Epidemiological studies of *Streptococcus pneumoniae* carriage in the post-vaccination era among two risk groups: children and the elderly.

Sónia das Neves Nicolau Nunes Leitão

Dissertation presented to obtain the Ph.D. degree
in Biology/Molecular Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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Knowledge Creation

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Aos meus filhos e ao meu marido…

ABSTRACT

Streptococcus pneumoniae is a global cause of disease including pneumonia, otitis media, conjunctivitis, sepsis, and bacterial meningitis. These infections are not essential to the transmission or long-term survival of the bacterium; indeed, *S. pneumoniae* depends on asymptomatic colonization of the human nasopharynx for its dissemination to additional hosts. Considering this, colonization studies are a good way to monitor changes in the pneumococcal epidemiology that may result from the use of antibiotics and vaccines. The molecular characterization of pneumococci is crucial to assess these changes which highlight the need for the development and validation of easier and faster methods of molecular typing.

Since 1996 our group has been monitoring the pneumococcal population colonizing children attending day care centers. However, for several years these studies have been confined to the Lisbon area. In this PhD we have addressed this situation by including other regions of Portugal in our study. In addition, we have started to study pneumococcal colonization in the elderly, the other age group where the incidence of pneumococcal infections is high.

This thesis summarizes five studies conducted during this PhD. The first four studies were focused on the pneumococcal epidemiology among the two age groups where the rates of pneumococcal disease are highest: children up to six years old and adults older than 60 years. The fifth and last study describes the evaluation and validation of a new genotyping strategy for pneumococci.

The first study was a retrospective study aimed to describe the epidemiology of the recently discovered serotype 6C colonizing healthy children attending day care centers between 1996 and 2007. In that study, using PCR serotyping and the Quellung reaction, pulsed-field gel electrophoresis (PFGE), multilocus

sequence typing (MLST), and antibiotyping, we observed that this serotype had been circulating in Portugal since at least 1996, it is genetically diverse, and often antibiotic resistant.

The second study was conducted in Coimbra and aimed to determine the prevalence of pneumococcal carriage and to characterize the pneumococcal strains colonizing children attending day care centers in this region in the era of pneumococcal vaccines. Between January and February 2007, nasopharyngeal swabs were obtained from 507 children (76.7% had received at least one dose of PCV7) and 61.3% were pneumococcal carriers. All 311 pneumococcal samples were antibiotyped and serotyped and the pneumococcal strains that were resistant to at least one antibiotic were also typed by PFGE. We have found in Coimbra similar rates of colonization and antimicrobial resistance patterns and similar genotypes to those previously described in the Lisbon area.

The aim of the third study was to compare the patterns of colonization among young children who attended day care centers and lived in two different areas: an urban area, Oeiras, and Montemor-o-Novo, a rural area in the Southern part of Portugal. In this study 1088 and 756 nasopharyngeal swabs from day care center attendees from urban and rural area, respectively, were collected. Pneumococcal strains were characterized by antibiotype and serotype. Similar rates of colonization (c.a. 61%) were found; however, in the urban area there were higher rates of antimicrobial resistance (32.4% vs 21.6%, $p<0.001$) and higher rates of antimicrobial consumption one month before sampling (16.7% vs 11.6%, $p=0.004$). Multivariable logistic regression analysis was performed to identify the factors associated with differences between the two regions. Antibiotic consumption during last month, being colonized with serotype 19A or non-typeable strains, and attending day care in Oeiras and being colonized with serotype 19A, were risk factors for being colonized by pneumococcal resistant strains. This study highlighted that in the era of widespread use of

pneumococcal conjugate vaccines in Portugal, antibiotic consumption remains a main driving force for the maintenance of antimicrobial resistant pneumococci in the community.

To gain insights into the pneumococcal carriage patterns among the elderly, the fourth study was performed in two distinct areas: Oeiras and Montemor-o-Novo. In this study, 1,298 nasopharyngeal samples were collected. All pneumococci were antibiotyped, serotyped and characterized by MLST. Association between pneumococcal carriage, socio-demographic and clinical factors was evaluated using a logistic regression. The rates of colonization in this age group were low (2.2%), but there were high variability of colonizing serotypes and genetic backgrounds. Out of the 30 pneumococcal isolated, sixteen showed antimicrobial resistance. Smoking was a risk factor for pneumococcal colonization and living in the rural area seemed to increase the rate of pneumococcal colonization in this population. This study provided an important baseline to monitor the impact of pneumococcal vaccines on the patterns of colonization among the elderly.

In the last part of this thesis, we described and validated a newly developed multiple-locus variable tandem repeat analysis (MLVA) method to easily and rapidly genotype pneumococcus. This method was compared to the current gold standards methods, MLST and PFGE. The three typing methods showed Simpson´s diversity indices of 98.5% or higher. The Wallace coefficient of MLVA and MLST was 0.874, meaning that if two strains had the same MLVA type they had an 88% chance of having the same MLST type. For some isolates belonging to a single MLST clonal complex, despite displaying different serotypes, MLVA was more discriminatory, generating groups according to serotype or serogroup. This study showed that MLVA is a promising genotyping method for *S. pneumoniae*.

Altogether, these studies have contributed to improve our knowledge on pneumococcal colonization in Portugal in two age groups, children and the elderly.

The main conclusions of this thesis were:

i) the recently described serotype 6C is frequently carried by healthy young children in Portugal, is genetically diverse, and has been circulating in our country at least since 1996;
ii) the patterns of pneumococcal colonization among healthy children living in Coimbra are similar to those living in the Lisbon area;
iii) antibiotic consumption remains a main cause for the maintenance of antimicrobial resistance, in the era of widespread use of pneumococcal conjugate vaccines;
iv) the rates of pneumococcal colonization in the elderly are low and the serotype and genotype diversity are high;
v) MLVA is a promising genotyping method to characterize pneumococci.

RESUMO

Streptococcus pneumoniae, ou pneumococos, é responsável por várias doenças em todo o mundo nas quais se incluem as infeções do trato respiratório, do ouvido médio, conjuntivite, meningite e sepsis. No entanto, é a elevada prevalência na nasogaringe em portadores assintomáticos que contribui para a sua disseminação na população. Assim, os estudos de colonização por pneumococos são importantes pois permitem monitorizar a flora da nasofaringe em vários grupos etários, e estudar a influência da utilização de vacinas e do uso de antibióticos. Igualmente importante é a possibilidade de otimização das vacinas de acordo com as características da população alvo. A caracterização molecular de pneumococos é crucial para a avaliação dessas alterações, o que alerta para a necessidade do desenvolvimento e validação de métodos de tipagem molecular fáceis e rápidos.

Desde 1996 que o nosso grupo tem vindo a estudar a população pneumocócica em crianças saudáveis que frequentam creches e jardins de infância. No entanto, esses estudos estiveram desde sempre confinados à área da grande Lisboa. Com o intuito de obter uma amostra populacional mais abrangente e variada, esta tese inclui estudos de outras regiões de Portugal não só em crianças mas também na população idosa, uma vez que este último constitui também um grupo etário de risco, com elevada incidência de doença pneumocócica.

Nesta tese são apresentados cinco estudos que foram realizados durante este doutoramento. Os primeiros quatro estudos focaram-se na epidemiologia de pneumococos, tendo sido envolvidas nos primeiros três, crianças até aos 6 anos de idade e no quarto estudo, adultos com mais de 60 anos. O quinto e

último estudo descreve e faz-se a validação de uma nova estratégia de tipagem molecular para pneumococos.
O primeiro trabalho é um estudo retrospectivo que descreve a epidemiologia de um serotipo recentemente descoberto, 6C. Este estudo utilizou amostras que foram recolhidas da nasofaringe de crianças saudáveis que frequentavam creches e jardins de infância entre 1996 e 2007. Nesse estudo, utilizou-se a serotipagem por PCR complementada com a reacção de Quellung, electroforese em campo pulsado (PFGE), “multilocus sequence typing” (MLST) e antibiograma, para caracterizar os isolados. Concluiu-se que este serotipo já circulava em Portugal pelo menos desde 1996, que é geneticamente diverso, e que frequentemente apresenta resistência a antibióticos.

O segundo estudo foi realizado em Coimbra e teve como objectivos determinar a prevalência de portadores de pneumococos e caracterizar as estirpes de pneumococos que colonizam crianças que frequentam creches e jardins de infância nesta região, na era das vacinas pneumocócicas. Entre Janeiro e Fevereiro de 2007 foram pesquisadas 507 crianças (76.7% tinham recebido pelo menos uma dose de vacina pneumocócica conjugada 7-valente) das quais 61.3% eram portadores de pneumococos. Todos os 311 pneumococos isolados foram serotipados e foi realizado o antibiograma. Às estirpes de pneumococcus que apresentavam resistência a pelo menos um agente antimicrobiano foi também realizada a tipagem por PFGE. Em Coimbra as taxas de colonização, os padrões de resistência a antibióticos e os genotipos encontrados foram semelhantes aos descritos previamente na área metropolitana de Lisboa.

O objectivo do terceiro estudo foi comparar os padrões de colonização em crianças que vivem e frequentam creches e jardins de infância em duas áreas distintas: uma área urbana, Oeiras, e uma área rural na região centro-sul de Portugal, Montemor-o-Novo. Neste estudo foram recolhidas 1088 e 756 zaragatoas da nasofaringe de crianças da área urbana e da área rural,

respectivamente. Os pneumococos foram caracterizados por serotipagem e antibiograma. As taxas de colonização encontradas foram semelhantes nas duas regiões (c.a. 61%). No entanto, na área urbana o nível de resistência a agentes antimicrobianos foi mais elevado (32.4% vs 21.6%, $p<0.001$), o mesmo acontecendo relativamente às taxas de consumo de antibióticos um mês antes da amostragem (16.7% vs 11.6%, $p=0.004$). Foi realizada uma análise estatística multivariada para identificar os fatores associados às diferenças encontradas nas duas regiões. O consumo de antibióticos durante o mês antecedente à recolha da amostra, estar colonizado com o serotipo 19A ou com uma estirpe não-tipável, e frequentar creche ou jardim de infância em Oeiras foram considerados fatores de risco para a colonização por pneumococos resistentes a antibióticos. Este estudo alerta para o facto do consumo de antibióticos continuar a ser a principal causa para a manutenção dos níveis de pneumococos resistentes a antibióticos encontrados na comunidade apesar da grande utilização de vacinas pneumocócicas conjugadas na comunidade infantil em Portugal.

O quarto estudo foi realizado com o objetivo de obter informação relativa aos padrões de colonização de pneumococos na população idosa. Neste estudo, 1298 amostras da nasofaringe foram recolhidas em duas áreas distintas: Oeiras e Montemor-o-Novo. Todos os pneumococos foram serotipados e caracterizados por MLST e foi também realizado o antibiograma. A associação entre portadores de pneumococos, fatores socio-demográficos e fatores clínicos foi avaliada usando uma regressão logística. As taxas de colonização neste grupo etário foram baixas (2.2%), mas encontrou-se uma grande variedade de serotipos e genotipos. Dos 30 pneumococos isolados, 16 mostraram resistência a agentes antimicrobianos. Foram identificados como fatores de risco para a colonização por pneumococos tabagismo e habitar em área rural. Este estudo possibilitou a criação de uma importante linha de

referência para monitorizar o impacto das vacinas pneumocócicas nos padrões de colonização nos idosos.

Na última parte desta tese foi descrita e validada uma nova estratégia de "multiple-locus variable tandem repeat analysis" (MLVA) aplicada a pneumococos que permite de uma forma fácil e rápida, fazer uma análise genotípica deste microrganismo. Este método foi comparado com os métodos mais utilizados atualmente MLST e PFGE. Os três métodos mostraram índices de diversidade de Simpson de 98.5% ou superiores. O coeficiente de Wallace para MLVA e MLST foi de 0.874, o que significa que se duas estirpes tiverem o mesmo tipo de MLVA tem 88% de probabilidade de terem o mesmo tipo de MLST. O MLVA foi mais discriminativo para alguns isolados do mesmo complexo clonal (por MLST), gerando grupos de acordo com o serotipo ou serogrupo. Este estudo mostrou que o MLVA é um método promissor para genotipar *S. pneumoniae*.

Em suma, os trabalhos apresentados contribuíram para melhorar o nosso conhecimento em relação à colonização por pneumococos em Portugal em duas faixas etárias, crianças e idosos. As principais conclusões desta tese foram:

i) o serotipo 6C, recentemente descrito, é frequentemente encontrado em crianças saudáveis em Portugal, é geneticamente diverso e tem estado em circulação no nosso país pelo menos desde 1996;
ii) os padrões de colonização por pneumococos em crianças saudáveis que vivem em Coimbra são semelhantes aos encontrados nas crianças que vivem na área de Lisboa;
iii) o consumo de antibióticos continua a ser a principal causa da manutenção da resistência antimicrobiana na era da grande utilização de vacinas pneumocócicas conjugadas;

iv) as taxas de colonização por pneumococos nos idosos são baixas mas a diversidade de serotipos e de genotipos é elevada;
v) o MLVA é um método de genotipagem promissor para caracterizar pneumococos.

