Massive shift in the pneumococcal nasopharyngeal flora after the 7-valent conjugate vaccine: epidemiological studies and testing pathogenic potential in animal models

Nelson Frazão

Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa

Oeiras 2010

Massive shift in the pneumococcal nasopharyngeal flora after the 7-valent conjugate vaccine: epidemiological studies and testing pathogenic potential in animal models

Nelson Frazão

Universidade Nova de Lisboa

Instituto de Tecnologia Química e Biológica

Oeiras 2010

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR Portugal

Financial support from Fundação para a Ciência e a Tecnologia, Portugal through grant SFRH/BD/30103/2006 awarded to Nelson Frazão.

Cover by Nelson Frazão

First edition, October 27, 2010

Second edition, January 03, 2011

Massive shift in the pneumococcal nasopharyngeal flora after the 7-valent conjugate vaccine: epidemiological studies and testing pathogenic potential in animal models

ISBN 978-989-20-2163-8

Supervisors

Hermínia de Lencastre, PhD

Alexander Tomasz, PhD

Co-Supervisor

Raquel Sá Leão, PhD

Chairman of Examiners

Luís Paulo S. N. M. Rebelo, PhD

Examiners

Peter Hermans, PhD

Josefina Liñares, PhD

José A. G. Melo Cristino, MD-PhD

Dissertation presented to obtain the PhD degree in Biology/Molecular Biology by Universidade Nova de Lisboa, Instituto de Tecnologia Química e Biológica.

Acknowledgments

First of all, I would like to thank my main supervisor, Professor Hermínia de Lencastre, for having pushed me “on board” for this adventure. Thank you for having confidence in me and letting me pursue my ideas, for your guidance and fantastic critical sense, and for making it possible for me to attend so many exciting conferences and meetings. Just for the record, besides all I have learned scientifically speaking, now I also know how to get a coffee.

I am deeply grateful to Professor Alexander Tomasz, also my supervisor. Your remarkable knowledge and crystal clear thinking helped me immensely when I was stuck, thinking everything was going wrong. Thank you for teaching me see the big picture. Your experience, astuteness and great sense of humor were crucial in not giving up during the hardest moments.

To Dr. Raquel Sá Leão, my co-supervisor, for her guidance, many fruitful discussions, and also for providing me with the opportunity to participate in interesting conferences.

Thanks to all friends and colleagues at the Laboratory of Genetics at the Instituto de Tecnologia Química e Biológica and the Laboratory of Microbiology at The Rockefeller University, who were vital in the development of my PhD thesis.

Thanks to the day-care center team, including directors, staff, parents, children, and all those who participated in the isolation and characterization of the pneumococcal isolates.

The work presented in this thesis would not have been possible without the excellent conditions provided at the Instituto de Tecnologia Química e Biológica and The Rockefeller University, and the financial support of Fundação para a Ciência e a Tecnologia (SFRH/BD/30103/2006).

Finally, I want to thank all my family, especially my parents, brother, and grandparents, who support me no matter what.

Last but not least, I dedicate this thesis to P. and M. who help me just by existing!

Abstract

Although it exists mostly as a commensal bacterium colonizing the human nasopharynx, particularly in children, the Gram-positive bacterium *Streptococcus pneumoniae*, is also a major human pathogen that can cause a wide range of diseases, which include otitis media, sinusitis, pneumonia, and such life-threatening afflictions as bloodstream infection and meningitis.

Created to protect children against pneumococcal disease, the 7-valent pneumococcal conjugate vaccine (PCV7) showed high efficacy in preventing disease caused by the serotypes included in the vaccine, the so-called vaccine types (VTs). Since colonization is an essential first step to develop pneumococcal disease, it is of importance to investigate the effect of this vaccine on the degree of colonization, on changes in the composition of the nasopharyngeal flora and the virulence potential of the non vaccine type (NVT) strains.

The present thesis is aimed at evaluating the impact of PCV7 on single and multiple colonization among Portuguese children by determining the serotypes and clonal types of pneumococci replacing the original flora under the influence of the conjugate vaccine. The thesis also includes experiments testing the virulence potential of the non vaccine serotype strains of pneumococci through the use of animal models.

First, we conducted a prospective study to evaluate the PCV7 effect on the nasopharyngeal colonization, with particular emphasis on drug resistant strains in children attending day-care centers in Lisbon, Portugal. Comparison of vaccinated and control groups revealed that PCV7 caused replacement of strains expressing vaccine serotypes by novel clonal types of pneumococci that produced capsular polysaccharides not included in the vaccine. On the other hand, the general rate of carriage of pneumococci and the rate of carriage of drug resistant strains has remained unaltered.

Next, a pilot study was performed to assess the effect of a single dose of the PCV7 vaccine on the composition of the colonizing flora of individual children. The fact that the same children were followed in two sampling periods just one month apart (i.e., before

and after vaccination) has allowed assessment of the actual mechanism of the vaccine's effect. In conclusion, in children immunized with a single PCV7 dose a serotype replacement phenomenon occurred, both at the population and individual levels. Moreover, the PCV7 mode of action relied on the prevention of the *de novo* acquisition of VTs on one hand, and unmasking NVTs on the other.

In a final study, murine models of colonization and virulence were used to characterize the most common penicillin nonsusceptible non vaccine serotypes colonizing the nasopharynx of Portuguese children vaccinated with PCV7. Additionally, the role of the capsule *versus* the genetic background in colonization and disease was also investigated. In brief, each one of the three major NVTs tested – i.e., strains expressing serotypes 6A, 15A, and 19A – were able to colonize and disseminate from the nasal epithelium to adjacent tissues, such as the olfactory bulbs, brain, lungs and the middle ear mucosa. Each serotype was able to cause lethal lung infection if high bacterial titers were reached in the lung. Serotype 19A (ST276) was the only one of the NVTs tested to show propensity to cause blood infection. Finally, colonization depended on both the capsule and the genetic background, while virulence (i.e. blood infection) was only dependent on the capsule.

Resumo

A bactéria Gram-positiva *Streptococcus pneumoniae* existe normalmente como um microrganismo comensal, colonizador da nasofaringe humana, especialmente em crianças. Contudo esta bactéria é também um importante agente patogénico humano capaz de causar um vasto leque de doenças incluindo otite média, sinusite, pneumonia, e ainda patologias que colocam em risco a vida humana como a infecção do sangue e a meningite.

A vacina pneumocócica conjugada 7-valente (PCV7) foi criada para proteger crianças contra a doença provocada por *S. pneumoniae* revelando enorme eficácia na prevenção de doença causada pelos serótipos incluídos na vacina, também chamados serótipos vacinais. Sendo a colonização um primeiro passo fundamental para o desenvolvimento da doença pneumocócica, reveste-se da maior importância a investigação do efeito desta vacina no grau de colonização, nas alterações da composição da flora da nasofaringe e no potencial de virulência das estirpes não vacinais.

Os objectivos propostos para esta tese levaram à execução de estudos epidemiológicos e de experimentação animal. Os estudos epidemiológicos que realizámos permitiram avaliar o impacto da vacina PCV7 em colonização simples e múltipla em crianças portuguesas identificando os serótipos e os tipos clonais que substituem a flora pneumocócica original sob a influência da vacina. Por outro lado, estudou-se o potencial de virulência das estirpes pneumocócicas não vacinais seleccionadas pela vacina usando modelos animais de colonização e doença.

Inicialmente realizámos um estudo epidemiológico prospectivo para avaliar o efeito da PCV7 na colonização da nasofaringe, com especial incidência nas estirpes resistentes a antimicrobianos isoladas de crianças a frequentar infantários na região de Lisboa em Portugal. A comparação do grupo vacinado com o grupo controlo mostrou que a PCV7 causou a substituição dos serótipos vacinais por novos tipos clonais que expressam na cápsula polissacáridos (serótipos) não incluídos na vacina. Por outro lado, a taxa de

colonização geral por *S. pneumoniae* e a taxa de colonização por estirpes resistentes a antimicrobianos permaneceu inalterada.

De modo a estudar o efeito de apenas uma dose da vacina PCV7, foi realizado um estudo-piloto no qual se estudou além da colonização pneumocócica simples a colonização pneumocócica múltipla. O estudo envolveu crianças que foram analisadas durante dois períodos de colheita com apenas um mês de intervalo (i.e., antes e depois da vacinação), o que permitiu identificar o mecanismo de acção da vacina. Os resultados obtidos neste estudo permitiram concluir que em crianças imunizadas com apenas uma dose de vacina ocorre um efeito de substituição de serótipos vacinais por não vacinais, tanto a nível da população como a nível do indivíduo. Foi possível ainda concluir que o mecanismo de acção da PCV7 consiste na prevenção da aquisição *de novo* dos serótipos vacinais por um lado e no "unmasking" de serótipos não vacinais por outro.

No último trabalho apresentado nesta tese foram utilizados modelos murinos de colonização e doença. Estes modelos animais serviram para caracterizar os serótipos não vacinais e não susceptíveis à penicilina mais comuns, que colonizam a nasofaringe de crianças portuguesas vacinadas com a PCV7. O papel da cápsula *versus* o património genético no que diz respeito aos estádios de colonização e doença foi também abordado neste estudo. Em resumo, os três serótipos não vacinais mais comuns que foram estudados – i.e., estirpes que expressam os serótipos 6A, 15A e 19A – colonizaram e disseminaram-se em seguida para os tecidos adjacentes à nasofaringe, designadamente o bolbo olfactivo, o cérebro, os pulmões e a mucosa do ouvido médio. Cada serótipo provocou infecção pulmonar letal quando atingidos títulos bacterianos elevados. O serótipo 19A (ST276) foi o único dos serótipos não vacinais testado a evidenciar propensão para causar infecção no sangue. Finalmente, demonstrou-se que a colonização dependeu da cápsula e do património genético, enquanto a virulência (isto é, a infecção do sangue) dependeu apenas da cápsula.

Thesis Outline

The work presented in this Doctoral Thesis describes the evaluation of the impact of the 7-valent pneumococcal conjugate vaccine (PCV7) on colonization among Portuguese children attending day care. This general objective was attained firstly by investigating the epidemiology of the pneumococcus, and secondly by assessing the virulence potential of non vaccine serotypes selected *in vivo* by the PCV7 vaccine.

Chapter I – General introduction. This chapter provides an outline of the *Streptococcus pneumoniae* epidemiology in colonization and disease. The pivotal role of the pneumococcal capsule and antibiotic resistance on the development and impact of the pneumococcal vaccines is also addressed. Moreover, mouse models are described as important and valuable systems to study pneumococcal colonization and pathogenesis.

Chapter II – Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers (DCCs) in Lisbon. This chapter reports, for the first time in Portugal, the impact of the PCV7 in colonization and drug resistance among DCC attendees.

Chapter III – Impact of a single dose of the 7-valent pneumococcal conjugate vaccine on colonization. This chapter describes the effect of one PCV7 dose on single and multiple carriers, assessing the actual mechanism of the vaccine's effect.

Chapter IV – Virulence potential of serotypes selected *in vivo* by the 7-valent pneumococcal conjugate vaccine (PCV7) in Portugal. This chapter presents a study on the virulence potential of colonizing non vaccine types selected *in vivo* by the PCV7 vaccine using several mouse models.

Chapter V – Conclusions and perspectives. This chapter combines the major findings of this Doctoral thesis, suggesting future follow-ups of these studies.

Chapters II and **III** are reproductions of the following publications:

Chapter II – **Frazão, N., A. Brito-Avô, C. Simas, J. Saldanha, R. Mato, S. Nunes, N. G. Sousa, J. A. Carriço, J. S. Almeida, I. Santos-Sanches, and H. de Lencastre.** 2005. Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon. Pediatr Infect Dis J **24:**243-52.

Chapter III – **Frazão, N., R. Sá-Leão, and H. de Lencastre.** 2010. Impact of a single dose of the 7-valent pneumococcal conjugate vaccine on colonization. Vaccine **28:**3445-52.

List of abbreviations

A

AOM – Acute otitis media

C

CDC – Centers for Disease Control

CFU – Colony forming units

CSF – Cerebrospinal fluid

D

DCC – Day-care center

DR – Drug resistant

DRPn – Drug resistant *Streptococcus pneumoniae*

I

i.cist. – Intracisternal

i.n. – Intranasal

IPD – Invasive pneumococcal disease

i.p. – Intraperitoneal

i.t. – Intratracheal

i.v. – Intravenous

M

MIC – Minimum inhibitory concentration

MLST – Multi-locus sequence typing

N

NCCLS – National Committee for Clinical Laboratory Standards

NCKP – Northern California Kaiser Permanente

NP – Nasopharyngeal

NT – Non typeable

NVT – Non vaccine type

P

PCV7 – 7-valent pneumococcal conjugate vaccine

PCV13 – 13-valent pneumococcal conjugate vaccine

PFGE – Pulsed-field gel electrophoresis

PMEN – Pneumococcal Molecular Epidemiology Network

Pn – *S. pneumoniae*

PNSPn – Penicillin nonsusceptible *S. pneumoniae*

PP23 – 23-valent pneumococcal polysaccharide vaccine

PS – Capsular polysaccharides

PSPn – Penicillin susceptible *S. pneumoniae*

R

RTI – Respiratory tract infections

S

SXT – Trimethoprim-sulfamethoxazole

V

VT – Vaccine type

TABLE OF CONTENTS

Chapter I

1. General introduction

1. General introduction

Streptococcus pneumoniae (the pneumococcus) has been studied for more than a century and has so far bypassed all the human initiatives targeted to eliminate this apparently unbeatable pathogen. The widespread belief that antimicrobial drugs would cure all pneumococcal infections waned the interest in using vaccines to prevent infections. Only the emergence of antimicrobial resistant strains led to a recurring interest in developing new pneumococcal vaccines that aim to eventually eradicate this bacterium. To attain this long-term goal it is essential to be aware of the epidemiological dynamics of the pneumococcus.

Since colonization is the first step to disease development, an in-depth understanding of the major pneumococcal serotypes and clones carried in the nasopharyngeal mucosa together with data on their virulence potential is critical. Based on this information, it may be possible to predict future pneumococcal disease serotypes/genotypes and include them in future vaccine formulations.

1.1. *Streptococcus pneumoniae*

Streptococcus pneumoniae, also known as the pneumococcus, is a lancet-shaped gram-positive bacterium which can grow in liquid medium as single cells, diplococci or in chains. It is a facultative anaerobic microorganism which shows α-hemolysis when growing in blood agar plates, solubility when in presence of bile salts, catalase negativity and usually susceptibility to optochin. All these characteristics together with common positive agglutination with specific antipneumococcal polysaccharide capsule antibodies are taken into consideration when classifying a bacterium as belonging to the *Streptococcus pneumoniae* species (Dowson, 2004; Murray, 1999).

1.2. Historical background

In 1875, while searching for a proof of the infectious nature of pneumonia, Edwin Klebs was probably the first to recognize *S. pneumoniae* in pneumonic lung tissue, describing the phenomenon as nonmotile, sometimes linked “monads” (White *et al.*, 1938).

Six years later, in 1881, Louis Pasteur in France and Georg Sternberg in the USA reported for the first time the isolation of *S. pneumoniae* in the laboratory (Pasteur, 1881; Sternberg, 1881).

After the discovery of the microorganism, its name changed several times. Pasteur called his isolate the “microbe septicémique du saliva” while Sternberg called his *Micrococcus pasteuri*. In 1886, Albert Fraenkel performed the first complete description of the microorganism and gave us the familiar name “pneumokokkus”. In the same year Anton Weichselbaum suggested the name *Diplococcus pneumoniae* (White *et al.*, 1938), which became the official name until 1974 when the organism was reclassified *Streptococcus pneumoniae* according to its characteristic property of growing as chains of cocci in liquid media (Bergey, 1974).

Isolated and characterized more than 120 years ago, *S. pneumoniae* has been one of the most extensively studied microorganisms leading to seminal discoveries in several scientific areas, including the putative use of polysaccharide antigens as vaccines (Avery *et al.*, 1917), bacterial gene transfer (Griffith, 1928), the isolation and chemical characterization of the first polysaccharide antigen (Goebel & Adams, 1943), the identification of the “transforming principle” (later named DNA) as the genetic material (Avery *et al.*, 1944), the therapeutic efficacy of penicillin (Tillett *et al.*, 1944), the role of the bacterial capsule in resistance to phagocytosis (Felton *et al.*, 1955), and the first bacterial quorum sensing factor (Tomasz, 1965).

1.3. Epidemiology of *Streptococcus pneumoniae*

Pasteur and Sternberg, although independently, performed similar experiments when they isolated for the first time *S. pneumoniae*. Both injected human saliva, from asymptomatic carriers, into rabbits that died shortly after from blood infection (Pasteur, 1881; Sternberg, 1881). Altogether, these pioneering experiments showed for the first time that *S. pneumoniae* on one hand can be asymptomatically carried and on the other has the potential to cause disease.

1.3.1. Pneumococcal colonization

The pneumococcus colonization is described as a commensal relationship with its principal host, man (Austrian, 1997). Asymptomatic pneumococcal colonization of the human nasopharyngeal mucosa starts immediately after birth (Aniansson *et al.*, 1992) and, at any given time, up to 30% of adults and 60% of children are colonized (Austrian, 1986). The children's nasopharynx constitutes the major reservoir for the pneumococcus, specially day-care center (DCC) attendees where pneumococcal colonization rate is particularly high. In Portugal up to 71% of the children up to 6 years old attending day-care centers are colonized with pneumococci (Mato *et al.*, 2005).

Depending on the host's age and the pneumococcal colonizing strain, the duration of carriage ranges between 1 to 17 months (Gray *et al.*, 1980). Pneumococci can present two patterns of colonization: i) single colonization, where the host carries only one pneumococcal strain or ii) multiple colonization, also called co-colonization, where the host carries simultaneously more than one pneumococcal strain, which can differ in phenotypic and/or genotypic characteristics (Auranen *et al.*, 2010; Frazão *et al.*, 2010; Gray *et al.*, 1980; O'Brien *et al.*, 2007; Sá-Leão *et al.*, 2002).

Pneumococcal transmission occurs through direct contact with respiratory secretions or inhalation of aerosols, from either asymptomatic carriers or from a person with pneumococcal disease (Antao & Hausdorff, 2009). Among children, the risk factors related to pneumococcal colonization include young age (less than 2 years old), having

a sibling and day-care center attendance. Among adults, recurrent contact with children and crowdness constitute the major risk factors to be colonized with pneumococci (Kristinsson, 1997).

Pneumococcal colonization is usually studied by performing the characterization of a single pneumococcal isolate for each colonized individual. However, studies show that individuals can be co-colonized with different pneumococcal strains (Auranen *et al.*, 2010; Frazão *et al.*, 2010; Gray *et al.*, 1980; O'Brien *et al.*, 2007; Sá-Leão *et al.*, 2002). Epidemiological studies based on a single isolate characterization are important and valuable tools to detect differences in the pneumococcal dynamics at a population level. However, studies where co-colonization is detected are crucial to identify differences at the individual level, such as *de novo* acquisition, clearance and unmasking of pneumococcal strains (Dagan *et al.*, 2003; Lipsitch, 1999; Rinta-Kokko *et al.*, 2009).

1.3.2. Pneumococcal disease

The pneumococcus is described as being a commensal human pathogen and colonization of the nasopharyngeal mucosa is often a transient process (Hill *et al.*, 2008; Hogberg *et al.*, 2007), constituting the initial event in the progression to disease (Henriques-Normark & Normark, 2010). As a pathogen *S. pneumoniae* can give rise to a wide variety of diseases that can be divided into invasive and non-invasive. In invasive pneumococcal disease (e.g. meningitis, bacteremic pneumonia and bloodstream infections – bacteremia and septicemia), pneumococcus can be isolated from blood or other normally sterile body fluids. In non-invasive pneumococcal disease, also known as mucosal infections such as sinusitis, conjunctivitis, non-bacteremic pneumonia and otitis media, pneumococcus can be isolated from mucosal excretions only (Bogaert *et al.*, 2004a; Feldman & Klugman, 1997; Musher, 1992).

The spectrum of pneumococcal diseases differs in different age groups and different populations (Bogaert *et al.*, 2004a; Hausdorff *et al.*, 2005; Musher, 1992; O'Brien & Santosham, 2004). Several risk factors for pneumococcal infection, such as viral infection, age (less than 5 and over 60 years of age), race, immunodeficiency, chronic

underlying illness, socio-economic status, previous antibiotic therapy and day-care attendance have been reported (O'Brien & Santosham, 2004).

Pneumococcus is a leading pathogen, causing infections with high mortality and morbidity (Austrian, 1977; Greenwood *et al.*, 2007; Hausdorff *et al.*, 2000a; Hausdorff *et al.*, 2000b; Mulholland, 2007; O'Brien & Santosham, 2004; Scott *et al.*, 1996). Up to one million children die annually from pneumococcal diseases, and most of them are young children in developing countries (O'Brien *et al.*, 2009; WHO, 1999; Williams *et al.*, 2002). In industrialized countries invasive pneumococcal infections occur, especially among children and elderly people (Eskola *et al.*, 1992; Hausdorff *et al.*, 2005; Sankilampi *et al.*, 1997; Scott *et al.*, 1996).

1.3.3. Capsule, antibiotic resistance, and vaccines

In the overwhelming majority of pneumococcal isolates the outside surface of the bacterium is covered by a polysaccharide capsule, which plays a major role in colonization and disease and constitutes the main virulence factor of *S. pneumoniae*. The chemical nature of the capsule and the amount of capsule produced are major factors inhibiting complement activity, neutrophil phagocytosis, and bacterial killing by neutrophil extracellular traps (Hyams *et al.*, ; Kim & Weiser, 1998; Kim *et al.*, 1999; Macleod & Krauss, 1950; Magee & Yother, 2001; Nelson *et al.*, 2007; Paton *et al.*, 1993; Quin *et al.*, 2007). The capsule also plays a major role in bacterial interactions with the epithelium in colonization (Bootsma *et al.*, 2007; Macleod & Krauss, 1950; Magee & Yother, 2001; Nelson *et al.*, 2007; Quin *et al.*, 2007; Wartha *et al.*, 2007).

The pneumococcal capsules are very diverse in chemical structure and a nomenclature system was created based on the antigenic differences of the capsular polysaccharides (PS) grouping the capsules into more than 90 serotypes (Austrian, 1981; Henrichsen, 1999; Sorensen, 1995). In recent years additional new serotypes 6C, 6D and 11E were discovered, suggesting that the diversity of pneumococcal capsules is even greater than was previously recognized (Calix & Nahm, 2010; Henrichsen, 1995; Jin *et al.*, 2009; Oftadeh *et al.*, 2010; Park *et al.*, 2007b).

Shortly after the pneumococcal capsule was identified as a major immunogen in the 1930's, vaccine development focused on purified PS culminating with the commercialization of two hexavalent polysaccharide vaccines in 1946 in the United States (Felton, 1938; Francis & Tillett, 1930). Concomitantly, sulfonamides and penicillin had become readily available and new antimicrobial drugs were on the way. The discovery of antimicrobials paved the way for better health for millions around the world. Before penicillin became a viable medical treatment in the early 1940's, no true cure for gonorrhea, strep throat, or pneumonia existed. Patients with infected wounds often had to have a wounded limb removed, or face death from infection. The dawn of antimicrobial drugs enabled all these fierce medical conditions to be cured with a short course of antimicrobial treatment. Thus, the vaccines were forgotten and were withdrawn from the market in 1954 for lack of demand (Fedson *et al.*, 1994). However, it did not take long before it became clear that antimicrobials had not eliminated pneumococcal disease.

Resistance to antimicrobials is closely linked to the fact that *S. pneumoniae* is a naturally transformable bacterium. Pneumococci, when in a competent state, have the capacity of taking up DNA and incorporate it into their genome (Claverys & Havarstein, 2007). Whenever the DNA incorporated into the genome has resistance genes the pneumococci become resistant to drugs.

In 1964, a study by Austrian and Gold reported that nearly one in four patients admitted with pneumococcal bacteremia died even with antimicrobial drug treatment (Austrian & Gold, 1964). In 1967, the first intermediately penicillin resistant pneumococcal isolate was reported in Australia (Hansman & Bullen, 1967). In the following years penicillin remained the drug of choice to treat pneumococcal infections as resistance to other drugs such as tetracyclines, chloramphenicol, macrolides, and trimethoprim-sulfamethoxazole (SXT) had emerged (Klugman, 1990). In 1978, the first pneumococcal strains resistant to all the above mentioned antimicrobial classes, as well as fully resistant to penicillin, were isolated (Jacobs *et al.*, 1978). It was the beginning of the pneumococcal multidrug resistance (resistance to three or more classes of drugs) era. During the 1980's, multidrug resistant strains were found in Spain and spread globally

(Klugman, 1990). In the 1990s, multidrug resistance continued to increase such that in the United States in 2000 nearly half of all invasive pneumococcal disease (IPD) was caused by pneumococcal isolates resistant to penicillin and/or macrolides (Whitney *et al.*, 2000).

The widespread belief that antimicrobials alone would "solve" all pneumococcal infections was definitely put aside as soon as pneumococcal drug resistance became a reality causing treatment failures (Kaplan & Mason, 2002). Consequently, a renewed interest in the prevention through vaccination was regained and led to clinical trials of a PS vaccine. In 1977 the Merck's 14-valent pneumococcal polysaccharide vaccine (Pneumovax) was licensed in the United States. Similar 14-valent vaccines were later produced by Lederle (Pnu-Immune) and by Pasteur Mérieux. The 14 PS types included in these vaccines (1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F, and 25F) were selected based on epidemiological information from the United States, parts of Europe, and South Africa, being the most common serotypes causing pneumococcal disease (Robbins *et al.*, 1983). In 1983 the 14-valent vaccine was expanded to include 23 PS types, and is currently the only approved formulation available for adults in the United States (Fedson & Musher, 2004; Siber, 1994).

The main disadvantage of the 23-valent vaccine is the fact that it is not effective in children less than two years old, who are at highest risk of life-threatening pneumococcal infection and acute otitis media (CDC, 2000; Makela & Butler, 2008). To overcome this major weakness the concept of pneumococcal conjugate vaccine was developed. The novelty of these conjugate vaccines is the fact that PS types instead of being "presented" alone to the child's immune system are attached to an immunogenic carrier protein that allows a highly efficient antibody production and induces immunological memory.

The 7-valent pneumococcal conjugate vaccine (PCV7) was the first conjugate vaccine to be licensed. It began to be marketed in 2000 and includes the seven most common serotypes isolated from the blood or cerebrospinal fluid (CSF) of children under 6 years old (i.e., 4, 9V, 6B, 14, 18C, 19F and 23F) in the United States (CDC, 2000). In Portugal

PCV7 was introduced in June 2001 and, despite widespread vaccination, it is still not part of the national vaccination plan.

Very recently, higher valency pneumococcal conjugate vaccines were licensed such as PCV10 (10-valent pneumococcal conjugate vaccine), which includes the PCV7 serotypes plus serotypes 1, 5, and 7F. PCV13 (13-valent pneumococcal conjugate vaccine) was the last to be licensed and includes PCV10 serotypes plus serotypes 3, 6A, and 19A.

1.4. Pneumococcal conjugate vaccine

The pneumococcal conjugate vaccine was a big step forward to win the apparently never-ending war against the *S. pneumoniae* pathogen, especially in children under two years of age who are at higher risk of contracting pneumococcal infections. This age group is not immunologically responsive to the 23-valent pneumococcal polysaccharide vaccine, but is protected by a pneumococcal conjugate vaccine.

1.4.1. Immunological basis

The primary pneumococcal antigens eliciting a host immune response are the pneumococcal capsular polysaccharides, which induce a T-cell independent immune response that is virtually absent in children until around two years of age. Conversely, when capsular polysaccharides are covalently coupled to immunogenic proteins such as the mutant diphtheria toxin CRM_{197} used in PCV7, a T-cell dependent response is elicited. This type of immunological response is already present in children aged up to two years, thus rendering conjugate vaccines a good immunogen for this age group. Another major characteristic of T-cell dependent responses is the induction of immunological memory characterized by affinity maturation and a booster response on subsequent exposure to the bacterial antigen. In summary, a conjugate vaccine induces T-cell dependent immunity in young children, which leads to effective antibody production and immunologic memory.

The pneumococcal conjugate vaccine can confer both systemic and mucosal immunity (Korkeila *et al.*, 2000; Nieminen *et al.*, 1999; Nurkka *et al.*, 2001a). The immunological protection is mediated through the production of specific antibodies. Mucosal immunity is mainly mediated through the production of serotype-specific immunoglobulin A (IgA) antibodies, which are locally produced at the nasopharyngeal mucosa (Choo *et al.*, 2000; Korkeila *et al.*, 2000; Nurkka *et al.*, 2001a). Serotype-specific immunoglobulin G (IgG) antibodies are mainly found in serum, conferring systemic immunity. However, they can also be detected in the nasopharyngeal mucosa, although more rarely than IgA (Choo *et al.*, 2000; Kauppi *et al.*, 1995; Korkeila *et al.*, 2000; Nurkka *et al.*, 2001a; Nurkka *et al.*, 2001b). It is believed that circulating IgG antibodies can passively cross the nasal mucous membrane and help prevent pneumococcal colonization, though mucosal IgG production has been suggested to happen as well (Berneman *et al.*, 1998; Ogra, 2000).

1.4.2. Impact on colonization

The impact of the 7-valent pneumococcal conjugate vaccine on colonization can have both a direct effect — protecting those successfully immunized from carriage — and an indirect effect — providing protection against carriage among unimmunized individuals by reducing transmission of the organism within the community.

The vaccination schedule varies between countries, but all the primary PCV7 immunization series include two or three vaccine doses between 6 weeks and 6 months of age. A booster dose in the second year of life is the recommended practice in economically developed countries, but is not included in the schedules of developing countries due to the high price of the vaccine (ACIP, 2000). Studies of the effect in colonization of fewer than the recommended doses are scarce. A mathematical model of vaccination suggested that a single dose given between 5 and 7 months of age could prevent up to one-third of invasive pneumococcal disease (Barzilay *et al.*, 2006). Bearing in mind that disease is preceded by colonization, studying the impact of less than the recommended vaccine doses in colonization may play a major role for the

development of less expensive vaccine schedules that eventually may foster the introduction of pneumococcal vaccines in developing countries.

The major conclusion concerning the direct effect of the conjugate vaccines is that in vaccinated individuals there is a reduction in the prevalence of nasopharyngeal colonization by the serotypes included in the pneumococcal conjugate vaccines. This general effect has been shown in children after a primary PCV immunization series (Dagan *et al.*, 1997; Mbelle *et al.*, 1999; O'Brien *et al.*, 2007), or after a boosting dose of either the 23-valent polysaccharide or conjugate vaccines (Dagan *et al.*, 1997; Dagan *et al.*, 2000; Kilpi *et al.*, 2001; Obaro *et al.*, 1996).

In the specific case of children attending DCCs, where colonization rates are normally very high (Dagan *et al.*, 1996a; Dagan *et al.*, 2002; Dagan *et al.*, 2005; Frazão *et al.*, 2005), the pneumococcal conjugate vaccine also showed efficacy in reducing the colonization by vaccine serotype strains. The same effect of reducing vaccine type colonization was also reported when immunization was carried out with fewer than the recommended conjugate vaccine doses (Frazão *et al.*, 2010; Jones *et al.*, 2005; van Gils *et al.*, 2009)

The above mentioned effect has been reported for all serotypes in the pneumococcal conjugate vaccine formulation. However, in some studies, the effect on serotype 19F is reported as being the lowest (Dagan *et al.*, 2002; Dagan *et al.*, 2005; Huang *et al.*, 2005; Millar *et al.*, 2006; Veenhoven *et al.*, 2003). Concerning the vaccine related serotypes, the ones belonging to the same serogroups, 6A prevalence decreases in vaccinated individuals whereas 19A and 23A prevalence increases (Huang *et al.*, 2005; Millar *et al.*, 2006; Sá-Leão *et al.*, 2009; Veenhoven *et al.*, 2003). Regarding the non vaccine serotypes, there is an increase in vaccinated children when compared to non vaccinated children (Dagan *et al.*, 1996a; Dagan *et al.*, 1997; Dagan, 2002; Frazão *et al.*, 2005; Frazão *et al.*, 2010; Sá-Leão *et al.*, 2009). The immune response to the pneumococcal conjugate vaccines confers direct protection against the vaccine types (VTs) in the nasopharynx. The ecological mechanism(s) by which the pneumococcal conjugate vaccines act directly against the vaccine types is not addressed in the majority of the

studies. However, there are strong indications that the primary mechanism of the vaccines' effect is the prevention of the *de novo* acquisition of VTs, rather than clearance, and additionally the reduction of the density of colonization by the vaccine serotypes (Dagan *et al.*, 2005; Frazão *et al.*, 2010; O'Brien *et al.*, 2007).

The reduction in the VT pneumococcal carriage due to the PCV thus opens a biological niche that is filled by non vaccine type (NVT) strains leading to a phenomenon which has been termed as serotype replacement colonization. The colonization of the nasopharynx with serotypes not included in the vaccine (NVTs) can happen through i) capsular serotype switching, such that previous vaccine type clones exhibit now NVT capsules, ii) the expansion and propagation of existing NVT clones in the community, and iii) the introduction of new NVT clones into the community.

Worth to be investigated should be the "unmasking" phenomenon in which a minority clone/serotype is masked by a dominant clone/serotype in hosts that carry more than one type of pneumococci at the same time. This can only be addressed if multiple colonization (co-colonization) studies are undertaken. These studies may clarify whether the new NVT clones/serotypes are in fact new or simply types already present but masked and unidentified by approaches where only a single pneumococcal isolate per sample was recovered.

As a rule of thumb, the final result of the direct impact of the pneumococcal conjugate vaccines in colonization is that serotype replacement carriage occurs, meaning that vaccine types are substituted by non vaccine serotypes. This massive direct effect of PCV in pneumococcal colonization among vaccinated children results in a low or inhibited transmission of vaccine types within households, day-care attendees, and communities as a whole, leading also to serotype replacement of vaccine by non vaccine types just as is known to happen in vaccinated children. This effect of PCV on unimmunized individuals is termed the indirect or herd immunity effect, which was shown in numerous studies. Indeed, immunization of children with a pneumococcal conjugate vaccine was shown to decrease vaccine serotype colonization in children too young to be immunized (under 2 months) and older children and adults who are not

eligible for vaccination, pointing at the same time to an increased prevalence of non vaccine serotypes colonizing the nasopharynx of unvaccinated individuals (Givon-Lavi *et al.*, 2003; Hammitt *et al.*, 2006; Hennessy *et al.*, 2005; Moore *et al.*, 2004; O'Brien *et al.*, 2007).

