2-2-1971
US013	Alaska	1993	167	2-2-1971
US014	Oklahoma	1996	167	2-2-1971
US015	Washington	1993	167	2-2-1971
US016	Washington	1995	167	2-2-1971
US017	Ohio	1993	167	2-2-1971
US018	United States	1990s	167	2-2-1971
US019	United States	1990s	167	2-2-1971
US020	United States	1990s	167	2-2-1971
US021	United States	1990s	167	2-2-1971
US022	United States	1990s	167	2-2-1971
US023	Maryland	1994	297	2-2-1971
US024	United States	1990s	297	2-2-1971
US025	California	1994	298	2-2-1971
US026	Wisconsin	1992	296	2-2-1971
US027	Oklahoma	1996	76	2-2-1971

***RFEL analysis*.** Typing of pneumococcal strains by RFEL analysis was performed as described by van Steenbergen et al. (33) and adapted by Hermans et al. (14). Briefly, purified pneumococcal DNA was digested with restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [-32P]dATP by using Taq DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. The gel was transferred to filter paper, vacuum dried (HBI, Saddle Brook, N.Y.), and exposed to ECL Hyperfilms (Amersham, Little Chalfont, Bucks, United Kingdom).

***PBP genotyping*.** Genetic polymorphisms of the penicillin resistance genes *pbpla*, *pbp2b*, and *pbp2x* were investigated by restriction fragment length polymorphism analysis. PCR

amplification of the PBP-encoding genes was performed with a 50-µl PCR buffer system containing 75 mM Tris-HCl (pH 9.0), 20 mM (NH4)2SO4, 0.01% (wt/vol) Tween 20, 1.5 mM MgCl2, 0.2 mM each deoxynucleoside triphosphate, 10 pmol of each primer, 0.5 U of Taq DNA polymerase (Goldstar), and 10 ng of purified chromosomal DNA. Cycling was performed with a PTC-100 programmable thermal controller (MJ Research, Watertown, Mass.) and consisted of the following steps: predenaturation at 94°C for 1 min; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C; and final extension at 72°C for 3 min. The primers used to amplify the genes *pbp1a*, *pbp2b*, and *pbp2x* were described previously (3, 6, 21). The amplification products (5 µl) were digested with restriction endonuclease *Hinf*I and separated by electrophoresis in 2.5% agarose gels (27). Gels were scanned and printed with a Geldoc 2000 system (Biorad, Veenendaal, The Netherlands). The different PBP genotypes are represented by a three-number code (e.g., 06-14-43), referring to the restriction fragment length polymorphism patterns of the genes *pbp1a* (pattern 6), *pbp2b* (pattern 14), and *pbp2x* (pattern 43), respectively.

Computer-assisted analysis of the DNA banding patterns. The RFEL types were analyzed with the Windows version of Gelcompar software, version 4 (Applied Maths, Kortrijk, Belgium), after imaging of the RFEL autoradiograms with Image Master DTS (Pharmacia Biotech, Uppsala, Sweden). DNA fragments in the molecular size range of 160 to 400 bp were documented. The DNA banding patterns were normalized with pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the banding patterns was performed by the unweighted pair-group method with arithmetic averages (26) and with the Jaccard similarity coefficient applied to peaks (31). Computer-assisted analysis and the methods and algorithms used in this study were in

Table 2. PBP genotypes of the 153 *S. pneumoniae* strains isolated from patients with meningitis in 1994 in The Netherlands

PBP genotype	No. of strains	Serotype(s) of the strains
02-02-03	67	21 distinct serotypes
02-02-71	54	14 distinct serotypes
02-02-02	22	10 distinct serotypes
02-02-14	4	Serotype 8
02-02-05	3	Serotype 19F
02-02-15	2	Serotype 5
02-02-16	1	Serotype 32A

Table 3. RFEL clusters consisting of strains with different stereotypes

RFEL cluster [a] (RFEL types)	Serotypes (no. of strains)
A(28)	14 (4), 15C (1), 19F (1), 24F (3)
B(101)	4(1), 18B (1), 18C (7)
C(23)	9V (3), 19F (1)
D(328,330)	23F (2), 23B (1)
E(119,342)	8 (2), 33F (1)
F(56,341)	14 (1), 19F (1)
G(321)	14 (1), 19F (1)
H(377)	18F (1), 18C (1)

[a] For definition of RFEL clusters and RFEL types, see Materials and Methods

accordance with the instructions of the manufacturer of Gelcompar. A tolerance of 1.2% in band positions was applied during comparison of the DNA patterns.

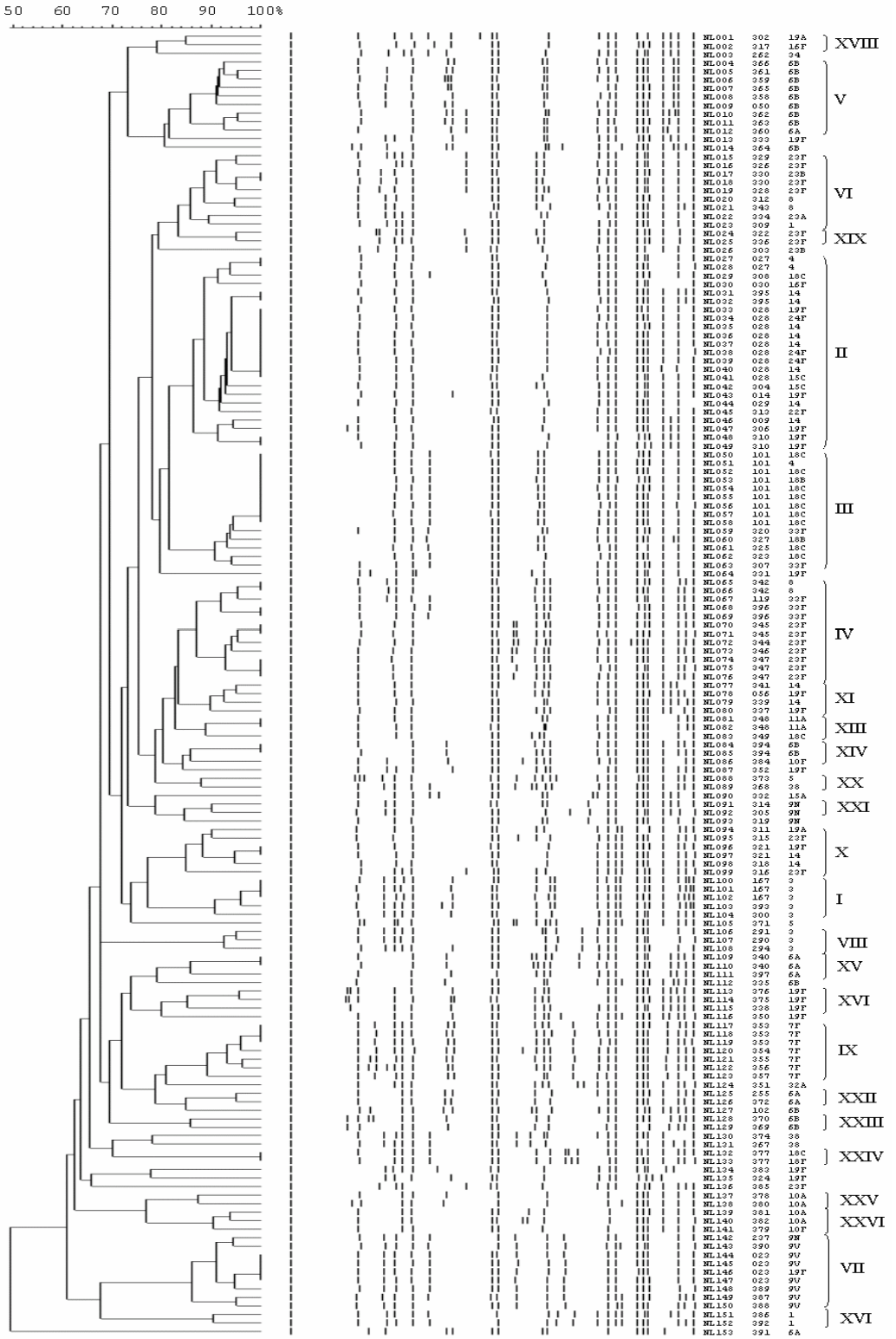
For evaluation of the genetic relatedness of the strains, we used the following definitions: (i) strains of a particular RFEL type are 100% identical on the basis of RFEL analysis, (ii) an RFEL cluster represents a group of RFEL types that differs by only one band (approximately 95% genetic relatedness), and (iii) an RFEL clade represents a group of RFEL types that differs by less than four bands (approximately 85% genetic relatedness). The genetic heterogeneity is defined as the number of RFEL clades representing one or more strains divided by the total number of strains.

RESULTS

Epidemiology of invasive pneumococcal isolates in The Netherlands. The epidemiology of *S. pneumoniae* strains isolated in a nationwide manner from patients with meningitis in 1994 in The Netherlands was investigated. These strains (n = 153) were all found to be penicillin susceptible and were analyzed by serotyping, PBP typing, and RFEL typing. The results are shown in Fig. 1 and Table 2. The invasive isolates represented 31 serotypes: 1 (n = 3), 3 (n = 8), 4 (n = 3), 5 (n = 2), 6A (n = 7), 6B (n = 15), 7F (n = 7), 8 (n = 4), 9N (n = 4), 9V (n = 7), 10F (n = 2), 10A (n = 4), 11A (n = 2), 14 (n = 12), 15A (n = 1), 15C (n = 2), 16F (n = 2), 18F (n = 1), 18B (n = 2), 18C (n= 12), 19F (n = 18), 19A (n = 2), 22F (n = 1), 23F (n = 16), 23A (n = 1), 23B (n = 2), 24F (n = 3), 32A (n = 1), 33F (n = 5), 34 (n = 1), and 38 (n = 3).

Seven distinct PBP genotypes displaying variations in the RFLP patterns of pbp2x only were observed. The PBP types 02-02-03, 02-02-71, and 02-02-02 occurred most frequently. In addition, all serotype 8 strains displayed PBP genotype 02-02-14, both serotype 5 strains displayed PBP genotype 02-02-15, and the single serotype 32A strain displayed PBP genotype 02-02-16. Finally, 3 of the 18 19F strains displayed PBP genotype 02-02-05 (Table 2).

RFEL analysis divided the 153 strains into 116 distinct RFEL types. These RFEL types represented 28 genetic clusters, i.e., strains showing over 95% genetic relatedness, and 73 RFEL types that were less than 95% related to other strains. RFEL clusters were represented by 80 strains (52%). The cluster size varied from two (19 clusters) to nine (2 clusters) strains. In addition, four clusters of three strains and three clusters of four strains were observed. RFEL types 28 (genetic clade II) and 101 (genetic clade III) were the most predominant types. They were each represented by nine isolates. Within genetic clusters, different serotypes were observed. Eight of the 28 RFEL clusters displayed two or more serotypes (Table 3). The strain collection could be divided into 25 genetic clades, i.e., strains


50
60
70
80
90
100%
NL001 302 19A
NL002 317 16F
NL003 262 34
NL004 366 6B
NL005 361 6B
NL006 359 6B
NL007 365 6B
NL008 358 6B
NL009 050 6B
NL010 362 6B
NL011 369 6B
NL012 360 6A
NL013 323 19F
NL014 364 6B
NL015 329 23F
NL016 326 23F
NL017 330 23B
NL018 330 23F
NL019 328 23F
NL020 012 8
NL021 349 8
NL022 334 23A
NL023 309 1
NL024 322 23F
NL025 336 23F
NL026 300 23B
NL027 027 4
NL028 027 4
NL029 308 18C
NL030 030 16F
NL031 395 14
NL032 395 14
NL033 028 19F
NL034 028 24F
NL035 028 14
NL036 028 14
NL037 028 14
NL038 028 24F
NL039 028 24F
NL040 028 14
NL041 028 15C
NL042 304 15C
NL043 014 19F
NL044 029 14
NL045 312 22F
NL046 009 14
NL047 306 19F
NL048 310 19F
NL049 310 19F
NL050 101 18C
NL051 101 4
NL052 101 18C
NL053 101 18B
NL054 101 18C
NL055 101 18C
NL056 101 18C
NL057 101 18C
NL058 101 18C
NL059 320 33F
NL060 327 18B
NL061 325 18C
NL062 323 18C
NL063 307 33F
NL064 321 19F
NL065 342 8
NL066 342 8
NL067 119 33F
NL068 396 33F
NL069 396 33F
NL070 345 23F
NL071 345 23F
NL072 344 23F
NL073 346 23F
NL074 347 23F
NL075 347 23F
NL076 347 23F
NL077 341 14
NL078 056 19F
NL079 339 14
NL080 327 19F
NL081 348 11A
NL082 348 11A
NL083 349 18C
NL084 394 6B
NL085 394 6B
NL086 384 10F
NL087 262 19F
NL088 379 5
NL089 368 38
NL090 332 15A
NL091 314 9N
NL092 305 9N
NL093 319 9N
NL094 311 19A
NL095 315 23F
NL096 321 19F
NL097 321 14
NL098 318 14
NL099 316 23F
NL100 167 3
NL101 167 3
NL102 167 3
NL103 393 3
NL104 300 3
NL105 371 5
NL106 291 3
NL107 290 3
NL108 294 3
NL109 340 6A
NL110 340 6A
NL111 397 6A
NL112 335 6B
NL113 376 19F
NL114 375 19F
NL115 338 19F
NL116 350 19F
NL117 353 7F
NL118 353 7F
NL119 353 7F
NL120 354 7F
NL121 355 7F
NL122 356 7F
NL123 357 7F
NL124 251 22A
NL125 255 6A
NL126 372 6A
NL127 102 6B
NL128 370 6B
NL129 369 6B
NL130 374 38
NL131 367 38
NL132 377 18C
NL133 377 18F
NL134 382 19F
NL135 324 19F
NL136 385 23F
NL137 378 10A
NL138 380 10A
NL139 381 10A
NL140 382 10A
NL141 379 10F
NL142 237 9N
NL143 390 9V
NL144 023 9V
NL145 023 9V
NL146 023 19F
NL147 023 9V
NL148 389 9V
NL149 387 9V
NL150 388 9V
NL151 386 1
NL152 392 1
NL153 391 6A
XVIII
V
VI
XIX
II
III
IV
XI
XIII
XIV
XX
XXI
X
I
VIII
XV
XVI
IX
XXII
XXIII
XXIV
XXV
XXVI
VII
XVI

with more than 85% RFEL homology. The genetic clades varied in size from 2 to 23 strains (Fig. 1).

Comparison of penicillin-susceptible invasive strains with penicillin-nonsusceptible strains representing 193 distinct RFEL types present in the international data library and representing 16 countries (13) revealed no overlap in RFEL types between penicillin-susceptible strains and penicillin-non-susceptible strains.

Genetic relatedness within serotypes in The Netherlands. The genetic relatedness of strains within the nine most predominant serotypes present in the collection was investigated. All strains of serotype 7F (n = 7) belonged to clade IX, and all strains of serotype 9V (n = 7) belonged to clade VII. Strains of serotype 3 (n = 8) belonged to two distinct genetic clades, I and VIII. Strains of serotype 14 (n = 12) represented three distinct genetic clades, III, X, and XI. Strains of serotypes 6B, 18C, and 23F were genetically more heterogeneous. However, most strains of serotypes 6B, 18C, and 23F belonged to one clade. Eight of the 15 serotype 6B strains belonged to clade V, 9 of the 12 serotype 18C strains belonged to clade III, and 7 of the 16 serotype 23F strains belonged to clade IV. Strains with serotypes 6A and 19F displayed the most heterogeneity in this collection of *S. pneumoniae* strains, as 7 serotype 6A strains were represented by 4 genetic clades and 18 serotype 19F strains were represented by 11 genetic clades (Fig. 1).

Genetic relatedness within serotype 3 isolates of distinct geographic origins. We investigated the molecular epidemiology of serotype 3 strains from The Netherlands (n = 8) and three additional countries: the United States (n = 27), Thailand (n = 9), and Denmark (n = 6). These 50 epidemiologically unrelated serotype 3 strains were characterized by RFEL analysis. Four distinct RFEL clades and seven RFEL types that were less than 85% related to other serotype 3 strains were observed among these strains (Fig. 2). The most predominant RFEL clade, I, represented 29 serotype 3 strains (58%). This RFEL clade was represented by 22 isolates from the United States, 2 isolates from Denmark, and 5 isolates from The Netherlands. RFEL cluster VIII was represented by eight strains (16%)two American, three Danish, and three Dutch strains. RFEL clade XII was represented by four Thai isolates. In addition, two Thai isolates formed a Thai-specific clade. Thus, 43 strains shared RFEL types with at least one other strain (86%). Seven serotype 3 strains with RFEL types 296, 295, 165, 105, 289, 76, and 242 did not match the four genetic clades, and six of them did not match any of the 153 Dutch invasive strains representing 116 RFEL types and

Figure 1 (left). Genetic relatedness of 153 penicillin-susceptible invasive pneumococcal isolates, based on the RFEL banding patterns of the isolates. The country code (NL, The Netherlands), strain codes, RFEL types, and serotypes are depicted. Codes I to XI refer to genetic clades of pneumococcal strains; genetic clusters are indicated by a grey box in the dendrogram (for definitions, see Materials and Methods).

31 serotypes. In contrast, the serotype 3 strain with RFEL type 105 was genetically related (90.9%) to a serotype 19F strain representing RFEL type 352.
The serotype 3 collection was also analyzed by PBP typing. PBP genotype 02-02-71 was invariably observed in the strains from the United States, Denmark, and The Netherlands. The Thai strains displayed three distinct PBP genotypes: 02-02-03 (n = 5), 02-02-71 (n = 3), and 09-02-71 (n = 1) (Table 1).

DISCUSSION

Few studies have documented genotype analyses of penicillin-susceptible strains (12, 29) and of serotype-specific strains (9, 18). We investigated the epidemiological characteristics of 153 penicillin-susceptible *S. pneumoniae* strains isolated from patients with meningitis in The Netherlands in 1994. The isolates represented 31 serotypes. The most predominant serotypes were 19F, 23F, 6B, 18C, 14, 3, 6A, 7F, and 9V. Various investigators have reported the occurrence of horizontal transfer of capsular genes (2, 11-13). In Dutch penicillin-susceptible isolates, horizontal transfer of capsular genes has occurred frequently. A high frequency of capsular exchange has been reported in molecular epidemiological studies of penicillin-resistant isolates from many countries (12, 13). This is the first study suggesting the frequent occurrence of horizontal transfer of capsular genes among penicillin-susceptible isolates.
RFEL analysis revealed that 52% of the strains belonged to genetic clusters. The amount of genetic clustering was substantially lower among the penicillin-susceptible isolates than among the penicillin-nonsusceptible isolates in other studies (2, 5, 11-13, 25). A comparison of the penicillin-susceptible invasive isolates studied here with 193 penicillin-nonsusceptible strains representing 193 distinct RFEL types in the international data library and representing 16 countries revealed no overlap (12, 13).
The PBP genotypes 02-02-03, 02-02-71, and 02-02-02 were found most frequently. This observation corresponds with the PBP typing results for penicillin-susceptible pediatric carriage isolates in the U.S. population (30). Interestingly, four additional PBP genotypes (02-02-14, 02-02-15, 02-02-16, and 02-02-05) were identified for serotypes 8, 5, 32A, and 19F, respectively. The serotype specificity of the latter PBP genotypes suggests a divergence of the PBP genotypes before the origin of the capsular types 8, 5, 32A, and 19F.
The genetic relatedness within the specific pneumococcal serotypes was highly variable. RFEL genotypes of serotype 6A and 19F strains displayed high levels of heterogeneity; i.e., strains of these serotypes represented many RFEL types that belonged to many genetic clusters and genetic clades. In contrast, the RFEL genotypes of serotype 7F, 9V, 14, and 3 strains were found to be genetically related. Interestingly and consistent with our

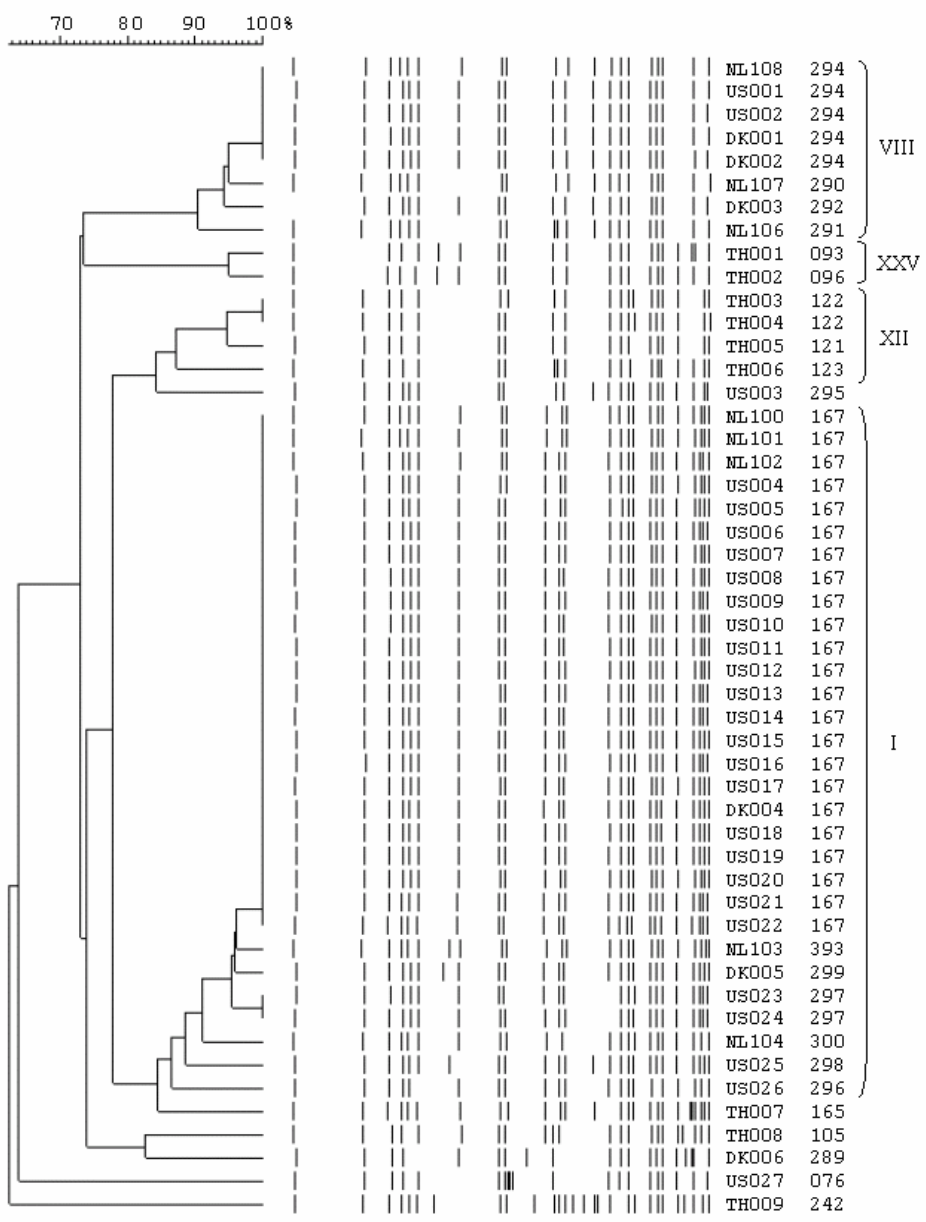


Figure. 2. Genetic relatedness of 50 penicillin-susceptible pneumococcal serotype 3 isolates, based on the RFEL banding patterns of the isolates. RFEL types are depicted. Codes I, VIII, XII, and XXVII refer to genetic clades of pneumococcal strains; genetic clusters are indicated by a grey box in the dendrogram (for definitions, see Materials and Methods). Bars represent the number of isolates per RFEL type.

observations, Canadian penicillin-susceptible isolates of serotypes 3 and 7F were also more genetically related than isolates of other serotypes (18). Moreover, invasive penicillin-susceptible serotype 3 isolates from the United Kingdom also tended to be more closely related to each other than to isolates of other serotypes (9).

We focused on the molecular epidemiological characteristics of epidemiologically unrelated serotype 3 pneumococci and extended our serotype 3 collection with isolates from theUnited States, Thailand, and Denmark. RFEL analysis demonstrated that serotype 3 strains isolated in these countries displayed a strong degree of genetic relatedness: the vast majority of the strains represented two distinct RFEL clades. Furthermore, both genetic clades harbored isolates from three countries: the United States, Denmark, and The Netherlands. These observations indicate that two serotype 3 clones have been disseminated internationally. In addition, six Thai serotype 3 isolates belonged to two RFEL clades (clades XII and XXVII). The data suggest strong genetic homogeneity within the serotype 3 pneumococci and support the observations for Canada and the United Kingdom (9, 18). Interestingly, the Canadian serotype 3 strains displayed two distinct genotypes, and the majority of the epidemiologically nonrelated serotype 3 strains from the United Kingdom displayed two genotypes. Moreover, MLST analysis of serotype 3 strains isolated in six countries identified two major genetic clusters (M. C. Enright and B. G. Spratt, http://mlst.zoo.ox.ac.uk). Since the strains have been characterized by distinct typing methods, i.e., pulsed-field gel electrophoresis, multilocus enzyme electrophoresis, MLST, and RFEL analysis, and since there is no overlap in the characterized strains, the genetic relatedness between the latter serotype 3 strains and the strains characterized in this study is currently unknown. The remaining six serotype 3 RFEL types each occurred once in our collection. Our observations suggest that these latter strains have been derived from horizontal transfer of the capsular type 3 gene locus to other pneumococcal genotypes.

PBP genotyping of the serotype 3 strains demonstrated limited variation in the *pbp1a*, *pbp2b*, and *pbp2x* genes. All serotype 3 strains from the United States, Denmark, and The Netherlands displayed PBP genotype 02-02-71. However, variation was demonstrated in the Thai serotype 3 isolates. PBP type 09-02-71 was represented by a single Thai isolate. This PBP type was also specific for the penicillin-susceptible phenotype, as there was no overlap with penicillin-nonsusceptible isolates from 16 countries (13).

In conclusion, pneumococcal strains belonging to serotype 3 display limited genetic heterogeneity despite the lack of epidemiological relatedness. We hypothesize that this serotype has recently evolved or has remained unchanged for a prolonged period. The few serotype 3 isolates not belonging to the main clusters are presumably derived from horizontal transfer of capsular genes.

ACKNOWLEDGMENTS

We thank Jay Butler (Centers for Disease Control and Prevention, Atlanta, Ga.), Gregory C. Gray (Emerging Illness Division, Naval Health Research Center, San Diego, Calif.), Jørgen Henrichsen (Statens Serum Institute, Copenhagen, Denmark), Surang Dejsirilert (National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand), and Lodewijk Spanjaard (Department of Medical Microbiology, University of Amsterdam, Amsterdam, The Netherlands) for providing us with pneumococcal isolates.

REFERENCES

1. Alonso DeVelasco, E., A. F. Verheul, J. Verhoef, and H. Snippe. 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. Microbiol. Rev. 59:591-603.
2. Barnes, D. M., S. Whittier, P. H. Gilligan, S. Soares, A. Tomasz, and F. W. Henderson. 1995. Transmission of multidrug-resistant serotype 23F *Streptococcus pneumoniae* in group day care: evidence suggesting capsular transformation of the resistant strain in vivo. J. Infect. Dis. 171:890-896.
3. Coffey, T. J., C. G. Dowson, M. Daniels, J. Zhou, C. Martin, B. G. Spratt, and J. M. Musser. 1991. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. Mol. Microbiol. 5:2255-2260.
4. Coffey, T. J., M. C. Enright, M. Daniels, P. Wilkinson, S. Berron, A. Fenoll, and B. G. Spratt. 1998. Serotype 19A variants of the Spanish serotype 23F multiresistant clone of *Streptococcus pneumoniae*. Microb. Drug Resist. 4:51-55.
5. Dejsirilert, S., K. Overweg, M. Sluijter, L. Saengsuk, M. Gratten, T. Ezaki, and P. W. Hermans. 1999. Nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae* among children with acute respiratory tract infections in Thailand: a molecular epidemiological survey. J. Clin. Microbiol. 37:1832-1838.
6. Dowson, C. G., A. Hutchison, and B. G. Spratt. 1989. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. Mol. Microbiol. 3:95-102.
7. Enright, M. C., and B. G. Spratt. 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 144:3049-3060.
8. Facklam, R. R., and J. A. Washington, II. 1991. Streptococcus and related catalase-negative gram-positive, cocci, p. 238-257. In A. Balows, W. J. Hausler, K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
9. Hall, L. M., R. A. Whiley, B. Duke, R. C. George, and A. Efstratiou. 1996. Genetic relatedness within and between serotypes of *Streptococcus pneumoniae* from the United Kingdom: analysis of multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and antimicrobial resistance patterns. J. Clin. Microbiol. 34:853-859.
10. Henrichsen, J. 1995. Six newly recognized types of *Streptococcus pneumoniae*. J. Clin. Microbiol. 33:2759-2762.
11. Hermans, P. W., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. Goessens, and R. de Groot. 1997. Molecular epidemiology of drug-resistant pneumococci: towards an international approach. Microb. Drug Resist. 3:243-251.
12. Hermans, P. W., M. Sluijter, K. Elzenaar, A. van Veen, J. J. Schonkeren, F. M. Nooren, W. J. van Leeuwen, A. J. de Neeling, B. van Klingeren, H. A. Verbrugh, and R. de Groot. 1997. Penicillin-resistant *Streptococcus pneumoniae* in The Netherlands: results of a 1-year molecular epidemiologic survey. J. Infect. Dis. 175:1413-1422.
13. Hermans, P. W. M., K. Overweg, M. Sluijter, and R. de Groot. 2000. Penicillin-resistant *Streptococcus pneumoniae*: an international molecular epidemiological study, p. 457-466. In A. Tomasz (ed.), *Streptococcus pneumoniae*: molecular biology and mechanisms of disease. Mary Ann Liebert, Inc., Publishers, New York, N.Y.
14. Hermans, P. W. M., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 33:1606-1612.
15. Hsueh, P. R., J. J. Wu, and T. R. Hsiue. 1996. Invasive *Streptococcus pneumoniae* infection associated with rapidly fatal outcome in Taiwan. J. Formos. Med. Assoc. 95:364-371.
16. Klugman, K. P. 1990. Pneumococcal resistance to antibiotics. Clin. Microbiol. Rev. 3:171-196.
17. Lawrenson, J. B., K. P. Klugman, J. I. Eidelman, A. Wasas, S. D. Miller, and J. Lipman. 1988. Fatal infection caused by multiply resistant type 3 pneumococcus. J. Clin. Microbiol. 26:1590-1591.
18. Louie, M., L. Louie, G. Papia, J. Talbot, M. Lovgren, and A. E. Simor. 1999. Molecular analysis of the genetic variation among penicillin-susceptible and penicillin-resistant *Streptococcus pneumoniae* serotypes in Canada. J. Infect. Dis. 179:892-900.

19. Martin, D. R., and M. S. Brett. 1996. Pneumococci causing invasive disease in New Zealand, 1987-94: serogroup and serotype coverage and antibiotic resistances. N. Z. Med. J. 109:288-290.
20. Mufson, M. A., D. M. Kruss, R. E. Wasil, and W. I. Metzger. 1974. Capsular types and outcome of bacteremic pneumococcal disease in the antibiotic era. Arch. Intern. Med. 134:505-510.
21. Munoz, R., T. J. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J. M. Musser, B. G. Spratt, and A. Tomasz. 1991. Intercontinental spread of a multidrug-resistant clone of serotype 23F *Streptococcus pneumoniae*. J. Infect. Dis. 164:302-306.
22. Munoz, R., J. M. Musser, M. Crain, D. E. Briles, A. Marton, A. J. Parkinson, U. Sorensen, and A. Tomasz. 1992. Geographic distribution of penicillin-resistant clones of *Streptococcus pneumoniae*: characterization by penicillin-binding protein profile, surface protein A typing, and multilocus enzyme analysis. Clin. Infect. Dis. 15:112-118.
23. Nesin, M., M. Ramirez, and A. Tomasz. 1998. Capsular transformation of a multidrug-resistant *Streptococcus pneumoniae* in vivo. J. Infect. Dis. 177:707-713.
24. Nielsen, S. V., and J. Henrichsen. 1992. Capsular types of *Streptococcus pneumoniae* isolated from blood and CSF during 1982-1987. Clin. Infect. Dis. 15:794-798.
25. Overweg, K., P. W. M. Hermans, K. Trzcinski, M. Sluijter, R. de Groot, and W. Hryniewicz. 1999. Multidrug-resistant *Streptococcus pneumoniae* in Poland: identification of emerging clones. J. Clin. Microbiol. 37:1739-1745.
26. Romesburg, H. C. 1990. Cluster analysis for researchers, p. 9-28. Krieger, Malabar, Fla.
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. Scott, J. A., A. J. Hall, R. Dagan, J. M. Dixon, S. J. Eykyn, A. Fenoll, M. Hortal, L. P. Jette, J. H. Jorgensen, F. Lamothe, C. Latorre, J. T. Macfarlane, D. M. Shlaes, L. E. Smart, and A. Taunay. 1996. Serogroup-specific epidemiology of *Streptococcus pneumoniae*: associations with age, sex, and geography in 7,000 episodes of invasive disease. Clin. Infect. Dis. 22:973-981.
29. Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck. 1992. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. Infect. Immun. 60:4119-4126.
30. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. F. Goessens, A. van Belkum, and P. W. M. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first years of life. J. Clin. Microbiol. 36:2248-2253.
31. Sneath, P. H. A., and R. R. Sokal. Numerical taxonomy, p. 131-132. Freeman, San Fransisco, Calif.
32. van Belkum, A., M. Sluijter, R. de Groot, H. Verbrugh, and P. W. M. Hermans. 1996. Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 34:1176-1179.
33. van Steenbergen, T. J., S. D. Colloms, P. W. M. Hermans, J. de Graaff, and R. H. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc. Natl. Acad. Sci. USA 92:5572-5576.

Chapter 6

Pneumococcal vaccines: an update on current strategies

D. Bogaert, P.W.M. Hermans, P.V. Adrian, H.C. Rümke and R. de Groot

Vaccine; in press

ABSTRACT

Streptococcus pneumoniae is a major cause of morbidity and mortality in infants, children and the elderly. Despite the availability of excellent antimicrobial therapy and adequate health care systems, respiratory diseases and invasive infections caused by pneumococci still comprise a major health problem. The emerging resistance to penicillin and other commonly used antibiotics underscores the importance of the development of novel vaccine strategies to combat pneumococcal disease. Although the 23-valent polysaccharide (PS) vaccine is immunogenic and protective in most adults and children over 5 years of age, they fail to protect children under two years of age. Fortunately, the recent conjugate vaccines have shown to be highly efficacious in preventing invasive diseases in this risk group. Moreover, promising results regarding prevention of pneumonia and acute otitis media have been published. Unfortunately, protection is raised against a limited number of pneumococcal serotypes, and serotype replacement and subsequent vaccine failure have become a serious concern. Currently, several pneumococcal surface proteins are considered as alternative vaccine candidates because of their serotype-independence. Thus far, PsaA has proven to be highly protective against colonization in animal models. Moreover, PspA and pneumolysin have shown to elicit protection against invasive diseases. Future research will elucidate their true potential in protecting humans. In this paper we discuss the present knowledge on pneumococcal vaccines and the current status of novel vaccine strategies.

INTRODUCTION

Streptococcus pneumoniae is a major cause of invasive diseases such as meningitis, septicaemia and pneumonia. Approximately, 1 million children under 5 years of age die of pneumococcal disease annually [1]. In countries where the incidence of *Neisseria meningitidis* and *Haemophilus influenzae* infections has drastically decreased through the introduction of vaccines against meningococci group C and *H. influenzae* type B, *S. pneumoniae* has become the major cause of meningitis and septicemia in children. In addition, the morbidity by *S. pneumoniae* through respiratory tract infections such as otitis media and sinusitis is enormous. 30-50% of all patients with otitis media and a substantial percentage of cases of sinusitis and pneumonia are caused by pneumococci. Risk groups for serious pneumococcal disease include children under the age of 2 years, elderly and patients with immunodeficiencies [2].

Nasopharyngeal colonization by *S. pneumoniae* is common: probably all humans are colonized with this organism at least once early in life. Especially in circumstances of crowding, as in day-care centers, nursing homes, hospitals and jails, the risk of colonization with pneumococci is high [3-5]. Colonization is not usually followed by disease, since this is prevented by the innate and adaptive immune system. However, disturbance of homeostasis between host and pathogen, for example through viral infections, malnutrition or local damage of the mucosa, is associated with the development of (invasive) diseases [6-8].

Since the discovery of the antibacterial properties of penicillin by Fleming in 1929, many antibiotics have been used for treatment of pneumococcal infections. Recently, antibiotic resistance has become a worldwide problem, which limits the choice of antimicrobial agents. Therefore, prevention of pneumococcal disease has become of great interest. Up till now, many research groups have focused on the development of new effective vaccines to be used in particular risk groups including immunocompromised patients and children. In this article we will discuss the history of pneumococcal vaccination, and we will evaluate the different vaccine strategies, which are currrently undertaken. Furthermore, we will discuss the recent developments in novel vaccine strategies.

HISTORY

Sternberg and Pasteur were the first to identify *S. pneumoniae*, initially described as the pneumococcus [9]. Effective therapy against pneumococcal disease was reported only a few years later by Klemperer and Klemperer, who discovered the protective potential of patient serum against homologous organisms [10]. The first preventive strategy was introduced by Sir Almroth E. Wright in 1911, who suggested that inoculation of killed, whole pneumococci

might induce a protective effect against pneumococcal infections [11]. Unfortunately this pioneering vaccine failed because only one of the two serotypes discovered t that time was included, and the maximum applicable vaccine dosage was insufficient because of the relatively large inocula required. In 1926 Felton and Bailey for the first time isolated pneumococcal capsular polysaccharides [12]. This directly led to the first capsular PS vaccine, which proved its effectiveness by successfully aborting an outbreak of pneumonia at a state hospital in Worcester, Massachusetts in 1931 [13]. Because of the subsequent development of successful antibiotic therapy that could more effectively deal with pneumococcal disease, the vaccine's popularity decreased and finally the PS vaccine was withdrawn from the market [14,15]. However, despite the development of new classes of antibiotics, morbidity and mortality stopped declining [16]. An additional problem was the rapidly emerging penicillin and multidrug resistance. The first reports on resistance to penicillin were reported in the 1930s in Australia and New Guinea [17]. It took until 1977 before highly resistant pneumococci were reported in South Africa (MIC >1,0 mg/L). In addition, these strains were also resistant to other penicillins and cephalosporins [18]. In the years following, penicillin resistant pneumococci rapidly spread throughout the world. The cause of this international spread was mostly due to a few multidrug-resistant clones with serotypes 6B, 9V, 14, 19A, 19F and 23F [19]. These problems have lead to renewed interest in vaccine strategies, resulting in 1977 in the production of a 14-valent pneumococcal PS vaccine by Robert Austrian and coworkers [16]. This vaccine was expanded in 1983 to a 23-valent vaccine and had a theoretical coverage of ≥ 80% of the pneumococci causing infections in adults. Unfortunately, the vaccine was less immunogenic in small children and immunocompromised patients [20]. This finding has accelerated the current search for new and promising vaccine candidates. In order to gain insight into the mechanisms of vaccine response we will first discuss the natural host defense mechanisms against pneumococcal disease.

HOST DEFENSE AGAINST PNEUMOCOCCAL DISEASE

The pneumococcal outer surface consists of a cell wall covered by a polysaccharide capsule. Capsule polysaccharides are highly heterogeneous and, thus far, almost 100 different capsular serotypes have been described. The polysaccharide capsule is the most important virulence factor of the pneumococcus as it protects the bacteria from phagocytosis. Capsular polysaccharides are highly immunogenic and antibodies against these polysaccharides protect against infection with the homologous serotype. The antigenicity of the capsule is type-specific, but cross-reactions occur because of shared polysaccharides [21]. The next layer, the cell wall, consists of polysaccharides, teichoic acid

and several cell wall associated surface proteins. The cell wall is responsible for the intense inflammatory reaction that accompanies pneumococcal infection since it stimulates the influx of inflammatory cells. In addition, it activates the alternative complement cascade and induces cytokine production [21]. The cell-surface associated proteins are believed to specifically contribute to virulence as well. The most immunogenic part of the cell wall is the phosphocholine part of the teichoic acid, which is also playing a major role in the inflammatory process. The cell wall is shielded from the host response by the capsule, which is completely covered.

Pneumococcal clearance from the lung mainly results from phagocytosis and intracellular killing of the bacteria by neutrophils and alveolar macrophages. This process can only occur in the presence of type-specific immunoglobulins (IgG1 and IgG2, IgM and IgA) and active complement. This antibody-initiated complement-dependent opsonization, which activates the classical complement pathway, is believed to be the major immune mechanism protecting the host against infections with pneumococci. Pneumococci may escape this mechanism in the absence of serotype-specific antibodies, and consequently, may enter the host through the interstitial tissue of the lung resulting in lymphatic spread and subsequent blood stream invasion causing bacteremia [22].

The mechanism of clearance from the blood appears to depend on the interaction of type-specific antibodies (IgG) complement and phagocytic cells in the liver and spleen. The absence of the spleen or cirrhosis of the liver predisposes for severe pneumococal infection. Congenital deficiencies in immunoglobulin or complement are also associated with predisposition to pneumococcal infection [21].

Non-capsular antibodies, for example immunoglobulins directed against cell wall components, may play a role in the host response to pneumococcal infection as well. Although most cell wall components are protected from opsonization and phagocytosis by the capsule, certain proteins may penetrate the capsule and may therefore be recognized by the immune system. So far, several animal studies have shown a protective effect of immunoglobulins directed against selected cell surface-associated proteins including PspA, PsaA, and pneumolysin [22]. This T-cell dependent (TD) immune response, which is present from birth, mainly induces IgG1 subclass immunoglobulins. Type-specific immunoglobulins may be more effective if from the IgG2 subclass. The adult antibody response against capsular polysaccharides mainly generates these IgG2 subclass antibodies. However, the antibody response to (pneumococcal) polysaccharides is relatively immature in young children and generates predominantly IgG1 antibodies, which may result in an enhanced susceptibility to pneumococcal infections [23]. Thus, with the type of vaccination, the type of immune response may differ, and so may the level of protection. One has to take this into account when evaluating different (future) preventive strategies.

POLYSACCHARIDE VACCINES

At present one pneumococcal PS vaccine is still licensed and marketed, i.e. Pneumovax 23 produced by Merck Research Laboratories, USA. These vaccines contain 23 purified capsular polysaccharide antigens (serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) accounting for 85-90% of pneumococcal diseases [20]. These vaccines are effective in adults and children above 2 years of age. Several studies have also shown a protective effect in risk groups such as patients with sickle cell disease or asplenia, and elderly with underlying conditions including chronic obstructive pulmonary disease [20,21,24]. Unfortunately, children under two years of age, who suffer the highest rates of pneumococcal carriage and disease, and immunocompromised patients show a severely impaired antibody response upon this vaccination [25, 27-28]. Furthermore, the PS vaccine is not effective against acute otitis media caused by *S. pneumoniae* [29]. In addition, the PS vaccines do not induce a T-cell dependent immune response. This implicates the absence of memory B-cells and limits the period of protection. Finally, several capsule polysaccharides are poorly immunogenic. These include several serotypes associated with penicillin resistance [30]. These factors have induced to the development of new pneumococcal vaccines, although this has not yet resulted in the complete replacement of the PS vaccines. The PS vaccines do have an efficacy of 61-75% in children over 2 years of age as well as in adults, and, thus, are still useful for various purposes [31,32]. Therefore, the Advisory Committee on Immunization Practices (ACIP) recommends this vaccine in a) persons aged ≥ 65 years, b) immunocompetent children ≥ 5 years who are at increased risk for illness and death associated with pneumococcal disease because of chronic illness, c) persons aged ≥ 5 years with functional or anatomic asplenia and d) immunocompromised patients aged ≥ 5 years who are at high risk for infection. They also recommend vaccination for people living in high-risk environments with emphasis on American Indians and Alaska natives [20,33].

Several studies have shown an additional effect of the PS vaccine when used as a booster after a primary conjugate vaccination [34,35]. For example, Breukels et al. (1999) showed a poor IgG2 response in otitis prone children when vaccinated with a conjugate vaccine only, but boosting with a PS vaccine could overcome this problem [36]. It has been suggested that administration of conjugate vaccines in infancy followed by the administration of a PS vaccine after the age of 10 years would offer the prospect of reducing the total burden of pneumococcal morbidity and mortality throughout life [9,37]. At this moment, the ACIP recommends to use the PS vaccine as a booster 2 months after conjugate vaccination in children 2-5 years of age [33].

An emerging risk group for pneumococcal infections is that of HIV positive patients. The pneumococcal attack rate is 40 times higher in these patients than in seronegative

individuals [38,39]. Therefore, the use of the PS vaccine is considered to be an important preventive strategy. However, due to a low peak concentration of protective Ig antibodies after vaccination, the protected period is significantly shorter (<3 years compared to 5 years in HIV negative adults) [19,40]. The impaired antibody response does not account for all serotypes. This is probably caused by the phenomenon that some anti-polysaccharide responses are T-cell dependent (TD), which is impaired in HIV infected patients, while others are T-cell independent (TI) [19]. Vaccination with conjugate vaccine, therefore, offers no solution either. This is because of the effectiveness of this vaccine, which depends mainly on the impaired T-cell dependent immune response in this patient group. So until new, more promising alternatives are found, the best strategy for this risk group appears to be revaccination with polysaccharides every three years. Recently though, in sub-Saharan Africa this strategy has proven to be inefficient. French et al. (2000) did not only show a lack of efficacy of the 23-valent PS vaccine in HIV-1-infected Ugandan adults, but they also described evidence of deleterious vaccine-associated side-effects. Significantly more cases of pneumonia were seen in the vaccine recipients compared to controls. This phenomenon may be explained by transient stimulation of HIV-1 transcription after pneumococcal vaccination, but also destruction of polysaccharide-responsive B-cell clones by pneumococcal polysaccharides has been suggested [41].

CONJUGATE VACCINES

Already in 1929, Avery and coworkers showed that covalent binding of capsular polysaccharides to proteins increases the immunogenicity of the polysaccharides [30]. This comprises linkage of capsular polysaccharides to a protein carrier, either by covalent binding or through reactive groups [30,42]. The proteins that have proven to be good immunocompetent carriers in the case of *H. influenzae* type b vaccines are tetanus toxoid, diphtheria toxoid and outer membrane protein from meningococcus group B [30,43]. The difference in the immune response towards pure PS vaccines is the observed switch to a TD response promoted by the protein. This leads to the induction of memory B-cells and an improved B-cell response. In addition, it leads to a better immune response early in childhood. This is explained by the stepwise maturation of the human immune system with the earlier maturation of the TD response compared to anti- (poly) saccharide antibodies (TI-2 response). Unfortunately, linkage of polysaccharides to proteins is restricted: too much carrier antigen may impair the antibody response to the polysaccharides by antigen competition or carrier-mediated epitope suppression [44,45]. Thus, protection is restricted to a certain number of serotypes. Conjugate vaccine variants with 4 to 11 serotypes linked to variable protein carriers have been designed, and all showed to be safe and immunogenic in

infants and toddlers (Table 1) [35,46-48]. The most prevalent serotypes, correlated with invasive diseases and antibiotic resistance, were chosen as vaccine candidates. Thus far, the 7-valent pneumococcal conjugate vaccine Prevnar (Wyeth, USA) or Prevenar (Wyeth, Europe), containing polysaccharides of serotype 4, 6B, 9V, 14, 18C, 19F and 23F, has been approved by the Food and Drug Administration (USA), and the Committee on Proprietary Medicinal Products (Europe) for the prevention of invasive diseases in children. These vaccines have a potential coverage of over 85% of the pneumococcal isolates for the USA, 60-70% for Europe and around 55% for Asia [49]. Clinical efficacy of the 7-valent conjugate vaccine against invasive diseases has been convincingly shown by Black and coworkers in the northern California Kaiser Permanente trial. Besides an efficacy of 97.4% against invasive diseases caused by vaccine serotypes, they also showed a significant impact on pneumonia and otitis media (Table 1) [50-52]. This was supported by data of a Finnish vaccination trial [53]. Moreover, several investigators have shown a significant reduction in nasopharyngeal carriage of vaccine type pneumococci in infants as a result of different conjugate vaccinations (Table 1) [47,54-56]. Mucosal immunity in addition to systemic immunity would explain this decline in nasopharyngeal colonization. This is supported by the results of a second Finnish study, showing that a tetravalent pneumococcal conjugate vaccine induces both mucosal and systemic antibody responses in toddlers [57]. It was suggested that diminished nasopharyngeal carriage could lead to reduced person to person spread of the pneumococcal serotypes most often associated with disease and antibiotic resistance, and even to herd immunity [47,58,59]. Because of these promising data, the ACIP recommended pneumococcal conjugate vaccination for all children under the age of 24 months, and children 24-59 months of age when at risk of pneumococcal disease [20,33]. Unfortunately, the Dutch OMAVAX study investigating the efficacy of the 7-valent conjugate vaccine against recurrent otitis media in children 12 months to 7 years of age, showed no effect on the number of otitis media events despite good antibody responses [60]. The authors suggested that interference in an existing balance between host and pathogen by means of vaccination may induce pneumococcal strain replacement. The appearance of a new pathogen, in turn, increases the risk of acute otitis media [61,62]. This might explain the different results of the two landmark studies in California and Finland investigating the effect of conjugate vaccination on otitis media in infants: at infant age the conjugate vaccination may prohibit or delay nasopharyngeal acquisition of the most prevalent pneumococcal serotypes, thus preventing or delaying early pneumococcal AOM until a later age. Replacement is therefore not possible in young children whereas the OMAVAX colonization data showed full replacement of vaccine type pneumococci by non-vaccine type strains [63]. With ongoing follow-up, more studies have reported this phenomenon [53,55 ,64]. The effect of replacement to the burden of invasive diseases still remains unknown, although one

Table 1. Vaccine efficacy by means of reduction in disease (invasive disease, pneumonia, RTI, otitis media), secondary outcome (ventilatory tube placement, antibiotic prescriptions), carriage, cross protection, replacement and resistance.

Country †	year	Number of children	age in months	Vaccine schedule	follow up in months	invasive pneumococcal diseases	pneumonia (radiograph confirmed)	LRTI	URTI	otitis media episodes	Ventilatory tubes	antibiotic pre-scriptions	carriage of vaccine serotypes	cross protection	Replace-ment	resistance colonizing strains
The Netherlands	2003	383	12-72	1/ 2x 7-valent + PS booster	26					-			++ < 24 mths No > 24 mths	No	Yes No	
California	2002/ 2003	37868	2	3 x 7-valent + booster	24-42	+++	+			+	+	+				
Finland	2001	1662	2	3 x 7-valent + booster	18					+				Yes	Yes	
United Kingdom	2003	293	2	3 x 7-valent + PS booster	12-48								No		No	
Israel	2002	264	12-35?	1x 9-valent	24								++	6A yes 19A no	Yes	
Israel	2001	264	12-35	1 x 9-valent	22			+	+	+		+				
Soth Africa	1999	500	2	1 x 9-valent	9								++		Yes	++*
Israel	1996	263	12-18	1/ 2 x 7-valent	3								++		No	+++**

† Data are retrieved from Dutch OMAVAX trial [60,63]; California Kaiser Permanente trial [48,50-52]; Finnish otitis media study group [53,61,66]; Sheffield Institute for Vaccine studies, UK [54]; Soroka University Medical Center, Beer Sheva, Israel [47,55,56,58]; Pneumococcal Diseases Research Unit, Johannesburg, South Africa [64].
* significant reduction in penicillin and cotrimoxazole resistant strains. ** large reduction in all antibiotic resistant strains.

conjugate vaccination trial in the US recently reported 25% increase in invasive diseases caused by non-vaccine serotypes. Though this was not yet statistically significant, in the future it may become clinically relevant [65].

Meanwhile, the conjugate vaccine has been implemented nation wide in the United States [66]. For the European union, only Austria is currently (January 2003) recommending country-wide childhood vaccination [67]. However, most other countries, including Belgium, The Netherlands, Denmark, Finland, France, Germany, Ireland, Italy, Spain and the UK limit vaccine application to high-risk groups including patients with functional asplenia, immunodeficiencies, diabetes and chronic diseases like kidney, liver, lung, and heart diseases and children with cochlear implants [67]. The Achilles heel as presented by the Finnish national expert group appears to be the less favourable cost-effectiveness of the recommended 4-dose vaccination schedule [66].

IMMUNOGENIC PROTEINS

Though conjugate vaccines are highly protective against pneumococcal diseases, long-term vaccine failure due to the limited serotype coverage, and consequently, induction of replacement disease is realistic. Moreover, the vaccine is rather expensive, prohibiting implementation in most developing countries. [53,55,60,64,65,68,69]. In the last decade, several groups have investigated the use of pneumococcal proteins as potential vaccine candidates and promising results have been reported. Although many proteins including pneumolysin, pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), Choline binding protein A (CbpA), neuraminidase, and autolysin have been suggested as potential candidates, the proteins PspA, PsaA and pneumolysin are currently the leading vaccine candidates (Table 2) [70].

Pneumococcal surface protein A (PspA) is a member of a family of structurally related choline-binding surface proteins, which are able to interfere with complement fixation. They block the initiation of the alternative pathway through reduction of the amount of C3b deposited on the pneumococci. These proteins reduce the effectiveness of the complement receptor mediated pathways of clearance [71-74]. Although PspA shows considerable antigenic heterogeneity between different strains, Nabors at al. (2000) found broadly cross-reactive antibodies to heterologous PspA molecules after immunization with a single recombinant PspA preparation [75,76]. This is supported by findings of Kolberg et al. who showed that a combination of two monoclonals identify 94% of clinical isolates [77]. Moreover, active immunization with PspA in animal models has proven protective against invasive infections and nasopharyngeal carriage [78-82]. In addition, Nabors and coworkers have performed the first phase I vaccination trial with a single recombinant PspA variant in

Table 2. Evidence for protective role of the vaccine candidate proteins PsaA, PspA and Pneumolysin against colonization, sepsis, pneumonia and otitis media.

	Colonization			**Sepsis**			**Pneumonia**			**Otitis media**		
	PsaA	PspA	Ply	PsaA	PspA	Ply	PsaA	PspA	Ply	PsaA	PspA	Ply
Animal Model*												
-knock out			+++[92]		++[94]	++[94]			+++[92]			
mutagenesis						+++[93]						
-monoclonal antibodies	++[87]											
-animal antibodies					+++[82,117]							
-human antibodies	+/+++[87]				+++[115]	++[118]						
-active immunisation	++/+++[87]	+[78]	- [83]	+[88,83]	++[83]	++[83]	+++[90]	+[83]	+[83]		+++[116]	
		++[82]		+++[90]	++[81]							
Human												
-immunogenic (+ or -):												
serum IgG	+[112]	+[112]	+[112]							+[113]	+[112]	+[110]
salivary IgA	+[114]	+[114]	+[114]							+[114]	+[114]	+[114]
-protective role		+[115]								+[113]		

+ partial (protective) effect (< 50%), ++ protective effect of 50-90%, and +++ 90-100% protection

humans. This vaccine showed to elicit broadly cross-reactive antibodies to heterologous PspA molecules [76]. Furthermore, these antibodies were found to protect mice challenged intraperitoneally with pneumococci [83,84].

Another inportant candidate is pneumococcal surface adhesin A (PsaA), a member of the family of metal binding lipoproteins. This protein is part of an ABC transporter complex thought to be involved in the transport of manganese into pneumococci [85,86]. The first immunization studies with PsaA showed significant protection against colonization but little to modest protection against invasive infections [70,87-89]. Recently though, Seo et al. showed that oral vaccination with PsaA encapsulated in microalginate microspheres elicits significant protection against colonization, pneumonia as well as septicemia in mice [90]. Because PsaA and PspA have different functions in virulence, an additive role for these proteins in vaccination was suggested. Indeed, promising results have been found for the combination of PsaA and PspA in prevention of colonization and otitis media in animal models [79,85].

Pneumolysin, also containing a choline-binding domain, is suggested to interfere with host immunity and inflammatory responses by a variety of functions, including complement fixation and inhibition of phagocyte function. It also inhibits ciliary activity in the bronchus and is therefore important in pathogenesis of pulmonary infection [3,91]. Knock-out mutagenesis of pneumolysin has proven its role in virulence in case of colonization as well as infection [92-94]. Several investigators have described the protective properties of pneumolysin against challenge with pneumococci in mice, be it against invasive diseases only [95,96]. PspA has shown complementary protection to invasive diseases in animals when used in combination with pneumolysin [70,79].