THESIS OUTLINE

The studies presented in this thesis provide an overview of pneumococcal colonization in Portugal in two age groups: children and the elderly.

Chapter I provides an outline of *Streptococcus pneumoniae* as a bacterium that frequently colonizes the nasopharynx but can be also responsible for serious diseases. Topics such as historical highlights, epidemiology, identification, typing methods and the effect of pneumococcal vaccines are addressed.

Chapter II describes the temporal trends and molecular epidemiology of the recently described serotype 6C of *Streptococcus pneumoniae.* The study analyses the evolution of this serotype from 1996 to 2007, by antibiotyping, serotyping and also using PFGE and MLST.

Chapter III describes the pneumococcal population colonizing the nasopharynx of children attending day care centers in Coimbra, a city in the central region of Portugal.

Chapter IV compares the patterns of pneumococcal colonization of young children attending day care centers in two distinct areas of Portugal: one rural, Montemor-o-Novo, and other urban, Oeiras.

Chapter V is a study of pneumococcal colonization in the elderly in Portugal that was also performed in an urban area, Oeiras, and a rural area, Montemor-o-Novo.

Chapter VI describes and validates a novel genotyping strategy applied to pneumococci: multiple-locus variable tandem repeat analysis (MLVA).

Chapter VII presents general conclusions and futures perspectives.

Chapters II to **VI** are reproductions of the publications indicated below. They can be read independently.

Chapter II

Nunes S.*, C. Valente*, R. Sá-Leão, and H. de Lencastre. 2009. Temporal trends and molecular epidemiology of recently described serotype 6C of *Streptococcus pneumoniae*. J Clin Microbiol. **47:**472-4. *These authors contributed equally.

Chapter III

Rodrigues F.*, S. Nunes *, R. Sá-Leão, G. Gonçalves, L. Lemos, H. de Lencastre. 2009. *Streptococcus pneumoniae* nasopharyngeal carriage in children attending day-care centers in the central region of Portugal, in the era of 7-valent pneumococcal conjugate vaccine. Microb Drug Resist. **15:**269-77. *These authors contributed equally.

Chapter IV

Nunes S., C. Valente, A. S. Simões, A. C. Paulo, A. Brito-Avô, H. de Lencastre and R. Sá-Leão. 2012. Antibiotic consumption remains a main driving force of antimicrobial resistance in the era of pneumococcal conjugate vaccines. *In preparation*.

Chapter V

Nunes S., S. Almeida, A. C. Paulo, I. Valadares, S. Martins, F. Breia, A. Brito-Avô, A. Morais, H. de Lencastre, and R. Sá-Leão. 2012. Low pneumococcal carriage and high serotype diversity among elderly living in Portugal. *In preparation.*

Chapter VI

Elberse KE*, S. Nunes *, R. Sá-Leão, H.G. van der Heide, and L.M. Schouls. 2011. Multiple-locus variable number tandem repeat analysis for *Streptococcus pneumoniae*: comparison with PFGE and MLST. PLoS One. 6:19668. *These authors contributed equally.

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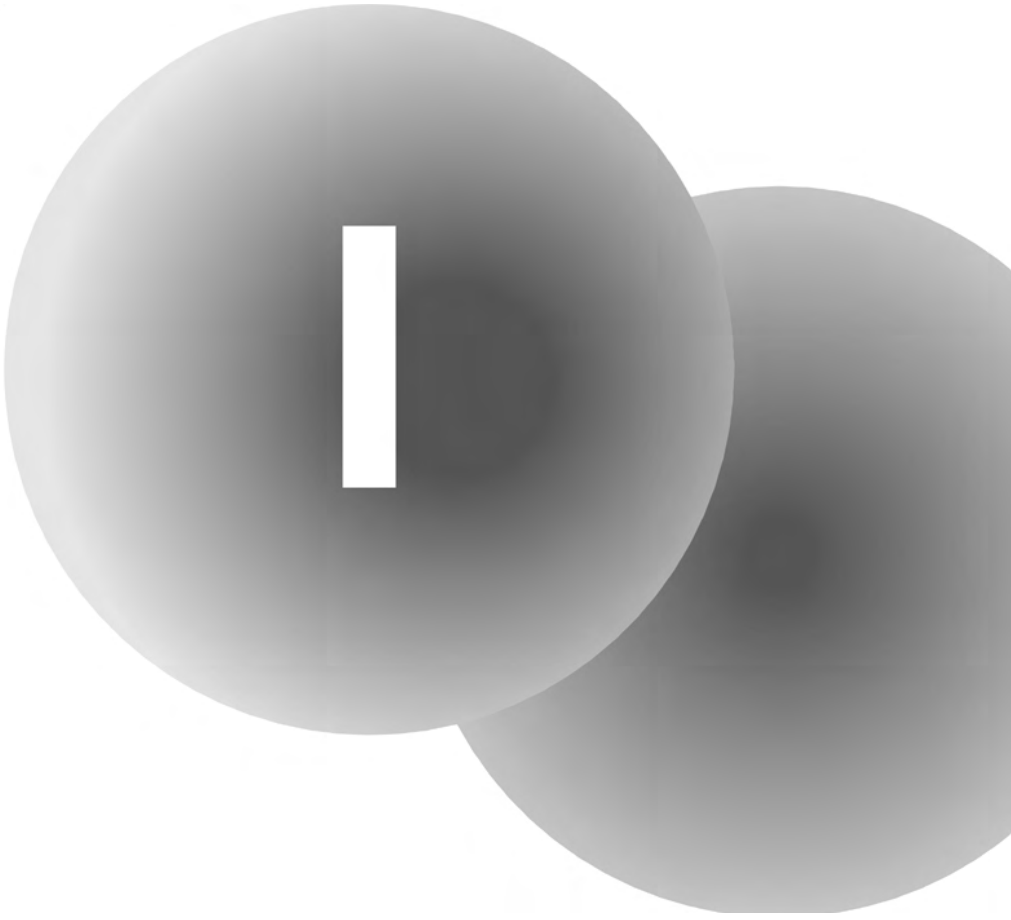
General Introduction



INTRODUCTION

Streptococcus pneumoniae, or pneumococcus, is a Gram-positive bacterium responsible for high rates of mortality and morbidity worldwide. It is a leading cause of disease particularly among young children, the elderly, and the immuno-compromised of all ages. Although it colonizes asymptomatically the nasopharynx, *S. pneumoniae* can cause otitis media, sinusitis, pneumonia and more severe diseases such as bacteremia and meningitis. Pneumococcal disease remains a great concern worldwide despite great advances aiming to decrease it, such as the use of antibiotic therapy and prevention through vaccination.

Since colonization precedes disease, epidemiological studies monitoring the nasopharynx flora are valuable tools to anticipate the emergence of serotypes that may became responsible for disease in the future.

HISTORICAL HIGHLIGHTS

In 1875 Edwin Klebs was the first to recognize *Streptococcus pneumoniae* in lung tissue and in infected sputum (reviewed in (54)), but only six years later this bacterium was isolated by two researchers working in France and the USA. In January 1881, Louis Pasteur in France reported for the first time the isolation of *S. pneumoniae*, after finding bacteria in the saliva of a youngster with rabies. George Miller Sternberg was in New Orleans studying malaria fever when he sampled his own saliva (as a control for his experiments) and injected it subcutaneously in a rabbit, which quickly died. This observation was in September 1880, but did not publish his report until April 1881(reviewed in (54)).

More than 90 years went by before this bacterium was named as *Streptococcus pneumoniae.* Pasteur called his isolate the “microbe septicémique du saliva” and

Sternberg called his "*Micrococcus pasteuri".* In 1883 Mátray applied the term "pneumoniekokken" and Albert Fraenkel in 1886, after the first complete description of the bacterium, gave us the familiar name "pneumokokkus". In the same year, Anton Weichselbaum suggested the name *Diplococcus pneumoniae*, which was used until the reclassification, on the basis of its growth in chains in liquid medium, as *Streptococcus pneumoniae,* in 1974 (reviewed in (54)).

After its isolation and identification, *S. pneumoniae* was the model organism used in important discoveries, including Gram staining in 1884 and the putative use of polysaccharide antigens as vaccines (reviewed in (54)). The identification of DNA as the source of genetic information was done using pneumococcus. Griffith in 1928 showed that avirulent strains could be transformed into virulent strains. Avery, MacLeod and McCarty demonstrated in 1944 that DNA was responsible for the transformation and thus the carrier of genetic information (reviewed in (54)). The therapeutic efficacy of penicillin, the role of bacterial capsule in resistance to phagocytosis (reviewed in (54)), and the first quorum sensing factor (149) were also described for the first time in pneumococci.

EPIDEMIOLOGY OF PNEUMOCOCCI

Diseases caused by the pneumococcus constitute a major global public health problem. According to the World Health Organization (WHO), in 2005, about 1.6 million deaths were caused by this agent annually, from which 0.7-1 million were among children under five years. In developing countries, pneumococcal disease is common in children under two years; in the elderly population the burden of disease is largely unknown. HIV infection and other conditions associated with immunodeficiency greatly increase the likelihood of contracting pneumococcal disease. Of the deaths occurring in HIV-negative children, over 60% occur in ten African and Asian countries (109) (Figure 1).

In the USA and Europe *S. pneumoniae* is the most common cause of pneumonia in adults. The annual incidence of invasive pneumococcal disease (IPD) in these regions ranges from 10 to 100 cases per 100,000 (WHO, www.who.int/ith/disease/pneumococcal).

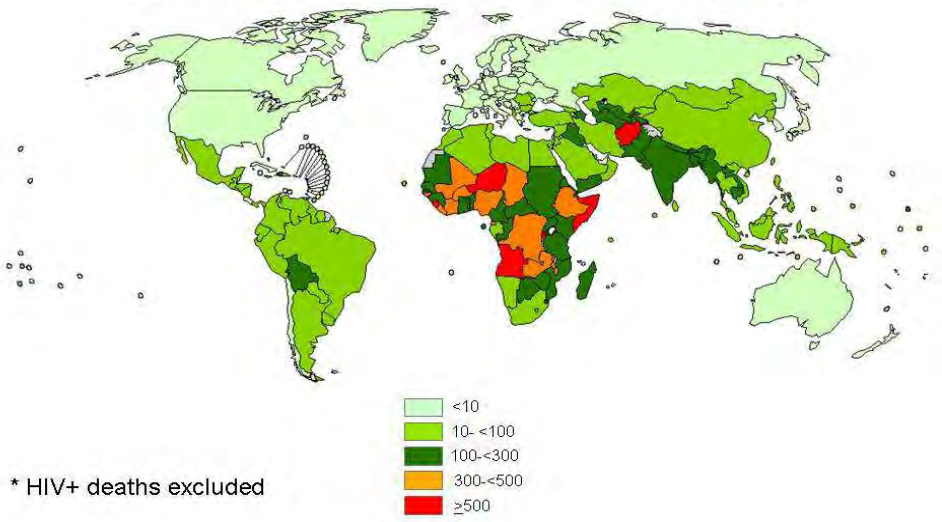


Figure 1. – Rates of *Streptococcus pneumoniae* death (per 100,000 children under age 5). Data from WHO August, 2009.

Pneumococcal carriage

The pneumococcus is a normal component of the nasopharyngeal mucous membrane microflora. Acquisition is the first stage towards carriage and possible infection with pneumococcus. Carriage is usually asymptomatic. Transmission occurs by airborne droplets or direct contact with respiratory secretions (102). Recently, it has been suggested that environmental surfaces may also serve as sources of pneumococcal infection as pneumococci were found to be able to survive long-term desiccation (157).

Children are the major carriers and colonization occurs soon after birth (6). The peak in incidence is more or less at three years of age (15) and then declines with increasing age, until 10 years of age, remaining low in adulthood (15). Few studies have studied nasopharyngeal colonization in the elderly, but the carriage rates are typically low, ranging between 2 and 5% (2, 15, 68, 121).

Since it is in young children where the highest frequency of pneumococcal colonization and the highest crowding index are found, this group is considered the most important vector for horizontal dissemination of pneumococci in the community (88). Consequently, part of the strategy to prevent pneumococcal disease focuses on prevention of nasopharyngeal colonization.

In Portugal, data of pneumococcal carriage in healthy children attending day care centers, have been published by our group since 1996 and rates have been generally close to 60-65% (42, 43, 95, 104, 125, 128, 137). Concerning colonization in the elderly in Portugal, no studies had been conducted until recently. This topic was addressed for the first time in the pioneer study described in Chapter V of this thesis.