The effect of the pneumococcal conjugate vaccine on antibiotic resistance is explained by the fact that the global pandemic of pneumococcal resistance was dominated by a relatively small number of resistant and multidrug resistant clones that express mainly five (6B, 9V, 14, 19F, and 23F) of the seven serotypes included in the PCV7 vaccine (Dagan & Klugman, 2008; Kyaw *et al.*, 2006; McGee *et al.*, 2001). Since both the direct and indirect effects of the pneumococcal conjugate vaccine on colonization lead to the decrease of the VTs, which are strongly associated with resistance, particularly high-level penicillin resistance and multidrug resistance, one can expect that vaccination with a pneumococcal vaccine will reduce the prevalence of antibiotic resistant *S. pneumoniae* strains. In fact, studies in Israel, where multi-valent pneumococcal conjugate vaccines were used to immunize children, reported a reduction in the drug resistant pneumococcal strains colonizing the nasopharynx (Dagan *et al.*, 1996a; Dagan *et al.*, 1997; Dagan *et al.*, 2000; Dagan *et al.*, 2003; Mbelle *et al.*, 1999). Nevertheless, in some locations the increasing prevalence of NVTs, also drug resistant, as a result of the serotype replacement phenomenon led to a compensatory effect that allows the maintenance of pneumococcal drug resistance in the populations. In Portugal, after vaccination with the PCV7 the carriage rate of drug resistant pneumococcal strains exhibiting vaccine capsular types decreased, but was compensated by a gradual increase in the prevalence of drug resistant NVTs, nonsusceptible to penicillin (6A, 6C, 10A, 15A, 15B/C, 19A, 24F and 33F) (Frazão *et al.*, 2005; Sá-Leão *et al.*, 2009). In the United States, a similar trend was found (Huang *et al.*, 2005). In PCV7 vaccinated children, parallel to the decrease of drug resistant VTs, a substantial increase in NVTs was seen, namely in the most commonly carried penicillin nonsusceptible serotypes: 6A, 19A, 23A and serogroups 15 and 29 (Huang *et al.*, 2005).

Moore and co-workers also found the same serotype dynamics in vaccinated children where the carriage of penicillin nonsusceptible strains was maintained after vaccination with PCV7 (Moore *et al.*, 2004).

Possible explanations for the rise of drug resistant strains after vaccination with a pneumococcal conjugate vaccine include the *de novo* acquisition of resistance, serotype switching, the introduction of new clones, and the expansion of existing clones. In Portugal the increase of drug resistance among the non vaccine type population (6A, 10A, 15A, 15B/C, 19A, 23A, and 33F) seems to be due to the *de novo* acquisition of resistance (a rare phenomenon only observed for serotype 10A) and, most frequently, the introduction of new clones and the expansion of existing ones (Frazão *et al.*, 2007). Hanage and co-workers, observed a similar scenario where resistance increased mainly through the expansion of pre-existent clones of non vaccine serotypes, particularly 15A, 19A, and 35B (Hanage *et al.*, 2007).

1.4.3. Impact on disease

The direct effect of the 7-valent pneumococcal conjugate vaccines translates into a sharp decline of invasive disease among immunized children caused by *S. pneumoniae* serotypes included in its formulation (Aguiar *et al.*, 2008; Aguiar *et al.*, 2010; Bettinger *et al.*, 2010; Black *et al.*, 2004; Dagan *et al.*, 2001; Hennessy *et al.*, 2005; Hsu *et al.*, 2005; Kellner *et al.*, 2005; Munoz-Almagro *et al.*, 2008; Pilishvili *et al.*, 2010).

Concerning the efficacy of PCV7, data from the Northern California Kaiser Permanent health system (NCKP), which conducted the first clinical trial to evaluate PCV7, demonstrated an efficacy of 97% against invasive pneumococcal disease (IPD) caused by the vaccine serotypes (Black *et al.*, 2000). After the PCV7 licensure in 2000 in the United States, a population-based study from the Active Bacterial Core Surveillance of the Centers for Disease Control (CDC) and Prevention reported a large IPD reduction from 24.3 cases in 1998-1999 to 17.3 per 100000 in 2001. The main decline was observed in children younger than two years old showing a 78% decrease in the rate of IPD caused by the vaccine serotypes (Whitney *et al.*, 2003).

Regarding the pneumococcal non-invasive mucosal disease, the impact of the pneumococcal conjugate vaccine was confirmed although considered moderate. The estimated efficacy of PCV7 in preventing first episodes of radiographic pneumonia was 30% (Hansen *et al.*, 2006). Additionally, studies based on data from the NCKP showed a 7% efficacy in preventing episodes of otitis media, a disease with high burden and associated related costs. The same was found in the FinOM trial (Black *et al.*, 2000; Eskola *et al.*, 2001; Kilpi *et al.*, 2001).

Just like in the colonization phenomenon, in disease too, the pneumococcal conjugate vaccine was also accompanied by an indirect effect. The rationale for this relies on the fact that by immunizing a subset of a population (children) one can reduce significantly the circulation of vaccine types, reducing carriage and even more the disease caused by these serotypes, among the non-vaccinated individuals. Evidence for this indirect or herd immunity effect, described early after PCV7 licensure, highlights an important component of the public health profit of pneumococcal conjugate vaccine use. A report from the NCKP study indicated that the reduction in disease was greater than the percentage of children vaccinated (Black *et al.*, 2001). A similar pattern was observed in the CDC data where the rate of vaccine type disease among children less than 5 years old dropped 94%, even though only 68% of children had been vaccinated (CDC, 2005). Reported decrease of IPD caused by vaccine types among children too young to be immunized (under 2 months) and older children and adults who are not eligible for vaccination further confirm the herd immunity effect of the pneumococcal conjugate vaccine (Black *et al.*, 2004; Poehling *et al.*, 2006; Whitney *et al.*, 2003).

The overall reduction of the disease-causing vaccine serotypes in settings where pneumococcal conjugate vaccines were administered raised an expectancy of decreasing the overall pneumococcal drug resistance. This expectation is based on the fact that five of the serotypes included in the conjugate vaccine (6B, 9V, 14, 19F, and 23F) were responsible for the prevalence of drug resistance among pneumococci. Indeed, several studies reported large decreases in disease episodes caused by drug resistant pneumococci (Hennessy *et al.*, 2005; Kaplan *et al.*, 2004; Klugman *et al.*, 2003; Kyaw *et al.*, 2006). However, at the same time that the decline in disease in general,

and that caused by drug resistant pneumococci in particular, were observed, an increase in disease caused by non vaccine types, namely resistant to drugs, was reported (Byington *et al.*, 2005; Eskola *et al.*, 2001; Hicks *et al.*, 2007; Porat *et al.*, 2004). The substitution of vaccine by non vaccine serotypes in disease due to the selective pressure exerted by the conjugate vaccine is called replacement disease. Early studies of the impact of vaccination found that, among children, increases in the rate of disease due to non vaccine serotypes were either small in magnitude or limited to a single geographic location (Byington *et al.*, 2005; Flannery *et al.*, 2004; Kaplan *et al.*, 2004). It was hoped that IPD caused by non vaccine types such as 6A, and 19A could be prevented through cross protection with serotypes 6B and 19F included in the PCV7. Although this reduction has been reported for serotype 6A (Millar *et al.*, 2006; Whitney *et al.*, 2003), an increase rather than a decrease in serotype 19A IPD has been observed (Beall *et al.*, 2006; Hennessy *et al.*, 2005; Pai *et al.*, 2005). More recent data showed larger increases in the incidence of serotype 19A and serogroup 15 strains among children and of multiple non vaccine serotypes among adults, specially with HIV/AIDS, where the infection with *S. pneumoniae* occurs up to 100 times more frequently than among the general population (Aguiar *et al.*, 2010; Dworkin *et al.*, 2001; Flannery *et al.*, 2006; Hicks *et al.*, 2007; Munoz-Almagro *et al.*, 2008; Nuorti *et al.*, 2000; Pai *et al.*, 2005).

In just a few years after the licensing of the PCV7 vaccine, serotype 19A has become the predominant cause of invasive disease in children and, not less importantly as it can lead to medical treatment failures, increasingly drug resistant (Aguiar *et al.*, 2010; Hicks *et al.*, 2007; Munoz-Almagro *et al.*, 2008; Pai *et al.*, 2005).

1.5. Mouse models

Experimental animal models have been used extensively to understand vital processes of the pneumococcal colonization and disease (Chiavolini *et al.*, 2008; Malley & Weiser, 2008). Mouse models have been playing a vital role concerning much of what has been learned on these topics, serving as surrogates for the same phenomena in humans.

1.5.1. Mouse model of colonization

The pneumococcal colonization is a crucial step for invasive disease development, rendering the *in vivo* study of this phenomenon fundamental to complement epidemiological and *in vitro* studies.

The mouse model is the most widely used for studying pneumococcal colonization, for a number of reasons: i) mice are colonized by pneumococci of multiple serotypes, ii) there is availability of reagents and diversity of mice strains (such as CD1, C57BL/6, BALB/c, and CBA/n), iii) the intranasal inoculation of anesthetized mice with selected strains can subsequently lead to disease, in a way that mimics natural pneumococcal infections in humans (Balachandran *et al.*, 2002; Takashima *et al.*, 1997; Wu *et al.*, 1997), and iv) the persistence of colonization in mice was shown to last several weeks, which again may be viewed as representative of what may happen in humans (Gray *et al.*, 1980; Hogberg *et al.*, 2007; McCool & Weiser, 2004). Moreover, the characterization of experimental nasal colonization in humans provided a basis for assessing the applicability of the mouse models of colonization. An example of this is the fact that the same isolate used in human studies was found to colonize mice in association with a similar inoculum dose, duration, and immune response (McCool & Weiser, 2004).

Several variations of the model have been used, but in the majority of cases, a 10-µl bacterial inoculum is intranasally dropped atraumatically into the nares of the mice. After inoculation the presence and density of pneumococcal colonization can be investigated over the subsequent days, generally by obtaining nasopharyngeal washes from recently euthanized animals (Wu *et al.*, 1997).

Several limitations have been attributed to the mouse model, such as the fact that different laboratories have used different mouse strains, which can vary in susceptibility to colonization and may prevent reproducibility of results. More specifically, much of the research work has been carried out using mainly the D39 isolate (Iannelli *et al.*, 1999), which is useful for studying invasive disease but colonizes mice relatively inefficiently

when compared to other pneumococcal isolates (Lipsitch *et al.*, 2007; van Ginkel *et al.*, 2003; Wu *et al.*, 1997).

Mouse models have provided important information on the pneumococcal commensal state, colonization. For example, using a mouse model it was shown that a minimum amount of capsular polysaccharide is needed for efficient colonization, and that unencapsulated mutants remain capable of colonizing but at a lower density and duration when compared with the capsulated parental strains (Magee & Yother, 2001; Nelson *et al.*, 2007). Regarding prevention strategies to avoid colonization by using protein vaccines, it was shown in a mouse model that the choline-binding protein A (CbpA), also named PspC or SpsA, is crucial for pneumococcal colonization and that another choline-binding surface protein, PspA, can elicit a mucosal immune response that may confer protection against colonization (Balachandran *et al.*, 2002; Rosenow *et al.*, 1997).

Also the ease of use of genetically modified mice allows the characterization of host factors essential in colonization. Studies which examined the time course of colonization using genetically modified mice showed that pneumococcal clearance in the nasopharynx is dependent on members of a family of Toll-like receptors, which recognize pathogen associated patterns and lead to efficient clearance of colonization (van Rossum *et al.*, 2005). In recent years, McCool and Weiser showed, using a mouse model of colonization, that pneumococcal colonization occurs in an antibody-independent manner (McCool & Weiser, 2004).

1.5.2. Mouse models of pneumococcal disease

Pneumococcal disease can range from mild to life-threatening infections. Similarly to what happens with colonization, the most commonly used animal model for pneumococcal disease is the mouse.

A multiplicity of mouse strains that includes inbred mice, BALB/c, C57BL/6, DBA, and CBA and outbred mice, MF1, CD1, Swiss-Wbster and NMRI is used to study the widely

diverse scenarios of pneumococcal disease, such as pneumonia, septicemia, meningitis, and otitis media (Briles *et al.*, 1981; Canvin *et al.*, 1995; Iizawa *et al.*, 1996; Melhus & Ryan, 2003; Shapiro *et al.*, 2000; Wang *et al.*, 2001; Zwijnenburg *et al.*, 2001). The mouse outbred strains have become increasingly popular because they maintain the maximum heterozygosity, which leads to a phenotypic diversity resembling that of humans. This feature is important as it mimics the natural variation in response to infection in humans. Moreover, the lower cost of these outbred mice strains makes them attractive alternatives to inbred strains.

1.5.2.1. Pneumonia model

Mouse models of pneumococcal pneumonia allow the analysis of various parameters such as survival after challenge, bacterial presence in lungs and blood, inflammation levels, and histology of lung tissue. Additionally, quantification of antibody titers and antimicrobials performed in vaccine and drug pharmacokinetic studies, respectively, are also feasible. Two main routes of infection are currently used to induce pneumonia: the intratracheal (i.t.) and the intranasal (i.n.) route.

The i.t. model is a complex and invasive technique, but has the advantage of allowing the delivery of the entire bacterial inoculum into the lower respiratory tract directly causing pneumonia (Rubins *et al.*, 1996). Briefly, the bacterial suspension is injected trough the mouse oropharynx or through the mouse exposed or cannulated trachea into the lungs (Azoulay-Dupuis *et al.*, 1991; Iwasaki *et al.*, 1999; Tasaka *et al.*, 2002).

The i.t. model is still used in studies of drug efficacy, host response to infection, and the role of pneumococcal virulence factors in the disease process (Abgueguen *et al.*, 2007; Mohler *et al.*, 2003; Rubins *et al.*, 1996).

The i.n. model is a simple and non-invasive technique which includes both the standard aspiration method (Canvin *et al.*, 1995) and the aerosol nebulizer system (Nuermberger *et al.*, 2005). The model of i.n. aspiration to cause pneumonia is the most commonly used because it is fast and easy to perform and, most importantly, mimics the natural

route of infection in humans. Briefly, a volume of bacterial inoculum higher than 10 µL is intranasally dropped atraumatically into the nares of anesthetized mice. The model of i.n. aerosol system requires an exposure chamber equipped with a nebulizer. This aerosol technique less closely resembles the development of pneumococcal pneumonia in humans, where the disease generally follows aspiration of bacteria from the upper respiratory tract.

Studies on the host immune response to lung infection, the role of virulence determinants, mice susceptibility and resistance to disease, the efficacy of antibiotics and anti-inflamatory drugs and even studies on protection from i.n. challenge after immunization with vaccine candidates have been carried out using the pneumonia mouse model (Alexander *et al.*, 1994; Bergeron *et al.*, 1998; Blue *et al.*, 2003; Coil *et al.*, 1978; Gingles *et al.*, 2001; Wang *et al.*, 2000; Wang *et al.*, 2005).

1.5.2.2. Septicemia model

The mouse is the most common experimental animal used to study septicemia induced by *S. pneumoniae*. The occurrence of pneumococcal septicemia in mice is assessed mostly by determining the presence of bacteria in the blood and by observing post-challenge survival. Pneumococcal septicemia is induced in the mouse either by injecting bacteria directly into the bloodstream (i.v.) or by injection into the peritoneal cavity (i.p.).

In brief, the i.v. model consists of an injection of the bacterial suspension into the mouse tail vein. Due to the reduced size of the mouse vein, an infrared lamp is used to allow vasodilatation and facilitate the procedure. The i.p. route of infection is one of the earliest techniques used to induce septicemia and consists of an injection of the bacterial suspension into the peritoneal cavity, which leads to posterior infection of the blood. The i.v. model of infection is a more direct system than the i.p. model. However, the former can be time-consuming and difficult to perform comparing to the i.p. route, which is technically easier but presents disadvantages including the risk of tissue damage or pain to the animal (Briles *et al.*, 1992; Iannelli *et al.*, 2004).

Studies that aimed to investigate the role of virulence factors during septicemia, the efficacy of potential vaccines and of novel antimicrobials, as well as blood clearance mechanisms have been performed using the septicemia mouse model (Benton *et al.*, 1995; Briles *et al.*, 1981; Briles *et al.*, 1992; Brown *et al.*, 2001; Cao *et al.*, 2007; Casal *et al.*, 2002; Iannelli *et al.*, 2004; Loeffler *et al.*, 2003; Ogunniyi *et al.*, 2007; Swiatlo *et al.*, 2003).

1.5.2.3. Meningitis model

Only recently has the mouse become an experimental system for studying meningitis induced by *S. pneumoniae*.

There are two main types of procedures to induce pneumococcal meningitis in mice – a direct infection by intracerebral or the intracisternal (i.cist.) route and infection via the blood route (i.p. or i.n.). A third route was recently described by van Ginkel and co-workers, in which pneumococci colonize the nasopharynx and enter the central nervous system along the olfactory nerves without causing blood infection. The latter contradicts the assumption that pneumococci induce meningitis only by invasion via the blood (van Ginkel *et al.*, 2003).

The direct infection of the central nervous system mimics the contiguous spread of pneumococci infecting the sinuses or middle ear or the direct infection due to trauma. It is an experimental system that allows the study of host-pneumococci interactions once the disease is established, but does not allow the study of the previous steps that occur from colonization to disease (Koedel *et al.*, 2002). Conversely, meningitis induced via i.n. or i.p. routes allows the analysis of pathogenesis according to what is believed to be the natural route of infection. However, a major disadvantage is the fact that using this technique ~50% of the mice die due to septicemia even before developing meningitis (Tsao *et al.*, 2002; Zwijnenburg *et al.*, 2001).

The characterization of meningitis in the mouse model has been carried out by assessing different parameters, including mice survival, clinical scores, bacterial

presence in the brain and cerebrospinal fluid, histological analysis, and determination of leukocyte and cytokine levels (Gerber *et al.*, 2001; Grandgirard *et al.*, 2007; Koedel *et al.*, 2001; Marra & Brigham, 2001; Tan *et al.*, 1995).

Studies on the efficacy of antimicrobials against meningitis, host and pneumococcal virulence factors involved in meningitis pathogenesis, and the assessment of postinfectious sequelae have been performed using the meningitis mouse model (Bottcher *et al.*, 2003; Echchannaoui *et al.*, 2002; Iizawa *et al.*, 1998; Kostyukova *et al.*, 1995; Nau *et al.*, 1999; Shapiro *et al.*, 2000; Wellmer *et al.*, 2000).

1.5.2.4. Otitis media model

The interest in using the mouse as a model for otitis media has been growing in recent years as mice present advantages when compared to other animal systems, including lower price and a better characterization regarding immunological and genetic information. Drawbacks of using the mouse as an otitis media model include its small size together with the reduced accessibility of the middle ear for inoculation and sampling purposes.

In the mouse model of otitis media two different routes are used: direct injection (intratympanic or intrabullar) into the middle ear, and the i.n. route that mimics the natural mode of middle ear infection. Briefly, in the direct injection there is a direct application of the inoculum into the middle ear cavity either through the tympanic membrane or by exposing the bulla and injecting the bacterial suspension through the bony wall using a thin needle (Ryan *et al.*, 2006; Sabirov & Metzger, 2006). In the i.n. route, an intranasal inoculation is performed to establish colonization of the nasopharynx, which is followed by invasion of the middle ear cavity by the pneumococci in about 50% of the cases (Ryan *et al.*, 2006; Sabirov & Metzger, 2008).

Recently, in 2009, a very elegant model was proposed by Stol and co-workers, in which anesthetized mice receive 10 µL of the pneumococcal suspension in a similar way as in the colonization model. By using a pressure cabin, a 40 kPa pressure increase is

applied to translocate pneumococci from the nasopharyngeal cavity into both mouse middle ears (Stol *et al.*, 2009)

Studies on potential vaccines and new therapies, pneumococcal virulence factors, inflammation, and histology have been carried out using the otitis media mouse model (MacArthur *et al.*, 2006; McCoy *et al.*, 2005; McCullers *et al.*, 2007; Melhus & Ryan, 2003; Sabirov & Metzger, 2006; Sabirov & Metzger, 2008; Stol *et al.*, 2009).

1.6. Aim of the work

S. pneumoniae is a colonizer of the nasopharyngeal mucosa in young children. Pneumococcal disease takes place after colonization and can range from mild to fatal infections. PCV7 was the first pneumococcal conjugate vaccine created to overcome the lack of efficacy of the 23-valent polysaccharide vaccine in young children. The aim of this thesis was two-fold: i) to evaluate the effect of the PCV7 on the pneumococcal single and multiple colonization flora of young children in Portugal, and ii) to assess the colonization and virulence potential of non-PCV7 serotypes using mouse models.

1.7. References

Abgueguen, P., Azoulay-Dupuis, E., Noel, V., Moine, P., Rieux, V., Fantin, B. & Bedos, J. P. (2007). Amoxicillin is effective against penicillin-resistant *Streptococcus pneumoniae* strains in a mouse pneumonia model simulating human pharmacokinetics. *Antimicrob Agents Chemother* **51**, 208-214.

ACIP (2000). Preventing pneumococcal disease among infants and young children: recommendations of the Advisory Commitee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* **49**, 1-35.

Aguiar, S. I., Serrano, I., Pinto, F. R., Melo-Cristino, J. & Ramirez, M. (2008). Changes in *Streptococcus pneumoniae* serotypes causing invasive disease with non-universal vaccination coverage of the seven-valent conjugate vaccine. *Clin Microbiol Infect* **14**, 835-843.

Aguiar, S. I., Brito, M. J., Gonçalo-Marques, J., Melo-Cristino, J. & Ramirez, M. (2010). Serotypes 1, 7F and 19A became the leading causes of pediatric invasive pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. *Vaccine* **28**, 5167-5173.

Alexander, J. E., Lock, R. A., Peeters, C. C., Poolman, J. T., Andrew, P. W., Mitchell, T. J., Hansman, D. & Paton, J. C. (1994). Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. *Infect Immun* **62**, 5683-5688.

Aniansson, G., Alm, B., Andersson, B., Larsson, P., Nylen, O., Peterson, H., Rigner, P., Svanborg, M. & Svanborg, C. (1992). Nasopharyngeal colonization during the first year of life. *J Infect Dis* **165 Suppl 1**, S38-42.

Antao, V. C. & Hausdorff, W. P. (2009). Global epidemiology of pneumococcal disease--new prospects for vaccine control. *Adv Exp Med Biol* **634**, 19-29.

Auranen, K., Mehtala, J., Tanskanen, A. & M, S. K. (2010). Between-strain competition in acquisition and clearance of pneumococcal carriage--epidemiologic evidence from a longitudinal study of day-care children. *Am J Epidemiol* **171**, 169-176.

Austrian, R. & Gold, J. (1964). Pneumococcal Bacteremia with Especial Reference to Bacteremic Pneumococcal Pneumonia. *Ann Intern Med* **60**, 759-776.

Austrian, R. (1977). Pneumococcal infection and pneumococcal vaccine. *N Engl J Med* **297**, 938-939.

Austrian, R. (1981). Pneumococcus: the first one hundred years. *Rev Infect Dis* **3**, 183-189.

Austrian, R. (1986). Some aspects of the pneumococcal carrier state. *J Antimicrob Chemother* **18 Suppl A**, 35-45.

Austrian, R. (1997). The enduring pneumococcus: unfinished business and opportunities for the future. *Microb Drug Resist* **3**, 111-115.

Avery, O. T., Chickering, H. T., Cole, R. & Dochez, A. R. (1917). Acute lobar pneumonia, prevention and serum treatment. *Monogr Rockefeller Inst* **7**, 1-110.

Avery, O. T., Macleod, C. M. & McCarty, M. (1944). Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type III. *J Exp Med* **79**, 137-158.

Azoulay-Dupuis, E., Bedos, J. P., Vallee, E., Hardy, D. J., Swanson, R. N. & Pocidalo, J. J. (1991). Antipneumococcal activity of ciprofloxacin, ofloxacin, and temafloxacin in an experimental mouse pneumonia model at various stages of the disease. *J Infect Dis* **163**, 319-324.

Balachandran, P., Brooks-Walter, A., Virolainen-Julkunen, A., Hollingshead, S. K. & Briles, D. E. (2002). Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. *Infect Immun* **70**, 2526-2534.

Barzilay, E. J., O'Brien, K. L., Kwok, Y. S., Hoekstra, R. M., Zell, E. R., Reid, R., Santosham, M., Whitney, C. G. & Feikin, D. R. (2006). Could a single dose of pneumococcal conjugate vaccine in children be effective? Modeling the optimal age of vaccination. *Vaccine* **24**, 904-913.

Beall, B., McEllistrem, M. C., Gertz, R. E., Jr., Wedel, S., Boxrud, D. J., Gonzalez, A. L., Medina, M. J., Pai, R., Thompson, T. A., Harrison, L. H., McGee, L. & Whitney, C. G. (2006). Pre- and postvaccination clonal compositions of invasive pneumococcal serotypes for isolates collected in the United States in 1999, 2001, and 2002. *J Clin Microbiol* **44**, 999-1017.

Benton, K. A., Everson, M. P. & Briles, D. E. (1995). A pneumolysin-negative mutant of *Streptococcus pneumoniae* causes chronic bacteremia rather than acute sepsis in mice. *Infect Immun* **63**, 448-455.

Bergeron, Y., Ouellet, N., Deslauriers, A. M., Simard, M., Olivier, M. & Bergeron, M. G. (1998). Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infect Immun* **66**, 912-922.

Bergey, D. H. (1974). *Bergey's manual of determinative bacteriology*, 8th edn. Baltimore: Williams & Wilkins Co.

Berneman, A., Belec, L., Fischetti, V. A. & Bouvet, J. P. (1998). The specificity patterns of human immunoglobulin G antibodies in serum differ from those in autologous secretions. *Infect Immun* **66**, 4163-4168.

Bettinger, J. A., Scheifele, D. W., Kellner, J. D., Halperin, S. A., Vaudry, W., Law, B. & Tyrrell, G. (2010). The effect of routine vaccination on invasive pneumococcal infections in Canadian children, Immunization Monitoring Program, Active 2000-2007. *Vaccine* **28**, 2130-2136.

Black, S., Shinefield, H., Fireman, B., Lewis, E., Ray, P., Hansen, J. R., Elvin, L., Ensor, K. M., Hackell, J., Siber, G., Malinoski, F., Madore, D., Chang, I., Kohberger, R., Watson, W., Austrian, R. & Edwards, K. (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.

Black, S., Shinefield, H., Baxter, R., Austrian, R., Bracken, L., Hansen, J., Lewis, E. & Fireman, B. (2004). Postlicensure surveillance for pneumococcal invasive disease after use of heptavalent pneumococcal conjugate vaccine in Northern California Kaiser Permanente. *Pediatr Infect Dis J* **23**, 485-489.

Black, S. B., Shinefield, H. R., Hansen, J., Elvin, L., Laufer, D. & Malinoski, F. (2001). Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine. *Pediatr Infect Dis J* **20**, 1105-1107.

Blue, C. E., Paterson, G. K., Kerr, A. R., Berge, M., Claverys, J. P. & Mitchell, T. J. (2003). ZmpB, a novel virulence factor of *Streptococcus pneumoniae* that induces tumor necrosis factor alpha production in the respiratory tract. *Infect Immun* **71**, 4925-4935.

Bogaert, D., De Groot, R. & Hermans, P. W. (2004). *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* **4**, 144-154.

Bootsma, H. J., Egmont-Petersen, M. & Hermans, P. W. (2007). Analysis of the *in vitro* transcriptional response of human pharyngeal epithelial cells to adherent *Streptococcus pneumoniae*: evidence for a distinct response to encapsulated strains. *Infect Immun* **75**, 5489-5499.

Bottcher, T., Spreer, A., Azeh, I., Nau, R. & Gerber, J. (2003). Matrix metalloproteinase-9 deficiency impairs host defense mechanisms against *Streptococcus pneumoniae* in a mouse model of bacterial meningitis. *Neurosci Lett* **338**, 201-204.

Briles, D. E., Nahm, M., Schroer, K., Davie, J., Baker, P., Kearney, J. & Barletta, R. (1981). Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. *J Exp Med* **153**, 694-705.

Briles, D. E., Crain, M. J., Gray, B. M., Forman, C. & Yother, J. (1992). Strong association between capsular type and virulence for mice among human isolates of *Streptococcus pneumoniae*. *Infect Immun* **60**, 111-116.

Brown, J. S., Ogunniyi, A. D., Woodrow, M. C., Holden, D. W. & Paton, J. C. (2001). Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. *Infect Immun* **69**, 6702-6706.

Byington, C. L., Samore, M. H., Stoddard, G. J., Barlow, S., Daly, J., Korgenski, K., Firth, S., Glover, D., Jensen, J., Mason, E. O., Shutt, C. K. & Pavia, A. T. (2005). Temporal trends of invasive disease due to *Streptococcus pneumoniae* among children in the intermountain west: emergence of non vaccine serogroups. *Clin Infect Dis* **41**, 21-29.

Calix, J. J. & Nahm, M. H. (2010). A new pneumococcal serotype, 11E, has a variably inactivated wcjE gene. *J Infect Dis* **202**, 29-38.

Canvin, J. R., Marvin, A. P., Sivakumaran, M., Paton, J. C., Boulnois, G. J., Andrew, P. W. & Mitchell, T. J. (1995). The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. *J Infect Dis* **172**, 119-123.

Cao, J., Chen, D., Xu, W., Chen, T., Xu, S., Luo, J., Zhao, Q., Liu, B., Wang, D., Zhang, X., Shan, Y. & Yin, Y. (2007). Enhanced protection against pneumococcal infection elicited by immunization with the combination of PspA, PspC, and ClpP. *Vaccine* **25**, 4996-5005.

Casal, J., Aguilar, L., Jado, I., Yuste, J., Gimenez, M. J., Prieto, J. & Fenoll, A. (2002). Effects of specific antibodies against *Streptococcus pneumoniae* on pharmacodynamic parameters of beta-lactams in a mouse sepsis model. *Antimicrob Agents Chemother* **46**, 1340-1344.

CDC (2000). Preventing pneumococcal disease among infants and young children: recommendations from the Advisory Committee on Immunizations Practices. *MMWR Morb Mortal Wkly Rep* **49**, 1-35.

CDC (2005). Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease: United States, 1998–2003. *MMWR Morb Mortal Wkly Rep* **54**, 893-897.

Chiavolini, D., Pozzi, G. & Ricci, S. (2008). Animal models of *Streptococcus pneumoniae* disease. *Clin Microbiol Rev* **21**, 666-685.

Choo, S., Zhang, Q., Seymour, L., Akhtar, S. & Finn, A. (2000). Primary and booster salivary antibody responses to a 7-valent pneumococcal conjugate vaccine in infants. *J Infect Dis* **182**, 1260-1263.

Claverys, J. P. & Havarstein, L. S. (2007). Cannibalism and fratricide: mechanisms and raisons d'etre. *Nat Rev Microbiol* **5**, 219-229.

Coil, J. A., Dickerman, J. D. & Boulton, E. (1978). Increased susceptibility of splenectomized mice to infection after exposure to an aerosolized suspension of type III *Streptococcus pneumoniae*. *Infect Immun* **21**, 412-416.

Dagan, R., Melamed, R., Muallem, M., Piglansky, L., Greenberg, D., Abramson, O., Mendelman, P. M., Bohidar, N. & Yagupsky, P. (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.

Dagan, R., Muallem, M., Melamed, R., Leroy, O. & Yagupsky, P. (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.

Dagan, R., Zamir, O., Tirosh, N., Belmaker, I., Guy, L., Greenberg, D. & Fraser, D. (2000). Nasopharyngeal carriage of *Streptococcus pneumoniae* in toddlers vaccinated during infancy with an 11-valent pneumococcal vaccine conjugated to diphtheria and tetanus toxoids. In *40th Annu Intersci Conf on Antimicrob Agents Chemother*. Toronto, Canada.

Dagan, R., Sikuler-Cohen, M., Zamir, O., Janco, J., Givon-Lavi, N. & Fraser, D. (2001). Effect of a conjugate pneumococcal vaccine on the occurrence of respiratory infections and antibiotic use in day-care center attendees. *Pediatr Infect Dis J* **20**, 951-958.

Dagan, R. (2002). Immunisation with a pneumococcal 7-valent conjugate vaccine. *Int J Clin Pract* **56**, 287-291.

Dagan, R., Givon-Lavi, N., Zamir, O., Sikuler-Cohen, M., Guy, L., Janco, J., Yagupsky, P. & Fraser, D. (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.

Dagan, R., Givon-Lavi, N., Zamir, O. & Fraser, D. (2003). Effect of a nonavalent conjugate vaccine on carriage of antibiotic-resistant *Streptococcus pneumoniae* in day-care centers. *Pediatr Infect Dis J* **22**, 532-540.

Dagan, R., Givon-Lavi, N., Fraser, D., Lipsitch, M., Siber, G. R. & Kohberger, R. (2005). Serum serotype-specific pneumococcal anticapsular immunoglobulin g concentrations after immunization with a 9-valent conjugate pneumococcal vaccine correlate with nasopharyngeal acquisition of pneumococcus. *J Infect Dis* **192**, 367-376.

Dagan, R. & Klugman, K. P. (2008). Impact of conjugate pneumococcal vaccines on antibiotic resistance. *Lancet Infect Dis* **8**, 785-795.

Dowson, C., E. Tuomanen, T. Mitchel, D. A. Morrison and B. G. Spratt (2004). What Is a Pneumococcus. In *The Pneumococcus*. Edited by E. Tuomanen, T. Mitchel, D. A. Morrison & B. G. Spratt. Washington, DC: ASM Press.

Dworkin, M. S., Ward, J. W., Hanson, D. L., Jones, J. L. & Kaplan, J. E. (2001). Pneumococcal disease among human immunodeficiency virus-infected persons: incidence, risk factors, and impact of vaccination. *Clin Infect Dis* **32**, 794-800.

Echchannaoui, H., Frei, K., Schnell, C., Leib, S. L., Zimmerli, W. & Landmann, R. (2002). Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis* **186**, 798-806.

Eskola, J., Takala, A. K., Kela, E., Pekkanen, E., Kalliokoski, R. & Leinonen, M. (1992). Epidemiology of invasive pneumococcal infections in children in Finland. *Jama* **268**, 3323-3327.

Eskola, J., Kilpi, T., Palmu, A., Jokinen, J., Haapakoski, J., Herva, E., Takala, A., Kayhty, H., Karma, P., Kohberger, R., Siber, G. & Makela, P. H. (2001). Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* **344**, 403-409.