Other pneumococcal proteins that have shown potential as vaccine candidates are PspC (CbpA), the Pht family, PpmA, autolysin and neuraminidase. PspC either contains a choline-binding domain like PspA and pneumolysin or a LPXTG motif like other Gram-positive bacteria [97]. This protein is supposed to bind secretory IgA and to interact with human epithelial and endothelial cells [97,98]. Vaccination with PspC has shown to be protective against sepsis in mice. Moreover, antibodies directed against this protein have shown cross-reactivity against PspA [99]. It is not yet clear though whether vaccination with PspC elicits protection against heterologous PspC type strains. The Pht family is one of cell surface-exposed homologous proteins representing histidine triad motifs of which several members have shown to elicit protection against different pneumococcal serotypes in a mouse sepsis model [100]. Importantly, these proteins appear to be highly conserved among pneumococal strains, implicating a potential broad coverage. The putative proteinase maturation protein A (PpmA) is a highly conserved surface-associated protein which shows homology to members of the family of peptidyl-prolyl cis/trans isomerases. Mutagenesis of PpmA

reduced virulence of pneumococci in a mouse pneumonia model. However, further in vivo studies are necessary to investigate the potential protective properties of this protein [101]. Pneumococci also produce two neuroaminidases, NanA and NanB. These pneumococcal enzymes cleave N-acetylneuraminic acid from mucin, glycolipids, glycoproteins and oligosaccharides on host cell surfaces. Although the precise role in the pathogenesis of *S. pneumoniae* has not yet been identified, neuraminidase is presumed to contribute to adherence to mucosal surfaces through decreasing the viscosity of the mucuslayer or through exposing cell surface receptors for pneumococci [102]. However, mutagenesis of neuraminidaseA did not affect virulence of pneumococci after intraperitoneal challenge [103], which suggests a limited role in invasive disease. Pneumococcal autolysin (LytA) is, like PspA, CbpA and pneumolysin, a choline-binding protein, which contributes to virulence by mediating the release of pneumolysin and possibly inflammatory cell wall degradation products [94,104]. The exact role of autolysin in pathogenesis is still unclear, and controversial data have been reported regarding the protective properties of this protein [105].

So far, none of the proteins are considered to elicit species-wide pneumococcal protection. This can be explained by the occurence of allelic variation within most individual proteins [75,97,105]. Antibodies raised against a single protein may not recognize allelic variants. Therefore, immunological interference using multiple variants of a single protein or using multiple proteins will limit immunological escape by the pneumococcus. Therefore, a combination of proteins should be considered in future protein vaccine strategies. Which combination of proteins should be chosen remains open for discussion. Although a combination of pneumolysin and PspA is suggested because of their protective effect against invasive diseases in animal models, the route of infection investigated in these models is either directly intravascular or via intraperitoneal challenge, and therefore not physiological. The natural route of infection with *S. pneumoniae* is believed to start with colonization, which progresses to (invasive) disease, by crossing of the natural physical and immunological barriers. Therefore, it seems rational to aim for prevention of colonization. By preventing nasopharyngeal colonization of *S. pneumoniae*, horizontal spread of pneumococcal strains will be diminished as well, thereby enhancing herd-immunity. This theory supports the usage of PsaA as vaccine candidate as this protein has shown to elicit significant protection against pneumococcal colonization. In combination with PspA full protection may even become reality.

Several recent studies suggest that administrating the protein vaccine via the oral or nasal route is as effective as systemic application [78,81,82,90]. One of these routes is strongly preferable because of the high number of vaccines already administered intramuscularly or subcutaneously to children as part of community vaccination programs. Moreover, in

contrast to pneumococcal conjugate vaccines and PS vaccines, protection is also expected in children with HIV/AIDS, even in a later stadium, because of the intact mucosal immune response in these patients [90].

The addition of an adjuvant will be inevitable when full protection is pursued; recent studies have all shown a significant increase in protection when adjuvants are used [81,82,85,88-90]. Which adjuvant elicits the best protection should be further investigated. Finally, encapsulating the proteins should be considered because this may elicit additional systemical protection due to prevention of degradation in the stomach [90].

Care should be taken that the vaccine elicits a high level of cross-reactivity against the heterogeneous spectrum of subtypes to avoid escape by recombination events. Several of the potential proteins display complex mosaic structure as a result of horizontal (inter-species) exchange of gene parts [97,105,106, 107]. However, for the PspA molecules, cross-reactivity between the different variants is present because they share cross-reactive epitopes [76,77].

On the other hand, cross-reactivity with species other than *S. pneumoniae* should be closely monitored; for example, Jado et al. have shown close homology between the PsaA molecules of *S. pneumoniae* and PsaA molecules in three different viridans streptococci [108]. The eradication of these species is undesirable because they may protect humans from respiratory tract infections by other streptococci and species [109-111]. All in all, thorough investigation of these proteins still is necessary before large-scale immunization studies in humans will become within reach.

CONCLUSION

At the moment, prevention of pneumococcal diseases is possible through 23-valent PS vaccines and a 7-valent conjugate vaccine. Although the PS vaccine has several disadvantages, of which the most important one is its minimal efficacy in children < 2 years and immunodeficient patients, they are still useful for immunocompetent individuals ≥ 5 years of age who are at risk of pneumococcal infections [20 ,33]. The more recently approved conjugate vaccine, which contains the capsule polysaccharides of the 7 most prevalent serotypes conjugated to a carrier protein, is highly immunogenic in children < 2 years of age, showing better immune responses and immunological memory. Unfortunately, the efficacy is limited due to the restricted number of serotypes included, and in the long run the efficacy will be threatened by serotype replacement. Therefore pneumococcal proteins have been studied thoroughly to evaluate their possible role in future pneumococcal vaccination. First of all, it is suggested that these proteins can be used as carrier protein in a conjugate vaccine, eliciting protection against the remaining serotypes thus preventing

serotype replacement. In this respect, the results from Kuo et al., showing significant protection in mice by a conjugate vaccine containing polysaccharides of serotype 18C conjugated to pneumolysin, look promising [26]. Secondly, if the proteins turn out to elicit sufficient protection by themselves or in combination, a pure protein vaccine could also be optional. Because of the additive roles in virulence and protection of pneumolysin, PspA and most importantly PsaA, a combination of these proteins is most promising. A major advantage of this combination is serotype-independent protection. In addition, this concept will limit the costs due to the relatively simple production of these recombinant proteins [22]. Distribution of this vaccine to the third world countries would become possible, thus reaching a broader target group then ever before. Importantly, if the vaccine can be administered via the oral or intranasal route a major risk group, the HIV/AIDS patients, will also benefit of the protection elicited by the mucosal immune-system which is unimpaired in these patients [90]. On the other hand, escape mechanisms of the pathogen in response to the immunological pressure is not unlikely if full eradication cannot be achieved. Moreover, cross-responsiveness to commensal species is realistic.

The effect of vaccination on the bacterial dynamics depends the kind of protection that is elicited. When vaccination leads to the prevention of pneumococcal carriage, the horizontal spread of pneumococcal strains will also be diminished. This may lead to herd immunity resulting in protection of unvaccinated individuals against pneumococcal diseases. In addition, the prevention of nasopharyngeal colonization indicates the presence of mucosal immunity, which may also reduce mucosal diseases caused by *S. pneumoniae*. On one hand, when eradication of pneumococcal carriage at the nasopharynx occurs, replacement of colonization and infection with non-vaccine serotype pneumococci and competitive species might easily happen. On the other hand, this may also lead to herd susceptibility towards non-vaccine sero- or genotypes and even alternative pathogenic species, and hence, disease burden. In contrast, when aiming for the prevention of invasive diseases without disturbing the nasopharyngeal niche, the risk for replacement disease is minimal. Systemic antibodies against proteins involved in invasion but not adherence may accomplish this. Unfortunately, positive ‘side-effects’ such as prevention of mucosal disease and the induction of herd immunity will not occur with this strategy.

In conclusion, thorough investigation of the efficacy and safety of these vaccine candidates and monitoring of side-effects have to be performed before human use is optimized. Until then, the available vaccines can be used in the prevention of invasive diseases in infants but close monitoring of escape mechanisms and vaccine failures remains necessary.

REFERENCES

1. Jaffar, S., Leach, A., Hall, A.J. *et al.* Preparation for a pneumococcal vaccine trial in The Gambia: individual or community randomisation? *Vaccine* 1999, 18(7-8), 633-640.
2. Pichichero, M.E., Shelly, M.A. & Treanor, J.J. Evaluation of a pentavalent conjugated pneumococcal vaccine in toddlers. *Pediatr Infect Dis J* 1997, 16(1), 72-74.
3. Kellner, J.D. & Ford-Jones, E.L. *Streptococcus pneumoniae* carriage in children attending 59 Canadian child care centers. Toronto Child Care Centre Study Group. *Arch Pediatr Adolesc Med* 1999, 153(5), 495-502.
4. Nuorti, J.P., Butler, J.C., Crutcher, J.M. *et al.* An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. *N Engl J Med* 1998, 338(26), 1861-1868.
5. Principi, N., Marchisio, P., Schito, G.C. & Mannelli, S. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. *Pediatr Infect Dis J* 1999, 18(6), 517-523.
6. Hament, J.M., Kimpen, J.L., Fleer, A. & Wolfs, T.F. Respiratory viral infection predisposing for bacterial disease: a concise review. *FEMS Immunol Med Microbiol* 1999, 26(3-4), 189-195.
7. Mulholland, K. Strategies for the control of pneumococcal diseases. *Vaccine* 1999, 17 Suppl 1, S79-84.
8. Plotkowski, M.C., Puchelle, E., Beck, G., Jacquot, J. & Hannoun, C. Adherence of type I *Streptococcus pneumoniae* to tracheal epithelium of mice infected with influenza A/PR8 virus. *Am Rev Respir Dis* 1986, 134(5), 1040-1044.
9. Austrian, R. The pneumococcus at the millennium: not down, not out. *J Infect Dis* 1999, 179 Suppl 2, S338-341.
10. Klemperer, G. & Klemperer, F. Versuche uber Immunisierung und Heilung bei der Pneumokokkeninfection. *Berl Klin Wochenstr* 1891, 28, 833-835, 869-875.
11. Watson, D.A. & Musher, D.M. A brief history of the pneumococcus in biomedical research. *Semin Respir Infect* 1999, 14(3), 198-208.
12. Felton, L. & Baily, G. Biologic significance of the soluble specific substance of pneumococci. *J Infect Dis* 1926, 38, 131-144.
13. Smillie, W., Warnock, G. & White, H. A study of a type I pneumococcus epidemicat the state hospital in Worcester, Mass. *American Journal Public Health* 1938, 28, 293-302.
14. Austrian, R. Pneumococcus: the first one hundred years. *Rev Infect Dis* 1981, 3(2), 183-189.
15. Austrian, R. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. *Rev Infect Dis* 1981, 3 Suppl, S1-17.
16. Austrian, R. & Gold, J. Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. *Ann Intern Med* 1964, 60, 759-776.
17. Meis, J.F. & Neeleman, C. [Problems of resistance in *Streptococcus pneumoniae* Resistentieproblemen bij *Streptococcus pneumoniae*. *Ned Tijdschr Geneeskd* 1996, 140(3), 141-144.
18. Appelbaum, P.C., Bhamjee, A., Scragg, J.N., Hallett, A.F., Bowen, A.J. & Cooper, R.C. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol. *Lancet* 1977, 2(8046), 995-997.
19. Kroon, F.P., van Dissel, J.T., de Jong, J.C., Zwinderman, K. & van Furth, R. Antibody response after influenza vaccination in HIV-infected individuals: a consecutive 3-year study. *Vaccine* 2000, 18(26), 3040-3049.
20. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 1997, 46(RR-8), 1-24.
21. Bruyn, G.A., Zegers, B.J. & van Furth, R. Mechanisms of host defense against infection with *Streptococcus pneumoniae*. *Clin Infect Dis* 1992, 14(1), 251-262.
22. Paton, J.C., Andrew, P.W., Boulnois, G.J. & Mitchell, T.J. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. *Annu Rev Microbiol* 1993, 47, 89-115.
23. Rijkers, G.T., Sanders, E.A., Breukels, M.A. & Zegers, B.J. Infant B cell responses to polysaccharide determinants. *Vaccine* 1998, 16(14-15), 1396-1400.
24. Rubins, J.B., Alter, M., Loch, J. & Janoff, E.N. Determination of antibody responses of elderly adults to all 23 capsular polysaccharides after pneumococcal vaccination. *Infect Immun* 1999, 67(11), 5979-5984.
25. Koskela, M., Leinonen, M., Haiva, V.M., Timonen, M. & Makela, P.H. First and second dose antibody responses to pneumococcal polysaccharide vaccine in infants. *Pediatr Infect Dis* 1986, 5(1), 45-50.
26. Kuo, J., Douglas, M., Ree, H.K., &Lindberg, A.A. Characterization of a recombinant pneumolysin and its use as a protein carrier for pneumococcal type 18C conjugate vaccines. *Infect Immun* 1995, 63(7), 2706-13.
27. Leinonen, M., Sakkinen, A., Kalliokoski, R., Luotonen, J., Timonen, M. & Makela, P.H. Antibody response to 14-valent pneumococcal capsular polysaccharide vaccine in pre-school age children. *Pediatr Infect Dis* 1986, 5(1), 39-44.
28. O'Brien, K.L., Steinhoff, M.C., Edwards, K., Keyserling, H., Thoms, M.L. & Madore, D. Immunologic priming of young children by pneumococcal glycoprotein conjugate, but not polysaccharide, vaccines. *Pediatr Infect Dis J* 1996, 15(5), 425-430.
29. Wadwa, R.P. & Feigin, R.D. Pneumococcal vaccine: an update [see comments]. *Pediatrics* 1999, 103(5 Pt 1), 1035-1037.
30. Poland, G.A. The burden of pneumococcal disease: the role of conjugate vaccines. *Vaccine* 1999, 17(13-14), 1674-1679.
31. Butler, J.C., Shapiro, E.D. & Carlone, G.M. Pneumococcal vaccines: history, current status, and future directions. *Am J Med* 1999, 107(1A), 69S-76S.
32. Fedson, D.S. Pneumococcal vaccination in the United States and 20 other developed countries, 1981-1996. *Clin Infect Dis* 1998, 26(5), 1117-1123.
33. Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory

Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2000, 49(RR-9), 1-35.
34. Chan, C.Y., Molrine, D.C., George, S. *et al.* Pneumococcal conjugate vaccine primes for antibody responses to polysaccharide pneumococcal vaccine after treatment of Hodgkin's disease. *J Infect Dis* 1996, 173(1), 256-258.
35. Obaro, S.K., Huo, Z., Banya, W.A. *et al.* A glycoprotein pneumococcal conjugate vaccine primes for antibody responses to a pneumococcal polysaccharide vaccine in Gambian children. *Pediatr Infect Dis J* 1997, 16(12), 1135-1140.
36. Breukels, M.A., Rijkers, G.T., Voorhorst-Ogink, M.M., Zegers, B.J. & Sanders, L.A. Pneumococcal conjugate vaccine primes for polysaccharide-inducible IgG2 antibody response in children with recurrent otitis media acuta. *J Infect Dis* 1999, 179(5), 1152-1156.
37. Austrian, R. Pneumococcal polysaccharide vaccines. *Rev Infect Dis* 1989, 11 Suppl 3, S598-602.
38. Madhi, S.A., Petersen, K., Madhi, A., Khoosal, M. & Klugman, K.P. Increased disease burden and antibiotic resistance of bacteria causing severe community-acquired lower respiratory tract infections in human immunodeficiency virus type 1-infected children. *Clin Infect Dis* 2000, 31(1), 170-176.
39. Nuorti, J.P., Butler, J.C., Gelling, L., Kool, J.L., Reingold, A.L. & Vugia, D.J. Epidemiologic relation between HIV and invasive pneumococcal disease in San Francisco County, California. *Ann Intern Med* 2000, 132(3), 182-190.
40. Nielsen, H., Kvinesdal, B., Benfield, T.L., Lundgren, J.D. & Konradsen, H.B. Rapid loss of specific antibodies after pneumococcal vaccination in patients with human immunodeficiency virus-1 infection. *Scand J Infect Dis* 1998, 30(6), 597-601.
41. French, N., Nakiyingi, J., Carpenter, L.M. *et al.* 23-valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial. *Lancet* 2000, 355(9221), 2106-2111.
42. Klein, J.O. The pneumococcal conjugate vaccine arrives: a big win for kids. *Pediatr Infect Dis J* 2000, 19(3), 181-182.
43. Sorensen, R.U., Leiva, L.E., Giangrosso, P.A. *et al.* Response to a heptavalent conjugate *Streptococcus pneumoniae* vaccine in children with recurrent infections who are unresponsive to the polysaccharide vaccine. *Pediatr Infect Dis J* 1998, 17(8), 685-691.
44. Di John, D., Wasserman, S.S., Torres, J.R. *et al.* Effect of priming with carrier on response to conjugate vaccine. *Lancet* 1989, 2(8677), 1415-1418.
45. Peeters, C.C., Tenbergen-Meekes, A.M., Haagmans, B. *et al.* Pneumococcal conjugate vaccines. *Immunol Lett* 1991, 30(2), 267-274.
46. Ahman, H., Kayhty, H., Tamminen, P., Vuorela, A., Malinoski, F. & Eskola, J. Pentavalent pneumococcal oligosaccharide conjugate vaccine PncCRM is well-tolerated and able to induce an antibody response in infants. *Pediatr Infect Dis J* 1996, 15(2), 134-139.
47. Dagan, R., Muallem, M., Melamed, R., Leroy, O. & Yagupsky, P. Reduction of pneumococcal nasopharyngeal carriage in early infancy after immunization with tetravalent pneumococcal vaccines conjugated to either tetanus toxoid or diphtheria toxoid. *Pediatr Infect Dis J* 1997, 16(11), 1060-1064.
48. Shinefield, H.R., Black, S., Ray, P. *et al.* Safety and immunogenicity of heptavalent pneumococcal CRM197 conjugate vaccine in infants and toddlers. *Pediatr Infect Dis J* 1999, 18(9), 757-763.
49. Pelton, S.I., Dagan, R., Gaines, B.M. *et al.* Pneumococcal conjugate vaccines: proceedings from an Interactive Symposium at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy. *Vaccine* 2003, 21(15), 1562-1571.
50. Black, S.B., Shinefield, H.R., Ling, S. *et al.* Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. *Pediatr Infect Dis J* 2002, 21(9), 810-815.
51. Black, S., Shinefield, H., Fireman, B. *et al.* Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000, 19(3), 187-195.
52. Fireman, B., Black, S.B., Shinefield, H.R., Lee, J., Lewis, E. & Ray, P. Impact of the pneumococcal conjugate vaccine on otitis media. *Pediatr Infect Dis J* 2003, 22(1), 10-16.
53. Eskola, J., Kilpi, T., Palmu, A. *et al.* Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* 2001, 344(6), 403-409.
54. Lakshman, R., Murdoch, C., Race, G., Burkinshaw, R., Shaw, L. & Finn, A. Pneumococcal nasopharyngeal carriage in children following heptavalent pneumococcal conjugate vaccination in infancy. *Arch Dis Child* 2003, 88(3), 211-214.
55. Dagan, R., Givon-Lavi, N., Zamir, O. *et al.* Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J Infect Dis* 2002, 185(7), 927-936.
56. Dagan, R., Melamed, R., Muallem, M. *et al.* Reduction of nasopharyngeal carriage of pneumococci during the second year of life by a heptavalent conjugate pneumococcal vaccine. *J Infect Dis* 1996, 174(6), 1271-1278.
57. Nieminen, T., Kayhty, H., Leroy, O. & Eskola, J. Pneumococcal conjugate vaccination in toddlers: mucosal antibody response measured as circulating antibody-secreting cells and as salivary antibodies. *Pediatr Infect Dis J* 1999, 18(9), 764-772.
58. Dagan, R. & Fraser, D. Conjugate pneumococcal vaccine and antibiotic-resistant *Streptococcus pneumoniae*: herd immunity and reduction of otitis morbidity. *Pediatr Infect Dis J* 2000, 19(5 Suppl), S79-87; discussion S88.
59. Daum, R.S. Pneumococcal vaccines for children: an update. *Pediatr Infect Dis J* 1998, 17(9), 823-824.
60. Veenhoven, R., Bogaert, D., Uiterwaal, C. *et al.* Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. *Lancet* 2003, 361, 2189-2195.
61. Kilpi, T., Palmu, A., Jokinen, J., Kähty, H. & Mäkela. Efficacy of conjugate vaccine against acute otitis media (AOM) - Finnish Experience. In: Program and abstracts of the 3rd International Symposium on Pneumococci and

Pneumococcal Diseases, Anchorage, May 5-8, 2002:110. Abstract., 2002.
62. Gray, B.M., Converse, G.M., 3rd & Dillon, H.C., Jr. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* 1980, 142(6), 923-933.
63. Veenhoven, R. Impact of combined pneumococcal conjugate and polysaccharide vaccination on nasopharyngeal carriage in children with recurrent acute otitis media: Program and abstracts of the 3rd International Symposium on Pneumococci and Pneumococcal Diseases, Anchorage, May 5-8, 2002. 2003.
64. Mbelle, N., Huebner, R.E., Wasas, A.D., Kimura, A., Chang, I. & Klugman, K.P. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis* 1999, 180(4), 1171-1176.
65. Hsu, K., Pelton, D., Heisey-Grove, S., Hashemi, J., Klein, J. & Health, a.M.o.t.M.D.o.P. Conjugate vaccine era serotype-specific surveillance for invasive pneumococcal disease in massachusetts children:Program and abstracts of the 21st Annual Meeting of the European Society for Paediatric Infectious Diseases, Giardini Naxos, April 9-11, 2003. 2003.
66. Nohynek, H.M., Kilpi, T.M., Nuorti, J.P. *et al.* Recommending use of pneumococcal conjugate vaccine for children in Finland based on scientific evidence and cost effectiveness analysis: Program and abstracts of the 21st Annual Meeting of the European Society for Paediatric Infectious Diseases, Giardini Naxos, April 9-11, 2003. 2003.
67. Fara, G.M. Pneumococcal conjugate vaccine immunization: targeting at-risk groups or universal vaccination? Pros and cons: Program and abstracts of the 21st Annual Meeting of the European Society for Paediatric Infectious Diseases, Giardini Naxos, April 9-11, 2003. 2003.
68. Dagan, R., Givon, N., Yagupsky, P. & al., e. Effect of a 9-valent pneumococcal vaccine conjugated to CRM 197 (PnCRM9) on nasopharyngeal carriage of vaccine type and non-vaccine type *Streptococcus pneumoniae* (Pnc) strains among day care attendees(abstract G-552). Presented at. in *the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, September 24 to 27, 1998. Washington, DC: American Society for Microbiology, 1998.*, 1998.
69. Obaro, S.K., Adegbola, R.A., Banya, W.A. & Greenwood, B.M. Carriage of pneumococci after pneumococcal vaccination. *Lancet* 1996, 348(9022), 271-272.
70. Briles, D.E., Hollingshead, S.K., Nabors, G.S., Paton, J.C. & Brooks-Walter, A. The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. *Vaccine* 2000, 19 Suppl 1, S87-95.
71. Neeleman, C., Geelen, S.P., Aerts, P.C. *et al.* Resistance to both complement activation and phagocytosis in type 3 pneumococci is mediated by the binding of complement regulatory protein factor H. *Infect Immun* 1999, 67(9), 4517-4524.
72. Tu, A.H., Fulgham, R.L., McCrory, M.A., Briles, D.E. & Szalai, A.J. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. *Infect Immun* 1999, 67(9), 4720-4724.
73. Yother, J. & Briles, D.E. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. *J Bacteriol* 1992, 174(2), 601-609.
74. Yother, J., Handsome, G.L. & Briles, D.E. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the pspA gene. *J Bacteriol* 1992, 174(2), 610-618.
75. Briles, D.E., Tart, R.C., Swiatlo, E. *et al.* Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). *Clin Microbiol Rev* 1998, 11(4), 645-657.
76. Nabors, G.S., Braun, P.A., Herrmann, D.J. *et al.* Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine* 2000, 18(17), 1743-1754.
77. Kolberg, J., Aase, A., Michaelsen, T.E. & Rodal, G. Epitope analyses of pneumococcal surface protein A: a combination of two monoclonal antibodies detects 94% of clinical isolates. *FEMS Immunol Med Microbiol* 2001, 31(3), 175-180.
78. Wu, H.Y., Nahm, M.H., Guo, Y., Russell, M.W. & Briles, D.E. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. *J Infect Dis* 1997, 175(4), 839-846.
79. Ogunniyi, A.D., Folland, R.L., Briles, D.E., Hollingshead, S.K. & Paton, J.C. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 2000, 68(5), 3028-3033.
80. Briles, D.E., King, J.D., Gray, M.A., McDaniel, L.S., Swiatlo, E. & Benton, K.A. PspA, a protection-eliciting pneumococcal protein: immunogenicity of isolated native PspA in mice. *Vaccine* 1996, 14(9), 858-867.
81. Kang, H.Y., Srinivasan, J. & Curtiss, R., 3rd. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica serovar typhimurium vaccine. *Infect Immun* 2002, 70(4), 1739-1749.
82. Arulanandam, B.P., Lynch, J.M., Briles, D.E., Hollingshead, S. & Metzger, D.W. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infect Immun* 2001, 69(11), 6718-6724.
83. Briles, D.E., Hollingshead, S., Brooks-Walter, A. *et al.* The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. *Vaccine* 2000, 18(16), 1707-1711.
84. Briles, D.E., Hollingshead, S.K., King, J. *et al.* Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *J Infect Dis* 2000, 182(6), 1694-1701.
85. Briles, D.E., Ades, E., Paton, J.C. *et al.* Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect Immun* 2000, 68(2), 796-800.
86. Dintilhac, A., Alloing, G., Granadel, C. & Claverys, J.P. Competence and virulence of *Streptococcus pneumoniae*:

Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol Microbiol* 1997, 25(4), 727-739.
87. Romero-Steiner, S., Pilishvili, T., Sampson, J.S. *et al.* Inhibition of Pneumococcal Adherence to Human Nasopharyngeal Epithelial Cells by Anti-PsaA Antibodies. *Clin Diagn Lab Immunol* 2003, 10(2), 246-251.
88. Gor, D.O., Ding, X., Li, Q., Schreiber, J.R., Dubinsky, M. & Greenspan, N.S. Enhanced immunogenicity of pneumococcal surface adhesin A by genetic fusion to cytokines and evaluation of protective immunity in mice. *Infect Immun* 2002, 70(10), 5589-5595.
89. Johnson, S.E., Dykes, J.K., Jue, D.L., Sampson, J.S., Carlone, G.M. & Ades, E.W. Inhibition of pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin a. *J Infect Dis* 2002, 185(4), 489-496.
90. Seo, J.Y., Seong, S.Y., Ahn, B.Y., Kwon, I.C., Chung, H. & Jeong, S.Y. Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin a encapsulated in microspheres. *Infect Immun* 2002, 70(3), 1143-1149.
91. Hirst, R.A., Sikand, K.S., Rutman, A., Mitchell, T.J., Andrew, P.W. & O'Callaghan, C. Relative roles of pneumolysin and hydrogen peroxide from *Streptococcus pneumoniae* in inhibition of ependymal ciliary beat frequency. *Infect Immun* 2000, 68(3), 1557-1562.
92. Kadioglu, A., Taylor, S., Iannelli, F., Pozzi, G., Mitchell, T.J. & Andrew, P.W. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect Immun* 2002, 70(6), 2886-2890.
93. Wellmer, A., Zysk, G., Gerber, J. *et al.* Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis. *Infect Immun* 2002, 70(11), 6504-6508.
94. Berry, A.M., Lock, R.A., Hansman, D. & Paton, J.C. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. *Infect Immun* 1989, 57(8), 2324-2330.
95. Lock, R.A., Paton, J.C. & Hansman, D. Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*. *Microb Pathog* 1988, 5(6), 461-467.
96. Paton, J.C., Lock, R.A. & Hansman, D.J. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. *Infect Immun* 1983, 40(2), 548-552.
97. Iannelli, F., Oggioni, M.R. & Pozzi, G. Allelic variation in the highly polymorphic locus pspC of *Streptococcus pneumoniae*. *Gene* 2002, 284(1-2), 63-71.
98. Rosenow, C., Ryan, P., Weiser, J.N. *et al.* Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol Microbiol* 1997, 25(5), 819-829.
99. Ogunniyi, A.D., Woodrow, M.C., Poolman, J.T. & Paton, J.C. Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. *Infect Immun* 2001, 69(10), 5997-6003.
100. Adamou, J.E., Heinrichs, J.H., Erwin, A.L. *et al.* Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infect Immun* 2001, 69(2), 949-958.
101. Overweg, K., Kerr, A., Sluijter, M. *et al.* The putative proteinase maturation protein A of *Streptococcus pneumoniae* is a conserved surface protein with potential to elicit protective immune responses. *Infect Immun* 2000, 68(7), 4180-4188.
102. Tong, H.H., Blue, L.E., James, M.A. & DeMaria, T.F. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase- deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infect Immun* 2000, 68(2), 921-924.
103. Berry, A.M. & Paton, J.C. Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect Immun* 2000, 68(1), 133-140.
104. Lock, R.A., Hansman, D. & Paton, J.C. Comparative efficacy of autolysin and pneumolysin as immunogens protecting mice against infection by *Streptococcus pneumoniae*. *Microb Pathog* 1992, 12(2), 137-143.
105. Jedrzejas, M.J. Pneumococcal virulence factors: structure and function. *Microbiol Mol Biol Rev* 2001, 65(2), 187-207 ; first page, table of contents.
106. Hollingshead, S.K., Becker, R. & Briles, D.E. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun* 2000, 68(10), 5889-5900.
107. Claverys, J.P., Prudhomme, M., Mortier-Barriere, I. & Martin, B. Adaptation to the environment: *Streptococcus pneumoniae*, a paradigm for recombination-mediated genetic plasticity? *Mol Microbiol* 2000, 35(2), 251-259.
108. Jado, I., Fenoll, A., Casal, J. & Perez, A. Identification of the psaA gene, coding for pneumococcal surface adhesin A, in viridans group streptococci other than *Streptococcus pneumoniae*. *Clin Diagn Lab Immunol* 2001, 8(5), 895-898.
109. Faden, H., Stanievich, J., Brodsky, L., Bernstein, J. & Ogra, P.L. Changes in nasopharyngeal flora during otitis media of childhood. *Pediatr Infect Dis J* 1990, 9(9), 623-626.
110. Ghaffar, F., Muniz, L.S., Katz, K. *et al.* Effects of large dosages of amoxicillin/clavulanate or azithromycin on nasopharyngeal carriage of *Streptococcus pneumoniae*, Haemophilus influenzae, nonpneumococcal alpha-hemolytic streptococci, and Staphylococcus aureus in children with acute otitis media. *Clin Infect Dis* 2002, 34(10), 1301-1309.
111. Ghaffar, F., Friedland, I.R., Katz, K. *et al.* Increased carriage of resistant non-pneumococcal alpha-hemolytic streptococci after antibiotic therapy. *J Pediatr* 1999, 135(5), 618-623.
112. Rapola, S., Jantti, V., Haikala, R. *et al.* Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. *J Infect Dis* 2000, 182(4), 1146-1152.
113. Rapola, S., Kilpi, T., Lahdenkari, M., Takala, A.K., Makela, P.H. & Kayhty, H. Do antibodies to pneumococcal surface adhesin a prevent pneumococcal involvement in acute otitis media? *J Infect Dis* 2001, 184(5), 577-581.

114. Simell, B., Korkeila, M., Pursiainen, H., Kilpi, T.M. & Kayhty, H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin a, pneumolysin, and pneumococcal surface protein a in children. *J Infect Dis* 2001, 183(6), 887-896.
115. McCool, T.L., Cate, T.R., Moy, G. & Weiser, J.N. The immune response to pneumococcal proteins during experimental human carriage. *J Exp Med* 2002, 195(3), 359-365.
116. White, P., Hermansson, A., Svanborg, C., Briles, D. & Prellner, K. Effects of active immunization with a pneumococcal surface protein (PspA) and of locally applied antibodies in experimental otitis media. *ORL J Otorhinolaryngol Relat Spec* 1999, 61(4), 206-211.
117. Wortham, C., Grinberg, L., Kaslow, D. *et al.* Enhanced protective antibody responses to PspA after intranasal or subcutaneous injections of PspA genetically fused to granulocyte-macrophage colony-stimulating factor or interleukin-2. *Infect Immun* 1998, 66(4), 1513-1520.
118. Musher, D.M., Phan, H.M. & Baughn, R.E. Protection against bacteremic pneumococcal infection by antibody to pneumolysin. *J Infect Dis* 2001, 183(5), 827-830.

Chapter 7

The impact of vaccination on colonization and infection with *Streptococcus pneumoniae*

Chapter 7.1

Effect of pneumococcal conjugate vaccine followed by pneumococcal polysaccharide vaccine on recurrent acute otitis media

R.H. Veenhoven, D. Bogaert, C.S.P.M. Uiterwaal, C. Brouwer, H.H. Kiezebrink, J. Bruin, E. IJzerman , P.W.M. Hermans, R. de Groot, B. Zegers, W. Kuis, G.T. Rijkers, A.G.M. Schilder and E.A.M. Sanders

Lancet 2003; 361:2189-95

ABSTRACT

Background. Pneumococcal conjugate vaccine prevents recurrent acute otitis media (AOM) in infants immunised at 2, 4, 6, and 12–15 months of age. We aimed to find out whether this vaccine also prevents AOM in older children who have had previous episodes of AOM.

Methods. In this double-blind, randomised study, we enrolled 383 patients aged 1–7 years who had had two or more episodes of AOM in the year before entry. Randomisation was stratified in four groups according to age (12–24 months *vs* 25–84 months) and the number of previous AOM episodes (two or three episodes *vs* four or more episodes). Children received either 7-valent pneumococcal conjugate vaccine followed by 23-valent pneumococcal polysaccharide vaccine, or hepatitis A or B vaccines. They were followed up for 18 months for recurrence of AOM. We also cultured samples of middle-ear fluid and nasopharyngeal swabs to assess association of pneumococcal serotypes with AOM after vaccination.

Findings. We noted no reduction of AOM episodes in the pneumococcal vaccine group compared with controls (intention-to-treat analysis: rate ratio 1·25, 95% CI 0·99–1·57). Although nasopharyngeal carriage of pneumococci of serotypes included in the conjugate-vaccine was greatly reduced after pneumococcal vaccinations, immediate and complete replacement by non-vaccine pneumococcal serotypes took place.

Interpretation. These data do not lend support to the use of pneumococcal conjugate vaccine to prevent otitis media in previously unvaccinated toddlers and children with a history of recurrent AOM.

INTRODUCTION

The American Academy of Pediatrics has recommended immunisation with 7-valent pneumococcal conjugate vaccine (PCV7) for children with recurrent or severe acute otitis media (AOM) and children who have tympanostomy tubes because of recurrent AOM. [1] This advice was based on the results of two clinical trials with PCV7. The trials included almost 40000 healthy infants, who were immunised at 2, 4, and 6 months of age, and had booster vaccinations at 12–15 months of age. [2 and 3] These children were followed up for the occurrence of AOM up to their second birthday. The pneumococcal vaccine reduced the number of infants with recurrent episodes of AOM by 9%. The largest effect was a reduction of 23% in the number of children developing a severely otitis-prone condition (five episodes in 6 months or six episodes per year). [2] Furthermore, the number of children receiving tympanostomy tubes was reduced by 20%. [2]

However, the benefits of pneumococcal conjugate vaccine have not been investigated in previously unvaccinated toddlers and older children who have documented episodes of AOM before vaccination. Assessment of the vaccine's effectiveness is especially important in this group, since children with recurrent AOM can have subtle immunodeficiencies that alter the vaccine's immunogenicity. [4, 5 and 6] Genetically determined factors in innate and adaptive immunity may also affect the effectiveness of the vaccine. [7 and 8] Furthermore, vaccine effectiveness in older children might differ from that in infants due to differences in pneumococcal serotype coverage and environmental factors. [9] Therefore, the efficacy of pneumococcal conjugate vaccine needs to be assessed in randomised trials to support recommendations that these children should also be immunised.

We investigated whether combined vaccination with PCV7 followed by 23-valent pneumococcal polysaccharide vaccine (PPSV23) could prevent AOM in children aged 1–7 years, with two or more documented episodes of AOM before vaccination. This combination was chosen because of the booster effect of the polysaccharide vaccine after priming with conjugate vaccine both in infants and in children prone to otitis. [10 and 11] Furthermore, the broad pneumococcal serotype coverage by the 23-valent vaccine could benefit children older than 2 years of age. We assessed the protective efficacy of pneumococcal vaccination against recurrent AOM, and the effect of vaccination on culture-confirmed pneumococcal AOM and nasopharyngeal carriage.

METHODS

We did a randomised, double-blind trial between April, 1998, and January, 2002, at a general hospital (Spaarne Hospital, Haarlem) and a tertiary care hospital (Wilhelmina Children's Hospital of the University Medical Center Utrecht) in the Netherlands. Parents

were informed about the study by primary care physicians, paediatricians, and otolaryngologists from across the Netherlands. Parents who were willing to participate signed a consent form to enrol their child in the study.

Inclusion criteria for the study were two or more episodes of AOM in the year before study entry, and age 1–7 years. The number of previous AOM episodes was based both on parental report—with AOM defined as having one or more of the symptoms: acute earache, new-onset otorrhea, irritability, and fever—and on clinical confirmation of the diagnosis by a physician. Exclusion criteria were primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities such as cleft palate, chromosomal abnormalities such as Down's syndrome, and severe adverse events during previous vaccinations.

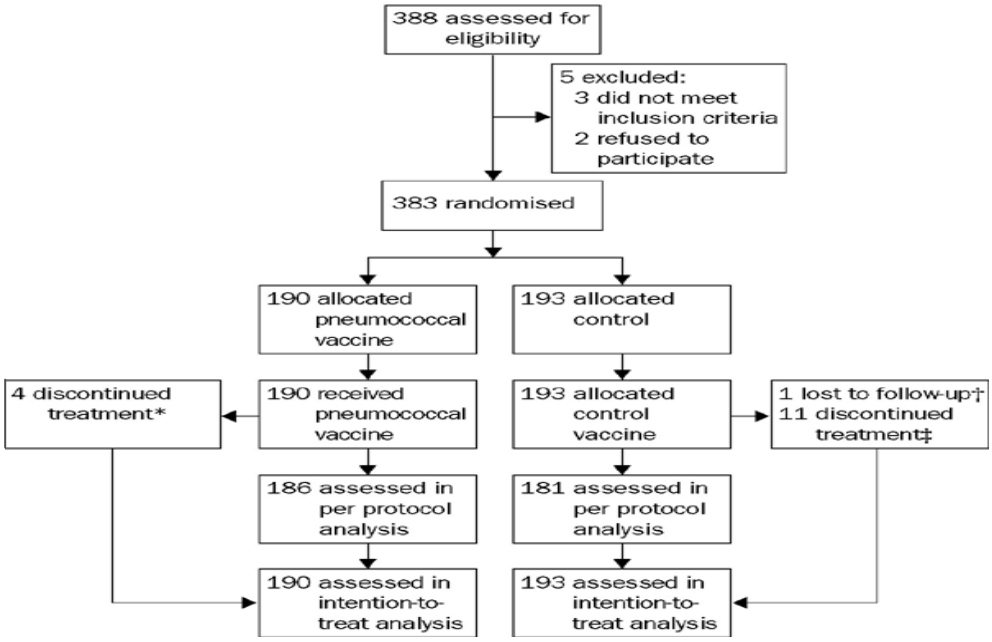


Figure 1. Trial profile

*One child discontinued treatment because of gastroenteritis directly after first vaccination (link with vaccination questionable); three discontinued because parents were not motivated. † The patient moved and we did not know their new address. ‡Four discontinued because of fears about vaccination and venous sampling; two because of disappointment of parents about efficacy of vaccine; two because parents were not motivated; one because the patient's mother was disappointed about communication with study physicians; one because common variable immune deficiency was diagnosed immedately after first vaccination; one for unknown reasons.

The children were randomised to receive either PCV7 followed by PPSV23, or hepatitis A or B vaccines. PCV7 (Prevnar®, Wyeth, Rochester, NY, USA) consisted of 2 µg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 µg of serotype 6B polysaccharide, and µ2 g of serotype 18C oligosaccharide, each conjugated individually to the CRM197 protein. PPSV23 (Pneumune®, Wyeth) consisted of 25 µg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Control vaccines were recombinant hepatitis B vaccine (Engerix-B = AE Junior®, GlaxoSmithkline, Rixenart, Belgium) and hepatitis A vaccine (Havrix = AE Junior®, GlaxoSmithkline).

Since we expected that age at baseline and the number of episodes of AOM in the year before study entry would be important prognostic indicators for AOM, we randomised the children within four groups according to age (12–24 months *vs* 25–84 months) and number of previous AOM episodes per year (two or three episodes *vs* four or more episodes). The children were assigned a number from a table of random numbers that identified the vaccine scheme. The vaccine was administered to the child by a study nurse, so that parents and physicians were unaware of treatment. Children aged 12–24 months in the pneumococcal vaccine group were immunised with PCV7 twice (with a 1-month interval between immunisations) followed 6 months later by PPSV23. The control vaccine group aged 12–24 months received three hepatitis B vaccinations according to a similar time schedule. Children aged 25–84 months in the pneumococcal vaccine group received one dose of PCV7, followed 7 months later by PPSV23. The control group aged 25–84 months received hepatitis A vaccine twice.

The primary endpoint was the efficacy of pneumococcal vaccination against clinical episodes of AOM during a follow-up period of 18 months, starting 1 month after completion of the vaccination scheme. AOM episodes occurring during the 6–7 month period beginning 1 month after PCV7 or control vaccinations and ending 1 month after the last vaccination were also recorded. We instructed parents to visit the study clinics or their family physician, otolaryngologist, or paediatrician to assess symptoms suggesting AOM. Physicians registered signs and symptoms of every AOM episode on standard registration forms. Guidelines issued by the Dutch College of General Practitioners define AOM as the presence of an abnormal tympanic membrane on otoscopy (red, dull, or bulging), or otorrhoea and at least one of these signs or symptoms of acute infection: acute earache, new-onset otorrhoea, irritability, or fever greater than 38·5°C rectally or 38·0°C axillary. [12] New episodes of AOM were recorded after a minimum 7-day interval free of AOM-related symptoms and treatment.

Additional outcomes in our study included number of AOM episodes due to the seven pneumococcal serotypes included in the conjugate vaccine and nasopharyngeal carriage of

conjugate vaccine serotypes. Bacterial cultures from middle-ear fluid were obtained only once in every child, at the time of the first AOM episode arising at least 1 month after the last vaccination. Parents had been asked to bring their child to the study clinic within 24 h after the onset of symptoms suggesting AOM. After clinical confirmation of the diagnosis of AOM, middle-ear fluid was collected by myringotomy or by spontaneous drainage near the perforation site with an aspirator (Juhn Tym-Tap collector, Xomed, Jacksonville, USA) or sterile dry cotton-wool swab (Copan Italia, Transwab, Medical Wire and Equipment Company, Corsham, England). At study entry and follow-up visits, we took nasopharyngeal samples transnasally with a flexible, sterile, dry cotton-wool swab. After sampling, we immediately placed swabs in Stuart's transport medium. Samples of middle-ear fluid and nasopharyngeal swabs were plated within 6 h onto two 5% sheep blood agar plates, a 5% sheep blood agar plate with 5 mg/L gentamicin, and a chocolate agar plate. Agar plates were incubated at 37°C for 48 h; the blood agar plates aerobically and anaerobically, the blood agar plate with gentamicin and the chocolate agar plate with raised CO_2. Identification of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* was based on colony morphology and conventional methods of determination. When *S pneumoniae* was isolated, we undertook serotyping with the capsular swelling method (Quellung reaction) by microscopy with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

All baseline and follow-up visits took place at the study clinics. At entry into the study, parents filled out a standard questionnaire on the medical history of their child and risk factors for AOM. For every episode of AOM diagnosed by a physician during follow-up, parents were asked to record all AOM-related symptoms and treatment in a diary for as long as symptoms persisted. At follow-up visits scheduled at 7, 14, 20, and 26 months after randomisation, AOM registration forms filled in by the physicians and diaries were brought in and checked with the parents. We recorded information on ear, nose, and throat operations. Between these scheduled visits, study physicians contacted the parents by telephone every 3 months. Previous to and 1 month after each vaccination, a blood sample was taken for immunological assessment.

Concentrations of IgG to the seven pneumococcal serotypes in the conjugate vaccine were measured in serum by ELISA. [13] All laboratory work was done by individuals who were unaware of treatment allocation.

The study was undertaken in accordance with the European statements for good clinical practice, which includes the provisions of the Declaration of Helsinki of 1989. The medical ethics committees of both participating hospitals approved the study protocol.

Statistical analysis. On the basis of data from previous studies in the Netherlands, we estimated that 55% of our high-risk patients in the control group would have at least one episode of AOM during the 18 months of follow-up after completion of vaccinations. In view of the multifactorial causes of AOM and comparison of the expected benefit of vaccinations to that of antibiotic prophylaxis and tympanostomy tubes, we judged a reduction of at least 25% to a recurrence rate of 40% of one or more AOM episodes in the pneumococcal vaccine group to be clinically relevant. In order to detect such a reduction, with a (2-sided) 0-05 and power 80%, 176 patients would have to be included in each group. To compensate for an estimated dropout of about 10%, 388 patients would have to be randomised.

Vaccine efficacy was assessed with Cox-type proportional hazards regression models, including a frailty term allowing for differences between individuals in numbers of recurrent AOM episodes. We undertook this analysis in S-plus, version 2000; all other analyses were done with SPSS 10·1. Results are presented as rate ratios with 95% CI; we judged significance to be reached when CI did not include 1. We did both intention-to-treat and per-protocol analyses.

The differences in conjugate and non-conjugate nasopharyngeal pneumococcal carriage between the treatment groups were assessed as follows: children were classified as having had a positive culture for any pneumococcal serotype included in PCV7 or any pneumococcal serotype not included in PCV7 if they had such a positive culture at any of the scheduled follow-up visits after complete vaccination. Proportional differences in pneumococcal carriage and pathogens causing AOM were analysed with Chi^2 tests or Fisher's exact tests when appropriate. We judged $p<0·05$ to be significant. Differences in diary data between groups were assessed with the Mann-Whitney *U* test.

All values are medians (range) or means (SD) unless otherwise indicated. * Atopy is defined as suffering from eczema, hay fever, or recurrent wheezing/asthma.

RESULTS

We enrolled 383 children between April, 1998, and January, 2001; 190 children were randomised to receive pneumococcal vaccinations and 193 to receive control hepatitis vaccinations (figure 1). Age, sex, number of previous AOM episodes, and other risk factors for AOM did not differ between the groups (table 1). In the pneumococcal vaccine group, 186 of 190 children (98%) completed the vaccination scheme, as did 181 of 193 controls (94%). The median follow-up after complete vaccination was similar in the pneumococcal vaccine group (18·1 months, range 2·4–23·0) and control group (18·0 months, range 0·5–23·0). One patient was lost to follow-up immediately after the first vaccination. No serious adverse events were noted after pneumococcal or hepatitis vaccinations.

Table 1. Baseline characteristics, ear, nose, and throat history, and risk factors for AOM.

Variable	Pneumococcal vaccine group (n=190)		Control vaccine group (n=193)	
Male sex	118	(62%)	119	(62%)
Median age, years (range)	2·09	(1-6·86)	2·36	(1-6·99)
Age				
12-24 months	83	(44%)	79	(41%)
25-84 months	107	(56%)	114	(59%)
Number of AOM episodes in preceding year (%)				
2-3	72	(38%)	69	(36%)
4-5	55	(29%)	63	(33%)
6 or more	63	(33%)	61	(32%)
Ventilation tube placement				
None	90	(47%)	96	(50%)
Once	63	(33%)	63	(33%)
Twice or more	37	(20%)	34	(18%)
Adenoidectomy	90	(48%)	89	(46%)
Mean gestational age in weeks	39·3	(SD 2·1)	39·4	(SD 2·1)
Mean birthweight (g)	3358·4	(SD 603·5)	3334·8	(SD 637·1)
Day care (%)				
At age 12-24 months	38 of 83	(46%)	35 of 79	(44%)
At age 25-48 months	53 of 66	(80%)	55 of 65	(85%)
Mean number of siblings	1.05	(SD 0·81)	1.11	(SD 0·93)
Median age at first AOM episode, months (range)	8.0	(1-54)	9.0	(1-48)
Breastfeeding $\geq$ 3 months	83	(44%)	85	(44%)
Atopy*				
Patient history	94	(50%)	100	(52%)
Family history	110	(58%)	115	(60%)
Family history of recurrent AOM				
Parents	107	(56%)	117	(61%)
Siblings	83	(44%)	72	(37%)
Tobacco smoke exposure indoors	58	(31%)	63	(33%)

*Atopy defined as having eczema, hay fever, or recurrent wheezing or asthma.

Of the 475 AOM episodes diagnosed during follow-up after the final vaccination, 275 episodes were recorded in 107 of 186 children (58%) in the pneumococcal vaccine group who completed all vaccinations (recurrence rate 1·1 episodes per person-year) and 200 episodes in 101 of 181 controls (56%; recurrence rate 0·83 episodes per person-year). In this per-protocol analysis after complete vaccination, the rate ratio of recurrence of AOM for the pneumococcal vaccine group versus controls was 1·29 (95% CI 1·02–1·62). The results of the intention-to-treat analysis did not differ from those of the per-protocol analysis over the same period (rate ratio 1·25, 95% CI 0·99–1·57). The cumulative hazard function for AOM of the fully vaccinated pneumococcal vaccine group and controls is shown in figure 2. Subgroup analysis suggested a slightly higher rate ratio of recurrence of AOM in the pneumococcal vaccine group than in controls in children older than 2 years at the time of first vaccination (rate ratio 1·45, 95% CI 1·09–1·94), compared with the group aged 1–2 years (1·07, 0·72–1·60). The rate ratio also seemed higher in children who had two or three episodes of AOM in the year preceding the study (1·66, 1·11–2·49) compared with those who had four or more episodes (1·20, 0·92–1·56). However, since neither of the interactions between age and treatment effect (1·37, 0·87–2·14) and between previous AOM episodes and treatment effect (0·74, 0·45–1·22) was significant, we were not able to conclude that rate ratios differed across subgroups. Excluding the severely otitis-prone children with six or more AOM episodes in the year before study entry from the analyses did not change the outcome of the study (1·30, 0·83–2·06).

We recorded a total of 840 episodes of AOM during the investigation, including those that arose in the period of 6–7 months between first study vaccinations and 1 month after the last vaccination. 445 episodes were in 135 of the 190 children (71%) in the pneumococcal vaccine group (recurrence rate 1·23 episodes per person-year), and 395 episodes in 139 of the 192 controls (72%; recurrence rate 1·08 episodes per person-year). During this whole period, the intention-to-treat analysis also showed no decrease of AOM in the pneumococcal vaccine group compared with controls (rate ratio 1·11, 95% CI 0·92–1·33).

We used data from the diaries to assess the severity and duration of the AOM episodes. Parents of 179 of 208 children with AOM during follow-up completed diaries for 399 of the 475 episodes. We noted no differences between pneumococcal vaccine group and controls in median days per episode for ear-related symptoms such as earache, otorrhea, irritability, and fever, and ear-related treatment such as use of analgesics, antibiotics, and ototopical medications. The number of children treated with tympanostomy tubes during follow-up was similar in the pneumococcal vaccine and control groups (33 and 39, respectively; P = 0·36).

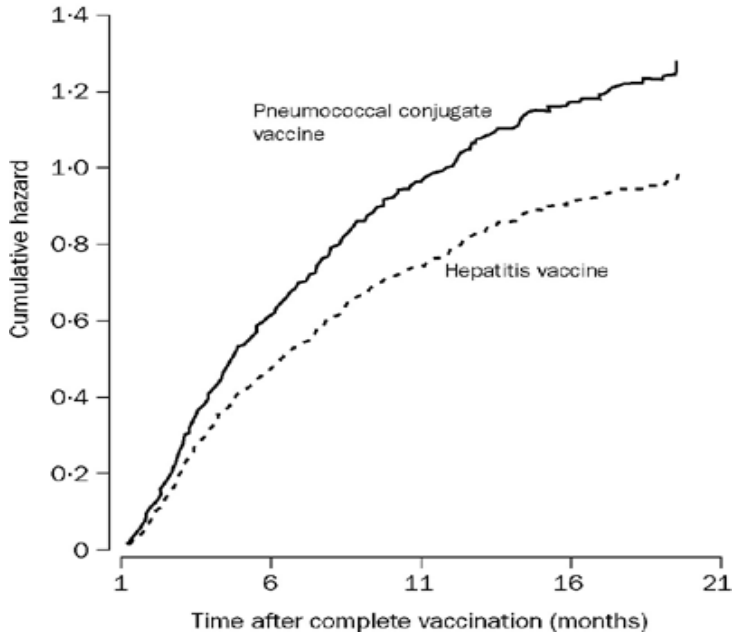


Figure 2. Cumulative hazard function for risk of AOM

Nasopharyngeal swabs were taken at baseline, just before the last vaccination, and at 7, 13, and 19 months after complete vaccinations in 375, 358, 346, 282, and 240 of the children, respectively. At baseline, nasopharyngeal carriage of *S pneumoniae* was found in 49% of all children, regardless of age. Of these nasopharyngeal pneumococcal serotypes, 53% had been included in PCV7; these were serotypes 19F (13%), 6B (12%), 23F (11%), 14 (9%), 9V (5%), 18C (1%), and 4 (1%). In the pneumococcal vaccine group the nasopharyngeal carriage of the conjugate vaccine serotypes fell substantially after complete vaccination compared with the control group ($p<0·001$). However, overall nasopharyngeal carriage of pneumococci was not affected by pneumococcal vaccination, because of a concurrent significant increase in non-conjugate-vaccine serotypes ($p=0·04$; figure 3). Booster vaccination with PPSV23 did not seem to prevent carriage of serotypes not included in the conjugate vaccine. The largest reduction in carriage of conjugate vaccine serotypes (69%) was noted for serotype 18C ($p=0·03$); the lowest reduction (30%) was found for serotype 6B ($p=0·29$). Replacement by non-conjugate vaccine serotypes was mainly caused by serotypes 11 ($p=0·01$) and 15 ($p=0·02$), even though these serotypes were included in PPSV23, and by serotype 16 ($p=0·03$), which was not included in PPVS23. Carriage rate of cross-reacting pneumococcal serotype 6A did not differ between the pneumococcal vaccine and control groups ($p=0·47$).

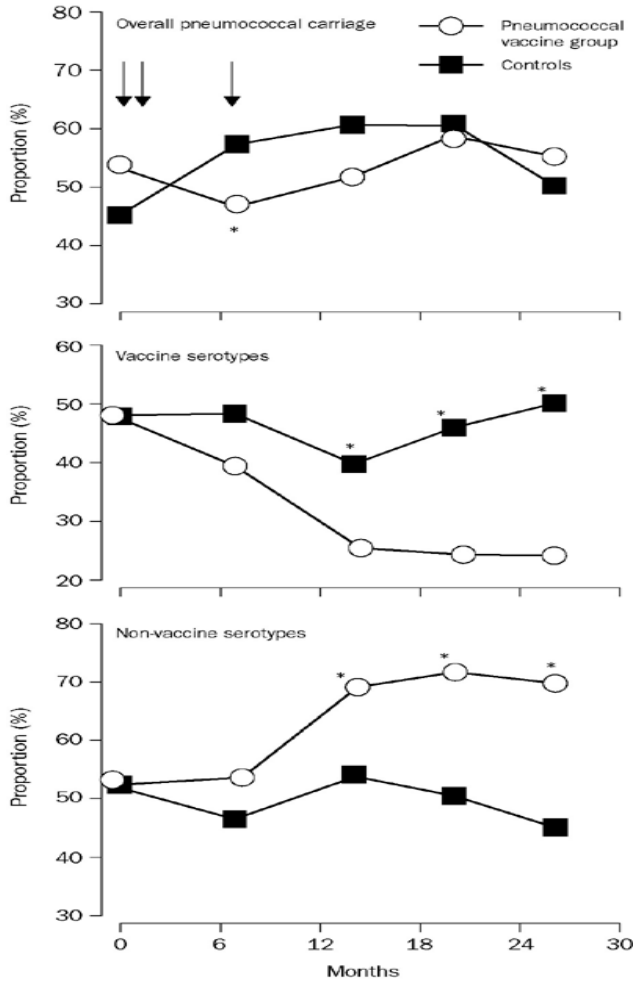


Figure 3. Nasopharyngeal carriage of pneumococci

*Differences in nasopharyngeal carriage of conjugate vaccine and non-conjugate-vaccine pneumococcal serotypes were significant between the two treatment groups ($p<0{\cdot}05$, see results section).