Pneumococcal carriage is not a permanent state. In fact, once established in the nasopharynx, pneumococci are cleared within weeks to months; subsequently the nasopharynx can be recolonized by another strain. The duration of carriage is variable (5 to 290 days) depending on the serotype and the age of the carrier (53, 141, 143). Risk factors for colonization among others are young age (lower than 6 yrs), having young siblings, crowding (e.g. attending day-care centers, prisons, military training campus, sports with contact, retirement homes), season, low socioeconomic status, other respiratory infections such as influenza, immunosuppression such as HIV-infection, exposure to cigarette smoke, and asthma (1, 7, 23, 51, 53, 80).

Most studies on pneumococcal colonization studies are based on the characterization of a single isolate of each individual. However, more than one pneumococcal serotype or clone can coexist in the nasopharynx of an individual (50, 53, 108, 129, 151). For this reason, studies on co-colonization are very important to identify changes at the individual level, such as *de novo* acquisition, clearance and unmasking of pneumococcal strains (which can include serotypes or clones) (37, 90), particularly in the era of multivalent pneumococcal conjugate vaccines.

Pneumococcal disease

S. pneumoniae may spread from the nasopharynx into the respiratory tract or the bloodstream to cause infections. These infections can be divided in two groups: invasive (isolated from sterile body sites) and non-invasive (isolated from non-sterile body sites). Otitis media and sinusitis are non-invasive infections. The nasopharynx is connected with the middle ear cavity via the Eustachian tube, which is a pressure regulator and prevents the entrance of the bacteria in the middle ear cavity. However, if the Eustachian tube is blocked, the bacteria may be trapped in the middle ear and cause otitis media. Sinusitis is caused when the bacteria spread locally into the sinuses and cause an accumulation of fluids in the sinuses due to an obstruction. The pneumococcus can also be responsible for more severe infections such as pneumonia, bacteremia and meningitis. From the nasopharynx, the bacteria may spread into the lungs or directly to the bloodstream. If bacteria spread into the lungs the immune system reacts and the accumulation of fluids and bacteria in the alveoli decreases oxygen transport and causes pneumonia (74). In adults, pneumonia without bacteremia is the most common non-invasive pneumococcal infection, however if the pneumococcus spreads into bloodstream, the infection becomes invasive. Pneumonia with bacteremia and meningitis are the most frequently recognized invasive disease. Pneumococcal meningitis occurs when the bacteria spread directly to bloodstream into the meninges (membranes that surround and protect the brain and spinal

cord). The meninges are filled with a liquid called cerebrospinal fluid where the bacteria can multiply freely, causing inflammation and swelling of the meninges and the brain tissue. Other invasive diseases are sepsis, peritonitis and arthritis.

Risk factors for pneumococcal infection are numerous and include, among many others, extremes of age (under 5 years of age and over 60 years old), immunosuppression, presence of underlying medical conditions, defects in host immune responses, low socioeconomic status, malnutrition, cigarette smoking and alcoholism (29, 91, 106, 107).

In Portugal, the report of pneumococcal infectious disease is not mandatory and, to my knowledge, no study has been carried out to calculate the incidence of invasive pneumococcal disease (IPD) over time. However, epidemiological studies on pneumococcal infections using data from several hospitals in different areas of Portugal have been conducted (3, 5, 63, 134, 135).

PROPERTIES OF STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae is a lancet-shaped Gram-positive bacterium, whose diameter ranges between 0.5 and 1.25 µm for an individual cell. Pneumococci are frequently seen as pairs of cocci (diplococci), but they can also be in single cell and in short chains. It is a fastidious organism, growing best in media containing blood and in an enriched atmosphere with 5% CO_2. On blood agar, colonies characteristically produce alpha hemolysis, form an inhibition halo ≥14 mm around a 5 mg optochin disc, and are soluble in bile salts (e.g. deoxycholate). Addition of a few drops of 10% deoxycholate at 37°C, lyses the entire culture in minutes. The majority of pneumococci have a polysaccharide capsule and the assignment of a capsule type through serotyping is also used for identification of this bacterium. However, some pneumococci do not have a capsule and others give an atypical

result in one or more of the assays described above (4, 32, 105, 111, 117, 127, 138).

The capsule is the main virulence factor of the pneumococcus and, with the exception of serotype 3, is covalently linked to the peptidoglycan of the cell wall (146). The capsule is 200 to 400nm thick (140), protects against opsonophagocytosis, and plays an important role in the interaction between the bacteria and the epithelium in colonization of the upper respiratory tract (103, 158). In addition, the pneumococcus may shift from transparent-phase (capsule less dense and thicker cell wall) to opaque-phase and vice-versa. This allows for attachment to the epithelial cells in carriage. The pneumococci in opaque-phase have denser capsules, providing better protection against opsonization and killing in invasive disease (159).

Based on the reaction of the capsular polysaccharide with polyclonal factor sera and more recently with the use of monoclonal antibodies, 94 serotypes are recognized up to now (16, 21, 22, 61, 115).
The first “novel” serotype recognized using monoclonal antibodies was 6C (115). As it was previously classified as 6A by the Quellung reaction, the real prevalence of this serotype and its characteristics were not known. In order to obtain insights on its epidemiology in colonization in Portugal, a retrospective study was performed and is described in this thesis in Chapter II.

Distribution of serotypes

Although 94 distinct pneumococcal serotypes, varying in capsular polysaccharide structure, have been described, not all seem to have the same capacity to cause disease (18, 60, 126, 136). Some serotypes can be carried in the nasopharynx asymptomatically and cause disease in a small proportion of infected individuals only. Others are rarely identified in the nasopharynx but are frequently associated with disease. Serotypes 1 and 5 are examples of serotypes that rarely are

detected in colonization, but are responsible for a high proportion of invasive disease and are frequently associated with outbreaks (8, 13, 38, 96, 123). Before the introduction of pneumococcal conjugate vaccines 10 to 12 serogroups were responsible for the majority of pneumococcal invasive disease worldwide, including 1, 3, 4, 6A, 6B, 7F, 8, 9V, 14, 18C, 19F, and 23F (62, 112).

The prevalence of pneumococcal serotypes/serogroups causing IPD depends on several factors, such as geographic location, disease manifestation, and age of the host (15, 18, 19, 60). In young children the serogroups/serotypes 6, 14, 18, 19 and 23F are predominant due to the lower immunogenicity of these serotypes (20).

Several authors have been looking at the distribution of serotypes in colonization and disease to estimate the invasive potential (18, 19, 55, 131, 141). According to Brueggeman *et al.* (18) and Sleeman *et al.* (141) serotypes 1, 4, 5, 7F, 9V, 14, 18C, and 19A are classified as highly invasive serotypes. In a study reported by Greenberg *et al.* (55) the authors estimated the disease potential in pediatric community-acquired alveolar pneumonia. In this study the nasopharyngeal samples were also taken from healthy children during disease and the serotype-specific odds ratios were calculated. They observed that serotypes 1, 5, 7F, 9V, 14, 19A, and 22F have a higher disease potential than serotypes 6A, 6B, 23A, and 35B in childhood pneumonia. In Israel another study was conducted to assess the invasive disease potential of pneumococcal serotypes causing IPD, acute otitis media and acute conjunctivitis in children. Odds ratios for each disease were calculated and a significant positive association with IPD was found for serotypes 1, 5 and 12F. Also, a significant positive association was found in otitis media for serotypes 1, 3, 5, 12F, 19A and 19F, and in acute conjunctivitis, for serotype 3 and NT strains. On the other hand, a significant negative association with IPD was found in NT strains and in acute otitis media in serotypes 6A, 6B, 15A and NT strains (136).

In Portugal, a study was published (126) where the invasive disease potential of serotypes and clones circulating in Portugal before the introduction of PCV7 was calculated. In this study serotypes 1, 3, 4, 5, 7F, 8, 9N, 9L, 12B, 14, 18C and 20 were found to have a high propensity to cause invasive disease, whilst serotypes 6A, 6B, 11A, 15B/C, 16F, 19F, 23F, 34, 35F, and 37 were associated with carriage. Additionally, significant differences in invasiveness were found between clones that shared the same serotype, namely among serotypes 3, 6A, 6B, 11A, 14, 19A, 19F, 22F, 23F, 34 and NT, which highlights the importance of the genetic background when analyzing the invasive disease potential of certain serotypes.

The distribution of serotypes is also affected by temporal changes, inherent of secular trends of specific serotypes, and this has been reported in several studies (57, 59, 71, 120).

Serotype distribution can be altered by antibiotic consumption and vaccination patterns. Following the introduction of the first pneumococcal conjugate vaccine, the increase of non-vaccine serotypes has been reported in several countries, in disease and also in colonization (more details are presented in the next topic).

Use of antibiotics and pneumococcal vaccines

Nowadays, the main tool to control pneumococcal disease, besides antibiotic therapy, is vaccination. Efforts to develop effective pneumococcal vaccines began in 1911, when the first clinical trial on the effectiveness of a whole-cell pneumococcal vaccine was conducted by Wright *et al*. in South-Africa (reviewed in (93)). Several decades were needed before the effectiveness of pneumococcal vaccines was demonstrated. Only at the end of World War II in 1945 (92), the first capsular polysaccharide vaccine trials were undertaken.

Antimicrobial resistance

Before penicillin became available for medical treatment in the early 1940s, no cure for pneumonia existed and many people died with pneumococcal infection. The introduction and use of antimicrobial drugs changed this scenario and led to the withdrawn from the market of a pneumococcal vaccine that was licensed and in usage. However, it did not take long before it became clear that antimicrobials *per se* had not eliminated pneumococcal disease.

In 1964, Austrian and Gold reported that one in four patients admitted with pneumococcal bacteremia died even with antimicrobial therapy (12). In 1967, the first pneumococcal isolate with intermediate resistance to penicillin was described by Hansman *et al.* (58) in Australia. Within a decade, similar strains were found in South Africa, Europe and North America (77). The first multiresistant pneumococcal isolates were reported by Jacobs *et al*. in 1978 (70) and since then we have been observing a widespread of multidrug resistance has been observed (33, 97).

The rates of antimicrobial resistance differ from country to country and depend on several factors as antibiotic consumption habits, use of pneumococcal vaccines, and geographic areas. The last report from the European Antimicrobial Resistance Surveillance Network (EARS-Net Database) from 2010 indicated that, in Europe, pneumococcal resistance to macrolides ranges between 4%, in Sweden and UK, and around 30% in Spain and France. Concerning the prevalence of penicillin non-susceptible pneumococci, Belgium showed the lower rate (0.4%) and, in contrast, Spain and France appear in the top of the ranking, with values of 27.6% and 29.8%, respectively (45). In the USA, in 2008 CDC reported that 10.7% of the pneumococcal isolates in invasive disease showed decreased susceptibility to penicillin. Resistance to macrolides increased in all parts of the USA since 2000 and in 2006 the rate was 30% (72). In Asian countries in non-meningeal isolates recovered from 2008-2009, 5.3% of the isolates showed a MIC≥4 µg/mL to

penicillin. Resistance to erythromycin was very prevalent, 72.7%, and multidrug resistance was observed in 59.3% of the isolates (76) (reviewed in (144)).

In Portugal, among invasive disease isolates in adults, the latest data (2006-2008) showed rates of penicillin non-susceptibility around 17% and the resistance to macrolides was 18% (63). In children (including children until 17 years) during the same period, around 19% of the isolates expressed non-susceptibility to penicillin and resistance to erythromycin was found in 22.9% of the isolates. Additionally 11.6% of the isolates showed simultaneously resistance to both antimicrobial agents. In young healthy children data from 2006/2007 showed rates of macrolides resistance of 25.3% and 26.6% of the strains showed decreased susceptibility to penicillin (137).

Pneumococcal vaccines

In 1977, Austrian *et al.* reinforced the interest of pneumococcal prevention and a pneumococcal polysaccharide vaccine including 14 serotypes was licensed (11). This vaccine was extended to 23 serotypes and licensed in 1983 (Pneumovax23, Merck&Co, PPV23).

In children, responses induced by polysaccharide vaccines are low (44). Conjugation of the polysaccharides with a carrier protein induced better antibody responses in infants due to the T-cell dependent immune pathway. In 2000, the first pneumococcal conjugate vaccine was licensed (Prevnar™; Wyeth-Lederle, PCV7). It included the seven serotypes most common in pneumococcal disease in children under 2 years old in the United States (4, 6B, 9V, 14, 18C, 19F and 23F). In PCV7 the polysaccharide of each serotype is individually conjugated to the protein carrier, CRM_{197}. In Portugal, this vaccine was neither introduced in the National Vaccination Plan nor subsidized by the state. However, the majority of pediatricians recommended this vaccine what explained high intake of the vaccine; the most frequently used was three doses at 2, 4, 6 months and a booster after 12

months. Data obtained from Pfizer, Portugal, and our data from colonization studies in the Lisbon area, until 2007 (137), around 70% of the children under six years old were vaccinated suggested that at least one vaccine dose.
In 2009, a new pneumococcal conjugate vaccine was introduced in the market (Synflorix®, GlaxoSmithKline Inc., PCV10). It included the same serotypes in PCV7 plus three additional serotypes (1, 5, 7F; serotypes 18C and 19F are conjugated to tetanus and diphtheria toxoids, respectively, and the remaining 8 serotypes are conjugated to the non-typeable *Haemophilus influenzae* protein D).