Fedson, D. S., Shapiro, E. D., LaForce, F. M., Mufson, M. A., Musher, D. M., Spika, J. S., Breiman, R. F. & Broome, C. V. (1994). Pneumococcal vaccine after 15 years of use. Another view. *Arch Intern Med* **154**, 2531-2535.

Fedson, D. S. & Musher, D. M. (2004). Pneumococcal polysacharide vaccine. In *Vaccines*, pp. 529-588. Edited by S. A. Plotkin & W. A. Orenstein. W. B. Saunders, Philadelphia, Pa.

Feldman, C. & Klugman, K. (1997). Pneumococcal infections. *Current Opinion in Infectious Diseases* **10**, 109-115.

Felton, L. (1938). Studies on immunizing substances in pneumococci. VII. Response in human beings to antigenic pneumococcal polysacharides, types I and II. *Public Health Rep* **53**, 1855-1877.

Felton, L. D., Kauffmann, G., Prescott, B. & Ottinger, B. (1955). Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides. *J Immunol* **74**, 17-26.

Flannery, B., Schrag, S., Bennett, N. M., Lynfield, R., Harrison, L. H., Reingold, A., Cieslak, P. R., Hadler, J., Farley, M. M., Facklam, R. R., Zell, E. R. & Whitney, C. G.

(2004). Impact of childhood vaccination on racial disparities in invasive *Streptococcus pneumoniae* infections. *Jama* **291**, 2197-2203.

Flannery, B., Heffernan, R. T., Harrison, L. H., Ray, S. M., Reingold, A. L., Hadler, J., Schaffner, W., Lynfield, R., Thomas, A. R., Li, J., Campsmith, M., Whitney, C. G. & Schuchat, A. (2006). Changes in invasive Pneumococcal disease among HIV-infected adults living in the era of childhood pneumococcal immunization. *Ann Intern Med* **144**, 1-9.

Francis, T. & Tillett, W. S. (1930). Cutaneous Reactions in Pneumonia. The Development of Antibodies Following the Intradermal Injection of Type-Specific Polysaccharide. *J Exp Med* **52**, 573-585.

Frazão, N., Brito-Avô, A., Simas, C., Saldanha, J., Mato, R., Nunes, S., Sousa, N. G., Carriço, J. A., Almeida, J. S., Santos-Sanches, I. & de Lencastre, H. (2005). Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon. *Pediatr Infect Dis J* **24**, 243-252.

Frazão, N., Sá-Leão, R., Nunes, S., Sousa, N. G., Brito-Avo, A., Saldanha, J., Mato, R., Santos-Sanches, I. & Lencastre, H. (2007). *Streptococcus pneumoniae* drug resistance in non vaccine serotypes isolated from children vaccinated with the heptavalent conjugate vaccine (PCV7). In *3rd Conference on New Frontiers in Microbiology and Infection*. Villars-sur-Ollon, Switzerland.

Frazão, N., Sá-Leão, R. & de Lencastre, H. (2010). Impact of a single dose of the 7-valent pneumococcal conjugate vaccine on colonization. *Vaccine* **28**, 3445-3452.

Gerber, J., Raivich, G., Wellmer, A., Noeske, C., Kunst, T., Werner, A., Bruck, W. & Nau, R. (2001). A mouse model of *Streptococcus pneumoniae* meningitis mimicking several features of human disease. *Acta Neuropathol* **101**, 499-508.

Gingles, N. A., Alexander, J. E., Kadioglu, A., Andrew, P. W., Kerr, A., Mitchell, T. J., Hopes, E., Denny, P., Brown, S., Jones, H. B., Little, S., Booth, G. C. & McPheat, W. L. (2001). Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. *Infect Immun* **69**, 426-434.

Givon-Lavi, N., Fraser, D. & Dagan, R. (2003). Vaccination of day-care center attendees reduces carriage of *Streptococcus pneumoniae* among their younger siblings. *Pediatr Infect Dis J* **22**, 524-532.

Goebel, W. F. & Adams, M. H. (1943). The Immunological Properties of the Heterophile Antigen and Somatic Polysaccharide of Pneumococcus. *J Exp Med* **77**, 435-449.

Grandgirard, D., Steiner, O., Tauber, M. G. & Leib, S. L. (2007). An infant mouse model of brain damage in pneumococcal meningitis. *Acta Neuropathol* **114**, 609-617.

Gray, B. M., Converse, G. M., 3rd & Dillon, H. C., Jr. (1980). Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* **142**, 923-933.

Greenwood, B. M., Weber, M. W. & Mulholland, K. (2007). Childhood pneumonia--preventing the worlds biggest killer of children. *Bull World Health Organ* **85**, 502-503.

Griffith, F. (1928). The Significance of Pneumococcal Types. *J Hyg (Lond)* **27**, 113-159.

Hammitt, L. L., Bruden, D. L., Butler, J. C., Baggett, H. C., Hurlburt, D. A., Reasonover, A. & Hennessy, T. W. (2006). Indirect effect of conjugate vaccine on adult carriage of *Streptococcus pneumoniae*: an explanation of trends in invasive pneumococcal disease. *J Infect Dis* **193**, 1487-1494.

Hanage, W. P., Huang, S. S., Lipsitch, M., Bishop, C. J., Godoy, D., Pelton, S. I., Goldstein, R., Huot, H. & Finkelstein, J. A. (2007). Diversity and antibiotic resistance among non vaccine serotypes of *Streptococcus pneumoniae* carriage isolates in the post-heptavalent conjugate vaccine era. *J Infect Dis* **195**, 347-352.

Hansen, J., Black, S., Shinefield, H., Cherian, T., Benson, J., Fireman, B., Lewis, E., Ray, P. & Lee, J. (2006). Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than 5 years of age for prevention of pneumonia: updated analysis using World Health Organization standardized interpretation of chest radiographs. *Pediatr Infect Dis J* **25**, 779-781.

Hansman, D. & Bullen, M. (1967). A resistant pneumococcus. *Lancet* **ii**, 264-265.

Hausdorff, W. P., Bryant, J., Kloek, C., Paradiso, P. R. & Siber, G. R. (2000a). 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.

Hausdorff, W. P., Bryant, J., Paradiso, P. R. & Siber, G. R. (2000b). Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* **30**, 100-121.

Hausdorff, W. P., Feikin, D. R. & Klugman, K. P. (2005). Epidemiological differences among pneumococcal serotypes. *Lancet Infect Dis* **5**, 83-93.

Hennessy, T. W., Singleton, R. J., Bulkow, L. R., Bruden, D. L., Hurlburt, D. A., Parks, D., Moore, M., Parkinson, A. J., Schuchat, A. & Butler, J. C. (2005). Impact of heptavalent pneumococcal conjugate vaccine on invasive disease, antimicrobial

resistance and colonization in Alaska Natives: progress towards elimination of a health disparity. *Vaccine* **23**, 5464-5473.

Henrichsen, J. (1995). Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol* **33**, 2759-2762.

Henrichsen, J. (1999). Typing of *Streptococcus pneumoniae*: past, present, and future. *Am J Med* **107**, 50S-54S.

Henriques-Normark, B. & Normark, S. (2010). Commensal pathogens, with a focus on *Streptococcus pneumoniae*, and interactions with the human host. *Exp Cell Res* **316**, 1408-1414.

Hicks, L. A., Harrison, L. H., Flannery, B., Hadler, J. L., Schaffner, W., Craig, A. S., Jackson, D., Thomas, A., Beall, B., Lynfield, R., Reingold, A., Farley, M. M. & Whitney, C. G. (2007). Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998-2004. *J Infect Dis* **196**, 1346-1354.

Hill, P. C., Cheung, Y. B., Akisanya, A., Sankareh, K., Lahai, G., Greenwood, B. M. & Adegbola, R. A. (2008). Nasopharyngeal carriage of *Streptococcus pneumoniae* in Gambian infants: a longitudinal study. *Clin Infect Dis* **46**, 807-814.

Hogberg, L., Geli, P., Ringberg, H., Melander, E., Lipsitch, M. & Ekdahl, K. (2007). Age- and serogroup-related differences in observed durations of nasopharyngeal carriage of penicillin-resistant pneumococci. *J Clin Microbiol* **45**, 948-952.

Hsu, K., Pelton, S., Karumuri, S., Heisey-Grove, D. & Klein, J. (2005). Population-based surveillance for childhood invasive pneumococcal disease in the era of conjugate vaccine. *Pediatr Infect Dis J* **24**, 17-23.

Huang, S. S., Platt, R., Rifas-Shiman, S. L., Pelton, S. I., Goldmann, D. & Finkelstein, J. A. (2005). Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. *Pediatrics* **116**, e408-413.

Hyams, C., Camberlein, E., Cohen, J. M., Bax, K. & Brown, J. S. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun* **78**, 704-715.

Iannelli, F., Pearce, B. J. & Pozzi, G. (1999). The type 2 capsule locus of *Streptococcus pneumoniae*. *J Bacteriol* **181**, 2652-2654.

Iannelli, F., Chiavolini, D., Ricci, S., Oggioni, M. R. & Pozzi, G. (2004). Pneumococcal surface protein C contributes to sepsis caused by *Streptococcus pneumoniae* in mice. *Infect Immun* **72**, 3077-3080.

Iizawa, Y., Kitamoto, N., Hiroe, K. & Nakao, M. (1996). *Streptococcus pneumoniae* in the nasal cavity of mice causes lower respiratory tract infection after airway obstruction. *J Med Microbiol* **44**, 490-495.

Iizawa, Y., Hiroe, K., Nakao, M. & Okonogi, K. (1998). Therapeutic efficacy of cefozopran in a murine model of haematogenous pneumococcal meningitis. *Chemotherapy* **44**, 265-271.

Iwasaki, K., Ohrui, T., Wang, Q., Sekizawa, K., Akaike, T., Maeda, H. & Sasaki, H. (1999). Combined effects of both bacteria and gastric juice on pneumonia in mice. *Respir Physiol* **116**, 201-209.

Jacobs, M. R., Koornhof, H. J., Robins-Browne, R. M., Stevenson, C. M., Vermaak, Z. A., Freiman, I., Miller, G. B., Witcomb, M. A., Isaacson, M., Ward, J. I. & Austrian, R. (1978). Emergence of multiply resistant pneumococci. *N Engl J Med* **299**, 735-740.

Jin, P., Kong, F., Xiao, M., Oftadeh, S., Zhou, F., Liu, C., Russell, F. & Gilbert, G. L. (2009). First report of putative *Streptococcus pneumoniae* serotype 6D among nasopharyngeal isolates from Fijian children. *J Infect Dis* **200**, 1375-1380.

Jones, V. F., Harrison, C., Stout, G. G. & Hopkins, J. (2005). Nasopharyngeal colonization with heptavalent pneumococcal conjugate vaccine serotypes of *Streptococcus pneumoniae* with prolonged vaccine dosing intervals. *Pediatr Infect Dis J* **24**, 969-973.

Kaplan, S. L. & Mason, E. O., Jr. (2002). Mechanisms of pneumococcal antibiotic resistance and treatment of pneumococcal infections in 2002. *Pediatr Ann* **31**, 250-260.

Kaplan, S. L., Mason, E. O., Jr., Wald, E. R., Schutze, G. E., Bradley, J. S., Tan, T. Q., Hoffman, J. A., Givner, L. B., Yogev, R. & Barson, W. J. (2004). Decrease of invasive pneumococcal infections in children among 8 children's hospitals in the United States after the introduction of the 7-valent pneumococcal conjugate vaccine. *Pediatrics* **113**, 443-449.

Kauppi, M., Eskola, J. & Kayhty, H. (1995). Anti-capsular polysaccharide antibody concentrations in saliva after immunization with Haemophilus influenzae type b conjugate vaccines. *Pediatr Infect Dis J* **14**, 286-294.

Kellner, J. D., Church, D. L., MacDonald, J., Tyrrell, G. J. & Scheifele, D. (2005). Progress in the prevention of pneumococcal infection. *Cmaj* **173**, 1149-1151.

Kilpi, T., Syrjanen, R., Palmu, A., Herva, E., Eskola, J., Makela, P. H. & Group, F. s. (2001). Parallel evaluation of the effect of a 7-valent pneumococcal conjugate vaccine (PncCRM) on pneumococcal (Pnc) carriage and acute otitis media (AOM). In *19th Annu Meet Eur Soc Paediatr Infect Dis*, pp. p. 4. Istanbul, Turkey.

Kim, J. O. & Weiser, J. N. (1998). Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J Infect Dis* **177**, 368-377.

Kim, J. O., Romero-Steiner, S., Sorensen, U. B., Blom, J., Carvalho, M., Barnard, S., Carlone, G. & Weiser, J. N. (1999). Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of *Streptococcus pneumoniae*. *Infect Immun* **67**, 2327-2333.

Klugman, K. P. (1990). Pneumococcal resistance to antibiotics. *Clin Microbiol Rev* **3**, 171-196.

Klugman, K. P., Madhi, S. A., Huebner, R. E., Kohberger, R., Mbelle, N. & Pierce, N. (2003). A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *N Engl J Med* **349**, 1341-1348.

Koedel, U., Paul, R., Winkler, F., Kastenbauer, S., Huang, P. L. & Pfister, H. W. (2001). Lack of endothelial nitric oxide synthase aggravates murine pneumococcal meningitis. *J Neuropathol Exp Neurol* **60**, 1041-1050.

Koedel, U., Scheld, W. M. & Pfister, H. W. (2002). Pathogenesis and pathophysiology of pneumococcal meningitis. *Lancet Infect Dis* **2**, 721-736.

Korkeila, M., Lehtonen, H., Ahman, H., Leroy, O., Eskola, J. & Kayhty, H. (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.

Kostyukova, N. N., Volkova, M. O., Ivanova, V. V. & Kvetnaya, A. S. (1995). A study of pathogenic factors of *Streptococcus pneumoniae* strains causing meningitis. *FEMS Immunol Med Microbiol* **10**, 133-137.

Kristinsson, K. G. (1997). Effect of antimicrobial use and other risk factors on antimicrobial resistance in pneumococci. *Microb Drug Resist* **3**, 117-123.

Kyaw, M. H., Lynfield, R., Schaffner, W., Craig, A. S., Hadler, J., Reingold, A., Thomas, A. R., Harrison, L. H., Bennett, N. M., Farley, M. M., Facklam, R. R., Jorgensen, J. H., Besser, J., Zell, E. R., Schuchat, A. & Whitney, C. G. (2006). Effect of introduction of the pneumococcal conjugate vaccine on drug resistant *Streptococcus pneumoniae*. *N Engl J Med* **354**, 1455-1463.

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

Lipsitch, M., O'Neill, K., Cordy, D., Bugalter, B., Trzcinski, K., Thompson, C. M., Goldstein, R., Pelton, S., Huot, H., Bouchet, V., Reid, R., Santosham, M. & O'Brien, K. L. (2007). Strain characteristics of *Streptococcus pneumoniae* carriage and invasive disease isolates during a cluster-randomized clinical trial of the 7-valent pneumococcal conjugate vaccine. *J Infect Dis* **196**, 1221-1227.

Loeffler, J. M., Djurkovic, S. & Fischetti, V. A. (2003). Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. *Infect Immun* **71**, 6199-6204.

MacArthur, C. J., Hefeneider, S. H., Kempton, J. B., Parrish, S. K., McCoy, S. L. & Trune, D. R. (2006). Evaluation of the mouse model for acute otitis media. *Hear Res* **219**, 12-23.

Macleod, C. M. & Krauss, M. R. (1950). Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed *in vitro*. *J Exp Med* **92**, 1-9.

Magee, A. D. & Yother, J. (2001). Requirement for capsule in colonization by *Streptococcus pneumoniae*. *Infect Immun* **69**, 3755-3761.

Makela, P. H. & Butler, J. (2008). History of Pneumococcal Immunization. In *Pneumococcal Vaccines: the Impact of Conjugate Vaccine*, pp. 19-29. Edited by G. Siber, K. Klugman & P. H. Makela. Washington, DC: ASM.

Malley, R. & Weiser, J. N. (2008). Animal models of pneumococcal colonization. In *Pneumococcal vaccines: the impact of conjugate vaccine*, pp. 59-66. Edited by G. R. Siber, K. Klugman & P. H. Makela. Washington, DC: ASM Press.

Marra, A. & Brigham, D. (2001). *Streptococcus pneumoniae* causes experimental meningitis following intranasal and otitis media infections via a nonhematogenous route. *Infect Immun* **69**, 7318-7325.

Mato, R., Sanches, I. S., Simas, C., Nunes, S., Carrico, J. A., Sousa, N. G., Frazao, N., Saldanha, J., Brito-Avo, A., Almeida, J. S. & Lencastre, H. D. (2005). Natural history of drug resistant clones of *Streptococcus pneumoniae* colonizing healthy children in Portugal. *Microb Drug Resist* **11**, 309-322.

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

McCool, T. L. & Weiser, J. N. (2004). Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. *Infect Immun* **72**, 5807-5813.

McCoy, S. L., Kurtz, S. E., Macarthur, C. J., Trune, D. R. & Hefeneider, S. H. (2005). Identification of a peptide derived from vaccinia virus A52R protein that inhibits cytokine

secretion in response to TLR-dependent signaling and reduces *in vivo* bacterial-induced inflammation. *J Immunol* **174**, 3006-3014.

McCullers, J. A., Karlstrom, A., Iverson, A. R., Loeffler, J. M. & Fischetti, V. A. (2007). Novel strategy to prevent otitis media caused by colonizing *Streptococcus pneumoniae*. *PLoS Pathog* **3**, e28.

McGee, L., McDougal, L., Zhou, J., Spratt, B. G., Tenover, F. C., George, R., Hakenbeck, R., Hryniewicz, W., Lefevre, J. C., Tomasz, A. & Klugman, K. P. (2001). Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. *J Clin Microbiol* **39**, 2565-2571.

Melhus, A. & Ryan, A. F. (2003). A mouse model for acute otitis media. *Apmis* **111**, 989-994.

Millar, E. V., O'Brien, K. L., Watt, J. P., Bronsdon, M. A., Dallas, J., Whitney, C. G., Reid, R. & Santosham, M. (2006). Effect of community-wide conjugate pneumococcal vaccine use in infancy on nasopharyngeal carriage through 3 years of age: a cross-sectional study in a high-risk population. *Clin Infect Dis* **43**, 8-15.

Mohler, J., Azoulay-Dupuis, E., Amory-Rivier, C., Mazoit, J. X., Bedos, J. P., Rieux, V. & Moine, P. (2003). *Streptococcus pneumoniae* strain-dependent lung inflammatory responses in a murine model of pneumococcal pneumonia. *Intensive Care Med* **29**, 808-816.

Moore, M. R., Hyde, T. B., Hennessy, T. W., Parks, D. J., Reasonover, A. L., Harker-Jones, M., Gove, J., Bruden, D. L., Rudolph, K., Parkinson, A., Butler, J. C. & Schuchat, A. (2004). Impact of a conjugate vaccine on community-wide carriage of nonsusceptible *Streptococcus pneumoniae* in Alaska. *J Infect Dis* **190**, 2031-2038.

Mulholland, K. (2007). Childhood pneumonia mortality--a permanent global emergency. *Lancet* **370**, 285-289.

Munoz-Almagro, C., Jordan, I., Gene, A., Latorre, C., Garcia-Garcia, J. J. & Pallares, R. (2008). Emergence of invasive pneumococcal disease caused by non vaccine serotypes in the era of 7-valent conjugate vaccine. *Clin Infect Dis* **46**, 174-182.

Murray, P. (1999). *Manual of Clinical Microbiology.* Washington, D. C.: American Society for Microbiology.

Musher, D. M. (1992). Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity, and treatment. *Clin Infect Dis* **14**, 801-807.

Nau, R., Wellmer, A., Soto, A., Koch, K., Schneider, O., Schmidt, H., Gerber, J., Michel, U. & Bruck, W. (1999). Rifampin reduces early mortality in experimental *Streptococcus pneumoniae* meningitis. *J Infect Dis* **179**, 1557-1560.

Nelson, A. L., Roche, A. M., Gould, J. M., Chim, K., Ratner, A. J. & Weiser, J. N. (2007). Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect Immun* **75**, 83-90.

Nieminen, T., Kayhty, H., Leroy, O. & Eskola, J. (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.

Nuermberger, E., Helke, K. & Bishai, W. R. (2005). Low-dose aerosol model of pneumococcal pneumonia in the mouse: utility for evaluation of antimicrobial efficacy. *Int J Antimicrob Agents* **26**, 497-503.

Nuorti, J. P., Butler, J. C., Gelling, L., Kool, J. L., Reingold, A. L. & Vugia, D. J. (2000). Epidemiologic relation between HIV and invasive pneumococcal disease in San Francisco County, California. *Ann Intern Med* **132**, 182-190.

Nurkka, A., Ahman, H., Korkeila, M., Jantti, V., Kayhty, H. & Eskola, J. (2001a). Serum and salivary anti-capsular antibodies in infants and children immunized with the heptavalent pneumococcal conjugate vaccine. *Pediatr Infect Dis J* **20**, 25-33.

Nurkka, A., Ahman, H., Yaich, M., Eskola, J. & Kayhty, H. (2001b). Serum and salivary anti-capsular antibodies in infants and children vaccinated with octavalent pneumococcal conjugate vaccines, PncD and PncT. *Vaccine* **20**, 194-201.

Obaro, S. K., Adegbola, R. A., Banya, W. A. & Greenwood, B. M. (1996). Carriage of pneumococci after pneumococcal vaccination. *Lancet* **348**, 271-272.

O'Brien, K. L. & Santosham, M. (2004). Potential impact of conjugate pneumococcal vaccines on pediatric pneumococcal diseases. *Am J Epidemiol* **159**, 634-644.

O'Brien, K. L., Millar, E. V., Zell, E. R., Bronsdon, M., Weatherholtz, R., Reid, R., Becenti, J., Kvamme, S., Whitney, C. G. & Santosham, M. (2007). Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. *J Infect Dis* **196**, 1211-1220.

O'Brien, K. L., Wolfson, L. J., Watt, J. P., Henkle, E., Deloria-Knoll, M., McCall, N., Lee, E., Mulholland, K., Levine, O. S. & Cherian, T. (2009). Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* **374**, 893-902.

Oftadeh, S., Satzke, C. & Gilbert, G. L. (2010). Identification of the newly described *Streptococcus pneumoniae* serotype 6D using Quellung reaction and PCR. *J Clin Microbiol*.

Ogra, P. L. (2000). Mucosal immune response in the ear, nose and throat. *Pediatr Infect Dis J* **19**, S4-8.

Ogunniyi, A. D., LeMessurier, K. S., Graham, R. M., Watt, J. M., Briles, D. E., Stroeher, U. H. & Paton, J. C. (2007). Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. *Infect Immun* **75**, 1843-1851.

Pai, R., Moore, M. R., Pilishvili, T., Gertz, R. E., Whitney, C. G. & Beall, B. (2005). Postvaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States. *J Infect Dis* **192**, 1988-1995.

Park, I. H., Pritchard, D. G., Cartee, R., Brandao, A., Brandileone, M. C. & Nahm, M. H. (2007). Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. *J Clin Microbiol* **45**, 1225-1233.

Pasteur, L. (1881). Note sur la maladie nouvelle provoqueé par la salive d'un enfant mort de la rage. *Comptes Rendus* **92**, 159-165.

Paton, J. C., Andrew, P. W., Boulnois, G. J. & Mitchell, T. J. (1993). Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. *Annu Rev Microbiol* **47**, 89-115.

Pilishvili, T., Lexau, C., Farley, M. M., Hadler, J., Harrison, L. H., Bennett, N. M., Reingold, A., Thomas, A., Schaffner, W., Craig, A. S., Smith, P. J., Beall, B. W., Whitney, C. G. & Moore, M. R. (2010). Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* **201**, 32-41.

Poehling, K. A., Talbot, T. R., Griffin, M. R., Craig, A. S., Whitney, C. G., Zell, E., Lexau, C. A., Thomas, A. R., Harrison, L. H., Reingold, A. L., Hadler, J. L., Farley, M. M., Anderson, B. J. & Schaffner, W. (2006). Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. *Jama* **295**, 1668-1674.

Porat, N., Barkai, G., Jacobs, M. R., Trefler, R. & Dagan, R. (2004). Four antibiotic-resistant *Streptococcus pneumoniae* clones unrelated to the pneumococcal conjugate vaccine serotypes, including 2 new serotypes, causing acute otitis media in southern Israel. *J Infect Dis* **189**, 385-392.

Quin, L. R., Moore, Q. C., 3rd & McDaniel, L. S. (2007). Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. *Infect Immun* **75**, 2067-2070.

Rinta-Kokko, H., Dagan, R., Givon-Lavi, N. & Auranen, K. (2009). Estimation of vaccine efficacy against acquisition of pneumococcal carriage. *Vaccine* **27**, 3831-3837.

Robbins, J. B., Austrian, R., Lee, C. J., Rastogi, S. C., Schiffman, G., Henrichsen, J., Makela, P. H., Broome, C. V., Facklam, R. R., Tiesjema, R. H. & et al. (1983). Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. *J Infect Dis* **148**, 1136-1159.

Rosenow, C., Ryan, P., Weiser, J. N., Johnson, S., Fontan, P., Ortqvist, A. & Masure, H. R. (1997). Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol Microbiol* **25**, 819-829.

Rubins, J. B., Charboneau, D., Fasching, C., Berry, A. M., Paton, J. C., Alexander, J. E., Andrew, P. W., Mitchell, T. J. & Janoff, E. N. (1996). Distinct roles for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia. *Am J Respir Crit Care Med* **153**, 1339-1346.

Ryan, A. F., Ebmeyer, J., Furukawa, M., Pak, K., Melhus, A., Wasserman, S. I. & Chung, W. H. (2006). Mouse models of induced otitis media. *Brain Res* **1091**, 3-8.

Sabirov, A. & Metzger, D. W. (2006). Intranasal vaccination of neonatal mice with polysaccharide conjugate vaccine for protection against pneumococcal otitis media. *Vaccine* **24**, 5584-5592.

Sabirov, A. & Metzger, D. W. (2008). Mouse models for the study of mucosal vaccination against otitis media. *Vaccine* **26**, 1501-1524.

Sá-Leão, R., Tomasz, A., Santos Sanches, I. & de Lencastre, H. (2002). Pilot study of the genetic diversity of the pneumococcal nasopharyngeal flora among children attending day-care centers. *J Clin Microbiol* **40**, 3577-3585.

Sá-Leão, R., Nunes, S., Brito-Avô, A., Frazão, N., Simões, A. S., Crisóstomo, M. I., Paulo, A. C., Saldanha, J., Santos-Sanches, I. & de Lencastre, H. (2009). Changes in pneumococcal serotypes and antibiotypes carried by vaccinated and unvaccinated day-care centre attendees in Portugal, a country with widespread use of the seven-valent pneumococcal conjugate vaccine. *Clin Microbiol Infect* **15**, 1002-1007.

Sankilampi, U., Isoaho, R., Bloigu, A., Kivela, S. L. & Leinonen, M. (1997). Effect of age, sex and smoking habits on pneumococcal antibodies in an elderly population. *Int J Epidemiol* **26**, 420-427.

Scott, J. A., Hall, A. J., Dagan, R., Dixon, J. M., Eykyn, S. J., Fenoll, A., Hortal, M., Jette, L. P., Jorgensen, J. H., Lamothe, F., Latorre, C., Macfarlane, J. T., Shlaes, D. M., Smart, L. E. & Taunay, A. (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.

Shapiro, M. A., Donovan, K. D. & Gage, J. W. (2000). Comparative therapeutic efficacy of clinafloxacin in a pneumococcal meningitis mouse model. *J Antimicrob Chemother* **45**, 489-492.

Siber, G. R. (1994). Pneumococcal disease: prospects for a new generation of vaccines. *Science* **265**, 1385-1387.

Sorensen, U. B. (1995). Pneumococcal polysaccharide antigens: capsules and C-polysaccharide. An immunochemical study. *Dan Med Bull* **42**, 47-53.

Sternberg, G. M. (1881). A fatal form of septicaemia in the rabbit, produced by the subcutaneous injection of human saliva. *Natl Board of Health Bull* **2**, 781-783.

Stol, K., van Selm, S., van den Berg, S., Bootsma, H. J., Blokx, W. A., Graamans, K., Tonnaer, E. L. & Hermans, P. W. (2009). Development of a non-invasive murine infection model for acute otitis media. *Microbiology* **155**, 4135-4144.

Swiatlo, E., King, J., Nabors, G. S., Mathews, B. & Briles, D. E. (2003). Pneumococcal surface protein A is expressed *in vivo*, and antibodies to PspA are effective for therapy in a murine model of pneumococcal sepsis. *Infect Immun* **71**, 7149-7153.

Takashima, K., Tateda, K., Matsumoto, T., Iizawa, Y., Nakao, M. & Yamaguchi, K. (1997). Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice. *Infect Immun* **65**, 257-260.

Tan, T. Q., Smith, C. W., Hawkins, E. P., Mason, E. O., Jr. & Kaplan, S. L. (1995). Hematogenous bacterial meningitis in an intercellular adhesion molecule-1-deficient infant mouse model. *J Infect Dis* **171**, 342-349.

Tasaka, S., Richer, S. E., Mizgerd, J. P. & Doerschuk, C. M. (2002). Very late antigen-4 in CD18-independent neutrophil emigration during acute bacterial pneumonia in mice. *Am J Respir Crit Care Med* **166**, 53-60.

Tillett, W. S., Cambier, M. J. & McCormack, J. E. (1944). The Treatment of Lobar Pneumonia and Pneumococcal Empyema with Penicillin. *Bull N Y Acad Med* **20**, 142-178.

Tomasz, A. (1965). Control of the competent state in Pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria. *Nature* **208**, 155-159.

Tsao, N., Chang, W. W., Liu, C. C. & Lei, H. Y. (2002). Development of hematogenous pneumococcal meningitis in adult mice: the role of TNF-alpha. *FEMS Immunol Med Microbiol* **32**, 133-140.

van Gils, E. J., Veenhoven, R. H., Hak, E., Rodenburg, G. D., Bogaert, D., Ijzerman, E. P., Bruin, J. P., van Alphen, L. & Sanders, E. A. (2009). Effect of reduced-dose schedules with 7-valent pneumococcal conjugate vaccine on nasopharyngeal pneumococcal carriage in children: a randomized controlled trial. *Jama* **302**, 159-167.

van Ginkel, F. W., McGhee, J. R., Watt, J. M., Campos-Torres, A., Parish, L. A. & Briles, D. E. (2003). Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection. *Proc Natl Acad Sci U S A* **100**, 14363-14367.

van Rossum, A. M., Lysenko, E. S. & Weiser, J. N. (2005). Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. *Infect Immun* **73**, 7718-7726.

Veenhoven, R., Bogaert, D., Uiterwaal, C., Brouwer, C., Kiezebrink, H., Bruin, J., E, I. J., Hermans, P., de Groot, R., Zegers, B., Kuis, W., Rijkers, G., Schilder, A. & Sanders, E. (2003). Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. *Lancet* **361**, 2189-2195.

Wang, E., Simard, M., Ouellet, N., Bergeron, Y., Beauchamp, D. & Bergeron, M. G. (2000). Modulation of cytokines and chemokines, limited pulmonary vascular bed permeability, and prevention of septicemia and death with ceftriaxone and interleukin-10 in pneumococcal pneumonia. *J Infect Dis* **182**, 1255-1259.

Wang, E., Ouellet, N., Simard, M., Fillion, I., Bergeron, Y., Beauchamp, D. & Bergeron, M. G. (2001). Pulmonary and systemic host response to *Streptococcus pneumoniae* and Klebsiella pneumoniae bacteremia in normal and immunosuppressed mice. *Infect Immun* **69**, 5294-5304.

Wang, E., Bergeron, Y. & Bergeron, M. G. (2005). Ceftriaxone pharmacokinetics in interleukin-10-treated murine pneumococcal pneumonia. *J Antimicrob Chemother* **55**, 721-726.

Wartha, F., Beiter, K., Albiger, B., Fernebro, J., Zychlinsky, A., Normark, S. & Henriques-Normark, B. (2007). Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell Microbiol* **9**, 1162-1171.

Wellmer, A., Noeske, C., Gerber, J., Munzel, U. & Nau, R. (2000). Spatial memory and learning deficits after experimental pneumococcal meningitis in mice. *Neurosci Lett* **296**, 137-140.

White, B., Robinson, E. S. & Barnes., L. A. (1938). *The biology of pneumococcus.* Cambridge, Massachusetts: Commonwealth Fund, Harvard University Press.

Whitney, C. G., Farley, M. M., Hadler, J., Harrison, L. H., Lexau, C., Reingold, A., Lefkowitz, L., Cieslak, P. R., Cetron, M., Zell, E. R., Jorgensen, J. H. & Schuchat, A. (2000). Increasing prevalence of multidrug resistant *Streptococcus pneumoniae* in the United States. *N Engl J Med* **343**, 1917-1924.

Whitney, C. G., Farley, M. M., Hadler, J., Harrison, L. H., Bennett, N. M., Lynfield, R., Reingold, A., Cieslak, P. R., Pilishvili, T., Jackson, D., Facklam, R. R., Jorgensen, J. H. & Schuchat, A. (2003). Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* **348**, 1737-1746.

WHO (1999). Pneumococcal vaccines. *Wkly Epidemiol Rec* **74**, 177-183.

Williams, B. G., Gouws, E., Boschi-Pinto, C., Bryce, J. & Dye, C. (2002). Estimates of world-wide distribution of child deaths from acute respiratory infections. *Lancet Infect Dis* **2**, 25-32.

Wu, H. Y., Virolainen, A., Mathews, B., King, J., Russell, M. W. & Briles, D. E. (1997). Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb Pathog* **23**, 127-137.

Zwijnenburg, P. J., van der Poll, T., Florquin, S., van Deventer, S. J., Roord, J. J. & van Furth, A. M. (2001). Experimental pneumococcal meningitis in mice: a model of intranasal infection. *J Infect Dis* **183**, 1143-1146.

Chapter II

2. Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon.

Published by Nelson Frazão, A. Brito-Avô, C. Simas, J. Saldanha, R. Mato, S. Nunes, N. G. Sousa, J. A. Carriço, J. S. Almeida, I. Santos-Sanches, and H. de Lencastre *in* Pediatric Infectious Disease Journal (2005); 24:243-52.