We took no more than one sample of middle-ear fluid during an episode of AOM from any child. Middle-ear fluid was obtained from 92 of 107 children (86%) with AOM in the pneumococcal vaccine group and 89 of 101 controls (88%; table 2). *S pneumoniae* was isolated more often in middle-ear fluid samples in controls (21%) than in the pneumococcal vaccine group (14%). 4% of middle-ear fluid samples from the pneumococcal vaccine group were positive for pneumococcal serotypes included in PCV7, compared with 9% of controls. These numbers were too small for meaningful statistical analysis. 30% of our bacterial

cultures were negative. Numbers of middle-ear fluid cultures of untypable *H influenzae, M catarrhalis,* group A streptococci, and *P aeruginosa* did not differ between the groups. However, we isolated *S aureus* more often in the pneumococcal vaccine group than in the control group (26 *vs* nine children, P=0·002). All *S aureus* cultures and *P aeruginosa* cultures were derived from spontaneous drainage of ears; 75% of the children had ventilation tubes.

Table 2. Pathogens cultured at the first AOM episode after completion of the vaccination scheme

	Pneumococcal vaccination	**Control vaccination**	**p-value**
No. of children with at least one AOM episode	107	101	
No. of AOM episodes MEF obtained	92	89	
MEF obtained by:			
- spontaneously draining ear	71	66	
- myringotomy	21	23	
Culture confirmed S. pneumoniae	13	19	0·22
PCV7 pneumococcal serotypes	4	8	0·21
Other pneumococcal serotypes	9	11	0·44
H. influenzae	21	23	0·64
M. catarrhalis	8	6	0·62
Group A streptococcus	6	4	0·75
Negative cultures	32	35	0.53
Others (all from spontaneously draining ears)			
P. aerigunosa	9	6	0·46
S. aureus	26	9	0·002*

* p-value was considered statistically significantly different at p<0·05. MEF= middle ear fluid.

IgG anti-pneumococcal antibody concentrations were measured for 126 randomly selected children, 24 from each of the four randomisation groups who received pneumococcal vaccines and 30 controls. Geometric mean concentrations of these antibodies were consistently higher in the pneumococcal vaccine group than in controls, and reached values far above 1·5 mg/L, apart from concentrations of serotype 6B, which remained below 0·2 mg/L (table 3).

Table 3. Geometric mean concentrations (mg/L) of IgG anti-pneumococcal antibodies against conjugate vaccine pneumococcal serotypes

Pneumococcal Serotype	Pre-vaccination		1 month after PCV7		1 month after PPSV23	
	PV	CV	PV	CV	PV	CV
4	0·04	0·05	0·38	0·11	2·48	0·11
6B	0·04	0·04	0·06	0·02	0·19	0·05
9V	0·16	0·16	0·51	0·32	4·94	0·30
14	1·44	1·85	6·24	4·02	30·44	8·01
18C	0·17	0·16	6·61	0·29	10·62	0·27
19F	0·25	0·27	1·25	0·37	8·49	0·40
23F	0·47	0·45	5·67	0·50	24·32	0·65

Pneumococcal vaccine group (PV), control vaccine group (CV), 7-valent pneumococcal conjugate vaccine (PCV7), 23-valent pneumococcal polysaccharide vaccine (PPSV23).

DISCUSSION

Our results show that combined pneumococcal conjugate and polysaccharide vaccination is not effective in prevention of AOM in children older than 1 year of age with recurrent AOM. Exclusion of children who were severely prone to otitis from the analysis did not change the outcome of the investigation.

During the trial we saw a marked reduction in AOM episodes both in the pneumococcal vaccine and control groups to an average of one episode per child per year. This decrease could be the result of overestimation of the number of AOM episodes by parents before study entry; such overestimation has been reported previously in studies of children with recurrent AOM.[14] Furthermore, spontaneous recovery of recurrent AOM with increasing age would have had a role in our investigation, [15] since the recurrence rate of AOM episodes per person-year decreased in the total group of patients from 1·63 in the interval between first and last vaccination to 0·97 between the last vaccination and the end of the study. Finally, evidence suggests that medical outcomes can improve substantially due to trial participation itself, which is assumed to be related to expectation of future benefit, improved clinical follow-up, and other aspects of management of the condition. [16 and 17]

In accord with our assumptions, 101 of 181 (56%) children in the control group had at least one episode of AOM during follow-up. On the basis of results from previous trials with PCV7 in healthy infants,[2 and 3] we assumed the efficacy of the vaccine to be higher in children with increased baseline risk of AOM. The children in our study had already had recurrent episodes of AOM and were followed up for a sufficiently long period to detect the reduction

of AOM episodes by PCV7 that we intended. Our results do not show any beneficial effect of this vaccination scheme in terms of reduction of AOM. Since randomisation was successful, loss to follow-up was very low, and AOM episodes were meticulously recorded, we believe that this outcome is valid and that a further increase of precision (more included children) would be unlikely to change these estimates.

We noted very good IgG antibody responses to pneumococcal vaccination in our group of children with recurrent otitis. These responses were significantly higher than those reported in the California and Finnish infant studies,[2 and 3] except for those to serotype 6B. Recent data from the Finnish otitis media study group also show higher concentrations of antibody in infants after booster vaccination with the polysaccharide vaccine at 14 months of age, compared with PCV7 booster vaccination, which was associated with a better clinical protection against AOM caused by serotype 19F. [18] The deficient response to serotype 6B in our study might be due to a subtle immune deficiency, which is characteristic of children who are prone to otitis. [19] Results of other studies have shown that when healthy infants and toddlers were vaccinated with PCV, they were less likely to carry serotype 6B and cross-reactive serotype 6A or have AOM caused by these pathogens. [3 and 20] By contrast, we found a low effect of pneumococcal vaccination against carriage of serotype 6B and no effect against 6A. This finding is probably the result of the low titres of antibody against serotype 6B, and might have influenced the outcome of our study, since serotypes 6B and 6A are among the most common AOM serotypes. [3]

Our findings of no beneficial effect of pneumococcal vaccinations contrast with those of the two landmark studies on prevention of AOM by PCV7 in infants.[2 and 3] These investigations both showed a small but beneficial effect on AOM and improved results in prevention of frequent recurrent AOM. Apart from the booster vaccination with PPSV23, the most important difference between these two studies and ours is that the former studies include healthy infants, who were vaccinated as early as 2 months of age. At this age the child has not yet developed AOM and does not have fully established nasopharyngeal pneumococcal carriage. [21] *S pneumoniae* is a frequent pathogen in early AOM.[9] Because of inflammation and subsequent damage to the middle-ear mucosa and eustachian tube, early pneumococcal AOM could predispose infants to recurrent AOM caused by other pathogens such as *H influenzae*, which was shown to become increasingly important in recurrent AOM episodes.[9] Arguably, conjugate vaccination at infant age might prohibit or delay nasopharyngeal acquisition of the most frequent pneumococcal serotypes, preventing or delaying pneumococcal AOM until a later age, at which time the child is immunologically and anatomically more mature and more capable of handling an AOM infection than in infancy. Thus, prevention of early pneumococcal AOM could be especially important for the prevention of the otitis-prone condition.

In our study, pneumococcal carriage was noted in 50% of children at study entry. This proportion remained constant throughout follow-up, both in the pneumococcal vaccine group and in controls. Although pneumococcal vaccinations did reduce nasopharyngeal carriage of the seven conjugate vaccine serotypes, including serotype 6B, this reduction was accompanied by an increase in pneumococcal serotypes not included in the conjugate vaccine. This shift in nasopharyngeal pneumococcal carriage after conjugate vaccination is consistent with observations in other studies[22 and 23] and is most probably the result of replacement. [24 and 25] The finding that replacement by serotypes 11 and 15 cannot be prevented by PPSV23, which includes these serotypes, lends support to previous results showing that polysaccharide vaccine did not affect nasopharyngeal carriage. [26 and 27] Although children aged 2–7 years showed better responses to the polysaccharides 11 and 15 compared with the younger group, nasopharyngeal carriage was still unaffected by vaccination (data not shown). By induction of nasopharyngeal replacement with non-conjugate pneumococcal serotypes, PCV could even induce recurrence of AOM, because newly acquired carriage is associated with an increased risk for AOM compared with the risk associated with established carriage. [28] This risk might account for the increased number of AOM episodes in the pneumococcal vaccine group in our study. The potentially pathogenic capacity of non-conjugate-vaccine pneumococcal serotypes was previously shown in the Finnish infant study on AOM; [3] the conjugate vaccine reduced AOM caused by conjugate-vaccine-type pneumococci by 57%, but AOM caused by non-conjugate-vaccine pneumococcal serotypes was increased by 34%.

We were not able to confirm that replacement took place in middle-ear fluid. For ethics reasons, we obtained middle-ear fluid only in the first episode of AOM after vaccination. Therefore, the number of middle-ear fluid cultures investigated was small. We noted a 51% reduction in AOM caused by conjugate-vaccine-serotype pneumococci, and overall pneumococcal AOM was reduced by 34%; this finding was similar to that of the Finnish study.[3] We noted no difference between the groups in presence of other middle ear pathogens, apart from *S aureus.* This species was noted more often in middle-ear fluid cultures from the pneumococcal vaccine group, although only in samples taken from spontaneously draining ears. Whether *S aureus* is a true AOM pathogen or is the result of contamination from the external ear canal is uncertain,[29 and 30] but the double-blind nature of our study suggests that pneumococcal vaccination has an effect on the isolation of *S aureus* in samples from spontaneously draining ears.

To summarise, we found that pneumococcal conjugate vaccination combined with pneumococcal polysaccharide vaccination does not prevent AOM in children older than 1 year who have had recurrent episodes of AOM before vaccination. Therefore, pneumococcal vaccinations are not indicated in the management of recurrent AOM in

toddlers and older children. In view of the results of other studies, we might conclude that to prevent pneumococcal AOM in general, and to protect children from developing the otitis-prone condition, pneumococcal vaccinations should be given early in life, at least before 12 months of age and preferably before two or more episodes of AOM have occurred.

ACKNOWLEDGMENTS

We thank all children and parents who participated in the study. We also thank Steve Lockhart, Wyeth Vaccines Research, Berkshire, UK, and Klaas Duijn, Wyeth Pharmaceuticals, Hoofddorp, The Netherlands, for the supply of the pneumococcal vaccines, which were kindly provided by Wyeth Lederle Vaccines and Pediatrics, Rochester, NY, USA. Hepatitis vaccines were kindly provided by GlaxoSmithKline BV, Rixensart, Belgium. This investigation was financed by the Netherlands Organisation for Health Research and Development ZonMw (grant number 002828480) and the Netherlands Health Insurance Company Zilveren Kruis-Achmea.

REFERENCES

1. Policy statement: recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine (Prevnar), pneumococcal polysaccharide vaccine, and antibiotic prophylaxis (RE9960). *Pediatrics* 2000; 106: 362–66.
2. S. Black, H. Shinefield, B. Fireman *et al.*, Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. *Pediatr Infect Dis J* 19 (2000), pp. 187–195.
3. J. Eskola, T. Kilpi, A. Palmu *et al.*, Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* 344 (2001), pp. 403–409.
4. H.G. Herrod, S. Gross and R. Insel, Selective antibody deficiency to *Haemophilus influenzae* type B capsular polysaccharide vaccination in children with recurrent respiratory tract infection. *J Clin Immunol* 9 (1989), pp. 429–434.
5. K. Prellner, G. Harsten, B. Lofgren, B. Christenson and J. Heldrup, Responses to rubella, tetanus, and diphtheria vaccines in otitis-prone and non-otitis-prone children. *Ann Otol Rhinol Laryngol* 99 (1990), pp. 628–632.
6. E.A.M. Sanders, G.T. Rijkers, A.M. Tenbergen-Meekes, M.M. Voorhorst-Ogink and B.J.M. Zegers, Immunoglobulin isotype specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent respiratory tract infections. *Pediatric Research* 37 (1995), pp. 812–819.
7. E.A.M. Sanders, J.G. Van de Winkel, G.T. Rijkers *et al.*, Fc receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. *J Infect Dis* 170 (1994), pp. 854–861.
8. Koch, M. Melbye, P. Sørensen *et al.*, Acute respiratory tract infections and mannose-binding lectin insufficiency. *JAMA* 285 (2001), pp. 1348–1355.
9. T. Kilpi, E. Herva, T. Kaijalainen, R.K. Syrjänen and A.K. Takala, Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life. *Pediatr Infect Dis J* 20 (2001), pp. 654–662.
10. K.L. O'Brien, M.C. Steinhoff, K. Edwards, H. Keyserling, M.L. Thomas and D. Madore, Immunologic priming of young children by pneumococcal glycoprotein conjugate, but not polysaccharide vaccines. *Pediatr Infect Dis J* 15 (1996), pp. 425–430.
11. M.A. Breukels, G.T. Rijkers, M.M. Voorhorst-Ogink, B.J.M. Zegers and E.A.M. Sanders, Pneumococcal conjugate vaccine primes for polysaccharide-inducible IgG2 antibody response in children with recurrent otitis media acuta. *J Infect Dis* 179 (1999), pp. 1152–1156.
12. C.L.M. Appelman, P.C. Bossen, J.H.M. Dunk, E.H. van de Lisdonk, R.A. de Melkert and H.C.P.M. van Weert, Standaard M09, Nederlands Huisartsen Genootschap: otitis media acuta. *Huisarts Wet* 33 (1990), pp. 242–245.
13. D.M. Musher, M.J. Luchi, D.A. Watson, R. Hamilton and R.E. Baughn, Pneumococcal polysaccharide vaccine in young adults and older bronchitics: determination of IgG responses by ELISA and the effect of adsorption of serum with non-type-specific cell wall polysaccharide. *J Infect Dis* 161 (1990), pp. 728–735.
14. O.P. Alho, The validity of questionnaire reports of a history of acute otitis media. *Am J Epidemiol* 132 (1990), pp. 1164–1170.
15. O.P. Alho, E. Laara and H. Oja, What is the natural history of recurrent acute otitis media in infancy?. *J Fam Pract*

43 (1996), pp. 258–264.
16. J. Kleijnen, A.J. de Craen, J. van Everdingen and L. Krol, Placebo effect in double-blind clinical trials: a review of interactions with medications. *Lancet* 344 (1994), pp. 1347–1349.
17. R.C. Maly, L.B. Bourque and R.F. Engelhard, A randomized controlled trial of facilitating information giving to patients with chronic medical conditions: effects on outcomes of care. *J Fam Pract* 48 (1999), pp. 356–363.
18. Kilpi T, Palmu A, Jokinen J, Kähty H, Mäkela. Efficacy of conjugate vaccine against acute otitis media (AOM)—Finnish Experience. In: Program and abstracts of the 3rd International Symposium on Pneumococci and Pneumococcal Diseases, Anchorage, May 5–8, 2002: 110 (abstr).
19. O. Kalm, K. Prellner, A. Freijd and B. Rynnel-Dagoo, Antibody activity before and after pneumococcal vaccination of otitis-prone and non-otitis-prone children. *Acta Otolaryngol* 101 (1986), pp. 467–474.
20. R. Dagan, N. Givon-Lavi, O. Zamir *et al.*, Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J Infect Dis* 185 (2002), pp. 927–936.
21. R.K. Syrjänen, T.M. Kilpi, T.H. Kaijalainen, E.E. Herva and A.K. Takala, Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish Children younger than 2 years old. *J Infect Dis* 184 (2001), pp. 451–459.
22. S.K. Obaro, R.A. Adegbola, W.A. Banya and B.M. Greenwood, Carriage of pneumococci after pneumococcal vaccination. *Lancet* 348 (1996), pp. 271–272.
23. R. Dagan, M. Sikuler-Cohen, O. Zamir, J. Janco, N. Givon-Lavi and D. Fraser, Effect of conjugate pneumococcal vaccine on the occurrence of respiratory infections and antibiotic use in day-care center attendees. *Pediatr Infect Dis J* 20 (2000), pp. 951–958.
24. M. Lipsitch, J.K. Dykes, S.E. Johnson, E.W. Ades, J. King, D.E. Briles and G.M. Carlone, Competition among Streptococcus pneumoniae for intranasal colonization in a mouse model. *Vaccine* 18 (2000), pp. 2895–2901.
25. B.G. Spratt and B.M. Greenwood, Prevention of pneumococcal disease by vaccination: does serotype replacement matter?. *Lancet* 356 (2000), pp. 1210–1211
26. R.M. Douglas and H.B. Miles, Vaccination against *Streptococcus pneumoniae* in childhood: lack of demonstrable benefit in young Australian children. *J Infect Dis* 149 (1984), pp. 861–870.
27. C. Rosen, P. Christensen, B. Hovelius and K. Prellner, A longitudinal study of the nasopharyngeal carriage of pneumococci as related to pneumococcal vaccination in children attending day-care centres. *Acta Otolaryngol* 98 (1984), pp. 524–532.
28. H. Faden, L. Duffy, R. Wasielewski, J. Wolf, D. Krystofik and Y. Tung, Relationship between nasopharyngeal colonization and the development of otitis media in children. *J Infect Dis* 175 (1997), pp. 1140–1145.
29. B.M. Gray, G.M. Converse and H.C. Dillon, Epidemiologic Studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first months of life. *J Infect Dis* 142 (1980), pp. 923–933.
30. G. Giebink, The microbiology of otitis media. *Pediatr Infect Dis J* 8 (1989), pp. S18–S20.

Chapter 7.2

Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media

R.H. Veenhoven, D. Bogaert, A.G.M. Schilder, G.T. Rijkers, C.S.P.M. Uiterwaal, H.H. Kiezebrink, M.J.P. van Kempen, I.J. Dhooge, J. Bruin, E.P.F. IJzerman, R. de Groot, W. Kuis, P.W.M. Hermans and E.A.M. Sanders

Clin Infect Dis; accepted for publication

ABSTRACT

The impact of 7-valent pneumococcal conjugate followed by 23-valent polysaccharide vaccination on pneumococcal nasopharyngeal carriage was studied in a prospective, randomized trial, including 383 children with previous acute otitis media, aged 1-7 years. Nasopharyngeal swabs were collected at baseline and at 6-7 months intervals during 26 months follow-up. Overall pneumococcal carriage rates did not diminish, remaining around 50%. A significant shift from conjugate vaccine to non-conjugate vaccine type pneumococci was observed in children aged 1-2 years, who received twice the conjugate vaccine before polysaccharide vaccination. Conjugate vaccine serotype carriage was not influenced in older children, who received the conjugate vaccine once before the polysaccharide booster. Vaccination with the conjugate vaccine at least twice also after 2 years of age may be mandatory for carriage reduction of conjugate vaccine serotypes in children with recurrent otitis media. Polysaccharide booster vaccination did not affect nasopharyngeal colonization of serotypes not included in the conjugate vaccine.

INTRODUCTION

Streptococcus pneumoniae is one of the leading causes of bacterial infections worldwide, mucosal disease like acute otitis media (AOM) being a thousand times more frequent as life-threatening invasive disease like meningitis. *S. pneumoniae* commonly colonizes the nasopharynx with carriage being highest in infants and toddlers [1-3]. From ages 3 to 5 years carriage rates decrease, coinciding with a natural increase of antibodies against the capsular polysaccharide antigens of pneumococci and natural decrease of pneumococcal diseases [4].

Pneumococcal conjugate vaccines have been shown to reduce nasopharyngeal (NP) carriage of conjugate vaccine type (CVT) *S. pneumoniae* in healthy infants and toddlers [5-8]. This may partly explain the observed reduction of upper and lower respiratory tract infections and AOM [9-12]. In several studies, however, decreased carriage of CVT *S. pneumoniae* after vaccination coincided with an increase of non-conjugate vaccine serotypes (NCVT) [5,6,8]. Most likely, this shift from CVT to NCVT *S. pneumoniae* is due to replacement [13,14]. So far, no significant increase in NVCT *S. pneumoniae* in invasive disease has been reported [15]. For mucosal infections like acute otitis media however, infants vaccinated with heptavalent pneumococcal conjugate vaccine showed a 27-33% higher rate of AOM caused by NCVT *S. pneumoniae* than controls, alongside a significant decrease of AOM caused by CVT *S. pneumoniae* [10,16]. Serotype replacement at the NP level therefore may have a larger impact on mucosal infections as compared to invasive disease [17].

So far, reported clinical efficacy on AOM and carriage studies of pneumococcal conjugate vaccines mainly focused on healthy infants and toddlers [9-11,16]. One study published data of the clinical efficacy of a 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar®, Wyeth) in children with recurrent AOM [18]. In this study combined vaccination with PCV7 and 23-valent pneumococcal polysaccharide vaccine (PPSV23) had no effect on the clinical recurrence rate of AOM. Possibly, particularly in children prone to recurrent respiratory tract infections, phenomena like pneumococcal replacement at the NP level after pneumococcal vaccination strongly influence the clinical outcome in the prevention of mucosal infections. We now describe in detail the impact of vaccination with PCV7 followed by a booster PPSV23 on NP carriage of *S. pneumoniae* in children aged 1 to 7 years with a history of recurrent AOM with respect to the different pneumococcal vaccination schemes used in the study.

SUBJECT, MATERIALS, METHODS

Nasopharyngeal carriage of *S. pneumoniae* was studied in a double blind, randomized, controlled trial, conducted between April 1998 and January 2002. This study was part of a larger study on the clinical efficacy of pneumococcal vaccination on AOM in children with recurrent AOM. Overall results of this study have recently been published [18]. Subjects, materials and methods of the current study were also described.

Inclusion criteria for the study were two or more AOM episodes in the year before study entry, and age 1-7 years [18]. Exclusion criteria for the study were primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial malformation such as cleft palate, chromosomal abnormalities such as Down syndrome, and severe adverse events during previous vaccinations. Vaccination schemes and timing of NP sampling and antipneumococcal antibody evaluation are shown in figure 1.

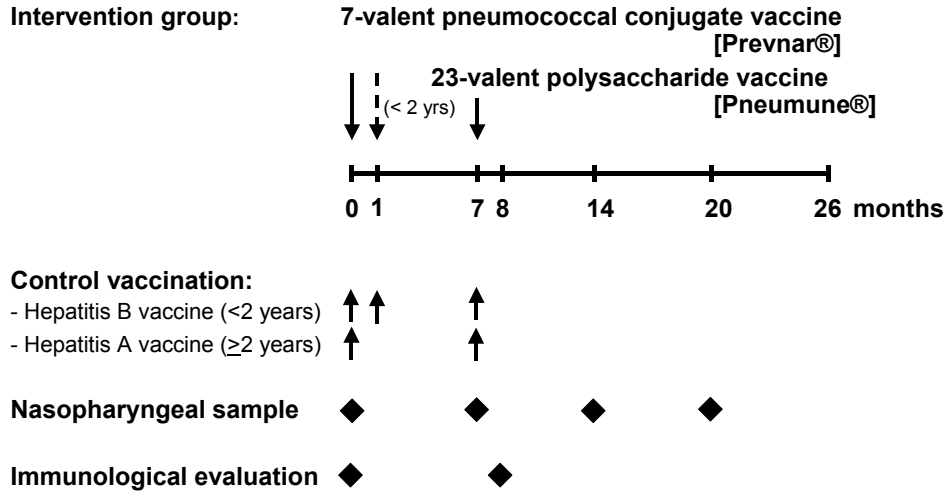


Figure 1. Vaccination scheme and time points at which nasopharyngeal sampling and immunological evaluation (antipneumococcal antibodies against PCV7 serotypes) were performed. Children aged 12-24 months received PCV7 twice and children aged 25-84 months once before the booster PPSV23. The controls received hepatitis vaccines in a similar time-schedule.

Vaccinations. The children were randomized to receive either PCV7 followed by PPSV23, or hepatitis A or B vaccines. PCV7 (Prevnar®, Wyeth) contained 2 µg each of saccharides of pneumococcal serotypes 4, 9V, 14, 18C, 19F and 23F and 4 µg of 6B, coupled to the protein carrier CRM197. PPSV23 (Pneumune®, Wyeth) consisted of 25 µg each of

polysaccharides of pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Control vaccines were recombinant hepatitis B vaccine (Engerix®,-B=AE Junior, GlaxoSmithkline) and hepatitis A vaccine (Havrix®,=AE Junior, GlaxoSmithkline).

Nasopharyngeal swabs. NP samples were cultured for *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis.* NP samples were obtained by the study physicians by using a flexible sterile dry cotton-wool swab (Copan Italia, Transwab, Medical Wire & Equipment Co. Ltd., Corsham, England) induced into the nostrils and advanced until resistance was found. The swabs were inoculated directly in Stuart's transport medium and plated within 6 h onto two 5% sheep blood agar plates, a 5% sheep blood agar plate with 5 mg/L gentamicin, and a chocolate agar plate. Agar plates were incubated at 37^0C for 48 h; the blood agar plates aerobically and anaerobically, the blood agar plate with gentamicin and the chocolate agar plate with raised CO_2. Identification of *S. pneumoniae, H. influenzae,* and *M. catarrhalis* was based on colony morphology and conventional methods of determination. Initially, 4 *S. pneumoniae* colonies of each plate were serotyped. Since the 4 colonies were found to be identical in the first 20 patients, we later serotyped one colony of each plate. Serotyping was performed with the capsular swelling method (Quellung reaction) by microscopy with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

Vaccine immunogenicity. Blood samples for serum IgG to the seven pneumococcal serotypes in the conjugate vaccine were analyzed by ELISA in randomly selected patients in the pneumococcal vaccine group [19].

Statistical Analysis. In children with a positive culture for *S. pneumoniae,* carriage of CVT and NCVT *S. pneumoniae* and individual serotypes were analyzed both at 7 months after conjugate/control vaccination(s) and after booster polysaccharide/control vaccination. After this last vaccination, for each individual only the first pneumococcal culture occurring at 14, 20 or 26 months was included in the analysis.

Proportional differences in carriage of *S. pneumoniae, H. influenzae,* and *M. catarrhalis* at enrolment, after conjugate vaccination alone and after booster with the polysaccharide vaccine were analyzed using Chi-square tests or Fisher's exact tests when appropriate. Differences between IgG anti-pneumococcal antibodies between children aged 12-24 months and children aged 25-84 months were analyzed with Mann-Whitney U test. We judged $P<0.05$ to be significant.

The study was undertaken in accordance with the ethical standards of the responsible committees of both participating hospitals and with the Helsinki Declaration of 1975, as revised in 1983.

RESULTS

A total of 383 children were enrolled; 190 children received pneumococcal vaccinations and 193 control hepatitis vaccinations. Both groups were comparable for age, sex, number of previous AOM episodes and environmental risk factors for AOM, like out-of home care (table 1). More than 95% of all children completed the vaccination scheme and the median follow-up in both groups was similar [18].

Nasopharyngeal swabs were obtained in a similar number of children of the pneumococcal vaccine and control group (respectively 185, 182, 175, 141, and 121 children *vs.* 190, 174, 169, 140, and 118 children) at study entry and 7, 14, 20, and 26 months after the first vaccine dose.

Pneumococcal carriage at baseline. At enrolment 185 (49%) of the 375 NP swabs of children were positive for *S. pneumoniae.* Of all pneumococcal cultures obtained, 53%

Table 1. Baseline data and clinical characteristics

Variable	Pneumococcal vaccine group (n=190)		Control vaccine group (n=193)	
Male sex (%)	118	(62%)	119	(62%)
Median age in years (range)	2.1	(1-6.9)	2.4	(1-7.0)
Age (%)				
12-24 months	83	(44%)	79	(41%)
25-36 months	42	(22%)	35	(18%)
37-84 months	65	(34%)	79	(41%)
No. of AOM episodes in preceding year (%)				
2-3	72	(38%)	69	(36%)
4 or more	118	(62%)	124	(64%)
Day care (%)				
At age 12-24months	38/83	(46%)	35/79	(44%)
At age 25-48 months	53/66	(80%)	55/65	(85%)
Mean no. of siblings (SD)	1.1	(0.8)	1.1	(0.9)
Tobacco smoke exposure indoors (%)	58	(31%)	63	(33%)
No. of children who completed the vaccination scheme (%)	186	(98%)	181	(94%)
Median months of follow-up after completion of vaccination scheme (range)	18.1	(2.4-23.0)	18.0	(0.5-23.0)

belonged to CVT: serotype 19F 13%; serotype 6B 12%; serotype 23F 11%; serotype 14 9%; serotype 9V 6%; serotypes 18C and 4 both 1%. Of the remaining isolates, 45% were found to be NCVT, most frequently cross-reactive serotypes 6A (11%) and 19A (3%) and non-cross-reactive serotypes 11 (4%) and 3 (3%). Furthermore, 2% of all pneumococcal isolates were not typable. Pneumococcal carriage rate at baseline was not influenced by age (12-24 months *vs.* 25-84 months) or the number of previous AOM episodes (2-3 AOM *vs.* 4 or more) in the year prior to first vaccination (results not shown). However, the relative contribution of CVT pneumococci tended to be less in children 25 months and older (49/102 pneumococcal isolates; 48%) compared with the younger group aged 1-2 years (49/83 pneumococcal isolates; 59%, *P*=0.14). Even after the age of 36 months still 25 of the 60 isolated pneumococci (42%) belonged to the CVT. Regardless of age the relative contribution of CVT and NCVT *S. pneumoniae* was not influenced by the number of previous AOM episodes (results not shown).

Impact of vaccination on pneumococcal carriage. Seven months after the first PCV7, the number of children carrying *S. pneumoniae* (85 of 182 children; 47%) was found to be decreased compared with controls (101 of 174 children; 58%, *P*=0.04). With respect to age, this difference was found to be significant only in children older than 2 years of age at the time of the first vaccination (47% *vs.* 57%; *P*=0.04). After PPSV23 overall pneumococcal carriage was not reduced compared with controls, remaining around 50% in both age groups during follow-up (figure 2).

The relative contribution of CVT *S. pneumoniae* to all pneumococcal isolates 7 months after the first vaccination was lower in pneumococcal vaccinees (33/85 pneumococcal isolates; 39%) compared with controls (49/101; 49%). This difference was not significant (*P*=0.19) and not influenced by age (table 2). After PPSV23 however, the contribution CVT *S. pneumoniae* to all pneumococcal cultures was now markedly decreased (35/141 pneumococcal isolates; 25%) compared with control vaccinees (64/144; 44%, *P*=0.001). This difference was most pronounced in those children first vaccinated at age 12-24 months (25% *vs.* 52%; *P*=0.002) (table 2). Among children enrolled between 2-7 years, CVT carriage rates fell over time among both treatment groups and less difference in CVT contribution was observed between pneumococcal vaccinees and controls (25% *vs.* 38%; *P*=0.06).

Both after PCV7 vaccination(s) alone at 7 months as well as after PPSV23, the decrease in carriage rates of CVT *S. pneumoniae* occurred in parallel with an increase of carriage of NCVT *S. pneumoniae* (table 2). Seven months after PCV7, 45 of 85 pneumococcal isolates (53%) proved to be NVCT compared with 46 of 101 pneumococcal isolates (46%) in controls (*P*=0.31). After PPSV23, 103 of 141 pneumococcal isolates (73%) were NCVT compared with 78 of 144 pneumococcal isolates (54%) in controls (*P*=0.001). The difference in NCVT

carriage between most treatment groups was more pronounced in children aged 12-24 months (73% *vs.* 48%; *P*=0.004) than in older children (73% *vs.* 59%; *P*=0.07).
Figure 3 shows the relative contributions of CVT and NCVT *S. pneumoniae* to overall pneumococcal carriage during total follow-up according to age at the time of first

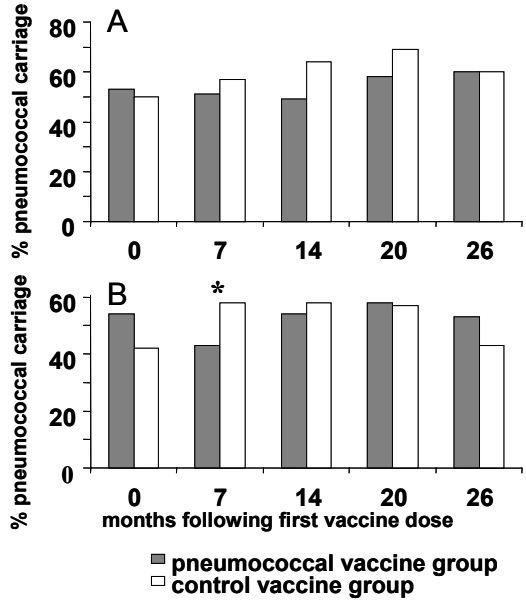


Figure 2. Overall pneumococcal carriage percentages at study entry and at 7, 14, 20 and 26 months after thefirst vaccination in children according to age 12-24 months (A) and 25-84 months (B) at the time of first vaccination. For the number of vaccinees at various time points see results section. Differences in carriage of *S. pneumoniae* were analyzed using Chi-square tests.
* Statistical significance was considered reached at *P*<0.05.

vaccination; the lower impact of pneumococcal vaccination on pneumococcal NP carriage among children enrolled between 2-7 years of age held true when this group was stratified further into those 25 to 36 months and those 37 to 84 months at the time of first vaccination
With respect to the impact of pneumococcal vaccinations on the individual CVT pneumococci in children carrying *S. pneumoniae* 7 months after the first dose of PCV7, only the reduction of the conjugate vaccine serotype 14 was found to be significant (*P*=0.04) in children aged 25-84 months at the time of first vaccination (table 2). After PPSV23 the reduction of serotype 14 and other individual CVT serotypes did not reach significance. The

Table 2. Relative contribution of CVT and NCVT *S. pneumoniae*

	7 months after first vaccination				**Follow-up after last vaccination**			
	Age first vaccination				**Age first vaccination**			
	12-24 months		**25-84 months**		**12-24 months**		**25-84 months**	
	PV	**CV**	**PV**	**CV**	**PV**	**CV**	**PV**	**CV**
	PI, n=41	**PI, n= 41**	**PI, n=44**	**PI, n=60**	**PI, n=60**	**PI, n=63**	**PI, n=81**	**PI, n=81**
All PCV7 serotypes	**18 (44%)**	**21 (51%)**	**15 (34%)**	**28 (47%)**	**15 (25%)**	**33 (52%)***	**20 (25%)**	**31 (38%)**
4	0	0	1	0	0	1	1	0
6B	5	7	2	6	4	9	5	5
9V	0	2	2	2	1	3	0	2
14	0	4	0	6 *	1	3	3	6
18C	0	1	1	1	0	5	1	3
19F	8	3	3	7	3	4	6	9
23F	5	4	6	6	6	8	4	6
Other serotypes	**19 (46%)**	**17 (41%)**	**26 (59%)**	**29 (48%)**	**44 (73%)***	**30 (48%)**	**59 (73%)**	**48 (59%)**
6A **	4	8	5	3	10	10	10	7
19A **	0	1	1	2	0	2	2	3
23A **	1	1	3	0	1	2	2	1
23B **	1	0	2	1	3	0	4	4
3	1	0	1	1	1	2	3	4
10	1	0	1	1	1	4	2	3
11	3	1	3	4	10 *	1	10	6
15	3	2	3	4	9	4	5	3
16	0	1	0	1	2	2	6	3
21	3	0	0	0	1	0	2	2
Various	2	3	7	12	6	3	13	12
Non-Typeable	**4 (10%)**	**3 (8%)**	**3 (7%)**	**3 (5%)**	**1 (2%)**	**0 (0%)**	**2 (2%)**	**2 (3%)**

Note. In children positive for pneumococcal isolates (PI, n) the number of children with a conjugate vaccine serotype (CVT) or non-conjugate vaccine serotype (NCVT) are shown for pneumococcal vaccinees (PV) and control vaccinees (CV). Children are stratified according to age at the time of the first vaccination. Results are shown for time points 7 months after the first PCV7/control vaccinations and pooled after booster PPSV23/control vaccination. Data of results at 14, 20 and 26 months are pooled, including only the first pneumococcal isolate in a child analysis. Differences in carriage of the individual CVT and NCVT *S. pneumoniae* between both vaccine groups were analyzed using Chi-square tests or Fisher's exact tests when appropriate. * $P<0.05$ was considered significant. ** Cross-reactive serotypes with PCV7.

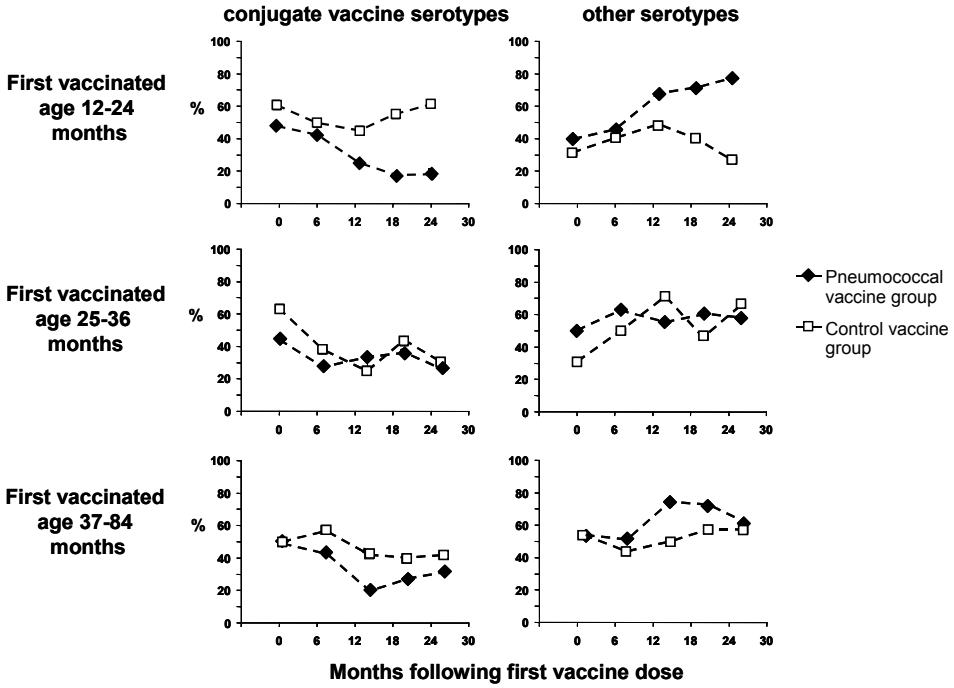


Figure 3. Carriage of CVT and NCVT *S. pneumoniae* in pneumococcal and control vaccinees.
Note. Relative contribution of CVT and NCVT *S. pneumoniae* to overall pneumococcal carriage according to age at first vaccination. Percentages of CVT and NCVT at study entry and at 7, 14, 20 and 26 months after the first vaccine dose are shown for pneumococcal vaccinees (solid squares) and control vaccinees (open squares).

increase of NCVT *S. pneumoniae* was significant for serotype 11 (*P*=0.007) only after PPSV23 in the youngest children (table 2). With respect to possible cross-protection between different serotypes of the same serogroup, no protective effect of pneumococcal vaccination for serotype 6A was observed. Serotypes 19A, 23A and 23B were only rarely cultured.

Vaccine immunogenicity. After complete vaccination, geometric mean IgG concentrations reached values far above 1.0 mg/L and were roughly similar in children first vaccinated at age 12-24 months and older children for all CVT serotypes with the exception of 6B, which remained below 1.0 mg/L in the youngest group (table 3).

Nasopharyngeal carriage of H. influenzae and M. catarrhalis. At study entry *H. influenzae* and *M. catarrhalis* were found in the nasopharynx of 58% and 50% of children, respectively. With respect to age and history of AOM, only carriage of *M. catarrhalis* was influenced by

age; 12-24 months 57% and 25-84 months 44% (*P*=0.02). Pneumococcal vaccinations did not affect carriage of *H. influenzae* or *M. catarrhalis* during follow-up as compared to control vaccinations (data not shown).

Table 3. Antipneumococcal antibodies

PCV7 serotypes	Pre vaccination		p-value	After last vaccination		p-value
	Age first vaccine dose			Age first vaccine dose		
	12-24 months	≥25 months		12-24 months	≥ 25 months	
4	0.05	0.04	0.02 *	5.85	3.33	0.02 *
6B	0.04	0.04	0.70	0.56	1.52	0.01 *
9V	0.18	0.20	0.61	28.40	24.94	0.64
14	1.03	2.01	0.09	78.06	75.89	0.86
18C	0.15	0.29	0.003 *	9.63	9.01	0.97
19F	0.22	0.36	0.04 *	12.34	13.05	0.98
23F	0.42	0.60	0.02 *	1.71	3.69	0.05

Note. Geometric mean concentrations (mg/L) of IgG antibodies against the 7 conjugate vaccine pneumococcal serotypes at baseline and one month after booster PPSV23 are shown separately for children first vaccinated at age 12-24 months (n=42) and age 25-84 months (n=51) Statistical differences between the youngest and older children were analyzed with Mann-Whitney U test. * We judged *P*<0.05 to be significant.

DISCUSSION

We describe for the first time the impact of 7-valent pneumococcal conjugate followed by 23-valent polysaccharide vaccination on NP carriage of *S. pneumoniae* in a group of toddlers and children aged 1-7 years with a history of recurrent AOM. Overall pneumococcal NP carriage rates in these children did not diminish after pneumococcal vaccinations during 2 years of follow-up. However, in particular in children first vaccinated at age 1-2 years who received PCV7 twice before PPSV23, a shift was induced from CVT to NCVT *S. pneumoniae*. In older children, who received PCV7 only once before PPSV23, the impact of pneumococcal vaccinations on pneumococcal carriage was far less pronounced. Since both age groups showed a similar contribution of CVT *S. pneumoniae* to NP carriage at start of the study, this does not explain the lower vaccine influence after 2 years of age. Also both age groups achieved roughly similar IgG serum levels against pneumococcal serotypes by pneumococcal vaccinations except for IgG titers against serotype 6B, which remained below

1 mg/L in the youngest group. We therefore assume that the greater shift from CVT to NCVT in children aged 1-2 years is primarily explained by the difference in vaccine schemes; the younger patients receiving two and the older patients one PCV7. This is underlined by the fact that vaccination influence was already nearly absent in pneumococcal vaccinees aged 25 to 36 months.

The T-cell dependent characteristics of a conjugate vaccine result in recruitment of new memory B-cells, which increase after each vaccination whereas a polysaccharide vaccine may only trigger pre-existing memory cells [20]. At the mucosal level, the extra B-cell recruitment may result in better opportunity to boost mucosal immunity by transient natural contacts with conjugate vaccine serotypes. Nurkka *et al*. previously described an increase in salivary IgG several months after a last vaccination with PCV7 in several children whereas only one child showed a late increase in salivary IgG after a booster polysaccharide vaccine [21]. Thus, although both PCV7 and PPSV23 booster vaccinations significantly increase serum quantitative IgG responses, the conjugate vaccine may be superior in inducing mucosal memory for the conjugate vaccine serotypes. Also other qualities like antibody affinity may be superior after extra conjugate vaccinations as compared to the polysaccharide booster, which may also lead to better mucosal immunity [22]. Pneumococcal polysaccharide vaccine is known to have little influence on NP pneumococcal colonization [23,24]. Our study shows it also fails to prevent the increase of nasopharyngeal carriage of NCVT pneumococci such as serotype 11, despite induction of good serum IgG levels [18] and apparently does not affect the NP flora of those serotypes included in the PPSV23 but not in PCV7. Hypothetically, a polysaccharide booster might even frustrate future antibody responses at the mucosal site on subsequent natural challenge with polysaccharides [25]. After the conjugate vaccinations, we saw an initial decrease of pneumococcal carriage just before booster vaccination with the 23-valent polysaccharide vaccine, particularly in children over 2 years of age. After the polysaccharide booster however, pneumococcal carriage rates subsequently increased again to levels similar to before vaccinations and identical to controls. We do not think however, that this failure to bring down pneumococcal carriage is due to the polysaccharide booster because after pneumococcal vaccination in healthy infants and toddlers overall pneumococcal carriage also was not affected [6-8].

In our risk group for AOM, baseline NP pneumococcal carriage rate was around 50%, which is in line with average carriage rates for similar age groups worldwide [1]. PCV7 serotypes comprised 53% of the pneumococcal strains isolated from the nasopharynx, which is similar to recent Finnish data on NP pneumococcal carriage in children [26]. Whether otitis-prone children do have higher pneumococcal carriage rates during health as compared to non otitis-prone children is still a matter of debate [27]. However, in contrast to a recent report in

the Netherlands [28], in our study older children aged 3-7 years did not show decreasing overall pneumococcal colonization rates even after 2 years of follow-up. It may be speculated that older children suffering from recurrent otitis still have high pneumococcal carriage rates because they are unable to eradicate pneumococci from their nasopharynx or prevent new acquisition due to minor immunodeficiency [18,29-33].

Despite low mean serum IgG concentrations for serotype 6B (< 1 mg/L), a significant decrease in carriage of serotype 6B by 50% was noted in the youngest age group. We found a lack of influence of pneumococcal vaccinations on carriage of the cross-reactive serotype 6A, most probably related to the low anti-6B antibody response. Väkeväinen *et al*. showed that on average, 2-6 times more anti-6B antibodies were needed for 50% opsonophagocytic killing of the type 6A than the type 6B strain [34]. A low IgG response against serotype 6B upon PCV7 as in both younger and older patients of our study group was also reported in other small groups of infection–prone patients [35,36]. A third study in otitis-prone children could not confirm this observation [37].

The observed nasopharyngeal replacement from CVT to NCVT pneumococci after pneumococcal vaccination in our study population seems to be due largely to replacement [Bogaert *et al.* submitted]. Apart from replacement by NVCT pneumococci, pneumococcal vaccinations did not influence carriage of other in potential AOM pathogens, like *H. influenzae* and *M. Catarrhalis*.

Clinically, we previously reported that no protective effect of combined pneumococcal conjugate and polysaccharide vaccination was found for the prevention of AOM after 1 year of age in children with a history of AOM [18]. Particularly in the youngest children this may be due to the replacement of CVT by NCVT *S. pneumoniae.* After two years of age, pneumococcal carriage was less influenced by vaccination and therefore may not have affected the incidence of AOM.

The results of our study show that in high risk groups not only vaccine immunogenicity studies should be performed but also clinical efficacy studies combined with the evaluation of the bacterial changes in nasopharyngeal colonization to provide insight in the impact of vaccinations. Despite adequate quantitative serum IgG levels, at least two doses of conjugate vaccinations seem to be mandatory for reduction of nasopharyngeal carriage of CVT serotypes. To obtain higher serum IgG levels for serotype 6B, a third or perhaps fourth conjugate vaccination may be required at all ages, which may only than result in reduction of carriage of serotype 6A. Booster vaccination with a polysaccharide vaccine does not seem to enhance mucosal immunity. The results of this study should be kept in mind when recommending pneumococcal vaccinations for mucosal disease like AOM but also for lower respiratory tract infections like pneumonia in children after 1 year of age, particularly for risk groups.

ACKNOWLEDGEMENTS

Pneumococcal vaccines were kindly provided by Wyeth Lederle Vaccines and Pediatrics, Rochester, N.Y. Hepatitis vaccines were kindly provided by GlaxoSmithkline BV, Rixensart, Belgium. K. Elzenaar and Dr. W.J.B. Wannet from the Laboratory for Infectious Diseases, Diagnostics and Screening, National Institute of Public Health and the Environment, The Netherlands are acknowledged for serotyping of pneumococci.

Other members of the Dutch Otitis Media Study group include: *study nurses* – I. Weers, A. Haan; *Pediatric residents* – C. Brouwer, A. Janse, and Y. van de Berg; *Bacteriology* – M. Sluijter, M. Sonke; *Data management* – B. Slotboom.

Grant support: the Netherlands Organization for Health, Research and Development (project 28-2848-0) and the Health Company Zilveren Kruis-Achmea.

CONFLICT OF INTEREST

None of the authors have a financial and personal relationship with other people or organisation that could have inappropriately influenced this work.

REFERENCES

1. Ghaffar F, Friedland IR, McCracken GH. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. Pediatr Infect Dis J 1999; 18:638-46.
2. Bogaert D, Engelen MN, Timmer-Reker AJM, et al. Pneumococcal carriage in children in the Netherlands: a molecular epidemiological study. J Clin Microb 2001; 39:3316-20.
3. O'Brien KL, Nohynek H. Report from a WHO Working Group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*. Pediatr Infect Dis J 2003; 22:133-40.
4. Rubin LG. Pneumococcal vaccine. Pediatr Clin North Am 2000; 47:269-85.
5. Obaro SK, Adegbola RA, Banya WAS, Greenwood BM. Carriage of pneumococci after pneumococcal vaccination. Lancet 1996; 348:271-2.
6. Mbelle N, Huebner RE, Wasas AD, Kimura A, Chang I, Klugman KP. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. J Infect Dis 1999; 180:1171-6.
7. Dagan R, Muallem M, Melamed R, Leroy O, Yagupsky P. Reduction of pneumococcal nasopharyngaeal carriage in early infancy after immunization with tetravalent pneumococcal vaccines conjugated to either tetanus toxoid or diphteria toxoid. Pediatr Infect Dis J 1997; 16:1660-4.
8. Dagan R, Givon-Lavi N, Janco J, et al. Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J Infect Dis 2002; 927-36.
9. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Pediatr Infect Dis J 2000; 19:187-95.
10. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344:403-9.
11. Dagan R, Sikuler-Cohen M, Zamir O, Janco J, Givon-Lavi N, Fraser D. Effect of a conjugate pneumococcal vaccine on the occurrence of respiratory infections and antibiotic use in day-care attendees. Pediatr Infect Dis J 2001; 20:951-8.
12. Black SB, Shinefield HR, Ling S, et al. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumoniae. Pediatr Infect Dis J 2002; 21:810-15
13. Lipsitch M, Dykes JK, Johnson SE, et al. Competition among Streptococcus pneumoniae for intranasal colonization in a mouse model. Vaccine 2000; 18:2895-901.
14. Spratt BG, Greenwood BM. Prevention of pneumococcal disease by vaccination: does serotype replacement matter? Lancet 2000; 356:1210-1.
15. Whitney CG, Farley MM, Hadler J, et al. Decline in invasive disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med 2003; 348 :1737-46.
16. Kilpi T, Ahman H, Jokinen J, et al. Protective efficacy of a second pneumococcal conjugate vaccine against

pneumococcal acute otitis media in infants and children. A randomized, controlled trial of a7-valent pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine in 1666 children. Clin Infect Dis 2003; 37:1155-64.
17. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal Relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone specifik differences in invasive disease potential. J Infect Dis 2003; 187:1424-32.
18. Veenhoven R, Bogaert D, Uiterwaal C, et al. Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. Lancet 2003; 361:2189-95.
19. Musher DM, Luchi MJ, Watson DA, Hamilton R, Baughn RE. Pneumococcal polysaccharide vaccine in young adults and older bronchitics: determination of IgG responses by ELISA and the effect of adsorption of serum with non-type-specific cell wall polysaccharide. J Infect Dis 1990; 161: 728-35.
20. Antilla M, Eskola J, Åhman H, Käyhty H. Avidity of IgG for *Streptococcus pneumoniae* type 6B and 23F polysaccharides in infants primed with pneumococcal conjugates and boosted with polysaccharide or conjugate vaccines. J Infect Dis 1998; 177:1614-21.
21. Nurkka A, Åhman H, Korkeila M, Jäntti V, Käyhty H, Eskola J. Serum and salivary anti-capsular antibodies in infants and children immunized with the heptavalent pneumococcal conjugate vaccine. Pediatr Infect Dis J 2001; 20:25-33.
22. Wuorimaa T, Dagan R, Vakevainen M, et al. Avidity and subclasses of IgG after immunization of infants with an 11-valent pneumococcal conjugate vaccine with or without aluminum adjuvant. J Infect Dis 2001; 184:1211-5.
23. Rosen C, Christensen P, Hovelius B, Prellner K. A longitudinal study of the nasopharyngeal carriage of pneumococci as related to pneumococcal vaccination in children attending day-care centers. Acta Otolaryngol 1984; 98:524-532.
24. Douglas RM, Hansman D, Miles HB, Paton JC. Pneumococcal carriage and type-specific antibody. Am J Dis Child 1986; 140:1183-5.
25. MacLennan J, Obaro S, Deeks J, et al. Immunologic memory 5 years after meningococcal A/C conjugate vaccination in infancy. J Infect Dis 2001; 183:97-104.
26. Syrjänen RK, Kilpi TM, Kaijalainen TH, Herva EE, Takala AK. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish Children younger than 2 years old. J Infect Dis 2001; 184:451-9.
27. Faden H, Brodsky L, Waz MJ, Stanievich J, Bernstein JM, Ogra PL. Nasopharyngeal flora in the first three years of life in normal and otitis-prone children. Ann Otol Laryngol 1991; 100:612-5.
28. Bogaert D, Koppen S, Boelens H, et al. Epidemiology and risk factor analysis of nasopharyngeal carriage of bacterial pathogens in healthy Dutch children. In: Program and abstracts of the 21st Annual Meeting of the European Society for Paediatric Infectious Diseases, Giardini Naxos, Sicily, Italy, April 9-11, 2003:51. Abstract.
29. Sanders EAM, Rijkers GT, Tenbergen-Meekes AM, Voorhorst Ogink MM, Zegers BJ. Immunoglobulin isotype specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent respiratory tract infections. Pediatric Research 1995; 37:812-9.
30. Herrod HG, Gross S, Insel R. Selective antibody deficiency to Haemophilus influenzae type B capsular polysaccharide vaccination in children with recurrent respiratory tract infection. J Clin Immunol 1989; 9 :429-34.
31. Sanders EA, Van de Winkel JG, Rijkers GT, et al. Fc gamma receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. J Infect Dis 1994; 170:854-61.
32. Koch A, Melbye M, Sorensen, et al. Acute respiratory tract infections and mannose-binding lectin insufficiency. JAMA 2001; 285:1348-55.
33. Immunoglobulins in otitis-prone children. Veenhoven R, Rijkers G, Schilder A, et al. Pediatr Res 2004; 55:159-62
34. Vakevainen M, Eklund C, Eskola J, Kayhty. Cross-reactivity of antibodies to type 6B and 6A polysaccharides of Streptococcus pneumoniae, evoked by pneumococcal conjugate vaccines, in infants. J Infect Dis 2001; 184:789-93.
35. Zielen S, Bühring I, Strnad N, Reichenbach J, Hofmann D. Immunogenicity and tolerance of a 7-valent pneumococcal conjugate vaccine in nonresponders to the 23-valent pneumococcal vaccine. Infection and Immunity 2000; 68:1435-40.
36. Sorensen RU, Leiva LE, Giangrosso PA, et al. Response to a heptavalent conjugate *Streptococcus pneumoniae* vaccine in children with recurrent infections who are unresponsive to the polysaccharide vaccine. Pediatr Infect Dis J 1988; 17:685-91.
37. Barnett ED, Pelton SI, Cabral HJ, et al. Immune response to pneumococcal conjugate and polysaccharide vaccines in otitis-prone and otitis-free children. Clin Infect Dis 1999; 29:191-2.

Chapter 7.3

Molecular epidemiology of pneumococcal colonization in response to pneumococcal conjugate vaccination in children with recurrent acute otitis media

D. Bogaert, R.H. Veenhoven, M. Sluijter, W.J.W. Wannet, G. T. Rijkers, T.J. Mitchell, S.C. Clarke, W.H.F. Goessens, A.G. Schilder, E.A.M. Sanders, R. de Groot and P.W.M. Hermans

J Clin Microbiol; accepted for publication

ABSTRACT

Introduction. A randomized double-blind trial with a 7-valent pneumococcal conjugate vaccine was conducted in The Netherlands among 383 children, aged 1-7 years, with a history of recurrent acute otitis media. No effect of vaccination was found on the pneumococcal colonization rate. However, a shift in serotype distribution was clearly observed (Veenhoven, 2003. Lancet: 361:2189-95).

Methods. We investigated the molecular epidemiology of 921 pneumococcal isolates retrieved from both the pneumococcal vaccine (PV) and control vaccine (CV) group during the vaccination study.

Results. Within individuals a high turnover rate of pneumococcal genotypes was observed, which was unaffected by vaccination. Comparison of the genetic structure before and after completion of the vaccination scheme revealed, despite a shift in serotypes, clustering between 70% of the pneumococcal populations. The remaining isolates (30%) were equally observed in the PV and the CV group. In addition, the degree of genetic clustering was unaffected by vaccination. However, within the population genetic structure, non-vaccine serotype clusters with the serotypes 11, 15 and 23B became predominant over vaccine-type clusters after vaccination. Finally, overall pneumococcal resistance was low (14%), and, albeit not significant, a reduction in pneumococcal resistance as a result of pneumococcal vaccination was observed.