In 2010, PCV7 was replaced by PCV13 (Prevenar®; Pfizer). This vaccine included serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F. These vaccines have not been introduced in the National Vaccination Plan, and are not subsidized by the state in Portugal.

According to the Global Alliance for Vaccines and Immunisation (GAVI), in July of 2012, 18 countries in the developing world started the introduction of pneumococcal conjugate vaccine and 36 GAVI eligible countries have been approved for GAVI support to introduce pneumococcal conjugate vaccine into the national immunization programmes. GAVI and partners hope that by 2015, 90 million children can be immunized with pneumococcal vaccines and the pneumococcal vaccine rollout has been achieved in 58 countries (http://www.gavialliance.org).

Recent data from Active Bacterial Core Surveillance (ABCs) in the USA indicated a decreased in the incidence of IPD among children <5 years from approximately 99 cases per 100,000 during 1998-1999 to 21 cases per 100,000 in 2008 (28). Data from the last report of ABCs from 2012 (25), indicated 12,8 cases of pneumococcal infection per 100,000, with higher rates in children under one year of age and adults over 65. Considering the rates of death by this bacterium, in total

1,24 cases per 100,000 were reported, with higher rates in adults aged more than 50 years.

Considering the high serotype diversity within *S. pneumoniae*, the high cost of these vaccines, and the fact that serotype coverage varies between geographic regions, there is the need of developing vaccines that confer broader coverage, preferentially independent of the capsular polysaccharide and with a lower cost.

Currently a 15-valent pneumococcal conjugate vaccine (PCV13 serotypes plus serotypes 22F and 33F) is in evaluation in an infant-rhesus monkey model (139) and a new approach based on whole-cell is also in development (100).

A vaccine which goal was protection against pneumococcal nasopharyngeal colonization was also designed. This vaccine was based in specific antigens that activated $CD4^+$ T cells to secrete the cytokine interleukin-17A that mediate resistance to mucosal colonization. Recently, was reported that this vaccine was tested in a mouse model and showed protection against pneumococcal infection (99, 101).

EFFECTS OF PNEUMOCOCCAL VACCINATION

Decrease of vaccine serotypes

In the USA the introduction of PCV7 in the vaccination schedule began shortly after the vaccine was licensed, resulting in a decrease of 77% of IPD in vaccinated children aged up to 5 years old by 2005 (26). Two years later, Pilishvili *et al.* analyzed IPD cases, identified between January of 1998 and December of 2007, in eight participating ABCs in the USA. They reported a decline in IPD incidence of 76% in children under 5 years old, with a decline around 100% of PCV7 types (118).

In Europe, Isaacman *et al.* observed the impact of PCV7 use on the incidence of IPD in children from eight countries. The results were variable across studies, with the decrease of IPD incidences ranging from 28% to 68%, depending on the country, vaccine uptake and the type of disease manifestation (69).

Herd immunity

The extensive vaccination with PCV7 has also resulted in an indirect protection of the non-vaccinated population in all age groups, an effect known as herd immunity (26). This was shown for example in a surveillance study conducted in eight areas of the USA with adults older than 50 years old, from which Lexau *et al.* concluded that the use of conjugate vaccine in children also benefited the adults included in the study, with a decline of 28% in IPD in this age group (89). Herd immunity was also reported in newborns, which are too young to receive the vaccine, as described by Poehling *et al.* (119). Also in other countries such as England and Wales (98) and Australia (73), herd immunity was detected in the population.

Serotype replacement

Another effect of pneumococcal vaccination was serotype replacement that results from the ability of non-PCV7 serotype strains to fill the niche left vacant by the PCV7 serotypes. In several countries, although there was a reduction in IPD cases caused by PCV7 serotypes, cases associated to non-PCV7 serotypes increased post-PCV7 introduction (3, 5, 63, 64, 69, 86, 118).

The increase of IPD associated to non-PCV7, especially to serotype 19A, was first documented by a surveillance study conducted in Massachusetts during 2001 to 2007 (64). Pilishvili and co-authors also reported that in the USA, between 1998 and 2007, in ABCs surveillance, the incidence of IPD caused by serotype 19A and also other non-PCV7 types increased in all age groups. Serotype 19A increased from 0.8 to 2.7 cases per 100,000 and other non-PCV7 types increased from 6.1 to 7.9 cases per 100,000 (118). However, this increase remains low comparing

with the substantial decrease of PCV7 types. In this study the increase in IPD caused by non-PCV7 types was more pronounced in meningitis and invasive pneumonia, whereas in primary bacteremia there was no change (118).
Isaacman and co-authors showed also serotype replacement in Europe in a review that describes the trends of IPD in European children between 1990 and 2008. Before the use of PCV7 the serotypes most common recovered in IPD were 6B, 14, 19F and 23F, although with some variation among countries. After the widespread use of PCV, the most common IPD serotypes in Europe were 1, 19A, 3, 6A and 7F (69).

Recently, a study from Alberta, Canada between 1998 and 2010 encompassing PCV7 introduction in the routine vaccination plan, reported that PCV7 types were eradicated from IPD in children under two years old and almost eliminated in all other age groups (86). On the other hand, they observed an increase in non-PCV7 serotype IPD, mainly 19A in children and serotypes 5 and 19A in adults (86). The authors justify this increase in serotype 5 incidence with the occurrence of an outbreak in 2007 (154). Nevertheless, after exclusion of epidemiological data from the year 2007, the increase in the IPD cases associated with serotype 5 was still significant, compared to the pre-PCV7 era (0.03 per 100,000 versus 0.51 per 100,000; $p<0.001$) (86).

However, decrease in IPD incidence and serotype replacement were not observed in particular populations. As an example, among the White Mountain Apache in Arizona, despite the use of PCV7, high rates of IPD continue to be seen (83). One explanation could be the low rates of coverage of this vaccine, only 56.2%, due to the fact that the serotypes that were more prevalent in that population are not included in PCV7 (83). Interestingly, in this population the rate of 19A has been stable or has decreased after vaccination.

These observations highlight that replacement of pneumococcal serotypes may be related to other factors apart from the introduction of a vaccine. For example, in Israel and South Korea, an increase of 19A in IPD was noted before the introduction of PCV7 in the market (30, 36). Also, in the study referred to above, in Canada, the authors mention that a significant increase of serotype 19A in all ages was noticed before the widespread use of PCV7 (86).

The use of PCV7 had also an effect in otitis media, and a global review has been recently published by Rodgers *et al.* (122). In the USA after the introduction of PCV7 it has been observed a decrease in the number of PCV7 cases and an increase in the number of infections produced by non-PCV7 serotypes, such as 19A, 3, 6A (122). Another important aspect reported in some studies from countries where PCVs were introduced was the decrease in the number of medical visits mainly due to otitis and also the number of antibiotic prescriptions (14, 114, 161).
In a study conducted in Israel, with children attending day care centers, children vaccinated with PCV9 needed significantly fewer days of antibiotic uptake for treating respiratory illnesses and otitis than the control group, vaccinated with meningococcal group C vaccine (48).

Antimicrobial resistance

Antimicrobial resistance rates also suffered some alterations with the introduction of PCV7, since the serotypes included in PCV7 tended to have high rates of resistance (39). But, as described before, the decreased of PCV7 serotypes varied according to the geographic location and vaccine uptake (39, 64, 82).

A good example of how PCV7 could be used to help decreasing antimicrobial resistance rates happened in France. In this country, a campaign for the rationalization of antimicrobial prescription paralleled vaccination of young children with PCV7. Although serotype replacement was observed in carriage and in

disease, the rates of IPD in children less than two years decreased as well as the rates of pneumococcal antimicrobial resistance (130, 155).

Invasive pneumococcal disease in Portugal

In Portugal, serotype replacement in pediatric IPD was also reported (3, 5). However, this replacement was not as pronounced as in the USA, where PCV7 was introduced in the national vaccination program unlike what happens in Portugal. Another important aspect was the low coverage of PCV7 in IPD in Portugal, mainly due to the high prevalence of serotype 1 compared to the USA. The fact that PCV7 is not included in the national vaccination plan and the low coverage of this vaccine in IPD in Portugal affect also herd immunity: despite a marked reduction in the proportion of IPD in adults caused by PCV7 serotypes, they still accounted for 18% of the cases between 2006 and 2008 (63). In the same study a decline of serotypes 4, 8 and 14, and an increase of serotypes 1, 7F and 19A were observed in adult IPD in the post-PCV7 period. Similar with adults, in pediatric IPD, serotypes 1, 19A, 7F, 14 and 3 were the serotypes more prevalent in 2006-2008 (3).

Concerning antimicrobial resistance, it remained stable after a small decrease of penicillin non-susceptibility in IPD in children under five years old in the first post-PCV7 years (5). Penicillin non-susceptibility was observed in 18.7% of the isolates and resistance to erythromycin was found in 22.9% of the isolates, from which 18% were NVT (3). On the other hand, in IPD in adults, serotypes 19A and 14 accounted for the majority of erythromycin and penicillin non-susceptible isolates in 2008. Although penicillin non-susceptibility remained stable (17%), resistance to erythromycin has been increasing in the post-PCV7 years (63).

7-valent pneumococcal vaccine and carriage

The first report to show that conjugate pneumococcal vaccines could decrease pneumococcal carriage and pneumococcal antibiotic resistance was published in

1996. In that study carried out by Dagan *et al.* the effect of a seven valent pneumococcal conjugate vaccine (which included the serotypes: 4, 6B, 9V, 14, 18C, 19F and 23F conjugated to the outer-membrane protein complex of *Neisseria meningitis* group B) was evaluated in children 12-18 months of age (40). The authors observed a decrease of the serotypes included in the vaccine among the vaccinated while no changes occurred in the control group. One year after vaccination, carriage of antibiotic resistant vaccine-types was still lower in vaccinated children than in the control group. After that, a second study conducted by the same group was published and both studies showed clearly that conjugate vaccine could reduce the carriage and the resistance of pneumococcal serotypes included in conjugate vaccines (41).

Later, Obaro *et al.*, studied the effect of a pentavalent pneumococcal vaccine (including serotypes 6B, 14, 18C, 19F and 23F conjugated to CRM_{197}) in infants in Gambia and observed the maintenance of high pneumococcal carriage of serotypes included in vaccine in control group and a decreased of these serotypes in vaccinees. However, an increased of serotypes not included in the vaccine was observed in vaccinated children and this effect was not observed in the control group (110). This phenomenon of replacement was also observed in a study conducted in toddlers attending day-care centers in Southern Israel with 9-valent pneumococcal vaccine conjugate with CRM_{197} (35).

After introduction of PCV7, multiple studies worldwide have demonstrated a decrease in nasopharyngeal carriage of PCV7 types and an increased of non-PCV7 types (39, 49, 66, 116, 125, 137, 156). As observed in IPD the introduction of PCV7 interfered with pneumococcal antimicrobial resistance rates, but also varied with geographic location, the levels antibiotic consumption and vaccine uptake. A pediatric cross-sectional study conducted in Massachusetts, USA (65) showed that the vaccine serotypes virtually disappeared in young children, with a rapid replacement of non-PCV7 types, which were frequently penicillin non susceptible, mainly 19A and 35B. Pelton *et al.* (116) reported that in two Boston

communities serotypes 19F and 6B were the PCV7 types most frequently isolated post-PCV7 vaccination, whereas serotypes 4, 9V, 14 and 18C were rarely recovered. In Norway, after vaccine introduction, the pneumococcal carriage remained high, around 80%, but a decrease in PCV7 serotypes was observed (156). In Calgary, Canada a study conducted in seven community health centers where routinely PCV7 vaccination began in 2002, a decrease of PCV7 serotypes and an increased of non-PCV7 was also observed. In this study the pneumococcal carriage rate was low, 20%, and the largest increased of non-PCV7 type was noticed in serotypes 6A, 15C, and 11A (75).