2.1. Abstract

Aims: Prospective study to evaluate the impact of the 7-valent pneumococcal conjugate vaccine (Prevenar) on the nasopharyngeal (NP) carriage of drug resistant *Streptococcus pneumoniae* (DRPn), by healthy children attending day-care centers (ages 6 months–6 years). **Methods**: Vaccinees (238 children) who received vaccine and controls (457 children) were followed for carriage of total *S. pneumoniae* and DRPn and for the serotypes and genetic backgrounds of DRPn during 6 consecutive sampling periods between May 2001 and February 2003. **Results**: We detected no significant differences between vaccinees and the control group in the total carriage rate of Pn (average, 68%) or in the frequency of carriage of DRPn (average, 38%), including the frequency of penicillin nonsusceptible strains (average, 24%). In contrast, there was a decline in the carriage of DRPn with vaccine serotypes, which was compensated by the appearance and gradual increase in the frequency of DRPn expressing unusual serotypes (6A, 10A, 15A and 15C, 19A, 23A, 33F), which were not present in the vaccine as well as an increase in non typeable strains. The majority of the DRPn with unusual serotypes showed different pulsed-field gel electrophoresis patterns indicating replacement of the original resistant flora by other clonal types of drug resistant bacteria. Antibiotic consumption and the frequency of respiratory tract infections were similar among the vaccinees and controls. **Conclusions**: Pneumococcal vaccination did not change the frequency of carriage of drug resistant strains, being the initially dominant vaccine serotypes replaced by others expressing non vaccine serotypes. Reduction in the carriage of DRPn may require a combination of the conjugate vaccine and a decrease in antibiotic pressure.

2.2. Introduction

The main reservoir of *Streptococcus pneumoniae* is the nasopharynx of young children (Aniansson *et al.*, 1992; Austrian, 1986). When the balance between host and pathogen

is disturbed, the pneumococcus (Pn) can spread to adjacent mucosal tissues to cause infections such as acute otitis media (AOM) and pneumonia or enter the bloodstream causing invasive infections such as sepsis and meningitis (Bogaert *et al.*, 2004a). Young children attending day-care centers (DCCs) commonly carry pneumococci, especially drug resistant strains that can easily be transmitted by person-to-person contacts in the environment of DCCs where antibiotic use is frequent and leads to selection for resistant strains (Kristinsson, 1997; Munford & Murphy, 1994; Reichler *et al.*, 1992).

β-Lactam antibiotics have been the most commonly used antimicrobials for treatment of pneumococcal disease. Since the mid-1960s, the prevalence of penicillin resistant strains increased worldwide complicating disease management (Appelbaum, 1992) and providing an incentive to address prevention of pneumococcal disease through immunization.

Pneumococci comprise more than 90 serotypes defined by the capsular polysaccharide (Hausdorff *et al.*, 2000b). The pneumococcal conjugate vaccine, Prevenar, is a 7-valent formulation that includes polysaccharides of types 4, 6B, 9V, 14, 18C, 19F and 23F linked to the nontoxic diphtheria variant protein carrier CRM_{197} (7-valent pneumococcal conjugate vaccine). The vaccine was introduced in the United States in 2000 (CDC, 2000) and became available in Portugal in July 2001. This conjugated protein carrier, CRM_{197}, elicits a T-cell dependent response, making it immunogenic and efficacious in children younger than 2 years (Black *et al.*, 2000; Eskola *et al.*, 2001).

In clinical trials, the conjugate vaccine induced high concentrations of serum anticapsular antibodies (Shinefield *et al.*, 1999) and reduced nasopharyngeal carriage of serotypes included in the vaccine [vaccine types (VT)] (Dagan *et al.*, 1996a; Dagan *et al.*, 1997; Dagan & Fraser, 2000; Dagan, 2002; Dagan *et al.*, 2002; Dagan *et al.*, 2003).

Conjugate vaccines are efficacious against invasive disease and modestly beneficial against acute otitis media (Black *et al.*, 2000; Black & Shinefield, 2002; Eskola *et al.*, 2001). Antibacterial-resistant strains of pneumococci most often are serotypes included in the 7-valent pneumococcal conjugate vaccine (Joloba *et al.*, 2001; Wuorimaa &

Kayhty, 2002). Vaccination has a direct effect on reducing carriage of drug resistant pneumococci (DRPn) and an indirect effect on preventing dissemination of resistant strains in the community (Black *et al.*, 2001; Givon-Lavi *et al.*, 2003; O'Brien & Dagan, 2003). Children protected through vaccination against VT strains are more likely to carry non vaccine type (NVT) strains (Lipsitch, 2001). The capacity of these NVT pneumococci to acquire antimicrobial resistance traits and cause disease is not known.

The purposes of our study conducted between 2001 and 2003 were to evaluate the impact of the 7-valent conjugate vaccine on the nasopharyngeal carriage of drug susceptible and drug resistant pneumococci and to evaluate the serotypes, drug resistance pattern and genetic backgrounds of drug resistant pneumococci colonizing healthy children attending DCCs in the Lisbon area of Portugal.

2.3. Materials and Methods

Population. The target population consisted of 695 healthy children 6 months-6 years old attending 8 DCCs in the Lisbon area of Portugal. The DCCs were selected to reflect the demography of the community. DCCs were divided into 2 groups: an intervention group (vaccinees) with 238 children in 5 DCCs; and a control group with 457 children in 3 DCCs. Written and signed informed consent for vaccination and sampling procedures was obtained from parents or guardians of all participating children. The absolute number of samples in the intervention group from the first to the sixth sampling period (2003) decreased to about one-half, because every year older children who had been vaccinated in the first period (2001) left the DCC and consequently could not be followed.

Study design. This prospective study was conducted between 2001 and 2003 (3-year surveillance period). The vaccination was performed in the intervention group (vaccinees) in May, June and November 2001. The nasopharyngeal (NP) flora of the vaccinees was analyzed during 6 sampling periods: in May, June and November 2001,

immediately before the vaccine dose administration; and afterward in February and November 2002 and February 2003. Children of the control group were sampled for pneumococcal carriage during the same sampling periods except in the second period (June 2001) when they were not sampled.

Antibiotic consumption and numbers of episodes of respiratory tract infections (RTI) were evaluated through questionnaires filled out by parents or guardians before each sampling period.

All Pn isolates were tested for antimicrobial resistance and all further characterizations were restricted to drug resistant *S. pneumoniae* (DRPn) strains. All data including antimicrobial consumption, frequency of RTI as well as serotype and genetic data of *S. pneumoniae* isolates were introduced into a database of special design (Silva *et al.*, 2003) for further analysis.

Vaccine administration. The 7-valent pneumococcal conjugate vaccine, Prevenar, was provided by Wyeth Lederle Portugal (Farma), Lda. According to the specifications of the manufacturer, the vaccine contains 2 µg of each of the polysaccharides of serotypes 4, 9V, 14, 18C, 19F and 23F and 4 µg of 6B coupled to the nontoxic diphtheria-toxin analogue CRM_{197} and absorbed on aluminum phosphate. Infants 6 –11 months of age received 3 vaccine doses, children 12–24 months of age received 2 doses and children older than 2 years received a single dose as recommended by the vaccine manufacturer. The 238 children enrolled in the intervention group received a total number of 305 doses of vaccine during the 3 vaccination periods. Intramuscular injection of 0.5 mL of vaccine was performed by a pediatric nurse in the deltoid muscle of the upper arm of each child.

Specimen collection and identification. Sampling was performed by trained pediatric nurses. A single nasopharyngeal flexible swab (BBL Culture Swab; Becton-Dickinson, Sparks, MD) was inserted through each child's nostril until it touched for a few seconds the posterior wall of the nasopharynx. The swab was removed, then introduced into the transport medium (Stuart medium) and transported at ambient temperature to the

Laboratory of Molecular Genetics at Instituto de Tecnologia Química e Biológica. Bacterial samples were processed within 4 hours after arrival at the laboratory as described previously (de Lencastre *et al.*, 1999). Single colonies streaked on blood agar plates and identified as *S. pneumoniae* were grown in tryptic soy agar (Difco, Detroit, MI), and isolates were frozen and stored at -70℃ (de Lencastre *et al.*, 1999).

Antimicrobial susceptibility testing. All Pn strains were tested by the Kirby-Bauer technique, according to the National Committee for Clinical Laboratory Standards recommendations (NCCLS, 2000), against oxacillin, chloramphenicol, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole and levofloxacin (Becton-Dickinson). Minimum inhibitory concentrations (MICs) of penicillin and ceftriaxone were determined by *E*-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations.

Pneumococcal strains resistant to at least one of the antimicrobial agents tested were designated as DRPn. DRPn (except strains resistant only to oxacillin and/or tri-methoprim-sulfamethoxazole) were further characterized for serotype and genetic background [pulsed-field gel electrophoresis (PFGE) pattern].

Serotyping. DRPn were serotyped by the Quellung reaction with the use of commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark) (Sorensen, 1993).

Strains that gave negative or positive reaction for agglutination with all the pooled sera were named non typeable (NT). Pn strains with serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, included in the vaccine, were defined as VT strains. Strains with other serotypes, were defined as non vaccine type (NVT) strains.

PFGE. PFGE was performed after digestion of genomic DNA with *Sma*I using Pn strain R6 and PFGE λ marker (New England Biolabs) as molecular weight standards according to an earlier protocol (Sá-Leão *et al.*, 2000b).

Classification of PFGE patterns was performed by visual comparison according to

accepted criteria (Tenover *et al.*, 1995). We compared our patterns with the ones identified in studies from our group conducted between 1996 and 1999 (S. Nunes *et al.*, unpublished data) (Sá-Leão *et al.*, 2000a; Sá-Leão *et al.*, 2000b) and also with the patterns of 26 international clones included in the Pneumococcal Molecular Epidemiology Network (PMEN) (McGee *et al.*, 2001).

Statistical analysis. The effect of vaccination was evaluated on vaccinated and nonvaccinated cohorts. Contingency table analysis was conducted with the χ^2 test, both for individual sampling periods and for the pairwise comparison of all periods. The χ^2 test was performed for association between vaccination and carriage of Pn or occurrence of afflictions episodes. A maximum error type I of 0.05 was considered for recognition of a significant vaccine effect.

2.4. Results

Demographic and clinical data. We enrolled 695 children between May 2001 and February 2003. There were no significant differences concerning demographic and clinical characteristics between the intervention group and control group.

The intervention group (n=238) included 118 male and 120 female subjects. The control group (n=457) included 237 boys and 220 girls.

Antibiotic consumption at sampling or 1 month before sampling was high in both groups during all sampling periods, as reported by the parents through the questionnaires. Twenty-eight children (4%) in the intervention group and 32 children (5%) in the control group had taken antibiotics at the time of sampling. One hundred and nine children (17%) in the intervention group and 74 children (11%) in the control group took antibiotics 1 month before sampling.

Otitis media, throat infection and low respiratory infections were indicated as the afflictions that most frequently caused antimicrobial consumption in both groups. Forty-

seven children (34%) in the intervention group and 40 children (29%) in the control group took antibiotics because of otitis media. Forty-seven children (34%) in the intervention group and 58 children (42%) in the control group took antibiotics because of throat infection. Concerning lower respiratory infection, only 9% (13 children) and 6% (9 children) of children included, respectively, in the intervention and control groups took antibiotic because of this affliction.

Carriage of *S. pneumoniae* strains. Of the 1,950 NP samples obtained, 1,014 were from children in the intervention group (vaccinees) and 936 from the control group. Total carriage rate of Pn strains was similar in intervention and control groups during all sampling periods. As many as 68% of the NP samples carried *S. pneumoniae* in both the control (633 Pn strains isolated from 936 NP samples) and the intervention (692 Pn strains isolated from 1,014 NP samples) groups. The greatest difference in Pn carriage between the 2 groups was 9% (Table 1). The carriage rate of Pn fluctuated in a very similar manner among controls and vaccinees during the sampling periods.

The carriage rate of DRPn was similar in intervention and control groups throughout the sampling periods. Among the total of 692 Pn strains collected from the intervention group, 37% were DRPn; among the 633 Pn strains recovered from the control group, 39% were DRPn (Table 1).

Carriage rates of penicillin nonsusceptible strains were also similar and constant during the sampling periods and no significant differences were found between intervention and control groups. Of 692 Pn strains isolated in the intervention group, 24% were nonsusceptible to penicillin; of 633 Pn strains isolated in the control group, 23% were penicillin nonsusceptible (Table 1).

Table 1. Nasopharyngeal carriage of *Streptococcus pneumoniae* strains isolated in the Intervention and Control groups during the 6 sampling periods.

NP Sampling Periods	Carriage rate								
	Intervention group					Control group			
	NP samples	Pn strains*	DRPn strains†	PNSPn strains‡	PSPn strains§	NP samples	Pn strains*	DRPn strains†	PNSPn strains‡	PSPn strains§
May 2001, 1st	243	182 (75.0) ‖	84 (46.0)	59 (32.4)	25 (13.7)	233	163 (70.0)	81 (50.0)	46 (28.2)	35 (21.5)
June 2001, 2nd	196	132 (67.0)	53 (40.0)	35 (26.5)	18 (13.6)	NA	NA	NA	NA	NA
November 2001, 3rd	152	97 (64.0)	41 (42.0)	24 (24.7)	17 (17.5)	214	143 (67.0)	58 (41.0)	34 (23.7)	24 (16.8)
February 2002, 4th	168	105 (63.0)	32 (30.5)	18 (17.1)	14 (13.3)	182	105 (58.0)	31 (29.5)	21 (20.0)	10 (9.5)
November 2002, 5th	128	94 (73.0)	28 (30.0)	18 (19.1)	10 (10.6)	132	93 (70.5)	30 (32.0)	19 (20.4)	11 (11.8)
February 2003, 6th	127	82 (65.0)	21 (26.0)	15 (18.2)	6 (7.3)	175	129 (74.0)	49 (38.0)	28 (21.7)	21 (16.3)
Total	1014	692 (68.2)	259 (37.4)	169 (24.4)	90 (13.0)	936	633 (67.6)	249 (39.3)	148 (23.3)	101 (15.9)

*Carriage of *S. pneumoniae* (both drug resistant and drug susceptible) as percent of NP samples collected.

†Carriage DRPn: strains resistant to any of the antimicrobial agents, alone or in combination (except strains only resistant to oxacillin).

‡Carriage PNSPn: strains nonsusceptible to penicillin (MIC ≥ 0.094 µg/mL) alone or in combination with other antimicrobial agents.

§Carriage PSPn: strains susceptible to penicillin (MIC < 0.094 µg/mL) but resistant to other antimicrobial agents. These numbers are derived by subtracting the numbers in column ‡ from the numbers in column † (§ = † - ‡).

‖Numbers in parentheses, percent.

NA indicates data not available.

Within the intervention group, a decline was observed in penicillin nonsusceptibility from > 24% of isolates in 2001 to 17–19% in 2002–2003, which was not observed in the control group, although that difference was not statistically significant (*P* >0.05). All pneumococcal strains were susceptible to levofloxacin and the overall prevalence of strains resistant to the other antimicrobials tested was similar between control and intervention samples. The differences in susceptibility to each one of the antimicrobial

tested between intervention and control were not statistically significant ($P > 0.05$) except for chloramphenicol (Table 2).

Table 2. *Streptococcus pneumoniae* strains resistant to antimicrobial agents*.

Resistance to	No. of DRPn	
	Intervention group	Control group
Penicillin	169 (24.4) †	148 (23.3)
Erythromycin	170 (24.5)	151 (23.8)
Clindamycin	159 (22.9)	139 (21.9)
Tetracycline	144 (20.8)	132 (20.8)
Trimethoprim-sulfamethoxazole	119 (17.1)	101 (15.9)
Chloramphenicol	16 (2.3)	29 (4.5)
Ceftriaxone	7 (1.0)	6 (1.0)

*Carriage rate was calculated over the total number of Pn strains isolated in the intervention (n = 692) and control (n = 633) groups.
† Number in parentheses, percent.

Serotypes of DRPn. Although the total number of DRPn strains was similar between the control and intervention samples, differences became apparent once the strains were separated into bacteria expressing VT and NVT capsular polysaccharides (Fig. 1). In the intervention group (vaccinees), the frequency of VT strains decreased significantly, from 81% to 5%, throughout the 6 sampling periods ($P < 0.05$). At the same time, the frequency of NVT strains increased significantly from 19% to 95% ($P < 0.05$). In the control group, the carriage rate of VT strains remained high (between ~59 and 75%) and the carriage rate of NVT strains remained between 25 and 46% and showed no trends of consistent increase or decline during the surveillance period (Table 3 and Fig. 1).

Table 3. Serotypes of DRPn isolated in the Intervention and Control groups during the 6 sampling periods.

Nasopharyngeal sampling periods	Carriage rate of vaccine and non vaccine type DRPn strains*					
	DRPn strains	Intervention group		DRPn strains	Control group	
		VT strains†	NVT strains‡		VT strains†	NVT strains‡
May 2001, 1st	79	64 (81.0)§	15 (19.0)	70	41 (59.0)	29 (41.0)
June 2001, 2nd	47	32 (68.0)	15 (32.0)	NA	NA	NA
November 2001, 3rd	31	21 (68.0)	10 (32.0)	51	38 (75.0)	13 (25.0)
February 2002, 4th	27	14 (52.0)	13 (48.0)	28	19 (68.0)	9 (32.0)
November 2002, 5th	26	8 (31.0)	18 (69.0)	28	15 (54.0)	13 (46.0)
February 2003, 6th	21	1 (5.0)	20 (95.0)	46	31 (67.0)	15 (33.0)
Total DRPn strains	231‖	140	91	223‖	144	79

*Carriage rate was calculated over the DRPn strains isolated, respectively, in the intervention or control groups and selected for serotyping.

†VT strain: strains with serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, included in the vaccine.

‡NVT strains: strains with other serotypes different from the ones included in the vaccine and NT strains.

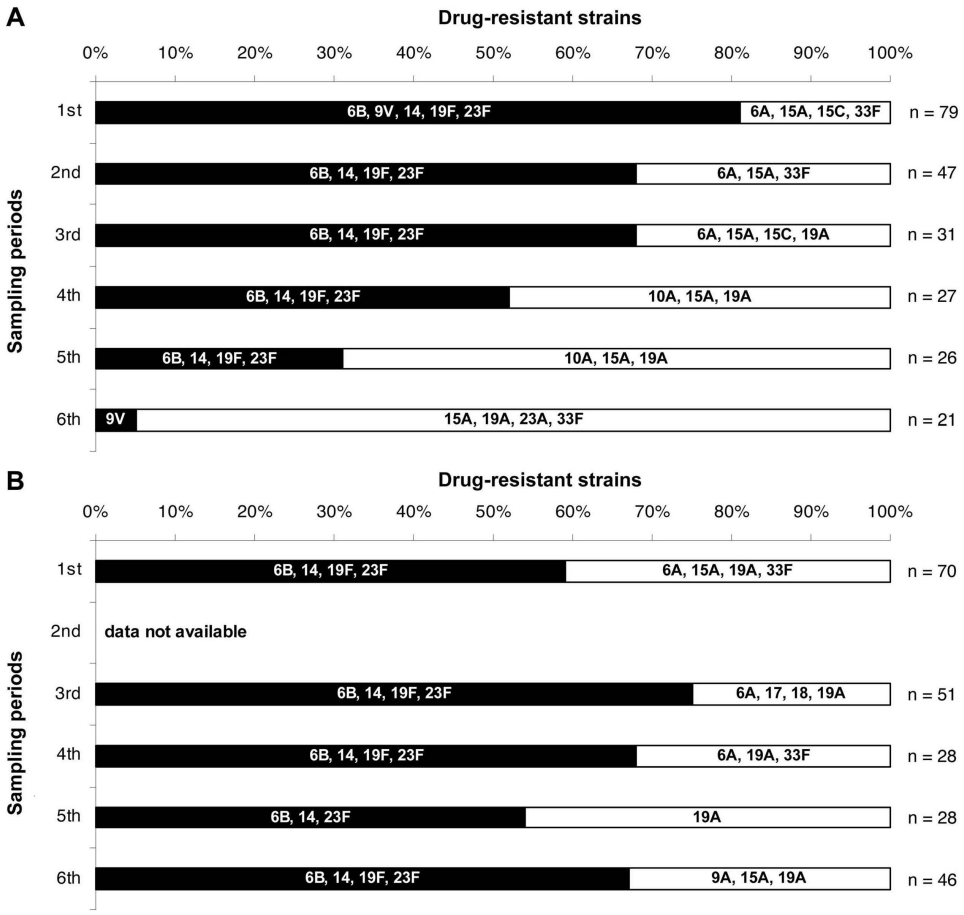
§Numbers in parentheses, percent.

‖All DRPn strains were selected for serotyping (except strains only resistant to oxacillin and/or trimethoprim-sulfamethoxazole).

NA indicates not available.

Before vaccine administration (first sampling period), the carriage rate of VT DRPn strains was 81% in the intervention group and 59% in the control group. During the remaining sampling periods, carriage rates of DRPn with vaccine serotypes continued to decrease in the intervention group reaching 5%; whereas the frequency of these strains remained above 53% in the controls. The prevalence of NVT strains in the first sampling period was 19% in the intervention group and 41% in the control group.

Figure 1. Distribution of VT and NVT strains in the Intervention (A), and Control (B) groups along time. ■ indicates VT strains with respective serotypes; □, NVT strains with respective serotypes.



During the remaining sampling periods, carriage rates of NVT strains increased in the intervention group, reaching 95% in the sixth sample, whereas the frequency of these serotypes remained on average ~33% of the DRPn strains in the control samples (Table 3). Of all the DRPn strains with VT serotypes that were present in the first sample from the intervention group, a single serotype 9V remained by the final sampling period. DRPn strains expressed a wider range of NVT serotypes that included serotypes 6A, 10A, 15A, 15C, 19A, 23A and 33F. Serotype 15A was recovered in all sampling periods (Fig. 1). In the control samples, the NVT serotypes were 6A, 9A, 15A, 17, 18, 19A and 33F with the most frequent one being 19A (Fig. 1). NT strains were detectable in the intervention (25 strains) and control (28 strains) groups.

Penicillin nonsusceptible *S. pneumoniae* strains expressing vaccine and non vaccine serotypes. Among DRPn strains, penicillin nonsusceptible strains with VTs decreased from 59% to 5% and NVT strains increased from 15% to 67% in the intervention group. In the control group, penicillin nonsusceptible strains most frequently expressed VT serotypes (Fig. 2). High level resistance to penicillin (MIC ≥ 1.5 µg/mL) was only observed in few VT isolates. Penicillin MIC_{50} was 0.094 µg/mL in VT isolates, either in the intervention or control, and among the NVT isolates it was 0.125 µg/mL in the intervention and 0.032 µg/mL in the control. Penicillin MIC_{90} was 1 µg/mL in VT isolates of the intervention and 0.75 µg/mL in the control; the MIC_{90} was 0.5 µg/mL among the NVT isolates of intervention and control groups.

PFGE patterns of DRPn strains. Most of the vaccine type DRPn, either in the intervention (vaccinees) or control groups, belonged to 9 internationally spread epidemic clones identified and deposited in the collection of the PMEN (Figs. 3 and 4). These clonal types were as follows: Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, England^{14}-9, Poland^{6B}-20, Portugal^{19F}-21, Greece^{6B}-22, Sweden^{15A}-25 and Colombia^{23F}-26.

Figure 2. Penicillin nonsusceptible strains (MIC ≥ 0.094 µg/mL) and penicillin-susceptible strains (MIC < 0.094 µg/mL) with vaccine (VT) and non vaccine (NVT) serotypes in the intervention (A) and control (B) groups along time. Dashed black bars indicate penicillin nonsusceptible (VT) strains; dashed gray bars, penicillin nonsusceptible (NVT) strains; black bars, penicillin susceptible but resistant to other antimicrobials (VT) strains; gray bars, penicillin susceptible but resistant to other antimicrobials (NVT) strains.

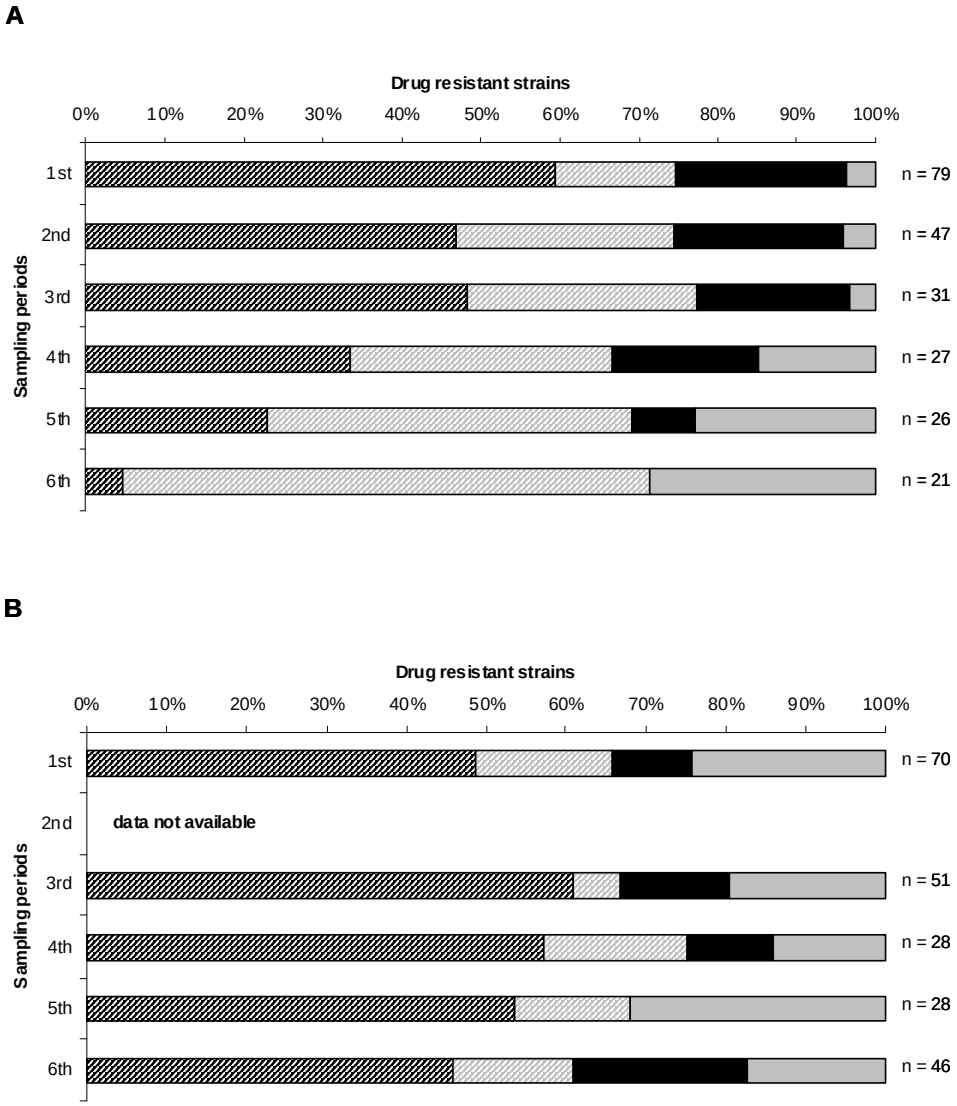


Figure 3. Clonal distribution among VT and NVT strains isolated in the intervention (A) and control (B) groups along time. Black bars indicate VT strains (PMEN clonal types); gray bars, VT strains (non-PMEN clonal types); dashed black bars, NVT strains (PMEN clonal types); dashed gray bars, NVT strains (non-PMEN clonal types).

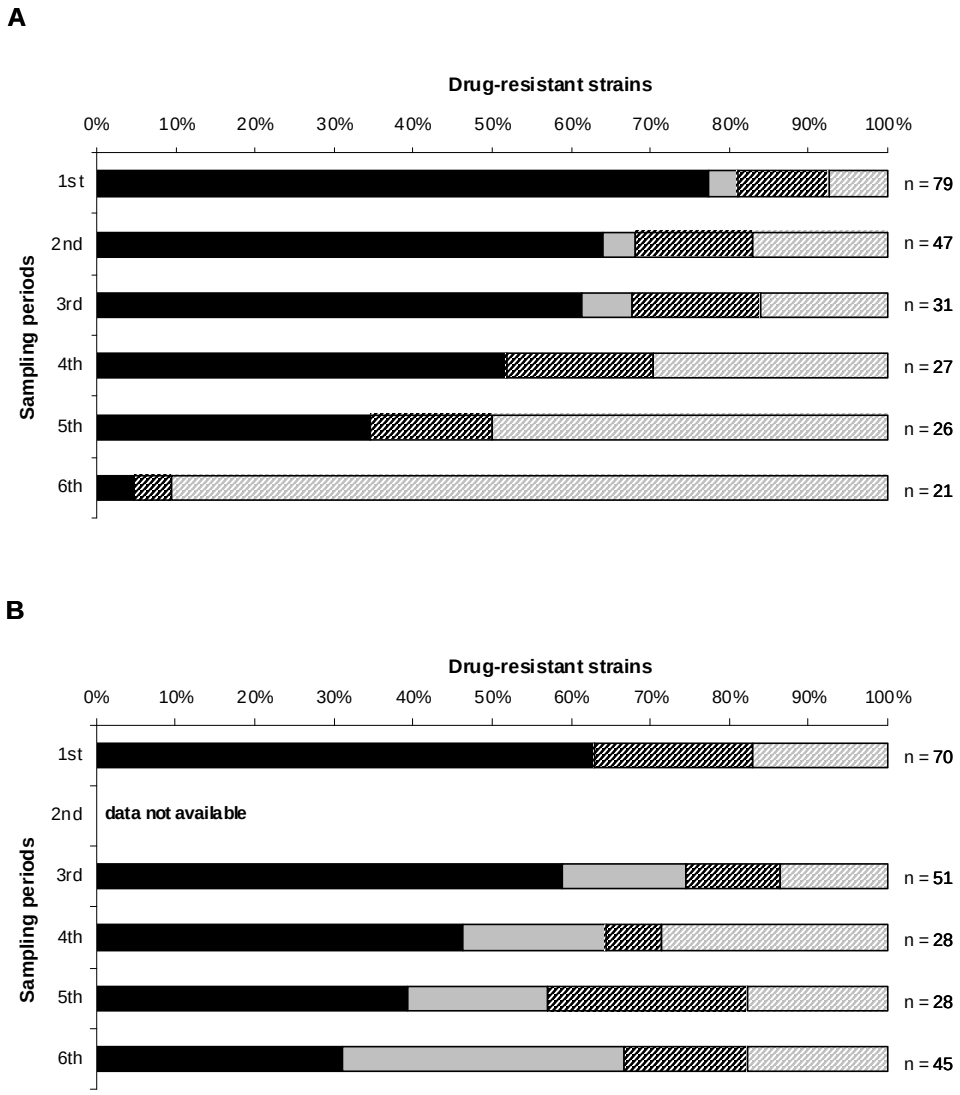
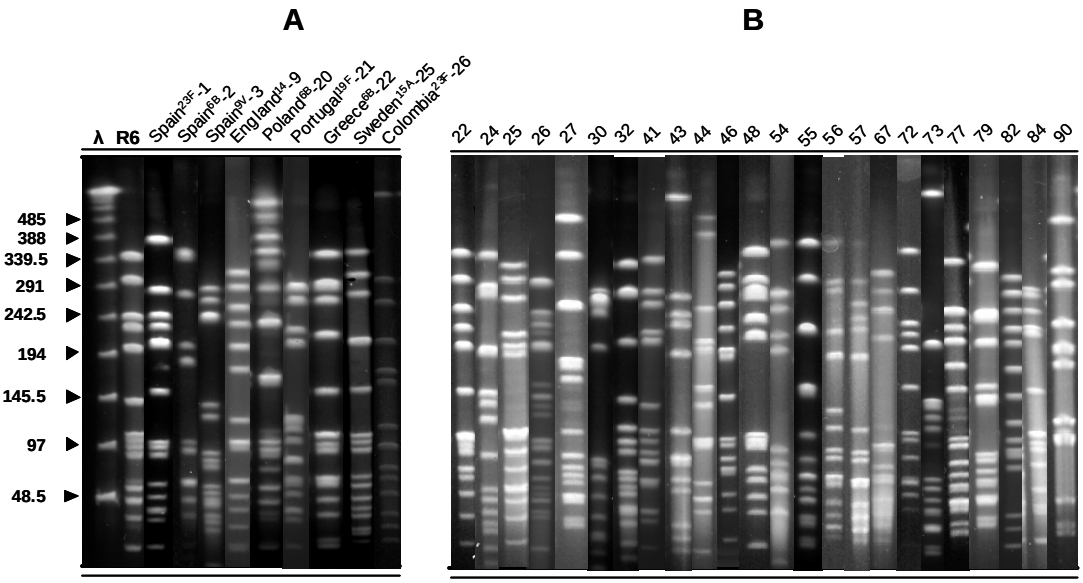


Figure 4. PFGE patterns identified in the study (Control and intervention). A, PMEN clones; B, non-PMEN clones. The λ ladder and the reference strain R6 are indicated and were used as molecular weight markers. Ordinate shows molecular sizes in kilobases.



In the intervention group, during the first sampling period, 81% of the 79 DRPn isolates expressed VT capsules. The majority of vaccine type strains belonged to one or the other of 9 international epidemic clones (PMEN clones), and only 3 VT strains showed PFGE types different from the PMEN clones. The rest of the 15 DRPn from this sampling period expressed NVT capsules: 5 strains belonged to the serotype 15A clone of Sweden^{15A}-25; and 6 of the other 10 strains showed 4 non-PMEN clonal types.

In this period, epidemic PMEN clonal types were dominant over the non-PMEN clonal types. Of 79 DRPn strains isolated, 89% (70 strains) belonged to 8 epidemic PMEN clonal types and 11% only (9 strains) belonged to 7 non-PMEN clonal types. By the last sampling period, the number of VT strains in the intervention group was reduced to a single serotype 9V strain with the PFGE pattern of the international clone Spain^{9V}-3. All

the rest of the 20 DRPn expressed NVT capsules and were represented by 7 PFGE patterns of which only 1 is a PMEN clone. In this last period, the non-PMEN clones became dominant over the epidemic PMEN clones. Of 21 DRPn strains isolated, 2 strains (10%) belonged to PMEN clonal types (2 clones) and 19 strains (90%) belonged to 7 non-PMEN clonal types.

In the control group during the first sampling period, when the majority (41 of 70) of DRPn strains expressed VT capsules, all of these belonged to 7 internationally spread clones identified in the PMEN. The representation of these internationally spread epidemic clones remained high among the DRPn strains with VT capsular types collected throughout the sampling periods.