Conclusion. Molecular surveillance of colonization in Dutch children shows no effect of pneumococcal conjugate vaccination on the degree of genetic clustering, and the genetic structure of the pneumococcal population. However, within the genetic pneumococcal population structure, a clear shift was observed towards non-vaccine serotype clusters.

INTRODUCTION

Streptococcus pneumoniae is worldwide one of the major bacterial causes of invasive disease and respiratory tract infections in children. Risk groups for pneumococcal infections are young children, elderly and immune-deficient patients. Despite adequate antibiotic treatment, morbidity and mortality due to pneumococcal disease remains high (6). Moreover, the increasing (multi) drug resistance among pneumococcal isolates hampers adequate treatment (1, 11, 21, 30). New pneumococcal conjugate vaccines, have shown to be effective against invasive diseases in young children (4). Furthermore, a protective effect against respiratory tract infections such as (recurrent) otitis media has been observed (4, 15). Thus far, the 7-valent pneumococcal conjugate vaccine Prevnar (Wyeth, USA) has been approved by the Food and Drug Administration (USA), and the Committee on Proprietary Medicinal Products (Europe), and is recommended by the Advisory Committee on Immunization Practices (USA) for the prevention of invasive diseases in children under 2 years of age. Recommendations are also made for older children at increased risk for invasive disease, like those with HIV and asplenia, and those with increased risk for pneumococcal mucosal disease, such as children with recurrent acute otitis media (2). We recently studied the effect of a 7-valent pneumococcal conjugate vaccine followed by a 23-valent polysaccharide vaccine in children aged 1-7 years with a history of recurrent acute otitis media (32). Clinically, no protective effect of the pneumococcal vaccines on recurrence of acute otitis media was found. At the nasopharyngeal level, however, a significant reduction of colonization with vaccine-type pneumococci was found after vaccination, whereas a simultaneous increase in colonization with non-vaccine serotypes was observed (32).

In this study, we investigated the molecular epidemiological dynamics and resistance profiles of the pneumococcal isolates collected from both children in the pneumococcal vaccine (PV) and the hepatitis control vaccine group (CV) in order to obtain insight in the effect of conjugate vaccination on the genetic pneumococcal population structure.

MATERIAL AND METHODS

Bacterial isolates. In total, 383 children, aged 1-7 years, suffering from recurrent acute otitis media, were enrolled in this double-blind randomized vaccination trial in the period April 1998 to December 2001 (32). Hundred-ninety children received once a 7-valent pneumococcal conjugate vaccine when 24 months of age and older (Prevenar®, Wyeth Lederle), or twice in children 12-24 months of age, followed by a 23-valent pneumococcal polysaccharide vaccine after 6 months for all children (Pneumune®, Wyeth Lederle). The 193 control children received, depending on the age, three times Hepatitis B (Engerix-B®,

Smithkline Beecham) or twice Hepatitis A vaccine (Havrix junior®, Smithkline Beecham). Nasopharyngeal cultures were performed at study entry, just before booster vaccination at 7 months and at 14, 20 and 26 months. An additional nasopharyngeal sample was obtained at the first acute otitis media event after full vaccination. Pneumococcal carriage was observed in around 50% of all children. This carriage rate was maintained in both study groups in the 26 months follow-up period. Thus, no influence on overall colonization was observed during the study. Instead a decline in vaccine serotype carriage was observed in the PV group whereas the non-vaccine serotype carriage increased (32). Nine hundred twenty-one isolates (95%) from 353 out of 383 patients participating in this study were available for further analysis by genotyping and susceptibility testing.

Bacteriological procedures. Isolation and identification of the *S. pneumoniae* isolates were performed by standard methods as described previously (32). Susceptibility testing was performed by the agar-dilution method (25). Resistance was defined by the breakpoint concentrations for the respective antibiotics as defined by the NCCLS (26). Multidrug resistance was defined as resistance to ≥3 classes of antimicrobial agents.

Serotyping. Pneumococci were serotyped by the capsular swelling method (Quellung reaction) observed microscopically using commercially available antisera (Statens Serum institute, Copenhagen, Denmark).

Restriction fragment end labeling (RFEL) typing. Pneumococcal strain typing by RFEL was done as described by van Steenbergen et al. (31) and adapted by Hermans et al. (19). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72^{o}C with [α-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 *M* urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, NY), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, UK).

Computer-assisted analysis of DNA band patterns. RFEL autoradiographs were converted to images (Image Master DTS; Pharmacia Biotech, Uppsala Sweden) and analyzed by computer (Windows version Gelcompar software version 4; Applied Math. Kortrijk, Belgium). DNA fragments were analyzed as described previously (27). For evaluation of the genetic relatedness of the isolates we used the following definitions: (1) isolates of a particular RFEL type are 100% identical by RFEL analysis; (2) an RFEL cluster represents a group of RFEL types that differ in only one band (approximately >95% genetic relatedness).

Multi locus sequence type (MLST) analysis. The genotypes of 38 isolates representing different serotypes were investigated by MLST analysis. Within the 23 largest clusters

representing 29 RFEL genotypes, the most prevalent serotypes were analyzed. For this purpose, a fully automated method for MLST was used as described previously (20). The MLST types were compared with the global PMEN database (http://www.pneumo.com/physician/pmen/pmen_history.asp).

Data-analysis. P-values for differences were calculated with the Chi-square test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, USA).

RESULTS

Nine hundred twenty-one pneumococcal isolates from nasopharyngeal samples of 351 children participating in the study were available for molecular analysis by RFEL. In total, 450 of these 921 pneumococcal isolates were isolated from children in the pneumococcal vaccine group, whereas 471 isolates were isolated in the control group. Of the 921 isolates, 180 isolates were isolated at the start of the study (T=0 months; day of (first) conjugate vaccination), 176 isolates were retrieved at 7 months (day of booster vaccination) and 186, 163 and 119 isolates were isolated after completion of the vaccination scheme at 14, 20 and 26 months of study duration, respectively (Table 1). The remaining 97 isolates were retrieved at the first AOM events after full vaccination (>7 months). The serotype distribution of all pneumococcal isolates collected during this study was discussed previously (32). In summary, the contribution of vaccine serotype pneumococci to colonization gradually declined from 46% at study entry to 26% at the end of the study compared to the control group in which the contribution of the conjugate-vaccine serotypes remained approximately 50%, whereas the total pneumococcal carriage rate remained unaffected.

Table 1. Pneumococcal isolates with regard to study group and study phase.

	Total	Vaccine group	% vaccine-type strains	Control group	% vaccine-type strains
All phases	921	451		470	
0 months	180	96	46%	84	58%
7 months	176	81	41%	95	46%
14 months	186	84	26%	102	40%
20 months	163	81	25%	82	49%
26 months	119	64	26%	55	54%
First AOM	97	45	24%	52	44%

All 921 isolates were characterized by RFEL. We identified 275 different genotypes representing one to 49 isolates per genotype with an average of 3.35 isolates per genotype. Analysis of the per-patient follow-up revealed a high turnover rate of pneumococcal

Table 2. Pneumococcal colonization dynamics of the 351 children according to study and age group. Persistent carriage is shown in grey. Vaccine type pneumococci are depicted with the number code “0”. Non-vaccine type pneumococci are depicted with the number code “1”.

		Months								Months								Months								Months							
Vaccine group		0	7	14	20	26	1st AOM			Vaccine group		0	7	14	20	26	1st AOM			Control group		0	7	14	20	26	1st AOM			Control group		0	7	14	20	26	1st AOM	
12-24 months	1	1	1	1		0				2-7 years	75	0		1	0	0	1			12-24 months	1	0	0	0	0					2-7 years	77	0			1	0	1		
	2	1		1	1	1	1				76	1	1								2	0	0		0	1					78			1					
	3				1	1					77	0									3	0									79		0						
	4			1	1						78	0		1							4			1	1		1				80		1						
	5	1	1		1	1					79			1							5		0	0	0	1					81				1				
	6		1	1		1					80	0	0	0	0	1					6		0			0					82	1	0	0					
	7	1	0								81	1	0		1						7	0			1	0					83	1		0					
	8	0	0	0	0	1					82			1		0	1				8	0	1		1	0					84	0	0		0	1	1		
	9	0		1							83	1	0								9			1	0						85		0		0	1			
	10					1					84		1	1	1						10			1	0						86		1				0		
	11	0	1		0						85				1	0					11		1		1						87		1	0	1		1		
	12		0		0						86	1		1	1		1				12	1	0		0		0				88			0		1			
	13		1	0		1					87			1		0	1				13		1	1	0	0					89	1	0	0					
	14		1			1	0				88		1	1	1	1	1				14					0					90		1		0	1			
	15	1		1		0					89			1	1		1				15		0		0						91	1		1	1				
	16	1									90						1				16	0		1	0	1	1				92	1	0						
	17	0	0	1							91	1					1				17	0		1		0					93			1	1	0			
	18	0									92	0									18		1	1		0					94					1			
	19	1	0	1	1						93	0	1		1	1					19	1	1	1	1	0					95	1		1	0	1			
	20			0	1						94				1						20			1		0					96	0	1		0		1		
	21	0			1						95	1		1		1					21	0		1		1					97	0	1		1				
	22	0	0		1		0				96				1						22			0							98	1	1	1					
	23	0			1		1				97				1	1					23			0		0					99	0	1	1	1				
	24	0	0								98				0						24	1		0	1						100				1	0			
	25	1	1	0							99	1	1	1			1				25			1							101				0				
	26	0		1							100	0		1	1	0					26	1	1								102		0	0	0		0		
	27		1	1							101	0	0	0							27				1	0					103		1						
	28	1		1	1		1				102	1	0		1	1					28	1	1	1	0	1	1				104			1	0	1	0		
	29		1	0	1	1					103	1	0								29		1	0		1	0/1				105			1					
	30		0			0	1				104	0									30			0	1		1				106	1	0	1					
	31					0	1				105	0	0				0				31		1	0		0					107			0			1/0		
	32				1						106		1	1	1						32		1	0	1	0	1				108	1	1	0/1	0/0				
	33		1	1	1	1					107	1	0								33					0					109			1	1				
	34	0	0	1	0	1					108	0		0							34			1			1				110	0	0						
	35	0			1						109	1				1					35			0							111	0							
	36	0	0		0						110	1			1	1					36		0			0					112	1							
	37	0	1	1	1		1				111			0			1				37	0	1		0						113		0						
	38	0	0	1	1	1	1				112			1		1					38	0	0	1	0	0	1				114			0	1				
	39	1				1	1				113	0	1	1		1	0				39			1							115		0						
	40		1								114					1					40		0		0		0/1				116	0	0	0	0				
	41	1			1	1					115		1		0						41	0	1	0	1	0					117		1	0	1				
	42			1							116	1		1	1						42		1	0	0		1				118				1	0			
	43	1									117		1	0	0	0					43	1	1		0	1					119	0	1	1		0			
	44			0							118	1	1	1	1						44				0	0	0				120		1						
	45		0	0	1	1					119	1			1	1					45				0	1	0				121						0		
	46	1	1				1				120		1	1	0						46	0	0	1	0	0	0				122	1		1					
	47					1					121		1		1	1					47		0	0	0	0	0				123		0		0				

48		1			1	
49				1	1	
50			1		1	
51	1	0	1	0		
52	0	0				
53	1		0	1	0	
54						0
55		1	1	1	1	
56		0		1		
57	0		1			
58		1		1	1	
59						0
60	0	1		1		
61	1		0			
62		1	1			
63		1	1			
64	0					
65				1		
66	1	1				1
67	0	1	1			
68		0				0
69			0			
70			1			
71	0					1
72	0	1	1			1
73	1	0				
74	1	0				

122	0				0	
123		1	1	1	1	1
124	1	0	1	1	1	
125					0	1
126	0	0	0			1
127				1	1	
128	0		1	1	1	1
129		1	1			
130	0		1	0	1	1
131	1					
132	1		1	0	0	
133		1	1			
134	1	1	1	0	1	
135	0			1		
136	1			1	1	
137	1	1		0	0	
138	0			1		
139	0				1	
140	1		1	1		
141		1		1	0	
142		1	1	1	1	1
143	1	1		1		0
144	1		0			
145		1	1		1	
146			1		1	
147	0				1	1
148	0					0
149		0		1		
150		1		1	1	
151						1
152			0	1		
153			0	1		
154			1	0		
155		1				
156				1		0
157	1		1			
158				1		
159	1		1		1	
160	1	0	0	1		
161	1	1		1		
162	0	0	1	0		1
163	1			0		0
164	0	1				
165			0			
166	1	0	1			
167	0		0			
168		1				1
169		1	0			
170	1					
171		1	0			

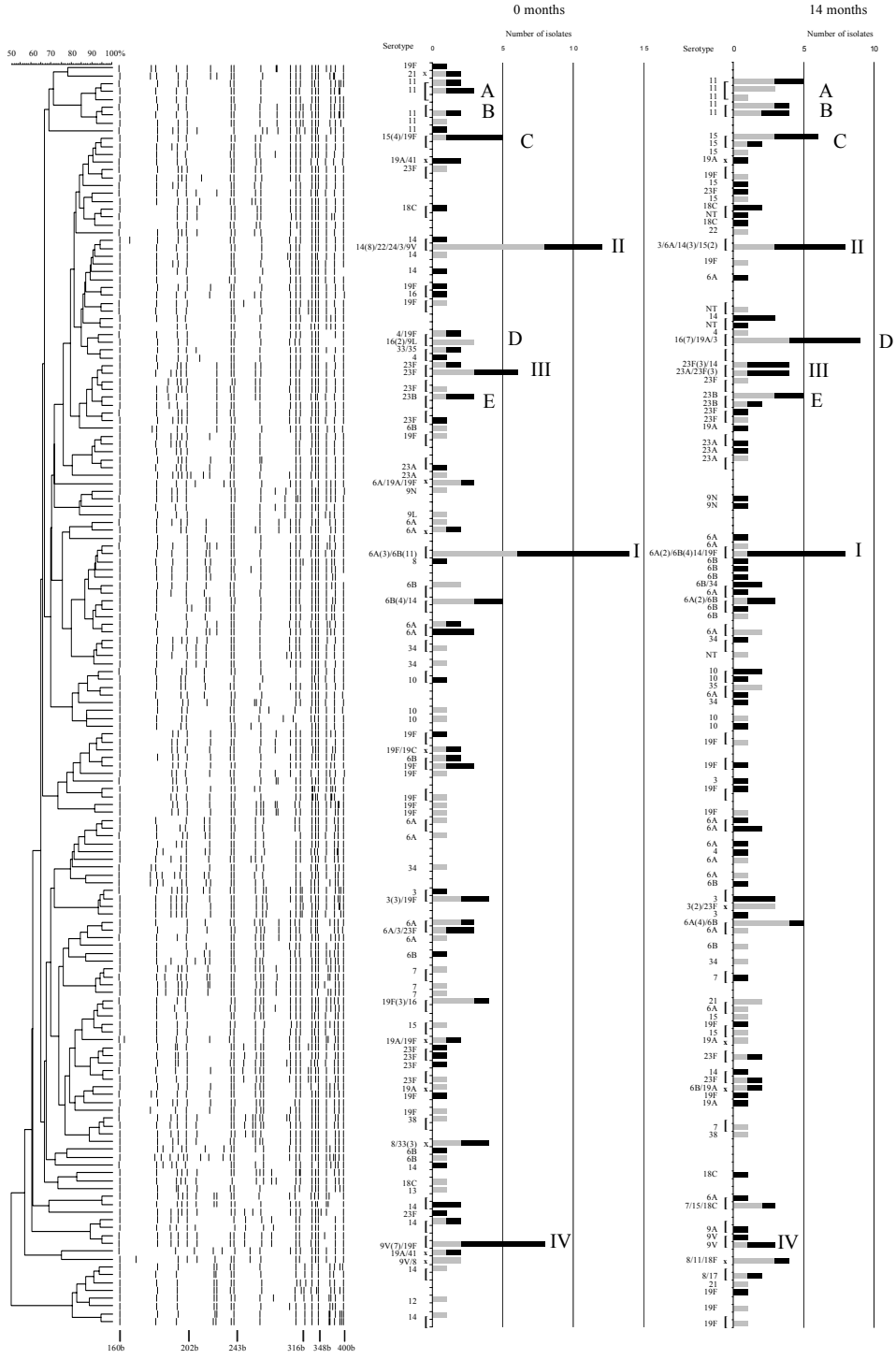
48	1	1	0		0	
49			1			
50	0			0		
51	0					1
52			1			
53			0			
54	0	1		0		
55			1	0		0/1
56	0	0	0	0		0
57	0					
58	0					
59			1			
60	0		1			
61	0	0		1		
62			0			
63	1		0	0		0
64	1					
65	0			1		
66		1	0			1
67		0				
68	0		1			
69	1					1
70	1	1	1			
71	0	0				0
72	1	1				
73	0	0	0			0
74	1					
75		1	0			
76	1	0				

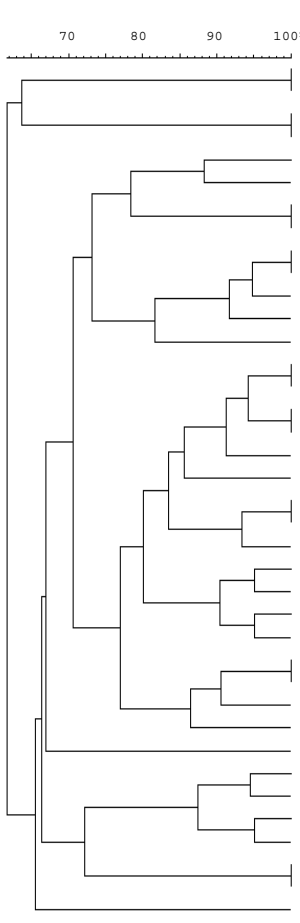
124	0	0	1	1		1
125	0	1		0	1	
126		0	1	0		
127	1		0			
128	1	0	0	0		
129			1	1	1	
130			1	1		
131	0			1		1
132		1	1	1		1
133		0	1	1	1	
134	0	0	0			
135		0	0	0		
136	0		1			
137	1	0		0		
138				0		
139		1		1	1	1
140	0	1	1	1	1	0
141			0	1	1	
142	1	1			0	0
143		0				
144	1	1		1		1
145	0					
146	0	0		1	1	
147		1	1			
148			1		0	1
149			0			
150	1		1		1	
151					0	
152			0			
153	0		1		0	
154	1				1	
155		0			1	
156			1	1		
157			1			
158	0	1	1			40
159	0	1	0			1
160	0					
161			1	0		
162		1	1			0
163		0				
164		0	1			
165	0	1	1			
166	0	0	0	1		
167	1		1	1		
168		0				1
169		1				1
170			1			
171		1	1	1		
172			1			
173	0	0	1			
174	1					0
175		1				
176		1				0
177	0					
178	1	1	1			

genotypes; only in 54 out of the 351 children persistent carriage was found for at most 3 consecutive samples (recurrence twice after a 6-7 months interval) (Table 2). No statistical difference in the rate of persistent carriage was found between the pneumococcal vaccine group and the control group children (15% versus 16%; p = 0.67). In both groups, the majority of the persistent isolates (60%) were non-vaccine serotypes. On three occasions, persistence of a specific genotype was accompanied by a switch in serotype. In one case, colonization with one particular genotype which is closely related to MLST type displaying serotype 15 was 6 months later followed by colonization with a second genotype of serotype 19A. Another 6 months later the initial genotype with a capsular switch to serotype 19A was identified. In the second case a switch was observed from serotype 14 to serotype 8 within the same genotype after a 6 months interval. The third case represented a capsular switch from serotype 6A to serotype 19F observed after a 6 months interval.

We analyzed the genetic relatedness of the pneumococcal isolates retrieved at start of the study and 14 months after the initial vaccination from both study groups. The 180 pneumococci isolated at start of the study displayed 93 genotypes, representing 52 unique genotypes and 30 clusters (128 isolates) with an average cluster size of 4.3. The 186 isolates isolated 14 months after start of the study displayed 105 genotypes, representing 54 unique genotypes and 29 clusters (132 isolates) with an average cluster size of 4.6. Close homology (≥ 95% genetic relatedness) was found between 70% of the isolates at both time points (T=0 and T=14 months). The remaining genotypes equally represented strains from either PV or CV children (49% and 51%, respectively). The 4 most predominant clusters at start of the study were cluster I (7.8% of all isolates; serotypes 6A and 6B), cluster II (7.3%; serotype 14), cluster III (4.5%; serotype 23F) and cluster IV (4.5%; serotype 9V). These clusters were still predominant 14 months after vaccination, though slightly reduced in size (4.8%, 4.3%, 4.3% and 2.2%, respectively) and mainly observed in control group children (Figure 1). In addition, five minor clusters observed in the initial phase of the study, cluster A (2.8% of all isolates; serotype 11), cluster B (1.1%; serotype 11), cluster C (2.8%; serotype 15), cluster D (2.8%; serotype 16) and cluster E (1.7%; serotype 23B), became predominant clusters 14 months after vaccination with a prevalence of 4.8%, 4.3%, 4.3%, 5.4% and 3.8%, respectively. The first 2 clusters, which resembled 85% homology, were predominantly present in PV children (89% and 63%, respectively) (Figure 1).

Figure 1 (right). Population genetic structure of the 180 and 186 pneumococcal isolates retrieved before vaccination (T=0 months) and after pneumococcal conjugate vaccination (T=14 months), respectively. Genetic relatedness is depicted in percentages. The serotypes and number of isolates per genotype are shown in bars for the two periods separately. The contribution of vaccinated children and control children is shown in grey and black, respectively. The numbers I-IV represent predominant clusters at the initial phase of the study. The letters A-E represent emerging clusters after conjugate vaccination. Clusters consisting of two or more genotypes are shown in brackets. Clusters of one genotype are depicted with x.


0 months
14 months
Number of isolates
Serotype
50 60 70 80 90 100%
160b 202b 243b 316b 348b 400b
A
B
C
D
E
I
II
III
IV



RFEL	sero type	MLST	*aroE*	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
241	9V	941	8	10	15	27	2	28	71
241	NT	941	8	10	15	27	2	28	71
23	15	162	7	11	10	1	6	8	14
23	9V	162	7	11	10	1	6	8	14
599	10	097	5	7	4	2	10	1	27
535	35	446	5	7	4	19	10	40	27
12	23F	1040	1	8	9	9	6	26	6
12	23A	NEW	1	8	9	9	6	28	6
50	19F	176	7	13	8	6	10	6	14
50	3	176	7	13	8	6	10	6	14
430	14	138	7	5	8	5	10	6	14
467	6A	207	10	8	30	5	6	1	9
457	19F	NEW	7	14	4	12	1	14	14
304	15	1200	8	13	14	4	17	4	5
304	15	199	8	13	14	4	17	4	14
28	15	199	8	13	14	4	17	4	14
28	14	124	7	5	1	8	14	11	14
443	19A	416	1	13	14	4	17	51	14
101	18C	113	7	2	1	1	10	1	21
30	16	414	1	5	27	5	1	28	1
30	6A	030	1	5	27	20	1	1	1
27	4	205	10	5	4	5	13	10	18
438	23F	176	7	13	8	6	10	6	14
330	23B	439	1	8	9	2	6	4	6
329	23A	036	1	8	4	1	1	4	6
326	23F	036	1	8	4	1	1	4	6
347	19A	440	7	5	1	1	13	31	14
347	23F	440	7	5	1	1	13	31	14
56	19F	309	8	10	2	5	9	48	6
119	33	100	5	12	29	12	9	39	18
426	NT	NEW	2	5	2	NEW	2	19	14
422	11	062	2	5	29	12	16	3	14
442	11	062	2	5	29	12	16	3	14
581	6B	176	7	13	8	6	10	6	14
490	11	NEW	2	5	29	12	9	NEW	14
420	19F	395	1	5	7	12	17	1	35
420	6A	395	1	5	7	12	17	1	14
167	3	180	7	15	2	10	6	1	22

Figure 2. Genetic relatedness of the 38 MLST sequence types observed within the 23 largest RFEL clusters.

We investigated 38 isolates representing different serotypes by MLST analysis. Within the 23 largest clusters representing 29 RFEL genotypes, the most prevalent serotypes were analyzed. (Figure 2). We found no PMEN clones in our collection of pneumococci. In addition, we observed 4 new MLST genotypes. In general, the observed homology by RFEL genotyping was confirmed by MLST, except for RFEL genotype 028 for which RFEL genotyping showed to be less discriminatory than MLST analysis (Figure 2).

We determined the MICs for penicillin, cotrimoxazole, tetracycline, erythromycin, rifampicin, vancomycin and ciprofloxacin of 919 isolates. Resistance to at least one antibiotic was found in 128 pneumococcal isolates (14%). High level resistance to penicillin, cotrimoxazole,

tetracycline, erythromycin and ciprofloxacin was found in 2 (0.2%), 37 (4.0%), 28 (3.0%), 29 (3.2%), and 2 (0.2%) isolates, respectively. Furthermore, intermediate resistance to penicillin and cotrimoxazole was found in 8 (0.9%) and 61 (6.6%) isolates, respectively. Intermediate resistance to tetracycline, erythromycin and cefotaxime was seen only occasionally (Table 3).

Table 3. Antibiotic resistance rates for the *S. pneumoniae* collection

	Nr. of resistant strains	Percentage of total	Nr. of intermediate resistant strains	Percentage of total
Penicilline	2	0.2%	8	0.9%
Cotrimoxazole	37	4.0%	61	6.6%
Tetracycline	28	3.0%	5	0.5%
Erythromycin	29	3.2%	1	0.1%
Ciprofloxacin	2	0.2%	0	
Cefotaxim	0		2	0.2%

Table 4. *S. pneumoniae* antibiotic resistance profiles, profile rates, number of genotypes and their serotype distribution.

Resistance Profile*	Nr. of strains	(%)	Nr. of genotypes	Serotypes
Co(I)	53	(5.7)	29	3/ 7/ 8/ 10/ 11/ 12/ 14/ 18C/ 18F/ 6A/ 6B/ 9A/ 9V/ 19A/ 19F/ 23F/ 31/ 33D /NT
Co	25	(2.7)	16	6A/ 6B/ 9V/ 18C/ 21/ 23F/ NT/ 34
T	7	(0.8)	6	3/ 19C/ 19F
T(I)	4	(0.4)	4	23F/18C/ 11/ 38
T(I)E	1	(0.1)	1	33
Tci	1	(0.1)	1	19F
TE	7	(0.8)	6	9N/ 19F
E	8	(0.9)	6	11/ 14/ 15/ 33
E(I)	1	(0.1)	1	23B
Co(I)E	2	(0.2)	2	6A/ 8
Co(I)T	1	(0.1)	1	6B
Co(I)TE	1	(0.1)	1	6B
CoT	2	(0.2)	2	23A/ 19F
CoTE	5	(0.5)	4	6A/6B
P(I)	1	(0.1)	1	11
P(I)CoCf(I)	1	(0.1)	1	14
P(I)Co(I)ECf(I)	1	(0.1)	1	6B
P(I)CoTE	3	(0.3)	3	19F/ 6B/ 14
PCo(I)	1	(0.1)	1	15
P(I)Co(I)	2	(0.2)	2	14/ 23F
PcoTE	1	(0.1)	1	6B
PE	1	(0.1)	1	6A
Total	128	(13.8)		

*Co: cotrimoxazole; P: penicilline; T: tetracyclin; E: erythromycin; Cf: cefotaxim; Ci: ciprofloxacin.
(I): intermediate resistance

No cases of resistance to rifampicin and vancomycin were identified. In Table 4, the resistance profiles and their rates, serotypes and number of genotypes are depicted. In total, 21 different resistance profiles were observed. We observed (intermediate) resistance to a single drug in 99 isolates (10.7% of all isolates), dual resistance in 17 isolates (1.8%) and multidrug resistance (resistance to 3 or more antibiotics) in 12 isolates (1.3%).
To evaluate the effect of vaccination on pneumococcal resistance, we compared resistance rates before full vaccination (samples at study entry plus at 7 months study duration) and after full vaccination (samples at 14, 20 and 26 months). In the PV children resistance declined from 17.5% before full vaccination to 11.8% after full vaccination, whereas in the CV children resistance was stable (14.5% and 14.3% before and after full vaccination, respectively). This difference did not reach statistical significance. We also evaluated the serotype distribution among the resistant isolates. 57% of all resistant isolates displayed vaccine serotypes. In addition, 10 out of the 12 multidrug-resistant isolates (83%) were vaccine serotype isolates, whereas the remaining two isolates displayed the cross-reactive serotype 6A.

DISCUSSION

Between 1998 and 2002 a large randomized, double blind vaccination trial with a 7-valent pneumococcal conjugate vaccine followed by a 23-valent polysaccharide vaccine was performed among 383 children, aged 1-7 years, with a history of recurrent acute otitis media. Surprisingly, no beneficial effect was observed on the frequency of acute otitis media after pneumococcal vaccination nor on the overall colonization rate of *S. pneumoniae*. However, a shift of vaccine type pneumococci to non-vaccine type pneumococci was observed among nasopharyngeal colonization isolates. Emerging non-conjugate vaccine serotypes were serotypes 11, 15 and 16 (32). We questioned whether this shift occurred within specific genotypes or whether replacement took place with genetically different strains. If the latter was true, we wondered if these different genotypes were equally capable of horizontal dissemination and whether they represented comparable antibiotic resistance profiles.
Therefore, we analyzed the 921 pneumococcal isolates retrieved from 351 of the 383 participating children. We observed 275 different genotypes, representing 106 genetic clusters and 75 unique genotypes. Analyzing the per-patient follow-up revealed few episodes of persistent carriage. This implicates that pneumococcal colonization is a dynamic process with a high turnover rate of colonizing strains. No effect of vaccination was found on the limited rate of persistent strains. This was to be expected, because the majority of the persistent strains in both PV and CV group were non-vaccine serotypes.

Remarkably, in three cases of persistent carriage, a serotype switch was observed. However, one could argue whether the consecutive colonization with the serotype 15 and serotype 19A variant of a strain closely related to MLST 199 suggests the recruitment of a second isolate with identical genotype rather than a capsular switch. Importantly, in contrast to many countries including the US, this genotype is not very common in The Netherlands (3%) and a 19A serotype variant has not been previously observed. Therefore, our findings strongly suggest a capsular switch. So far, this phenomenon was only reported in vivo twice by Barnes et al. (3) and Sluijter et al. (27). Indirect proof for capsular switch was previously shown by other investigators who demonstrated the true recombinational exchanges at the capsular locus (7-9, 24). One might argue that our observations are events enhanced by conjugate vaccination due to the induction of a selective immunological pressure. Indeed, the serotype 15/19A switch was observed in a PV child but no conjugate vaccine type pneumococci were involved. The two additional cases (serotype 6A/19F and 14/8 switch) were observed in CV children. Although our data support the theory that serotype switch is a natural process which can be observed occasionally within an individual, a large quantity of data will be required to study the impact of conjugate vaccination on this process.

Comparison of the genetic structure of the pneumococci isolated at study entry and at 14 months after pneumococcal conjugate vaccination showed 70% homology between the pneumococcal isolates at the two time points. The non-overlapping isolates were equally distributed among PV and CV children. Furthermore, initially predominant clusters displaying vaccine serotypes had been partially replaced by non-vaccine serotype clusters after vaccination, which displayed a similar capability to spread horizontally. Though replacement of vaccine types by non-vaccine serotypes has has been described as a result of growing age (12), we observed this shift significantly more often in children who received the pneumococcal vaccines, indicating this process is enhanced by vaccination.

Our most predominant clusters represented multiple vaccine and non-vaccine serotypes. Since the observed genetic homology was confirmed by MLST analysis, our data suggest that a large number of recombinational events at the capsular loci have occurred within these clusters. This is in line with previous data from the USA and Latin America where the major (resistant) clones also show multiple serotypes as a result of capsular serotype switch (9, 10, 16, 22, 29). Wolf et al. (33) have shown that these events occur even more often in susceptible pneumococcal clones, which is in line with our findings.

Our data support the hypothesis that serotype replacement observed after conjugate vaccination does not directly indicate a shift in the genetic structure of the pneumococcal population. Shifts towards and predominance of non-vaccine serotype variants are likely to occur within genetic clusters displaying both vaccine and non-vaccine serotypes. However, MLST analysis of the most predominant clusters of our collection of pneumococcal strains

showed the presence of new genotypes and the absence of PMEN homologous clones. Therefore, this collection of strains might not be representative for countries where multidrug resistant clones are predominantly present.

To evaluate whether vaccination will have an effect on the presence of antibiotic resistance, we determined the antibiotic resistance profiles of all 921 isolates. Susceptibility testing of 919 of the 921 pneumococcal isolates was performed for penicillin, cotrimoxazole, tetracycline, erythromycin, rifampicin, vancomycin, cefotaxime and ciprofloxacin. In agreement with previous studies performed in The Netherlands, the overall resistance is low (14% of the isolates) compared to other European countries (5, 13, 14, 17, 18). Penicillin resistance was found rarely in our study. In contrast, we most frequently observed resistance to cotrimoxazole, tetracyclin and erythromycin. We compared our data with a previous study performed in The Netherlands, where 10,489 clinical pneumococcal isolates have been tested for drug susceptibility (14). Compared to this study (reference year 1999), we noted a higher incidence in cotrimoxazole resistance (4.4 versus 10.6) and a lower incidence in tetracyclin resistance (3.5% versus 6.6%). Both observations can be explained by the age difference between the study groups; our study was performed in children under 7 years of age, whereas the surveillance study represented all age groups including adults. In contrast to adults, tetracyclines are contraindicated in children whereas cotrimoxazole is often first choice treatment.

We found an equal percentage of (intermediate) resistance to a single drug in our study population compared to the surveillance study (77% of the resistant isolates), comparable dual resistance (13% an 19%, respectively) and significantly higher multidrug resistance (9% and 4%, respectively; $p < 0.01$). We hypothesize that our children might select for multidrug resistant strains because of higher antibiotic consumption which is in accordance with previous findings (23, 28).

In addition, we analyzed changes in the incidence of pneumococcal resistance. To this end, we compared resistance among the pneumococcal isolates between the initial phases of the study (before full vaccination) and the post-vaccination phases. Although a trend was seen with a decline in resistance from 17.5% to 11.8%, this was not statistically significant. Because of the low resistance rates, no subsidiary analysis could be performed for the separate sample dates. Therefore, we analyzed the serotype distribution among the resistant isolates, which showed that 57% of the resistant isolates depict a vaccine serotype, which is comparable to the overall serotype distribution. However, all multidrug-resistant isolates were vaccine types or cross-reactive serotypes. Although resistance is low among *S. pneumoniae* in The Netherlands, these data suggest that vaccination with the 7-valent conjugate vaccine may reduce pneumococcal resistance in the population, particularly multidrug resistance.

In conclusion, pneumococcal conjugate vaccination did not induce a shift in the population-based structure of the pneumococci, nor decreased their tendency to spread horizontally. Our observations combined with the vaccine efficacy data of Veenhoven et al. (32) suggest that pneumococcal conjugate vaccination is not very useful for prevention of pneumococcal colonization in children above 1 year of age. Moreover, we strongly advice continuous and close monitoring of the pneumococcal genetic structure in areas with a conjugate vaccination policy.

ACKNOWLEDGMENTS

We would like to thank CP. Elzenaar (National Institute for Public Health and the Environment, Bilthoven, The Netherlands), J. Bruin (Regional Laboratory of Public Health, Haarlem, The Netherlands) and N. Lemmens-den Toom (Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands) for their technical assistance. Furthermore, we express our gratitudes to EP. IJzerman (Regional Laboratory of Public Health, Haarlem, The Netherlands) for microbiological advice.

This study was sponsored by the Sophia Foundation for Medical Research, The Netherlands (grant 268), and the Dutch Science Foundation (grant SGO-Inf. 005).

REFERENCES

1. 1997. From the Centers for Disease Control and Prevention. Surveillance for penicillin-nonsusceptible *Streptococcus pneumoniae*--New York City, 1995. Jama 277:1585-6.
2. 2000. Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 49:1-35.
3. Barnes, D. M., S. Whittier, P. H. Gilligan, S. Soares, A. Tomasz, and F. W. Henderson. 1995. Transmission of multidrug-resistant serotype 23F *Streptococcus pneumoniae* in group day care: evidence suggesting capsular transformation of the resistant strain in vivo. J Infect Dis 171:890-6.
4. Black, S., H. Shinefield, B. Fireman, E. Lewis, P. Ray, J. R. Hansen, L. Elvin, K. M. Ensor, J. Hackell, G. Siber, F. Malinoski, D. Madore, I. Chang, R. Kohberger, W. Watson, R. Austrian, and K. Edwards. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr Infect Dis J 19:187-95.
5. Bogaert, D., M. N. Engelen, A. J. Timmers-Reker, K. P. Elzenaar, P. G. Peerbooms, R. A. Coutinho, R. de Groot, and P. W. Hermans. 2001. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J Clin Microbiol 39:3316-20.
6. Butler, J. C., E. D. Shapiro, and G. M. Carlone. 1999. Pneumococcal vaccines: history, current status, and future directions. Am J Med 107:69S-76S.
7. Coffey, T. J., M. Daniels, M. C. Enright, and B. G. Spratt. 1999. Serotype 14 variants of the Spanish penicillin-resistant serotype 9V clone of *Streptococcus pneumoniae* arose by large recombinational replacements of the cpsA-pbp1a region. Microbiology 145 (Pt 8):2023-31.
8. Coffey, T. J., C. G. Dowson, M. Daniels, J. Zhou, C. Martin, B. G. Spratt, and J. M. Musser. 1991. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. Mol Microbiol 5:2255-60.
9. Coffey, T. J., M. C. Enright, M. Daniels, J. K. Morona, R. Morona, W. Hryniewicz, J. C. Paton, and B. G. Spratt. 1998. Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*. Mol Microbiol 27:73-83.
10. Coffey, T. J., M. C. Enright, M. Daniels, P. Wilkinson, S. Berron, A. Fenoll, and B. G. Spratt. 1998. Serotype 19A variants of the Spanish serotype 23F multiresistant clone of *Streptococcus pneumoniae*. Microb Drug Resist 4:51-5.
11. Crook, D. W., and B. G. Spratt. 1998. Multiple antibiotic resistance in *Streptococcus pneumoniae*. Br Med Bull

54:595-610.
12. Dagan, R., N. Givon-Lavi, O. Zamir, M. Sikuler-Cohen, L. Guy, J. Janco, P. Yagupsky, and D. Fraser. 2002. Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J Infect Dis 185:927-36.
13. de Neeling, A. J., and R. v. V. e. Milieu. 1999. Resistentiepeiling in 9 Nederlandse streeklaboratoria in 1995 en 1998.
14. de Neeling, A. J., B. P. Overbeek, A. M. Horrevorts, E. E. Ligtvoet, and W. G. Goettsch. 2001. Antibiotic use and resistance of *Streptococcus pneumoniae* in The Netherlands during the period 1994-1999. J Antimicrob Chemother 48:441-4.
15. Eskola, J., T. Kilpi, A. Palmu, J. Jokinen, J. Haapakoski, E. Herva, A. Takala, H. Kayhty, P. Karma, R. Kohberger, G. Siber, P. H. Makela, S. Lockhart, and M. Eerola. 2001. Efficacy of a Pneumococcal Conjugate Vaccine against Acute Otitis Media. N Engl J Med 344:403-409.
16. Gherardi, G., C. G. Whitney, R. R. Facklam, and B. Beall. 2000. Major related sets of antibiotic-resistant Pneumococci in the United States as determined by pulsed-field gel electrophoresis and pbp1a-pbp2b-pbp2x-dhf restriction profiles. J Infect Dis 181:216-29.
17. Gruneberg, R. N., and D. Felmingham. 1996. Results of the Alexander Project: a continuing, multicenter study of the antimicrobial susceptibility of community-acquired lower respiratory tract bacterial pathogens. Diagn Microbiol Infect Dis 25:169-81.
18. Hermans, P. W., M. Sluijter, K. Elzenaar, A. van Veen, J. J. Schonkeren, F. M. Nooren, W. J. van Leeuwen, A. J. de Neeling, B. van Klingeren, H. A. Verbrugh, and R. de Groot. 1997. Penicillin-resistant *Streptococcus pneumoniae* in the Netherlands: results of a 1-year molecular epidemiologic survey. J Infect Dis 175:1413-22.
19. Hermans, P. W., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J Clin Microbiol 33:1606-12.
20. Jefferies, J., S. C. Clarke, M. A. Diggle, A. Smith, C. Dowson, and T. Mitchell. 2003. Automated pneumococcal MLST using liquid-handling robotics and a capillary DNA sequencer. Mol Biotechnol 24:303-8.
21. Klugman, K. P. 1996. Epidemiology, control and treatment of multiresistant pneumococci. Drugs 52:42-6.
22. Lipsitch, M., J. K. Dykes, S. E. Johnson, E. W. Ades, J. King, D. E. Briles, and G. M. Carlone. 2000. Competition among *Streptococcus pneumoniae* for intranasal colonization in a mouse model. Vaccine 18:2895-901.
23. McCormick, A. W., C. G. Whitney, M. M. Farley, R. Lynfield, L. H. Harrison, N. M. Bennett, W. Schaffner, A. Reingold, J. Hadler, P. Cieslak, M. H. Samore, and M. Lipsitch. 2003. Geographic diversity and temporal trends of antimicrobial resistance in *Streptococcus pneumoniae* in the United States. Nat Med.
24. Meats, E., A. B. Brueggemann, M. C. Enright, K. Sleeman, D. T. Griffiths, D. W. Crook, and B. G. Spratt. 2003. Stability of serotypes during nasopharyngeal carriage of *Streptococcus pneumoniae*. J Clin Microbiol 41:386-92.
25. NCCLS. 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Fourth edition; approved standard, M7-A4, NCCLS, Wayne, PA.
26. NCCLS. 2002. Performance standards for antimicrobial susceptibility testing: twenfth informational supplement, M 100-S12, 19 (1). NCCLS, Wayne, PA.
27. Sluijter, M., H. Faden, R. de Groot, N. Lemmens, W. H. Goessens, A. van Belkum, and P. W. Hermans. 1998. Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. J Clin Microbiol 36:2248-53.
28. Soriano, F., and V. Rodriguez-Cerrato. 2002. Pharmacodynamic and kinetic basis for the selection of pneumococcal resistance in the upper respiratory tract. J Antimicrob Chemother 50 Suppl C:51-8.
29. Spratt, B. G., and B. M. Greenwood. 2000. Prevention of pneumococcal disease by vaccination: does serotype replacement matter? Lancet 356:1210-1.
30. Tomasz, A. 1997. Antibiotic resistance in *Streptococcus pneumoniae*. Clin Infect Dis 24 Suppl 1:S85-8.
31. van Steenbergen, T. J., S. D. Colloms, P. W. Hermans, J. de Graaff, and R. H. Plasterk. 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc Natl Acad Sci U S A 92:5572-6.
32. Veenhoven, R., D. Bogaert, C. Uiterwaal, C. Brouwer, H. Kiezebrink, J. Bruin, P. Hermans, R. de Groot, W. Kuis, G. Rijkers, A. Schilder, and L. Sanders. 2003. Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. Lancet 361:2189-95.
33. Wolf, B., L. C. Rey, S. Brisse, L. B. Moreira, D. Milatovic, A. Fleer, J. J. Roord, and J. Verhoef. 2000. Molecular epidemiology of penicillin-resistant *Streptococcus pneumoniae* colonizing children with community-acquired pneumonia and children attending day-care centres in Fortaleza, Brazil. J Antimicrob Chemother 46:757-765.

Chapter 7.4

Colony blot assay: a useful method to detect multiple pneumococcal serotypes within clinical specimens

D. Bogaert, R.H. Veenhoven, M. Sluijter, E.A.M. Sanders, R. de Groot and P.W.M. Hermans

FEMS Immunol Med Microbiol; accepted for publication

ABSTRACT

The efficacy of pneumococcal conjugate vaccines in young children may be complicated by serotype replacement. We developed a colony blot assay which enables the identification of re-colonization with novel serotypes (replacement), overgrowth by minor co-colonizing serotypes or suppression of previously predominant vaccine serotype strains as a result of vaccination. This method allows the identification of multiple serotypes in a single specimen in a ratio of 1:1000. In order to demonstrate the potential of our method, we investigated the consecutive nasopharyngeal samples of 26 children who had shown a shift in pneumococcal colonization after conjugate vaccination. Mixed colonization was found once in 15 pre-vaccination samples and 4 times in 26 post-vaccination samples. In the remaining children 'true replacement' had presumably occurred. Hence, we conclude that the colony blot assay is an easy to apply method, which allows the identification of different pneumococcal serotypes within single clinical specimens.

INTRODUCTION

Current pneumococcal conjugate vaccines are protective against invasive diseases in children [1]. However, the impact of vaccination on otitis media and nasopharyngeal colonization is limited [2,3]. Several studies demonstrated a shift in carriage from vaccine-type pneumococci towards non-vaccine types after conjugate vaccination [4]. Whether this is caused by unmasking of pneumococcal serotypes which are already present at the nasopharynx or by replacement, i.e. the acquisition of other serotypes, remains unknown [5]. We therefore developed a colony blot assay, which allows the detection of multiple serotypes, i.e. mixed colonization, within clinical specimens. Such a method is a useful tool to investigate the pneumococcal serotype dynamics, e.g. changes in pneumococcal population structure during colonization. Furthermore, this method has important applications with respect to the monitoring of pneumococcal vaccination.

MATERIALS AND METHODS

Clinical specimens. We studied the nasopharyngeal cultures from 26 children participating in a pneumococcal conjugate vaccine trial in The Netherlands [2]. Inclusion criteria for this study were (1) two or more AOM episodes prior to study entry, and (2) age of 1 to 7 years. The number of previous AOM episodes was based on parental report. Children aged 12-24 months were immunized with PCV7 (Prevnar®, Wyeth) twice at a one-month interval followed 6 months later by PPSV23 (Pneumune®, Wyeth). Children aged 25-84 months received one dose of PCV7 followed 7 months later by PPSV23.

Nasopharyngeal swabs. At study entry, at 7 months (just before booster PPSV23 vaccination) and at 7, 14, 20 and 26 months after the last vaccination a nasopharyngeal sample was obtained for culture of *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis.* The nasopharyngeal samples were taken transnasally by the study physicians using a flexible sterile dry cotton-wool swab (Copan Italia, Transwab, Medical Wire & Equipment Co. Ltd., Corsham, England). After sampling, swabs were placed directly in Stuart's transport medium. Samples of nasopharyngeal swabs were plated within 6 hours. Isolation and identification of the isolates was performed by standard methods [2]. In addition, in case of positive cultures for *S. pneumoniae* the gentamycin plates were rinsed with 1.0 ml THY broth containing 18.5% glycerol and stored at -80ºC (primary culture). Pneumococci were microscopically serotyped by the capsular swelling method (Quellung reaction) using commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

Colony blot assay. The colony blot assay is based on the dot blot method of Fenoll et al. [6]. This method, used for serotyping of pneumococcal isolates, was adjusted to determine single colonies of a second serotype among predominant serotypes. In summary, serial

dilutions of primary cultures in 0.9% phosphate buffered saline (PBS) were plated onto blood-agar plates and grown overnight in 5-10% CO2 at 37ºC to obtain colony densities of approximately 1000 individual colonies per plate. The colonies were blotted onto a nitrocellulose membrane (Optitran, Schleicher & Schuell, 's-Hertogenbosch, Netherlands) by applying the membrane on top of the plate for 20 minutes. After careful colony lifting, the membranes were dried for 30 minutes at room temperature (RT). The membrane was incubated in PBS for 30 minutes at 56ºC to inhibit pneumococcal phosphatase activity. After incubation in blocking buffer (5% skim milk in PBS) for 1 hour at RT, the membranes were washed 3 times for 10 minutes in PBS containing 0.05% Tween 20 (PBST). To detect serotype-specific colonies the membranes were incubated with anti-capsule-specific rabbit sera (Statens Seruminstitut, Copenhagen, Denmark) diluted 1: 10,000 in PBS for 1 hour at RT. For the detection of serotype 6B pneumococci, we used a 1: 4,000 dilution of monoclonal mouse antibodies (14A2), which were kindly provided by PG van der Dobbelsteen, Netherlands Vaccine Institute, Bilthoven, The Netherlands. Prior to this incubation, these sera were preadsorbed with R6 pneumococci to remove aspecific non-capsular antibodies. The plates were washed 3 times with PBST after which antibody binding was detected by alkaline-phospatase-conjugated anti-rabbit IgG (Sigma, USA) or anti-mouse IgG (Serotec, UK) for 1 hour at RT. Conjugate binding was identified by the substrate *p*-nitrophenyl phosphate (Sigma). A colony blot using a pneumococcal strain with an identical serotype as searched for served as a positive control. After the membranes were dried at room temperature, positive colonies were identified and compared to the colonies on the original plate. The corresponding colonies were picked from the plate and re-grown overnight. Subsequently, the strains were serotyped and characterized by RFEL genotype analysis.

Cross-reactivity of the capsule-specific antisera of the Statens Seruminstitut was tested by means of colony blot analysis of at least two pneumococcal strains of each of the 7 conjugate vaccine serotypes 4, 6B, 9V, 14, 18C, 19F, 23F, the cross-reactive serotypes 6A and 23B, and the serotypes 3, 10, 11, 15, and 16. Pre-incubation of the sera with cell wall polysaccharides of strain R6 eliminated cross-reactivity for most sera. Only the serotype 6B anti-serum showed cross-reactivity with all remaining serotypes. Therefore, this antiserum was replaced by monoclonal mouse antibodies raised against serotype 6B capsule polysaccharides (14A2). Cross-reactivity remained for 19F antiserum and the pneumococcal serotypes 6A and 16 (data not shown).

In order to test the sensitivity of the colony blot method, we mixed cultures of different pneumococcal serotypes in various ratios. We were able to identify minor pneumoccocal serotypes representing 0.1% of the bacterial population.

The colony blot analysis was performed in a group of children who had shown a shift in colonization from a vaccine serotype A to a (non-) vaccine serotype B strain during 2 consecutive nasopharyngeal samples. Mixed colonization was defined as the presence of serotype A colonies among primarily serotype B colonies, or as the presence of serotype B colonies among primarily serotype A colonies. Replacement was defined as the absence of serotype A in the serotype B culture or the absence of serotype B in the serotype A culture.

For the 26 children included in this analysis at least two primary cultures from consecutive specimens following pneumococcal vaccination were present. Only from 15 children a primary culture was available at study entry. Children displaying a shift in colonization but with less than two primary cultures available for analysis were excluded from analysis (n=5).

RFEL analysis. Pneumococcal strain typing by RFEL was performed as described previously [7]. Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72^{o}C with [α-32P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 *M* urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, NY), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, UK). RFEL autoradiographs were converted to images (Image Master DTS; Pharmacia Biotech, Uppsala Sweden) and analyzed by computer (Windows version Gelcompar software version 4; Applied Math. Kortrijk, Belgium). DNA fragments were analyzed as described previously [8]. The genetic relatedness of the isolates was evaluated by computerized comparison of the individual banding patterns.

RESULTS

We developed a colony blot assay which enables the identification of re-colonization with novel serotypes (replacement), overgrowth by or suppresion of minor co-colonizing serotypes as a result of vaccination. This method allows the identification of multiple serotypes in a single specimen in a ratio of 1:1000 (Figure 1).

To validate the colony blot assay, we applied the technology to clinical specimens from children vaccinated with a 7-valent pneumococcal conjugate vaccine, who were colonized with pneumococci before and after vaccination. Samples were only included when a shift in pneumococcal serotype and genotype from a vaccine-type strain before vaccination to a non-vaccine type strain after vaccination had occurred. We re-grew the primary cultures using serial dilutions to obtain densely grown bacterial plates but with individual bacterial colonies (approximately 1000 colonies per plate). Initial cultures were tested for the presence of the post-vaccination serotype strain, whereas post-vaccination

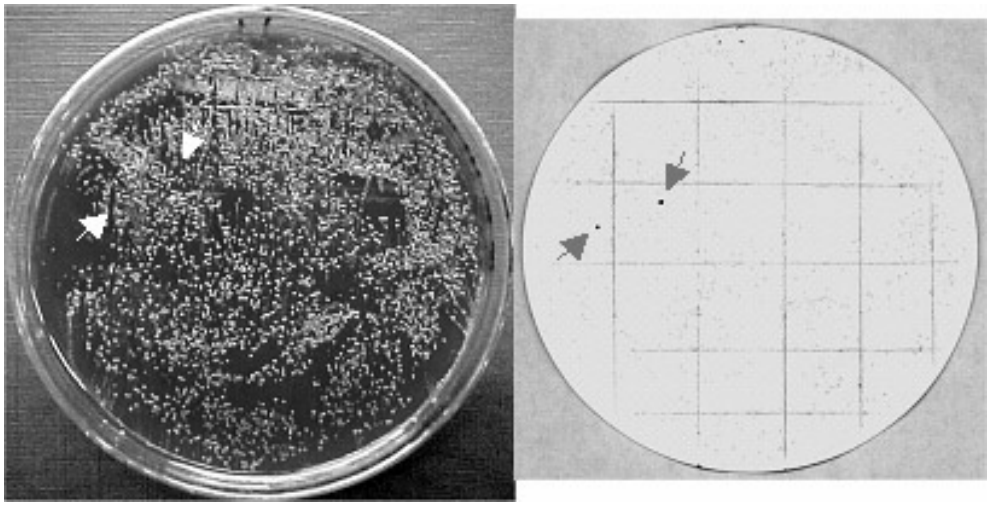


Figure 1. Culture containing multiple pneumococcal serotypes. The colonies were blotted onto a nitrocellulose membrane. The two colored colonies marked by arrows represent a minor serotype 6A colony among predominant serotype 38 colonies.

cultures were tested for the presence of pre-vaccination serotype strains. In case the intended serotype was found, we confirmed the genetic similarity with the original strain by RFEL genotyping (figure 2). Post-vaccination cultures were available for all 26 children and were tested for the presence of the pre-vaccination serotype (Table 1). In 4 instances representing 3 children the pre-vaccination serotype strain was observed as a minor strain in the post-vaccination culture. For only 15 children pre-vaccination cultures were available and could be tested for the presence of the post-vaccination serotype as a minor strain (Table 1). This phenomenon was observed in 1 child only (patient 20). The latter child showed an initial carriage of a serotype 19F strain followed 7 months later by colonization with a serotype 11 strain. Another 6 months later a serotype 19F strain with identical genotype was cultured as predominant strain again. With the colony blot assay we were able to detect the presence of the serotype 19F strain as minor strain among the serotype 11 pneumococci. The suppression of serotype 19F in specimen 14 with the subsequent predominance one culture later in specimen 20 is depicted in Table 1. In the remaining 14 children re-colonization (replacement) of the vaccine serotype strains by the newly acquired non-vaccine type pneumococci had most likely occurred.

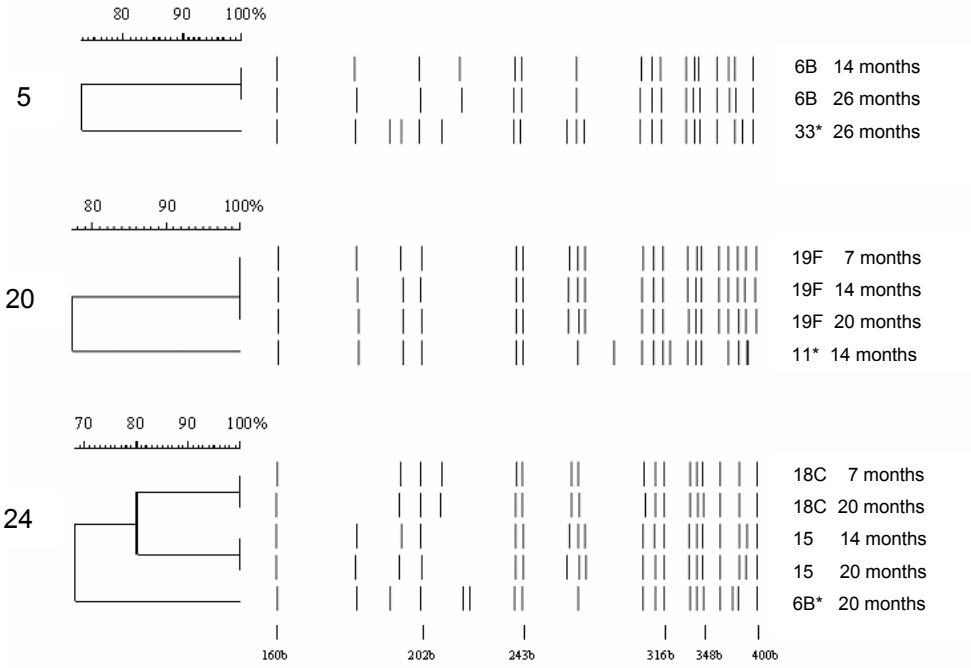


Figure 2. RFEL patterns of the consecutive isolates of the children who displayed mixed colonization by colony blot screening. Patient numbers, moment of sampling (months) and serotypes of the pneumococcal isolates are depicted. *predominant strain.