Serotype replacement has also been observed in pneumococcal colonization in Portugal. The first evidence was in a controlled study conducted before the introduction of PCV7 in the market, which included 236 vaccinated children and 354 control children that attended the same day care center (49). Additionally, a reduction on pneumococcal resistance PCV7 serotypes was replaced by an increase of resistance in non-PCV7 serotypes (49). Surveillance studies conducted after the availability of PCV7 in Portugal also described a marked serotype replacement effect in the population (125). Despite serotype replacement, maintenance of the antimicrobial resistance rates was observed, due to expansion of pre-existent non-PCV7 resistant clones (125, 137). In 2006, five years after the introduction of PCV7 in the Portuguese market, non-vaccine types 1, 6C, 7F, 15A, 16F, 21, 23A, 29, and non-typeable strains (NT) increased significantly. A non-significant increase of serotype 19A was also noticed (125). In 2006 and 2007, the major serotypes were 6A, 6C, 14, 15A, 19A, 19F, 23F and non-typeable strains. A significant decrease in clonal diversity was observed for serotypes 14 and 19F, whereas a significant increase in clonal diversity was observed for serotype 6C and non-typeable strains. For serotypes 6A and 19A no significant changes in clonal diversity occurred with introduction of PCV7 (137).

All the above mentioned studies were conducted in the Lisbon area; in order to evaluate if this data would be representative of the country, surveillance initiatives were expanded to other areas of Portugal and the results are in Chapters III and IV of this thesis.

Recently, a study published by Valente *et al.* suggested a novel potential benefit of conjugate vaccines. In the study the effect of PCV7 on pneumococcal co-colonization was evaluated. Lower co-colonization rates were observed among fully vaccinated children when compared with unvaccinated children. Since a decrease of co-colonization could translate in lower opportunities for horizontal gene transfer, this effect may function as a "brake" for capsular switch or acquisition of resistant genes (151).

PERSPECTIVES FOR PCV10 AND PCV13

The introduction of PCV10 and PCV13 is expected to have an impact on pneumococcal disease and carriage. In 2010, a study conducted in the USA predicted the effectiveness of PCV13 from observed PCV7 data. According to their model PCV13 would prevent 106,000 invasive pneumococcal disease cases and 2.9 million pneumonia cases over a 10-year period (124). Recently, another study examined public-health and economic impacts of PCV pediatric national immunization programs in Germany, Greece and The Netherlands and estimated that PCV13 would eliminate 31.7%, 46.4%, and 33.8% of IPD in Germany, Greece, and The Netherlands, respectively. PCV13 was found to be cost-effective or cost saving when compared to PCV7 and PCV10 (147).

The use of the polysaccharide vaccine (PPV23) remains controversial, as described by Huss *et al.* in a recent meta-analysis (67). In this meta-analysis 22 trials were included. The authors analyzed several databases, all reports of reviews and meta-analysis for clinical trials that compared the use of PPV with a

control. The vaccine efficacy on clinical outcomes and the quality of the methodology used in the trials were evaluated. For the authors in all trials analyzed there was little evidence of protection of this vaccine against the elderly or in adults with chronic disease. This vaccine seems not to be effective for prevention of pneumonia, even in countries where it is currently recommended. Recently, PCV13 has been approved for prevention of pneumonia and invasive disease caused by PCV13 serotypes among adults aged 50 years and older (27). However, the efficacy of this vaccine in this age group has not been established yet. A trial is in progress in The Netherlands involving 85,000 persons aged 65 years and older (56). Nevertheless, the full impact of PCV13 in children and in adults is not known yet.

PNEUMOCOCCAL TYPING METHODS

In the era of pneumococcal vaccines, with several and rapid changes in the pneumococcal population is important and essential to have good and adequate typing methods.

The choice of a typing method will depend upon the needs, skill level, and the resources of the laboratory. An optimal typing method should show high typeability, adequate stability, high reproducibility and high discriminative power. Additionally, ease of use and interpretation, quick, and low cost are also convenient criteria.

Typing pneumococcus has been useful for understanding the evolution of the species, in epidemiological studies and also in outbreak cases (153).
Characteristics expressed by the microorganism permit to classify them by phenotyping methods. Methods that involve direct DNA-based analysis of chromosomal or extra-chromossomal genetic determinants are considering

genotyping methods. In general, genotyping methods have higher discriminative power and higher typeability than phenotyping methods and constitute the best approach for bacterial comparison.

Antibiotyping

Antibiotyping consists of an antimicrobial susceptibility testing where the isolates are tested by diffusion or dilution methods against a panel of antimicrobial agents. The results are interpreted according to international guidelines. This technique is easy to perform, gives rapid results, it is cheap and readily available in the routine microbiology laboratories. However, the major disadvantage is the poor discriminative power for typing proposes. Antibiotic resistance patterns are, to some extent, influenced by the local environment and the antibiotic pressure (81).

Pneumococcal serotyping

This method consists of a reaction of the capsule polysaccharides with polyclonal factor sera (145) and recently, also with the use of monoclonal antibodies (21). Classical serotyping is performed using the Quellung or Neufeld reaction (10). With this assay the swelling of the capsule of the pneumococcus is observed using a contrast phase microscope after mixing the bacteria with serotype specific antiserum. The assay includes one chessboard sequential testing of antisera of a pool that gives a serogroup, and then a specific factor serum that gives the serotype. The bacteria will frequently also agglutinate. The subjectivity in interpretation, the high cost of antisera, the need for a complete set of control strains, and technical expertise requirements associated with these serologic methods have resulted in development of PCR based serotyping systems (17).

Several other methods for pneumococcal serotyping have been described, such as coagglutination, enzyme–linked immunosorbent assay and countrimmunoelectrophoresis (9, 85, 160). However, the sensitivity, specificity and the fact that they are very laborious do not make these methods a good alternative

to Quellung reaction. Latex agglutination tests also have been developed since at least 1988 (84). However, only in 2004 Slotved *et al*. (142) developed a simple, rapid latex agglutination test (Pneumotest-Latex), which is used nowadays in several laboratories.

More recently, a PCR scheme using a sequential series of multiplex PCRs to assess serotypes or related sets of serotypes has been developed by the CDC (113) (www.cdc.org). The use of this approach to assess the serotype is convenient because of the relatively low cost compared to the classic method and the time consumed.

Recently, Elberse *et al*., described a new genotyping method, capsular sequence typing (CST), based on the determination of the partial sequence of the capsular *wzh* gene, using a single PCR reaction with multiple primer sets (46). Another promising methodology published for serotyping pneumococci is based on three multiplex PCR combined with fragment analysis and automated fluorescent capillary electrophoresis (FAF-mPCR) which detects a great number of serotypes/serogroups. Although it does not differentiate some groups of serotypes, this automatic method allows the analysis of 30 samples in a few hours and at low cost (133).

Additionally, some isolates do not react with any commercially available antiserum. These atypical isolates are called non-typeable *S. pneumoniae* (NTPn). These isolates are difficult to identify as their differentiation from closely related species such as *Streptococcus pseudopneumoniae* and other streptococcus of the mitis group is not always straightforward. Recently, Simões *et al*. (138) developed a low cost and easy assay to detect NTPn. The strategy is based on a multiplex PCR targeting *lytA*, *cpsA*, *aliB*-like ORF2 and 16S rDNA genes, plus a RFLP assay to differentiate typical from atypical *lytA.*

PFGE and MLST

Currently, the gold standard genotyping methods for pneumococci are pulsed-field gel electrophoresis (PFGE) (132) and multilocus sequence typing (MLST) (47).

Pulsed-field gel electrophoresis (PFGE) was developed in 1984 by Schwartz and Cantor (132) and is based on the digestion of the total chromosomal DNA with a restriction endonuclease that cleaves the DNA infrequently. The macro-restriction fragments are separated by gel electrophoresis according to molecular weight in an apparatus which voltage is periodically switched among three directions. The different orientation of the electric pulses during electrophoresis allows the separation of large DNA fragments. Normally the total chromosomal DNA of *S. pneumoniae* is digested with *Sma*I and 10 to 19 bands of 20 to 300 kb are obtained (87). This technique is inexpensive, shows a good typeability, reproducibility, and a good discriminative power. However, it is time consuming and standardization of the method and good quality of the gels are pre-requisites to compare results between laboratories. Despite established interpretation criteria by visual comparison (148) and the standardization of the method (31) the comparison of results remains sometimes difficult. As an alternative to visual classification, automatic methods that use band-based similarity coefficient to classify by type/subtype the microbial isolates analyzed by PFGE have been developed. Carriço *et al.* demonstrated a good correspondence between visual and automatic classification for *S. pneumoniae* (24).

MLST for pneumococci is a nucleotide sequence based approach that includes PCR amplification and sequencing of ~450-bp internal fragments of seven housekeeping genes: *aroE* - shikimate dehydrogenase, *gdh* - glucose-6-phosphate dehydrogenase*, gk*i – glucose kinase*, recP* - transketolase, *spi* – signal peptidase, *xpt* – xanthine phosphoribosyltransferase, and *ddl* – D-alanine-D-alanine ligase (47). The sequence of each allele is compared to all know alleles available online in an international database (www.mlst.net), and each allele is

assigned with a number. The combination of the seven numbers defines the allelic profile or sequence type (ST). The major advantage of this technique is the unambiguous ability to compare the results obtained in different laboratories, using the online database. However, this method is time consuming if need to analyze a big collection and expensive for laboratories without a sequencer. MLST has a good discriminative power and is a good tool for local and global epidemiology.

MLVA

Multiple-Locus Variable number tandem repeat Analysis (MLVA) is based in repetitive DNA localized in multiple loci of all bacterial genome. The number of repeat sequences and the sizes of these units, named BOX elements, may vary for different isolates of the same species. These BOX elements were identified in the genome of the pneumococcus in 1992 (94). In the genome of prototype strains R6 and TIGR4 115 and 127 BOX elements, have been found, respectively. The function and origin of BOX elements are unknown; however, it is thought that they may be involved in regulating the expression of virulence-associated genes when they are located in the promoter region of genes (78, 94). Van Belkum and colleagues were the first to show the usefulness of these repeats for genotyping *S. pneumoniae* using PCR-BOX. However the banding profiles were difficult to interpret and often lacked reproducibility (152). The first MLVA scheme based on BOX typing applied to pneumococcus was developed by Koeck *et al.* in 2005 (79). In this scheme 16 BOX loci are analyzed after amplification in a single PCR reaction and the product are visualized by agarose gel electrophoresis. This technique has high congruence with MLST and shows high discriminative power, providing a good alternative to MLST. Although it allows for the comparison of data between laboratories using a website (www.mlva.eu), this scheme and the use of agarose gel electrophoresis makes this method very fastidious. A new scheme and easier protocol was needed and is described in chapter VI of this thesis.

Whole genome sequencing

The sequencing of whole genomes (WGS) represents a great advance for the study of bacterial populations because current approaches such as MLST are based on the analysis of only seven housekeeping gene fragments amounting only less than 0,2% of the total length of the genome. Horizontal gene transfer (HGT) is a fundamental process in bacterial genome evolution where a great portion of DNA can be incorporated from another species genome. Since *S. pneumoniae* is naturally transformable these events occur frequently. Small-scale mutations as substitutions, deletions or insertions could also occur. With this technique all these events can be identified by comparing the sequences with a reference genome. These differences in the core genome allow the construction of phylogenetic trees to show the evolutionary relationships among the isolates of the same species. WGS can also be useful in epidemiological studies, namely to follow the spread of a clone which isolates seem to be identical in several countries. One example of this was demonstrated with the genome sequencing of several isolates of the international multiresistant Spain^{23F}-1 clone, which showed that the genome sequence of each isolate differs slightly and these differences represent an evolution of the specie, which was impossible to evaluate without WGS (34).

In addition WGS has the capacity to simultaneously elucidate serotype (if the capsule is expressed), sequence type and details of genetic information, which provide a good approach to monitor the impact of pneumococcal vaccines.

Recently, Golubchick *et al.* reported that using whole-genome resequencing allowed them to identify five independent occurrences of vaccine escape through capsular switches between serotype 4, which is included in PCV7, by serotype 19A that is not. By using WGS the authors were able to search for putative donor and recipient genomes and demonstrated the independent origin of each recombinant lineage. Their results also reinforced the idea that during a single

episode of recombination multiple and large additional DNA fragments can be imported (52).

Although very promising the price of WGS remains high and the bioinformatic tools for data analysis need further development for routine use by hospitals and by scientific community in general like as mention by Török *et al.* (150).

AIM OF THE WORK

The aims of this thesis were to gain insights into: (i) the epidemiology of the recently discovered serotype 6C, (ii) the pneumococcal colonization patterns in different regions of Portugal among healthy children and (iii) the pneumococcal colonization patterns in the elderly. Additionally, the need for the development and validation of easier and faster methods of molecular typing was also addressed.