The epidemic PMEN clonal types were dominant over the non-PMEN clonal types in the first period. Of 70 DRPn strains isolated, 84% (59 strains) belonged to 8 epidemic PMEN clonal types and 16% (11 strains) belonged to 8 non-PMEN clonal types. By the last sampling period, PMEN clonal types and non-PMEN clones were detected in similar ratios, concerning both the number of clonal types and the number of strains. Of 45 DRPn strains isolated, 49% (22 strains) belonged to PMEN clonal types (8 clones) and 51% (23 strains) belonged to 12 non-PMEN clonal types.

In summary, of 231 DRPn strains from the intervention group, 71% (165 strains) had PFGE patterns identical with those of 9 international clones included in the PMEN (McGee *et al.*, 2001). The remaining 66 strains (29%) presented 19 different PFGE patterns (non-PMEN clones). In the control group, 143 of 222 DRPn strains (64%) had PFGE patterns identical with those of 8 international clones included in the PMEN (McGee *et al.*, 2001). The remaining 79 strains (36%) presented 29 different PFGE patterns (non-PMEN clones).

Serotyping and molecular typing by PFGE identified a few cases that might represent capsular transformation. Among the isolates recovered from the control group at the first sampling period, 6 strains showed the PFGE pattern of the international clone Spain^{23F}-1 that typically expresses serotype 23F capsule. These particular 6 strains expressed

serotype 19A. Another group of 10 strains showed the PFGE pattern of the international clone Poland^{6B}-20, which typically expresses serotype 6B. The 10 particular strains expressed serotype 6A. Additional examples for putative capsular switch were also detected among some strains with familiar PFGE type but expressing no identifiable capsular types (non typeable strains). There was no evidence for an increased frequency of strains with such suspected capsular changes among the DRPn isolated from the nasopharyngeal samples of vaccinees. Therefore capsular switch did not appear to be a major mechanism contributing to the change from the vaccine to the non vaccine serotype of the DRPn isolates.

2.5. Discussion

We evaluated the impact of the 7-valent pneumococcal vaccine on the carriage of drug resistant *S. pneumoniae* strains in the nasopharynx of children attending DCCs in Portugal. Because most drug resistant pneumococci express a limited number of serotypes that are present in the 7-valent vaccine, the carriage rates of drug resistant pneumococci was expected to decrease in the vaccinees as part of the reduction in all pneumococcal VTs that colonized the nasopharynx of the children.

In agreement with several studies on the impact of pneumococcal conjugate vaccines, our results also showed that vaccination with 7-valent pneumococcal vaccine did not affect the global carriage of Pn strains (susceptible and resistant). Also in agreement with previous studies (Dagan *et al.*, 1996a; Dagan *et al.*, 1997; Dagan, 2002; Dagan *et al.*, 2002; Dagan *et al.*, 2003), the carriage of vaccine type DRPn strains decreased markedly from 81% to 5% in the vaccinated children. In contrast to findings reported in some earlier studies (Dagan *et al.*, 1996a; Dagan *et al.*, 1997; Dagan, 2002; Dagan *et al.*, 2002; Dagan *et al.*, 2003; Klugman, 2001; Piffer, 2002), the total carriage rate of DRPn was not reduced. In parallel with the decline in the frequency of DRPn expressing the vaccine serotypes, there was an increase in DRPn with non vaccine serotypes. As a result, the carriage rate of DRPn strains has remained virtually unchanged during the 3 years of follow-up sampling of the NP flora of vaccinees. Furthermore the DRPn with the

non vaccine serotypes were represented in almost all cases by bacteria exhibiting genetic backgrounds different from the PMEN clones, as identified by their unusual PFGE patterns.

We explain the replacement of vaccine type DRPn by drug resistant pneumococci with different genetic backgrounds and expressing NVT serotypes as the combined effect of vaccine pressure and undiminished use of antibacterial agents. Vaccination against the VT serotypes must exert selective pressure on the colonizing flora of pneumococci preventing the cross-infection and multiplication of the vaccine type strains, which provides a competitive advantage to pneumococci expressing other capsular types not present in the vaccine. The emergence of drug resistant strains among these NVT type bacteria could be because of the acquisition of drug resistant determinants from the DRPn strains with the VT capsules that are receding under the vaccine pressure. Alternatively drug resistant strains with the NVT capsules may preexist as minor components of the nasopharyngeal flora because of multiple carriage of pneumococci (Sá-Leão *et al.*, 2002). This latter alternative appears likely given that DRPn with serotypes 6A, 15A, 15C and 33F were identified in the NP flora at the onset of the study.

Vaccine efficacy is closely linked to a decrease in vaccine type strains that normally implies a decrease in resistance and virulence (Joloba *et al.*, 2001; Wuorimaa & Kayhty, 2002). Drug resistance, especially to penicillin, is found almost exclusively among isolates of the vaccine serotypes (Klugman, 2001; Wuorimaa & Kayhty, 2002). In our study, we found that penicillin nonsusceptible strains with non vaccine serotypes became frequent in vaccinees with time (Fig. 2). Our findings are consistent with the predictions of mathematical modeling studies suggesting that the probability for the occurrence of long term replacement of DRPn would increase after vaccination (Temime *et al.*, 2004).

The pathogenic potential of non vaccine serotypes is not known, although the capacity of these strains for effective colonization might imply pathogenic potential (Hava & Camilli, 2002). This has been shown by the Finnish Otitis Media Study Group (Eskola *et al.*, 2001), who found a 33% increase in AOM episodes caused by NVT in a group of

vaccinated children. In our study, non vaccine serotypes isolated from vaccinees became prominent in the nasopharynx (95%) with some serotypes (e.g., 15A, 33F) repeatedly recovered from children along the sampling periods (Fig. 1). High frequency and prolonged duration of carriage has been associated with frequent pneumococcal disease (Smith *et al.*, 1993). The serogroups and serotypes identified in our study, such as 6A, 10A, 15A/C, 19A, 23A and 33F, have been associated with AOM episodes in Finland, the United States and Israel (Eskola *et al.*, 2001; McEllistrem *et al.*, 2003; Porat *et al.*, 2004) and with invasive disease episodes in France (6A, 10A, 15C, 19A) (Doit *et al.*, 2002), United Kingdom (6A, 15C, 19A, 33F) (Brueggemann *et al.*, 2003) and Portugal (6A, 10A, 19A, 23A, 33F) (Serrano *et al.*, 2004).

The nasopharyngeal flora of DRPn of vaccinated children in our setting appeared to undergo a progressive compositional change over time because of replacement of the initially dominant epidemic DRPn clones by other clonal types of pneumococci expressing new serotypes that are not present in the vaccine.

The origin and nature of resistance determinants and pathogenic potential of the DRPn clones replacing the vaccine type pneumococci in the nasopharyngeal flora are unknown. However, the results of our study seem to imply that reduction of the carriage of drug resistant strains of *S. pneumoniae* requires a combination of the conjugate vaccine and reduction in the use of antimicrobial agents.

2.6. Acknowledgments

We thank the directors and staff of the participating day-care centers as well as all the parents and children who collaborated in this study. We also thank the 2 experienced state registered pediatric nurses, Anabela Gonc¸alves and Paula Gonzaga, for their excellent vaccination and sampling procedures and also all the members of the Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa who participated in this work.

We thank Sara Silva, Rodrigo Oliveira and António Maretzek for building the database and the web interface; Francisco Pinto and Susana Vinga for discussion on statistical analysis; and Alexander Tomasz for helping with the study design, interpretation of the results and extensive revision of the manuscript.

N. Frazão was responsible for the global data analysis and experimental work except collection of the isolates.

2.7. References

Aniansson, G., Alm, B., Andersson, B., Larsson, P., Nylen, O., Peterson, H., Rigner, P., Svanborg, M. & Svanborg, C. (1992). Nasopharyngeal colonization during the first year of life. *J Infect Dis* **165 Suppl 1**, S38-42.

Appelbaum, P. C. (1992). Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. *Clin Infect Dis* **15**, 77-83.

Austrian, R. (1986). Some aspects of the pneumococcal carrier state. *J Antimicrob Chemother* **18 Suppl A**, 35-45.

Black, S., Shinefield, H., Fireman, B., Lewis, E., Ray, P., Hansen, J. R., Elvin, L., Ensor, K. M., Hackell, J., Siber, G., Malinoski, F., Madore, D., Chang, I., Kohberger, R., Watson, W., Austrian, R. & Edwards, K. (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.

Black, S. & Shinefield, H. (2002). Safety and efficacy of the seven-valent pneumococcal conjugate vaccine: evidence from Northern California. *Eur J Pediatr* **161 Suppl 2**, S127-131.

Black, S. B., Shinefield, H. R., Hansen, J., Elvin, L., Laufer, D. & Malinoski, F. (2001). Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine. *Pediatr Infect Dis J* **20**, 1105-1107.

Bogaert, D., De Groot, R. & Hermans, P. W. (2004). *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* **4**, 144-154.

Brueggemann, A. B., Griffiths, D. T., Meats, E., Peto, T., Crook, D. W. & Spratt, B. G. (2003). Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* **187**, 1424-1432.

CDC (2000). Preventing pneumococcal disease among infants and young children: recommendations from the Advisory Committee on Immunizations Practices. *MMWR Morb Mortal Wkly Rep* **49**, 1-35.

Dagan, R., Melamed, R., Muallem, M., Piglansky, L., Greenberg, D., Abramson, O., Mendelman, P. M., Bohidar, N. & Yagupsky, P. (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.

Dagan, R., Muallem, M., Melamed, R., Leroy, O. & Yagupsky, P. (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.

Dagan, R. & Fraser, D. (2000). Conjugate pneumococcal vaccine and antibiotic-resistant *Streptococcus pneumoniae*: herd immunity and reduction of otitis morbidity. *Pediatr Infect Dis J* **19**, S79-87; discussion S88.

Dagan, R. (2002). Immunisation with a pneumococcal 7-valent conjugate vaccine. *Int J Clin Pract* **56**, 287-291.

Dagan, R., Givon-Lavi, N., Zamir, O., Sikuler-Cohen, M., Guy, L., Janco, J., Yagupsky, P. & Fraser, D. (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.

Dagan, R., Givon-Lavi, N., Zamir, O. & Fraser, D. (2003). Effect of a nonavalent conjugate vaccine on carriage of antibiotic-resistant *Streptococcus pneumoniae* in day-care centers. *Pediatr Infect Dis J* **22**, 532-540.

de Lencastre, H., Kristinsson, K. G., Brito-Avô, A., Sanches, I. S., Sá-Leão, R., Saldanha, J., Sigvaldadottir, E., Karlsson, S., Oliveira, D., Mato, R., Aires de Sousa, M. & Tomasz, A. (1999). Carriage of respiratory tract pathogens and molecular epidemiology of *Streptococcus pneumoniae* colonization in healthy children attending day-care centers in Lisbon, Portugal. *Microb Drug Resist* **5**, 19-29.

Doit, C., Loukil, C., Geslin, P. & Bingen, E. (2002). Phenotypic and genetic diversity of invasive pneumococcal isolates recovered from French children. *J Clin Microbiol* **40**, 2994-2998.

Eskola, J., Kilpi, T., Palmu, A., Jokinen, J., Haapakoski, J., Herva, E., Takala, A., Kayhty, H., Karma, P., Kohberger, R., Siber, G. & Makela, P. H. (2001). Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* **344**, 403-409.

Givon-Lavi, N., Fraser, D. & Dagan, R. (2003). Vaccination of day-care center attendees reduces carriage of *Streptococcus pneumoniae* among their younger siblings. *Pediatr Infect Dis J* **22**, 524-532.

Hausdorff, W. P., Bryant, J., Paradiso, P. R. & Siber, G. R. (2000). Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* **30**, 100-121.

Hava, D. L. & Camilli, A. (2002). Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol Microbiol* **45**, 1389-1406.

Joloba, M. L., Windau, A., Bajaksouzian, S., Appelbaum, P. C., Hausdorff, W. P. & Jacobs, M. R. (2001). Pneumococcal conjugate vaccine serotypes of *Streptococcus pneumoniae* isolates and the antimicrobial susceptibility of such isolates in children with otitis media. *Clin Infect Dis* **33**, 1489-1494.

Klugman, K. P. (2001). Efficacy of pneumococcal conjugate vaccines and their effect on carriage and antimicrobial resistance. *Lancet Infect Dis* **1**, 85-91.

Kristinsson, K. G. (1997). Effect of antimicrobial use and other risk factors on antimicrobial resistance in pneumococci. *Microb Drug Resist* **3**, 117-123.

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

McEllistrem, M. C., Adams, J., Mason, E. O. & Wald, E. R. (2003). Epidemiology of acute otitis media caused by *Streptococcus pneumoniae* before and after licensure of the 7-valent pneumococcal protein conjugate vaccine. *J Infect Dis* **188**, 1679-1684.

McGee, L., McDougal, L., Zhou, J., Spratt, B. G., Tenover, F. C., George, R., Hakenbeck, R., Hryniewicz, W., Lefevre, J. C., Tomasz, A. & Klugman, K. P. (2001). Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. *J Clin Microbiol* **39**, 2565-2571.

Munford, R. S. & Murphy, T. V. (1994). Antimicrobial resistance in *Streptococcus pneumoniae*: can immunization prevent its spread? *J Investig Med* **42**, 613-621.

NCCLS (2000). Performance standards for antimicrobial disk susceptibility tests. Document M2-A7.

O'Brien, K. L. & Dagan, R. (2003). The potential indirect effect of conjugate pneumococcal vaccines. *Vaccine* **21**, 1815-1825.

Piffer, S. (2002). [7-valent conjugate Pneumococcal vaccine and nasopharyngeal cavity]. *Ann Ig* **14**, 31-37.

Porat, N., Barkai, G., Jacobs, M. R., Trefler, R. & Dagan, R. (2004). Four antibiotic-resistant *Streptococcus pneumoniae* clones unrelated to the pneumococcal conjugate vaccine serotypes, including 2 new serotypes, causing acute otitis media in southern Israel. *J Infect Dis* **189**, 385-392.

Reichler, M. R., Allphin, A. A., Breiman, R. F., Schreiber, J. R., Arnold, J. E., McDougal, L. K., Facklam, R. R., Boxerbaum, B., May, D., Walton, R. O. & et al. (1992). The spread of multiply resistant *Streptococcus pneumoniae* at a day-care center in Ohio. *J Infect Dis* **166**, 1346-1353.

Sá-Leão, R., Tomasz, A., Sanches, I. S., Brito-Avô, A., Vilhelmsson, S. E., Kristinsson, K. G. & de Lencastre, H. (2000a). 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.

Sá-Leão, R., Tomasz, A., Sanches, I. S., Nunes, S., Alves, C. R., Avô, A. B., Saldanha, J., Kristinsson, K. G. & de Lencastre, H. (2000b). Genetic diversity and clonal patterns among antibiotic-susceptible and -resistant *Streptococcus pneumoniae* colonizing children: day-care centers as autonomous epidemiological units. *J Clin Microbiol* **38**, 4137-4144.

Sá-Leão, R., Tomasz, A., Santos Sanches, I. & de Lencastre, H. (2002). Pilot study of the genetic diversity of the pneumococcal nasopharyngeal flora among children attending day-care centers. *J Clin Microbiol* **40**, 3577-3585.

Serrano, I., Ramirez, M. & Melo-Cristino, J. (2004). Invasive *Streptococcus pneumoniae* from Portugal: implications for vaccination and antimicrobial therapy. *Clin Microbiol Infect* **10**, 652-656.

Shinefield, H. R., Black, S., Ray, P., Chang, I., Lewis, N., Fireman, B., Hackell, J., Paradiso, P. R., Siber, G., Kohberger, R., Madore, D. V., Malinowski, F. J., Kimura, A., Le, C., Landaw, I., Aguilar, J. & Hansen, J. (1999). Safety and immunogenicity of heptavalent pneumococcal CRM197 conjugate vaccine in infants and toddlers. *Pediatr Infect Dis J* **18**, 757-763.

Silva, S., Gouveia-Oliveira, R., Maretzek, A., Carriço, J., Gudnason, T., Kristinsson, K. G., Ekdahl, K., Brito-Avô, A., Tomasz, A., Sanches, I. S., de Lencastre, H. & Almeida, J. (2003). EURISWEB: Web-based epidemiological surveillance of antibiotic-resistant pneumococci in day-care centers. *BMC Med Inform Decis Mak* **3**, 9.

Smith, T., Lehmann, D., Montgomery, J., Gratten, M., Riley, I. D. & Alpers, M. P. (1993). Acquisition and invasiveness of different serotypes of *Streptococcus pneumoniae* in young children. *Epidemiol Infect* **111**, 27-39.

Sorensen, U. B. (1993). Typing of pneumococci by using 12 pooled antisera. *J Clin Microbiol* **31**, 2097-2100.

Temime, L., Guillemot, D. & Boelle, P. Y. (2004). Short- and long-term effects of pneumococcal conjugate vaccination of children on penicillin resistance. *Antimicrob Agents Chemother* **48**, 2206-2213.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233-2239.

Wuorimaa, T. & Kayhty, H. (2002). Current state of pneumococcal vaccines. *Scand J Immunol* **56**, 111-129.

Chapter III

3. Impact of a single dose of the 7-valent pneumococcal conjugate vaccine on colonization

Published by Nelson Frazão, R. Sá-Leão, R., and H. de Lencastre *in* Vaccine (2010); 28: 3445-3452.

3.1. Abstract

The impact of the 7-valent pneumococcal conjugate vaccine (PCV7) on the pneumococcal flora has been mostly studied without evaluating multiple colonization and the mechanism(s) leading to serotype replacement. These issues are addressed here, while assessing the effect of a single PCV7 dose. A group of children received one PCV7 dose just after nasopharyngeal sampling, with the control receiving no vaccine and both groups being sampled again a month later. Up to 10 pneumococcal isolates were recovered per colonized child — 1,224 isolates were serotyped and representative ones characterized by pulsed-field gel electrophoresis. In vaccinated children, serotype replacement between vaccine (VT) and non vaccine (NVT) types occurred in single and multiple carriers, and VTs were less prone to be *de novo* acquired. NVT unmasking was only detected in the vaccinated group. One month after vaccination with a single dose, PCV7 prevents VT *de novo* acquisition and promotes NVT unmasking.

3.2. Introduction

Streptococcus pneumoniae remains one of the most important human pathogens in our era, together with malaria, TB and HIV (WHO, 1999). The primary ecological reservoir of *S. pneumoniae* is the nasopharynx of young children who are colonized asymptomatically early in life (Gray *et al.*, 1980). When the balance between host and pathogen is disturbed, the nasopharynx can become a launching pad for pneumococcal disease. Colonizing pneumococci may spread to adjacent mucosal tissues to cause infections such as acute otitis media and pneumonia, or enter the bloodstream causing invasive infections such as sepsis and meningitis (Bogaert *et al.*, 2004a; Faden *et al.*, 1997). The first two years of life are the period of greatest risk for pneumococcal disease (Gray *et al.*, 1979), and methods that could suppress nasopharyngeal colonization by disease-causing pneumococci are believed to represent means of preventing or decreasing the frequency of pneumococcal infections.

The majority of pneumococci causing life-threatening disease in children in the USA, and to a certain extent also in Europe, express on their surface seven chemically different capsular types (vaccine types — VT), which are included in the 7-valent pneumococcal conjugate vaccine (PCV7) (Black *et al.*, 2000). Several surveillance and randomized controlled studies have shown that routine vaccination with PCV7 is efficacious against VT pneumococcal invasive disease in children younger than 2 years old (Black *et al.*, 2000; CDC, 2005; O'Brien *et al.*, 2003; Whitney *et al.*, 2003). Concerning pneumococcal colonization, the foremost conclusion of several studies is that PCV7 reduces nasopharyngeal carriage of VT pneumococci but, in parallel, there is an increase in non vaccine type (NVT) carriage, a phenomenon termed serotype replacement carriage (Dagan *et al.*, 1996a; Dagan, 2002; Frazão *et al.*, 2005; Sá-Leão *et al.*, 2009).

Traditionally, the most common method used to study the pneumococcal colonizing flora has been the serotyping of a single isolate recovered from the nasopharynx of each individual carrier. However, studies have shown that most individuals carry simultaneously more than one pneumococcal isolate (co-colonization), which can differ in properties such as serotype and genotype (Gray *et al.*, 1980; Sá-Leão *et al.*, 2002). Characterizing a single isolate does not allow detection of qualitative and quantitative changes that may occur in the nasopharyngeal flora under the influence of strong selective pressures, such as conjugate pneumococcal vaccines. In order to overcome this problem, in the colonization study described here we serotyped up to ten isolates per child, selecting randomly and/or by isolate morphology in cases where morphological differences were apparent. Until consensus on a more suitable method for the evaluation of the nasopharyngeal flora of pneumococci is reached, a recent study proposed serotyping of multiple isolates selected on the basis of morphological variation plus random picking as a reasonable way of assessing the composition of the pneumococcal nasopharyngeal flora (Hare *et al.*, 2008).

The World Health Organization and UNICEF have recognized the safety and effectiveness of PCV7, recommending the inclusion of this vaccine in national immunization programs. Indeed, 35 high- and middle-income countries currently provide

routine childhood immunization against pneumococcal disease, and Rwanda has recently become the first developing nation to introduce PCV7 (Rowland *et al.*, April 2009). However, in developing countries the current high price of the vaccine doses hinders the introduction of PCV7 (ACIP, 2000). There are reasons to believe that a single PCV7 dose has the potential to prevent a significant amount of invasive pneumococcal disease in children (Barzilay *et al.*, 2006; Whitney *et al.*, 2006). As the nasopharynx is the launching pad for pneumococcal disease, it is also of utmost importance to understand the effect of one dose in this niche. If proven efficacious, the use of a single vaccine dose may reduce the cost of vaccination sufficiently to facilitate introduction of PCV7 in more developing countries. To our best knowledge, the efficacy of a single dose of PCV7 on single and multiple colonization has not been evaluated, and studies on the effect of fewer than the recommended doses are scarce (Jones *et al.*, 2005; Revai *et al.*, 2006; van Gils *et al.*, 2009; van Kempen *et al.*, 2006). This evaluation should rely not only on the pneumococcal prevalence comparison among vaccinated and control groups, but also on the identification of the actual mechanism of the vaccine's effect (Rinta-Kokko *et al.*, 2009).

In this study we evaluated the impact of one PCV7 dose on single and multiple pneumococcal colonization in a group of children attending day-care centers, identifying the mechanisms of the vaccine's effect.

3.3. Materials and Methods

Study population. Eighty-five healthy children attending 5 day-care centers in the Lisbon area of Portugal were enrolled in this observational study of the effect of a single dose of PCV7 on pneumococcal colonization.

Vaccinated and control group allocation was based on three criteria — age between 12 and 24 months, same geographical area, and same social background. Children fulfilling the three requirements were included in the study. Those that were immunized with a

single PCV7 dose (69 children) constituted the vaccinated group, and those that received no vaccine (16 children) formed the control group.

In the vaccinated group, 38 children (55%) were males and 31 (45%) were females. In the control, seven children (44%) were males and nine (56%) were females.

Written and signed informed consent was obtained from parents or guardians of participating children for vaccination and sampling procedures.

Vaccine administration. PCV7 was provided by Wyeth Lederle Portugal (Farma), Lda. The vaccinated group was immunized with a single dose of the vaccine in May 2001. The intramuscular injection of 0.5 mL of vaccine was performed by a pediatric nurse in the deltoid muscle of the upper arm of each child.

Specimen collection. Pediatric nurses collected the nasopharyngeal specimens by use of calcium alginate swabs (BBL Culture Swab; Becton-Dickinson, Sparks MD). Swabs were inserted through the child's nostril until they touched the posterior nasopharynx, rotated 180 degrees, removed, placed in transport media (Stuart medium) and transported at room temperature to the Laboratory of Molecular Genetics at Instituto de Tecnologia Química e Biológica. Bacterial samples were processed within 4 h of collection (de Lencastre *et al.*, 1999).

Study design. Each child from the vaccinated and control groups was sampled in May and June 2001. In the vaccinated group, the first nasopharyngeal sample was collected immediately before immunization with a single PCV7 dose, in May 2001.

Nomenclature definitions. Children carrying pneumococcal isolates expressing only one capsular type (serotype) were designated as single carriers and children carrying more than one serotype were designated as multiple carriers. Among the latter, the serotype found in the majority of the isolates (>50%) was designated as the dominant serotype and the remaining serotypes were named minor serotypes. The ecological mechanisms that could be identified in this study were defined as follows: (i) clearance (disappearance of a pneumococcal isolate of a given serotype); (ii) *de novo* acquisition

(acquisition of a new pneumococcal isolate of a given serotype); (iii) unmasking (expansion of a minor serotype that becomes the dominant serotype); (iv) maintenance (maintenance of a given serotype) and (v) capsular switch (an isolate maintains its genotype/PFGE pattern, but presents a different serotype).

Pneumococcal isolation procedures. Each nasopharyngeal swab was streaked onto 5 µg/mL gentamicin-5% sheep blood triptic soy agar plate and incubated at 37℃ in 5% CO_2 atmosphere. Whenever available, up to 10 pneumococcal colonies were picked from this primary plate. Colonies were chosen randomly and any morphologically distinct colony was also picked. Colonies were re-streaked and cultivated on 5% sheep blood triptic soy agar and frozen at -80℃ in Mueller-Hinton broth containing 15% glycerol (v/v). Phenotypic characteristics (optochin susceptibility, morphology, and α-hemolysis) were used for presumptive pneumococci identification. The bile solubility assay was performed on suspected pneumococcal cultures exhibiting decreased susceptibility to optochin. These purified cultures were used in the subsequent assays.

Serotyping. All pneumococcal isolates were serotyped by the Quellung reaction using specific capsular antisera (Statens Seruminstitut, Copenhagen, Denmark) (Sorensen, 1993). Distinction between serotypes 6A and 6C was achieved by PCR using previously described primers (Park *et al.*, 2007a). Capsular types targeted by PCV7 (4, 6B, 9V, 14, 18C, 19F, and 23F) were classified as VT. Isolates expressing capsular types not included in PCV7 and non typeable isolates were classified as NVT.

Pulsed-field gel electrophoresis (PFGE). PFGE was performed according to a previously described protocol (Sá-Leão *et al.*, 2000b) after digestion of total DNA with *Sma*I (New England Biolabs) using as molecular weight standards the pneumococcal isolate R6 and the PFGE λ marker (New England Biolabs). In order to screen for putative capsular switch events, PFGE patterns of representative isolates were compared. To this end, one isolate for each serotype observed in a given child per sampling period was randomly selected.

Statistical analysis. Analysis of association between vaccination state and pneumococcal colonization was performed by calculating the odds ratio (OR), and statistical significance was assessed with χ^2 test or Fisher's exact test when appropriate. A maximum type I error of 0.05 was considered for recognition of a significant vaccination effect.

3.4. Results

All children of the vaccinated and control groups enrolled in this study yielded two nasopharyngeal swabs, the first in May 2001 and the second in June 2001. The average number of isolates per swab was 9 (range, 1-10) and the mode was 10. Overall, we isolated and serotyped 1,224 pneumococci, and the PFGE profile for representative isolates of each serotype was determined.

Prevalence of carrier children and pneumococcal isolates. In both the vaccinated and control groups the overall prevalence of single and multiple carrier children, as well as the number of pneumococcal isolates, was similar ($P > 0.05$) in the two sampling periods (Table 1).

Prevalence of VT and NVT isolates in single carriers. Regarding the vaccinated group, in May 2001 (pre-vaccine sampling period), among the 430 pneumococcal isolates recovered from single carriers, 13 serotypes were identified although four VT serotypes (6B, 14, 19F, and 23F) accounted for the majority of the isolates (60%) (Table 2). In June 2001, 1 month after vaccination with a single PCV7 dose, 14 serotypes were identified among the 430 pneumococcal isolates recovered. The frequency of VT serotypes decreased from 60 to 39%, while the frequency of NVT isolates increased from 40 to 61% ($P < 0.001$) (Table 2). Concerning the control group, in May 2001, among the 110 pneumococcal isolates recovered from single carriers, five serotypes were identified of which three VT serotypes (6B, 19F, and 23F) accounted for the majority of the isolates (64%) (Table 2). In June 2001, six serotypes were identified

among the 100 pneumococcal isolates recovered. The frequency of VT serotypes (6B, 14, 19F, and 23F) increased from 64 to 70%, while the frequency of NVT isolates decreased from 36 to 30% (*P* = 0.328) (Table 2).

Prevalence of VT and NVT isolates in multiple carriers. In the vaccinated group, among the 65 pneumococcal isolates recovered from multiple carriers in May 2001 (pre-vaccine), 10 serotypes were identified, of which four VT serotypes (6B, 14, 19F, and 23F) represented 45% of the isolates (Table 3). In June 2001, one month after the single dose, nine serotypes were identified among the 59 pneumococcal isolates, with VT serotypes (6B, 14, and 19F) decreasing from 45 to 19%, while NVT isolates increased from 55 to 81% (*P* = 0.002) (Table 3). In the control group, among the 20 pneumococcal isolates recovered from multiple carriers during May 2001, four serotypes were identified, of which VT serotypes (6B, 19F, and 23F) represented 95% of the isolates (Table 3). In June 2001, two serotypes were identified among the 10 pneumococcal isolates, with VT serotypes increasing from 95 to 100%, while NVT isolates decreased from 5 to 0% (*P* = 1) (Table 3).

Table 1. Children and pneumococcal isolates of the vaccinated and control groups.

Carriage status	No. of children (%)			No. of pneumococcal isolates (%)		
	May 2001	June 2001	*p*-Value	May 2001	June 2001	*p*-Value
Vaccinated group						
Single carriers (1 serotype)	48 (70)	48 (70)	1[a]	430 (87)	430 (88)	0.896[a]
Multiple carriers (2 serotypes)	5 (7)	4 (6)	1[b]	46 (9)	39 (8)	0.657[a]
Multiple carriers (3 serotypes)	2 (3)	2 (3)		19 (4)	20 (4)	
Non carriers	14 (20)	15 (21)	0.865[a]	0 (0)	0 (0)	-
Total	69 (100)	69 (100)	-	495 (100)	489 (100)	-
Control group						
Single carriers (1 serotype)	11 (69)	10 (63)	1[b]	110 (85)	100 (91)	0.704[a]
Multiple carriers (2 serotypes)	1 (6)	1 (6)	1[b]	10 (8)	10 (9)	0.193[a]
Multiple carriers (3 serotypes)	1 (6)	0 (0)		10 (8)	0 (0)	
Non carriers	3 (19)	5 (31)	1[b]	0 (0)	0 (0)	-
Total	16 (100)	16 (100)	-	130 (100)	110 (100)	-

[a] χ^2 test; .

[b] Fisher exact test.

Table 2. Serotypes found among pneumococcal isolates from single carriers of the vaccinated and control groups.

	No. of pneumococcal isolates (%)	
	May 2001 (%)	**June 2001 (%)**
Vaccinated group		
Vaccine types		
6B	54 (13)	50 (11.6)
14	53 (12)	37 (8.6)
19F	61 (14)	60 (14)
23F	88 (21)	22 (5.1)
Sub-total	256 (60)[a]	169 (39)[a]
Non vaccine types		
6A	20 (5)	30 (7)
7F	20 (5)	0 (0)
11A	20 (5)	49 (11.4)
15A	50 (12)	30 (7)
15B/C	10 (2)	17 (4)
16F	23 (5)	31 (7.2)
21	10 (1)	0 (0)
23A	0 (0)	10 (2.3)
23B	0 (0)	10 (2.3)
33F	10 (2)	20 (4.7)
38	0 (0)	20 (4.7)
NT	11 (3)	44 (10.2)
Sub-total	169 (40)[a]	261 (61)[a]
Total	430 (100)	430 (100)
Control group		
Vaccine types		
6B	30 (27.5)	20 (20)
14	0 (0)	30 (30)
19F	10 (9)	10 (10)
23F	30 (27.5)	10 (10)
Sub-total	70 (64)[b]	70 (70)[b]
Non vaccine types		
6A	20 (18)	20 (20)
19A	20 (18)	10 (10)
Sub-total	40 (36)[b]	30 (30)[b]
Total	110 (100)	100 (100)

[a] p-Value < 0.001 by χ^2 test.

[b] p-Value = 0.328 by χ^2 test.

Table 3. Serotypes found among pneumococcal isolates from multiple carriers of the vaccinated and control groups.

	No. of pneumococcal isolates (%)	
	May 2001 (%)	**June 2001 (%)**
Vaccinated group		
Vaccine types		
6B	8 (12.3)	5 (9)
14	5 (7.7)	4 (7)
19F	10 (15.4)	2 (3)
23F	6 (9.2)	0 (0)
Sub-total	29 (45)[a]	11 (19)[a]
Non vaccine types		
6A	15 (23)	14 (24)
11A	0 (0)	8 (14)
16F	0 (0)	12 (20)
17F	1 (1)	6 (10)
19A	2 (3)	0 (0)
23B	3 (5)	0 (0)
33F	9 (14)	1 (1)
38	6 (9)	0 (0)
NT	0 (0)	7 (12)
Sub-total	36 (55)[a]	48 (81)[a]
Total	65 (100)	59 (100)
Control group		
Vaccine types		
6B	9 (45)	0 (0)
19F	7 (35)	2 (20)
23F	3 (15)	8 (80)
Sub-total	19 (95)[b]	10 (100)[b]
Non vaccine types		
7F	1 (5)	0 (0)
Sub-total	1 (5)[b]	0 (0)[b]
Total	20 (100)	10 (100)

[a] *p*-Value = 0.002 by χ^2 test.

[b] *p*-Value = 1 by Fisher exact test.

VT and NVT co-colonization condition. Among the vaccinated group, in May 2001, co-colonization with VT isolates was detected in five out of seven multiple carriers, of which four presented the VT as the dominant serotype. In June 2001, co-colonization with VT isolates was detected in four out of six multiple carriers, with the VT being identified always as a minor serotype (Fig. 1, children A to K). Regarding the control, in May 2001, co-colonization with VT isolates was detected in two children who presented VTs as the dominant serotypes. In June 2001, co-colonization was detected only once and two VT serotypes were found in association (23F – dominant serotype; 19F – minor serotype) (Fig. 1, children L and M). Serotype 6A was the most common serotype found among multiple carriers — it was found co-colonizing with 19F (three occasions), 6B (two occasions), and 14, 19A and non typeable isolates (one occasion).

Comparison of PFGE profiles. Overall we compared 174 PFGE profiles of representative isolates of each of the serotypes found among the vaccinated (124 isolates) and control (50 isolates) groups and no capsular switch phenomenon was detected. In the group where the vaccine pressure was present, no vaccine escapee recombinant isolate was observed and the NVT PFGE profiles were found to differ from the preceding VT serotypes. A few examples of the PFGE profiles analyzed are shown in Figure 2.