DISCUSSION

In 2000 the Advisory Committee on Immunization Practices (ACIP) in the US has advised the 7-valent pneumococcal conjugate vaccine Prevnar (Wyeth, USA) to prevent invasive diseases in children under 2 years of age [9]. Moreover, they recommend conjugate vaccination for children 2-5 years of age at risk of developing pneumococcal diseases. However, as a result of vaccination, replacement of vaccine serotype pneumococci with non-vaccine serotype strains colonizing the nasopharynx occurs [10-12]. It has initially been suggested that replacement may be an innocent phenomenon: instead of true replacement outgrowth and thus detection of an already present strain may occur. In other words, by eradicating the predominant vaccine type strain due to vaccination a second minor strain is 'unmasked'. The latter strain might not be as harmful as a newly acquired strain [13]. It has also been suggested that replacement strains are less virulent and that the vaccine actually

patient	0 months (CV)		7 months (PV)		14 months		20 months		26 months		conclusion
	dominant serotype	minor serotype	dominant serotype	minor serotype	dominant serotype	minor serotype	dominant serotype	minor Serotype	dominant serotype	minor serotype	
1	23F	NA	23F	NA	10	23F(-)	18C	23F(-)	11	23F(-)	Replacement
2			19F	NA	19F	15(-)	15	19F(-)	15	19F(-)	Replacement
3	4	NA	9V	NA	4	NA	8	4(-)/9V(-)	16	4(-)/9V(-)	Replacement
4	23F	NA	23F	NA	23F	NA	19F	23F(-)/15(-)	15	23F(-)/19F(-)	Replacement
5			22	NA	6B	NA	6B	33(-)	33	6B(+)	Mixed colonization
6	7	NA			6B	3(-)/18C(-)	3	6B(-)/18C(-)	18C	6B(-)/3(-)	Replacement
7			23F	16(-)	16	23F(-)					Replacement
8	11	NA	9V	6B(-)/3(-)	6B	9V(-)/3(-)	3	9V(-)/6B(-)			Replacement
9	6A	23F(-)	23F	6A(-)/11(-)	11	6A(-)/23F(-)					Replacement
10	19F				NT	19F(-)					Replacement
11	6A	6B(-)	6B	6A(-)			6A	6B(-)	6A	6B(-)	Replacement
12	19F	NA	19F	23B(-)	23B	19F(-)					Replacement
13	19F	NA	11	NA	11	NA	16	19F(-)	16	19F(-)	Replacement
14	6A	NA	23F	6A(-)	6A	23F(-)	3	23F(-)/6A(-)			Replacement
15	6B	NA	6B	NA	15	NA	19A	6B(-)	19A	6B(-)	Replacement
16	6B	NA			11	NA	19F	23B(-)/6B(-)	23B	19F(-)/6B(-)	Replacement
17	6B	14(-)			14	6B(-)					Replacement
18	9V	NA			6A	9V(-)	19A	9V(-)	11	9V(-)	Replacement
19	9V	NA	6B	9V(-)/6A(-)	6B	9V(-)/6A(-)	6A	9V(-)/6B(-)			Replacement
20	3	NA	19F	NA	11	19F(+)	19F	11(-)	11	19F(-)	Mixed colonization
21	6B	NA	16	6B(-)	38	6B(-)	23F	6B(-)			Replacement
22	6B	NA	16	6B(-)	6B	16(-)					Replacement
23	6A	6B(-)	6B	6A(-)			6A	6B(-)			Replacement
24	6B	NA	18C	6B(-)/15(-)	15	6B(-)/18C(-)	6B	18C(+)/15(+)	23B	6B(-)/18C(-)/ 15(-)	Mixed colonization (2x)
25					6B	6A(-)	6A	6B(-)			Replacement
26	14	NA	21	14(-)			6A	14(-)			Replacement

NA, Initial culture not available; serotype (-), serotype not fiound as minor strain by colony blot method; Serotype(+), serotype found as minor serotype by colony blot method

protects the host from serious pneumococcal disease by replacing virulent strains with weaker variants [13]. However, recent reports have shown the emergence of replacement disease caused by non-vaccine serotype strains [2,3].

In order to test whether re-colonization (replacement) occurs, we developed a colony blot assay that can detect multiple and minor serotypes within a single specimen in a 1:1000 ratio. Alternative screening of individual colonies using the quellung method, is unsuitable because it is time and labor consuming. Moreover, it is inappropriate for high throughput screening of thousands of colonies.

To demonstrate the potential of the colony blot technique to answer this question, we selected a test cohort of 26 children who had shown a shift in pneumococcal carriage from vaccine to non-vaccine serotypes after pneumococcal conjugate vaccination. Among the 26 children who were investigated by this method, four events of persistence of vaccine type pneumococci as minor strains after vaccination were observed in the presence of a predominant non-vaccine serotype strain. In one child, predominance of an initially suppressed serotype strain was observed after vaccination. These data may implicate that in the majority of these cases replacement of serotypes occurs. However, the persistence of vaccine type strains accompanied by immunological pressure due to vaccination and the availability of non-vaccine serotype strains for replacement might create ideal circumstances for recombinational exchange of capsular genes [4]. Assuming that this phenomenon occurs as a result of vaccination, future vaccine failures with respect to prevention of disease and elimination of multidrug-resistant clones may take place. To study the true effect of conjugate vaccination on replacement, obviously a larger study should be performed. For such study, the colony blot assay will be a useful additional tool.

ACKNOWLEDGMENTS

We would like to thank J. Bruin (Regional Laboratory of Public Health, Haarlem, The Netherlands) and N. Zaynoun and N. Chung (Laboratory of Pediatrics, Erasmus MC-Sophia, Rotterdam, The Netherlands) for their technical assistance.

This study was financially supported by the Sophia Foundation for Medical Research, The Netherlands (grant 268), and the Dutch Science Foundation (grant SGO-Inf. 005).

Table 1 (left). Colony blot results of 26 patients vaccinated with the 7-valent pneumococcal conjugate vaccine (CV) followed by the 23-valent pneumococcal polysaccharide vaccine (PV) who displayed a serotype shift in nasopharyngeal colonization. The initial cultures (0, 7 and 14 months) were tested for the presence of the post-vaccination strain(s), and the post-vaccination cultures (14, 20 and 26 months) were tested for the presence of the initial strain(s)

REFERENCES

1 Black, S., Shinefield, H., Fireman, B. *et al.* Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group [In Process Citation]. *Pediatr Infect Dis J* 2000, 19(3), 187-195.

2 Veenhoven, R., Bogaert, D., Uiterwaal, C. *et al.* Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. *Lancet* 2003, 361, 2189-2195.

3 Eskola, J., Kilpi, T., Palmu, A. *et al.* Efficacy of a Pneumococcal Conjugate Vaccine against Acute Otitis Media. *N Engl J Med* 2001, 344(6), 403-409.

4 Spratt, B.G. & Greenwood, B.M. Prevention of pneumococcal disease by vaccination: does serotype replacement matter? *Lancet* 2000, 356(9237), 1210-1211.

5 Lipsitch, M. Bacterial vaccines and serotype replacement: lessons from Haemophilus influenzae and prospects for Streptococcus pneumoniae. *Emerg Infect Dis* 1999, 5(3), 336-345.

6 Fenoll, A., Jado, I., Vicioso, D. & Casal, J. Dot blot assay for the serotyping of pneumococci. *J Clin Microbiol* 1997, 35(3), 764-766.

7 Hermans, P.W., Sluijter, M., Hoogenboezem, T., Heersma, H., van Belkum, A. & de Groot, R. Comparative study of five different DNA fingerprint techniques for molecular typing of Streptococcus pneumoniae strains. *J Clin Microbiol* 1995, 33(6), 1606-1612.

8 Sluijter, M., Faden, H., de Groot, R. *et al.* Molecular characterization of pneumococcal nasopharynx isolates collected from children during their first 2 years of life. *J Clin Microbiol* 1998, 36(8), 2248-2253.

9 Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2000, 49(RR-9), 1-35.

10 Veenhoven, R. Impact of combined pneumococcal conjugate and polysaccharide vaccination on nasopharyngeal carriage in children with recurrent acute otitis media: Program and abstracts of the 3rd International Symposium on Pneumococci and Pneumococcal Diseases, Anchorage, May 5-8, 2002. in *Submitted*, 2003.

11 Mbelle, N., Huebner, R.E., Wasas, A.D., Kimura, A., Chang, I. & Klugman, K.P. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis* 1999, 180(4), 1171-1176.

12 Dagan, R., Givon-Lavi, N., Zamir, O. *et al.* Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J Infect Dis* 2002, 185(7), 927-936.

13 Lipsitch, M. Interpreting results from trials of pneumococcal conjugate vaccines: a statistical test for detecting vaccine-induced increases in carriage of nonvaccine serotypes. *Am J Epidemiol* 2001, 154(1), 85-92.

Chapter 8

Host immune response to pneumococcal colonization, infection and vaccination

Chapter 8.1

Pneumoccal conjugate vaccination does not induce a persisting mucosal IGA response in children with recurrent acute otitis media

D. Bogaert, R.H. Veenhoven, R. Ramdin, I.H.T. Luijendijk, G.T. Rijkers, E.A.M. Sanders, R. de Groot and P.W.M. Hermans

Submitted

ABSTRACT

Aim. In a prospective controlled study in young children with a history of recurrent acute otitis media, we analyzed the salivary IgA and IgG antibody titers upon vaccination with a 7-valent pneumococcal conjugate vaccine (PCV) given once or twice, followed by a 23-valent polysaccharide booster vaccination.

Methods. Salivary IgA and IgG antibody concentrations to vaccine serotype 6B, 14, 18C and 19F were measured by enzyme immunoassay in 38 samples of children vaccinated with PCV and 45 control samples. In the PCV group, 12 samples were taken prior to vaccination, 12 samples 4 weeks after the polysaccharide booster (8 months after the first conjugate vaccination) and 14 samples 7 months after the last vaccination (14 months after the first conjugate vaccination). In the control group 15 children were sampled at each of these 3 time points.

Results. We observed increased salivary IgG antibody concentrations against serotype 6B, 14, and 18C 14 months after the primary vaccination in the vaccinated children but not the controls, although this was significant for serotype 14 only. This increase was restricted to children vaccinated with PCV twice but not observed in children vaccinated once with PCV. IgA antibody titers increased significantly after 8 and after 14 months in both the pneumococcal vaccine recipients and the controls, however the observed increase in mean antibody titers was significantly higher in control children compared to the PCV group.

Conclusion. We suggest that repeated pneumococcal conjugate vaccination is necessary to induce an increase in salivary IgG antibodies and effectuate clearance of *S. pneumoniae* from the nasopharyngeal mucosa of children with recurrent acute otitis media. We hypothesize that the increase in salivary IgA is caused by the local boosting of the mucosal immune response by carriage and recurrent infections, which occurs less often in the PCV group compared to the control children.

INTRODUCTION

Clinical efficacy of the 7-valent conjugate vaccine (PCV7) against invasive pneumococcal diseases was recently demonstrated by Black and coworkers [1]. These and other investigators also showed that PCV7 has a significant impact on pneumonia and otitis media [1-3]. In a Finnish trial with PCV7, a 57% reduction in acute otitis media episodes caused by conjugate-vaccine serotypes was observed in children under the age of one year [4]. However, this study did not investigate whether pneumococcal vaccination also prevents AOM in older children with recurrent episodes of AOM. We therefore questioned whether combined vaccination with PCV7 followed by 23-valent pneumococcal polysaccharide vaccine (PPSV23) could prevent AOM in children 1 to 7 years of age, with two or more documented AOM episodes in the year prior to vaccination [5]. In this double blind randomized study, 383 patients aged 1 to 7 years received either once (> 24 months of age) or twice (< 24 months of age) a 7-valent pneumococcal conjugate vaccination followed by PPSV23 or hepatitis A or B vaccines. We observed a significant systemic IgG response as a result of pneumococcal vaccination. However, we found no reduction in AOM episodes in the pneumococcal vaccine group as compared to the control group. In addition, overall pneumococcal carriage rates did not diminish, although we observed a significant shift from conjugate vaccine to non-conjugate vaccine type pneumococci in the PCV group [5].

Because pneumococcal vaccine recipients showed decreased colonization with vaccine serotype strains but no effect on the occurrence of otitis media, we questioned whether pneumococcal vaccination had provoked a mucosal immune response. Therefore, we compared the IgG and IgA antibody concentration in saliva of children of the pneumococcal vaccine group with that in children of the control vaccine group.

MATERIAL AND METHODS

The study population of the OMAVAX study has previously been described in detail [5]. Shortly, a randomized double blind trial was conducted between April 1998 and January 2002. A signed informed consent was obtained from all participants. Inclusion criteria for the study were two or more AOM episodes in the year prior to entry, and age between 1 and 7 years. The children were randomized to receive either PCV7 (Prevnar®, Wyeth) followed by PPSV23 (Pneumune®, Wyeth), or hepatitis A (Havrix=AE Junior®, GlaxoSmithkline) or B (Engerix-B=AE Junior®, GlaxoSmithkline) control vaccines. Children aged 12-24 months of the pneumococcal vaccine group were immunized with PCV7 twice at a one-month interval followed 6 months later by PPSV23; the control vaccine group aged 12-24 months received 3 hepatitis B vaccinations according to a similar time schedule. Children aged 25-84 months

of the PCV group received one dose of PCV7 followed 7 months later by PPSV23. The CV group aged 25-84 months received hepatitis A vaccine twice.

From September 2000, saliva samples were obtained from children at enrollment, and one month and 7 months after the pneumococcal booster or control vaccination. Unstimulated saliva samples were collected by placing a sponge swab (Saliva Swab, Malvern, Medical Development, Worcester, UK) in the cheek area to allow uptake of saliva until saturation was reached. The saliva samples were immediately put on ice, transported and stored at -70°C until analysis. Samples were thawed and centrifuged at 19,000 g for 10 min prior to the immunological analysis. The supernatants were used for antibody measurement.

In the PCV group we had saliva samples available for analysis from 12 children at 0 months, 12 children at 8 months and 14 children at 14 months after the primary conjugate vaccination. For the CV group saliva samples from 15 children at each of these time points were available. Serotype specific IgG and IgA antibodies were measured by enzyme immuno assay (EIA) as described previously [6]. Briefly, microtiter plates (high binding polystyrene EIA plates; Costar, New York, USA) were coated overnight with 100 µl/well coating solution containing capsule polysaccharides at a concentration of 10 µg/ml (ATCC, LGC Promochem, Teddington, UK). The saliva samples were diluted 1:10 in PBS containing 5% cell wall polysaccharides (CPS; Statens Serum Institute, Copenhagen, Denmark) and incubated for 30 minutes at 4°C. A 3-fold serial dilution of the sputum sample was incubated for 2 hours at 37°C in duplicate. After washing, antibody binding was detected by addition of alkaline-phosphatase-conjugated anti-human IgG or anti-human IgA (Sigma, St Louis, USA). The color was developed by the substrate *p*-nitrophenyl phosphate (Sigma). Optical density (OD) was measured at 405 nm on an EIA reader (Molecular Devices SpectraMAX, Sunnyvale, USA). Phosphate-buffered saline (PBS)- treated plates were used to assess the unspecific binding of antibodies to the polystyrene wells. Optical density (OD) readings from the PBS wells were subtracted from the values of the antigen wells before calculation of antibody concentrations. The cut-off for positivity was set at 2 SD above the average OD of the blank wells. The antibody titers for saliva were calculated as µg IgA or IgG per mg of albumin (µg IgA/mg albumin or µg IgG/mg albumin calculated from the officially assigned IgG and IgA values of the 89-SF reference serum [7] and corrected for the excretory dilution component [8,9]. The albumin concentration in saliva was measured using the Human Albumin ELISA Core Kit (Koma Biotech, Seoul, Korea).

All statistical analyses were performed using the statistical software SPSS 11. Antibody concentrations are depicted as geometric mean concentrations (GMCs). Group differences between pneumococcal vaccinees and controls were analyzed with the independent sample t-test. Time-related differences within groups were analyzed with the one-way ANOVA test. Significance was set at a p-value of <0.05.

RESULTS

We measured IgG and IgA antibodies in serum as well as in saliva of 38 children of the PCV group and compared these to the data of 45 children in the CV group. The children were comparable with respect to age at study entry and frequency of AOM in the previous year. Their characteristics are depicted in Table 1.

We determined the GMC of IgG and IgA against the serotypes 6B, 14, 18C and 19F in saliva at 0, 8 and 14 months. An increase in IgG antibodies directed against the serotypes 6B, 14 and 18C was found 14 months after the first conjugate vaccination, although this was only significant for serotype 14 which showed an increase from 3.8 to 29.6 µg per mg of albumin (Table 2). The GMC for IgG directed against serotype 6B increased from 19.6 µg per mg of albumin to 31.2 µg per mg of albumin. For the IgG antibodies against serotype 18C an increase from 10.1 to 26.3 µg per mg of albumin was found. A baseline titer of 19.6 µg per mg of albumin was observed for IgG antibodies directed against capsular polysaccharides of serotype 19F. The GMC of anti-serotype 19F antibodies did not increase after conjugate and booster vaccination.

Table 1. Characteristics of the children of whom saliva samples were investigated. Number of samples, age of children, and history of AOM per study group are depicted.

	0 months (pre-vaccination)		7 months (1 months post booster)		14 months (7 months post booster)	
	PV	CV	PV	CV	PV	CV
Number of samples	12	15	12	15	14	15
Median age(years)[†]	2.3	2.7	1.9	2.0	2.0	2.0
Range (years)	1.3-6.9	1.2-6.7	1.1-3.6	1.0-6.2	1.0-4.2	1.0-6.2
> 4 AOM events[‡] in previous year	9/12	11/15	6/13	8/15	8/14	9/15

No significant differences were observed with respect to age [†] and history of AOM [‡] between the pneumococcal vaccine and control vaccine group.

Because the vaccination regime differed between children under two years of age, who received pneumococcal conjugate vaccination twice, and children older than two years of age, who received pneumococcal conjugate vaccination once, we performed separate analysis for both groups (Figure 1). We found that the observed increase in IgG antibodies directed against the serotypes 6B, 14 and 18C occurred only in children under two years, whereas no increase in salivary IgG was observed in the older children.

Table 2. GMC (in µg per mg of albumin) of IgG and IgA antibodies against the serotypes 6B, 14, 18C and 19F at 0, 8 and 14 months.

	Serotype 6B				Serotype 14			
	PV group		CV group		PV group		CV group	
	IgG	(SD)	IgG	(SD)	IgG	(SD)	IgG	(SD)
0 mths	19,63	(19,17)	23,64	(45,28)	3,85	(2,53)	4,43	(6,01)
8 mths	15,89	(20,66)	23,57	(34,25)	5,54	(8,91)	5,93	(5,00)
14 mths	31,23	(44,12)	15,12	(14,30)	29,55	(45,86)	14,16	(15,53)
	IgA		IgA		IgA		IgA	
0 mths	5,03	(5,27)	4,29	(3,863)	16,50	(19,63)	10,21	(11,84)
8 mths	5,25	(11,66)	12,87	(15,80)	10,88	(16,37)	14,01	(24,71)
14 mths	20,84	(24,36)	52,20*	(33,44)	36,20	(54,93)	54,61	(35,28)

	Serotype 18C				Serotype 19F			
	PV group		CV group		PV group		CV group	
	IgG	(SD)	IgG	(SD)	IgG	(SD)	IgG	(SD)
0 mths	10,11	(11,05)	13,39	(12,56)	19,59	(30,05)	9,68	(9,12)
8 mths	4,73	(4,83)	7,96	(7,12)	7,51	(6,89)	6,95	(8,15)
14 mths	26,26	(66,75)	12,21	(8,58)	18,84	(17,36)	17,86	(13,87)
	IgA		IgA		IgA		IgA	
0 mths	3,15	(3,23)	2,52	(2,78)	12,79	(16,23)	12,35	(7,69)
8 mths	3,56	(3,71)	13,05	(7,45)	10,74	(10,78)	27,36	(30,77)
14 mths	17,47	(21,93)	42,79*	(33,91)	37,09	(46,17)	80,46*	(67,96)

Significant difference (p-value < 0.05) between PV and CV group are marked by asteriks (independent samples t-test). A significant increase (p-value < 0.05) in antibodies over time is underlined (one-way ANOVA).

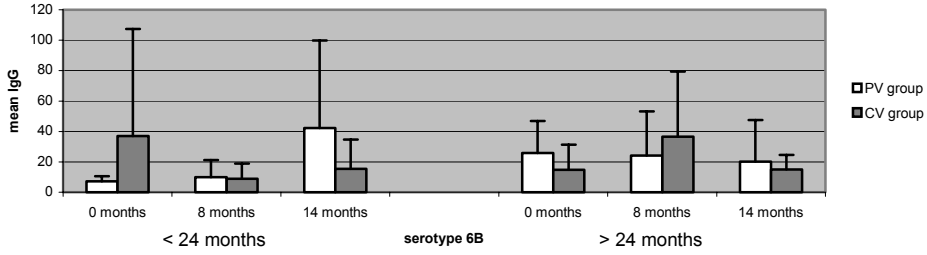
IgA titers increased significantly over time in both the PCV group as well as the control vaccine group (Table 2). For IgA antibodies against capsular polysaccharides of serotype 6B an increase of 5.0 to 20.8 µg per mg of albumin was observed, whereas for IgA against serotype 14 an increase from 16.5 to 36.2 µg per mg of albumin was found. The GMC for IgA directed against serotype 18C increased from 3.1 to 17.5 µg per mg of albumin, whereas for antibodies against serotype 19F an increase from 12.8 to 37.1µg per mg of albumin was observed. However, the IgA titer increased significantly more in the CV children

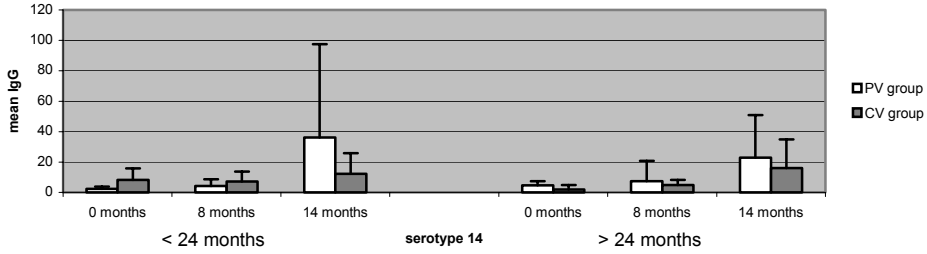
compared to the PCV group. The average increase in antibodies directed against serotype 6B was almost 3 times higher in control vaccinees compared to pneumococcal vaccinees (12.2- versus 4.2-fold increase, respectively). The IgA antibody titer against serotype 14 showed an over 2-fold higher increase in IgA antibodies in the control vaccinees compared to the pneumococcal vaccinees (5.1- and 2.2- fold increase, respectively), albeit not statistically significant. For the serotypes 18C and 19F the increase in IgA antibodies were 5.7 (pneumococcal vaccinees) and 17.5 (controls), and 2.9 (pneumococcal vaccinees) and 6.6 (controls), respectively. Again, we performed separate analyses for the children younger and older than two years of age. The observed increase in IgA antibodies directed against the four serotypes investigated as well as the larger increase in IgA antibodies in the CV group compared to the PCV group was found in both age groups (Figure 1).

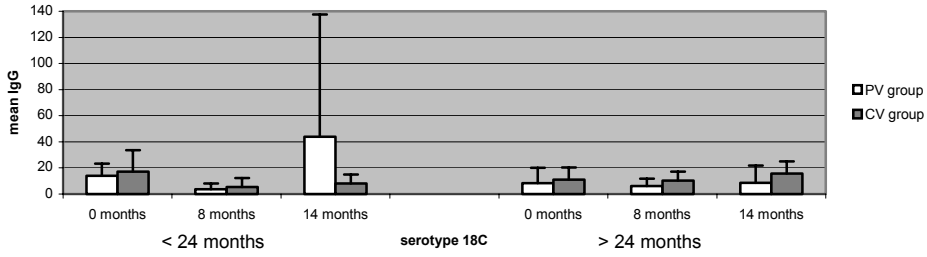
DISCUSSION

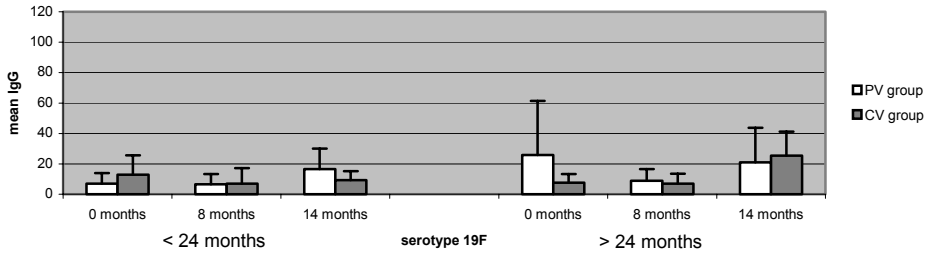
We studied serotype-specific mucosal IgG and IgA antibody levels in a subgroup of children who participated in our pneumococcal vaccination study. All children showed detectable levels of IgA and IgG against the serotypes 6B, 14, 18C and 19F in saliva. We also observed a rise in IgG antibody levels against the serotype 6B, 14 and 18C after vaccination. However, this increase was only significant for serotype 14. This might be due to the small number of children investigated and the large variability in initial antibody titers observed. Because the salivary IgG titers resembled the previous serological findings, they suggest that salivary IgG is a reflection of the serum IgG concentrations, thus supporting the hypothesis that salivary IgG is transudated from serum rather then being produced locally. Our study findings are in agreement with the results of previous trials, where similar low levels of anti-pneumococcal IgG in saliva corresponding to serum IgG concentrations were found [12,13]. Unfortunately, serum samples were not available from the children in our study. Therefore, we were unable to calculate the serum-mucosal IgG ratio.

In contrast to the mucosal IgG response, we observed a significant increase in salivary IgA levels 7 months after booster vaccination. Surprisingly, this increase was found both in the pneumococcal vaccine group and the control vaccine group. Furthermore, the IgA titers in the control vaccine group were significantly higher then in the pneumococcal vaccine group. These data suggest that a natural IgA response has occurred, rather then a vaccine-induced response. We previously showed a decrease in vaccine serotype carriage in the PCV group as compared to the controls, possibly explaining the more pronounced increase in the controls compared to the vaccinated children. Previous studies, however, have indicated the presence of a vaccine-induced mucosal IgA response upon pneumococcal conjugate vaccination [12-15]. The differences between these studies and our study are i) the age of


mean IgG
120
100
80
60
40
20
0
0 months
8 months
14 months
0 months
8 months
14 months
< 24 months
serotype 6B
> 24 months
PV group
CV group


mean IgG
120
100
80
60
40
20
0
0 months
8 months
14 months
0 months
8 months
14 months
< 24 months
serotype 14
> 24 months
PV group
CV group


mean IgG
140
120
100
80
60
40
20
0
0 months
8 months
14 months
0 months
8 months
14 months
< 24 months
serotype 18C
> 24 months
PV group
CV group


mean IgG
120
100
80
60
40
20
0
0 months
8 months
14 months
0 months
8 months
14 months
< 24 months
serotype 19F
> 24 months
PV group
CV group

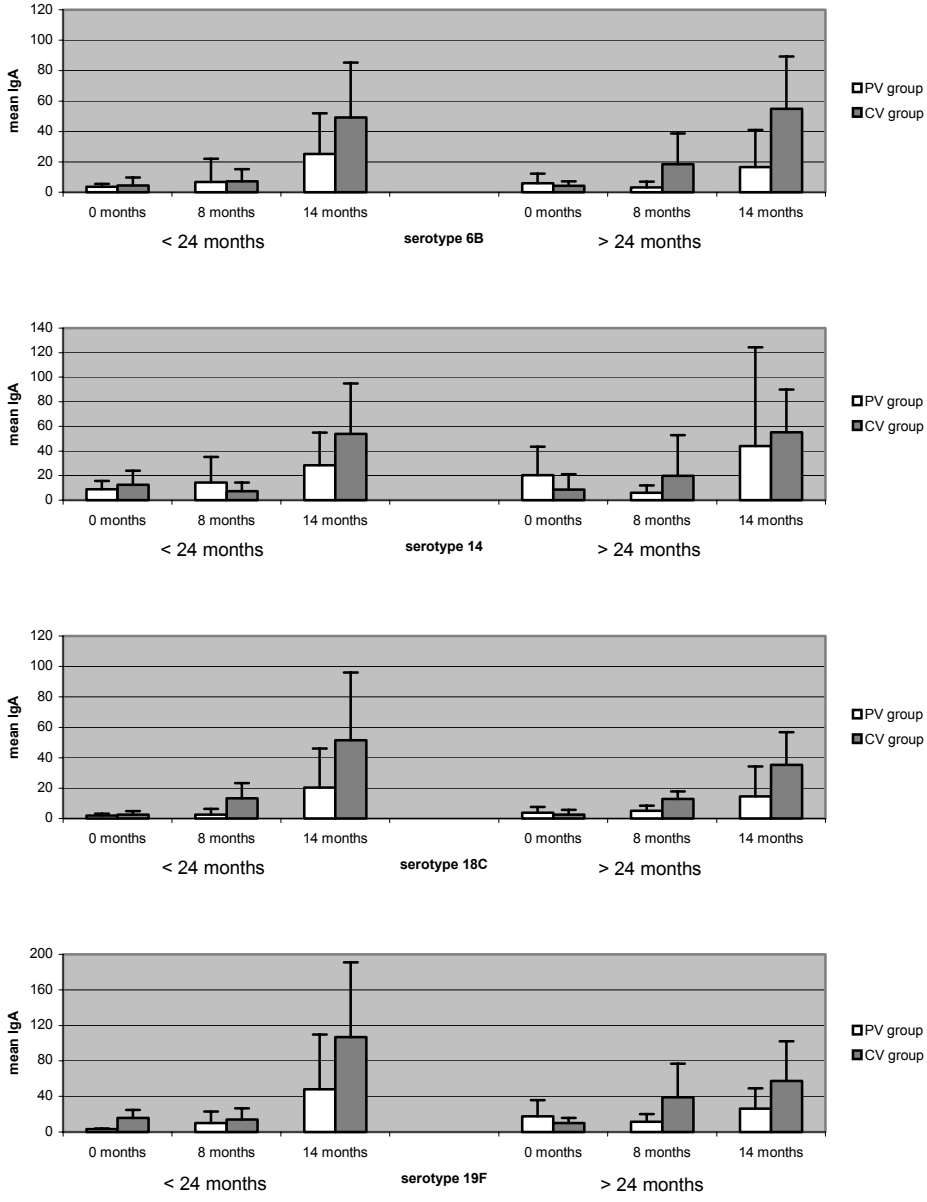


Figure 1. GMC of IgG and IgA anti-pneumococcal antibodies for the pneumococcal capsular polysaccharides 6B, 14, 18C and 19F at 0, 8 and 14 months in children vaccinated twice with pneumococcal conjugate vaccine (< 24 months of age) or once (> 24 months of age). Concentrations of IgG and IgA are depicted in µg per mg of albumin.

the vaccinees, ii) the vaccination scheme, iii) the medical history, and iv) the control group. The age of our children at study entry is on average 24 months compared to 0-12 months in the other studies. This age difference might result in differences in systemic IgG responses between the study groups, but less likely reflect differences in mucosal IgA responses, because the mucosal response has shown to mature early in life, i.e. from the age of 6 months [16,17].

Our vaccination scheme differs from previous studies, since we used one conjugate vaccination instead of a series of two, or three conjugate vaccinations in the children older then 24 months. Indeed, separate analysis of the two vaccination regimes, i.e. one or two conjugate vaccinations followed by a polysaccharide booster, showed a trend towards an increase in IgG antibodies in saliva after two conjugate vaccinations, but not after one conjugate vaccination. This is in agreement with data of Nurkka et al. who observed significantly more often salivary IgG antibodies after a booster with the pneumococcal conjugate vaccine but not with the polysaccharide vaccine [12]. In addition, these data support the observed shift from conjugate vaccine type to non-conjugate vaccine type pneumococci at the nasopharynx of children who received twice the PCV7 followed by PPS23, but not in the children who received PCV7 once (Veenhoven et al. submitted). These findings suggest that vaccination with at least twice the conjugate vaccine also after 2 years of age may be mandatory for carriage reduction of conjugate vaccine serotypes in children with recurrent otitis media. However, IgG serum titers do not explain this difference because similar serum IgG levels were found after one or two conjugate vaccinations, respectively. We hypothesize, that as a result of repeated conjugate vaccination additional B-cell recruitment occurs, which results in enhanced boosting of the mucosal immunity by natural contacts with conjugate vaccine serotype pneumococci, and consequently, in increased mucosal IgG antibody levels.

Furthermore, in our study a PPS23 booster was given after 6 months, whereas in most studies a booster with PCV7 was used. Several investigators however have compared both types of booster vaccination and have found no significant difference in immune response between these two regimens [6,13].

The children in our study have a history of recurrent acute otitis media. Therefore, they might differ immunologically from the healthy children participating in previous studies. However, the mucosal antibody response in our children prior to vaccination seemed adequate as is demontrated by the presence of detectable IgG and IgA levels in saliva. Importantly, none of the previous studies included a control group that did not receive a pneumococcal (booster) vaccination. Only one study used a control group without pneumococcal vaccination, however they sampled only at study entry and before booster

vaccination [15]. Without the results of the control group, the significant increase in secretory IgA in our study population could have been wrongly interpreted as vaccine-induced.

The follow-up period also varied between our study and previous studies. Whereas all previous studies evaluated the mucosal response of the booster vaccination one months after administration, we evaluated this response both one month and 7 months after the polysaccharide vaccination. The observed increase in antibodies occurred 7 months after the first vaccination, which has been missed by the other studies [6,12,13,15].

The main question remains why the CV group shows higher salivary IgA titers compared to the PCV group. As described previously, we found a decrease in vaccine serotype carriage in the pneumococcal vaccine group after vaccination [5]. This can be explained by elimination of the vaccine serotype strains by the passive transudation of serum IgG at the mucosa or by boosting of memory B-cells resulting in the local production of IgG. Though we were not able to show a significant increase in serotype-specific IgG in saliva presumably due to the low numbers of specimens, a trend was observed in increased GMCs for the serotypes 6B, 14 and 18C. Because pneumococcal carriage has been shown to induce a type-specific IgA response in saliva [17], the immediate drop in vaccine serotype carriage as a result of vaccination may explain the absence of an IgA response against the vaccine serotypes in these children. However, this cannot be the full explanation, since a difference in IgA titers between pneumococcal vaccine and control group was also observed for the children who were vaccinated once with pneumococcal conjugate vaccine, whereas these children did not show a significant decrease in vaccine serotype carriage as compared to controls.

In conclusion, our study suggests that pneumococcal conjugate vaccination followed by a polysaccharide booster does not elicit a persistent mucosal IgA antibody response and only a mucosal IgG response after repeated conjugate vaccinations. We hypothesize that mucosal vaccination, i.e. intranasally or orally, might sufficiently elicit a mucosal immune response that could protect against carriage as well as respiratory infection. This is supported by recent findings of Lynch et al. who have shown that intranasal vaccination with pneumococcal polysaccharides in the presence of IL-12 enhances systemic and mucosal immune responses to pneumococci and protects against pneumococcal carriage and invasive infection in a mouse model [20]. Further studies are needed to evaluate the effect of these mucosal vaccines against mucosal diseases in humans.

ACKNOWLEDGMENTS

We are thankful to S. Wiertsema for technical assistance.

REFERENCES

1. Black, S.B., Shinefield, H.R., Ling, S. *et al.* Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. *Pediatr Infect Dis J* 2002, 21(9), 810-815.
2. Black, S., Shinefield, H., Fireman, B. *et al.* Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group [In Process Citation]. *Pediatr Infect Dis J* 2000, 19(3), 187-195.
3. Fireman, B., Black, S.B., Shinefield, H.R., Lee, J., Lewis, E. & Ray, P. Impact of the pneumococcal conjugate vaccine on otitis media. *Pediatr Infect Dis J* 2003, 22(1), 10-16.
4. Eskola, J., Kilpi, T., Palmu, A. *et al.* Efficacy of a Pneumococcal Conjugate Vaccine against Acute Otitis Media. *N Engl J Med* 2001, 344(6), 403-409.
5. Veenhoven, R., Bogaert, D., Uiterwaal, C. *et al.* Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. *Lancet* 2003, 361, 2189-2195.
6. Korkeila, M., Lehtonen, H., Ahman, H., Leroy, O., Eskola, J. & Kayhty, H. Salivary anti-capsular antibodies in infants and children immunised with Streptococcus pneumoniae capsular polysaccharides conjugated to diphtheria or tetanus toxoid. *Vaccine* 2000, 18(13), 1218-1226.
7. Quataert, S.A., Kirch, C.S., Wiedl, L.J. *et al.* Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. *Clin Diagn Lab Immunol* 1995, 2(5), 590-597.
8. Hewson-Bower, B. & Drummond, P.D. Secretory immunoglobulin A increases during relaxation in children with and without recurrent upper respiratory tract infections. *J Dev Behav Pediatr* 1996, 17(5), 311-316.
9. Raux, M., Finkielsztejn, L., Salmon-Ceron, D. *et al.* Development and standardization of methods to evaluate the antibody response to an HIV-1 candidate vaccine in secretions and sera of seronegative vaccine recipients. *J Immunol Methods* 1999, 222(1-2), 111-124.
10. Dagan, R., Givon-Lavi, N., Zamir, O. *et al.* Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J Infect Dis* 2002, 185(7), 927-936.
11. Mbelle, N., Huebner, R.E., Wasas, A.D., Kimura, A., Chang, I. & Klugman, K.P. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis* 1999, 180(4), 1171-1176.
12. Nurkka, A., Ahman, H., Yaich, M., Eskola, J. & Kayhty, H. Serum and salivary anti-capsular antibodies in infants and children vaccinated with octavalent pneumococcal conjugate vaccines, PncD and PncT. *Vaccine* 2001, 20(1-2), 194-201.
13. Nurkka, A., Ahman, H., Korkeila, M., Jantti, V., Kayhty, H. & Eskola, J. Serum and salivary anti-capsular antibodies in infants and children immunized with the heptavalent pneumococcal conjugate vaccine. *Pediatr Infect Dis J* 2001, 20(1), 25-33.
14. Nieminen, T., Kayhty, H., Leroy, O. & Eskola, J. Pneumococcal conjugate vaccination in toddlers: mucosal antibody response measured as circulating antibody-secreting cells and as salivary antibodies. *Pediatr Infect Dis J* 1999, 18(9), 764-772.
15. Choo, S., Zhang, Q., Seymour, L., Akhtar, S. & Finn, A. Primary and booster salivary antibody responses to a 7-valent pneumococcal conjugate vaccine in infants. *J Infect Dis* 2000, 182(4), 1260-1263.
16. Virolainen, A., Vero, J., Kayhty, H., Karma, P., Leinonen, M. & Eskola, J. Nasopharyngeal antibodies to pneumococcal capsular polysaccharides in children with acute otitis media. *J Infect Dis* 1995, 172(4), 1115-1118.
17. Simell, B., Kilpi, T.M. & Kayhty, H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal capsular polysaccharides in children. *J Infect Dis* 2002, 186(8), 1106-1114.
18. Rynnel-Dagoo, B. & Agren, K. The nasopharynx and the middle ear. Inflammatory reactions in middle ear disease. *Vaccine* 2000, 19 Suppl 1, S26-31.
19. Faden, H.S. Immunology of the middle ear: role of local and systemic antibodies in clearance of viruses and bacteria. *Ann N Y Acad Sci* 1997, 830, 49-60.
20. Lynch, J.M., Briles, D.E. & Metzger, D.W. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. *Infect Immun* 2003, 71(8), 4780-4788.

Chapter 8.2

Host-pathogen interaction during pneumococcal infection in patients with chronic obstructive pulmonary disease

D. Bogaert, P. van der Valk, R. Ramdin, M. Sluijter, E. Monninkhof, R. Hendrix, R. de Groot and P. W. M. Hermans

Infect Immun 2004; 72(2): 818-23

ABSTRACT

Acute exacerbation is a frequent complication of chronic obstructive pulmonary disease (COPD). Recent studies suggested a role for bacteria such as *Streptococcus pneumoniae* in the development of acute exacerbation. For this study, we investigated the following in COPD patients: (i) the epidemiology of pneumococcal colonization and infection, (ii) the effect of pneumococcal colonization on the development of exacerbation, and (iii) the immunological response against *S. pneumoniae*. We cultured sputa of 269 COPD patients during a stable state and during exacerbation of COPD and characterized 115 pneumococcal isolates by use of serotyping. Moreover, we studied serum immunoglobulin G (IgG) antibody titers, antibody avidities, and functional antibody titers against the seven conjugate vaccine serotypes in these patients. Colonization with only pneumococci (monocultures) increased the risk of exacerbation, with a hazard ratio of 2.93 (95% confidence interval, 1.41 to 6.07). The most prevalent pneumococcal serotypes found were serotypes 19F, 3, 14, 9L/N/V, 23A/B, and 11. We calculated the theoretical coverage for the 7- and 11-valent pneumococcal vaccines to be 60 and 73%, respectively. All patients had detectable IgG levels against the seven conjugate vaccine serotypes. These antibody titers were significantly lower than those in vaccinated healthy adults. Finally, on average, a 2.5-fold rise in serotype-specific and functional antibodies in *S. pneumoniae*-positive sputum cultures was observed during exacerbation. Our data indicate that pneumococcal colonization in COPD patients is frequently caused by vaccine serotype strains. Moreover, pneumococcal colonization is a risk factor for exacerbation of COPD. Finally, our findings demonstrate that COPD patients are able to mount a significant immune response to pneumococcal infection. COPD patients may therefore benefit from pneumococcal vaccination.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality in adults. According to the Global Burden of Disease Study, COPD is the fifth most common disease and fourth leading cause of death in the world. Both the prevalence and mortality rate are still expected to increase in the coming decades (11). The chronic course of this disease is frequently interrupted by acute exacerbation, which has a major impact on the morbidity and mortality of COPD patients (17). Acute exacerbation of COPD is characterized by an acute sustained worsening of the patient's condition from a stable state, beyond normal day-to-day variations, which occurs one to three times a year and may warrant additional treatment (3, 18). Bacterial infections are thought to contribute to the pathogenesis and clinical course of COPD (20). Several recent studies have shown a clear association between the isolation of bacterial species such as *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae* and acute exacerbation (18). Patel et al. were the first investigators who observed a significant relation between lower airway colonization in the stable state and exacerbation frequency. However, their study population was too small to investigate the correlation for individual bacterial species (12). In contrast, Sethi et al. showed a significant increase in the occurrence of exacerbation when *S. pneumoniae* and *M. catarrhalis* were isolated (18).

We hypothesize that the prevention of bacterial growth in the airways of COPD patients will significantly decrease the occurrence of exacerbation and thus improve the quality of life for COPD patients. One of the pathogens that is frequently involved in colonization and infection of COPD patients is *S. pneumoniae*. For this pathogen, a 23-valent polysaccharide vaccine and a 7-valent conjugate vaccine are available. The 23-valent vaccine is protective against pneumococcal invasive disease in adults and elderly patients, but not in children under the age of two or immunocompromised patients (14). In addition, the 23-valent polysaccharide vaccine has not shown a significant reduction in cases of pneumonia in elderly patients (7). Moreover, the polysaccharide vaccine induces no immunological memory, which necessitates revaccination (2). In contrast, the conjugate vaccine is effective in young children against invasive as well as mucosal diseases and elicits immunological memory, but it covers fewer (7) serotypes. To investigate the relevance of pneumococcal colonization and infection in COPD patients, we determined the effect of pneumococcal colonization on the development of exacerbation. In addition, to assess the theoretical role of pneumococcal vaccination in COPD patients, we determined the serotype distribution among pneumococcal sputum isolates found during colonization and infection. Finally, we investigated the presence of immunoglobulin G (IgG) antibodies against pneumococcal capsular polysaccharides in COPD patients and the development of capsule-specific antibodies as a result of pneumococcal infection during exacerbation.

MATERIALS AND METHODS

Study population. Two hundred sixty-nine patients, aged 40 to 75 years, in the outpatient department of Medisch Spectrum Twente Hospital, Enschede, The Netherlands, participating in the COPE study and with a history of COPD, were recruited from May 1999 through March 2000 (19). The patients had to meet the following criteria: (i) a clinical diagnosis of stable COPD, as defined by American Thoracic Society criteria; (ii) no history of asthma; (iii) no exacerbation in the month prior to enrollment; (iv) current or former smoker; (v) age between 40 and 75 years; (vi) a baseline prebronchodilator forced expiratory volume in 1 s (FEV1) of 25 to 80% that predicted; (vii) a prebronchodilator ratio of FEV1 to inspiratory vital capacity value of 60% or less; (viii) reversibility value of FEV1 postinhalation of 80 μg of ipratropium bromide via a metered dose inhalator with Aerochamber of 12% the predicted value or less; (ix) a total lung capacity that was higher than the predicted total lung capacity minus 1.64 x the standard deviation; (x) no maintenance treatment with oral steroids or antibiotics; (xi) no medical condition with a low survival rate or serious psychiatric morbidity (e.g., cardiac insufficiency or alcoholism); (xii) absence of any other active lung disease (e.g., sarcoidosis). The use of medication such as nasal corticosteroids, theophyllines, chronic use of acetylcysteine, and all other bronchodilators was allowed (19).
None of the patients were vaccinated with pneumococcal vaccines before entering the study. Sputum samples were retrieved at 0, 4, 7, and 10 months, provided that the disease was stable, and during exacerbation. Serum was collected during acute exacerbation (acute-phase serum) and 2 weeks after exacerbation (convalescent-phase serum). The diagnosis of exacerbation was made based on the following criteria: (i) an objective exacerbation, with a >15% decrease in lung function (FEV1) and/or a 300-ml decrease in FEV1; (ii) a subjective exacerbation assessed by means of a symptom checklist and a 2-week diary; and (iii) a clinical exacerbation, as judged by the patient's own chest physician (19).

Sputum cultures. Sputa were collected at scheduled visits to the outpatient department and in cases of exacerbation. The sputum samples were collected in sterile vials and transported to the microbiology laboratory within 4 h. Sputum samples were judged microscopically with a low-magnification lens (x100) and had to contain <10 epithelial cells and >25 leukocytes per field to be considered representative bronchial samples. A fixed volume of a 1:100 dilution of sputum was plated onto blood agar, chocolate agar, MacConkey agar, and Sabouraud agar. The identification of bacterial growth was performed by standard techniques (10). *S. pneumoniae, H. influenzae, M. catarrhalis, Pseudomonas aeruginosa*, *Staphylococcus aureus, Aspergillus,* and *Candida* species and other gram-negative rods were considered to be potential pathogens. Other bacterial species were classified as normal flora.

Bacterial colonization was defined as the presence of bacteria in a culture retrieved from the lower respiratory tract of a clinically stable COPD patient (5). A bacterial infection was defined by the presence of one or more potential pathogens in a COPD patient with a clinical exacerbation. A monoculture was defined as the growth of a single bacterial species, whereas the presence of multiple bacterial species was defined as a mixed culture.

Serotyping of pneumococcal isolates. In total, 115 pneumococcal isolates were cultured and serotyped by the capsular swelling method (Quellung reaction), using commercially available antisera (Statens Serum Institute, Copenhagen, Denmark) and microscopic observation.

EIA for measuring anti-pneumococcal polysaccharide IgG concentrations.

Concentrations of IgG antibodies were measured by an enzyme immunoassay (EIA) method as described previously (9). The results are given in micrograms per milliliter, calculated from the officially assigned IgG values of the 89-SF reference serum (15), or were converted into units per milliliter by comparison with the 89-SF reference serum, which was considered to contain 100 U/ml, in cases for which no IgG values were available.

EIA for measuring the avidity of anti-pneumococcal polysaccharide antibodies.

The relative avidities of IgG antibodies for pneumococcal capsular polysaccharides were determined by the EIA method as described by Anttila et al. (1), with some minor modifications. Briefly, microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with 100 μl of coating solution, containing capsule polysaccharides at a concentration of 10 μg/ml (LGC Promochem, Teddington, United Kingdom), per well. Sera were diluted 1:50 in phosphate-buffered saline (PBS) containing 5% cell wall polysaccharides (Statens Serum Institute) and incubated at 4°C overnight. Sera were diluted 1:100 in PBS-0.05% Tween 20, and threefold serial dilutions were incubated for 2 h at 37°C. After washing, 0.5 M sodium thiocyanate in PBS was added to dissociate antibody-antigen complexes. After 15 min of incubation at room temperature, the plates were washed and antibody binding was detected by the addition of alkaline-phosphatase-conjugated anti-human IgG (Sigma, St. Louis, Mo.). The color was developed by the substrate p-nitrophenyl phosphate (Sigma). The absorbance was measured at 405 nm on an EIA reader (SpectraMAX; Molecular Devices, Sunnyvale, Calif.). Results were expressed as avidity index (AI) values, assigned as the percentages of antibodies that remained bound to the antigens after thiocyanate treatment. The high-avidity antibody concentration was calculated by multiplying the antibody concentration by the AI value. The reproducibility of the assay was checked by including a control serum in each plate.

Data analysis. The results were analyzed with the statistical software SPSS 10. The effect of pneumococcal colonization on the time to the first exacerbation episode was assessed by

using multivariate Cox proportional hazard analyses. We adjusted for potential confounding variables, including age, sex, smoking status, number of exacerbation episodes in the preceding year, and FEV1% predicted, as found previously (P. van der Valk, D. Bogaert, E. Monninkhof, J. van der Palen, P. Hermans, G. Zielhuis, C. van Herwaarden, and R. Hendriks, submitted for publication). The initial concentrations of antibodies are depicted as geometric mean concentrations (GMC). Avidities are given as the means of the AI values and high-avidity antibody concentrations. Statistical comparisons between antibody levels were performed by analysis of variance and independent sample t tests. The maximum increase in antibody titer was tested for significance by using the one-sample *t*-test. Statistical significance was set at P values of <0.05.

RESULTS

Sputum samples from 269 patients were obtained at 0, 4, 7, and 10 months in cases of stable disease. An additional sputum sample was collected at each hospital visit for an acute exacerbation of COPD. In total, 55% of the patient group developed at least one exacerbation episode during the follow-up period. In total, 918 stable state sputa and 241 exacerbation sputa were collected. Of the stable state sputa, 603 cultures were negative for potential pathogens, whereas 315 cultures were positive for at least one microorganism (34%). The exacerbation sputa showed significantly more positive cultures (49%). Mixed cultures were found in 9 and 5% of the sputum cultures during stable state and exacerbation, respectively. Monocultures were found significantly more often during exacerbation episodes (41%) than during stable states (26%). The three predominant bacterial species cultured during a stable state and exacerbation were *H. influenzae* (19 and 26%, respectively), *S. pneumoniae* (13 and 13%, respectively), and *M. catarrhalis* (9 and 7%, respectively).

We calculated the effect of the colonization status at the time of randomization on the time to the next exacerbation episode for 209 patients from whom sputum was available at that time. We adjusted for potential confounding variables, including age, sex, smoking status, number of exacerbations in the preceding year, and FEV1% predicted. Bacterial colonization in general did not increase the risk of a first exacerbation compared to noncolonized patients, nor did pneumococcal colonization in general (hazard ratio, 1.31; 95% confidence interval [CI], 0.743 to 2.305). However, the adjusted hazard ratio for the development of exacerbations in patients with a pneumococcal monoculture was 2.93 (95% CI, 1.41 to 6.07).

We investigated all 115 pneumococcal isolates in detail by means of serotyping. The most prevalent serotypes were serotypes 19F and 3 (13 and 10%, respectively), followed by

serotypes 14, 9L/N/V, 23A/B, and 11 (9% each) (Table 1). The theoretical vaccine coverages for the 7-valent and 11-valent pneumococcal conjugate vaccines and the 23-valent polysaccharide vaccine were 34, 47, and 70%, respectively. Inclusion of the expected cross-reactive serotypes increased the theoretical coverages to 60, 73, and 88%, respectively. A separate analysis of the potential vaccine coverage of pneumococcal isolates found during a stable state and exacerbation showed no significant difference (Table 1).

Table 1. Most prevalent pneumococcal serotypes observed in COPD patients.

VT serotype	Total number of isolates (%)		Stable state isolates (%)		Exacerbation Isolates (%)	
14	10	8.7%	5	5.5%	5	20.0%
19F	15	13.0%	12	13.3%	3	12.0%
23F	8	7.0%	7	7.8%	1	4.0%
NVT serotype						
3	12	10.4%	11	12.2%	1	4.0%
6A*	9	7.8%	7	7.7%	2	8.0%
9 L/N*	10	8.7%	5	5.6%	5	20.0%
11	9	7.8%	6	6.7%	3	12.0%
23A/B*	10	8.7%	9	10.0%	1	4.0%
7-valent	39	33.9%	30	33.3%	9	36.0%
7-valent**	69	60.0%	52	57.7%	17	68.0%
11-valent	54	47.0%	44	48.9%	10	40.0%
11-valent**	84	73.0%	66	73.3%	18	72.0%
23-valent	80	69.6%	61	56.8%	19	76.0%
23-valent**	101	78.8%	78	67.8%	23	92.0%
Total	115	100%	90	100%	25	100%

* cross-reactive serotypes
** including cross-reactive serotypes

We investigated the presence of IgG antibodies and the AI of these antibodies for the serotypes included in the 7-valent conjugate vaccine (4, 6B, 9V, 14, 18C, 19F, and 23F) for serum samples obtained from 92 patients. For this purpose, we included all first serum samples obtained during the study period. Antibody levels against all seven serotypes were detectable in all patients. The GMCs of antibodies against the seven serotypes for the COPD patients as well as for 10 healthy adult volunteers before and after vaccination with the 7-valent conjugate vaccine are depicted in Table 2. The GMCs ranged from 5.04 µg/ml for serotype 4 to 55.76 for serotype 6B, with a large variability (Fig. 1A). The GMCs for healthy adults ranged from 1.7 (serotype 4) to 21.9 (serotype 6B) µg/ml before vaccination to 13.4 (serotype 9V) to 76.7 (serotype 6B) µg/ml after conjugate vaccination. The mean AIs as well as the mean high-avidity antibody concentrations with 95% CIs are also shown in

Table 2. GMC (μg/ml) of anti-pneumococcal antibodies against the 7 conjugate vaccine pneumococcal serotypes.

Pneumococcal serotype	COPD patients	(95% CI)	healthy adults	(95% CI)	healthy adults after conjugate vaccination	(95% CI)
4	5.04	(3.93-6.15)	1.65*	(0.48-2.81)	19.95**	(5.99-33.92)
6B	55.76	(40.83-70.70)	21.9	(7.02-36.84)	76.70	(46.87-106.53)
9V	16.38	(12.22-20.54)	3.39*	(0.85-5.95)	13.41	(8.51-18.31)
14	7.06	(5.11-9.01)	3.37	(0.28-6.47)	30.74**	(14.22-47.26)
18C	17.49	(12.59-22.40)	9.37	(3.80-22.54)	56.53**	(27.23-85.83)
19F	36.34	(26.29-46.40)	18.40	(4.97-31.82)	66.76	(34.61-98.90)
23F	18.08	(13.13-23.04)	4.08	(0.64-7.50)	55.43**	(17.65-93.21)

* significantly lower antibody level compared to patient group
** significantly higher antibody level compared to patient group

Table 3. Serotype-specific IgG concentration in initial and convalescent serum and the maximum increase in serotype-specific IgG of 15 COPD patients with positive sputum cultures for *S.pneumoniae* during exacerbation

Patient	Serotype	Male/ Female	Age (years)	Flixotide (yes/no)	Serotype specific IgG (U/ml)		
					Initial serum	Convalescent serum	Increase
1	14	M	66	Yes	97,92	81,25	0,83
2	14	M	73	Yes	95,24	428,57	4,50
3	9N	M	47	No	156	146,43	0,94
4	19F	V	66	No	1000	1300	1,30
5	19F	M	57	No	75	100	1,33
6	9N	M	58	Yes	7000	9066,67	1,30
7	19F	M	73	No	1000	1800	1,80
8	9V	M	64	No	971,43	1540	1,59
9	9V	M	67	Yes	1500	9500	6,33
10	9N	M	56	No	500	5000	10,00
11	14	M	61	Yes	105,87	95,24	0,90
12	9N	M	61	Yes	15733,33	9066,67	0,58
13	11	M	60	Yes	950	450	0,47
14	14	M	63	Yes	31,67	50	1,58
15	9V	M	63	Yes	2000	5250	2,63

Fig. 1B and C. The AIs ranged from 55% for serotype 6B to 78% for serotype 18C. The high-avidity antibody titers ranged from 3.4 μg/ml for serotype 4 to 30.7 μg/ml for serotype 6B.