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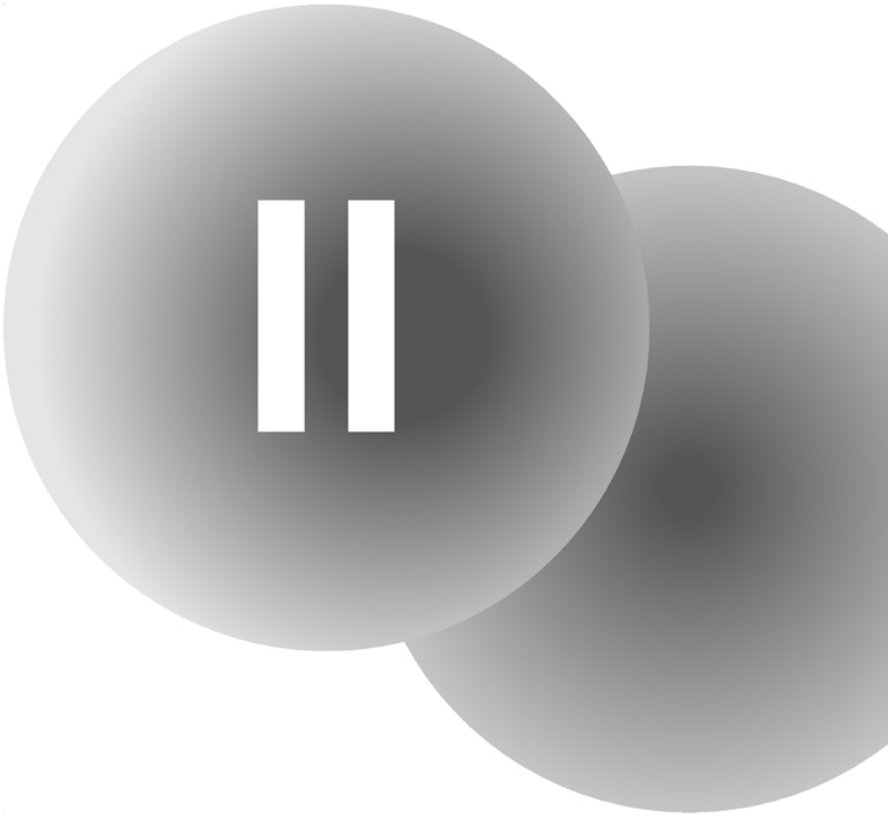
Temporal trends and molecular epidemiology of the recently described serotype 6C of **Streptococcus pneumoniae**



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Abstract

We studied the epidemiology of the recently described serotype 6C of *Streptococcus pneumoniae* among a collection of carriage isolates recovered between 1996 and 2007 in Portugal. Of 4,064 isolates, 106 (2.6%) were of serotype 6C of which 17.9% were multidrug resistant. The strains were genetically diverse.

Introduction

The polysaccharide capsule of *Streptococcus pneumoniae* is considered the most important virulence factor in this species. It is antigenically diverse and its identification by serology (Quellung reaction) has been used for decades as a primary criterion for classification of pneumococci (18). Up to now 91 serotypes have been described being 6C the most recent one (14). The latter is indistinguishable from 6A by the Quellung reaction although it contains a galactose molecule instead of a glucose in the capsular repeating oligosaccharide unit due to the presence of a different glycosyl transferase (*wciN*) gene (14). Serotypes 6A and 6C, together with serotype 6B constitute the serogroup 6 which has related capsular structures (1, 10). A putative serotype 6D that would result from the introduction of $wciN_{6C}$ in the 6B operon has been proposed although it remains unidentified (7).

The 7-valent pneumococcal conjugate vaccine (PCV7, targeting serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) was introduced in the USA in 2000 and became commercially available in several European countries during 2001. PCV7 confers protection, within serogroup 6, against serotype 6B. The most recent available data suggest it provides cross-protection to disease caused by serotype 6A but not serotype 6C (12).

Due to its recent identification, data on the epidemiology of serotype 6C are still very scarce (13). A recent study from South Africa found that serotype 6C strains had a higher propensity to cause meningitis than serotypes 6A or 6B (5). A study from the CDC in the United States reported that in 2006 the rate of invasive disease caused by serotype 6C was significantly higher than in 1999 but lower for serotype 6A (3). Another very recent publication from the USA also documented the increasing prevalence of this serotype after 2001 (8).

In this study, we describe the epidemiology of serotype 6C strains colonizing healthy preschool Portuguese children in studies conducted between 1996 and 2007.

Materials and Methods

We screened 4,064 *S. pneumoniae* strains isolated from a total of 6,559 nasopharyngeal samples obtained from children attending day-care centers (DCC) in Lisbon and Oeiras areas, Portugal, in studies conducted in nine different years, between 1996 and 2007: 1996-1999, 2001-2003, and 2006-2007. All samples were collected between January and March of each year. Identification of pneumococci was done as previously described (17). The serotypes of drug-resistant isolates (n=1,659) have been reported (9, 11, 16, 17).

Antimicrobial susceptibility testing was performed using the Kirby-Bauer technique, according to the Clinical and Laboratory Standards Institute (4) recommendations and definitions (4) for chloramphenicol, erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim (SXT). MICs of penicillin and ceftriaxone were determined with E-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations and interpreted according to CLSI guidelines (4). Multidrug resistance was defined as resistance to three or more antibiotics tested.

To identify strains of serogroup 6, all isolates lacking serotype assignment were screened by PCR for the presence of a specific region of *wzy* gene as described (2). Specifically, two primer pairs were used, 6Bwzy-f (5'-CGA CGT AAC AAA GAA CTA GGT GCT GAA AC-3') and 6Bwzy-r (5'- AAG TAT ATA ACC CTG TAA AAC TCT GAC-3'), generating a product of 200 bp for all serogroup 6 strains, and cpsA-f (5'-GGT GTT CTC TAT CCT TGT CAG CTC TGT GTC GCT C-3') and cpsA-r (5'-GTG TGA ATG GTC GAA TCA ACT CTA TAA ATG CC-3'), generating a product of 657 bp. The highly conserved gene *cpsA* exists in all but two capsular loci and was used as internal control (2). To assign serotypes 6A, 6B, and 6C (and the putative 6D) all serogroup 6 isolates were (i) typed by the Quellung reaction (18) and (ii) screened by PCR for a region of *wciN*, using previously described primers (5'-TACCATGCAGGGTGGAATGT-3' and 5'-CCATCCTTCGAGTATTGC-3') that result in a product size of 2.0 kb for serotypes 6A and 6B and of 1.8 kb for serotype 6C (13). The PCR reactions were done in a 10 µl volume with 1x GoTaq® Flexi Buffer (Promega, Madison, USA), 2.5 mM of $MgCl_2$, 0.08 mM of dNTPs, 1.0 µM of each primer, and 0.1 Uμl^{-1} of GoTaq® Flexi DNA Polymerase (Promega, Madison, USA). DNA was isolated from freshly grown bacterial cultures, picked with a sterile tip, and briefly immersed in the PCR reaction mix. Thermocycling was performed in My Cycler thermal cycler (Bio-Rad Laboratories, Hercules, California) with the following conditions: 94ºC for 4 min; 35 cycles of 94ºC for 30 s, 60ºC for 45 s and 72ºC for 45 s (for *wzy*) or 2 min (for *wciN*); and a final extension step at 72ºC for 5 min. PCR analysis was done by electrophoresis on 1% or 2% Seakem LE agarose gels in 1x TAE buffer. Gels were stained in a 0.1 µg ml^{-1} ethidium bromide solution.

Preparation of chromosomal DNA, restriction with SmaI endonuclease, PFGE, and analysis of patterns with Bionumerics software (version 5.10, Applied Maths, Gent, Belgium) were done as previously described (15, 17). Multilocus sequence typing (MLST) was performed as previously described (6) on selected strains of each

PFGE cluster, choosing within each group, at least one representative of each year.

Results and discussion

Out of 4,064 pneumococci strains recovered from colonization samples in studies conducted between 1996 and 2007, 809 (19.9%) were of serogroup 6: 7.3% were of serotype 6A, 9.6% were of serotype 6B, and 2.6% were of serotype 6C. Temporal fluctuations were observed for all serotypes (Table 1). Serotype 6B was dominant among serogroup 6 isolates from 1996 until 2002, when it started to decline, being almost absent by 2006. For serotype 6A a sharp decrease was observed in 1998 and no significant fluctuations were observed with the availability of PCV7 (since June 2001). Serotype 6C ranged from 0.2% to 5.8% reaching the highest value in 2007. There were no strains of the hypothetic serotype 6D.

Of the 106 serotype 6C strains identified, 35.8% were resistant to at least one of the antibiotics tested. The proportion of penicillin-resistant isolates according to parenteral meningeal and non-meningeal criteria was 30.2% and 0%, respectively, as 29.2% of the 106 isolates had a MIC of 0.12µg ml^{-1} and a single isolate (1%) had a MIC of 0.25 µg ml^{-1} (4). Resistance levels were 21.7% for erythromycin, 18.9% for clindamycin, 18.9% for tetracycline and 1.9% for SXT. Multidrug resistance was detected in 17.9% of the isolates and was observed in 2006 and 2007 only.

PFGE fingerprint clustered the 106 serotype 6C strains into eleven groups (Table 2). By MLST, the 27 strains representing all PFGE groups yielded 11 sequence types STs that, using eBURST (20) and the complete database of *S. pneumoniae* (available at http://spneumoniae.mlst.net/), fell into four clonal complexes based on a minimum similarity of five identical loci (numbered by ST of the predicted founder as CC138, CC395, CC386, and CC3034) and two singletons

(STs 3671 and 2789) (Table 2). Three novel STs were found in this study (3671, 3673, and 3711).

The dominant group 6C-1 (STs 395, 1692, 1714, and 3711) included 52 strains susceptible to all antibiotics that were recovered from several day-care centers and were detected in all but one sampling period (Figure 1 and Table 2). The next two most frequent groups 6C-2 (ST1150, 18 isolates) and 6C-10 (ST3396, 11 isolates) contained strains resistant to penicillin (according to meningeal criteria).

TABLE 1. Distribution of serogroup 6 pneumococci over time

Year	Total no. Pn isolates	No. of isolates (%)			
		SG 6	6A	6B	6C
1996	277	77 (27.8)	33 (11.9)	40 (14.4)	4 (1.4)
1997	353	84 (23.8)	26 (7.4)	46 (13.0)	12 (3.4)
1998	465	66 (14.2)	5 (1.1)	60 (12.9)	1 (0.2)
1999	596	137 (23.0)	45 (7.6)	68 (11.4)	24 (4.0)
2001	466	135 (29.0)	57 (12.2)	75 (16.1)	3 (0.6)
2002	559	97 (17.4)	30 (5.4)	61 (10.9)	6 (1.1)
2003	559	94 (16.8)	41 (7.3)	36 (6.4)	17 (3.0)
2006	392	64 (16.3)	46 (11.7)	2 (0.5)	16 (4.1)
2007	397	55 (13.8)	31 (7.8)	1 (0.3)	23 (5.8)
Total	4,064	809 (19.9)	314 (7.3)	389 (9.6)	106 (2.6)

Pn – pneumococci; SG - Serogroup

TABLE 2. Characteristics of serotype 6C strains

PFGE group (no. of isolates)	ST (no. of isolates)[a]	CC	Resistance pattern (no. of isolates)	No. of DCC
6C-1 (52)	ST395 (5)	395	Susceptible	12
	ST1714 (1)			
	ST1692 (1)			
	ST3711 (1)			
6C-2 (18)	ST1150 (4)	138	P (11)	3
			Susceptible (7)	
6C-3 (1)	ST1150 (1)	138	P	1
6C-4 (4)	ST3673 (2)	138	Ery	2
6C-5 (1)	ST2789 (1)	Singleton	Susceptible	1
6C-6 (2)	ST3671 (1)	Singleton	Susceptible	1
6C-7 (6)	ST1150 (1)	138	P (3)	3
	ST2689 (2)		Susceptible (3)	
6C-8 (2)	ST2185 (1)	3034	Ery, Clin, Tet, SXT	1
6C-9 (3)	ST395 (2)	395	Susceptible	1
6C-10 (11)	ST3396 (1)	386	P, Ery, Clin, Tet (8)	1
			Ery, Clin, Tet (3)	
6C-11 (6)	ST3396 (1)	386	P, Ery, Clin, Tet	1

P, penicillin G MIC of 0.12 μg ml^{-1} with the exception of a single isolate that had an MIC of 0.25 μg ml^{-1}; Ery, erythromycin; Clin, clindamycin; Tet, tetracycline; SXT, trimethoprim-sulfamethoxazole; No. of DCC, number of day-care centers where PFGE group was found. [a]Number of representative strains tested.