Ecological mechanism of the vaccine's effect. By observing the colonization pattern change from May to June 2001 among children of the vaccinated and control groups, we were able to assess the number of isolates that were cleared, *de novo* acquired, unmasked or maintained (Fig. 3).

Figure 1. Serotype associations found in the vaccinated and control groups. Vaccine types are represented in red and non vaccine types or non carriage in black. Between parentheses is the number of pneumococcal isolates per serotype. Children A through K are from the vaccinated group, L and M are from the control group.

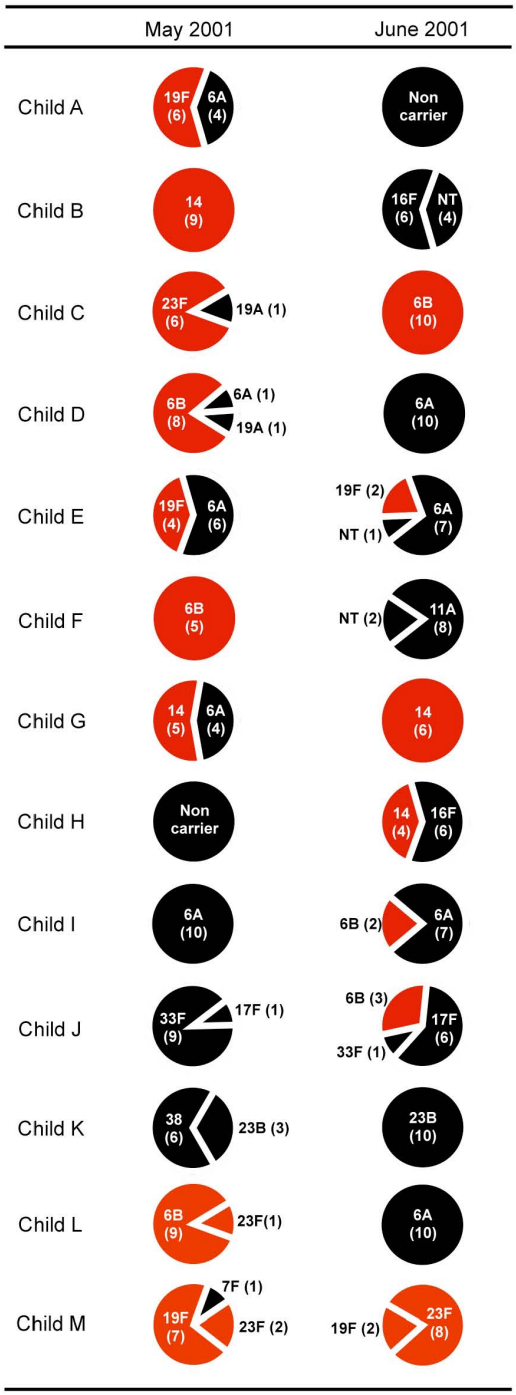


Figure 2. Examples of pulsed-field gel electrophoresis profiles found among children of the vaccinated group in May and June 2001. Children A, B, C and D are the same as in Figure 1.

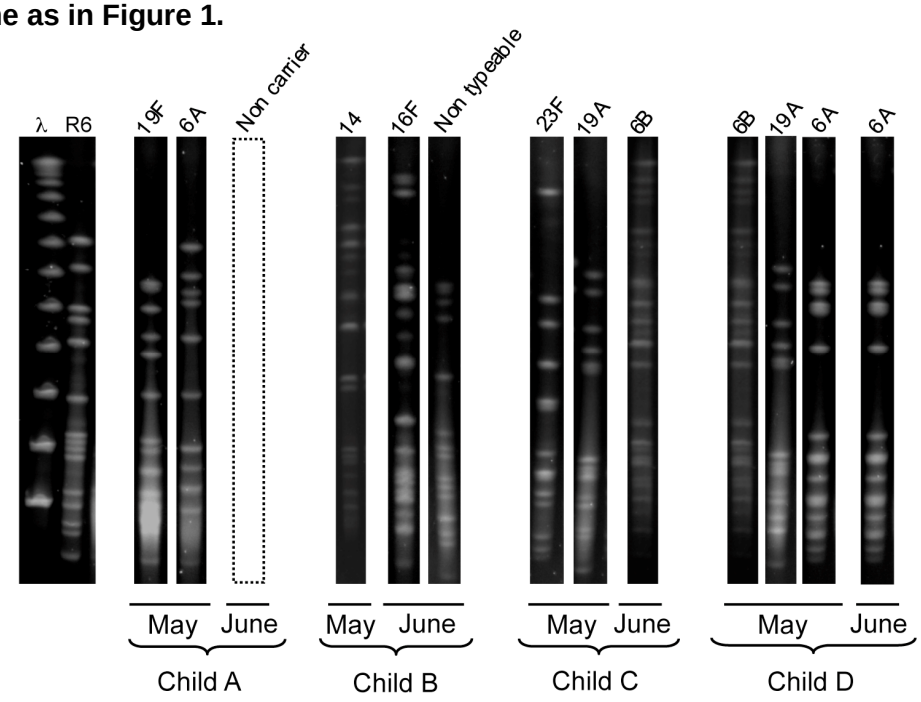


Figure 3. Examples of colonization pattern changes and respective ecological mechanisms. Red and black circles represent a given vaccine type (VT) or non vaccine type (NVT) pneumococcal isolate, respectively. Children N through Q are single carriers, child D is the same multiple carrier of Figure 1.

Pneumococcal isolates	Ecological mechanisms
May 2001 ●●●●●●●●●● June 2001 ●●●●●●●●●●	Child N – Maintenance of 10 VT isolates (serotype 14).
May 2001 ●●●●●●●●●● June 2001 Non carrier	Child O – Clearance of 10 VT isolates (serotype 6B).
May 2001 Non carrier June 2001 ●●●●●●●●●●	Child P – De novo acquisition of 10 NVT isolates (serotype 11A).
May 2001 ●●●●●●●●●● June 2001 ●●●●●●●●●●	Child Q – Clearance of 10 VT isolates (serotype 6B) and de novo acquisition of 10 NVT isolates (serotype 6A).
May 2001 ●●●●●●●●●● June 2001 ●●●●●●●●●●	Child D – Clearance of 8 VT isolates (serotype 6B) and 1 NVT isolate (serotype 19A). Maintenance of 1 NVT isolate (serotype 6A), and unmasking (serotype expansion) of 9 NVT isolates (serotype 6A).

Table 4. Ecological mechanisms of the vaccine's effect.

Pn isolates[a]	Clearance				*de novo* acquisition				Unmasking			
	Control (%)	Vaccinated (%)	OR (95% CI)	*p*-Value	Control (%)	Vaccinated (%)	OR (95% CI)	*p*-Value	Control (%)	Vaccinated (%)	OR (95% CI)	*p*-Value
VT	56(65)	212(62)	1.12 (0.68-1.84)	0.635[b]	40(67)	107(34)	4.80 (2.81-8.24)	<0.001[b]	6(100)	0(0)	-	<0.001[c]
NVT	30(35)	128(38)			20(33)	206(66)			0(0)	21(100)		
Total	86(100)	340(100)	-	-	60(100)	313(100)	-	-	6(100)	21(100)	-	-

[a] Pneumococcal isolates from single and multiple carriers from vaccinated and control groups were included in this analysis. OR, odds ratio; CI, confidence interval; VT, vaccine type; NVT, non vaccine type. [b] χ^2 test. [c] Fisher exact test.

Bearing in mind that PCV7 targets directly VT and indirectly NVT isolates, the effect of the vaccine on pneumococcal carriage was explored based on three potential mechanisms: prevention of VT *de novo* acquisition, enhancement of VT clearance, and enhancement of NVT unmasking. We compared these three mechanisms capable of affecting pneumococcal colonization between vaccinated and control groups to identify those that could explain the vaccine's effect.

Serotype clearance was similar between VT and NVT isolates among the vaccinated and control groups (P = 0.635). VT and NVT isolates were equally probable to be cleared in both groups (OR, 1.12; 95% confidence interval (CI), 0.68-1.84) (Table 4). *De novo* acquisition of VT isolates was drastically lower in the vaccinated group (34%) when compared with the control (67%). VT isolates were almost five times less likely to be acquired *de novo* in the vaccinated than in the control group (OR, 4.80; 95% CI, 2.81-8.24) (Table 4). Unmasking of NVTs was inexistent in the control and reached 100% in the vaccinated group (P < 0.001) (Table 4).

3.5. Discussion

Epidemiological studies in numerous countries have demonstrated the replacement of VT by NVT isolates in the nasopharynx of children immunized with a multi-valent pneumococcal conjugate vaccine (Cohen *et al.*, 2006; Dagan, 2002; Finkelstein *et al.*, 2003; Frazão *et al.*, 2005; O'Brien *et al.*, 2007; Sá-Leão *et al.*, 2009). The nasopharynx is the immediate source of disease-causing pneumococci and the appearance of NVT isolates with pathogenic potential has raised concerns (Gonzalez *et al.*, 2006; Pai *et al.*, 2005). In 2006, Barzilay et al. reported a 62% reduction in invasive pneumococcal disease caused by vaccine types in children immunized with a single PCV7 dose at 5-8 months of age (Barzilay *et al.*, 2006). In the same year, a matched case-control study observed a 93% effectiveness of a single PCV7 dose in children vaccinated at 12-23 months of age (Whitney *et al.*, 2006). However, the effect on nasopharyngeal colonization — the launching pad for pneumococcal disease — was not assessed.

The present study evaluated the effect of a single dose of PCV7 on the nasopharyngeal carriage of pneumococci in day-care center attendees in Lisbon, Portugal, i.e., a study population in which the pneumococcal carriage rates are known to be high (Cherian *et al.*, 1994; Dagan *et al.*, 1996b; Levine *et al.*, 1999; Takala *et al.*, 1995). Immunized children in this study were between 12 and 24 months, an age at which a single dose showed 93% effectiveness regarding invasive disease caused by vaccine types (Whitney *et al.*, 2006). Multiple pneumococcal isolates were analyzed, enabling the study of ecological phenomena that contribute to the serotype changes in the nasopharynx.

At the population level, although the overall number of pneumococcal isolates from single and multiple carriers was similar in both sampling periods in the vaccinated and control groups (Table 1), differences became apparent once the isolates were divided into VTs and NVTs. In the vaccinated group, within a month, a single PCV7 dose led to a serotype replacement phenomenon between VT and NVT isolates, both in single and multiple carriers, in contrast to the control where no replacement phenomenon was detected (Table 2 and 3).

At the individual level, a serotype replacement event could also be observed. After vaccination with a single dose, with the exception of two children, VT isolates were not present or were found as minor serotypes and, in parallel, NVTs were detected as dominant serotypes (Fig. 1, children A to K).

We show that a serotype replacement phenomenon took place one month after a single dose of PCV7, not only at the population but also at the individual level where vaccine types became minor serotypes co-colonizing with the emergent NVTs. Competition between serotypes in vaccinated children leads to serotype replacement of VT by NVT serotypes (Auranen *et al.*, 2010). The rise of NVT serotypes in colonization raises concern, particularly among children with underlying medical conditions, as they can indeed become disease agents rendering disease replacement a reality (Dagan, 2009).

Based on PFGE profile analyses, no capsular switch events were detected and thus no evidence was found in our study of vaccine escape recombinant isolates as reported by Bruegemann et al. in 2007 (Brueggemann *et al.*, 2007). However, it should be noted that the failure to detect capsular switch events could be linked to the relatively small sample size of 174 PFGE profiles.

In the present study, besides the pneumococcal prevalence comparisons that allowed detection of the known serotype replacement phenomenon between VT and NVT isolates (Tables 2 and 3), we actually identified the mechanism of the vaccine's effect in our setting. We show that within a month, in children aged between 12 and 24 months, a single dose of PCV7 decreases VT colonization as it prevents de novo acquisition, and conversely increases NVT colonization, namely by enhancing NVT unmasking (Table 4). Our data is in accordance with previous studies, which suggest that conjugate vaccines reduce VT carriage by preventing de novo acquisition rather than clearance (Bogaert *et al.*, 2004b; Dagan *et al.*, 2003; Klugman, 2001; Whitney *et al.*, 2006). Besides this major mechanism of the vaccine's effect we propose that an additional one is the enhancement of NVT unmasking (Table 4). Assessment of this last mechanism was only possible due to the study of multiple colonization.

As a result of the paucity of multiple carriers, we were unable to conclude about a specific tendency of serotype associations before and after a single vaccine dose. Nevertheless, we found that 13 serotypes (6A, 6B, 7F, 11A, 14, 16F, 17F, 19A, 19F, 23B, 23F, 33F, and 38) and non typeable isolates were able to co-colonize, associating with other serotypes in the children's nasopharynx. In the vaccinated group, serotype 6A was the most common serotype observed among multiple carriers. Worthy of note is the fact that in the PCV7 era, the nasopharynx of multiple carriers can constitute a reservoir for VT isolates. Some VTs (e.g., 6B, 14 and 19F) prevailed as minor serotypes "masked" by the dominant NVT isolates, in opposition to what occurred in the control. Whether or not the preferred co-existence of some serotypes reflects similarity of their chemical structures, similar nutritional requirements and/or bacteriocin compatibility (Dawid *et al.*, 2007) of the particular isolates remains to be determined.

In summary, the present study demonstrates that, as early as 1 month after vaccination with a single dose, PCV7 causes serotype replacement of VT by NVT isolates in single and multiple carriers, with the mechanisms of the vaccine's effect being the prevention of VT *de novo* acquisition and enhancement of NVT unmasking. In the PCV7 era, the assessment of nasopharyngeal changes among single and multiple carriers, together with insight on the mechanism of the vaccine's effect and information on pneumococcal virulence, will open new avenues to anticipate the long-term effect of pneumococcal conjugate vaccines on colonization and potentially prevent pneumococcal disease. Epidemiological studies accounting for multiple colonization can provide a more precise picture of the serotypes colonizing the nasopharynx, which can then be tested in developing animal models. This approach may help predict the virulence potential of these serotypes for their inclusion in pneumococcal vaccines even before they become major disease agents in humans.

3.6. Acknowledgments

This work was supported by projects GRACE (contract LSHMCT-2005-518226) and PNEUMOPATH (contract HEALTH-F3-2009-222983) from the European Commission and project PTDC/SAUESA/65048/2006 from Fundação para a Ciência e a Tecnologia (FCT). N.F. was supported by FCT grant SFRH/BD/30103/2006.

We gratefully acknowledge the directors, staff, parents and children at the participating day-care centers. We thank R. Mato, I. Santos-Sanches, A. Brito-Avô, J. Saldanha, S. Nunes, N. Sousa, C. Simas, A. Gonçalves and P. Gonzaga for participating in studies that led to the isolation and initial characterization of the pneumococcal collection described here. We would like to thank A. Tomasz for help with the study design, interpretation of the results and revision of the manuscript and F. Pinto for discussions on statistical analysis.

N. Frazão was responsible for the global data analysis and experimental work except collection of the isolates.

3.7. References

ACIP (2000). Preventing pneumococcal disease among infants and young children: recommendations of the Advisory Commitee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* **49**, 1-35.

Auranen, K., Mehtala, J., Tanskanen, A. & M, S. K. (2010). Between-strain competition in acquisition and clearance of pneumococcal carriage--epidemiologic evidence from a longitudinal study of day-care children. *Am J Epidemiol* **171**, 169-176.

Barzilay, E. J., O'Brien, K. L., Kwok, Y. S., Hoekstra, R. M., Zell, E. R., Reid, R., Santosham, M., Whitney, C. G. & Feikin, D. R. (2006). Could a single dose of pneumococcal conjugate vaccine in children be effective? Modeling the optimal age of vaccination. *Vaccine* **24**, 904-913.

Black, S., Shinefield, H., Fireman, B., Lewis, E., Ray, P., Hansen, J. R., Elvin, L., Ensor, K. M., Hackell, J., Siber, G., Malinoski, F., Madore, D., Chang, I., Kohberger, R., Watson, W., Austrian, R. & Edwards, K. (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.

Bogaert, D., De Groot, R. & Hermans, P. W. (2004a). *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* **4**, 144-154.

Bogaert, D., van Belkum, A., Sluijter, M., Luijendijk, A., de Groot, R., Rumke, H. C., Verbrugh, H. A. & Hermans, P. W. (2004b). Colonisation by *Streptococcus pneumoniae* and Staphylococcus aureus in healthy children. *Lancet* **363**, 1871-1872.

Brueggemann, A. B., Pai, R., Crook, D. W. & Beall, B. (2007). Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. *PLoS Pathog* **3**, e168.

CDC (2005). Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease: United States, 1998–2003. *MMWR Morb Mortal Wkly Rep* **54**, 893-897.

Cherian, T., Steinhoff, M. C., Harrison, L. H., Rohn, D., McDougal, L. K. & Dick, J. (1994). A cluster of invasive pneumococcal disease in young children in child care. *Jama* **271**, 695-697.

Cohen, R., Levy, C., de La Rocque, F., Gelbert, N., Wollner, A., Fritzell, B., Bonnet, E., Tetelboum, R. & Varon, E. (2006). Impact of pneumococcal conjugate vaccine and of reduction of antibiotic use on nasopharyngeal carriage of nonsusceptible pneumococci in children with acute otitis media. *Pediatr Infect Dis J* **25**, 1001-1007.

Dagan, R., Melamed, R., Muallem, M., Piglansky, L., Greenberg, D., Abramson, O., Mendelman, P. M., Bohidar, N. & Yagupsky, P. (1996a). Reduction of nasopharyngeal carriage of pneumococci during the second year of life by a heptavalent conjugate pneumococcal vaccine. *J Infect Dis* **174**, 1271-1278.

Dagan, R., Melamed, R., Muallem, M., Piglansky, L. & Yagupsky, P. (1996b). Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. *J Infect Dis* **174**, 1352-1355.

Dagan, R. (2002). Immunisation with a pneumococcal 7-valent conjugate vaccine. *Int J Clin Pract* **56**, 287-291.

Dagan, R., Givon-Lavi, N., Zamir, O. & Fraser, D. (2003). Effect of a nonavalent conjugate vaccine on carriage of antibiotic-resistant *Streptococcus pneumoniae* in day-care centers. *Pediatr Infect Dis J* **22**, 532-540.

Dagan, R. (2009). Pneumococcal conjugate vaccines probe studies: the solution points to the problem. *Adv Exp Med Biol* **634**, 69-77.

Dawid, S., Roche, A. M. & Weiser, J. N. (2007). The blp bacteriocins of *Streptococcus pneumoniae* mediate intraspecies competition both *in vitro* and *in vivo*. *Infect Immun* **75**, 443-451.

de Lencastre, H., Kristinsson, K. G., Brito-Avô, A., Sanches, I. S., Sá-Leão, R., Saldanha, J., Sigvaldadottir, E., Karlsson, S., Oliveira, D., Mato, R., Aires de Sousa, M. & Tomasz, A. (1999). Carriage of respiratory tract pathogens and molecular epidemiology of *Streptococcus pneumoniae* colonization in healthy children attending day-care centers in Lisbon, Portugal. *Microb Drug Resist* **5**, 19-29.

Faden, H., Duffy, L., Wasielewski, R., Wolf, J., Krystofik, D. & Tung, Y. (1997). Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. *J Infect Dis* **175**, 1440-1445.

Finkelstein, J. A., Huang, S. S., Daniel, J., Rifas-Shiman, S. L., Kleinman, K., Goldmann, D., Pelton, S. I., DeMaria, A. & Platt, R. (2003). Antibiotic-resistant *Streptococcus pneumoniae* in the heptavalent pneumococcal conjugate vaccine era: predictors of carriage in a multicommunity sample. *Pediatrics* **112**, 862-869.

Frazão, N., Brito-Avô, A., Simas, C., Saldanha, J., Mato, R., Nunes, S., Sousa, N. G., Carriço, J. A., Almeida, J. S., Santos-Sanches, I. & de Lencastre, H. (2005). Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon. *Pediatr Infect Dis J* **24**, 243-252.

Gonzalez, B. E., Hulten, K. G., Lamberth, L., Kaplan, S. L. & Mason, E. O., Jr. (2006). *Streptococcus pneumoniae* serogroups 15 and 33: an increasing cause of pneumococcal infections in children in the United States after the introduction of the pneumococcal 7-valent conjugate vaccine. *Pediatr Infect Dis J* **25**, 301-305.

Gray, B. M., Converse, G. M., 3rd & Dillon, H. C., Jr. (1979). Serotypes of *Streptococcus pneumoniae* causing disease. *J Infect Dis* **140**, 979-983.

Gray, B. M., Converse, G. M., 3rd & Dillon, H. C., Jr. (1980). Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* **142**, 923-933.

Hare, K. M., Morris, P., Smith-Vaughan, H. & Leach, A. J. (2008). Random colony selection versus colony morphology for detection of multiple pneumococcal serotypes in nasopharyngeal swabs. *Pediatr Infect Dis J* **27**, 178-180.

Jones, V. F., Harrison, C., Stout, G. G. & Hopkins, J. (2005). Nasopharyngeal colonization with heptavalent pneumococcal conjugate vaccine serotypes of *Streptococcus pneumoniae* with prolonged vaccine dosing intervals. *Pediatr Infect Dis J* **24**, 969-973.

Klugman, K. P. (2001). Efficacy of pneumococcal conjugate vaccines and their effect on carriage and antimicrobial resistance. *Lancet Infect Dis* **1**, 85-91.

Levine, O. S., Farley, M., Harrison, L. H., Lefkowitz, L., McGeer, A. & Schwartz, B. (1999). Risk factors for invasive pneumococcal disease in children: a population-based case-control study in North America. *Pediatrics* **103**, E28.

O'Brien, K. L., Moulton, L. H., Reid, R., Weatherholtz, R., Oski, J., Brown, L., Kumar, G., Parkinson, A., Hu, D., Hackell, J., Chang, I., Kohberger, R., Siber, G. & Santosham, M. (2003). Efficacy and safety of seven-valent conjugate pneumococcal vaccine in American Indian children: group randomised trial. *Lancet* **362**, 355-361.

O'Brien, K. L., Millar, E. V., Zell, E. R., Bronsdon, M., Weatherholtz, R., Reid, R., Becenti, J., Kvamme, S., Whitney, C. G. & Santosham, M. (2007). Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. *J Infect Dis* **196**, 1211-1220.

Pai, R., Moore, M. R., Pilishvili, T., Gertz, R. E., Whitney, C. G. & Beall, B. (2005). Postvaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States. *J Infect Dis* **192**, 1988-1995.

Park, I. H., Park, S., Hollingshead, S. K. & Nahm, M. H. (2007). Genetic basis for the new pneumococcal serotype, 6C. *Infect Immun* **75**, 4482-4489.

Revai, K., McCormick, D. P., Patel, J., Grady, J. J., Saeed, K. & Chonmaitree, T. (2006). Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. *Pediatrics* **117**, 1823-1829.

Rinta-Kokko, H., Dagan, R., Givon-Lavi, N. & Auranen, K. (2009). Estimation of vaccine efficacy against acquisition of pneumococcal carriage. *Vaccine* **27**, 3831-3837.

Rowland, J., Buss, J., Gordon, L. & Ngabo, F. (April 2009). Rwanda Becomes First Developing Nation to Introduce Vaccine for World's Leading Infectious Child Killer. http://www.wyeth.com/news?nav=display&navTo=/wyeth_html/home/news/pressreleases/2009/1240494621675.html.

Sá-Leão, R., Tomasz, A., Sanches, I. S., Nunes, S., Alves, C. R., Avô, A. B., Saldanha, J., Kristinsson, K. G. & de Lencastre, H. (2000). Genetic diversity and clonal patterns among antibiotic-susceptible and -resistant *Streptococcus pneumoniae* colonizing children: day-care centers as autonomous epidemiological units. *J Clin Microbiol* **38**, 4137-4144.

Sá-Leão, R., Tomasz, A., Santos Sanches, I. & de Lencastre, H. (2002). Pilot study of the genetic diversity of the pneumococcal nasopharyngeal flora among children attending day-care centers. *J Clin Microbiol* **40**, 3577-3585.

Sá-Leão, R., Nunes, S., Brito-Avô, A., Frazão, N., Simões, A. S., Crisóstomo, M. I., Paulo, A. C., Saldanha, J., Santos-Sanches, I. & de Lencastre, H. (2009). Changes in pneumococcal serotypes and antibiotypes carried by vaccinated and unvaccinated day-care centre attendees in Portugal, a country with widespread use of the seven-valent pneumococcal conjugate vaccine. *Clin Microbiol Infect* **15**, 1002-1007.

Sorensen, U. B. (1993). Typing of pneumococci by using 12 pooled antisera. *J Clin Microbiol* **31**, 2097-2100.

Takala, A. K., Jero, J., Kela, E., Ronnberg, P. R., Koskenniemi, E. & Eskola, J. (1995). Risk factors for primary invasive pneumococcal disease among children in Finland. *Jama* **273**, 859-864.

van Gils, E. J., Veenhoven, R. H., Hak, E., Rodenburg, G. D., Bogaert, D., Ijzerman, E. P., Bruin, J. P., van Alphen, L. & Sanders, E. A. (2009). Effect of reduced-dose schedules with 7-valent pneumococcal conjugate vaccine on nasopharyngeal pneumococcal carriage in children: a randomized controlled trial. *Jama* **302**, 159-167.

van Kempen, M. J., Vermeiren, J. S., Vaneechoutte, M., Claeys, G., Veenhoven, R. H., Rijkers, G. T., Sanders, E. A. & Dhooge, I. J. (2006). Pneumococcal conjugate vaccination in children with recurrent acute otitis media: a therapeutic alternative? *Int J Pediatr Otorhinolaryngol* **70**, 275-285.

Whitney, C. G., Farley, M. M., Hadler, J., Harrison, L. H., Bennett, N. M., Lynfield, R., Reingold, A., Cieslak, P. R., Pilishvili, T., Jackson, D., Facklam, R. R., Jorgensen, J. H. & Schuchat, A. (2003). Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* **348**, 1737-1746.

Whitney, C. G., Pilishvili, T., Farley, M. M., Schaffner, W., Craig, A. S., Lynfield, R., Nyquist, A. C., Gershman, K. A., Vazquez, M., Bennett, N. M., Reingold, A., Thomas, A., Glode, M. P., Zell, E. R., Jorgensen, J. H., Beall, B. & Schuchat, A. (2006). Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. *Lancet* **368**, 1495-1502.

WHO (1999). Pneumococcal vaccines. *Wkly Epidemiol Rec* **74**, 177-183.

Chapter IV

4. Virulence potential of serotypes selected *in vivo* by the 7-valent pneumococcal conjugate vaccine

4.1. Abstract

The first step for *Streptococcus pneumoniae* disease is the establishment of colonization. Using epidemiological studies to identify the major serotypes selected by the 7-valent pneumococcal conjugate vaccine (PCV7) in the nasopharynx of children is of utmost importance as this provides an indicator of the future pneumococcal disease causing serotypes.

The more prevalent and drug resistant non-PCV7 serotypes (6A-ST2191, 15A-ST63 and 19A-ST276) isolated from the nasopharynx of vaccinated children in Portugal were tested in mouse models to mimic colonization and predict virulence potential. Steady colonization with 6A, 15A and 19A serotypes was found to result in the invasion and infection of adjacent tissues, such as the olfactory bulbs, brain, lungs and the middle ear mucosa. If high bacterial concentrations were introduced into the lung, all the non-PCV7 serotypes were lethal. Serotype 19A-ST276 was the only strain to show propensity to cause blood infection. Experiments with laboratory-generated type 3 capsular transformants of the NVT strains 6A and 19A indicate that: i) colonization ability seems to depend not only on the capsular type, but also on the genetic background, and ii) the capsular type 3 has a major virulence enhancing effect, which is not dependent upon the genetic background of 6A and 19A parental strains.

4.2. Introduction

The first step for *Streptococcus pneumoniae* disease is the establishment of colonization, i.e., the creation of the carrier state, which can occur in over 70% of young children (Mato *et al.*, 2005). Worldwide, and particularly in developing countries, pneumococcal disease causes significant morbidity and mortality among children (Greenwood *et al.*, 2007; Mulholland, 2007). A recent report showed that *S. pneumoniae* causes around 11% of all deaths in children aged under 5 years old (O'Brien *et al.*, 2009). The spectrum of pneumococcal disease can range from non-invasive, mucosal

disease such as otitis media to severe, invasive infections such as sepsis (blood stream infection), bacteremic pneumonia and meningitis (Bogaert *et al.*, 2004a; Fletcher & Fritzell, 2007).

The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) had a dramatic impact on: i) disease caused by the PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F), which was greatly reduced in children, the population targeted by the vaccine, and also among the elderly as a consequence of herd immunity (CDC, 2005; Whitney *et al.*, 2003), and ii) colonization, where the PCV7 serotypes were replaced by non-PCV7 serotypes, that nowadays emerge as the dominant nasopharynx colonizers (Dagan *et al.*, 1996b; Dagan, 2002; Frazão *et al.*, 2005; Frazão *et al.*, 2010; Sá-Leão *et al.*, 2009). As shown by Lloyd-Evans and colleagues (Lloyd-Evans *et al.*, 1996), pneumococcal disease originates from nasopharyngeal colonization with the homologous serotype. Therefore, the non-PCV7 serotype distribution in the nasopharynx should be used as an indicator of potential disease-causing serotypes, as well as antibiotic resistance profiles and vaccine coverage.

In 2001-2003, the first epidemiological study in Portugal on the impact of the PCV7 on colonization and antimicrobial resistance shed light on the non-PCV7 serotype flora colonizing the nasopharynx of vaccinated children (Frazão *et al.*, 2005). Drug resistant pneumococcal strains isolated from the vaccinated group were serotyped and genetically characterized by pulsed-field gel electrophoresis (PFGE). After vaccination, drug resistant PCV7 serotypes were replaced by drug resistant non-PCV7 serotypes presenting different PFGE patterns. The most common drug resistant non-PCV7 capsular types were 6A, 15A, and 19A (Frazão *et al.*, 2005).

For the present study, these three serotypes – which together had accounted for 82% of the total capsulated drug resistant non-PCV7 strains isolated – were selected for characterization in experimental animal models. The goal was to reproduce the capacity to colonize and predict the infection potential of the pneumococcal non-PCV7 serotypes isolated from the nasopharynx of vaccinated children. Moreover, the effect of capsular

type on colonization and blood infection was investigated using otherwise isogenic capsular type 3 transformants.

We provide evidence that nasopharyngeal colonization with the non-PCV7 serotypes (6A, 15A, and 19A) results in invasion and infection of adjacent tissues, such as the olfactory bulbs, brain, lungs and the middle ear mucosa. All non-PCV7 serotypes caused fatal infection when a high bacterial load was reached in the lungs. Serotype 19A was the only serotype to show propensity to grow in the blood. Expression of the type 3 capsule in a 19A genetic background seems to diminish the capacity to colonize, while the 6A and 19A genetic backgrounds become highly virulent and cause fatal blood infection when expressing the capsular type 3.

4.3. Materials and Methods

Pneumococcal strains. Our studies used three *S. pneumoniae* representative strains exhibiting capsular types 6A, 15A, and 19A selected from the three major drug resistant serotypes found among PCV7 vaccinated children in Portugal (Fig.1 and Table 1). In order to select the above mentioned representative strains three selection criteria were used: i) belonging to one of the three major drug resistant non-PCV7 serotypes (Fig. 1), ii) belonging to the PFGE clonal type with the highest penicillin MIC range, and iii) strain with the highest penicillin MIC (Table 1). Capsular transformants exhibiting the type 3 capsule were generated using 6A and 19A as the parental strains. The transforming DNA for the above cited transformations was from the SV35T3 strain, which contains the capsular type 3 and a spectinomycin resistance cassette for selection. Bacteria were cultured in either C+Y broth (Kharat & Tomasz, 2006), Todd-Hewitt broth, Triptic Soy broth (TSB) or Triptic Soy agar (TSA) at 37℃ without shaking. For animal studies, bacteria were grown in C+Y medium to an OD_{590} of 0.8, centrifuged, and then resuspended in a sterile saline solution (0.9% NaCl) to obtain the desired bacterial concentration. In no case were the above mentioned pneumococcal isolates passaged in mice before use in any animal experiment.

Antimicrobial susceptibility testing. The non-PCV7 strains (6A, 15A, and 19A) were tested against a panel of nine antimicrobials by disk diffusion assay (oxacillin, chloramphenicol, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole and levofloxacin) and e-test (penicillin and ceftriaxone) as described previously (Frazão *et al.*, 2005). Drug resistant non-PCV7 strains are resistant to at least one of the antimicrobials tested: penicillin, chloramphenicol, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole, levofloxacin, and ceftriaxone.

Pulsed-field gel electrophoresis (PFGE). PFGE was performed after digestion of genomic DNA with *Sma*I using pneumococcal strain R6 and PFGE λ marker (New England Biolabs) as molecular weight standards according to an earlier protocol (Sá-Leão *et al.*, 2000b). PFGE-based clonal types were defined as isolates with ≥80% relatedness on the dendrogram analyzed using the Bionumerics software (Applied-Maths, Sint-Martens-Latem, Belgium).

Multi-locus sequence typing (MLST). MLST was performed as previously described (Sá-Leão *et al.*, 2006).

Capsule switch methods. Competent cells were prepared by growing bacteria in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) until OD_{590}=0.07-0.08. To carry out transformation, competent cells were diluted 1:20 in competence medium (TSB [pH 8.0], 0.16% bovine serum albumin, 0.01% $CaCl_2$) containing the competent stimulating peptide (CSP, ~500 ng) and whole genome transforming DNA (1,000 ng/mL). CSP1 and CSP2 were used to transform pneumococcal strains 6A and 19A, respectively. The transforming DNA was purified from strain SV35T3. The transformation reaction (1 mL) was held 4 h at 37℃ in an 1.5 mL eppendorf tube and then challenged in TSA plates supplemented with 5% sheep blood and spectinomycin (125 µg/mL). Plates were incubated overnight at 37°C with 5% CO_2. Transformants were confirmed by the typical type 3 mucoid colony morphology, serotyping with specific sera (Sorensen, 1993), and PCR amplification of the capsular type 3 operon using primers 3F – ATGGTGTGATTTCTCCTAGATTGGAAAGTAG and 3R –

CTTCTCCAATTGCTTACCAAGTGCAATAACG from Pai and co-workers (Pai *et al.*, 2006).