Because the studied serum samples were collected randomly, i.e., during both a stable state and exacerbation, we reanalyzed our data with respect to this variable. No significant difference in antibody titers against the seven tested serotypes was observed between samples obtained during exacerbation and during a stable state (data not shown).

We investigated the development of antibodies in response to colonization with pneumococci during acute exacerbation. In total, 15 patients, who were colonized with

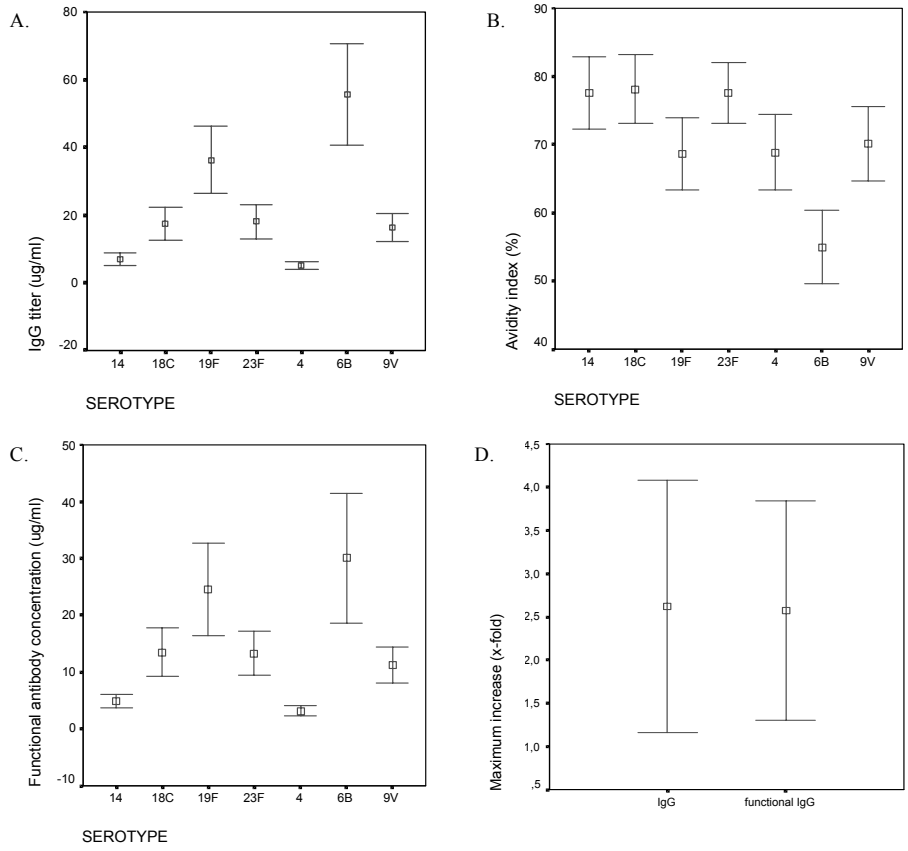


Figure 1. Capsular antibody titers against the seven pneumococcal conjugate vaccine serotypes in 92 COPD patients at the start of the study. Error bars show 95% CIs for the GMCs for IgG antibodies (A), AIs (B), and high-avidity antibody titers (C). (D) Error bars show 95% CIs for mean maximum antibody increases and mean maximum high-avidity antibody increases for the homologous serotype during exacerbation.

serotypes 14 (4), 9N (4), 9V (3), 19F (3), and 11 (1), were studied during an exacerbation. We investigated the antibody levels in serum and the AIs of the antibodies against the capsular polysaccharides of the pneumococcal serotypes isolated from the patients. When available, two or three consecutive serum samples were analyzed, as initial, acute-phase, and convalescent-phase samples. Furthermore, we calculated the maximum increase in antibody titer in the consecutive sera for individual patients (Table 3). We observed a significant rise in antibodies as well as in high-avidity antibodies, with an average 2.5-fold increase (Fig. 1D). The mean avidity, which was 70%, did not change significantly.

DISCUSSION

We investigated the effect of pneumococcal colonization on the development of exacerbation in COPD patients. Although patients colonized with bacteria in general did not show an increased risk of a first exacerbation episode compared to patients without bacterial colonization, colonization with *S. pneumoniae*, specifically when present as a monoculture, did increase the risk of a first exacerbation, with a hazard ratio of 2.91. Our data support the findings of Sethi et al., who also showed a significant increase in exacerbations when *S. pneumoniae* was isolated (18), which underscores the role of *S. pneumoniae* in the pathogenesis of exacerbations of COPD. Surprisingly, the presence of pneumococci in combination with other pathogens in sputum did not cause an increased risk of a next exacerbation. This might indicate that monocultures merely represent an infectious state, whereas mixed cultures are more representative of a stable state of colonization. This is supported by our data, which show that during exacerbations of COPD, monocultures of potential pathogenic microorganisms, in particular, are increased. Because this specific correlation has never been investigated, it will be worthwhile to include this variable in future analyses.

Subsequently, we calculated the theoretical coverage of the 7-valent, 11-valent, and 23-valent pneumococcal vaccines. The most prevalent serogroups among the 115 isolates investigated were serogroups 3, 9, 11, 14, 19, and 23 (10, 9, 8, 9, 14, and 16%, respectively). Because most of these serogroups are included in the conjugate vaccine, the theoretical coverage for these vaccines is 34 to 49% of all pneumococci, but is even higher (60 to 73%) when cross-reactive serotypes are considered. An analysis of just exacerbation cultures showed comparable coverage rates. This is in agreement with the data of Flamaing et al., who investigated the serotype distribution among pneumococcal isolates obtained from individuals 65 years of age and older with invasive disease in Belgium (6). The potential coverage of the 23-valent conjugate vaccine is even higher, with 70 to 88% coverage. Unfortunately, most previous studies could not find a protective effect of this vaccine against mucosal diseases.

Secondly, we investigated whether natural antibodies were already present in COPD patients but failed to protect these patients from pneumococcal colonization. GMCs for the 92 COPD patients investigated were normal to high when compared to healthy adults (M. Sluijter, unpublished observations). Moreover, as in healthy adults, GMCs for serotypes 4 and 14 were relatively low compared to the other serotypes. Finally, the quality of the antibodies, as measured by means of AIs, was sufficient. Therefore, we conclude that these patients are able to elicit an immunological response to pneumococcal challenge and thus are not immunologically deficient with respect to antibody development. The trend towards higher titers compared to healthy unvaccinated adults may represent the increased

pneumococcal contacts in this patient group, although a difference in gender (50 and 98% male, respectively) and smoking history (0 and 100%, respectively) between the healthy adults and the patient group may also be of influence (16).

Healthy adults vaccinated once with the 7-valent conjugate vaccine displayed higher GMCs than the patient group, although they were only significant for serotypes 4, 14, 18C, and 23F due to the relatively large CIs. Because a similar response from our patient group can be expected upon vaccination, we suggest that vaccination of our patient group may increase antibody levels and hence may elicit a higher level of protection against pneumococcal disease. This is in agreement with the results of Jonsson et al., who have shown that vaccination of COPD patients with a serotype 6B conjugate vaccine or a 23-valent polysaccharide vaccine elicits antibody levels comparable to those in vaccinated healthy adults (8). One might question, however, whether the present levels of antibodies prevent both colonization and infection with pneumococci in our patients. It has been hypothesized that the prevention of mucosal disease requires higher levels of anti-pneumococcal antibodies than the prevention of invasive disease. Previous work has shown that higher antibody levels are necessary to protect children against acute otitis media than against invasive diseases (13). Alternatively, one might suggest that anatomical damage to the lungs and the thick mucous layers prevents the immune system from recognizing or attacking colonizing bacteria. This is supported by the data of Davis et al., who have shown that vaccination of COPD patients with a 14-valent pneumococcal polysaccharide vaccine induces significant antibody responses but not protection against pneumonia and death (4). In contrast to our findings, most pneumococci observed during infection in that study were nonvaccine serotypes, which may explain the observed vaccine failure. We determined whether the presence of pneumococci during exacerbation elicits a natural immune response to the homologous serotype. Overall, a 2.5-fold increase in IgG antibody levels and high-avidity antibody levels was found. Although a small patient group was studied and a small number of consecutive sera per patient were available, these data suggest that the immune systems of two-thirds of the COPD patients were capable of mounting an antibody response towards pneumococci, whereas one-third of the patients did not show an increase in antibodies upon the presence of pneumococci. Obviously, one could argue whether these antibodies protect COPD patients against pneumococcal reinfection. Follow-up studies are necessary in which the protective potentials of these antibodies are investigated in order to speculate about the effect of pneumococcal conjugate vaccination in COPD patients.

In conclusion, our data indicate that pneumococcal colonization in COPD patients is frequently caused by vaccine serotype strains. Moreover, pneumococcal colonization is a risk factor for exacerbations in these patients. Finally, our findings show that the majority of COPD patients mount an anticapsular immune response during pneumococcal colonization

and infection. Clinical studies are needed to investigate the protective potentials of conjugate vaccination for COPD patients.

ACKNOWLEDGMENTS

This study was financially supported by the Sophia Foundation for Medical Research, Rotterdam, The Netherlands (grant 268), and the Dutch Science Foundation (grant SGO-Inf. 005).

REFERENCES

1. Anttila, M., J. Eskola, H. Ahman, and H. Kayhty. 1998. Avidity of IgG for *Streptococcus pneumoniae* type 6B and 23F polysaccharides in infants primed with pneumococcal conjugates and boosted with polysaccharide or conjugate vaccines. J. Infect. Dis. 177:1614-1621.
2. Artz, A. S., W. B. Ershler, and D. L. Longo. 2003. Pneumococcal vaccination and revaccination of older adults. Clin. Microbiol. Rev. 16:308-318.
3. Burge, S., and J. A. Wedzicha. 2003. COPD exacerbations: definitions and classifications. Eur. Respir. J. 41(Suppl.):46S-53S.
4. Davis, A. L., C. P. Aranda, G. Schiffman, and L. C. Christianson. 1987. Pneumococcal infection and immunologic response to pneumococcal vaccine in chronic obstructive pulmonary disease. A pilot study. Chest 92:204-212.
5. Ewig, S., R. Rodriguez-Roisin, and A. Torres. 2002. Indications for and choice of antibiotics in COPD, p. 201-220. In T. Similowski, W. Whitelaw, and J. Derenne (ed.), Clinical management of chronic obstructive pulmonary disease. Marcel Dekker, New York, N.Y.
6. Flamaing, J., J. Verhaegen, and W. E. Peetermans. 2002. *Streptococcus pneumoniae* bacteraemia in Belgium: differential characteristics in children and the elderly population and implications for vaccine use. J. Antimicrob. Chemother. 50:43-50.
7. Jackson, L. A., K. M. Neuzil, O. Yu, et al. 2003. Effectiveness of pneumococcal polysaccharide vaccine in older adults. N. Engl. J. Med. 348:1747-1755.
8. Jonsson, S., G. Vidarsson, H. Valdimarsson, G. Schiffman, R. Schneerson, and I. Jonsdottir. 2002. Vaccination of COPD patients with a pneumococcus type 6B tetanus toxoid conjugate vaccine. Eur. Respir. J. 20:813-818.
9. Kayhty, H., H. Ahman, P. R. Ronnberg, R. Tillikainen, and J. Eskola. 1995. Pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine is immunogenic in infants and children. J. Infect. Dis. 172:1273-1278.
10. Lenette, E., A. Balows, W. Hauser, Jr., and H. Shadomy (ed.). 1985. Manual of clinical microbiology. American Society for Microbiology, Washington, D.C.
11. Murray, C. J., and A. D. Lopez. 1997. Alternative projections of mortality and disability by cause. 1990-2020 Global Burden of Disease Study. Lancet 349:1498-1504.
12. Patel, I. S., T. A. Seemungal, M. Wilks, S. J. Lloyd-Owen, G. C. Donaldson, and J. A. Wedzicha. 2002. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. Thorax 57:759-764.
13. Pelton, S. I., R. Dagan, B. M. Gaines, K. P. Klugman, D. Laufer, K. O'Brien, and H. J. Schmitt. 2003. Pneumococcal conjugate vaccines: proceedings from an interactive symposium at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy. Vaccine 21:1562-1571.
14. Poland, G. A. 1999. The burden of pneumococcal disease: the role of conjugate vaccines. Vaccine 17:1674-1679.
15. Quataert, S. A., C. S. Kirch, L. J. Wiedl, D. C. Phipps, S. Strohmeyer, C. O. Cimino, J. Skuse, and D. V. Madore. 1995. Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. Clin. Diagn. Lab. Immunol. 2:590-597.
16. Sankilampi, U., R. Isoaho, A. Bloigu, S. L. Kivela, and M. Leinonen. 1997. Effect of age, sex and smoking habits on pneumococcal antibodies in an elderly population. Int. J. Epidemiol. 26:420-427.
17. Seemungal, T. A., G. C. Donaldson, A. Bhowmik, D. J. Jeffries, and J. A. Wedzicha. 2000. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 161:1608-1613.
18. Sethi, S., N. Evans, B. J. Grant, and T. F. Murphy. 2002. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. N. Engl. J. Med. 347:465-471.
19. van der Valk, P., E. Monninkhof, J. van der Palen, G. Zielhuis, and C. van Herwaarden. 2002. Effect of discontinuation of inhaled corticosteroids in patients with chronic obstructive pulmonary disease: the COPE study. Am. J. Respir. Crit. Care Med. 166:1358-1363.
20. Wedzicha, J. A. 2002. Exacerbations: etiology and pathophysiologic mechanisms. Chest 121:136S-141S.

Chapter 8.3

Multiplex opsonophagocytosis assay (MOPA): a useful tool for the monitoring of the 7-valent pneumococcal conjugate vaccine

D. Bogaert, M. Sluijter, R. de Groot and P.W.M. Hermans

Vaccine; accepted for publication

ABSTRACT

Pneumococcal conjugate vaccination is highly efficacious against invasive diseases in young children. Since host protection is mainly mediated by opsonin-dependent phagocytosis, the in vitro measurement of opsonophagocytic activity of the anti-capsular antibodies is assumed to be a reliable correlate of protection to monitor vaccine efficacy. Unfortunately, the methods used so far are all tedious to perform and material consuming. Therefore, we modified the multi-specificity opsonophagocytosis killing assay (MSOPKA) into a high throughput method, which simultaneously measures the opsonophagocytosis against the seven serotypes covered by the current conjugate vaccine in a single assay. In the so called multiplex opsonophagocytosis assay (MOPA), a mixture containing equal numbers of colony forming units (CFUs) of chloramphenicol-resistant serotype 4, spectinomycin-resistant serotype 6B, streptomycin-resistant serotype 9V, erythromycin-resistant serotype 14, rifampicin-resistant serotype 18C, tetracycline-resistant serotype 19F, and trimethoprim-resistant serotype 23F pneumococci was used as a target mixture and incubated with serial dilutions of test serum. After opsonophagocytosis by differentiated HL-60 cells in the presence of complement, the samples were spotted onto different blood agar plates containing the 7 selective antibiotics, respectively. Opsonophagocytosis was calculated as the highest serum dilution resulting in 90% or more reduction in CFUs. The data obtained by this assay correlated well with the data obtained by the MSOPKA. In conclusion, the MOPA simultaneously measures opsonophagocytosis capacity of serum against the capsular serotypes included in the 7-valent pneumococcal conjugate vaccine in a high-throughput fashion, requiring low volumes of patient sera.

INTRODUCTION

Streptococcus pneumoniae is one of the major pathogens causing serious respiratory tract and invasive infections [1]. Risk groups for these infections are young children under the age of two years, immunocompromised patients and elderly people. Therefore, prevention of pneumococcal infections by a 23-valent polysaccharide vaccine has been recommended by the Advisory Committee on Immunization Practices (ACIP) in these risk groups from the age of two years [2]. However, the 23-valent polysaccharide vaccine has shown to be less immunogenic in children under two years of age. Therefore, conjugate vaccines have been developed which contain the capsular polysaccharides of the seven to eleven most predominant pneumococcal serotypes linked to a protein carrier. These vaccines have shown to be immunogenic in risk groups and protect against invasive diseases in young children [3,4]. One of the vaccines, the 7-valent pneumococcal conjugate vaccine from Wyeth, has been released in the United States and Europe as preventive strategy against invasive pneumococal diseases.

Because host protection is mainly mediated by opsonin-dependent phagocytosis, the in vitro measurement of opsonophagocytic activity of the anti-capsular antibodies is believed to be a reliable correlate of protection to evaluate the vaccine efficacy [5,6]. Several techniques have been standardized and validated so far, and they all have shown to be comparable [7]. Replacement of human blood leucocytes with HL-60 cell-line derived granulocytes as effector cells and frozen aliquots of bacteria as target cells have improved the opsonophagocytosis assay significantly [8]. However, the current techniques are still time- and material consuming, and require large volumes of serum to test protection against all vaccine serotypes. The latter problem is certainly relevant for the main target group for the vaccines, i.e. young children. Therefore, Nahm *et al.* have modified the classical opsonophagocytosis assay (OPA) into a multi-specificity opsonophagocytic killing assay (MSOPKA) [9]. In this assay, a mixture of two antibiotic-resistant pneumococci of different serotypes is used as target in a single opsonophagocytosis assay, thus reducing the amount of labor time, volume of serum and reagents needed. However, this assay is still fairly time-, serum- and material consuming, and thus less suitable for high-throughput use in children. Therefore, we extended the assay to seven antibiotic-resistant serotypes, i.e. the seven serotypes covered by the 7-valent conjugate vaccine, which can be used in a single assay. Furthermore, we modified the read-out method by incorporating the method used in the classical complement-killing assay for *N. meningitidis* [10].

MATERIAL AND METHODS

Pneumococcal strains. Six *S. pneumoniae* strains of serotype 6B, 9V, 14, 18C, 19F and 23F, respectively, were kindly provided by R. Veenhoven, Spaarne Hospital, Haarlem, The Netherlands. These strains were nasopharyngeal isolates obtained from children participating in the OMAVAX study [11]. Strain 1124478 (serotype 19F), strain 1474912 (serotype 14), strain 1212458 (serotype 23F) and strain 1026409 (serotype 18C) were selected because of single drug resistance to tetracycline, erythromycin, trimethoprim and rifampicin, respectively.

Strain 1344387 representing serotype 6B and strain 1081748 representing serotype 9V were fully susceptible strains. A spectinomycin-resistant variant of strain 1344387 displaying a minimal inhibitory concentration (MIC) of > 400 mg/L (strain R6BSPCR) was obtained by natural selection on 5% blood agar plates with increasing concentrations of spectinomycin. A streptomycin-resistant variant of strain 1081748 was obtained by natural selection on 5% blood agar plates with increasing concentrations of streptomycin, resulting in a mutant strain with a MIC of > 400 mg/L (strain R9VSPCR). A chloramphenicol-resistant strain of serotype 4 (RT4CHLOR) was obtained by natural transformation of the serotype 4 strain KNR.7/87 with the chloramphenicol resistance cassette as described previously [12].

MICs of the pneumococcal strains were determined for the antibiotics tetracycline, erythromycin, trimethoprim, spectinomycin, chloramphenicol, rifampicin and streptomycin by the agar dilution test as described previously [13].

The serotypes of the pneumococci were confirmed by the capsular swelling method (Quellung reaction) observed microscopically using commercially available antisera (Statens Serum institute, Copenhagen, Denmark).

Pneumococcal cultures. The seven drug-resistant strains were plated on 5% sheep blood agar plates with the respective selective antibiotics and grown overnight under reduced oxygen conditions at 37ºC. The next day, several colonies were selected from the plate and grown to early logarithmic phase (OD 0.1-0.2) in Todd Hewitt broth supplemented with 0.5% yeast extract (THY broth). The bacteria were aliquoted in 17% glycerol and stored at -80ºC until use. Spot counts of the stocks were made on 5% blood agar plates as well as on the respective selective antibiotic plates to determine the number of viable CFUs/ml and to confirm the resistance profile. Pneumococci were stored for a maximum period of 3 months without any detectable decrease in viability.

Serum samples. Six adult volunteers were immunized with the 7-valent pneumococcal conjugate vaccine Prevenar (Wyeth, The Netherlands). Serum samples were obtained from the vaccinated volunteers 28 days after the vaccination was administered, as well as from 3 non-vaccinated adult controls.

Growth and differentiation of HL-60 cell line. Growth and differentiation of HL-60 cells was performed as described previously [8]. Briefly, undifferentiated HL-60 cells were grown in RPMI-1640 culture medium with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Differentiation was performed by incubating the cells in the presence of 0.8% dimethylformamide (Fisher Scientific, Pittsburgh, USA) for 7 days with renewal of the medium at day 5. Before use in the opsonophagocytosis assay, the cells were washed twice with and resuspended in RPMI-1640 containing 10% FCS.

Opsonophagocytosis assay. The opsonophagocytosis assay is modified from the MSOPKA described by Nahm et al. [9]. In summary, serial dilutions of test sera in the range of 1:2 to 1:128 were made in RPMI-1640 in a 96-well cell culture plate with 30 μl of (diluted) serum per well. Stocks of the seven pneumococcal serotype strains were washed once in RPMI-1640, and a bacterial mixture was made containing 2 x 10^5 CFUs/ml of each strain. 15 μl of this bacterial mixture was added to the serum dilutions and incubated for 20 minutes at 37°C. After incubation, 60 μl of HL-60 suspension and 15 μl of rabbit complement (Pelfreeze, Browndeer, USA) were added to each well. The mixtures containing final serum dilutions within the range of 1:16 to 1:1024, and bacteria and granulocytes in a ratio of 1:400 were incubated for 1 h at 37°C. After incubation, the mixtures were plated onto blood agar plates. Two read-out systems were tested: (i) 10 μl of each mixture was spotted onto each of 7 omni-trays (Nunc, Naperville, IL) containing 5% blood agar with 400 mg/L of streptomycin, 4 mg/L of chloramphenicol, 400 mg/L of spectinomycin, 1.5 mg/L of rifampicin, 16 mg/L of tetracycline, 1 mg/L of erythromycin and 32 mg/L of trimethoprim, i.e the multiplex opsonophagocytosis assay (MOPA), and (ii) 10 μl of mixture was spotted and carefully run out on the antibiotic-containing agar plates allowing counting of the individual colonies up to 100%, according to the original method, i.e. MSOPKA. In addition, 10 μl of the serial dilution of the bacterial mixture without serum was spotted onto each of the 7 different antibiotic plates to calculate the exact CFUs per well. Finally, the seven resistant strains were spotted individually onto the antibiotic trays to confirm monoresistance and selectivity of the antibiotic plates. The selective plates were incubated overnight under reduced oxygen conditions. Colonies counted on the streptomycin-containing blood agar trays were identified as those of the 9V serotype. Similarly, colonies present on the chloramphenicol, spectinomycin, rifampicin, tetracycline, erythromycin and trimethoprim trays were exclusively of the serotypes 4, 6B, 18C, 19F14 and 23F, respectively. Using the MOPA, the opsonophagocytosis titer was expressed as the reciprocal of the highest serum dilution with 90% killing of bacteria as compared to the number of bacteria counted in the controls without serum (range 16 to 1024). The MSOPKA method expressed the opsonophagocytosis titer as the reciprocal of the highest serum dilution with 50% killing as

compared to the bacterial growth in the controls without serum. Serum samples with titers of <16 were reported as a titer of 8 for the purpose of data analysis.

The assay was tested for non-specific killing of bacteria by serum, complement and HL-60 cells. Non-specific interference of serum or complement did not occur. Repeated washing of the HL-60 cells with RPMI-1640 was required to eliminate the toxic effect of the dimethylformamide and penicillin and/or streptomycin from the culture medium on pneumococcal growth. Washing of the bacterial stocks before use eliminated the interference of dead bacteria and/or degradation products during opsonophagocytosis.

Enzyme-immunoassay (EIA) for measuring anti-pneumococcal polysaccharide IgG concentrations. Concentrations of IgG antibodies were measured from the test sera by EIA method as described previously [14]. The results are given as μg/ml calculated from the officially assigned IgG values of the 89-SF reference serum [15].

Statistical analysis. Antibody titers from controls and vaccinees were compared using the Mann-Whitney U test. The opsonophagocytosis titers and ELISA IgG titers were converted to a log2 base and analyzed for correlation by the Pearson's correlation coefficient using the statistical software SPSS 11. Significance was set at a p-value < 0.05.

Table 1. MICs (mg/L) of the seven serotype strains for the seven selective antibitiotics used in the MOPA.

	MIC (mg/L)						
	14	4	19F	6B	18C	9V	23F
Erytromycin	**4.0**	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Chloramphenicol	1.0	**9.0**	1.0	1.0	2.0	1.0	1.0
Tetracycline	<1.0	<1.0	**32**	<1.0	<1.0	<1.0	<1.0
Spectinomycine	50	35	100	**>400**	50	25	100
Rifampicin	<0.25	0.5	0.5	0.5	**4.0**	0.25	0.25
Streptomycin	50	65	50	50	50	**>400**	50
Trimethoprim	4.0	<2.0	4.0	4.0	<2.0	2.0	**>32**

RESULTS

We developed a multiplex opsonophagocytosis assay to test the opsonophagocytosis activity of serum against the seven capsular serotypes included in the 7-valent pneumococcal conjugate vaccine.

The seven pneumococcal strains representing the vaccine serotypes used in the multiplex assay were first tested for cross resistance. The MICs of the seven vaccine serotype strains for the seven different antibiotics are depicted in Table 1. The concentration of antibiotics used for the selective growth of the seven strains was one-fourth of the MIC of the resistant strains. The culture plates were demonstrated to be selective for the individual strains.

We tested the MOPA using sera from nine adult volunteers. The sera were obtained from six volunteers one month after vaccination with the 7-valent pneumococcal conjugate vaccine

and from three non-vaccinated volunteers. We determined the serotype-specific IgG concentration against the seven conjugate vaccine serotypes for the sera of both the vaccinees and the controls (Figure 1).

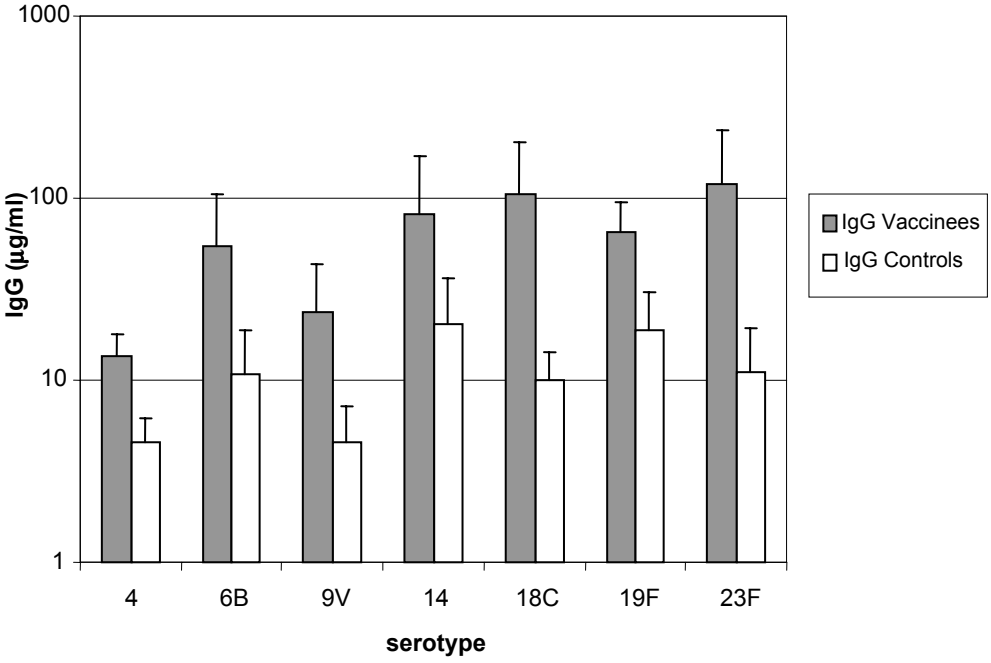


Figure 1. Average IgG antibody titers for vaccinees and controls for the 7 conjugate vaccine serotypes. The standard deviations are depicted. * Mann-Whitney U test: $p < 0.05$, ** Mann-Whitney U test: $p = 0.05$

gG antibodies were detectable for all sera. On average, the vaccinees showed 3-11 times higher serotype-specific IgG titers compared to the controls, which was significant for all serotypes except for the serotypes 9V and 23F.

We tested the opsonophagocytosis activity of the sera using the MOPA (Figure 2). The assay was performed twice. The reproducibility of the assay was high and varied within only one dilution for most analyses. Opsonophagocytosis activity was highest for the capsular serotypes 14, 18C, 19F and 23F, and lowest for the serotypes 6B and 9V (Table 2). From the vaccinated persons 6 out of 42 determinations were below the MOPA detection limit. From the unvaccinated volunteers 20 out of the 21 determinations were below the MOPA detection limit. Undetectable serotype-specific titers were seen merely in individuals with low serotype-specific IgG titers.

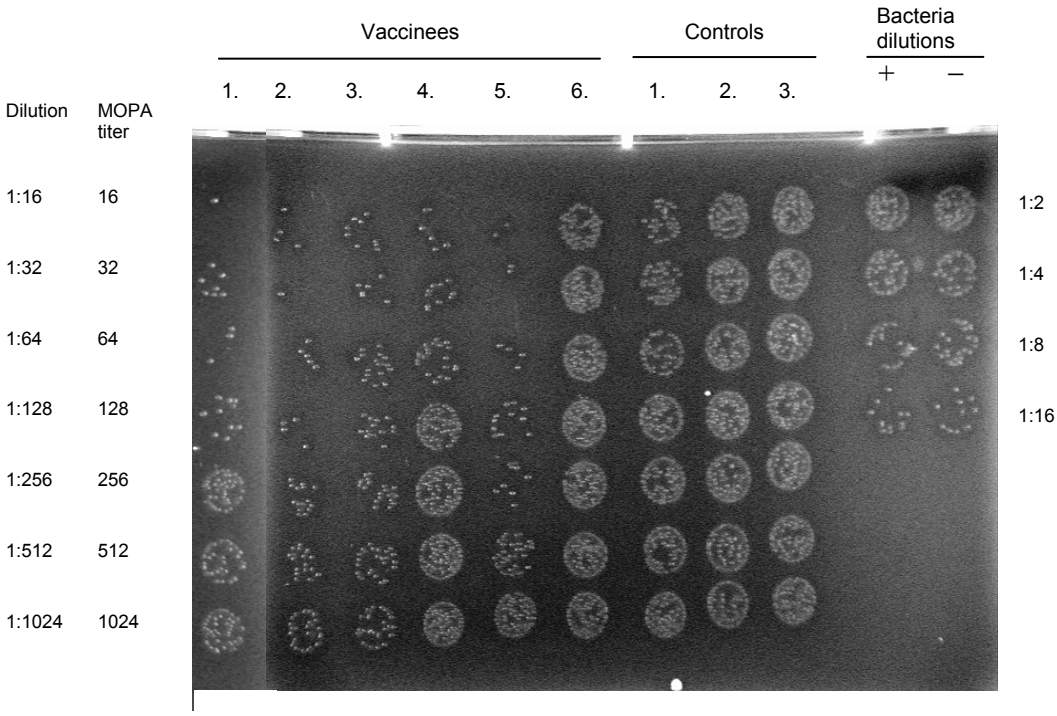


Figure 2. MOPA for serotype 23F. Opsonophagocytosis results for six vaccinees and three controls; two serial dilutions of bacteria incubated with (+) and without (-) complement and HL-60 cells are also depicted.

We determined the correlation between opsonophagocytosis and IgG antibody titer per capsular serotype. The R-values for the correlation between opsonophagocytosis and IgG for the serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were 0.70 ($p=0.05$), 0.17 ($p=0.70$), 0.58 ($p=0.13$), 0.52 ($p=0.19$), 0.83 ($p=0.01$), 0.43 ($p=0.29$) and 0.86 ($p<0.01$), respectively.

We also tested the read-out system used in the MSOPKA, i.e. the standard plating method allowing the counting of all CFUs in the suspension. By this method the opsonophagocytosis titer is defined as the highest dilution of serum resulting in 50% or more reduction in CFUs, in contrast to the MOPA, which requires the calculation of 90% or more reduction in CFUs. Higher titers were calculated using the MSOPKA compared to the MOPA (Table 2). We tested whether the results obtained by the MOPA and MSOPKA read-out systems correlated well. A scatter plot was made of all paired results obtained per serum and per serotype (Figure 3). The correlation between both methods was high with an R-value of 0.94. The difference in opsonophagocytosis titer between both methods was determined by calculating the best fit line drawn through the paired data. The best fit line for all serotypes is

defined by logMOPA = 0.86(logMSOPKA) + 0.3. This indicates that there is on average a difference of one log between both methods. Obviously, the MSOPKA method is slightly more sensitive because of the lower cut-off used. This is reflected in the number of undetectable serotype-specific opsonophagocytosis titers found in vaccinees: for the MSOPKA only 2 out of 40 determinations were below the detection limit, whereas for the MOPA 6 out of the 42 determinations were below the detection limit (Table 2).

Table 2. Opsonophagocytosis titers for the individual sera for both the vaccinees and controls.

Serotype	4		6B		9V		14	
	MOPA	MSOPKA	MOPA	MSOPKA	MOPA	MSOPKA	MOPA	MSOPKA
Vaccinees								
1	64	1024	16	48	16	64	128	1024
2	48	512	32	64	16	64	128	1024
3	24	256	16	64	8	8	128	1024
4	32	256	12	32	8	32	128	1024
5	12	48	12	48	8	16	128	1024
6	8	32	16	128	16	48	16	64
Controls								
1	8	16	8	8	8	8	8	128
2	8	32	8	8	8	8	12	64
3	8	8	8	8	8	8	8	8

Serotype	18C		19F		23F	
	MOPA	MSOPKA	MOPA	MSOPKA	MOPA	MSOPKA
Vaccinees						
1	128	ND	96	1024	96	768
2	128	1024	96	256	128	768
3	96	1024	128	1024	128	768
4	24	256	16	96	32	64
5	12	128	32	128	128	768
6	48	ND	8	8	8	16
Controls						
1	8	8	8	16	8	64
2	8	24	8	32	8	8
3	8	64	8	8	8	8

ND: not done

DISCUSSION

The 7-valent pneumococcal conjugate vaccine has shown to be highly immunogenic in young children and is highly protective against invasive pneumococcal diseases [4]. However, the efficacy of this vaccine against pneumonia and otitis media is limited [11,16,17]. Host protection against *Streptococcus pneumoniae* is mainly mediated by opsonin-dependent phagocytosis. Therefore, the in vitro measurement of opsonophagocytic activity of the anti-capsular antibodies is considered to be a reliable correlate of protection, and consequently of value to monitor unclear vaccine responses [6,7]. Unfortunately, this correlate of protection is seldom studied. This is mainly because the current techniques are

time-, and material consuming, and require large volumes of serum to test for the different pneumococcal serotypes. For example, the killing assay is very labor-intensive, and requires a separate titration curve of serum for each serotype tested [9]. The flow-cytometry assay is much less labor-intensive, but still requires 10 μl of serum per single titration curve [18]. In particular in the main target group for vaccination, i.e. young children, the amount of serum obtained is often limited. Therefore, Nahm *et al.* have developed a double serotype assay called the MSOPKA allowing the testing of opsonophagocytosis killing capacity of serum against two different serotypes in a single assay [9]. Nahm *et al.* have validated the double serotype assay method by direct comparison with the single opsonophagocytosis assay (OPA) and observed a high correlation between the two assays with R-values of 0.92 and 0.99. Furthermore, no specific 'bystander killing' was observed, and the MSOPKA provided equivalent results to those obtained with the single assays.

We developed a multiplex opsonophagocytosis assay, MOPA, extending the simultaneously investigated capsular serotypes from two to seven serotypes, hereby reducing the required volume of serum to 30 μl to test opsonophagocytosis against all vaccine serotypes. We tested the assay for non-specific killing of bacteria by serum, complement or by the HL-60 cells, and did not find non-specific interference of the individual components. Moreover, similarly to the previous opsonophagocytosis assays, the reproducibility of the test was high [8,9].

We simplified the read-out system using the method applied in the complement-killing assay for *N. meningtidis* [10]. Because the MOPA uses spotting of reaction mixture instead of plating, this assay requires the use of a higher degree of killing as cut-off (i.e. 90% killing instead of 50%), since only the growth of approximately 10-15 colonies can be distinguished, which matches with 85-90% killing of the initial CFUs. However, this method does not require detailed counting of large numbers of colonies for the individual serum dilutions, and therefore, the read-out time for the opsonophagocytosis capacity per serum is reduced significantly. In addition, spotting of droplets of reaction mixture is less time-consuming then plating the mixtures. Finally, the number of selective agar plates used is decreased significantly. To test whether the two read-out systems correlated well, we tested both systems within a single assay. A high correlation was found for all serotypes with an overall correlation coefficient of 0.94 ($p < 0.001$). Because the read-out system of the MOPA requires a cut-off for phagocytosis of 90%, the sensitivity of this test is lower compared to the read-out system of the MSOPKA, where the mixture is run out, allowing the exact counting of the individual colonies up to 100%, and, hence, the calculation of 50% reduction in phagocytosis. On average, a log difference between both methods is measured. As a result, opsonophagocytosis titers of vaccinees are more frequently found to be below the detection limit in case of the MOPA method (14%) compared to when MSOPKA method

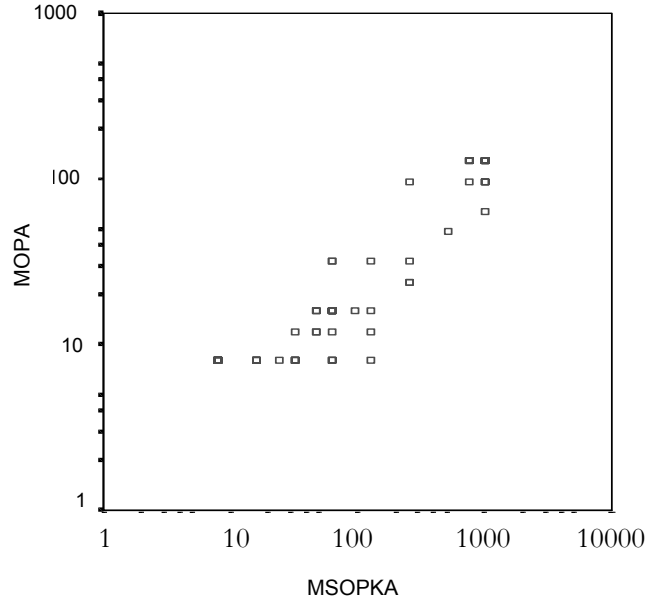


Figure 3. Scatter plot of the individual opsonization titers. MSOPKA titers are depicted on the x-axis and MOPA titers on the y-axis. Best fit line for all serotypes is logMOPA = 0.86(logMSOPKA)+0.3. The R-value is 0.94 ($p<0.001$).
Note: some paired results are identical and therefore multiple measurements can be represented by a single data point.

(5%). As no data are available regarding the minimal opsonophagocytic titers necessary for protection against pneumococcal disease, we cannot speculate about the consequence of this difference in detection limit. Therefore, we recommend further studies to evaluate both the spectrum of protective opsonophagocytic titers as well as the sensitivity of the assays to detect this spectrum.

We tested whether the phagocytosis capacity correlated with the serotype-specific IgG antibody titers. Possibly as a consequence of the limited number of sera tested, we only found high correlations for IgG antibody titers and opsonophagocytosis capacity (R-value >0.70, $p<0.05$) for the serotypes 4, 18C and 23F. The lowest correlations between serotype-specific IgG titers and opsonophagocytosis capacity were observed for the serotypes 6B and 19F, which is in line with the findings of Romero-Steiner et al. [8]. However, Jansen et al. have observed higher correlations for the latter serotypes using the flow-cytometry assay [19]. This might be explained by the difference in effector cells used. In the cytometric assay human leucocytes were used as effector cells instead of HL-60 cells. Leucocytes can be selected for a high-affinity IgG2 receptor allotype of FcγRIIa, whereas the HL-60 cells display the low-affinity variant of this receptor. Use of human leucocytes in the MOPA, however, is hardly possible due to the high number of cells required. Besides antibody titers, additional variables may influence opsonophagocytosis activity. Furthermore, our findings underline the importance of testing opsonophagocytosis activity in addition to the measurement of

antibody titers when monitoring vaccination. Obviously, the number of sera tested in this study is too small to draw conclusions about the exact correlations between the amount of serotype-specific antibodies and their capacity to elicit opsonophagocytosis.

In conclusion, the MOPA accurately measures the opsonophagocytosis capacity of serum against all serotypes included in the 7-valent conjugate vaccine in a high-throughput multiplex fashion, requiring low volumes of serum. Importantly, this assay is easily adjustable to measure the opsonophagocytosis capacity of antibodies raised against antigens other than the seven capsular polysaccharides, present in the current 7-valent conjugate vaccine.

REFERENCES

1. Pneumococcal vaccines: WHO position paper. *weekly epidemiological record* 1999, 74(23), 177-184.
2. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 1997, 46(RR-8), 1-24.
3. Black, S., Shinefield, H., Ray, P. *et al.* Efficacy of heptavalent conjugate pneumococcal vaccine in 37000 infants and children: results of the Northern California Kaiser Permanente efficacy trial. Kaiser Permanente Vaccine Study Center, Oakland, USA, 1998.
4. Black, S., Shinefield, H., Fireman, B. *et al.* Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000, 19(3), 187-195.
5. Parkkali, T., Vakevainen, M., Kayhty, H., Ruutu, T. & Ruutu, P. Opsonophagocytic activity against *Streptococcus pneumoniae* type 19F in allogeneic BMT recipients before and after vaccination with pneumococcal polysaccharide vaccine. *Bone Marrow Transplant* 2001, 27(2), 207-211.
6. Rodriguez, M.E., Van der Pol, W. & Van de Winkel, J.G. Flow cytometry-based phagocytosis assay for sensitive detection of opsonic activity of pneumococcal capsular polysaccharide antibodies in human sera. *J Immunol Methods* 2001, 252(1-2), 33-44.
7. Vakevanen, Jansen, Saeland, *et al* Are the opsonophagocytic activities of antibodies in infant sera measured by different pneumococcal phagocytosis assays comparable? *Clin Diagn Lab Immunol* 2001, 8(2), 363-369.
8. Romero-Steiner, S., Libutti, D., Pais, L.B. *et al.* Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. *Clin Diagn Lab Immunol* 1997, 4(4), 415-422.
9. Nahm, M.H., Briles, D.E. & Yu, X. Development of a multi-specificity opsonophagocytic killing assay. *Vaccine* 2000, 18(24), 2768-2771.
10. Borrow, R., Richmond, P., Kaczmarski, E.B. *et al.* Meningococcal serogroup C-specific IgG antibody responses and serum bactericidal titres in children following vaccination with a meningococcal A/C polysaccharide vaccine. *FEMS Immunol Med Microbiol* 2000, 28(1), 79-85.
11. Veenhoven, R., Bogaert, D., Uiterwaal, C. *et al.* Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. *Lancet* 2003, 361, 2189-2195.
12. Bricker, A.L. & Camilli, A. Transformation of a type 4 encapsulated strain of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* 1999, 172(2), 131-135.
13. Goessens, W.H., Lemmens-den Toom, N., Hageman, J. *et al.* Evaluation of the Vitek 2 system for susceptibility testing of *Streptococcus pneumoniae* isolates. *Eur J Clin Microbiol Infect Dis* 2000, 19(8), 618-622.
14. Kayhty, H., Ahman, H., Ronnberg, P.R., Tillikainen, R. & Eskola, J. Pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine is immunogenic in infants and children. *J Infect Dis* 1995, 172(5), 1273-1278.
15. Quataert, S.A., Kirch, C.S., Wiedl, L.J. *et al.* Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. *Clin Diagn Lab Immunol* 1995, 2(5), 590-597.
16. Black, S.B., Shinefield, H.R., Ling, S. *et al.* Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. *Pediatr Infect Dis J* 2002, 21(9), 810-815.
17. Eskola, J., Kilpi, T., Palmu, A. *et al.* Efficacy of a Pneumococcal Conjugate Vaccine against Acute Otitis Media. *N Engl J Med* 2001, 344(6), 403-409.
18. Jansen, W.T., Gootjes, J., Zelle, M. *et al.* Use of highly encapsulated Streptococcus pneumoniae strains in a flow-cytometric assay for assessment of the phagocytic capacity of serotype- specific antibodies. *Clin Diagn Lab Immunol* 1998, 5(5), 703-710.
19. Jansen, W.T., Vakevainen-Anttila, M., Kayhty, H. *et al.* Comparison of a classical phagocytosis assay and a flow cytometry assay for assessment of the phagocytic capacity of sera from adults vaccinated with a pneumococcal conjugate vaccine. *Clin Diagn Lab Immunol* 2001, 8(2), 245-250.

Chapter 8.4

Development of antibodies against pneumococcal proteins α-enolase, immunoglobulin A1 protease, streptococcal lipoprotein rotamase A, and putative proteinase maturation protein A in relation to pneumococcal carriage and otitis media

P.V. Adrian, D. Bogaert, M. Oprins, S. Rapola, M. Lahdenkari, T. Kilpi, R. de Groot, H. Käyhty and P.W.M. Hermans

Vaccine; in press

ABSTRACT

Surface associated pneumococcal proteins α-enolase (Eno), immunoglobulin A1 protease (Iga), streptococcal lipoprotein rotamase A (SlrA), and putative proteinase maturation protein A (PpmA) have potential as candidates for future protein-based anti-pneumococcal vaccines. The immunogenicity of these proteins were studied in a cohort of 329 children during their first two years of life. During the first recorded episode of otitis media, acute and convalescent phase sera were available from 151 children. Concentrations of antibodies against Eno, Iga, SlrA and PpmA were measured by EIA and detected in 99% (300/302), 95% (288/302), 95% (288/302), and 83% (251/302) of the sera, respectively. There were no statistically significant differences between the groups of children with and without a history of pneumococcal contact or with respect to the type of pneumococcal contact. Despite a mean overall decrease in the antibody titers in the convalescent sera following AOM, several children were able to respond with a more than 2-fold increase in antibody titer in response to AOM. The majority of the children with increased antibody concentrations appeared in the groups, which were colonized with pneumococci at the time of serum collection, but were recorded as having no prior contact with pneumococci. In conclusion, SlrA, PpmA, Eno and Iga are immunogenic proteins that elicit antibody responses early in life. No significant correlation between antibody titers to these proteins and pneumococcal carriage or infection was found. Presumably, this results from the presence of cross-reactive epitopes on commensal bacteria.

INTRODUCTION

Streptococcus pneumoniae is a commensal bacterium of the human nasopharynx (4). It is a leading etiological agent of mucosal disease such as acute otitis media (AOM), and of severe invasive disease such as septicemia, pneumonia and meningitis. Invasive pneumococcal disease may have fatal consequences: over three million deaths per year worldwide of which a million are young children (5). Little is known about the immunogenicity of pneumococcal proteins in relation to age, colonization, and disease status. Antibodies to pneumococcal proteins PspA, PsaA, and pneumolysin have been shown to develop early in life, moreover, adult levels of antibodies to PsaA, Pneumolysin, and PspA are reached by the ages of six months, two years and five years, respectively (10, 13). In addition, concentrations of antibodies against these proteins in young children are strongly associated with pneumococcal contact by colonization or infection (AOM) (10, 11). The relationship between antibodies against pneumococcal proteins and disease is less clear. In adults with severe pneumococcal infection, the immune response in the acute and convalescent phase serum to the pneumococcal proteins PspA, PrtA and SpuA shows no significant changes in relation to normal adult levels. A small increase in the titer was observed in the early convalescent phase serum to pneumococcal proteins PrtA and SpuA (16). In adults, titers of anti-PspA antibodies increase during colonization, and pre-existing low titers of anti-PspA antibodies were associated with pneumococcal colonization (6). In children over the age of nine months, higher titers of antibodies to PsaA were associated with a lowered risk of the development of pneumococcal AOM during pneumococcal carriage (12).

Pneumococcal proteins α-enolase (Eno), immunoglobulin A1 protease (Iga), streptococcal lipoprotein rotamase A (SlrA), and putative proteinase maturation protein A (PpmA) have been suggested to play a role in virulence of *S. pneumoniae* (1, 2, 8, 15). Due to their surface location they have been proposed as potential vaccine candidates. The glycolytic enzyme Eno has been identified both in the cytoplasm, and in significant quantities on the surface of pneumococci, and has been shown to be involved in virulence by the activation of plasmin into plasminogen, a host collagen-degrading enzyme (2). Iga has been shown to play an important role in pneumococcal colonization by cleaving the Fc receptor from secreted immunoglobulin A1, thus preventing opsonophagocytosis. The cleaved IgA1 fragments have been shown to increase the adherence properties of the pneumococcus (15). PpmA and SlrA are lipoproteins belonging to a class of chaperones, the peptidyl prolyl isomerases, which are thought to be involved in the secretion and activation of cell surface molecules (1, 8). PpmA shares homology with the parvulin group of peptidyl prolyl isomerases, and SlrA shares homology with the cyclophilins.

No data are available yet on the prevalence of antibodies to PpmA, SlrA, Eno and Iga in young infants or their natural development in response to pneumococcal colonization or

disease. There are also no data to indicate whether such antibodies are protective against pneumococcal infection. To fill such gaps in the background knowledge needed when considering the use of a future protein-based pneumococcal vaccine, we present data on the development of antibodies to PpmA, SlrA, Eno and Iga in young children and evaluate the association of the antibody titers with culture-proven pneumococcal carriage and disease.

METHODS

Study cohort and sample schedule. The study population comprised 329 children who were enrolled in the Finnish Otitis Media (FinOM) Cohort Study (10, 11). For the analysis, 151 pairs of serum samples from the acute and convalescent phase were taken from children, which presented with symptoms of a respiratory tract infection, and suspected AOM. The study population was divided into three groups according to whether or not they had a current contact with *S. pneumoniae* and according to the nature of the current contact. The three groups were: (1) current pneumococcal AOM: *S. pneumoniae* was cultured from the MEF and NPA sample obtained at the diagnosis of the evaluated AOM; (2) current pneumococcal carriage: *S. pneumoniae* was cultured from NPA but not from MEF; (3) no current pneumococcal contact: NPA and MPF cultures negative for *S. pneumoniae.* Each group was further divided into two subgroups according to whether the children had a history of previous pneumococcal contact (NP or NPA culture positive for *S. pneumoniae*). Thus the anti-PpmA, anti-SlrA, anti-Iga, and anti-Eno concentrations and responses were evaluated in the 6 groups of children.

Clinical samples and culture methods. The NP swabs, NPA samples and MEF were obtained as described previously (10, 11). Pneumococci were identified by standard methods (14). Serum samples were stored at -20°C at National Public Health Institute of Finland and a small aliquot was shipped frozen to the Laboratory of Pediatrics at Erasmus MC for antibody determinations.

Recombinant antigens. The PpmA and Iga protein antigens were prepared as described previously (7). The genes used for the production of the recombinant antigens Eno, and SlrA were amplified by PCR and cloned into a pET11a expression vector (Stratagene, LaJolla, CA). A His^6 tag was incorporated onto the C-terminal peptide sequences for Eno. The N-terminal signal peptide of SlrA was replaced with an N-terminal His^6 tag. The recombinant plasmids were electrotransformed into *E. coli* BL21 (DE3). The expression of recombinant protein was induced by the addition of IPTG, and the recombinant proteins were purified by Ni^+ affinity chromatography with the HisTrap™ Kit (Amersham Pharmacia, Uppsala, Sweden) according to the manufactures recommendations. The purity of the recombinant

proteins was determined by SDS-PAGE gel electrophoresis with CBB staining. The sequence of the purified peptides was confirmed with MALDI-TOF mass spectrometry.

Serologic methods. IgG antibodies to Eno, Iga, PpmA and SlrA were measured by EIA. Microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 100µl/well, with a coating concentration of 5µg/ml of antigen in PBS and incubated overnight at 37°C. The plates were blocked with 1% bovine serum albumin (BSA) and 0.05% Tween 20 in PBS at 37°C for 1 h. The samples were serially diluted (starting at 1:100) in the dilution buffer (1% BSA, 0.05% Tween 20 in PBS). Duplicates (100µl/well) were incubated at 37°C for 2 h. Polyclonal alkaline phosphatase-conjugated antisera to human IgG (Sigma Chemicals, St. Louis) was diluted 1:3000 in the dilution buffer, pipetted at 100µl/well, and incubated for 2 h at 37°C. Finally, 100µl/well of MUP (Sigma, CAT#M8883) substrate solution (0.2 mM 4-methylumbelliferyl phosphate, 0.05 M $NaCO_3$, 0.05 mM $MgCl_2$) was added, and the plates were incubated for 1 h at 37°C. The fluorescence was measured with an excitation wavelength of 365nm, and an emission wavelength of 450 nm with a Molecular Devices SpectraMAX (Sunnyvale, USA) microplate reader. The serum endpoint antibody titer was interpolated from the intersection of the absorbance-versus-serum dilution curve at the background fluorescence plus four times the standard deviation. The end-point titer was then converted into antibody concentration (EIA units of immunoglobulin per milliliter) by comparison with a standard serum consisting of pooled healthy adult volunteers, and was considered to contain 100 U/mL of anti-α-enolase, Iga, PpmA or SlrA. Samples with antibody concentrations below the detection limit (30 U/mL for Eno, 4 U/mL for Iga, 4 U/mL for SlrA, and 5 U/mL for PpmA) were assigned the values equivalent to half the detection limit.

Statistical methods. We used SPSS software (SPSS, USA) for analysis of log-transformed data. The antibody concentrations in the groups of children are reported as geometric mean concentrations (GMC, units/ml) with 95% confidence intervals (95% CI) and compared by one way ANOVA followed by a LSD post hoc test. Statistical comparisons between antibody levels in acute and convalescent phase sera were carried out using a paired Student's *t* test. Fisher's exact test was used when the proportions of variables were compared. A logistic regression was used to evaluate the effects of the acute phase antibody concentration, age and the nature of pneumococcal contact on the antibody response and to evaluate antibody concentrations (logarithm base *e* of antibody concentration) as a risk factor for the involvement of pneumococci as a risk factor for the development of AOM. Children were grouped into eight 3-month groups according to the age at which the acute serum was sampled. To determine whether there were any significant differences in relation to age, groups were analyzed by 1-way ANOVA followed by a Tukey's HSD test.

RESULTS

One hundred and fifty one children for whom a MEF sample and paired sera were available had at least one attack of AOM (Table 2). Of the 151 AOM events occurring at the median age of 267 days (range 49 to 723 days), 37 children had *S. pneumoniae* in the MEF culture, and in all of them it was also cultured from the NPA sample. *S. pneumoniae* was recovered only from the NPA sample in 43 cases, and in 71 cases, the MEF and NPA samples were negative for *S. pneumoniae*. Of the 37 children with current pneumococcal AOM, 15 had no known previous pneumococcal contact (Group 1) and 22 had at least one proven previous pneumococcal contact (Group 2). Of the 43 children with current pneumococcal carriage, 12 had no previous cultures positive for *S. pneumoniae* (Group 3), whereas 31 had a previous pneumococcal contact (Group 4). Of the 71 children with no current contact with *S. pneumoniae* (Group 5), 53 had no detected previous pneumococcal contacts, and 18 had a history of previous pneumococcal contact (Group 6) (Table 2). Measurable concentrations of antibodies against Eno, SlrA, Iga and PpmA were found in 99% (292/294), 97% (288/298), 95% (288/302), and 83% (251/302) of the sera respectively.

Antibodies in acute phase sera. Previous pneumococcal contact appeared to have no influence on antibodies against Eno in the acute phase serum (Table 1). Furthermore, there were no significant differences in anti-enolase concentrations in relation to the type of pneumococcal contact. The GMC of antibodies to IgA1 protease showed no statistically significant differences between pneumococcal contact groups. In general, mean anti-Iga concentrations tended to be higher in the groups who had previous contact with pneumococci, however, in the children from which pneumococci were isolated from MEF (Groups 1 and 2), antibody titers were 2-fold higher in the group with no previous pneumococcal contacts (Group 1). With respect to antibodies against SlrA in the acute phase sera, there were no statistically significant differences in the titers of anti-SlrA antibodies between groups in relation to the type of pneumococcal contact, and in relation to the history of detection of pneumococci in the nasopharynx prior to the development of AOM. The highest titers of anti-SlrA antibodies were recorded in the group of children from whom pneumococci were recovered from MEF, but from who no previous contact with pneumococci was detected (Group 1). In this group a more than 2-fold difference in the GMC of anti-SlrA antibodies was recorded in relation to the other pneumococcal contact groups. With respect to antibodies against PpmA in the acute phase sera, there were no statistically significant differences in the titers of anti-PpmA antibodies between groups in relation to the type of pneumococcal contact, and the history of detection of pneumococci in the nasopharynx prior to the development of AOM. There was, however, a trend towards higher titers of anti-PpmA antibodies being associated with previous pneumococcal contact. The GMC of anti-PpmA antibodies tended to be higher in the children from whom

Table 1. Geometric mean concentrations (GMCs) of anti-Eno, anti-Iga, anti-SlrA and anti-PpmA antibodies in acute and convalescent phase serum samples and the number of sero-conversions in children with AOM in relation to current and previous culture proven pneumococcal findings in MEF, NPA and NP samples.