The dominant group 6C-1 (STs 395, 1692, 1714, and 3711) included 52 strains susceptible to all antibiotics that were recovered from several day-care centers and were detected in all but one sampling period (Figure 1 and Table 2). The next two most frequent groups 6C-2 (ST1150, 18 isolates) and 6C-10 (ST3396, 11

isolates) contained strains resistant to penicillin (according to meningeal criteria). In addition, strains of group 6C-10 were also resistant to erythromycin, clindamycin and tetracycline (Table 2). While group 6C-2 was found in 1997 and between 2001 and 2003, group 6C-10 was identified in 2007 only (Figure 1). The remaining eight groups were sporadic representing 0.9-5.7% of the serotype 6C collection.

Our study shows that the recently described serotype 6C is frequently carried by healthy young children in Portugal. Overall, of all isolates that would have been conventionally typed by the Quellung reaction as serotype 6A, close to one-quarter (25.2%) were identified as serotype 6C; this value ranged between 5.0% and 42.6% depending on the sampling period.

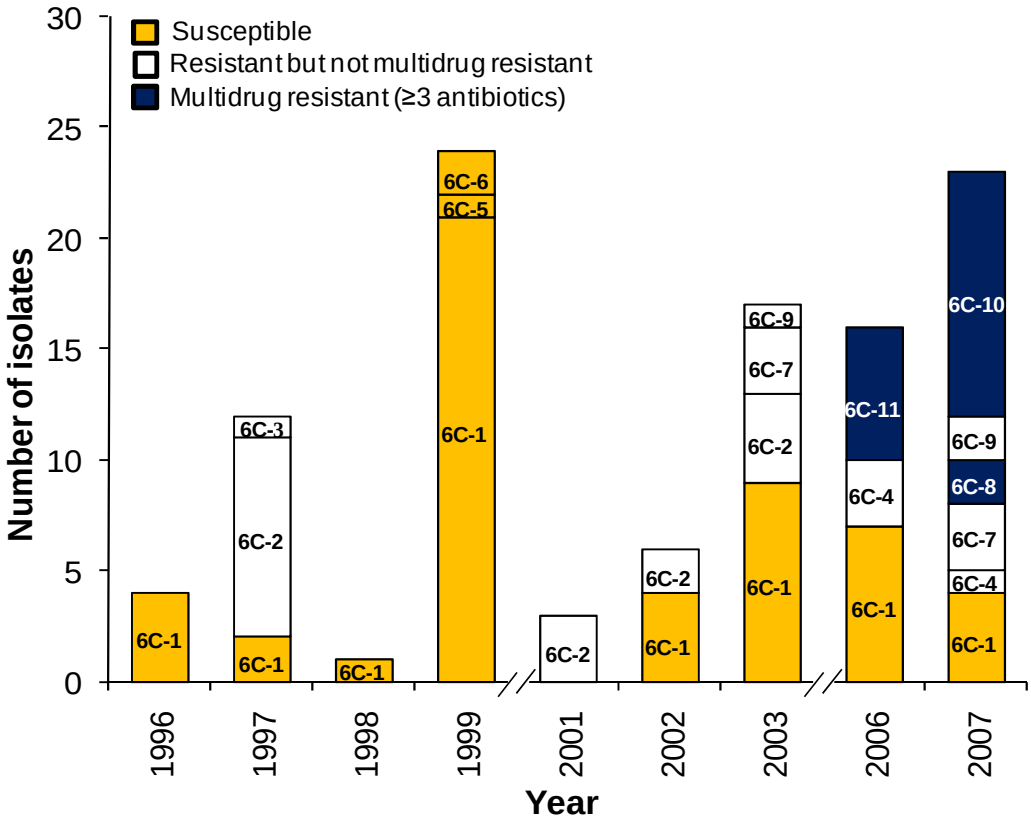

FIG 1. Distribution of serotype 6C PFGE clones through the nine study periods. MDR, multidrug resistant.

Serotype 6C strains circulating in Portugal are genetically diverse. Similar observations were recently reported in a USA study (8) and, of interest, there were no common STs among serotype 6C isolates from both studies. This observation implies that, if the introduction of *wciN*$_{6C}$ in the capsular operon of 6A occurred only once as suggested by Park *et al.*, it must have occurred a sufficiently long time ago to yield such lineage diversity (13). In our collection, one fully antibiotic-susceptible lineage (6C-1, STs 395, 1692, 1714, and 3711) persisted over one decade and accounted for close to half of all serotype 6C isolates suggesting it is well adapted even when considering the natural selection driven by antibiotic pressure. Still, strains of serotype 6C circulating in Portugal are often drug-resistant and, in particular, multidrug resistance has been detected in 2006 and 2007. These data contrast with recent observations from South Africa where most invasive strains of this serotype were fully susceptible or resistant to macrolides only, but are comparable to resistance rates reported in a recent study from the USA (8).

Conclusion

In summary, serotype 6C circulates in Portugal at least since 1996. It is genetically diverse and often antibiotic resistant. Continued surveillance of this serotype is important since it is not targeted by PCV7 and is not known if PCV13 (which targets not only 6B but also 6A) will confer cross-protection to it. Additional data from other countries and estimates of its invasive disease potential will help us understand the epidemiology of this serotype.

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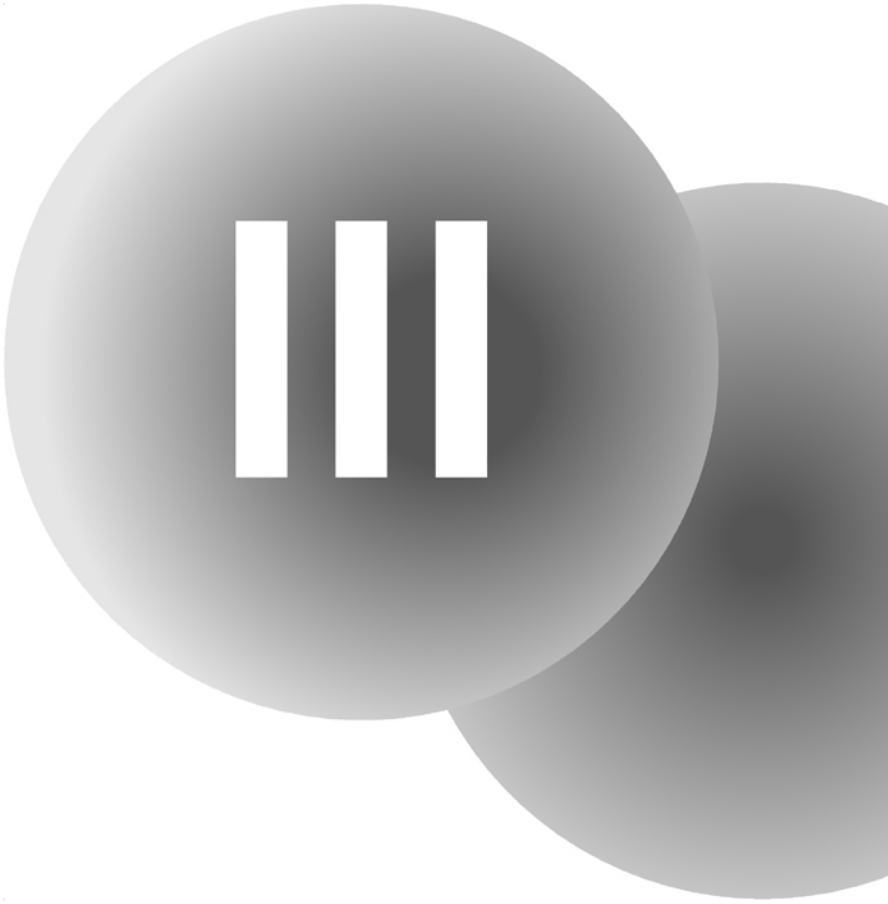
Streptococcus pneumoniae *nasopharyngeal carriage in children attending day-care centers in the central region of Portugal, in the era of 7-valent pneumococcal conjugate vaccine.*



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S. Nunes was responsible for the characterization of pneumococcal isolates.

ABSTRACT

The seven-valent pneumococcal conjugate vaccine (PCV7) became available in Portugal in 2001. Although not included in the national immunization program, vaccination coverage is high (over 60%). We studied for the first time the rates of nasopharyngeal (NP) carriage of pneumococci, antibiotic resistance patterns and serotypes among children attending day care centers (DCCs) in Coimbra, a city in the Central Region of Portugal. Between January and February 2007, a cross-sectional study was conducted among children aged 6 months to 6 years old attending eight DCC. NP swabs were obtained from 507 children: 76.7% had received at least one dose of PCV7 and 64.3% were age-appropriately vaccinated. The global pneumococcal carriage rate was 61.3%. Colonization proportions varied with age and number of children attending each DCC. Serotyping revealed that 20.7% of the pneumococci were vaccine types (VT), 70.8% were non-vaccine types (NVT), and 8.5% were non-typeable. Serotype 19F was the second most frequent serotype being detected in 10.5% of the samples. While global NP carriage was not associated with vaccination status, NVT were predominant among vaccinated children, who had significantly lower prevalence of VT. Of all isolates, 15.7% had penicillin MICs that ranged between 0.12 and 2 μg/ml. The proportion of resistant strains was significantly higher among VT and unvaccinated children. In conclusion, the rates of vaccination and prevalence of pneumococcal NP were high. Rates of antimicrobial resistance were similar to those found in studies conducted in Oeiras and Lisbon. This study is a platform for future surveillance activities.

INTRODUCTION

Pneumococcus is a leading cause of invasive and noninvasive disease among children worldwide. The importance of pneumococcal infections is highlighted by an increasing prevalence of drug-resistant strains (28, 34, 45).

A 7-valent pneumococcal conjugate vaccine (PCV7) was licensed in the USA in 2000. Pneumococcal conjugate vaccines (PCVs) have been shown to be highly efficacious against vaccine-type (VT) serotypes invasive pneumococcal disease (IPD). They are also efficacious against chest radiograph–confirmed pneumonia and otitis media (OM) (2, 6, 9, 12, 24, 31). Increase in NVT pneumococcal disease has been observed among some age groups in invasive-disease (16, 21) and in OM efficacy trials (12, 23).

Because NP carriage is a precondition for developing disease, and it is this condition, rather than pneumococcal disease, that results in person to person pneumococcal transmission, it is essential to thoroughly understand the impact of PCV on NP colonization. Conjugate vaccines are thought to reduce VT pneumococcal carriage by preventing new acquisitions, rather than by terminating existing carriage episodes (8), although the mechanism is incompletely understood. Reductions in VT pneumococcal carriage may prevent disease among unvaccinated individuals by decreasing the transmission of these serotypes in the community (7, 33, 44). Numerous studies have shown that conjugate vaccines protect against VT carriage in the months immediately after vaccination (27, 28, 32) and more recently Millar *et al*. showed that community-wide PCV7 vaccination in infancy reduces the prevalence of VT carriage and increases the prevalence of NVT carriage (serotype replacement) through at least 3 years of age (28). The indirect protective effects against VTs (herd immunity) and the indirect and direct replacement effects result in large part from the impact of PCV on NP colonization (30).

Day-care centers (DCCs), for preschool children are unique settings for the spread of pneumococcal carriage. DCC attendance has been found to be an important risk factor for development of IPD in children and infants (25, 43). The role of DCCs in the transmission of *S. pneumoniae* to the community is also well documented (19).

PCV7 became available in Portugal in 2001 but has not been included in the national vaccination program. Thus, vaccines are given after medical prescription and paid by the parents and not reimbursed by the National Health Service. Two studies reported vaccination coverage values (10, 35) among children within the age group attending DCC, but samples were not representative of the whole population. Portugal has high antibiotic consumption (17) and penicillin non-susceptible *S. pneumoniae* are frequently isolated (1, 37). Previous colonization studies in Portuguese DCCs, were conducted in the areas of Lisbon and Oeiras (26, 29, 36).

This study intended to estimate the prevalence of NP carriage of pneumococci, identifying serotype distribution and antibiotic resistance patterns among children attending DCCs, in the era of widespread use of PCV7, in Coimbra, a city in the central region of Portugal, 200 Km away from Lisbon.

METHODS

Study population

A convenience sample of four urban and four suburban DCCs was selected. The target population consisted of 660 children, aged 6 months to 6 years old, attending eight DCCs in Coimbra, a city in the central region of Portugal.

Study design and approval

A cross-sectional study was performed in January and February of 2007. The study was approved by the Ethical Committee of Hospital Pediátrico de Coimbra. Parents or guardians provided written informed consent for their child to participate.

Questionnaire

One week before sample collection, the following information was obtained from a questionnaire filled in by the parents or guardians of participating children: age of the child, number of children in the household who were ≤6 years of age, presence of any underlying illness, presence of a cigarette smoker at home, use of antibiotics in the preceding month, maternal educational level and dates of all PCV7 doses received. In the few cases where the number of doses was reported without vaccination dates, the parents were contacted either by one of the investigators by phone or by the nurses during the sample collection visit.