Mice. The protocol for the animal experiments was approved by the Institutional Review Board (IRB) of The Rockefeller University (Permit Number: 09073). Groups of 8-week-old female CD1 outbred mice obtained from the Charles River Laboratories (Charles River Laboratories, Wilmington, MA) were used in colonization, lung and blood infection models. During the experimental period all mice were monitored on a daily basis for survival. Whenever necessary, an intraperitoneal (i.p.) injection of 75 µL of a xylazine and ketamine mixture was used to anesthetize the animals (Kharat & Tomasz, 2006). CFU numbers inoculated in the mice were confirmed by colony count of serial dilutions on TSA supplemented with 5 µg/mL gentamicin. For sampling procedures mice were humanely euthanized by CO_2 asphyxiation. Mice were given food and water *ad libitum*.

Colonization model. Each anesthetized mouse was inoculated intranasally with 10 µL of inoculum containing 10^8 CFU using a 20-µL micropipette. At defined time points after the challenge (2, 7, 14, and 21 days) groups of mice were humanely euthanized and bacterial numbers were assessed in the nasopharyngeal and middle ear mucosa, olfactory bulbs, brain, lungs, and blood. Mouse perfusion was performed before the lungs, olfactory bulbs, and brain were harvested in order to avoid possible CFU contamination from the blood.

Lung infection model. Each anesthetized mouse was inoculated intranasally with 50 µL of inoculum containing 10^5, 10^7, or 10^8 CFU using a 100-µL micropipette. At defined time points (2 and 5 h), after the intranasal challenge with a 10^8 CFU inoculum, groups of mice were humanely euthanized and bacterial numbers were assessed in the lungs, and blood.

Blood infection model. Each mouse was injected intraperitoneally with 500 µL of inoculum containing 10^5 CFU. At defined time points after the intraperitoneal injection (0.5, 3, 6, 12, and 24 h) two to three mice were humanely euthanized and bacterial numbers were assessed in the blood.

Sample collection. To perform the nasopharyngeal wash the trachea was cannulated and 500 µL of a saline solution were collected through the mouse's nose. To sample the middle ear mucosa we adapted the procedure used by Lai and colleagues (Lai *et al.*, 1986). In brief, we injected into the middle ear cavity of each ear 10 µL of sterile saline solution by using a 10-µL micropipette which was inserted through the mouse tympanic bullae. The saline solution was withdrawn and reintroduced three times (final volume recovered, 13 µL/2 ears). Blood collection (300 µL) was done by heart puncture. Using a tissue homogenizer (PYREX® Potter-Elvehjem), the lungs, olfactory bulbs and brain were homogenized in 1000, 500 or 1000 µL of a sterile saline solution, respectively. Resulting solutions from all samples were diluted and plated for CFU counting.

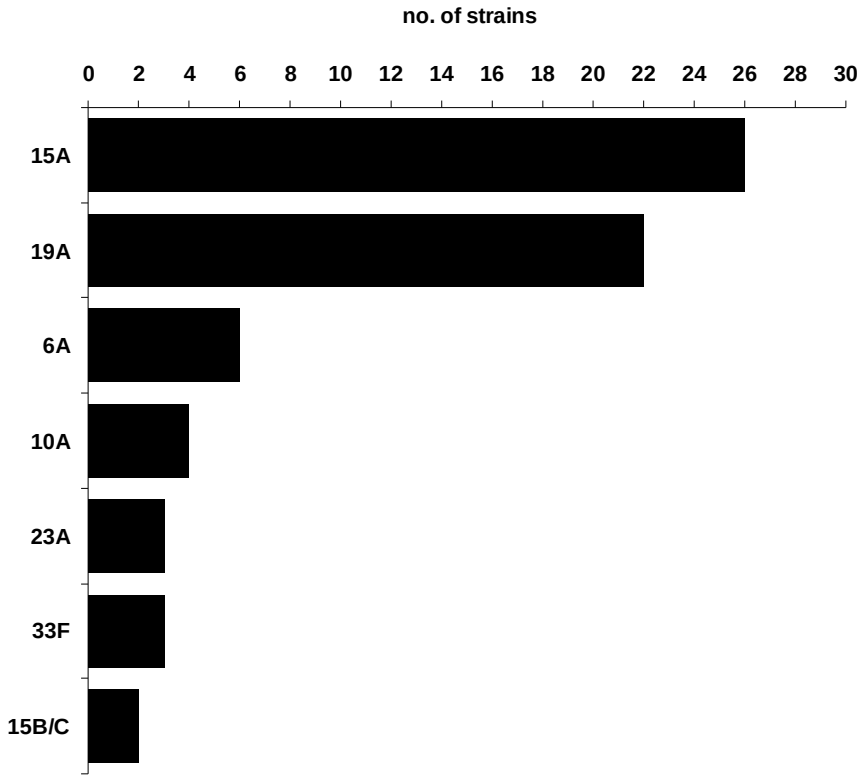
Definition of infection. Infection was defined as the presence of pneumococci in the following tissues: middle ear mucosa, olfactory bulbs, brain, lungs and blood.

Statistics. Significant differences in CFU numbers were analyzed by using One-way ANOVA with Tukey's multiple comparison test. Survival curves were analyzed using the log-rank (Mantel-Cox) test using Prism software from GraphPad Software Inc. In all analyses, a maximum error type I of 0.05 was considered for recognition of a significant difference.

4.4. Results

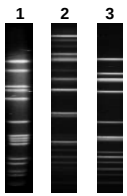
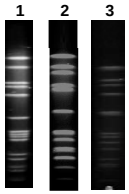
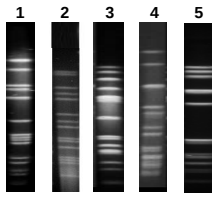
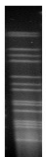
Genetic diversity of the non-PCV7 serotypes. Over a three-year (2001-2003) surveillance study in Portugal, a group of 238 children immunized with the PCV7 were investigated concerning the vaccine's impact on colonization and antimicrobial resistance (Frazão *et al.*, 2005). Out of 231 drug resistant strains isolated, 66 exhibited non-PCV7 capsular types. The most prevalent drug resistant non-PCV7 serotypes were 6A (6 strains), 15A (26 strains) and 19A (22 strains) that together accounted for 82% of the capsulated drug resistant non-PCV7 strains isolated (Fig. 1).

Figure 1. Drug resistant non-PCV7 serotypes (n = 66).



Serotype 6A and 15A were relatively homogenous concerning the genetic background, presenting only two PFGE patterns each throughout the study period. Serotype 19A was more heterogeneous, presenting up to four PFGE lineages along the study (Table 1). Nevertheless, among these clonal types we could recognize the dominant clone (the one with the higher number of strains), suggesting that a selection pressure of unknown source is selecting clones among the non vaccine serotypes. From the clonal types found within each one of the more common non-PCV7 serotypes (6A, 15A, and 19A), we selected the strains with the highest penicillin MIC for the present study. These representative strains were further characterized by MLST, with serotypes 6A, 15A and 19A exhibiting sequence types ST2191, ST63 and ST276, respectively (Table 1).

Table 1. Representative strains of the major drug resistant non-PCV7 serotypes.

Major drug resistant non-PCV7 serotypes	PFGE clones associated	Penicillin MIC associated	No. of strains	Representative strains		
				PFGE type	Antibiotype	Sequence Type
6A		**1** R6 **2** 6A-I – P(S) clone, MIC range: 0.023 – 0.064 µg/ml **3** 6A-II – P(I) clone, MIC: 0.19 µg/ml	(4 strains) (2 strains)	**6A-II** 	P(I) (MIC: 0.19 µg/ml) Tet	**2191**
15A		**1** R6 **2** 15A-I – P(I) clone, MIC range: 0.125 – 0.19 µg/ml **3** 15A-II – P(S) clone, MIC: 0.016 µg/ml	(25 strains) (1 strain)	**15A-I** 	P(I) (MIC: 0.19 µg/ml) Tet Da E	**63**
19A		**1** R6 **2** 19A-I – P(I) clone, MIC range: 0.5 – 0.75 µg/ml **3** 19A-II – P(S) clone, MIC range: 0.016 – 0.023 µg/ml **4** 19A-III – P(I) clone, MIC: 0.094 µg/ml **5** 19A-IV – P(I) clone, MIC: 0.19 µg/ml	(12 strains) (8 strains) (1 strain) (1 strain)	**19A-I** 	P(I) (MIC: 0.75 µg/ml) SXT Tet Da E	**276**

Non-PCV7 serotypes – Serotypes not included in the PCV7 (7-valent pneumococcal conjugate vaccine).
P(S) – Penicillin Susceptible (P<0.094 µg/ml); P(I) – Penicillin Intermediate resistant (0.094 µg/ml≤P<1.5 µg/ml) ; E – Erythromycin (E≤15 µg/ml); Da – Clindamycin (Da ≤15 µg/ml); Tet – Tetracycline (Te ≤18 µg/ml); SXT - Trimethoprim-sulfamethoxazole (SXT ≤15 µg/ml);
C – Chloramphenicol (C≤20µg/ml); L – Levofloxacin (L≤13µg/ml), and TX – Ceftriaxone (TX≤1µg/ml)
MIC – Minimal Inhibitory Concentration.

Colonization potential of non-PCV7 serotypes. A mouse colonization model was used to assess the colonization capacity of the representative strains of the non-PCV7 serotypes. Serotype 6A was maintained in the nasopharynx and olfactory bulbs at all time points over the 21-day experimental period. With the exception of day 14, it was always present in the brain. In the lungs, serotype 6A was found at days 2 and 21, whereas in the middle ear it was found at days 2, 7, and 14, disappearing by day 21. Serotype 6A was never found in the blood (Fig. 2). Throughout the experimental period, out of 24 mice sacrificed, 79% showed serotype 6A colonization in the nasopharynx, while 71% presented serotype 6A in the olfactory bulbs, 42% in the brain, 21% in the middle ear, and 17% in the lungs (Table 2). In the nasopharynx, olfactory bulbs, brain and lungs serotype 6A was present at average numbers of 10^1-10^4 CFU per mouse. By contrast, CFU numbers detected in the middle ear were much lower (~1 CFU per mouse) (Table 2). All the mice presenting pneumococci in the brain had higher or similar CFU numbers in the nasopharynx and olfactory bulbs, with no signs of bacteria in the blood.

Table 2. Colonization and infection with serotype 6A.

		Percentage of mice exhibiting colonization and infection					
Time (Days)	**Mice**	**Nasopharynx**	**Olfactory bulbs**	**Brain**	**Lungs**	**Middle ear**	**Blood**
2	**5**	100%	100%	60%	60%	60%	0%
7	**6**	100%	100%	83%	0%	17%	0%
14	**6**	67%	50%	0%	0%	17%	0%
21	**7**	57%	43%	29%	14%	0%	0%
Mean	**24**	79%	71%	42%	17%	21%	0%
		Mean CFU per mice exhibiting colonization and infection[a]					
Time (Days)	**Mice**	**Nasopharynx**	**Olfactory bulbs**	**Brain**	**Lungs**	**Middle ear**	**Blood**
2	**5**	$7x10^4$	$2x10^3$	$7x10^1$	$6x10^1$	4	0
7	**6**	$3x10^4$	$4x10^2$	$5x10^2$	0	<1	0
14	**6**	$9x10^2$**	$1x10^1$	0	0	<1	0
21	**7**	$1x10^3$**	$2x10^1$	7	3	0	0
Mean	**24**	$2x10^4$	$6x10^2$	$1x10^2$	$2x10^1$	1	0

[a]When mean CFU is less than 1, <1. The asterisks indicate a significant decrease in the number of CFUs compared to day 2 (One-way ANOVA with Dunnett's multiple comparison test; **p<0,01).

Serotype 15A was found in the nasopharynx, olfactory bulbs, lungs, brain, and middle ear at all time points over the 21-day experimental period, being only absent from blood (Fig. 2). Throughout the experimental period, out of 24 mice sacrificed, 96% presented serotype 15A colonizing the nasopharynx, 75% presented it in the olfactory bulbs, 67% in the lungs, 54% in the brain, and 46% in the middle ear (Table 3). In the nasopharynx, olfactory bulbs, brain and lungs, serotype 15A was present at average numbers of 10^2-10^4 CFU per mouse, whereas CFU numbers were much lower (~1 CFU per mouse) in the middle ear (Table 3). Again, all the mice presenting pneumococci in the brain had higher or similar CFU numbers in the nasopharynx and olfactory bulbs, with no signs of bacteria in the blood.

Table 3. Colonization and infection with serotype 15A.

		Percentage of mice exhibiting colonization and infection					
Time (Days)	**Mice**	**Nasopharynx**	**Olfactory bulbs**	**Brain**	**Lungs**	**Middle ear**	**Blood**
2	**6**	100%	100%	100%	83%	83%	0%
7	**6**	100%	83%	67%	83%	50%	0%
14	**6**	100%	67%	33%	83%	17%	0%
21	**6**	83%	50%	17%	17%	33%	0%
Mean	**24**	96%	75%	54%	67%	46%	0%
		Mean CFU per mice exhibiting colonization and infection[a]					
Time (Days)	**Mice**	**Nasopharynx**	**Olfactory bulbs**	**Brain**	**Lungs**	**Middle ear**	**Blood**
2	**6**	$7x10^4$	$2x10^3$	$5x10^2$	$8x10^3$	2	0
7	**6**	$7x10^4$	$5x10^2$	$1x10^2$	$5x10^4$	<1	0
14	**6**	$1x10^4$*	$7x10^1$	$2x10^1$*	$8x10^3$	<1	0
21	**6**	$1x10^4$*	$4x10^1$	$2x10^1$*	8	<1	0
Mean	**24**	$4x10^4$	$7x10^2$	$2x10^2$	$2x10^4$	<1	0

[a]When mean CFU is less than 1, <1. The asterisks indicate a significant decrease in the number of CFUs compared to day 2 (One-way ANOVA with Dunnett's multiple comparison test; *p<0,05).

Serotype 19A was found in the nasopharynx and the olfactory bulbs at all time points over the 21-day period. In the lungs, serotype 19A was always present, but disappeared at the last time point (day 21). In the brain and middle ear, this serotype was found only at days 2 and 7 after the challenge.

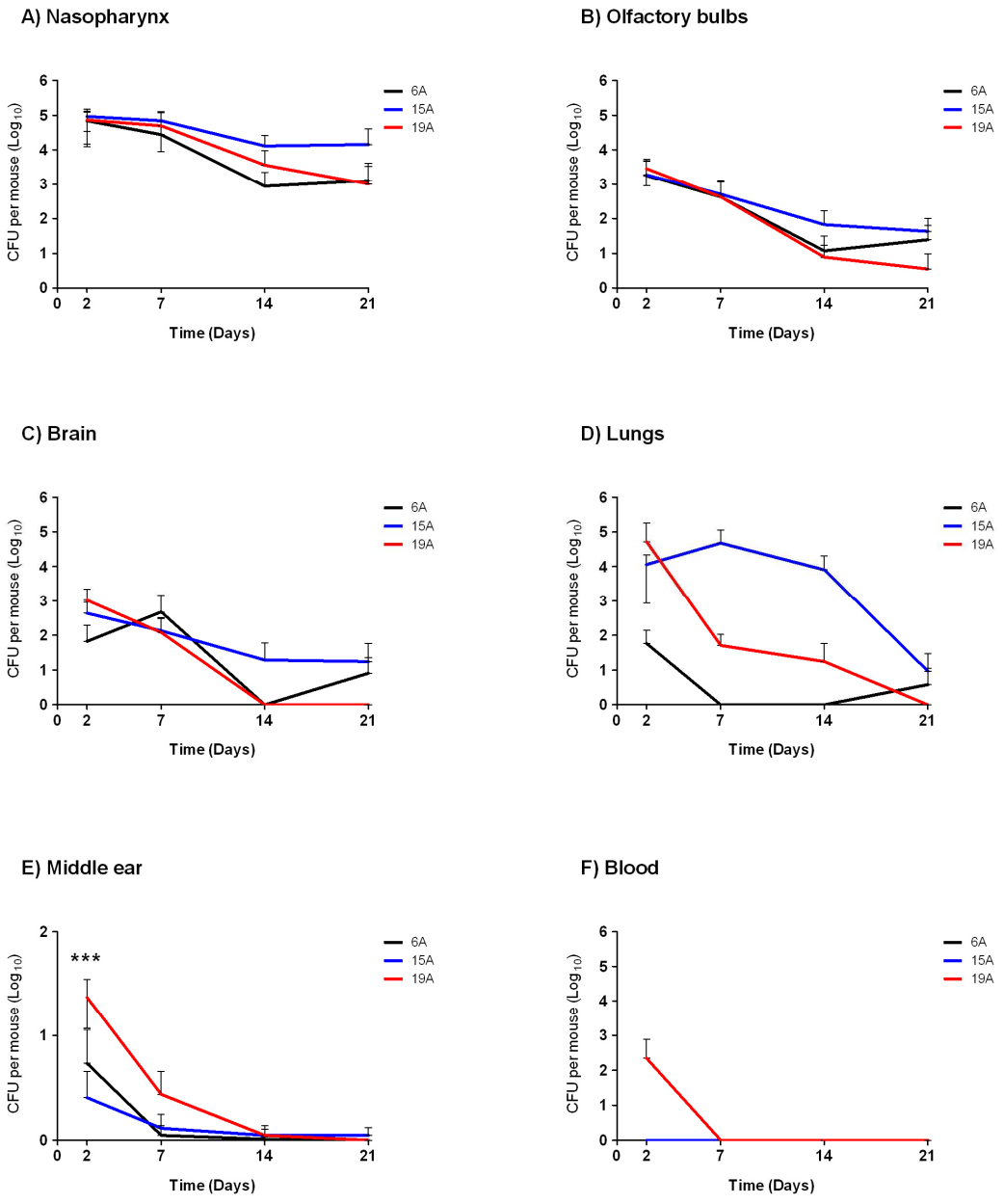
Concerning the blood, we only detected the presence of serotype 19A in a single mouse at the first experimental time point (day 2) (Fig. 2). Throughout the experimental period, out of 24 mice sacrificed, 79% showed serotype 19A colonizing the nasopharynx, 71% presented it in the olfactory bulbs, 50% in the middle ear, 38% in the brain and lungs, while 4% presented this serotype in the blood (Table 4). In the nasopharynx, olfactory bulbs, brain and lungs, serotype 19A was found at average numbers of 10^2-10^4 CFU per mouse, with the CFU numbers being much lower (6 CFU per mouse) in the middle ear. This was the only non-PCV7 serotype found in the blood. Numbers of $7x10^1$ CFU were found in a single mouse at day 2 of the experiment (Table 4). All the mice that presented pneumococci in the brain displayed higher or similar CFU numbers in the nasopharynx and olfactory bulbs, with no signs of bacteria in the blood. The sole exception was a single mouse that showed blood infection concomitantly with nasopharyngeal colonization and olfactory bulb and brain infection.

Table 4. Colonization and infection with serotype 19A.

		Percentage of mice exhibiting colonization and infection					
Time (Days)	**Mice**	**Nasopharynx**	**Olfactory bulbs**	**Brain**	**Lungs**	**Middle ear**	**Blood**
2	**6**	100%	100%	100%	83%	100%	17%
7	**6**	100%	100%	50%	50%	67%	0%
14	**6**	83%	67%	0%	17%	33%	0%
21	**6**	33%	17%	0%	0%	0%	0%
Mean	**24**	79%	71%	38%	38%	50%	4%
		Mean CFU per mice exhibiting colonization and infection[a]					
Time (Days)	**Mice**	**Nasopharynx**	**Olfactory bulbs**	**Brain**	**Lungs**	**Middle ear**	**Blood**
2	**6**	$8x10^4$	$3x10^3$	$1x10^3$	$6x10^4$	$2x10^1$	$3x10^2$
7	**6**	$5x10^4$	$4x10^2$	$1x10^2$	$5x10^1$	2***	0
14	**6**	$4x10^3$*	7	0	$2x10^1$	<1***	0
21	**6**	$1x10^3$*	3	0	0	0***	0
Mean	**24**	$3x10^4$	$8x10^2$	$3x10^2$	$2x10^4$	6	$7x10^1$

[a]When mean CFU is less than 1, <1. The asterisks indicate a significant decrease in the number of CFUs compared to day 2 (One-way ANOVA with Dunnett's multiple comparison test; *p<0,05; ***p<0,001).

Figure 2. Colonization model. Mice were intranasally challenged at day 0 with 10^8 CFU of the non-PCV7 serotypes 6A (black line), 15A (blue line) or 19A (red line) in a 10 µL volume. At days 2, 7, 14, and 21 after inoculation, nasal and middle ear washes were performed, and blood, lungs, brain, and olfactory bulbs were collected to assess the presence of live pneumococci. Values represent the $\log_{10}$ of the mean of CFU ± SD of 5 to 7 mice per serotype per time point. The 0 value on the y-axis represents the absence of detectable CFU. The asterisks indicate that 19A differs from 6A and 15A (One-way ANOVA with Tukey's multiple comparison test, p<0.001).



Lung infection potential of non-PCV7 serotypes. To investigate the potential of the non-PCV7 serotypes to cause infection in the lungs, mice were intranasally challenged with monocultures of each of the three selected non-PCV7 serotypes and survival was assessed over a 7-day period. When using 10^5 or 10^7 CFU inocula all mice survived the challenge (data not shown), whereas after inoculation with 10^8 CFU all mice tested (8 per serotype) died in 24 h (data not shown), showing signs of disease (e.g. piloerection, eyelid closure or hunched posture) as soon as 2 h after the intranasal challenge. To further evaluate non-PCV7 lung infection, pneumococcal titers were measured in the lungs and blood at pre-determined times (2 and 5 h). Lung and blood bacterial titers found for each serotype were maintained at the two time points (Table 5). In the lungs, the non-PCV7 bacterial titers were maintained fairly constant at high levels (10^8 CFU per mouse). In the blood the lower limit for the bacterial load was $7x10^4$ CFU (6A, 2 h after challenge), while the higher was $6x10^6$ CFU (19A, 5 h after challenge). The 19A serotype always presented the highest mean bacterial numbers, $4x10^6$ and $6x10^6$ CFU at 2 and 5 h, respectively (Table 5). Overall, in this model, the non-PCV7 serotypes presented constantly high bacterial titers in the lungs. This may lead to lung tissue lesions and a subsequent spill of pneumococci into the blood, where the bacterial numbers were always lower than in the lungs (Table 5).

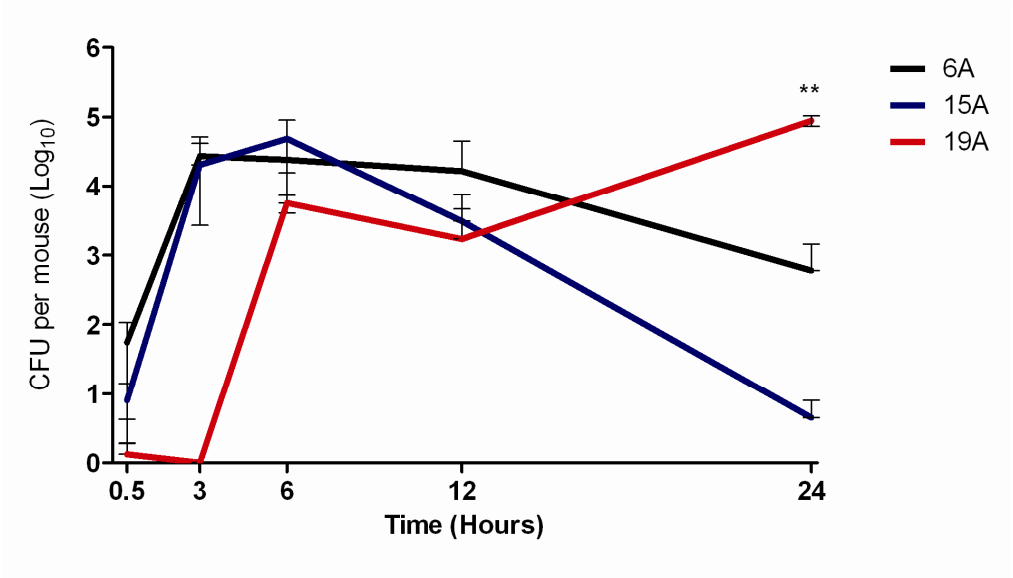
Table 5. Lung infection model.

		Mean CFU per mice[a]					
		6A		15A		19A	
Time (Hours)	Mice	Lungs	Blood	Lungs	Blood	Lungs	Blood
2	2	$1x10^8$	$7x10^4$	$3x10^8$	$7x10^5$	$1x10^8$	$4x10^6$
5	2	$3x10^8$	$2x10^6$	$2x10^8$	$2x10^5$	$2x10^8$	$6x10^6$

[a]We used two mice per serotype per time point.

Blood infection potential of non-PCV7 serotypes. Using an intraperitoneal blood infection model we evaluated the potential of non-PCV7 serotypes to cause infection. The pneumococcal titers were measured at pre-determined times (0.5, 3, 6, 12, and 24 h) after infection to assess the pneumococcal blood growth and clearance (Fig. 3). Three hours after infection, serotype 19A was not detected and no significant differences were found among serotypes 6A and 15A. In all other sampling points no significant differences were observed among the three serotypes. However, after the 12 h time point, serotype 19A showed a tendency to grow, in sharp contrast with the remaining two non-PCV7 serotypes, 6A and 15A, which presented propensity to be cleared. At 24 h the 19A serotype presented the highest mean number of bacteria in the blood ($8.8x10^4$ CFU per mouse) when compared to the remaining non-PCV7 serotypes (6A and 15A), which displayed mean titers of $6x10^2$ and 5 CFU per mouse, respectively ($p<0.01$) (Fig. 3). Thus, among the non-PCV7 serotypes analyzed, serotype 19A appears to be the more “fit” when growing in blood, denoting a higher potential to cause blood infection (Fig. 3).

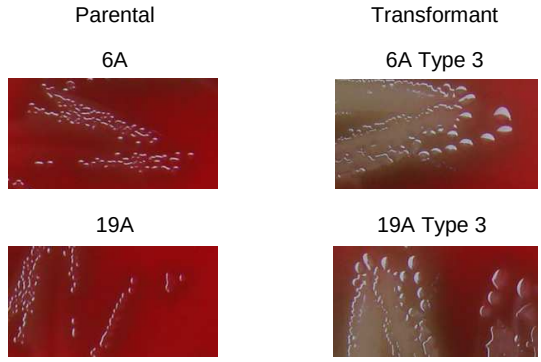
Figure 3. Blood infection model. Mice were intraperitoneally challenged at 0 hours with 10^5 CFU of the non-PCV7 serotypes, 6A (Black), 15A (Blue) or 19A (Red). At 0.5, 3, 6, 12, and 24 hours after injection, blood was collected to assess the presence of live pneumococci. Values represent the $\log_{10}$ of the mean of CFU ± SD of 2 to 3 mice per serotype per time sampling. The 0 value on the y-axis represents the absence of detectable CFU. The asterisks indicate that 19A differs from 6A and 15A (One-way ANOVA with Tukey's multiple comparison test, $p<0.01$).



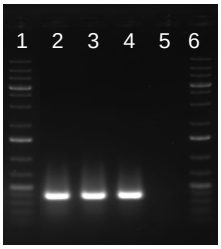
Pneumococcal capsule role in colonization and blood infection. The two non-PCV7 serotype strains, 6A and 19A, were the parental strains transformed to express the capsular type 3. The type 3 transformants were named 6A Type 3 or 19A Type 3 if the parental strain was 6A or 19A, respectively. Figure 4 shows the morphology of the parental and type 3 transformants (mucoid), as well as the PCR amplification results confirming the presence of the type 3 operon in the transformants' genome.

Figure 4. Colony morphology of parental and type 3 transformant strains (A), and PCR amplification results for the type 3 capsule operon (B).

A) Colony morphology



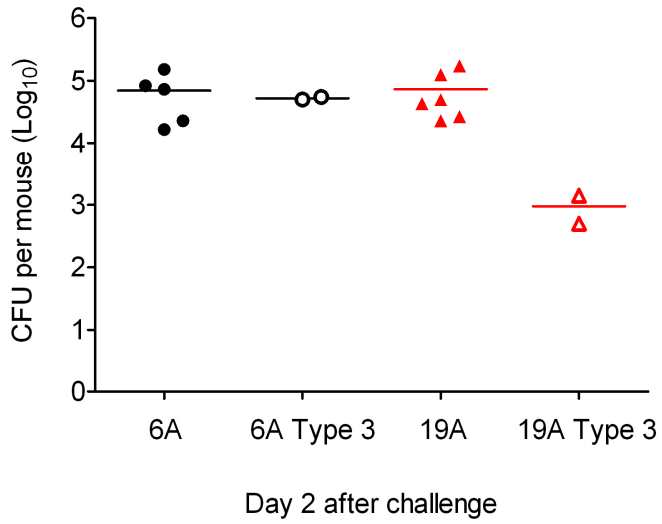
B) PCR amplification of the type 3 capsule operon



Legend:
1 – 1kb ladder
2 – 6A Type 3
3 – 19A Type 3
4 – Positive control (SV35T3)
5 – Negative control (No DNA)
6 – 1kb ladder

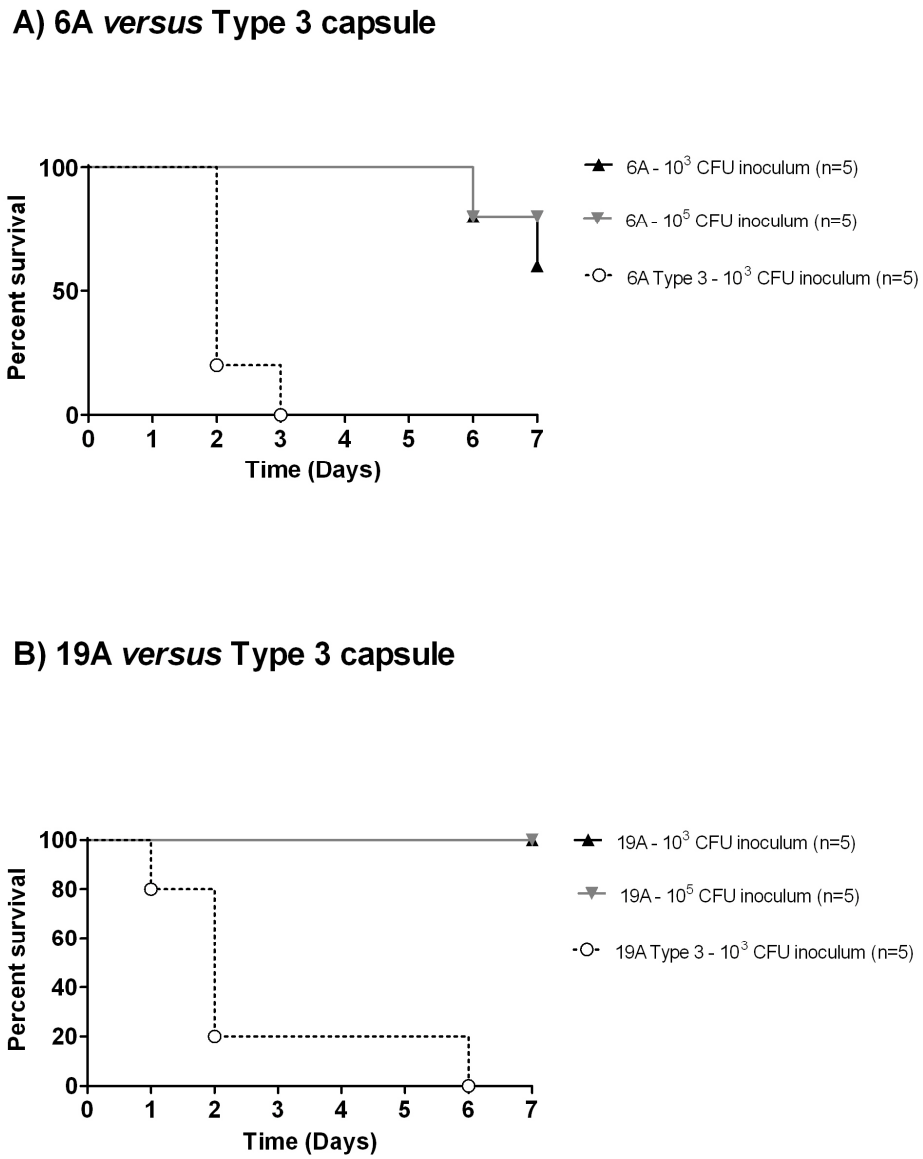
To test the hypothesis that capsular type affects the colonization potential of non-PCV7 serotypes, we colonized the nasopharynx of mice with serotypes 6A and 19A and the corresponding type 3 transformant strains (6A Type 3 and 19A Type 3). At day 2 after the challenge, the CFUs in the nasopharynx of the mice were quantified for comparison (Fig. 5). Serotype 6A presented on average $7x10^4$ CFU per mouse, while the corresponding type 3 transformant presented $5x10^4$ CFU per mouse. Regarding serotype 19A, on average each mouse was colonized with $7x10^4$ CFUs, with the corresponding type 3 transformant presenting $9x10^2$ CFU per mouse. Although no significant difference was found, it appears that the 19A genetic background is the only one negatively affected in the ability to colonize the mouse nasopharynx when expressing the type 3 capsule, because it presented a lower CFU number (Fig. 5).

Figure 5. Capsule role in colonization. Mice were intranasally challenged at day 0 with 10^8 CFU of 6A (full circles), 6A Type 3 transformant (open circles), 19A (full triangles) or 19A Type 3 transformant (open triangles) in a 10 µL volume. At day 2 after inoculation, nasal washes were performed to assess for the presence of live pneumococci. CFUs for each mouse are plotted. Horizontal bars indicate the mean CFU titer. The 0 value on the y-axis represents the absence of detectable CFU.



To investigate whether the capsular type affects the blood infection potential of the non-PCV7 serotypes, we infected groups of mice via the intraperitoneal route with serotypes 6A or 19A and the corresponding type 3 transformant strains (6A Type 3 and 19A Type 3). Following infection, mice survival was assessed over a 7-day period (Fig. 6). When the infection inoculum of serotypes 6A or 19A was 10^3 or 10^5 CFU per mouse, the vast majority of the animals survived the challenge without showing any signs of disease (Fig. 6). However, if the mice were challenged with only 10^3 CFU per mouse using the type 3 transformant strains, all animals died before day 7, presenting signs of disease as soon as one day after the intraperitoneal challenge (Fig. 6).

Figure 6. Capsule role in blood infection. Isogenic strains expressing either capsular types 6A or 19A and the corresponding capsular type 3 transformants were injected into the peritoneal space of CD1 mice. Survival was followed to assess the virulence potential of the capsular types. Five mice per serotype/inoculum were tested. Survival curves for parental strains (6A and 19A) were significantly different from the corresponding type 3 transformants (Mantel-Cox test, p<0.01).