				GMC (Units/ml) (95% CI)						
	Pneumococcal contact			Anti-Eno				Anti-Iga		
Group	Current	Previous	N	Acute	Convalescent	Response > 2 fold (%)	N	Acute	Convalescent	Response > 2 fold (%)
1	MEF, NPA	No	14	247.6 (153.9;367.0)	251.4 (171.0;369.6)	2 (14.3)	15	198.7 (115.4;342.2)	146.4 (83.0;258.3)	0 (0)
2	MEF, NPA	Yes	22	214.9 (141.3;327.0)	214.0 (150.5;304.2)	3 (13.6)	22	83 (49.3;139.5)	80.7 (53.3;122.3)	3 (13.6)
3	Only NPA	No	12	294.8 (133.9;649.1)	352.5 (190.6;651.8)	5 (41.7)	12	65.8 (28.2;153.3)	71.5 (32.8;156.2)	2 (16.7)
4	Only NPA	Yes	31	222.7 (169.7;292.3)	179.7 (133.2;242.6)	1 (3.2)	31	83.2 (57.8;119.9)	62.8 (44.1;89.3)	0 (0)
5	No	No	51	180.3 (147.4;220.5)	152.2 (124.2;186.5)	8 (15.7)	53	70.4 (51.7;95.9)	48.1 (34.5;67.1)	5 (9.4)
6	No	Yes	17	196.5 (150.3;256.8)	191.5 (119.2;307.6)	2 (11.8)	18	130 (76.9;219.9)	71.6 (36.9;139.0)	3 (16.7)

				GMC (Units/ml) (95% CI)						
	Pneumococcal contact			Anti-SlrA				Anti-PpmA		
Group	Current	Previous	N	Acute	Convalescent	Response > 2 fold (%)	N	Acute	Convalescent	Response > 2 fold (%)
1	MEF, NPA	No	14	143.4 (75.4;272.9)	103.7 (61.0;176.4)	0 (0)	15	66.8 (39.2;113.8)	49.8 (29.2;85.0)	2 (13.3)
2	MEF, NPA	Yes	21	52.7 (33.9;81.8)	68.2 (41.6;111.8)	4 (19.0)	22	64.5 (39.1;106.6)	51.2 (28.3;92.5)	2 (9.1)
3	Only NPA	No	12	65.7 (25.9;166.8)	51.3 (20.0;131.1)	3 (25.0)	12	36.1 (11.4;114.5)	49.6 (19.1;129)	4 (33.3)
4	Only NPA	Yes	31	64.7 (44.5;94.0)	47.0 (30.8;71.8)	1 (3.2)	31	56 (34.9;90.0)	60.6 (41.6;88.2)	6 (19.4)
5	No	No	53	54.4 (37.7;78.6)	46.2 (33.7;63.4)	5 (9.4))	53	35.6 (24.4;52.0)	25.0 (18.6;33.7)	7 (13.2)
6	No	Yes	18	53.1 (26.5;106.4)	60.2 (33.0;109.9)	3 (16.7)	18	54.8 (29.7;101.1)	36.1 (17.2;76.0)	1 (5.6)

pneumococci were isolated from MEF.

Antibody responses to AOM. Despite the fact that a number of children showed positive antibody responses to AOM, there was an overall decrease in the mean titer of antibodies against Eno, SlrA, Iga and PpmA antigens in the convalescent serum compared to the acute phase serum (Table 1). Of the different pneumococcal contact groups, the highest incidence of children, which showed a more than 2-fold increase in the response to AOM in the convalescent serum, was (Group 3), from which pneumococci were isolated from the NPA only, but which had no previous record of pneumococcal colonization. In this group, the percentage of children from which a more than 2-fold increase in the antibody titer following AOM in the convalescent serum was measured, was 42%, 25%, 17% and 33% for Eno, SlrA, Iga, and PpmA, respectively. In a logistic regression model, higher concentrations of antibodies to Eno, SlrA, Iga, and PpmA antigens were not associated with a decreased risk of pneumococcal AOM in any age groups (data not shown).

Age specific differences. There were no statistically significant differences between the antibody titers of the different age groups of children. However, for most pneumococcal antigens the GMC of antibodies were higher in the 0-3 months age group than for the remainder of the first year of life. This was most noticeable for anti-PpmA antibodies. Titers of antibodies against PpmA and SlrA antigens showed a trend towards increasing after 18 months of age.

DISCUSSION

A major contributing factor in the development of antibodies against pneumococcal antigens Eno, Iga, SlrA and PpmA, is the fact that several antigens that were tested may share common epitopes with the antigens present on the surface of other bacteria. This is especially true for Eno, which is a conserved essential glycolytic enzyme, which has epitopes that are able to ross-react with different species of streptococci and the human enzyme (3). Similarly, with Iga, despite it having a unique structure among bacterial proteases, homology searches have indicated that the Iga gene of *S. pneumoniae* is also found in the Viridans streptococci, the gene fragment used in this study shared >90% homology between viridans streptococci, and is unrelated to the serine IgA1 proteases of other commensal bacteria of the nasopharynx such as *Neisseria meningitidis* and *Haemophilus influenzae* (9). This suggests that the rapid colonization of children by commensal bacteria such as the viridans streptococci soon after birth is a likely source of cross-reactive epitopes of Eno and Iga. Furthermore, it helps to explain why anti-Eno and anti-Iga antibodies were present in almost all the serum samples, and indeed why the history of pneumococcal exposure, current pneumococcal colonization and AOM had little

impact on the serum concentrations of anti-Eno and -Iga antibodies in comparison to pneumococcal proteins PsaA, PspA and Pneumolysin for which the increase in antibody concentrations is associated with pneumcoccal contacts (10, 11). The influence of past or current pneumococcal contact appears to play little role in influencing concentrations of serum antibodies against SlrA. It is difficult to predict whether the presence of cross-reacting epitopes plays a hand in masking a noticeable response to pneumococcal contact. BLAST searches of all the bacterial genomes sequenced to date show that lipidated forms of the cyclophilin SlrA occur in all sequenced streptococcal genomes, and SlrA shares between 60% and 70% amino acid identity with its corresponding viridans streptococcal homologues.
PpmA is a member of the ubiquitous family of parvulin-like peptidyl prolyl isomerases, but despite being widely represented among bacteria, homology searches have shown that proteins of closely related bacteria share less than 52% homology with pneumococcal PpmA. In addition, polyclonal sera raised against purified PpmA has failed to recognize any epitopes of related streptococcal species by Western blot (unpublished data), and is unable to elicit opsonophagocytic activity against other streptococcal species (8). Anti-PpmA antibody titers showed a trend towards responsiveness to contact with pneumococci in a similar way as the pneumococcal antigens PspA, PsaA and Pneumolysin.
In conclusion, although the investigated proteins Iga. SlrA, Eno and PpmA showed to be immunogenic early in life, no correlation between colonization and infection with pneumococci and antibody titers directed against these proteins was found. These data suggest the presence of cross-reactive epitopes on commensal bacteria co-colonizing the nasopharyngeal niche. Since pathogen-specific epitopes are less likely to cross-react with epitopes of commensal bacteria of the nasopharynx, such proteins might have more potential as vaccine candidates. Finally, the effect of antibodies cross-reacting with vaccine epitopes on commensal bacteria warrants further study.

ACKNOWLEDGMENTS

This study was financially supported by STIGON, The Netherlands (project number 014-81-114).

REFERENCES

1. Adrian, P. V., A. Kerr, T. Hoogenboezem, R. de Groot, T. J. Mitchell, and P. W. M. Hermans. 2002. Development of antibodies against pneumococcal proteins α-enolase, immunoglobulin A1 protease, streptococcal lipoprotein rotamase A, and putative proteinase maturation protein A in relation to pneumococcal carriage and otitis media. Presented at the 3rd International Symposium on Pneumococci and Pneumococcal Diseases., Anchorage, Alaska, USA. 5-8th May.
2. Bergmann, S., M. Rohde, G. S. Chhatwal, and S. Hammerschmidt. 2001. alpha-Enolase of Streptococcus pneumoniae is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. Mol Microbiol 40:1273-87.
3. Fontan, P. A., V. Pancholi, M. M. Nociari, and V. A. Fischetti. 2000. Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. J Infect Dis 182:1712-21.
4. Ghaffar, F., I. R. Friedland, and G. H. McCracken, Jr. 1999. Dynamics of nasopharyngeal colonization by Streptococcus pneumoniae. Pediatr Infect Dis J 18:638-46.
5. Greenwood, B. 1999. The epidemiology of pneumococcal infection in children in the developing world. Philos Trans R Soc Lond B Biol Sci 354:777-85.
6. McCool, T. L., T. R. Cate, G. Moy, and J. N. Weiser. 2002. The immune response to pneumococcal proteins during experimental human carriage. J Exp Med 195:359-65.
7. McCool, T. L., T. R. Cate, E. I. Tuomanen, P. Adrian, T. J. Mitchell, and J. N. Weiser. 2003. Serum immunoglobulin G response to candidate vaccine antigens during experimental human pneumococcal colonization. Infect Immun 71:5724-32.
8. Overweg, K., A. Kerr, M. Sluijter, M. H. Jackson, T. J. Mitchell, A. P. de Jong, R. de Groot, and P. W. Hermans. 2000. The putative proteinase maturation protein A of Streptococcus pneumoniae is a conserved surface protein with potential to elicit protective immune responses. Infect Immun 68:4180-8.
9. Poulsen, K., J. Reinholdt, C. Jespersgaard, K. Boye, T. A. Brown, M. Hauge, and M. Kilian. 1998. A comprehensive genetic study of streptococcal immunoglobulin A1 proteases: evidence for recombination within and between species. Infect Immun 66:181-90.
10. Rapola, S., V. Jantti, R. Haikala, R. Syrjanen, G. M. Carlone, J. S. Sampson, D. E. Briles, J. C. Paton, A. K. Takala, T. M. Kilpi, and H. Kayhty. 2000. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. J Infect Dis 182:1146-52.
11. Rapola, S., T. Kilpi, M. Lahdenkari, P. H. Makela, and H. Kayhty. 2001. Antibody response to the pneumococcal proteins pneumococcal surface adhesin A and pneumolysin in children with acute otitis media. Pediatr Infect Dis J 20:482-7.
12. Rapola, S., T. Kilpi, M. Lahdenkari, A. K. Takala, P. H. Makela, and H. Kayhty. 2001. Do antibodies to pneumococcal surface adhesin a prevent pneumococcal involvement in acute otitis media? J Infect Dis 184:577-81.
13. Riddel, A. F., K. Sleeman, D. Crook, N. Day, C. Moore, T. Peto, B. Bartlett, S. Ferro, E. Wang, and E. R. Moxon. 2002. Presented at the 3rd International Symposium on Pneumococci and Pneumococcal Diseases, Anchorage, Alaska, USA. 5-8th May.
14. Takala, A. K., J. Eskola, M. Leinonen, H. Kayhty, A. Nissinen, E. Pekkanen, and P. H. Makela. 1991. Reduction of oropharyngeal carriage of Haemophilus influenzae type b (Hib) in children immunized with an Hib conjugate vaccine. J Infect Dis 164:982-6.
15. Weiser, J. N., D. Bae, C. Fasching, R. W. Scamurra, A. J. Ratner, and E. N. Janoff. 2003. Antibody-enhanced pneumococcal adherence requires IgA1 protease. Proc Natl Acad Sci U S A 100:4215-20.
16. Zysk, G., G. Bethe, R. Nau, D. Koch, V. C. Grafin Von Bassewitz, H. P. Heinz, and R. R. Reinert. 2003. Immune response to capsular polysaccharide and surface proteins of Streptococcus pneumoniae in patients with invasive pneumococcal disease. J Infect Dis 187:330-3.

Chapter 9

Summarizing discussion

Streptococcus pneumoniae is a major cause of invasive diseases such as meningitis, septicemia and pneumonia. Approximately 1 million children under 5 years of age die of pneumococcal disease annually [1]. In countries where the incidence of *Neisseria meningitidis* and *Haemophilus influenzae* infections has drastically decreased through the introduction of vaccines against meningococcus group C and *H. influenzae* type B, *S. pneumoniae* has become the major remaining cause of meningitis and septicemia in children. In addition, the morbidity by *S. pneumoniae* through respiratory tract infections such as otitis media and sinusitis is enormous. 30-50% of all patients with otitis media and a substantial percentage of cases of sinusitis and pneumonia are caused by pneumococci. Risk groups for serious pneumococcal disease include children under the age of 2 years, the elderly and patients with immunodeficiencies [2].

Nasopharyngeal colonization by *S. pneumoniae* is common: probably all humans are colonized with this organism at least once early in life. Especially in circumstances of crowding, in day-care centers, nursing homes, hospitals and jails, the risk of colonization with pneumococci is high [3-5]. Colonization is not usually followed by disease, since this is prevented by both the innate and adaptive immune systems. However, disturbance of homeostasis between host and pathogen, for example through viral infections, malnutrition or local damage of the mucosa, is associated with the development of (invasive) diseases [6-8].

In 1999, Engelen and coworkers investigated colonization in Amsterdam among 259 children attending 16 day-care centers (DCCs) and among 276 children who did not attend day-care centers (NDCCs). A 1.6-3.4 fold increased risk for nasopharyngeal colonization was observed in children attending day-care compared with non-day-care center children. We investigated the serotype and genotype distribution of 305 nasopharyngeal *S. pneumoniae* isolates collected in the latter study **(Chapter 3.1)**. The predominant serotypes in both the DCC and the NDCC group included serotypes 19F, 6B, 6A, 23F, and 9V. The theoretical vaccine coverage of the 7-valent conjugate vaccine was for both groups similar, i.e 56-59%. However, genetic analysis of the pneumococcal isolates revealed 75% clustering among pneumococci isolated from DCC attendees versus 50% among the NDCC children. The average pneumococcal cluster size in the DCC group was 3.8 and 4.6 isolates for two respective sample dates (range 2 to 13 isolates per cluster), while the average cluster size for the NDCC group was 3.0 (range 2 to 6 isolates per cluster). Similar to observations made in other countries, these results indicate a higher risk for horizontal spread of pneumococci in Dutch day-care centers in comparison with the general population, however this is not related to serotype distribution. A second risk factor for pneumococcal colonization is antibiotic consumption. However in our study, only 2% of the pneumococcal isolates were resistant to erythromycin and no resistance to penicillin,

cotrimoxazole, and tetracycline was found, therefore the enhanced horizontal spread within day-care centers was not related to antibiotic resistance. The RFEL patterns of the isolates were compared with the first sixteen international (multidrug-resistant) clones described by the pneumococcal epidemiology network (http://www.pneumo.com/ physician/pmen/pmen_ history.asp). Twenty-five isolates (10%) in the DCC group were homologous to three of the reference clones (100% identical), whereas six isolates (10%) in the NDCC group matched with four of these clones. In contrast to the multidrug-resistant reference clones, all Dutch isolates but one were fully susceptible or mono-resistant. These results suggest that the Dutch isolates represent members of the ancestor lineages of the resistant reference clones. The absence of the original resistant clones in The Netherlands may be explained by the reserved antibiotic prescription behavior of doctors in this country for many years, which starts with the hospitals of which over 50% have implemented restrictive formulary agreements. In addition, most general practioners follow guidelines of the Dutch College of General Practioners, which results in restrictive and selective prescription behavior compared to international colleagues.

In 2002, we had the opportunity to investigate the prevalence and determinants of nasopharyngeal carriage of *S. pneumoniae* and *S. aureus* in 3198 healthy children 1-19 years of age **(Chapter 3.2)**. Determinants of nasopharyngeal carriage of *S. pneumoniae* (19%) were age and day-care attendance. All of these factors were previously reported as determinants of carriage. Other variables which were reported in the literature to be determinants for pneumococcal carriage, such as passive smoking at home, large households and crowding among teenagers, did not correlate with carriage in our study, when corrected for the remaining determinants by means of multivariate logistic regression [9]. Analysis of the age-related distribution of vaccine and non-vaccine serotypes showed an initial predominance of vaccine-type pneumococci in the first 3 years of life, followed by a subtle shift towards non-vaccine type pneumococci after the age of 3 years. These findings are in agreement with those of Dagan et al., who showed a similar reversal after the age of 2 years in children who attended day-care centers [10]. Risk-factors for *Staphylococcus aureus* carriage (36%) were age (peak incidence: 10 years), sex, family size and passive smoking, whereas active smoking was inversely related to *S. aureus* carriage. Furthermore, passive smoking was associated with an increased risk while active smoking decreased this risk. We hypothesize, that tobacco smoke damages the epithelium of the upper airways. With initial exposure to smoke adherence of bacteria to host cell surfaces may be enhanced, while increased exposure may ultimately damage the surfaces such that colonization is prevented, as was shown by in vitro assays [11]. Pneumococcal serotype analysis showed 42% vaccine type and 58% non-vaccine type pneumococci. After correction for the independent determinants of colonization by multivariate logistic regression analysis, we observed a

negative correlation for co-colonisation of *S. aureus* and vaccine-type pneumococci, but not for *S. aureus* and non-vaccine serotypes. Because children under 5 years of age are considered to be representative for the target population for pneumococcal conjugate vaccination, bacterial interaction is particularly relevant in this age group. For this sub-cohort, the negative correlation for co-colonisation of vaccine type pneumococci and *S. aureus* was even stronger. Our findings are in agreement with the data of Veenhoven et al. (**Chapter 7.1**) [12], who demonstrated a significant increase in *S. aureus*-induced AOM episodes in otitis prone children vaccinated with the 7-valent conjugate vaccine. This interference has already been described in 1922 in an in vitro model by Mc Leod and Gordon. These investigators showed that *S. aureus* growth is inhibited in the presence of culture supernatant of *S. pneumoniae* [13]. At that time, this effect was thought to be caused by the presence of hydrogen peroxide in the supernatant. This hypothesis was later supported by studies of Dahiya et al. and Repine et al. [14,15]. Whether only pneumococci, or also other streptococcal species are involved in this competitive balance remains unclear, although the investigations of Uehara et al. suggest a similar competitive corrrelation between viridans streptococci and *S. aureus* [16]. This interspecies competition is also observed for streptococcal species and other nasopharyngeal bacteria [17,18]. These studies suggest that host factors are also involved in the complex process of inter-species competition and interaction. Why the observed interaction between *S. pneumoniae* and *S. aureus* is serotype-dependent remains uncertain. We hypothesise, that this interaction is primarily genotype related. This assumption is supported by findings of Pericone et al. who have shown strain variability in the expression of SpxB and consequently, in the production of hydrogen peroxide [19]. Differences in hydrogen peroxide concentrations generated by the pneumococcal strains might explain different inhibitory effects on other bacterial species. Extensive in vitro and in vivo research on the observed serotype-related interaction between *S. pneumoniae* and *S. aureus* is required to obtain insight in the molecular basis of the observed bacterial inhibitory interaction. In addition, the potential effect of pneumococcal conjugate vaccination on *S. aureus* carriage also requires further study.

We also investigated the prevalence and determinants of nasopharyngeal carriage of *N. meningitidis* in the same group of children in Rotterdam, The Netherlands. These children were vaccinated with a meningococcal group C vaccination because of a recent outbreak with meningococcal group C disease (**Chapter 3.3**). We observed meningococcal carriage in 46 children (1.5%). Subtyping of the meningococcal strains showed 8 group B isolates, 9 group C isolates, 24 type xyzw 135 isolates and 8 others. Peak-incidences were seen in the first year of life (3.2% carriage) and after the age of 15 years (3.7% carriage). We therefore concluded that nasopharyngeal carriage of *N. meningitidis* was low at the time of the Dutch national meningococcal vaccination campaign in the summer of 2002. One might argue that

this is caused by differences in the method of sampling, i.e. nasopharyngeal instead of oropharyngeal cultures. At present, there is still uncertainty about the most optimal method of meningococcal sampling [20-24]. More importantly, the time of sampling, i.e. the summer, might explain the low incidence in carriage. Most often, a peak in meningococcal colonization and infection is observed in autumn and winter when the crowding factor increases [25]. Independent determinants of *N. meningitidis* carriage included age, pneumococcal carriage and regular visits of youth clubs and dancings, as was also observed by Dominguez et al. [24]. Together with season, most of these determinants lead to the same consequence, i.e. enhanced horizontal spread of meningococci within the population.

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality in adults. According to the Global Burden of Disease Study, COPD is the fifth most common disease and fourth leading cause of death in the world. Both prevalence and mortality are still expected to increase in the coming decades [26]. The chronic course of this disease is frequently interrupted by acute exacerbations, which have a major impact on the morbidity and mortality of COPD patients [27]. Bacterial infections are thought to contribute to the pathogenesis and clinical course of COPD [28]. Therefore, we investigated the effect of bacterial colonization during stable disease on time to first exacerbation in COPD patients (Chapter 3.4). In a randomized controlled trial, 244 COPD patients were followed for period of 10 months. Sputum samples were collected at randomization and during exacerbations. We calculated the time to development of an exacerbation in colonized compared to non-colonized patients. At randomization 36% of the patients were colonized with potentially pathogenic microorganisms. We found that overall bacterial colonization in COPD patients is not an independent risk factor for the development of acute exacerbations. However, we hypothesized that monocultures might indicate a prestadium of infection in contrast to the presence of mixed cultures. Therefore, we performed a subsidiary analysis on monocultures of pneumococci, *H. influenzae* and *M. catarrhalis* in the latter study (Chapter 8.3). We found that colonization with merely pneumococci (monocultures) increased the risk of an exacerbation with a hazard ratio of 2.93. The most prevalent pneumococcal serotypes found were the serotypes 19F, 3, 14, 9L/N/V, 23A/B and 11 with a theoretical coverage for the 7-, and 11- valent pneumococcal vaccine of 60% and 73%, respectively. These results support a causative role of pneumococci in exacerbations of chronic obstructive pulmonary disease. In addition, we have shown that pneumococcal colonization in COPD patients is frequently caused by vaccine serotype strains. More detailed studies are necessary to determine a potential role for pneumococcal vaccination in the prevention of exacerbations in COPD patients.

Since the discovery of the antibacterial properties of penicillin by Fleming in 1929, many antibiotics have been used for treatment of pneumococcal infections. Recently, antibiotic resistance has become a worldwide problem, which limits the choice of antimicrobial agents. A majority of the resistant strains belongs to a limited number of multidrug-resistant pandemic clones, which have spread rapidly throughout Europe, Asia and the United States. To evaluate the genetic background of penicillin-resistant pneumococcal isolates from Greek children, we performed antibiotic susceptibility testing, serotyping, restriction fragment end labeling (RFEL), and penicillin binding protein (PBP) genotyping (Chapter 4.1). The serotypes 23A/23F, 19A/19F, 9V, 15A/15B/15C, 6A/6B and 21 were most prevalent in this collection. Fifty-three distinct RFEL types were identified. Sixteen different RFEL clusters, harboring 2 to 32 strains each, accounted for 82% of all strains. Eight of these genetic clusters representing 60% of the strains were previously identified in other countries. One predominant lineage (46% of the strains) representing serotype 19F and 23F isolates was closely related to the pandemic clone Spain^{23F}-1 (genetic relatedness ≥ 85%). Another lineage representing 11 strains, showed close genetic relatedness to the pandemic clone France^{9V}-3. Another lineage of 8 serotype 21 strains was Greece-specific as the RFEL types were not observed in an international collection of 193 genotypes from 16 different countries. Characterization of the PBP genes *pbp1a*, *pbp2b* and *pbp2x* revealed 20 distinct PBP genotypes of which PBP type 1-1-1, initially observed in the pandemic clones 23F and 9V, was predominantly present in 11 RFEL types in this Greek collection of penicillin-nonsusceptible strains (55%). Sixteen PBP types covering 52 strains (36%) were Greece-specific. This study underlines the strong contribution of penicillin-resistant international clones to the prevalence and spread of penicillin-nonsusceptible pneumococci among young children in Greece.

In addition, Syrogiannopoulos et al. observed a significant spread of serotype 6B multidrug-resistant (MDR) pneumococci, susceptible to penicillin and resistant to erythromycin, clindamycin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole in young carriers living in Central and Southern Greece since 1996. Using RFEL and PBP genotyping we studied 41 serotype 6B penicillin-susceptible MDR pneumococci isolated during two independent studies in Greece **(Chapter 4.2)**. Forty (98%) of these 41 isolates were strongly related, representing a single lineage (genetic relatedness ≥ 91%). The Greek isolates were closely related (genetic relatedness ~91%) to the penicillin-resistant MDR clone of serotype 6B that has spread from Spain to Iceland in the late 1980s. Moreover, the Greek group of isolates was genetically distinct (genetic relatedness ≤ 83%) from other penicillin-susceptible or -resistant serotype 6B strains from various parts of the world. All serotype 6B penicillin-susceptible MDR isolates displayed a penicillin-susceptible PBP 1A-2B-2X genotype. These data suggest that the penicillin-susceptible MDR 6B clone that was found in Greece

between the years 1996 and 1999 represents the ancestor of the penicillin-resistant pandemic MDR clone 6B, which already has a high potency to spread horizontally within the population.

To evaluate the genetic relatedness of pneumococcal isolates, susceptible to penicillin but resistant to non-β-lactam agents representing non-serotype 6B isolates, we also investigated hundred-twenty-eight *S. pneumoniae* isolates from young carriers in Greece by antibiotic susceptibility testing, serotyping, RFEL and antibiotic resistance genotyping (**Chapter 4.3**). The serotypes 6A/B, 14, 19A/F, 11A, 23A/F, 15B/C, and 21 were most prevalent in this collection. Of the isolates, 65% was erythromycin-resistant, while the remaining isolates were tetracycline- and/or trimethoprim-sulfamethoxazole-resistant. Fifty-nine distinct RFEL types were identified. Twenty different RFEL clusters, harboring 2 to 19 strains each, accounted for 76% of all strains. Confirmatory multi locus sequence typing (MLST) analysis of the genetic clusters showed the presence of 3 international clones (Tennessee^{23F}-4, England^{14}-9 and Greece^{6B}-22) representing 30% of the isolates. The *erm*(B), *mef*(A) and *mef*(E) genes were all present and belonged to distinct genetic clusters. The majority of the tetracycline-resistance was related to the *Tet*(M) gene. We concluded that penicillin-susceptible pneumococci resistant to non-β-lactams are a genetically heterogeneous group displaying a variety of genotypes, resistance markers and serotypes. This suggests multiple genetic events leading to non-β-lactam resistant pneumococci in Greece. Importantly, most of these genotypes are capable to disseminate within the community.

Until now, antibiotic resistance among pneumococci in The Netherlands is uncommon. In 1995 and 1996, a nation-wide molecular epidemiologic survey of penicillin-nonsusceptible *S.* was performed in The Netherlands, which showed less than 2% penicillin resistance. We analyzed the *pbp* genes from these clinical isolates in detail (**Chapter 4.4**). The pneumococcal strains were selected on the basis of differences in restriction fragment length polymorphism (RFLP) patterns of the complete genes *pbp1a, pbp2b* and *pbp2x*, representing 8, 7 and 10 distinct patterns, respectively. We characterized specific gene parts of *pbp1a*, *pbp2b* and *pbp2x*. Classification based upon sequence analysis of these *pbp* fragments correlated well with the classification according to RFLP analysis. Sequence analysis of *pbp2b* enables a refinement of the classification based on RFLP analysis. However, sequence analysis of *pbp1a* and *pbp2x* was less discriminatory compared to RFLP analysis. The mutations in the *pbp* sequences of the Dutch isolates invariably matched with the mutations described in *pbp* sequences of penicillin-nonsusceptible pneumococci isolated in other countries. This observation supports the hypothesis that multiple clones of penicillin-resistant pneumococci have been imported and spread in The Netherlands. Interestingly, novel combinations of mosaic structures were also identified

indicating horizontal exchange of *pbp* gene parts among penicillin-nonsusceptible pneumococci. This is in line with previous findings of Coffey et al. They concluded that the *pbp* mosaic genes have emerged by recombinational events between the *pbp* genes of pneumococci and their homologues in closely related streptococcal species. These recombinational genes may have disseminated among pneumococci by horizontal gene transfer, presumably mediated by genetic transformation between different pneumococcal lineages to produce new resistant clones [29]. However, these events seem to occur seldom as can be concluded from the persisting low percentages of penicillin resistance. Obviously, this can be explained by the limited antibiotic pressure within the community as a result of the restricted antibiotic prescription behavior of physicians in The Netherlands.

Because of the persistent high morbidity and mortality due to pneumococcal disease, and the rapid acquisition and spread of antibiotic resistance among pneumococci worldwide, prevention of pneumococcal disease has become of great interest. Until now, many research groups have focused on the development of new effective vaccines to be used in particular risk groups including immunocompromised patients and children. One of these vaccines, the 7-valent pneumococcal conjugate vaccine, has shown to be highly effective against invasive disease caused by the included pneumococcal serotypes [30]. The 7-valent conjugate vaccine has also shown to be partially effective against pneumonia and otitis media [31-34]. Moreover, several investigators have shown a significant reduction in nasopharyngeal carriage of vaccine type pneumococci in infants as a result of different conjugate vaccinations [10,35-37]. However, the coverage is limited and varies per country or continent [38]. In order to evaluate the theoretical coverage of the vaccines and the effect of vaccination on future serotype distribution we need to monitor in detail and at large scale the molecular epidemiology of pneumococcal colonization and infection before and after the implementation of these vaccines. So far, the coverage of the conjugate vaccine has been investigated in several parts of the world. Recently, the Asian Network for Surveillance of Resistant Pathogens (ANSORP) has studied nasal carriage of pneumococci in healthy children in Taiwan, Korea, Sri Lanka and Vietnam. The most common serogroups were serogroup 6, 23, 19, 14 and 15. Because no subtypes were determined, the exact vaccine coverage could not be calculated [39]. Therefore, we investigated the molecular epidemiology of 84 pneumococcal carriage isolates of children attending several outpatient departments in Hanoi, Vietnam, with acute respiratory tract infections **(Chapter 5.1).** The serotypes 23F, 19F, 6B and 14 were found most often. A significant number of strains was antibiotic-resistant. Fifty-two percent of the strains was (intermediate) resistant to penicillin, 87% (intermediate) resistant to cotrimoxazole, 76% resistant to tetracycline, 73% resistant to erythromycin and 39% intermediate resistant to cefotaxim. Seventy-five percent was resistant to three or more classes of antibiotics. A high degree of genetic heterogeneity

among the penicillin resistance genes was observed. In addition, the tetracycline resistance gene *tetM* and the erythromycin resistance gene *ermB* were predominantly observed among the isolates. Molecular analysis of the 84 isolates by RFEL revealed 35 distinct genotypes. Twelve of these genotypes represented in total 8 genetic clusters and consisted of 61 of the isolates (73%). The two largest clusters represented 24 isolates and 12 isolates were identical to the two internationally spreading multi-drug resistant clones Spain 23F-1 and Taiwan 19F-14 clones, respectively. The remaining RFEL types were Vietnam-specific. 93% of the horizontally spreading isolates were covered by the 7-valent conjugate vaccine, whereas this vaccine covered only 43% of the unique genotypes. According to the serotype distribution of the nasopharyngeal pneumococcal isolates, this study suggests a high potential benefit of the 7-valent pneumococcal conjugate vaccine for children in Hanoi, Vietnam.

We also characterized the potential vaccine coverage, the genetic background and resistance profiles of 578 pneumococcal isolates from healthy Dutch children aged 1-19 years by means of serotyping, RFEL genotyping and susceptibility testing (**Chapter 5.2**). The most prevalent serotypes found were the serotypes 6B, 19F, 23F, 6A, 3, 11 and 14. In total, 42% of the strains represented vaccine type and 58% non-vaccine type pneumococci. The vaccine and cross-reactive serotypes showed peak-incidences at the age of 1-2 years. The non-vaccine serotype 11 showed a peak-incidence at the age of 3-6 years whereas the non-vaccine serotypes 3, 8, and 10 showed peak-incidences at the age of 7-10 years. In total, 337 genotypes were observed, which consisted of 153 unique genotypes and 184 genotypes comprising 92 genetic clusters. In contrast to the observed age-related serotype distribution, the genetic background of the strains was not age-related. Comparison of our population to the PMEN database revealed the presence of 5 international clones, i.e. Spain^{9V}-3, England ^{14}-9, Tennesee^{23F}-4, CSR^{14}-10 and Sweden^{15A}-25 in 18 strains. In total, 19% of all strains showed resistance to one or more antibiotics. Resistance to cotrimoxazole, tetracycline, erythromycin and penicillin was found in 13.9%, 6.4%, 5.0% and 3.4%, respectively. Multidrug resistance was found in 2.4% of all strains. These data implicate that most pneumococci found have a high tendency to spread horizontally. Moreover, these data indicate that the internationally recognized pandemic clones do not significantly contribute to colonization and spread of pneumococci among children in The Netherlands. Finally, because the majority of the strains is fully susceptible to the most common antibiotics, these data underline that a high tendency to spread is not automatically related to (multi)drug resistance.

To illustrate the possible consequences of infections with multidrug-resistant pneumococci, we describe in **Chapter 5.3** the follow-up of a nosocomial outbreak with a multidrug-resistant strain of *S. pneumoniae* among 36 patients with chronic obstructive pulmonary disease in

The Netherlands. To limit the outbreak, barrier nursing and a short-term ceftriaxone-rifampin eradication therapy were started. Rifampin, a semisynthetic derivative of the rifamycins that is primarily prescribed for the treatment of tuberculosis was used because of broad-spectrum activity, efficient (mucosal) tissue penetration, and a low side-effect profile. Because of the possibility for rapid emergence of resistance, rifampin was prescribed in combination with a third-generation cephalosporins [40,41]. Although the epidemic rapidly ceased, eradication therapy failed in 3 patients, and follow-up investigation of these patients showed the emergence of rifampin-resistant isolates. Microbiologic analysis confirmed that the studied isolates were of clonal origin and were identical to the initial outbreak strain. The mutations occurred in 2 different regions in the *rpo*B gene and displayed 3 distinct amino acid substitutions. We conclude that inadequate therapy caused by inaccurate prescription resulted in a primary rpoB-mediated rifampin resistance on 2 occasions. The use of rifampin monotherapy in patients 1 and 3 contributed to the acquisition of rifampin resistance, and these patients did not respond to treatment. Patient 2 manifested primary resistance despite accurate therapy. We hypothesize that the pathological lung structure of these COPD patients has contributed to inadequacy of treatment by the treatment protocol. This study indicates the importance of accurate bacteriologic monitoring of patients with COPD who are colonized or infected with MDR pneumococci and suggests extension of the current eradication regimen with an additional (β-lactam) antibiotic.

As we discussed so far, next to the rapid horizontal dissemination of drug-resistant pneumococcal strains [42,43], several of our studies have suggested a high level of horizontal dissemination of susceptible pneumococci as well. To study this in more detail, we investigated the molecular epidemiological characteristics of *S. pneumoniae* from all patients with pneumococcal meningitis in The Netherlands in 1994 (**Chapter 5.4**). RFEL analysis demonstrated 52% genetic clustering among these penicillin-susceptible strains, a value substantially lower than the percentage of clustering among Dutch penicillin-nonsusceptible strains [42-46]. Different serotypes were found within 8 of the 28 genetic clusters, suggesting that horizontal transfer of capsular genes is common among penicillin-susceptible strains. The degree of genetic clustering was much higher among serotype 3, 7F, 9V, and 14 isolates than among isolates of other serotypes, i.e., 6A, 6B, 18C, 19F, and 23F. We further studied the molecular epidemiological characteristics of pneumococci of serotype 3, which is considered the most virulent serotype and which is commonly associated with invasive disease in adults. Fifty epidemiologically unrelated penicillin-susceptible serotype 3 invasive isolates originating from the United States (n = 27), Thailand (n = 9), The Netherlands (n = 8), and Denmark (n = 6) were analyzed. The vast majority of the serotype 3 isolates (74%) belonged to two genetically distinct clades that were observed in the United States, Denmark, and The Netherlands. In line with previous studies, these data indicate that two

penicillin-susceptible serotype 3 clones have been independently disseminated internationally, suggesting strong genetic homogeneity and limited genetic heterogeneity within the serotype 3 pneumococci [47,48]. We hypothesize that this serotype has recently evolved or has remained unchanged for a prolonged period. The few serotype 3 isolates not belonging to the main clusters are presumably derived from horizontal transfer of capsular genes.

As mentioned earlier, the 7-valent conjugate vaccine has shown, besides an efficacy of 97.4% against invasive diseases caused by vaccine serotypes, a significant though less efficient impact on pneumonia and otitis media [31-34]. The pneumococcal vaccine reduced the number of infants suffering from recurrent episodes of AOM by 9% in both the American and the Finnish trial. The largest effect, a reduction of 23%, was observed in the number of children developing a severely otitis-prone condition with 5 episodes in 6 months or 6 episodes per year. This was also reflected in a 20% reduction in the number of children receiving tympanostomy tubes. Moreover, several investigators have shown a significant reduction in nasopharyngeal carriage of vaccine type pneumococci in infants as a result of different conjugate vaccinations [10,35-37]. Mucosal immunity in addition to systemic immunity would explain these effects. This is supported by the results of a second Finnish study, showing that a tetravalent pneumococcal conjugate vaccine induces both mucosal and systemic antibody responses in toddlers [49]. Because of these promising data, the ACIP recommended pneumococcal conjugate vaccination for all children under the age of 24 months, and children 24-59 months of age when at risk of pneumococcal disease [50,51]. In addition, the American Academy of Pediatrics (AAP) issued the advice to immunize children with recurrent or severe acute otitis media (AOM) and children with tympanostomy tubes because of recurrent AOM with 7-valent pneumococcal conjugate vaccine (PCV7) [52]. However, the prevention of recurrent acute otitis media (AOM) by the pneumococcal conjugate vaccine was only proven in infants immunized at 2, 4, 6 and 12-15 months of age. It has not been established whether pneumococcal conjugate vaccine also prevents AOM in older children with previous episodes of AOM. Therefore, a double-blind, randomized study, including 383 patients aged 1 to 7 years with two or more episodes of AOM in the year before entry was performed (**Chapter 7.1**). Randomization was stratified in 4 groups according to age (12-24 months versus 25-84 months) and the number of previous AOM episodes (2-3 AOM versus 4 or more AOM). The children received either 7-valent pneumococcal conjugate vaccine followed by 23-valent pneumococcal polysaccharide vaccine or hepatitis A or B vaccines. They were followed for 18 months for recurrence of AOM. Additional outcomes included pneumococcal cultures of middle ear fluid and nasopharyngeal pneumococcal carriage. We observed no reduction in AOM episodes in the pneumococcal vaccine group as compared to the control group. Although nasopharyngeal

carriage of conjugate-vaccine pneumococcal serotypes was significantly reduced after pneumococcal vaccinations, we found immediate and complete replacement by non-vaccine pneumococcal serotypes. We suggest that interference in an existing balance between host and pathogen by means of vaccination may induce pneumococcal strain replacement. The appearance of a new pathogen, in turn, increases the risk of acute otitis media [53,54]. This might explain the different results of the two landmark studies in California and Finland investigating the effect of conjugate vaccination on otitis media in infants: at infant age conjugate vaccination may prohibit or delay nasopharyngeal acquisition of the most prevalent pneumococcal serotypes, thus preventing or delaying early pneumococcal AOM until a later age. Therefore replacement will not occur in young children, in contrast to older children where full replacement of vaccine type pneumococci with non-vaccine type strains was observed. With ongoing follow-up, more studies have reported this phenomenon [10,34,55]. The effect of replacement to the burden of invasive diseases still remains unknown, although one conjugate vaccination trial in the US recently reported 25% increase in invasive diseases caused by non-vaccine serotypes. Though this was not yet statistically significant, in the future it may become clinically relevant [56]. This hypothesis is supported by data of Brueggemann et al., who have shown a high invasive capacity for certain non-vaccine serotypes, which may also imply that replacement of carriage will lead to replacement of disease [57].

To evaluate the effect of 7-valent pneumococcal conjugate vaccination on carriage in the different age-groups, we performed additional analysis on the OMAVAX colonization data (**Chapter 7.2**). After vaccinations overall pneumococcal carriage rates did not change and remained around 50% in both pneumococcal and control group during 18 months of follow-up. However, in children who had received two conjugate vaccinations followed by a polysaccharide booster (age 12 to 24 months) a significant shift from conjugate vaccine type pneumococci to non-vaccine type pneumococci was observed. The most pronounced reduction in carriage was found for serotype 18C and the most important increase in colonization was noted for the serotypes 11, 15, and 16 as compared to the controls. In contrast, pneumococcal carriage was not influenced in children older than 24 months, who received the conjugate vaccine once followed by the polysaccharide booster. Therefore, we concluded that vaccination with at least twice the conjugate vaccine also after 2 years of age may be mandatory for carriage reduction of conjugate vaccine serotypes in children with recurrent otitis media. In addition, the natural shift in carriage of VT to non-VT *S. pneumoniae* by increasing age may limit the impact of the pneumococcal conjugate vaccine on pneumococcal carriage in older children, as concluded by previous studies [10].

We decided to study the molecular epidemiological dynamics and resistance profiles of the pneumococcal strains collected from both children in the pneumococcal vaccine (PV) and

the hepatitis control vaccine group (CV) of the latter study in order to obtain insight in the effect of conjugate vaccination on the genetic background of the pneumococcal population (**Chapter 7.3**). We investigated the molecular epidemiology of 921 pneumococcal isolates retrieved from both the pneumococcal vaccine (PV) and control vaccine (CV) group during the vaccination study. Within individuals a high turnover rate of pneumococcal genotypes was observed, which was unaffected by vaccination. Comparison of the genetic structure before and after completion of the vaccination scheme revealed, despite a shift in serotypes, genetic homology between 70% of the pneumococcal populations. The remaining isolates (30%) were equally observed in the PV and the CV group. In addition, the degree of genetic clustering was unaffected by vaccination. However, within the population genetic structure, non-vaccine serotype clusters with the serotypes 11, 15 and 23B became predominant over vaccine-type clusters as a result of vaccination. Finally, overall pneumococcal resistance was low (14%), and, albeit not significant, a reduction in pneumococcal resistance as a result of pneumococcal vaccination was observed. We therefore concluded that pneumococcal conjugate vaccination did not induce a shift in the population-based structure of the pneumococci, nor decreased their tendency to spread horizontally. Our observations combined with the vaccine efficacy data (**Chapter 7.1**) suggest that pneumococcal conjugate vaccination is not very useful for prevention of pneumococcal colonization in Dutch children older than 1 year of age.

In **Chapter 7.2**, we found replacement of colonizing strains at the nasopharynx as a result of pneumococcal conjugate vaccination, followed by a polysaccharide booster. This replacement may seriously complicate the efficacy of pneumococal conjugate vaccines. This depends on whether this phenomenon is caused by unmasking of pneumococcal serotypes which are already present at the nasopharynx or by replacement, i.e. the acquisition of other serotypes, remains unknown [58]. We therefore developed a colony blot assay, which allows the detection of multiple serotypes within clinical specimens (**Chapter 7.4**). This method can assess whether re-colonization with novel serotypes (replacement), overgrowth by minor co-colonizing serotypes (unmasking) or masking of previously predominant vaccine serotype strains occurs at the nasopharynx as a result of vaccination. We found that this method allows the identification of multiple serotypes in a single specimen in a ratio of 1:1000. In order to demonstrate the potential of our method, we investigated the consecutive nasopharyngeal samples of 26 children who had shown a shift in pneumococcal colonization after conjugate vaccination. Unmasking and masking were found in 1 out of 15 and 4 out of 26 cases, respectively. In the remaining children 'true replacement' had presumably occurred. We hypothesize, that the persistence of vaccine type strains accompanied by immunological pressure due to vaccination and the availability of non-vaccine serotype strains for replacement might create ideal circumstances for recombinational exchange of

capsular genes [59]. Assuming that this phenomenon occurs as a result of vaccination, future vaccine failures with respect to prevention of disease and elimination of multidrug-resistant clones may take place. To study the true effect of conjugate vaccination on replacement, obviously a larger study should be performed. For such study, the colony blot assay will be a useful additional tool.

So far, we have described studies with respect to the pathogen-related aspects of colonization and infection. However, pneumococcal colonization and infection as well as the efficacy of pneumococcal vaccination are determined by host, pathogen, and environmental factors. In Chapter 8, we describe several studies with respect to the host-response to pneumococcal vaccination and infection. First, because colonization with vaccine serotype strains had decreased in pneumococcal vaccine recipients but not otitis media, we questioned whether pneumococcal vaccination had provoked a mucosal immune response in our study population. We compared the IgG and IgA antibody concentration in saliva of children of the pneumococcal vaccine group with children of the control vaccine group (Chapter 8.1). IgA and IgG antibody concentrations to vaccine serotypes 6B, 14, 18C and 19F were measured by enzyme immunoassay in 38 saliva samples of children vaccinated with pneumococcal conjugate vaccine and 45 control samples. In the pneumococcal vaccine group, 12 samples were received prior to vaccination, 12 samples 4 weeks after the polysaccharide booster and 14 samples 7 months after the last vaccination. In the control group 15 children were sampled at each of these 3 time points. We observed a trend towards increased salivary IgG antibody concentrations 14 months after the primary vaccination for the serotypes 6B, 14, and 18C in the vaccinated children but not the control children. This increase in IgG antibodies was seen only in children vaccinated with pneumcoccal conjugate vaccine twice followed by a polysaccharide booster. The IgA antibody titers increased significantly after 8 and after 14 months for all serotypes in both pneumococcal vaccine recipients and controls. However, the mean antibody titer increased significantly more in control children compared to children vaccinated with pneumococcal conjugate vaccine. Our data suggest that repeated pneumococcal conjugate vaccination is obligatory for persisting elevated salivary IgG antibodies in children.. However, because a significantly higher increase in mucosal IgA response is observed in control children compared to vaccinated children, we hypothesize that this is provoked by ongoing carriage rather then by vaccination. Therefore, we conclude that combined pneumococcal conjugate and polysaccharide vaccination does not induce a mucosal IgA response.

Because recent studies suggest a role for bacteria like *Streptococcus pneumoniae* in the development of acute exacerbations of chronic obstructive pulmonary disease, we investigated the epidemiology of pneumococcal colonization and infection, the effect of pneumococcal colonization on the development of exacerbation, and the immunological

response against *S. pneumoniae* in COPD patients (Chapter 8.2). To this purpose, we cultured sputa of 269 COPD patients during stable state and exacerbations of COPD and we characterized the 115 pneumococcal isolates by means of serotyping. Moreover, we studied serum IgG antibodies, avidity and functional antibody titers against the seven conjugate-vaccine serotypes in these patients. Although overall carriage was not associated with exacerbations of COPD, we found that colonization with merely pneumococci (monocultures) increased the risk of an exacerbation with a hazard ratio of 2.93. The most prevalent pneumococcal serotypes found were the serotypes 19F, 3, 14, 9L/N/V, 23A/B and 11 with a theoretical coverage for the 7-, and 11- valent pneumococcal vaccine of 60% and 73%, respectively. These data indicate that pneumococal colonization in COPD patients is frequently caused by vaccine serotype strains. All patients had detectable IgG levels against the 7 conjugate-vaccine serotypes. These antibody titers were significantly lower than in vaccinated healthy adults. On average, a 2.5-fold rise in serotype-specific and functional antibodies was observed during exacerbations with *S. pneumoniae* positive sputum cultures. These data demonstrate that COPD patients are able to mount a significant immune response to pneumococcal infection. However, one could question whether these antibodies protect COPD patients against pneumococcal re-infection. Follow-up studies in which the protective potentials of these antibodies are investigated, are necessary in order to estimate the effect of pneumococcal conjugate vaccination of COPD patients.

Host protection against *S. pneumoniae* is mainly mediated by opsonin-dependent phagocytosis. Therefore, the in vitro measurement of opsonophagocytic activity of the anti-capsular antibodies is considered to be a reliable correlate of protection to evaluate vaccine responses [60,61]. Unfortunately, the methods used so far are all tedious to perform and material consuming. In **Chapter 8.3**, we describe the modification of the multi-specificity opsonophagocytosis killing assay (MSOPKA) into a high throughput method, which simultaneously measures the opsonophagocytosis against the seven serotypes covered by the current conjugate vaccine in a single assay. In the so called multiplex opsonophagocytosis assay (MOPA), a mixture containing equal numbers of colony forming units (CFUs) of chloramphenicol-resistant serotype 4, spectinomycin-resistant serotype 6B, streptomycin-resistant serotype 9V, erythromycin-resistant serotype 14, rifampicin-resistant serotype 18C, tetracycline-resistant serotype 19F, and trimethoprim-resistant serotype 23F pneumococci was used as a target mixture and incubated with serial dilutions of test serum. After opsonophagocytosis by differentiated HL-60 cells in the presence of complement, the samples were spotted onto different blood agar plates containing the 7 selective antibiotics, respectively. Opsonophagocytosis was calculated as the highest serum dilution resulting in 90% or more reduction in CFUs. We tested whether the phagocytosis capacity is correlated to the serotype-specific IgG antibody titers. Despite the limited number of sera tested, we

found high correlations for opsonophagocytosis capacity and IgG antibody titers for the serotypes 4, 6B, 9V, 18C and 23F. For the remaining serotypes, i.e. 14 and 19F, lower correlations were found for opsonophagocytosis and serotype-specific IgG. These data suggest that besides antibody titers, additional variables may influence opsonophagocytosis activity. Furthermore, our findings underline the importance of testing opsonophagocytosis activity in addition to the measurement of antibody titers when monitoring vaccination.

The current 7-valent conjugate vaccine is highly efficacious against invasive disease caused by the vaccine type strains. However, the vaccine coverage is limited and replacement by non-vaccine serotypes resulting in disease is a serious threat for the near future. Therefore, the search for new vaccine candidates that will elicit protection against a broader range of pneumococcal strains is still ongoing. To this purpose, several surface-associated protein vaccines are under investigation. Surface associated pneumococcal proteins α-enolase (Eno), immunoglobulin A1 protease (Iga), streptococcal lipoprotein rotamase A (SlrA), and putative proteinase maturation protein A (PpmA) have potential as candidates for future protein-based anti-pneumococcal vaccines. In **Chapter 8.4**, the immunogenicity of these proteins was studied in a cohort of 329 children during their first two years of life. During the first recorded episode of otitis media, acute and convalescent phase sera were available from 151 of these children. Concentrations of antibodies against Eno, Iga, SlrA and PpmA were measured by EIA and detected in 83-99% of the sera, respectively. There were no statistically significant differences between the groups of children with and without a history of pneumococcal contact or with respect to the type of pneumococcal contact. No significant correlation between antibody titers to these proteins and pneumococcal carriage or infection was found. These data suggest the presence of cross-reactive epitopes on commensal bacteria co-colonizing the nasopharyngeal niche. Since pathogen-specific epitopes are less likely to cross-react with epitopes of commensal bacteria of the nasopharynx, such proteins might have more potential as vaccine candidates.

REFERENCES

1. Jaffar S, Leach A, Hall AJ, et al. Preparation for a pneumococcal vaccine trial in The Gambia: individual or community randomisation? *Vaccine* 1999;18(7-8):633-40.
2. Pichichero ME, Shelly MA, Treanor JJ. Evaluation of a pentavalent conjugated pneumococcal vaccine in toddlers. *Pediatr Infect Dis J* 1997;16(1):72-4.
3. Kellner JD, Ford-Jones EL. *Streptococcus pneumoniae* carriage in children attending 59 Canadian child care centers. Toronto Child Care Centre Study Group. *Arch Pediatr Adolesc Med* 1999;153(5):495-502.
4. Nuorti JP, Butler JC, Crutcher JM, et al. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. *N Engl J Med* 1998;338(26):1861-8.
5. Principi N, Marchisio P, Schito GC, Mannelli S. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. *Pediatr Infect Dis J* 1999;18(6):517-23.
6. Hament JM, Kimpen JL, Fleer A, Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. *FEMS Immunol Med Microbiol* 1999;26(3-4):189-95.
7. Mulholland K. Strategies for the control of pneumococcal diseases. *Vaccine* 1999;17 Suppl 1:S79-84.
8. Plotkowski MC, Puchelle E, Beck G, Jacquot J, Hannoun C. Adherence of type I *Streptococcus pneumoniae* to tracheal epithelium of mice infected with influenza A/PR8 virus. *Am Rev Respir Dis* 1986;134(5):1040-4.