4.5. Discussion

As pneumococcal disease is initiated with the establishment of the carrier state, serotype distribution in the nasopharynx should constitute a good indicator of future pneumococcal disease agents. The impact of PCV7 in colonization was firstly investigated in Portugal in 2001-2003, with the major non-PCV7 serotypes being isolated and characterized regarding capsular type, genetic background and susceptibility to antimicrobials. Data showed that within the major drug resistant non vaccine serotypes a dominant clone could be identified suggesting that capsular type selection induced by PCV7 is accompanied by a clonal type selection (Table 1).

Within the major drug resistant non-PCV7 serotypes – 6A, 15A, and 19A – we identified the clonal types presenting the higher penicillin MIC range, from where we selected representative strains presenting the highest penicillin MIC. These representative strains, exhibiting serotypes, 6A, 15A, and 19A, were characterized using experimental animal models of colonization and disease. By testing these non-PCV7 serotypes in animal models, we aimed at mimicking their known capacity to colonize the human nasopharynx (Frazão *et al.*, 2005) and predicting their virulence potential.

Using a mouse model of colonization we were able to reproduce the known high colonization capacity of the non-PCV7 serotypes, as the three selected serotypes colonized the mice nasopharynx with a mean bacterial number of 10^4 CFU per mouse over a 21-day study period. The steady colonization of the nasopharynx with multi-drug resistant non-PCV7 serotypes during such a long experimental period may constitute a suitable system to test the efficacy of new antimicrobials or vaccines that target colonization (mucosal vaccines).

As a result of the establishment of colonization all three non-PCV7 serotypes behaved similarly and were capable of invading and infecting nasopharynx adjacent tissues such as the olfactory bulbs, brain, lungs and the middle ear mucosa. Indicating propensity to cause infection, serotype 19A presented significantly higher bacterial titers than the 6A and 15A serotypes in the middle ear mucosa ($p<0.001$). Additionally, it was the only

non-PCV7 serotype found in the blood of a mouse two days after nasopharynx colonization. Virulence potential was also exhibited by serotype 15A which grew easily in the lungs, presenting high bacterial numbers along the experimental period.

In accordance with previous data (Marra & Brigham, 2001; van Ginkel *et al.*, 2003), virtually all colonized mice with brain infection did not present bacteria in the blood, clearly showing that pneumococci can enter the brain directly from the nasopharynx in the absence of blood infection. The possibility that this direct route occurs in humans highlights the extreme importance of inhibiting nasopharyngeal colonization to prevent seeding of the brain with bacteria. Vaccines that do not target colonization would fail to prevent infection through this route as previously suggested (van Ginkel *et al.*, 2003).

To gain further insight into the potential to cause infections we tested the three non-PCV7 serotypes in lung and blood infection models. All serotypes tested in the lung infection model were lethal when using high inocula (10^8 CFU per mouse), killing all the mice as early as 24 h after the challenge. Moreover, the titer of bacteria in the lungs was always higher (~10^8 CFU per mouse) than in the blood. Such a high pneumococcal titer could enable lung tissue lesions leading to constant blood poisoning. With lower inocula (10^7 or 10^5 CFU per mouse), no mice were killed by any of the non-PCV7 serotypes, suggesting that death of the mice through lung infection is mainly dependent on the bacterial load reached in this organ. Using the blood infection model we found that serotypes 6A (clone ST2191) and 15A (clone ST63) were not fit to grow in blood, contrarily to what was observed for serotype 19A (clone ST276). This 19A clone was more fit than serotypes 6A and 15A clones when growing in blood, presenting higher bacterial numbers in the blood 24 h after challenge ($p<0.01$), while the latter serotypes started to be cleared from the blood after 12 h. These data suggest that serotype 19A (clone ST276), initially isolated in 2001 from the nasopharynx of healthy children vaccinated with PCV7 (Frazão *et al.*, 2005), has the capacity to cause blood infection. In fact, three recent studies released in 2009 and 2010 (Aguiar *et al.*, 2009; Ardanuy *et al.*, 2009b; Mahjoub-Messai *et al.*, 2009), have reported that this very same serotype 19A, presenting the same genetic background, ST276, is a major cause of invasive disease both in children and adults in Portugal, Spain and France. Moreover, in 2009, this 19A

clone was reported to be a major cause of otitis media in Israel, being associated with high levels of antibiotic consumption (Dagan *et al.*, 2009). This association of the 19A ST276 clone with high antibiotic consumption is a scenario that is mirrored in Portugal, specifically in children attending day-care center facilities from whom we isolated our multi-drug resistant 19A-ST276 strain in 2001 (Frazão *et al.*, 2005).

The variability in the capacity to cause site-specific disease has been reported not only among serotypes, but also across pneumococcal genetic backgrounds (Forbes *et al.*, 2008; Obert *et al.*, 2006; Pettigrew & Fennie, 2005; Pettigrew *et al.*, 2006; Sandgren *et al.*, 2004; Shouval *et al.*, 2006; Sjostrom *et al.*, 2006). Therefore, understanding the basis of pneumococcal virulence requires disentangling the impact of capsular and non-capsular genetic contributions. Here, we created otherwise isogenic capsular Type 3 transformants using 6A and 19A as the parental strains to overcome the variability issue and infer about the capsule role in colonization and blood infection. The 6A Type 3 transformant showed the same ability to colonize as the 6A parental strain, whereas the 19A Type 3 transformant showed diminished capacity to colonize when compared to its parental strain. We therefore conclude that colonization ability depends not only on the capsular type, but also on the genetic background that expresses it. Regarding blood infection, all mice intraperitoneally challenged with any of the type 3 transformants died in less than seven days, in sharp contrast to what was observed with the parental strains. Both the 6A and 19A parental genetic backgrounds allowed full expression of the typical virulence associated to the type 3 capsule causing fatal blood infection. Thus, it appears that the virulence potential, at least in this particular case, is more dependent on the capsular type exhibited than on the genetic background of the pneumococcal strain.

The present work reinforces the importance of coupling epidemiological and animal studies in order to predict pneumococcal virulence of the major serotypes selected after vaccine pressure. Evaluation of the virulence potential of other major serotypes colonizing the nasopharynx is required to anticipate the impact of the next generation of capsular vaccines. Additionally, further research will be crucial in the identification of

non-capsular virulence factors, which may aid in the development of universal strategies for prevention and control of pneumococcal disease.

4.6. Acknowledgments

This work was supported by a grant awarded to Dr. Tomasz from The Rockefeller University, "Bridges to Better Medicine Technology Inovation Fund", and PNEUMOPATH project (contract HEALTH-F3-2009-222983) from the European Commission. N.F. was supported by a PhD fellowship from Fundação para a Ciência e a Tecnologia (SFRH/BD/30103/2006).

We thank Garth Ehrlich (Allegheny-Singer Research Institute, Pittsburgh, USA) for generating and providing the SV35T3 strain, as well as Florian Gehre (The Rockefeller University, NY, USA), Susana Lima and Léa Zinck (Champalimaud Neuroscience Programme, Oeiras, Portugal) for assistance with the experimental animal model procedures.

N. Frazão was responsible for the global data analysis and all the experimental work.

4.7. References

Aguiar, S. I., Pinto, F. R., Nunes, S., Serrano, I., Melo-Cristino, J., Sá-Leão, R., Ramirez, M. & de Lencastre, H. (2009). Denmark14-230 clone as an increasing cause of pneumococcal infection in Portugal within a background of diverse serotype 19A lineages. *J Clin Microbiol* **48**, 101-108.

Ardanuy, C., Tubau, F., Pallares, R., Calatayud, L., Dominguez, M. A., Rolo, D., Grau, I., Martin, R. & Linares, J. (2009). Epidemiology of invasive pneumococcal disease among adult patients in barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997-2007. *Clin Infect Dis* **48**, 57-64.

Bogaert, D., De Groot, R. & Hermans, P. W. (2004). *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* **4**, 144-154.

CDC (2005). Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease: United States, 1998–2003. *MMWR Morb Mortal Wkly Rep* **54**, 893-897.

Dagan, R., Melamed, R., Muallem, M., Piglansky, L. & Yagupsky, P. (1996). Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. *J Infect Dis* **174**, 1352-1355.

Dagan, R. (2002). Immunisation with a pneumococcal 7-valent conjugate vaccine. *Int J Clin Pract* **56**, 287-291.

Dagan, R., Givon-Lavi, N., Leibovitz, E., Greenberg, D. & Porat, N. (2009). Introduction and proliferation of multidrug resistant *Streptococcus pneumoniae* serotype 19A clones that cause acute otitis media in an unvaccinated population. *J Infect Dis* **199**, 776-785.

Fletcher, M. A. & Fritzell, B. (2007). Brief review of the clinical effectiveness of PREVENAR against otitis media. *Vaccine* **25**, 2507-2512.

Forbes, M. L., Horsey, E., Hiller, N. L., Buchinsky, F. J., Hayes, J. D., Compliment, J. M., Hillman, T., Ezzo, S., Shen, K., Keefe, R., Barbadora, K., Post, J. C., Hu, F. Z. & Ehrlich, G. D. (2008). Strain-specific virulence phenotypes of *Streptococcus pneumoniae* assessed using the Chinchilla laniger model of otitis media. *PLoS One* **3**, e1969.

Frazão, N., Brito-Avô, A., Simas, C., Saldanha, J., Mato, R., Nunes, S., Sousa, N. G., Carriço, J. A., Almeida, J. S., Santos-Sanches, I. & de Lencastre, H. (2005). Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon. *Pediatr Infect Dis J* **24**, 243-252.

Frazão, N., Sá-Leão, R. & de Lencastre, H. (2010). Impact of a single dose of the 7-valent pneumococcal conjugate vaccine on colonization. *Vaccine* **28**, 3445-3452.

Greenwood, B. M., Weber, M. W. & Mulholland, K. (2007). Childhood pneumonia--preventing the worlds biggest killer of children. *Bull World Health Organ* **85**, 502-503.

Kharat, A. S. & Tomasz, A. (2006). Drastic reduction in the virulence of *Streptococcus pneumoniae* expressing type 2 capsular polysaccharide but lacking choline residues in the cell wall. *Mol Microbiol* **60**, 93-107.

Lai, W. C., Pakes, S. P., Stefanu, C. & Lu, Y. S. (1986). Comparison of Chalquest and Hayflick media, with and without ammonium reineckate, for isolating Mycoplasma pulmonis from rats. *J Clin Microbiol* **23**, 817-821.

Lloyd-Evans, N., O'Dempsey, T. J., Baldeh, I., Secka, O., Demba, E., Todd, J. E., McArdle, T. F., Banya, W. S. & Greenwood, B. M. (1996). Nasopharyngeal carriage of pneumococci in Gambian children and in their families. *Pediatr Infect Dis J* **15**, 866-871.

Mahjoub-Messai, F., Doit, C., Koeck, J. L., Billard, T., Evrard, B., Bidet, P., Hubans, C., Raymond, J., Levy, C., Cohen, R. & Bingen, E. (2009). Population snapshot of *Streptococcus pneumoniae* serotype 19A isolates before and after introduction of seven-valent pneumococcal Vaccination for French children. *J Clin Microbiol* **47**, 837-840.

Marra, A. & Brigham, D. (2001). *Streptococcus pneumoniae* causes experimental meningitis following intranasal and otitis media infections via a nonhematogenous route. *Infect Immun* **69**, 7318-7325.

Mato, R., Sanches, I. S., Simas, C., Nunes, S., Carrico, J. A., Sousa, N. G., Frazao, N., Saldanha, J., Brito-Avo, A., Almeida, J. S. & Lencastre, H. D. (2005). Natural history of drug resistant clones of *Streptococcus pneumoniae* colonizing healthy children in Portugal. *Microb Drug Resist* **11**, 309-322.

Mulholland, K. (2007). Childhood pneumonia mortality--a permanent global emergency. *Lancet* **370**, 285-289.

Obert, C., Sublett, J., Kaushal, D., Hinojosa, E., Barton, T., Tuomanen, E. I. & Orihuela, C. J. (2006). Identification of a Candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. *Infect Immun* **74**, 4766-4777.

O'Brien, K. L., Wolfson, L. J., Watt, J. P., Henkle, E., Deloria-Knoll, M., McCall, N., Lee, E., Mulholland, K., Levine, O. S. & Cherian, T. (2009). Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* **374**, 893-902.

Pai, R., Gertz, R. E. & Beall, B. (2006). Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. *J Clin Microbiol* **44**, 124-131.

Pettigrew, M. M. & Fennie, K. P. (2005). Genomic subtraction followed by dot blot screening of *Streptococcus pneumoniae* clinical and carriage isolates identifies genetic differences associated with strains that cause otitis media. *Infect Immun* **73**, 2805-2811.

Pettigrew, M. M., Fennie, K. P., York, M. P., Daniels, J. & Ghaffar, F. (2006). Variation in the presence of neuraminidase genes among *Streptococcus pneumoniae* isolates with identical sequence types. *Infect Immun* **74**, 3360-3365.

Sá-Leão, R., Tomasz, A., Sanches, I. S., Nunes, S., Alves, C. R., Avô, A. B., Saldanha, J., Kristinsson, K. G. & de Lencastre, H. (2000). Genetic diversity and clonal patterns among antibiotic-susceptible and -resistant *Streptococcus pneumoniae* colonizing children: day-care centers as autonomous epidemiological units. *J Clin Microbiol* **38**, 4137-4144.

Sá-Leão, R., Simões, A. S., Nunes, S., Sousa, N. G., Frazão, N. & de Lencastre, H. (2006). Identification, prevalence and population structure of non-typable *Streptococcus pneumoniae* in carriage samples isolated from preschoolers attending day-care centres. *Microbiology* **152**, 367-376.

Sá-Leão, R., Nunes, S., Brito-Avô, A., Frazão, N., Simões, A. S., Crisóstomo, M. I., Paulo, A. C., Saldanha, J., Santos-Sanches, I. & de Lencastre, H. (2009). Changes in pneumococcal serotypes and antibiotypes carried by vaccinated and unvaccinated day-care centre attendees in Portugal, a country with widespread use of the seven-valent pneumococcal conjugate vaccine. *Clin Microbiol Infect* **15**, 1002-1007.

Sandgren, A., Sjostrom, K., Olsson-Liljequist, B., Christensson, B., Samuelsson, A., Kronvall, G. & Henriques Normark, B. (2004). Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. *J Infect Dis* **189**, 785-796.

Shouval, D. S., Greenberg, D., Givon-Lavi, N., Porat, N. & Dagan, R. (2006). Site-specific disease potential of individual *Streptococcus pneumoniae* serotypes in pediatric invasive disease, acute otitis media and acute conjunctivitis. *Pediatr Infect Dis J* **25**, 602-607.

Sjostrom, K., Spindler, C., Ortqvist, A., Kalin, M., Sandgren, A., Kuhlmann-Berenzon, S. & Henriques-Normark, B. (2006). Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. *Clin Infect Dis* **42**, 451-459.

Sorensen, U. B. (1993). Typing of pneumococci by using 12 pooled antisera. *J Clin Microbiol* **31**, 2097-2100.

van Ginkel, F. W., McGhee, J. R., Watt, J. M., Campos-Torres, A., Parish, L. A. & Briles, D. E. (2003). Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection. *Proc Natl Acad Sci U S A* **100**, 14363-14367.

Whitney, C. G., Farley, M. M., Hadler, J., Harrison, L. H., Bennett, N. M., Lynfield, R., Reingold, A., Cieslak, P. R., Pilishvili, T., Jackson, D., Facklam, R. R., Jorgensen, J. H. & Schuchat, A. (2003). Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* **348**, 1737-1746.

Chapter V

5. Concluding remarks and future perspectives

5. Concluding remarks and future perspectives

The 7-valent pneumococcal conjugate vaccine produced a massive shift in the human pneumococcal nasopharyngeal flora. The high efficacy of the conjugate vaccine completely transformed the pneumococcal population structure, wiping out the vaccine serotypes and leading to the emergence of “new” serotypes, the so-called non vaccine types (non-PCV7 serotypes). The dramatic impact of the conjugate vaccine motivated numerous studies in a multitude of fields including immunology, epidemiology, drug resistance, and genetics.

This thesis aims to sum up the massive effect of this vaccine on pneumococcal colonization in Portugal, examining at the same time the virulence potential of the new pneumococcal players, the non-PCV7 serotypes. To achieve these objectives, a common set of techniques was used: serotyping, antimicrobial testing, pulsed-field gel electrophoresis (PFGE), multilocus sequence type (MLST), and mouse models of colonization and disease.

Before the study published in The Pediatric Infectious Disease Journal in 2005 and presented in Chapter II of this thesis, the effect of PCV7 on colonization was unknown in Portugal. This was the first work to evaluate the impact of PCV7 on colonization, with particular emphasis on drug resistance and genetic background of the non vaccine types (NVTs) carried by Portuguese children attending day-care centers (DCCs).

Similarly to what has been reported in several studies (Huang *et al.*, 2005; Huang *et al.*, 2009; Lakshman *et al.*, 2003; Pelton *et al.*, 2004), vaccination with PCV7 did not affect the global carriage of pneumococcal strains (susceptible and resistant). Due to the fact that in the pre-PCV7 era most drug resistant pneumococci expressed a limited number of serotypes included in the vaccine, it was expected that PCV7 could reduce drug resistance carriage rates (Joloba *et al.*, 2001; Wuorimaa & Kayhty, 2002). We therefore decided to study in more detail the impact of the PCV7 on drug resistant strains. In agreement with previous studies (Dagan *et al.*, 1996a; Dagan *et al.*, 1997; Dagan, 2002; Dagan *et al.*, 2002; Dagan *et al.*, 2003), the vaccine was found to reduce colonization

with vaccine type (VT) drug resistant strains. However, in contrast with earlier reports (Dagan *et al.*, 1996a; Dagan *et al.*, 1997; Dagan, 2002; Dagan *et al.*, 2002; Dagan *et al.*, 2003; Klugman, 2001; Piffer, 2002), total carriage rate of drug resistant strains did not decline after vaccination in Portugal. Concomitantly with the decrease of drug resistant VT strains there was also an increase of drug resistant NVTs.

The pressure exerted by the PCV7 vaccine led to a phenomenon termed serotype replacement colonization. Furthermore, drug resistant NVTs presented genetic backgrounds different from the ones of the VTs. The replacement of drug resistant VTs by NVTs, also drug resistant and presenting unusual genetic backgrounds, is very likely the result of a combined effect of vaccine pressure and undiminished use of antibacterial agents.

The emergence of drug resistant NVTs is still a matter of debate. Why are these NVTs also drug resistant? One may hypothesize that drug susceptible NVTs acquire drug resistant determinants from the receding drug resistant VTs, or that drug resistant NVTs that preexisted as minor components in the nasopharynx emerge when the more fit VTs disappear under the vaccine pressure. The latter alternative appears likely given that some drug resistant non vaccine serotypes were identified at the onset of the study.

As colonization is the initial step to pneumococcal disease, one may wonder if these NVTs can cause disease. Indeed, since the first studies after the PCV7 introduction, several pieces of evidence support the fact that serotype replacement occurs not only during colonization, but also in disease. In fact, the non vaccine serotypes and associated clonal types found in the present study (6A, 10A, 15A/C, 19A, 23A and 33F) have been associated with pneumococcal disease in the years following PCV7 introduction (Brueggemann *et al.*, 2003; Doit *et al.*, 2002; Eskola *et al.*, 2001; McEllistrem *et al.*, 2003; Porat *et al.*, 2004; Serrano *et al.*, 2004) and nowadays (Ardanuy *et al.*, 2009a; Ardanuy *et al.*, 2009b; Dagan *et al.*, 2009; Mahjoub-Messai *et al.*, 2009).

The studies described in Chapter II represent the first observations concerning the effect

of PCV7 on pneumococcal carriage in Portuguese children. The study provided unique and vital information on the most common drug resistant non vaccine serotypes and associated clonal types that emerged substituting the PCV7 serotypes. Worth of note is the fact that resistance maintenance in our setting seems to imply that reduction of the pneumococcal drug resistance rates requires a combination of pneumococcal vaccines and a reduction in the use of antimicrobial agents.

In Chapter III, the impact of PCV7 on colonization was evaluated by analyzing the effect of one dose of the vaccine, not only among single, but also among multiple carriers. The rationale underlying such a study was the fact that: i) in disease a single dose seems to be sufficient to prevent infections caused by VTs (Barzilay *et al.*, 2006; Whitney *et al.*, 2006), and ii) studying multiple colonization can provide a more complete view of the pneumococcal population structure to evaluate the real extent of the PCV7 effect.

The study design where the same child was sampled one month apart was quite advantageous as it allowed evaluation of the effect of the PCV7 not only at the population, but also at the individual level. Moreover, for the first time in Portugal, the effect of PCV7 on colonization was investigated not by analyzing a single colony by carrier, but by studying multiple colonization.

Our expectations were confirmed and similarly to what happens with more than one dose (Dagan *et al.*, 1996a; Dagan, 2002; Frazão *et al.*, 2005; Kilpi *et al.*, 2001; Mbelle *et al.*, 1999; O'Brien *et al.*, 2007), a single dose of PCV7 indeed decreased VT colonization. A replacement phenomenon was also found in the present study, with NVTs substituting serotypes included in the vaccine both at the population and the individual level.

Owing to the study design it was possible to evaluate the PCV7 impact on colonization, not only by prevalence comparison between vaccinated and control groups, but also by identifying the actual mechanism of the vaccine's effect. By observing the colonization patterns among vaccinated children, ecological mechanisms such as clearance, *de novo* acquisition, unmasking, and maintenance, were examined. In accordance with previous

studies (Bogaert *et al.*, 2004b; Dagan *et al.*, 2003; Klugman, 2001; Whitney *et al.*, 2006), the results obtained suggest that conjugate vaccines reduce VT carriage by preventing *de novo* acquisition rather than clearance. An additional mechanism of the vaccine's effect appears to be the enhancement of NVT unmasking in vaccinated children. Assessment of the latter mechanism was only possible due to the study of multiple colonization.

The studies described in Chapter III provide novel and valuable information on PCV7 single vaccine dose schedule. The findings were that, as early as one month after vaccination with a single dose, PCV7 causes serotype replacement of VT by NVT isolates at the population and individual levels, with the mechanisms of the vaccine's effect being the prevention of VT de novo *acquisition and enhancement of NVT unmasking.*

The study presented in Chapter IV is a complement to the epidemiological studies described above. As the first step to pneumococcal disease is the establishment of the carrier state, the pneumococcal population in the nasopharynx should constitute a good indicator of future pneumococcal disease agents. Therefore, representative strains of the most common non-PCV7 serotype/clonal types isolated from the nasopharynx of children included in the study presented in Chapter II were selected to investigate their virulence potential using animal models.

The three representative strains expressed capsular types, 6A, 15A and 19A. Using a mouse model of colonization the first finding in this study was the fact that shortly after colonization of the nasopharynx each of these three serotypes were capable of disseminating to adjacent tissues such as the olfactory bulbs, brain, lungs and the middle ear mucosa. Serotype 19A demonstrated a higher potential to cause blood infection, while serotype 15A was prone to cause lung infection. Worth of note was the fact that all these serotypes were able to invade the brain without causing blood infection. This finding is in agreement with previous studies that reported this very same phenomenon (Marra & Brigham, 2001; van Ginkel *et al.*, 2003), explaining it by retrograde axonal transport along olfactory neurons into the brain. The possibility that

this phenomenon can occur in humans highlights the importance of inhibiting nasopharyngeal colonization to prevent seeding of the brain.

When the virulence potential of the selected serotypes was assessed in a lung infection model, all three caused lethal infections at high bacterial loads and no killing at lower doses, indicating that lethal infection of the lungs was dose-dependent.

By using an intraperitoneal model of blood infection we found that serotype 19A was the most fit growing in blood, suggesting that serotype 19A (clone ST276), initially isolated in 2001 from the nasopharynx of healthy children vaccinated with PCV7 (Frazão *et al.*, 2005), has the capacity to cause blood infection. In fact, serotype 19A associated to ST276 is currently a major cause of invasive disease both in children and adults in Portugal, Spain and France (Aguiar *et al.*, 2009; Ardanuy *et al.*, 2009b; Mahjoub-Messai *et al.*, 2009). In 2009, this very same 19A clone was reported to cause otitis media in Israel, being associated with high levels of antibiotic consumption (Dagan *et al.*, 2009). Interestingly, high antibiotic consumption is a scenario mirrored in Portugal, specifically in children attending DCC facilities from whom we isolated our 19A (ST276) strain, which is multidrug resistant (Frazão *et al.*, 2005).

To assess the role of the capsule versus the genetic background in colonization and disease, otherwise isogenic strains of serotypes 6A and 19A expressing type 3 capsule were generated. In our setting we found that the colonization ability depended not only on the capsular type, but also on the genetic background that expresses it. Concerning blood infection potential, virulence appears to be more dependent on the capsular type expressed than on the genetic background.

In Chapter IV natural colonization isolates selected by the PCV7 vaccine were tested in mouse models, enabling conclusions on the virulence traits of these emergent serotypes selected by the vaccine pressure. This reinforces the importance of coupling epidemiological and animal studies in order to predict the virulence potential of serotypes selected by present and future pneumococcal vaccines.

In summary, the studies presented in this thesis i) documented – for the first time – the massive shift in colonization operated by the pneumococcal conjugate vaccine in Portugal; ii) provided insights into the mechanisms of serotype change using a model study with short exposure to the vaccine and iii) allowed testing virulence traits of the emergent non vaccine type pneumococci using animal models.

The foremost conclusion of the effect of PCV7, not only at the colonization but also at the disease level, is that there is a serotype replacement phenomenon. If, on one hand, we attribute the vaccine serotype decline to the effect of PCV7, the subsequent emergence of only certain specific non vaccine serotypes/genotypes is not yet fully explained. What are the characteristics that make certain non vaccine serotypes and specific genotypes within them so successful after vaccination?

For years, pneumococcal studies have been centered on the study of single isolates. However, in nature, inter and intraspecies interactions exist. Bearing this in mind, one can wonder what the role of these interactions on colonization and disease is. Finally, future efforts must not focus exclusively on the microorganism side as the development of systems designed to better understand the interaction between host and microbe is of utmost importance.

5.1. References

Aguiar, S. I., Pinto, F. R., Nunes, S., Serrano, I., Melo-Cristino, J., Sá-Leão, R., Ramirez, M. & de Lencastre, H. (2009). Denmark14-230 clone as an increasing cause of pneumococcal infection in Portugal within a background of diverse serotype 19A lineages. *J Clin Microbiol* **48**, 101-108.

Ardanuy, C., Rolo, D., Fenoll, A., Tarrago, D., Calatayud, L. & Linares, J. (2009a). Emergence of a multidrug resistant clone (ST320) among invasive serotype 19A pneumococci in Spain. *J Antimicrob Chemother* **64**, 507-510.

Ardanuy, C., Tubau, F., Pallares, R., Calatayud, L., Dominguez, M. A., Rolo, D., Grau, I., Martin, R. & Linares, J. (2009b). Epidemiology of invasive pneumococcal disease among adult patients in barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997-2007. *Clin Infect Dis* **48**, 57-64.

Barzilay, E. J., O'Brien, K. L., Kwok, Y. S., Hoekstra, R. M., Zell, E. R., Reid, R., Santosham, M., Whitney, C. G. & Feikin, D. R. (2006). Could a single dose of pneumococcal conjugate vaccine in children be effective? Modeling the optimal age of vaccination. *Vaccine* **24**, 904-913.

Bogaert, D., van Belkum, A., Sluijter, M., Luijendijk, A., de Groot, R., Rumke, H. C., Verbrugh, H. A. & Hermans, P. W. (2004). Colonisation by *Streptococcus pneumoniae* and Staphylococcus aureus in healthy children. *Lancet* **363**, 1871-1872.

Brueggemann, A. B., Griffiths, D. T., Meats, E., Peto, T., Crook, D. W. & Spratt, B. G. (2003). Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* **187**, 1424-1432.

Dagan, R., Melamed, R., Muallem, M., Piglansky, L., Greenberg, D., Abramson, O., Mendelman, P. M., Bohidar, N. & Yagupsky, P. (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.

Dagan, R., Muallem, M., Melamed, R., Leroy, O. & Yagupsky, P. (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.

Dagan, R. (2002). Immunisation with a pneumococcal 7-valent conjugate vaccine. *Int J Clin Pract* **56**, 287-291.

Dagan, R., Givon-Lavi, N., Zamir, O., Sikuler-Cohen, M., Guy, L., Janco, J., Yagupsky, P. & Fraser, D. (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.

Dagan, R., Givon-Lavi, N., Zamir, O. & Fraser, D. (2003). Effect of a nonavalent conjugate vaccine on carriage of antibiotic-resistant *Streptococcus pneumoniae* in day-care centers. *Pediatr Infect Dis J* **22**, 532-540.

Dagan, R., Givon-Lavi, N., Leibovitz, E., Greenberg, D. & Porat, N. (2009). Introduction and proliferation of multidrug resistant *Streptococcus pneumoniae* serotype 19A clones that cause acute otitis media in an unvaccinated population. *J Infect Dis* **199**, 776-785.

Doit, C., Loukil, C., Geslin, P. & Bingen, E. (2002). Phenotypic and genetic diversity of invasive pneumococcal isolates recovered from French children. *J Clin Microbiol* **40**, 2994-2998.

Eskola, J., Kilpi, T., Palmu, A., Jokinen, J., Haapakoski, J., Herva, E., Takala, A., Kayhty, H., Karma, P., Kohberger, R., Siber, G. & Makela, P. H. (2001). Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* **344**, 403-409.

Frazão, N., Brito-Avô, A., Simas, C., Saldanha, J., Mato, R., Nunes, S., Sousa, N. G., Carriço, J. A., Almeida, J. S., Santos-Sanches, I. & de Lencastre, H. (2005). Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon. *Pediatr Infect Dis J* **24**, 243-252.

Huang, S. S., Platt, R., Rifas-Shiman, S. L., Pelton, S. I., Goldmann, D. & Finkelstein, J. A. (2005). Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. *Pediatrics* **116**, e408-413.

Huang, S. S., Hinrichsen, V. L., Stevenson, A. E., Rifas-Shiman, S. L., Kleinman, K., Pelton, S. I., Lipsitch, M., Hanage, W. P., Lee, G. M. & Finkelstein, J. A. (2009). Continued impact of pneumococcal conjugate vaccine on carriage in young children. *Pediatrics* **124**, e1-11.

Joloba, M. L., Windau, A., Bajaksouzian, S., Appelbaum, P. C., Hausdorff, W. P. & Jacobs, M. R. (2001). Pneumococcal conjugate vaccine serotypes of *Streptococcus pneumoniae* isolates and the antimicrobial susceptibility of such isolates in children with otitis media. *Clin Infect Dis* **33**, 1489-1494.

Kilpi, T., Syrjanen, R., Palmu, A., Herva, E., Eskola, J., Makela, P. H. & Group, F. s. (2001). Parallel evaluation of the effect of a 7-valent pneumococcal conjugate vaccine (PncCRM) on pneumococcal (Pnc) carriage and acute otitis media (AOM). In *19th Annu Meet Eur Soc Paediatr Infect Dis*, pp. p. 4. Istanbul, Turkey.

Klugman, K. P. (2001). Efficacy of pneumococcal conjugate vaccines and their effect on carriage and antimicrobial resistance. *Lancet Infect Dis* **1**, 85-91.

Lakshman, R., Murdoch, C., Race, G., Burkinshaw, R., Shaw, L. & Finn, A. (2003). Pneumococcal nasopharyngeal carriage in children following heptavalent pneumococcal conjugate vaccination in infancy. *Arch Dis Child* **88**, 211-214.

Mahjoub-Messai, F., Doit, C., Koeck, J. L., Billard, T., Evrard, B., Bidet, P., Hubans, C., Raymond, J., Levy, C., Cohen, R. & Bingen, E. (2009). Population snapshot of *Streptococcus pneumoniae* serotype 19A isolates before and after introduction of seven-valent pneumococcal Vaccination for French children. *J Clin Microbiol* **47**, 837-840.

Marra, A. & Brigham, D. (2001). *Streptococcus pneumoniae* causes experimental meningitis following intranasal and otitis media infections via a nonhematogenous route. *Infect Immun* **69**, 7318-7325.

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

McEllistrem, M. C., Adams, J., Mason, E. O. & Wald, E. R. (2003). Epidemiology of acute otitis media caused by *Streptococcus pneumoniae* before and after licensure of the 7-valent pneumococcal protein conjugate vaccine. *J Infect Dis* **188**, 1679-1684.

O'Brien, K. L., Millar, E. V., Zell, E. R., Bronsdon, M., Weatherholtz, R., Reid, R., Becenti, J., Kvamme, S., Whitney, C. G. & Santosham, M. (2007). Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. *J Infect Dis* **196**, 1211-1220.

Pelton, S. I., Loughlin, A. M. & Marchant, C. D. (2004). Seven valent pneumococcal conjugate vaccine immunization in two Boston communities: changes in serotypes and antimicrobial susceptibility among *Streptococcus pneumoniae* isolates. *Pediatr Infect Dis J* **23**, 1015-1022.

Piffer, S. (2002). [7-valent conjugate Pneumococcal vaccine and nasopharyngeal cavity]. *Ann Ig* **14**, 31-37.

Porat, N., Barkai, G., Jacobs, M. R., Trefler, R. & Dagan, R. (2004). Four antibiotic-resistant *Streptococcus pneumoniae* clones unrelated to the pneumococcal conjugate vaccine serotypes, including 2 new serotypes, causing acute otitis media in southern Israel. *J Infect Dis* **189**, 385-392.

Serrano, I., Ramirez, M. & Melo-Cristino, J. (2004). Invasive *Streptococcus pneumoniae* from Portugal: implications for vaccination and antimicrobial therapy. *Clin Microbiol Infect* **10**, 652-656.

van Ginkel, F. W., McGhee, J. R., Watt, J. M., Campos-Torres, A., Parish, L. A. & Briles, D. E. (2003). Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection. *Proc Natl Acad Sci U S A* **100**, 14363-14367.

Whitney, C. G., Pilishvili, T., Farley, M. M., Schaffner, W., Craig, A. S., Lynfield, R., Nyquist, A. C., Gershman, K. A., Vazquez, M., Bennett, N. M., Reingold, A., Thomas, A., Glode, M. P., Zell, E. R., Jorgensen, J. H., Beall, B. & Schuchat, A. (2006). Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. *Lancet* **368**, 1495-1502.

Wuorimaa, T. & Kayhty, H. (2002). Current state of pneumococcal vaccines. *Scand J Immunol* **56**, 111-129.