9. Ghaffar F, Friedland IR, McCracken GH, Jr. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. *Pediatr Infect Dis J* 1999;18(7):638-46.
10. Dagan R, Givon-Lavi N, Zamir O, et al. Reduction of nasopharyngeal carriage of *Streptococcus pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J Infect Dis* 2002;185(7):927-36.
11. El Ahmer OR, Essery SD, Saadi AT, et al. The effect of cigarette smoke on adherence of respiratory pathogens to buccal epithelial cells. *FEMS Immunol Med Microbiol* 1999;23(1):27-36.
12. Veenhoven R, Bogaert D, Uiterwaal C, et al. Effect of pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. *Lancet* 2003;361:2189-95.
13. Mc Leod JW, Gordon J. Production of hydrogen peroxide by bacteria. *Biochemistry Journal* 1922;16:499-506.
14. Repine JE, Fox RB, Berger EM. Hydrogen peroxide kills *Staphylococcus aureus* by reacting with staphylococcal iron to form hydroxyl radical. *J Biol Chem* 1981;256(14):7094-6.
15. Dahiya RS, Speck ML. Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *J Dairy Sci* 1968;51(10):1068-72.
16. Uehara Y, Kikuchi K, Nakamura T, et al. Inhibition of methicillin-resistant *Staphylococcus aureus* colonization of oral cavities in newborns by viridans group streptococci. *Clin Infect Dis* 2001;32(10):1399-407.
17. Faden H, Stanievich J, Brodsky L, Bernstein J, Ogra PL. Changes in nasopharyngeal flora during otitis media of childhood. *Pediatr Infect Dis J* 1990;9(9):623-6.
18. Ghaffar F, Muniz LS, Katz K, et al. Effects of large dosages of amoxicillin/clavulanate or azithromycin on nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, nonpneumococcal alpha-hemolytic streptococci, and *Staphylococcus aureus* in children with acute otitis media. *Clin Infect Dis* 2002;34(10):1301-9.
19. Pericone CD, Overweg K, Hermans PW, Weiser JN. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. *Infect Immun* 2000;68(7):3990-7.
20. Bakir M, Yagci A, Ulger N, Akbenlioglu C, Ilki A, Soyletir G. Asymtomatic carriage of *Neisseria meningitidis* and *Neisseria lactamica* in relation to *Streptococcus pneumoniae* and *Haemophilus influenzae* colonization in healthy children: apropos of 1400 children sampled. *Eur J Epidemiol* 2001;17(11):1015-8.
21. Cartwright KA, Stuart JM, Jones DM, Noah ND. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. *Epidemiol Infect* 1987;99(3):591-601.
22. Sim RJ, Harrison MM, Moxon ER, Tang CM. Underestimation of meningococci in tonsillar tissue by nasopharyngeal swabbing. *Lancet* 2000;356(9242):1653-4.
23. Jordens JZ, Williams JN, Jones GR, Heckels JE. Detection of meningococcal carriage by culture and PCR of throat swabs and mouth gargles. *J Clin Microbiol* 2002;40(1):75-9.
24. Dominguez A, Cardenosa N, Izquierdo C, et al. Prevalence of *Neisseria meningitidis* carriers in the school population of Catalonia, Spain. *Epidemiol Infect* 2001;127(3):425-33.
25. Neal KR, Nguyen-Van-Tam JS, Jeffrey N, et al. Changing carriage rate of *Neisseria meningitidis* among university students during the first week of term: cross sectional study. *Bmj* 2000;320(7238):846-9.
26. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 1997;349(9064):1498-504.
27. Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ, Wedzicha JA. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161(5):1608-13.
28. Wedzicha JA. Exacerbations: etiology and pathophysiologic mechanisms. *Chest* 2002;121(5 Suppl):136S-141S.
29. Coffey TJ, Dowson CG, Daniels M, Spratt BG. Genetics and molecular biology of beta-lactam-resistant pneumococci. *Microb Drug Resist* 1995;1(1):29-34.
30. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000;19(3):187-95.
31. Black SB, Shinefield HR, Ling S, et al. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. *Pediatr Infect Dis J* 2002;21(9):810-5.
32. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000;19(3):187-95.
33. Fireman B, Black SB, Shinefield HR, Lee J, Lewis E, Ray P. Impact of the pneumococcal conjugate vaccine on otitis media. *Pediatr Infect Dis J* 2003;22(1):10-6.
34. Eskola J, Kilpi T, Palmu A, et al. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* 2001;344(6):403-9.
35. Lakshman R, Murdoch C, Race G, Burkinshaw R, Shaw L, Finn A. Pneumococcal nasopharyngeal carriage in children following heptavalent pneumococcal conjugate vaccination in infancy. *Arch Dis Child* 2003;88(3):211-4.
36. Dagan R, Melamed R, Muallem M, et al. Reduction of nasopharyngeal carriage of pneumococci during the second year of life by a heptavalent conjugate pneumococcal vaccine. *J Infect Dis* 1996;174(6):1271-8.
37. Dagan R, Muallem M, Melamed R, Leroy O, Yagupsky P. Reduction of pneumococcal nasopharyngeal carriage in early infancy after immunization with tetravalent pneumococcal vaccines conjugated to either tetanus toxoid or diphtheria toxoid. *Pediatr Infect Dis J* 1997;16(11):1060-4.
38. Pelton SI, Dagan R, Gaines BM, et al. Pneumococcal conjugate vaccines: proceedings from an Interactive Symposium at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy. *Vaccine*

2003;21(15):1562-71.
39. Lee NY, Song JH, Kim S, et al. Carriage of antibiotic-resistant pneumococci among Asian children: a multinational surveillance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP). *Clin Infect Dis* 2001;32(10):1463-9.
40. Bradley JS, Scheld WM. The challenge of penicillin-resistant *Streptococcus pneumoniae* meningitis: current antibiotic therapy in the 1990s. *Clin Infect Dis* 1997;24 Suppl 2:S213-21.
41. Friedland IR, McCracken GH, Jr. Management of infections caused by antibiotic-resistant *Streptococcus pneumoniae*. *N Engl J Med* 1994;331(6):377-82.
42. Hermans PW, Sluijter M, Dejsirilert S, et al. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. *Microb Drug Resist* 1997;3(3):243-51.
43. Hermans PWM, Overweg K, Sluijter M, Groot R. Penicillin-Resistant *Streptococcus pneumoniae*: An International Molecular Epidemiological Study. In: Tomasz A, ed. *Streptococcus pneumoniae*; Molecular Biology & mechanisms of Disease, 2000: 457-466.
44. Hermans PW, Sluijter M, Elzenaar K, et al. Penicillin-resistant *Streptococcus pneumoniae* in the Netherlands: results of a 1-year molecular epidemiologic survey. *J Infect Dis* 1997;175(6):1413-22.
45. Barnes DM, Whittier S, Gilligan PH, Soares S, Tomasz A, Henderson FW. Transmission of multidrug-resistant serotype 23F *Streptococcus pneumoniae* in group day care: evidence suggesting capsular transformation of the resistant strain in vivo. *J Infect Dis* 1995;171(4):890-6.
46. Overweg K, Hermans PW, Trzcinski K, Sluijter M, de Groot R, Hryniewicz W. Multidrug-resistant *Streptococcus pneumoniae* in Poland: identification of emerging clones. *J Clin Microbiol* 1999;37(6):1739-45.
47. Hall LM, Whiley RA, Duke B, George RC, Efstratiou A. Genetic relatedness within and between serotypes of *Streptococcus pneumoniae* from the United Kingdom: analysis of multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and antimicrobial resistance patterns. *J Clin Microbiol* 1996;34(4):853-9.
48. Louie M, Louie L, Papia G, Talbot J, Lovgren M, Simor AE. Molecular analysis of the genetic variation among penicillin- susceptible and penicillin-resistant *Streptococcus pneumoniae* serotypes in Canada. *J Infect Dis* 1999;179(4):892-900.
49. Nieminen T, Kayhty H, Leroy O, Eskola J. Pneumococcal conjugate vaccination in toddlers: mucosal antibody response measured as circulating antibody-secreting cells and as salivary antibodies. *Pediatr Infect Dis J* 1999;18(9):764-72.
50. Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2000;49(RR-9):1-35.
51. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 1997;46(RR-8):1-24.
52. Policy statement: recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine (Prevnar), pneumococcal polysaccharide vaccine, and antibiotic prophylaxis (RE9960). *Pediatrics* 2000;106:362-66.
53. Kilpi T, Palmu A, Jokinen J, Kähty H, Mäkela. Efficacy of conjugate vaccine against acute otitis media (AOM) - Finnish Experience. In: Program and abstracts of the 3rd International Symposium on Pneumococci and Pneumococcal Diseases, Anchorage, May 5-8, 2002:110. Abstract. 2002.
54. Gray BM, Converse GM, 3rd, Dillon HC, Jr. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* 1980;142(6):923-33.
55. Mbelle N, Huebner RE, Wasas AD, Kimura A, Chang I, Klugman KP. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis* 1999;180(4):1171-6.
56. Hsu K, Pelton D, Heisey-Grove S, Hashemi J, Klein J, Health aMotMDoP. Conjugate vaccine era serotype-specific surveillance for invasive pneumococcal disease in massachusetts children:Program and abstracts of the 21st Annual Meeting of the European Society for Paediatric Infectious Diseases, Giardini Naxos, April 9-11, 2003. 2003.
57. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* 2003;187(9):1424-32.
58. Lipsitch M. Bacterial vaccines and serotype replacement: lessons from *Haemophilus influenzae* and prospects for *Streptococcus pneumoniae*. *Emerg Infect Dis* 1999;5(3):336-45.
59. Spratt BG, Greenwood BM. Prevention of pneumococcal disease by vaccination: does serotype replacement matter? *Lancet* 2000;356(9237):1210-1.
60. Vakevainen M, Jansen W, Saeland E, et al. Are the opsonophagocytic activities of antibodies in infant sera measured by different pneumococcal phagocytosis assays comparable? *Clin Diagn Lab Immunol* 2001;8(2):363-9.
61. Rodriguez ME, Van der Pol W, Van de Winkel JG. Flow cytometry-based phagocytosis assay for sensitive detection of opsonic activity of pneumococcal capsular polysaccharide antibodies in human sera. *J Immunol Methods* 2001;252(1-2):33-44.

Chapter 10

Conclusions and future perspectives

In this final chapter we seek to provide answers to the five research objectives, which were put forward in the introductory chapter (Chapter 1). We have summarized our most relevant findings and propose future directions for clinical and laboratory research.

Question 1. What is the prevalence and which are the determinants of nasopharyngeal colonization with *S. pneumoniae* and other pathogenic bacteria in children?

Major conclusions. Nasopharyngeal carriage with *S. pneumoniae* is most frequently observed during the first three years of live with a peak incidence of 55% at the age of three years followed by a general decline to 10% after the age of 10 years. Nasopharyngeal colonization with *S. aureus* also shows an age-related distribution with the highest colonization rate of 50% at the age of 10 years. *N. meningitidis* was found rarely among Dutch children in the summer of 2002. The highest prevalence was seen in the second year of life and after the age of 15. Determinants of nasopharyngeal carriage with these bacteria are mainly environmental and include smoking (passive and active), and crowding (day-care, discotheque). Importantly, we observed a natural competitive balance between vaccine-type pneumococci and *S. aureus* during colonization, which may explain the increase in *S. aureus*-related otitis media events found after vaccination. In addition, we found a positive correlation between *N. meningitidis* and *S. pneumoniae* for colonization of the nasopharynx (Chapter 3.1, Chapter 3.2, Chapter 3.3).

Future directions. Age-related distributions of and species-specific interactions between the colonizing bacteria of the nasopharynx should be further investigated. To this respect, both extensive in vitro assays (competition and interaction) and follow-up studies (natural dynamics and interference) should be performed. Importantly, host, pathogen, as well as environmental factors should be considered in such studies.

Question 2. Is nasopharyngeal colonization with *S. pneumoniae* during health and (mucosal) infection a static or dynamic process?

Major conclusions. Pneumococcal colonization during asymptomatic colonization and disease is a highly dynamic process allowing the rapid acquisition, spread and elimination of pneumococcal isolates in individuals and within the population. This process is enhanced in case of crowding as occurs in day-care centers and hospitals. The tendency to spread horizontally is serotype and genotype related (Chapter 3.1, Chapter 5.1, Chapter 5.2, Chapter 5.3, Chapter 5.4).

Future directions. Continued molecular surveillance of nasopharyngeal colonization is indicated in risk groups for pneumococcal colonization and infection allowing us to study the

dissemination of pneumococcal clones, possible changes in serotype distribution and the acquisition and spread of resistance among pneumococci.

Question 3. How efficient do antibiotic-resistant pneumococci spread within the population and which events are involved in the acquisition and spread of resistance and resistance genes in pneumococci?

Major conclusions. Antibiotic-resistant pneumococci, specifically the internationally recognized multidrug-resistant clones, have shown a rapid worldwide spread. However, their non-resistant relatives of similar chromosomal genotype also display a high capacity to spread within the community, which suggests a prevailing genotype-related influence. Antibiotic resistance has evolved by the horizontal transfer of resistance genes between related species and pneumococcal strains and recombinational events within resistance genes of pneumococci. The acquisition of antibiotic resistance in individuals is enhanced by the level and type of antibiotic pressure as well as by the condition of the patient (Chapter 4.1, Chapter 4.2, Chapter 4.3, Chapter 4.4, Chapter 5.2).

Future directions. Restrictive and selective antibiotic prescription behavior is warranted to minimize the selection and spread of (multi)drug resistance among pneumococcal isolates. Close monitoring of (multi)drug resistance among pneumococcal isolates is necessary to provide a timely change in therapy and to prevent treatment failures. In case of a nosocomial outbreak with a multidrug resistant strain among COPD patients, adequate combination therapy should be given to ensure eradication and to avoid persistent carriage and horizontal spread.

Research question 4. What is the effect of pneumococcal vaccination on colonization and infection with *S. pneumoniae*?

Major conclusions. Administration of a pneumococcal conjugate vaccine followed by a polysaccharide booster has no effect on the prevalence of otitis media in children with recurrent acute otitis media. In addition, this vaccination regimen induces nasopharyngeal replacement of vaccine serotype strains with non-vaccine serotype pneumococci. This phenomenon occurs specifically in children under two years of age. In older children no effect of vaccination on colonization was observed. The genetic make-up of the replacement strains is closely related to the pneumococcal vaccine serotype strains. Therefore, pneumococcal conjugate vaccination followed by a polysaccharide booster has no effect on colonization, otitis media and genetic background of the pneumococcal population. (Chapter 7.1, Chapter 7.2, Chapter 7.3, Chapter 7.4).

Future directions. Future research should focus on the effect of pneumococcal conjugate vaccination on the first acquisition of pneumococci, and the potential influence on the occurrence of the first otitis media event. Moreover, such a study should investigate whether a delay in colonization with pneumococci is of influence on the composition of the nasopharyngeal flora.

Question 5. What is the response of the host immune system and what type of response occurs with respect to pneumococcal colonization, infection and vaccination?

Conclusions. Airway colonization with *S. pneumoniae* is a risk factor for the development of exacerbations in patients with chronic obstructive pulmonary disease (COPD). Colonization of sputum is frequently observed in this patient group and often involves vaccine serotype strains. The majority of the COPD patients mount an anti-capsular immune response during pneumococcal colonization and infection, which suggests a potential role for conjugate vaccination in the prevention of exacerbations of COPD.

In children, a significant systemic IgG response can be found in response to pneumococcal conjugate vaccination followed by a polysaccharide booster. Small increases in IgG antibody titers can be found in saliva of these children, suggesting the transudation of IgG from blood to saliva. This is only found after repeated conjugate vaccinations. We observed a significant increase in mucosal IgA antibodies after pneumococcal conjugate vaccination. Since this increase was higher in the control children we conclude this is the result of colonization and infection rather than of vaccination.

In the search for potential candidates for future protein-based anti-pneumococcal vaccines we found several pneumococcal proteins to elicit antibody responses early in life. However, no significant correlation between antibody titers to these proteins and pneumococcal carriage or infection could be found, which presumably results from the presence of cross-reactive epitopes on commensal bacteria (Chapter 8.1, Chapter 8.2, Chapter 8.4).

Future directions. Future research should be performed to evaluate what type of immune response protects against pneumococcal colonization and mucosal infection. In addition, detailed research has to be done with respect to mucosal vaccination (oral/nasal) and prevention of carriage and mucosal diseases. The exact role of pneumococcal colonization on exacerbations of COPD should be investigated. Moreover, a pneumococcal conjugate vaccine study should be performed to evaluate the potential effect of vaccination on exacerbations of COPD. Finally, future studies investigating the effect of (new) conjugate vaccines on colonization and infection with *S. pneumoniae* should consider side effects with respect to co-colonizing pathogens and the commensal flora.

Nederlandse samenvatting

Streptococcus pneumoniae is een belangrijke verwekker van hersenvliesontsteking, sepsis (bloedvergiftiging) en longontsteking. Deze bacterie, bekend onder de naam pneumokok, is de belangrijkste oorzaak van hersenvliesontsteking en sepsis bij kinderen sinds deze als onderdeel van het Rijksvaccinatie programma worden ingeënt tegen *Neisseria meningitidis* serogroep C en *Haemophilus influenzae* type B. Gemiddeld sterven er elk jaar wereldwijd 1 miljoen kinderen onder de leeftijd van 5 jaar ten gevolge van infecties door pneumokokken. Daarnaast veroorzaakt de bacterie veelvuldig bovenste luchtweginfecties zoals middenoorontsteking en voorhoofd- en bijholteontsteking. Ook ouderen en patiënten met afweerstoornissen hebben een verhoogd risico op het oplopen van infecties met deze bacterie.

Pneumokokken komen vaak asymptomatisch voor in de neus-en-keel holte. Vrijwel alle kinderen worden reeds in hun vroege jeugd 'gekoloniseerd' met deze bacterie. De kans op kolonisatie is met name groot op plaatsen waar veel mensen langdurig samenkomen, zoals in kinderdagverblijven, ziekenhuizen, verpleeghuizen of gevangenissen. De mens heeft van nature voldoende afweer om niet ziek te worden ten gevolge van dragerschap met deze bacterie. Echter, in geval van een (tijdelijk) verminderde afweer kunnen pneumokokken infectie en ziekte veroorzaken. Personen met een verhoogd risico op dragerschap hebben derhalve een verhoogde kans op infecties met pneumokokken.

De pneumokok is een diplokok; de bacterie komt in paren van twee voor omgeven door een suikerkapsel. Er zijn inmiddels 90 verschillende kapseltypen en subtypen bekend, ook wel serotypen genoemd. In de laatste decennia zijn er vaccins ontwikkeld om infecties met de meest voorkomende kapseltypen te voorkomen. Het 23-valente suikervaccin, waarin de meest voorkomende kapseltypen zijn opgenomen, is echter niet effectief in kinderen onder de leeftijd van 2 jaar omdat deze nog niet in staat zijn antistoffen te maken tegen suikers. Sinds kort is er echter een conjugaatvaccin op de markt dat werkzaam is tegen de 7 meest voorkomende serotypen. De suikers zijn hierbij gekoppeld aan een eiwit waardoor jonge kinderen wel antistoffen tegen de suikers kunnen maken. Dit vaccin heeft echter als nadeel dat het werkzaam is tegen slechts 7 van de 90 serotypen.

In 1999 werd de invloed van kinderdagverblijfbezoek op dragerschap met pneumokokken in Nederland bestudeerd (**hoofdstuk 3.1**). Hiervoor werden 259 kinderen uit 16 kinderdagverblijven in Amsterdam onderzocht op dragerschap met pneumokokken. Daarnaast onderzochten we een controle groep van 276 kinderen die geen kinderdagverblijf bezochten. De kinderen uit de kinderdagverblijven bleken 2 tot 3 keer zo vaak gekoloniseerd te zijn met pneumokokken dan de controle populatie. We hebben ook de verdeling van de serotypen bestudeerd om te kunnen berekenen hoeveel pneumokokkeninfecties voorkomen zouden kunnen worden met de introductie van het 7-valente conjugaatvaccin in Nederland. Verder werd de genetische achtergrond van de pneumokokken bestudeerd middels het DNA. De zogenaamde genetische 'vingerafdruk' die met deze techniek wordt verkregen wordt ook wel genotype genoemd (**hoofdstuk 3.1**). Bij zowel de kinderdagverblijfkinderen als bij de controle kinderen werden de serotypen 19F, 6B, 6A, 23F, and 9V het meest frequent gevonden. De theoretische bescherming van het 7-valente conjugaatvaccin was voor beide groepen kinderen 55 tot 59%. Bestudering van de genetische achtergrond van de pneumokokken toont aan dat de pneumokokken bij de kinderdagverblijfkinderen veel vaker genetisch op elkaar lijken dan de pneumokokken in de controle groep. Dit wijst er op dat pneumokokken zich sneller van kind tot kind verspreiden binnen kinderdagverblijven dan erbuiten, maar dat dit fenomeen niet afhankelijk is van het kapseltype.

In 2002 werden de risicofactoren voor dragerschap met pneumokokken en *S. aureus* onderzocht in 3200 gezonde kinderen in de leeftijd van 1 tot 19 jaar (**hoofdstuk 3.2**). De belangrijkste variabelen voor dragerschap met pneumokokken bleken leeftijd en het bezoeken van kinderdagverblijven. In de groep kinderen tot 3 jaar kwamen vooral pneumokokken serotypen voor die in het 7-valente vaccin vertegenwoordigd zijn. Bij de oudere kinderen werden vooral niet-vaccinserotypen gevonden. We concludeerden hieruit dat er gedurende de jeugd een natuurlijke verschuiving plaatsvindt van vaccintype pneumokokken naar niet-vaccinserotypen met de leeftijd.

S. aureus is een bacterie die een breed scala aan infecties kan veroorzaken waaronder wondinfecties. Risicofactoren voor dragerschap met *S. aureus* bleken leeftijd, het hebben van het mannelijk geslacht, het leven in grote gezinnen en passief roken, terwijl actief roken (gemeten vanaf de leeftijd van 12 jaar) de kans op dragerschap verminderde. Tevens werden pneumokokken en *S. aureus* minder vaak samen in de neus-en-keelholte gevonden dan ieder afzonderlijk. Deze resultaten suggereren dat er een natuurlijke vorm van competitie tussen deze twee bacteriën bestaat. Bovendien blijkt deze competitie alleen te bestaan tussen vaccintype pneumokokken en *S. aureus*, maar niet tussen niet-vaccinserotypen en *S. aureus*. Deze bevindingen komen overeen met de resultaten van eerder onderzoek, waarbij kinderen met recidiverende middenoorontstekingen gevaccineerd werden met het 7-valente conjugaatvaccin (**hoofdstuk 7.1**). Deze studie toont niet alleen aan dat de vaccintype pneumokokken na vaccinatie worden vervangen door niet-vaccinserotypen, maar ook dat er in de gevaccineerde groep meer oorontstekingen veroorzaakt worden door *S. aureus*. Verder onderzoek is nodig om het mechanisme van deze interactie en de consequenties hiervan voor vaccinatie te bestuderen.

In dezelfde groep Nederlandse kinderen werd tevens dragerschap met *N. meningitidis* ofwel de meningokok bestudeerd (**hoofdstuk 3.3**). Omdat in de periode voorafgaand aan deze studie een toename van hersenvliesontsteking door meningokokken was geconstateerd, werden alle kinderen op het moment van ons onderzoek gevaccineerd tegen groep C meningokokken. We vonden echter relatief weinig dragerschap met deze bacterie: bij slechts 1.5% van de kinderen werd een meningokok gekweekt. Dit werd mogelijk veroorzaakt door de invloed van het zomerseizoen. Het hoogste risico op dragerschap werd gevonden bij kinderen onder de leeftijd van 2 jaar en bij jongeren ouder dan 15 jaar. Daarnaast bleken pneumokokkendragerschap en regelmatig bezoek van uitgaansgelegenheden (gemeten vanaf de leeftijd van 12 jaar) de kans op dragerschap met meningokokken te verhogen. Samenvattend kan gesteld worden dat leeftijd en “crowding” de belangrijkste risicofactoren vormen voor dragerschap van meningokokken.

Een andere risicogroep voor het krijgen van pneumokokkeninfecties is de groep volwassen patiënten met chronisch obstructief longlijden (COPD). Dit is een veel voorkomende en ernstige ziekte onder volwassenen met een hoog risico op overlijden. Bij deze ziekte treden regelmatig acute verslechteringen (exacerbaties) op die invaliderend zijn voor de patiënt en tevens het ziekteverloop kunnen versnellen. Er waren aanwijzingen dat bacteriële ontstekingen een rol spelen bij het ontstaan van deze exacerbaties. Daarom hebben we in 244 COPD patiënten de relatie onderzocht tussen de aanwezigheid van bacteriën in de longen tijdens stabiele perioden en het ontstaan van exacerbaties (**hoofdstuk 3.4**). We vonden bij aanvang van de studie dat 36% van de COPD patiënten gekoloniseerd was met potentieel ziekmakende bacteriën. In het algemeen bleek dit geen invloed te hebben op het ontstaan van exacerbaties. Echter, wanneer pneumokokken dominant aanwezig waren bleken de patiënten wel een verhoogd risico te hebben op exacerbaties van COPD, waarbij het relatieve risico 3

maal groter was (**hoofdstuk 8.2**). De meest voorkomende kapseltype pneumokokken in deze patiëntengroep waren de serotypen 19F, 3, 14, 9L/N/V, 23A/B en 11. Het 7-valente pneumokokkenvaccin beschermt theoretisch tegen 60% van de infecties door deze bacteriën. Ook bleek dat alle COPD patiënten IgG antistoffen maakten tegen de pneumokok, en dat de concentratie aan IgG in bloed stijgt bij aanwezigheid van pneumokokken tijdens exacerbaties. Verder onderzoek naar het effect van pneumokokkenvaccinatie op het ontstaan van exacerbaties van COPD is derhalve gewenst.

De behandeling van infecties door pneumokokken wordt gecompliceerd door de ontwikkeling van ongevoeligheid van de bacterie voor antibiotica, ook wel resistentie genoemd. De resistentie tegen antibiotica is wereldwijd enorm toegenomen: in sommige landen zijn 60% van de pneumokokkenstammen ongevoelig geworden voor het meest gebruikte antibioticum, penicilline. Veel van deze pneumokokken behoren tot een beperkt aantal genetische 'families'.

Wij hebben de genetische achtergrond van de voor penicilline resistente pneumokokken onderzocht in een groep kinderen uit Griekenland (**hoofdstuk 4.1**). We vonden dat een hoog percentage van de pneumokokken tot een beperkt aantal genetische families ofwel clusters behoord. Tevens werd vastgesteld dat een beperkt aantal internationaal aanwezige clusters ook in Griekenland voor een groot deel verantwoordelijk zijn voor de verspreiding van voor penicilline resistente pneumokokken. Tijdens de studie werd een pneumokokkenfamilie gevonden die gevoelig was voor penicilline maar resistent tegen 5 andere klassen van antibiotica (**hoofdstuk 4.2**). Met behulp van aanvullende genetische studies bleek deze stam zich breed verspreid te hebben in Griekenland. Wij stelden tevens vast, dat deze bacteriestam een voorouder is van de penicillineresistente stam die zich in de tweede helft van de jaren 90 zeer snel verspreid heeft in IJsland. Hieruit kan geconcludeerd worden dat deze endemische stam reeds de potentie had zich snel te verspreiden vóór ongevoeligheid voor penicillines werd verkregen.

Er wordt veel onderzoek gedaan naar de preventie van pneumokokkeninfecties door middel van vaccinatie. Het reeds eerder genoemde 7-valente vaccin bleek in kinderen zeer effectief bij het voorkomen van invasieve ziekten zoals sepsis en hersenvliesontsteking, redelijk efficiënt in het voorkomen van longontsteking en slechts beperkt effectief tegen middenoorontstekingen. Daarnaast bleek dit vaccin ook het dragerschap met pneumokokken te verminderen. Zoals reeds vermeld is het belangrijk om vóór het introduceren van dit vaccin te onderzoeken hoeveel circulerende pneumokokken theoretisch door dit vaccin kunnen worden geëlimineerd. In Azië was slechts weinig bekend over de serotypeverdeling onder pneumokokken. Daarom werd een collectie pneumokokken afkomstig van jonge kinderen met een bovenste luchtweginfectie in Vietnam getypeerd en nader bestudeerd met behulp van moleculaire technieken (**hoofdstuk 5.1**). Ook in dit onderzoek werden vooral de serotypen 23F, 19F, 6B en 14 gekweekt. In 52% van de pneumokokken werd penicillineresistentie aangetoond. Resistentie tegen andere antibiotica zoals cotrimoxazol, tetracycline, erytromycine en cefotaxim werd gevonden bij 39-87% van de stammen. 75% van de pneumokokken was resistent tegen 3 of meer soorten antibiotica. De meeste pneumokokken in Vietnam behoren tot een beperkt aantal families. Twee van de grootste clusters bleken onderdeel te zijn van internationaal bekende en wijdverspreide families. Tevens bleek 93% van de stammen uit deze clusters een kapseltype te hebben dat behoort tot de serotypen uit het 7-valente vaccin. Van de unieke genotypen was dit percentage veel lager. Samenvattend kan geconcludeerd worden dat introductie van het 7-valente vaccin in Vietnam potentieel een significante reductie van het aantal infecties zal geven.

Hetzelfde onderzoek werd tevens uitgevoerd met de pneumokokken afkomstig van Nederlandse kinderen (**hoofdstuk 5.3**). De meest voorkomende kapseltypen in ons land zijn de serotypen 6B, 19F, 23F, 6A, 3, 11 en 14. In totaal behoorden 42% van de gevonden pneumokokken tot de 7-valente vaccintypen. Vaccintype pneumokokken werden vooral aangetroffen bij de kinderen tussen 1 en 5 jaar terwijl niet-vaccintypen pas na de leeftijd van 3 tot 6 jaar werden gevonden. In de studie werd een groot aantal verschillende genetische typen pneumokokken gevonden, waaronder veel genetische clusters. Met andere woorden, veel van de pneumokokken bleken in staat zich makkelijk onder kinderen te verspreiden. In de Nederlandse studie werden maar zelden internationaal bekende pneumokokkenfamilies aangetroffen. Resistentie werd gevonden in 19% van de isolaten, hetgeen internationaal gezien zeer laag is.

In de jaren 1995 en 1996 was in Nederland slechts 2% van de pneumokokken resistent tegen penicilline. De genetische achtergrond van deze stammen is verder onderzocht met behulp van diverse methoden (**hoofdstuk 5.4**). Het bleek dat de genen verantwoordelijk voor penicillineresistentie zeer divers waren, maar duidelijk overeenkwamen met die in andere landen. De import van meerdere penicillineresistente stammen uit het buitenland bleek vooral verantwoordelijk te zijn voor het optreden van penicillineresistentie in Nederland. Er werden echter ook nieuwe combinaties van genen gevonden hetgeen suggereert dat pneumokokken deze genen of fragmenten hiervan onderling uitwisselen. Het lage aantal pneumokokken dat resistent is tegen penicilline is waarschijnlijk te danken aan het restrictieve voorschrijfgedrag van antibiotica door artsen in Nederland.

In **hoofdstuk 5.2** hebben we de gevolgen beschreven van een ziekenhuisuitbraak onder patiënten met COPD in Veldhoven met een pneumokokkenstam die resistent was voor meerdere antibiotica. Na behandeling met het antibioticum rifampicine bleken 3 patiënten nog steeds dezelfde stam bij zich te dragen, ditmaal ook resistent tegen dit antibioticum. Met behulp van moleculaire technieken werd aangetoond dat de resistentie tegen rifampicine in de drie stammen op een afzonderlijke plaats in het hiervoor verantwoordelijke gen, het *rpoB* gen, was ontstaan. De resultaten van deze studie tonen aan dat ongevoeligheid voor rifampicine snel ontstaat, en dat behandeling met dit antibioticum alleen mag plaatsvinden indien dit wordt gecombineerd met meerdere klassen van antibiotica.

Zoals eerder besproken beschermt het 7-valente conjugaat vaccin bij toediening aan zuigelingen tegen invasieve ziekten, longontsteking en, zij het beperkt, middenoorontsteking. De effectiviteit van vaccinatie bij oudere kinderen was echter nog niet aangetoond. Daarom werd er een studie verricht naar het effect van vaccinatie met het 7-valente vaccin op het aantal middenoorontstekingen bij kinderen tussen 1 en 7 jaar oud met een voorgeschiedenis van recidiverende middenoorontstekingen (OMAVAX studie) (**hoofdstuk 7.1**). Hierbij kreeg de helft van de kinderen één of twee conjugaat vaccinaties gevolgd door een booster injectie met het 23-valente suikervaccin. De andere helft van de kinderen, de controle groep, kreeg een vaccin tegen hepatitis A of B. Voor zowel de behandelaars als de kinderen en hun ouders was het niet bekend welk vaccin er bij welk kind werd toegediend. Na het beëindigen van de studie bleken de kinderen die het pneumokokken vaccin gekregen hadden niet minder, maar zelfs meer middenoorontstekingen te hebben dan de kinderen uit de controle groep. Er werd een reductie gevonden in het aantal dragers van vaccintype pneumokokken, maar tevens een stijging in het aantal dragers met niet-vaccintypen. Met andere woorden, er was een verschuiving opgetreden van vaccintype dragerschap naar niet-vaccintype dragerschap, waarbij het totale percentage dragers gelijk was gebleven.

Bij nadere bestudering van dragerschap binnen de verschillende groepen kinderen in het OMAVAX onderzoek bleek dat de verschuiving zich vooral had voorgedaan bij de jongste groep kinderen (**hoofdstuk 7.2**). Omdat het conjugaatvaccin bij deze groep twee keer in plaats van één keer was toegediend concluderen wij dat herhaalde vaccinatie nodig is om eliminatie van pneumokokken in de neus-en-keelholte te realiseren. Dit wordt ondersteund door de aanwezigheid van antistoffen tegen de vaccintype pneumokokken in het speeksel van de gevaccineerde kinderen (**hoofdstuk 8.1**). Opnieuw hadden de jongste kinderen een verhoogde concentratie van IgG antistoffen na vaccinatie, terwijl bij de oudere kinderen geen toename in IgG antistoffen gevonden werd. De concentraties van een ander antistof, het pneumokokken-specifieke IgA, steeg eveneens in de gevaccineerde kinderen. Echter, in de controle groep steeg de hoeveelheid IgA antistoffen nog sterker dan bij de gevaccineerde kinderen. Op basis hiervan werd geconcludeerd dat dit veroorzaakt wordt door dragerschap in plaats van door vaccinatie.

De geobserveerde verschuiving in dragerschap van vaccinserotypen naar niet-vaccinserotypen werd door ons bestudeerd met behulp van de colony blot methode (**hoofdstuk 7.4**). Deze methode werd ontwikkeld om de aanwezigheid van verschillende serotypen pneumokokken in één monster aan te tonen. Door meerdere pneumokokkenserotypen aan te tonen wilden wij de vraag oplossen of de verschuiving in dragerschap na vaccinatie een verschuiving van dominante stam binnen een bestaand evenwicht van meerdere pneumokokken serotypen betreft, of een daadwerkelijke vervanging van de aanwezige stam. De eerste situatie heeft mogelijk minder consequenties dan de tweede, omdat in het eerste geval het immuunsysteem van het kind de aanvankelijk onderdrukte stam al herkent en er daarom geen ernstige infecties met deze stam meer kunnen ontstaan, terwijl in de tweede situatie mogelijk sprake is van het verkrijgen van een nieuwe en potentieel schadelijke bacterie. Wij hebben de verschuiving van een vaccinserotype pneumokok naar een niet-vaccinserotype stam onderzocht in 29 gevaccineerde kinderen. Bij de meerderheid van de kinderen was de vaccintype pneumokok daadwerkelijk vervangen door een niet-vaccinserotype en werd alleen sporadisch gemengd dragerschap gevonden.

Uit het onderzoek kan geconcludeerd worden dat vaccinatie met het 7-valente pneumokokkenvaccin middenoorontstekingen bij kinderen ouder dan één jaar niet voorkomt. Verder onderzoek zal nodig zijn om het effect van verschuiving in dragerschap van vaccintype naar niet-vaccintype pneumokokken op de frequentie van voorkomen van ernstige infecties te bestuderen.

Dankwoord

Het is zover, dit proefschrift is nu toch echt een feit! Een mijlpaal eerder dan een einde, want het onderzoek is nog zeker niet af! In tegendeel, voor mijn gevoel moet het allemaal nog beginnen. Toch is het een mooi moment om stil te staan bij wat we tot nu toe bereikt hebben en hoe dat alles tot stand is gekomen. Een dankwoord hoort daarbij maar is tegelijkertijd zo ontoereikend: wie moet je bedanken? Net als wanneer je bij een wielerwedstrijd terugblikt zie je ook in dit geval dat vroeg in de race al vele mensen terdege invloed op het koersverloop hebben gehad. Naarmate de finish meer in zicht komt, wordt de menigte wellicht steeds groter, maar niet persé belangrijker, met andere woorden alle supporters en toeschouwers verdienen dank! Het zijn bovendien niet alleen de mensen die je direct aan het hart gaan, maar ook vele (on)opvallende passanten zonder wie het resultaat wel degelijk anders zou zijn geweest. Terug naar 'mijn koers'. Misschien moet ik al beginnen bij de kleuterjuffrouw Zuster Augusta die middels de vouwblaadjes en ontwerpjes mijn 'exacte kant' heeft gestimuleerd. Ook de lagere en middelbare school zijn een thuishaven geweest met stimulerende en motiverende leraren. Dan spreek ik nog niet over de stimulans van de dubbeltjes, kwartjes en guldens die gul werden gedoneerd door familie, kennissen en buren bij een goed rapport. Naarmate ik verder ga zie ik een steeds grotere massa van professoren, leraren, student-assistenten, vrijwilligers, begeleiders, amenuensissen, cateringjuffrouwen, treinconducteurs en buschauffeurs (bedankt voor het altijd eerder stoppen dan de echte bushalte op Overvecht, Bunnik), collega's van weekend baantjes (bedankt voor het vertrouwen in mij), medestudenten, buren en huisbazen voorbijtrekken. Vergeet daarnaast ook niet de invloed van alle mensen die mijn buitenlandse stages hielpen regelen en verwezenlijken, en de mensen die daar terplekke tijdelijk maar onvoorwaardelijk mijn vrienden zijn geweest (bedankt voor barbecues, kaartjes, winkelmiddagen, tripjes, en troostende woorden (Texas), de dierbare warmwater douche, 'echte cola', het uitwisselen van ervaringen en niet te vergeten maaltijden met echt vlees (Zimbabwe). Op het moment dat je student af bent en het 'echte leven' instapt realiseer je je misschien niet altijd hoeveel mensen daar zorgen dat je het toch allemaal net wel volhoudt: de portier (bedankt voor de vriendelijke goedemorgens), de schoonmaakster, de secretaresse, de verpleegkundigen (bedankt voor de nachtelijke snoepfestijnen en film uurtjes), laboranten, caissières, ouders, studenten, patiënten (bedankt voor het surprise ei en de tekeningen), paramedici, typisten (voor het tot ? maal toe herstellen van brieven) en secretaresses. Collega's van buiten, collega's van binnen, chefs, mentoren, medeassistenten en onderzoekers, en ook die toevallige passant. En natuurlijk, niet te vergeten, mijn (schoon)familie! Een offer was nodig, ook van jullie kant! Dat in ogenschouw nemend lijkt het moeilijk om te besluiten wie ik nu daarvan in het bijzonder moet noemen. Maar omdat ik toch ook maar een mens ben, kan (durf) ik het niet laten om wat mensen persoonlijk te danken.

Prof. Dr. R. de Groot, beste Ronald, 5½ jaar geleden kwam ik solliciteren en toen zag je het wel zitten met mij. Dankzij jou heb ik de mogelijkheid gekregen om kindergeneeskunde en wetenschap te combineren. Ik hoop dan ook van harte dat ik aan je verwachtingen heb voldaan! Jouw enthousiasme werkt absoluut aanstekelijk en is zelfs, tegen wil en dank, besmettelijk!

Dr. P.W.M. Hermans, beste Peter, diezelfde 5½ jaar geleden moest je het nog maar zien. Wie had toen kunnen denken dat medici een significant onderdeel zouden gaan vormen van het laboratorium kindergeneeskunde in het algemeen en jouw groep in het bijzonder. Ik weet zeker dat ik jou niet heb teleurgesteld. We hebben tot op heden een aantal mooie studies gerealiseerd en " who knows what the

future will bring…….? Thanks so far!" (En zeker voor de vele marathon sessies manuscripten corrigeren!).

Marcel Sluijter, lieve Marcel, mijn paranimf, mijn buddy, mijn partner in crime (research)! Alles hebben we samen gedaan. Uniek denk ik. Ik zal je de komende jaren missen, maar wie weet komt er nog eens een nieuwe uitdaging waar we samen onze tanden in kunnen zetten.....Dank!

Mijn 2e paranimf en grote broer, lieve Roy, vroeger een twee-eenheid, nu rijdt ieder van ons zijn eigen koers. Desondanks absoluut wederzijds respect. Ik ben trots op jou en op je gezin, enneh..... die twee roodharige monstertjes van je zijn goud waard!

Drs. R.H. Veenhoven, beste Reinier, nog even en je bent een zeergeleerde heer! Voor mij ben je dat toch al. Ik heb bewondering voor de manier waarop en het enthousiasme waarmee je de OMAVAX studie en alles eromheen hebt gerund. Bedankt dat ik daar onderdeel van mocht zijn, ik had het voor geen goud willen missen!!

Prof. Dr. E.A.M. Sanders, beste Lieke, een mens kiest altijd bewust of onbewust zijn rolmodel, en jij bent de mijne! Dank voor je onvoorwaardelijke hulp, je persoonlijke benadering was een enorme steun!

Prof. Dr. A. van Belkum, beste Alex, maar weinigen hebben het vermogen om puur op zichzelf te vertrouwen en tegelijkertijd zichzelf te zijn, maar jij bent zo'n persoon! Het was een eer om jouw advies en commentaar te mogen ontvangen. Bedankt dat je in de commissie wilde plaatsnemen.

Prof. Dr. H. Hooijkaas en Prof. Dr. A. Finn, thank you for being committee members! I'm honored....

Dr. D.A.Watson and Dr. F.N. Watson. Dear Fay and David, beyond all doubt you were the supporting bystanders in the beginning of 'my race'. Even in one of the toughest periods of my live, you kept on believing in me. Without any personal benefit, you teached, motivated and supported me. Without you, this work would not have been initiated. I will always be grateful for that! All the best to you, Emily, Ross, Jon and Zach.

De samenwerkende groepen uit binnen en buitenland, zonder hen was dit boekje er niet geweest en was het werk zeker niet zo uitdagend en leuk geweest. Bedankt alle mensen van OMAVAX, en COPE; in het bijzonder dank aan de groep van Prof. Dr. H.A. Verbrugh, Hans Rümke en medewerkers van Vaxinostics en alle anderen die zich voor onze eigen AHOY studie hebben ingezet; bedankt Kees Elzenaar en collega's van het LIS, RIVM; thanks to our foreign colleagues Nguen Ha en coworkers from Vietnam, Prof. Dr. George Syrogiannopoulos and colleagues from Greece, Dr. Helena Kayhty and co-workers from Finland and Dr. Tim Mitchell and collegues from Scotland. Maybe we will be able to continue our cooperation in the future....

Dan kom ik toch ook op een aantal groepen mensen uit. Allereerst alle mensen van de subgroep infectieziekten en immunologie, bedankt voor de gemoedelijke sfeer tijdens zowel de maandagochtendbesprekingen als onze congressen en uitjes! De medewerkers van het lab kindergeneeskunde, het was leuk, leerzaam en gezellig. Ik mis ze nu al, de lab dagen, de kerstfeestjes, de taartenbakwedstrijden, de wijnproeverijen en de toertoto's. Ik hoop dat ik af en toe nog eens langs mag komen.....

'De partij', opgericht uit baldadigheid, uitgegroeid tot vriendenclub. Bedankt voor de vele borrels, met als hoogtepunt onze Hurricanes. Dat er nog vele vergaderingen mogen volgen.....

De AIO's, arts-onderzoekers en tegenwoordig ook AGIKO's; een zeer heterogene groep gemotiveerde mensen die de afgelopen jaren een behoorlijk verloop heeft gekend. Vanwege de fysieke afstand was er geen frequent contact, maar op de weekendjes, borrels en promoties hebben we heel wat gedeeld met elkaar. Ik zou zeggen.....tot de volgende promotie en 'we will meet' in de kliniek!

De arts-assistenten groep; ook jullie hebben een enorm verloop gekend, velen zijn gekomen, vanuit kliniek of onderzoek, en velen zijn gegaan, als kinderarts of anderszins. Als je samen assistent bent merk je dat dat allemaal niet uitmaakt, samen moet je het tenslotte doen. Die bewustwording heeft de laatste jaren geresulteerd in een hechte groep mensen, die met elkaar kunnen lachen en huilen, die elkaar steunen en in elkaar geïnteresseerd zijn. Ik hoop dat dat nog lang zo blijft!

Dan ons eigen clubje, Lieve Peter A., je bent nu alweer bijna een jaar weg maar we missen je nog steeds! Dank voor de mopperpraatjes, brainstormsessies en gezelligheid. Laten we snel weer gaan zeilen..... Beste Anja, ook jij bent al weer een paar jaar weg! Wat gaat de tijd toch snel..... Bedankt voor al je hulp! Theo, kamergenoot en hart van de groep, bedankt voor alles. Ingrid, ook wij hebben het laatste half jaar samen mogen werken. Bedankt voor je hulp en 'keep up the good work'. Lieve Sylvia, ondertussen ook alweer een aantal jaren in het lab. Een constante factor die zo nu en dan verrassend uit de hoek kan komen. Bedankt voor onder andere onze petekind praatjes! Beste Marieke, opvolgster in de lijn der promovendi en in de lijn der AGIKO's. Ik hoop dat ik het pad een beetje voor je geëffend heb. Succes met alles, en ik zie je..... Vishal en Wouter, succes met de staven en kokken! Saskia, helaas hebben onze wegen elkaar net gekruist, maar ik zal zeker met interesse 'de competitie' volgen....! De stagiaires die de afgelopen jaren allemaal op eigen wijze hebben bijgedragen aan dit boekje; Peter van T, Nayat, Maartje, Michiel, Nicole en Reshmi, ook jullie enorm bedankt voor al het mooie werk. Veel succes met jullie toekomst en geniet ervan!

De stafleden en mede assistenten van het MCRZ locatie Zuider, ik had geen betere perifere opleidingsplek kunnen bedenken! Bedankt voor alle leermomenten, blijken van interesse en gezelligheid.

Dan heb je natuurlijk met sommige mensen nog een bijzondere band: Marjolein, Marli, en Natasja, bedankt voor onze gezellige 'stedentripjes', hopelijk volgen er nog vele. Ingrid, Wendy en Esther, een gemeenschappelijke jeugd verbind ons, fantastisch toch? Ik hoop dat we deze band nog lang volhouden. Simone en Martijn, het is leuk om samen met jullie op te gaan in het leven. Onze etentjes zou ik dan ook niet willen missen! Lieve Clementien, de rust en tegelijkertijd openheid waarmee je in het leven staat, knap hoe jij moeder, dokter en onderzoeker zijn combineert! Ik hoop dat onze vriendschap, nog lang mag blijven bestaan. Lieve Annemarie, we zijn erachter gekomen dat we toch echt hetzelfde willen! Dat schept een band die we ondertussen omgezet hebben in vriendschap. Op onze toekomst duo fio! Lieve Mariëlle, recent werden we maatjes, ik mis je enorm nu jij in het Sophia zit! Ik ben blij dat we collega's zijn.....

Lieve mam. Het leven is niet altijd even rooskleurig voor je geweest, maar het feit dat je kinderen en zeker ook je kleinkinderen het zo goed doen geven je zichtbaar energie! Alhoewel je waarschijnlijk niet altijd meer begrijpt waar je dochter mee bezig is, ben je toch onvoorwaardelijk trots op me. Bedankt daarvoor, enneh..... geniet van het leven!

Mijn lieve vader, jij bent een van de weinigen die de laatste jaren mijn ups en downs, mijn onzekerheden, zorgen, maar ook mijn overwinningen heeft gekend. Bedankt voor je altijd luisterende oor, de momenten van reflectie en de motiverende woorden. Samen met Marjolein en Pim wens ik je veel geluk!

Lieve Tonny, deze koers hebben we samen gereden, dit keer liet ik de wielen draaien en reed jij ernaast, adviserend, steunend en nu en dan stiekem duwend als ik er even doorheen zat. Vandaag is de finish bereikt. Nu even nagenieten, en morgen ..... gewoon weer verder. Een nieuwe dag, een nieuwe etappe, nieuwe kansen en nieuwe uitdagingen. De wereld is mooi. Let's go on .....

Curriculum Vitae

Debby Bogaert was born in Goes (Zld), on July 15, 1974. She passed her secondary school exam (VWO) in 1992 at the Sint Willibrord College in Goes. From 1992 to 1996 she studied medicine at the University of Utrecht where she graduated Cum Laude for her doctoral exam. In 1996 she participated for 4 months in a research project on the role of pneumococcal surface-associated molecules in invasion under the supervision of Dr. D.A. Watson, University of Texas, Medical Branch, Galveston. During her senior internship she participated in a 'Red Cross' project at the Zvishavane Community Hospital in Zimbabwe, where she trained volunteers in home-based care for terminally ill patients. In October 1998 she obtained her Medical Degree, after which she started her PhD project on 'the host-pathogen interaction during *S. pneumoniae* colonization and infection in adults and children' under the guidance of Dr. P.W.M. Hermans and Prof.dr. R. de Groot. From January until July 1999 she worked as a Junior Registrar (AGNIO) at the Sophia's Children Hospital. Subsequently, she started with a combined residency in Pediatrics and research training program (AGIKO) (Head pediatrics: Prof.dr. H.A. Büller and Prof.dr. A.J. van der Heijden). During her PhD study, she followed courses in Molecular Biology and Recombinant DNA Technology (Department of Infectious Diseases and Immunology, University of Utrecht), Clinical Infectious Diseases (Boerhaave), Molecular Immunology (Department of Immunology, Erasmus MC, Rotterdam) and Biostatistics and Epidemiology (Nihes Summer School, Erasmus MC, Rotterdam). She received the Prof.dr. J.C. Birkenhäger award for best scientific presentation at the Erasmus MC "wetenschapsdag" 2003. At present she is member of the resident committee of the Paediatric Association of The Netherlands (NVK). She will finish her residency in December 2006. She has a long-term relationship with Tonny Moonen, a line-manager in a data- and telecom company.

List of publications

International

1. Watson D.A., **D. Bogaert**. What is behind antibiotic resistance? Laboratory Medicine. 1997; **28**(5).

2. Watson D.A., **D. Bogaert**. A Battle of Wills, antibiotics and bacteria lock horns in a struggle for survival. Laboratory Medicine. 1997; **28**(6).

3. **Bogaert D.**, G.A. Syrogiannopoulos, I.N. Grivea, R. de Groot, N.G. Beratis, P.W.M. Hermans. Molecular epidemiology of penicillin-nonsusceptible *Streptococcus pneumoniae* among children in Greece. J Clin Microbiol 2000 Dec; **38**(12):4361-4366.

4. Overweg K., **D. Bogaert**, M. Sluijter, Y. Yother, J. Dankert, R. de Groot, P.W.M. Hermans. Genetic relatedness within serotypes of penicillin-susceptible *Streptococcus pneumoniae* isolates. J Clin Microbiol 2000 Dec; **38**(12): 4548-4553.

5. **Bogaert D.**, M.N. Engelen, A.J. Timmers-Reker, C.P. Elzenaar, P.G.H. Peerbooms, R.A. Coutinho, R. de Groot, P.W.M Hermans. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J Clin Microbiol 2001;**39**(9)**:**3316-20.

6. Syrogiannopoulos G.A., **D. Bogaert**, I.N. Grivea, N.G. Beratis, R. de Groot, P.W.M. Hermans. Molecular epidemiology of penicillin-susceptible, multidrug-resistant serotype 6B pneumococci isolated from children in Greece. J Clin Microbiol 2001;**39**(2)**:**581-5.

7. Tilburg P.M van., **D. Bogaert**, M. Sluijter, A.R. Jansz, R. de Groot, P.W.M. Hermans. Emergence of rifampin-resistant Streptococcus pneumoniae as a result of antimicrobial therapy for penicillin-resistant strains. Clin Infect Dis 2001;**33**(8)**:**e93-6.

8. Overweg K., **D. Bogaert**, M. Sluijter, R. de Groot, P.W.M. Hermans. Molecular characteristics of penicillin-binding protein genes of penicillin-nonsusceptible Streptococcus pneumoniae isolated in the Netherlands. Microb Drug Resist 2001;**7**(4)**:**323-34.

9. **Bogaert D.**, N.T. Ha, M. Sluijter, N. Lemmens, R. de Groot, P.W.M. Hermans. Molecular epidemiology of pneumococcal carriage among children with upper respiratory tract infections in Hanoi, Vietnam. J Clin Microbiol 2002;**40**(11)**:**3903-8.

10. Veenhoven R.H., **D. Bogaert**, C.S. Uiterwaal, P.W. Hermans, R. de Groot, W. Kuis, G.T. Rijkers, A.G.M. Schilder and E.A.M. Sanders. Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media. Lancet 2003;**361**;2189-95.

11. Sanders E.A.M., R. Veenhoven, **D. Bogaert**, A. Schilder, and P.W.M. Hermans. Effect of conjugate pneumococcal vaccine on recurrent acute otitis media. Lancet 2003; 362:1081.

12. Hermans P.W.M., **D. Bogaert**, M. Sluijter and A. Van Belkum. Restriction fragment end labeling analysis: high-resolution genomic typing of *Streptococcus pneumoniae* isolates. In Experimental approaches for assessing genetic diversity among microbial pathogens. (Editors van Belkum, A., Duim, B. and Hays, J.), 2003. 33-46.

13. **Bogaert D.**, P.W.M. Hermans, I.N. Grivea, G.S. Katopodis, T.J. Mitchell, M. Sluijter, R. de Groot, N.G. Beratis, G.A. Syrogiannopoulos. Molecular epidemiology of penicillin-susceptible non-beta-lactam-resistant *Streptococcus pneumoniae* isolates from Greek children. J Clin Microbiol 2003 Dec;41(12):5633-9.

14. **Bogaert D.**, P. van der Valk, R. Ramdin, M. Sluijter, E. Monninkhof, R. Hendrix, R. de Groot, P.W.M. Hermans. Host-pathogen interaction during pneumococcal infection in patients with chronic obstructive pulmonary disease. Infect Immun 2004 Feb;72(2):818-23.

15. **Bogaert D.**, R. de Groot, P.W.M. Hermans. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis 2004 Mar 1;4(3):144-154.

16. **Bogaert D.**, R.H. Veenhoven, M. Sluijter, W.J.W. Wannet, G.T. Rijkers, T.J. Mitchell, S.C. Clarke, W.H.F. Goessens, A.G. Schilder, E.A.M. Sanders, R. de Groot and P.W.M. Hermans. Molecular dynamics of pneumococcal colonization in response to pneumococcal conjugate vaccination in children with recurrent acute otitis media. J Clin Microbiol 2004; in press.

17. **Bogaert D.**, M. Sluijter, L. Sanders, R. de Groot, and P.W.M. Hermans. Multiplex opsonophagocytosis assay (MOPA) is a useful tool for the monitoring of the 7-valent pneumococcal conjugate vaccine. Vaccine 2004; in press.

18. **Bogaert D.**, P.V. Adrian, H. Rumke, P.W.M. Hermans, R. de Groot. Pneumococcal vaccines: an update on current strategies. Vaccine 2004; in press.

19. Adrian P.V., **D. Bogaert**, M. Oprins, S. Rapola, H. Kayhty, P.W.M. Hermans. Development of antibodies against pneumococcal proteins α-enolase, IgA1 proteinase, SlrA, PpmA in relation to pneumococcal carriage and otitis media. Vaccine 2004; in press.

20. **Bogaert D.**, A. van Belkum, M. Sluijter, A. Luijendijk, R. de Groot, H.C. Rümke, H.A. Verbrugh and P.W.M. Hermans Natural competition between *Streptococcus pneumoniae* and *Staphylococcus aureus* during colonisation in healthy children. Lancet 2004; in press.

21. **Bogaert D.**, R.H. Veenhoven, M. Sluijter, W.J.B. Wannet, G.T. Rijkers, W.H. Goessens, E.A.M. Sanders, R. de Groot and P.W.M. Hermans. Molecular dynamics of pneumococcal colonization in response to pneumococcal conjugate vaccination in children with recurrent acute otitis media. J Clin Microbiol 2004; accepted for publication.

22. **Bogaert D.**, R.H. Veenhoven, M. Sluijter, E.A.M. Sanders, R. de Groot and P.W.M. Hermans.

Colony blot assay: a useful method to detect multiple pneumococcal serotypes within clinical specimens. FEMS Immunol Med Microbiol. 2004; accepted for publication.

23. Veenhoven R.H., **D. Bogaert**, A.G.M. Schilder, G.T. Rijkers, C.S.P.M. Uiterwaal, H. Kiezebrink, M.J.P. Van Kempen, I.J. Dhooge, J. Bruin, E.P.F. IJzerman, R. de Groot, W. Kuis, P.W.M. Hermans, and E.A.M. Sanders. Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media. Clin Infect Dis 2004; accepted for publication.

24. **Bogaert D.**, M. Sluijter, N. Lemmens-den Toom, W.H.F. Goessens, S.C. Clarke, R. de Groot and P.W.M. Hermans Molecular epidemiology of pneumococcal colonization in healthy Dutch children. 2004; submitted.

25. **Bogaert D.**, R.H. Veenhoven, R. Ramdin, I.H.T. Luijendijk, G.T. Rijkers, E.A.M. Sanders, R. de Groot and P.W.M. Hermans. Pneumococcal conjugate vaccination does not induce a mucosal IgA response in children with recurrent acute otitis media. 2004; submitted.

26. **Bogaert D.**, S. Koppen, M. Sluijter, A. Luijendijk, H.C. Rümke, P.W.M. Hermans, A. van Belkum, R. de Groot and H.A. Verbrugh. Epidemiology of nasopharyngeal carriage of *Neisseria meningitidis* in healthy Dutch children. 2004; submitted.

27. Valk P. van der, **D. Bogaert**, E. Monninkhof, J. van der Palen, P.W.M. Hermans, G. Zielhuis, C. van Herwaarden and R. Hendrix. Bacterial Colonization, Inhaled Corticosteroids and Risk of Exacerbations in COPD in the COPE Study. 2004; submitted.

National

28. **Bogaert D.**, R. de Groot, P.W.M. Hermans. Infecties door antibioticum-resistente pneumokokken in epidemiologisch perspectief. Ned. Tijdschr. Med. Microbiol. 2000;1: 9-12.

29. Rumke H.C., P.W.M. Hermans, **D. Bogaert**, R. de Groot. Pneumokokken-vaccinatie van jonge kinderen in het Rijksvaccinatieprogramma? Infectieziekten bulletin. 2001 juli;7: 217-22.

30. Veenhoven R.H., **D. Bogaert**, A.G.M. Schilder, C.S.P.M. Uiterwaal, G.T. Rijkers, P.W.M. Hermans, and E.A.M. Sanders. 2003. Klinische ineffectiviteit van gecombineerde vaccinatie met 7-valent pneumokokkenconjugaatvaccin en 23-valent pneumokokkenpolysacharidevaccin bij kinderen met recidiverende acute otitis media; een gerandomiseerd dubbelblind onderzoek. Ned. Tijdsch. Geneesk. 147:2220-2226.

31. **Bogaert D.**, R. de Groot, P.W.M. Hermans. Prevention of *Streptococcus pneumoniae* infections: currrent and future vaccine strategies. Werkboek Boerhaave (na)scholigscursus infectieziekten, maart 2004:287-307.

kinderdokter
zijn, worden, blijven
idealisme, nieuwsgierigheid
kennis, veel en weinig
soms ook te
mens
vooral, allemaal
Voor het kind geen spel of fantasie, maar echt
geluk of pech, droom maar weg, zoveel je kunt
Sophietje
groeit gelukkig door
met haar de kennis en de rest
de ouders, het broertje, de dokter ook
compleet, complex, eerlijk, echt, dus daarom
poppendokter
toch niet echt
dokter voor
het kleine
het grote
en de rest