Vaccination status

Vaccination with PCV7 was considered appropriate if it was done according to one of the following schemes: a 4-dose series if starting vaccination at 2 months of age; a 3-dose series if starting vaccination at 7-11 months of age; a 2-dose series if starting vaccination at 12-24 months of age and 1 dose if starting vaccination at ≥ 24 months, as recommended by the vaccine manufacturer. A child was defined as age-appropriately vaccinated if at the time of sampling had received all recommended doses, according to the age at initiation of vaccination. Those with fewer doses than recommended by age at initiation of vaccination were classified as partially vaccinated. Doses of PCV7 received during the four weeks that preceded sampling were not considered for the vaccination status classification.

Sample collection and isolation of pneumococci

Nasopharyngeal samples were obtained by trained nurses with a small flexible swab (BBL Culture Swab; Becton-Dickinson, Sparks, MD) inserted through the nostril until resistance was encountered. The swab was then removed and inoculated into the transport medium (Stuart medium) and transported at ambient temperature to the Laboratory of Microbiology at Centro Hospitalar de Coimbra and was stored at 4°C for a maximum of 12 hours before samples were plated. Specimens were streaked onto tryptic soy 5% sheep blood agar plates (Becton Dickinson) and incubated overnight at 37°C in 5% CO_2. Phenotypic characteristics (morphology and α-hemolysis) were used for the presumptive identification of pneumococci. Pneumococcal identification was confirmed by optochin susceptibility and bile solubility assays (29). When suspected pneumococcal colonies with more than one morphology were observed, each type was purified for further testing. Isolates were frozen and stored at -70ºC.

Antimicrobial susceptibility testing

Susceptibility of all isolates was tested by the disk diffusion method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (5), against erythromycin, clindamycin, tetracycline, chloramphenicol, sulfamethoxazole-trimethoprim (SXT), levofloxacin, and vancomycin (Becton-Dickinson). Minimum inhibitory concentrations (MICs) of penicillin and cefotaxime were determined by E-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations. Interpretation of penicillin and cefotaxime MICs followed CLSI criteria (5).

Serotyping

Pneumococci were serotyped by the Quellung reaction using commercially available antisera (Statens Serum Institut, Copenhagen, Denmark) (41). Serotypes were grouped into one of three categories: (1) vaccine type (VT; i.e., types 4, 6B,

9V, 14, 18C, 19F, and 23F); (2) non-vaccine type (NVT) and (3) non-typeable (NT).

Pulsed-field gel electrophoresis (PFGE)

Pneumococci strains that were resistant to at least one of the antibiotics tested, were also typed by PFGE. All strains with penicillin MICs ≥0.12 μg/ml were analyzed. Preparation of chromosomal DNA, restriction with *Sma*I endonuclease, and PFGE were done as previously described by Sá-Leão *et al.* (39). PFGE patterns were analyzed with Bionumerics software (version 5.10, Applied Maths, Gent, Belgium). Patterns were clustered by UPGMA, and a dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 1.0% and a tolerance of 1.5%. PFGE clusters were defined as isolates with a similarity of 80% or higher on the dendrogram (38).

Statistical analysis

Univariate analysis was done using Epi Info 2000 (11). Chi-square tests with Yates correction were used. Multivariate analysis, with carriage status of *S. pneumoniae* as the dependent variable, was done using a logistic regression model fitted by Stata (42). Population diversity was measured by calculating the Simpson´s Index of Diversity (SID) with 95% confidence intervals (CI) for drug-resistant NVT and VT strains. Congruence between PFGE clones and DCCs was evaluated with the Adjusted Rand (AR) coefficients (4).

RESULTS

General characteristics of the study group

A NP swab was obtained in 507 (77%) of the 660 children attending the eight DCC. Between 19 and 113 children were tested at each DCC. General characteristics of the study group at the time of sampling are described in Table 1. The mean age of participants was 40.6 months (range 6.1 to 80.9 months) and 262 (51.7%) were male; 148 (29%) had siblings ≤ 6 years of age and none had more than two siblings in that age group. Using maternal educational level as a proxy for socio-economic level, it was observed that more than 74.2% of the mothers had more than the compulsory schooling level in Portugal (nine years). The use of an antibiotic within one month prior to study enrollment was reported for 126 (24.9%) children; 29.9% lived with one or more cigarette smoker.

Vaccination status

Three hundred and eighty nine children (76.7%) had received one to four doses of PCV7; 64.5% (327/507) were classified as age-appropriately vaccinated and 12.3% participants had not completed the schedule recommended by the manufacturer and were classified as partially vaccinated (Table 2). A higher proportion of age-appropriately vaccinated children was observed among children aged 6-12 months, decreasing thereafter to a minimum at the age group older than 60 months (Figure 1).

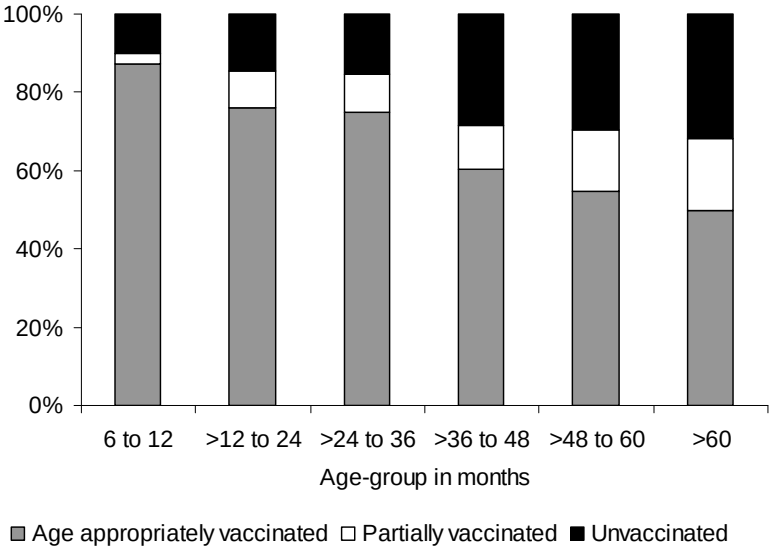

Fig. 1. Vaccination status according to the age group

Carriage

The global carriage rate was 61.3% (311/507). Pneumococcal colonization proportions varied with age ($p<0.0001$); among children below one year of age, 57.9% were colonized, and the peak (76.2%) was observed among children 12–24 months; from this age group on the proportion of colonized children decreased gradually to the older age groups ($p<0.000001$ in Chi-square test for linear trend), reaching the minimum level of 47.0% among the older age group (Table 1 and Figure 2).

Table 1. Characteristics of the study group and univariate analysis of factors associated with *Streptococcus pneumoniae* colonization

Characteristics / factors	Category	Non-SP colonization (n=196)	SP colonization (n=311)	Total SP (n=507)	p-value (χ2 test)
Sex	Male	99 (40.4%)	146 (59.6%)	245	0.490
	Female	97 (37.0%)	165 (62.9%)	262	
Age group	06-12	16 (42.1%)	22 (57.9%)	38	<0.001
(in months)	>12-24	20 (23.8%)	64 (76.2%)	84	
	>24-36	27 (29.7%)	64 (70.3%)	91	
	>36-48	33 (33.3%)	66 (66.7%)	99	
	>48-60	47 (49.5%)	48 (50.5%)	95	
	>60	53 (53.0%)	47 (47.0%)	100	
Maternal educational	≤9th grade	50 (42.7%)	67 (57.3%)	117	0.65
level	9th – 12th grade	58 (38.6%)	93 (61.6%)	151	
	> High school	85 (37.8%)	140 (62.2%)	225	
	No data			14	
No. of siblings ≤6	0	139 (37.1%)	220 (62.9%)	359	0.99
years of age	1	54 (37.9%)	86 (61.4%)	140	
	2	3 (37.5%)	5 (62.5%)	8	
Living with a smoker	No	144 (41.1%)	206 (58.9%)	350	0.08
	Yes	49 (32.5%)	102 (67.5%)	151	
	No data			6	
Antibiotic use in the	No	147 (38.6%)	234 (61.4%)	381	0.96
preceding month	Yes	49 (38.8%)	77 (61.1%)	126	
Vaccine (PCV7)	Unvaccinated	45 (38.1%)	73 (61.9%)	118	0.98
	≥1 dose	151 (38.8%)	238 (61.2%)	389	
Vaccination status	Unvaccinated	45 (38.1%)	73 (61.9%)	118	0.98
	Partially vaccinated	24 (38.1%)	39 (61.9%)	63	
	Age-appropriately vaccinated	127 (39.0%)	199 (61.0%)	326	

PCV7, 7-valent pneumococcal conjugate vaccine; SP, *Streptococcus pneumoniae*

Table 2. Vaccination status by number of doses of 7-valent pneumococcal conjugate vaccine received

No. of doses of PCV7	Vaccination status			Total
	Unvaccinated	Partially vaccinated	Age appropriately vaccinated	
0	118	0	0	118
1	0	18	4	22
2	0	13	28	41
3	0	31	86	117
4	0	0	209	209
Total	118	62	327	507

Pneumococcal colonization status was not associated with maternal education, number of siblings below six years of age, living with a smoker, or use of antibiotics in the previous month (Table 1). Pneumococcal colonization *per se* was also not associated with vaccination status no matter the criteria used to classify vaccination with PCV7: number of doses (P=0.510); one or more doses (P=0.980, Table 1); vaccination status (P=0.983, Table 1).

Logistic regression analysis showed that being colonized with *S. pneumoniae* depended on children's age (as described before) and the number of children attending each DCC; the likelihood of being colonized increased with the number of children attending each DCC (P=0.038).

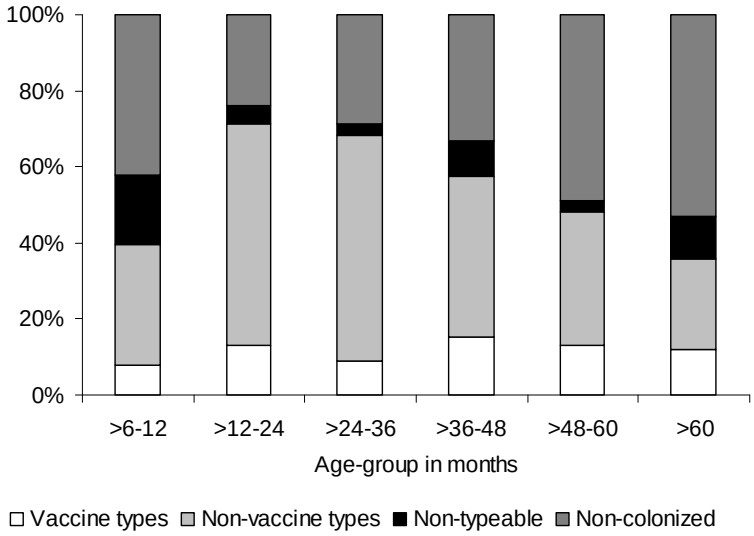

Fig. 2. *S. pneumoniae* colonization according to age group

Serotype distribution

The serotype distribution is shown in Table 3. Of the 315 isolates (311 carriers of which four had two strains of pneumococci), ten were lost. Thus, the information on specific serotype was available for 305 isolates. Among these, 63 (20.7%) were VT, 216 (70.8%) NVT and the remaining 26 (8.5%) were NT. Serotypes 19F, 6B and 14 were the most prevalent among VTs. Several NVT (23 types) were isolated being the most prevalent (in decreasing order) 23A, 16F, 23B, 19A, 21, 17F, 35F, 11A, and 6A (Table 3).

Table 3. Serotype distribution of *S. pneumoniae* isolates according to the vaccination status

Serotype	Unvaccinated children (n = 71)	Vaccinated children (≥1 doses PCV7; n = 234)	Total[a] (n = 305)
Vaccine Types (VT)			
19F	12	20	32
6B	11	8	19
14	8	3	11
23F	0	1	1
Total VT	31 (43.7%)	32 (13.7%)	63 (20.7%)
Non-Vaccine Types (NVT)			
23A	5	30	35
16F	2	20	22
23B	4	16	20
19A	3	15	18
21	1	16	17
17F	4	12	16
35F	1	13	14
11A	1	11	12
6A	4	8	12
10A	2	6	8
24F	0	8	8
15A	1	5	6
38	0	6	6
1	1	4	5
29	0	4	4
15B/C	2	2	4
16A	1	2	3
15F	0	1	1
18A	1	0	1
34	0	1	1
3	0	1	1
7F	0	1	1
9L	0	1	1
Total NVT	33 (46.5%)	183 (78.2%)	216 (70.8%)
Non-typeable	7 (9.9%)	19 (8.1%)	26 (8.5%)

[a]305 isolates from 301 children (2 different serotypes were isolated in 4 children)

Although colonization *per se* was not associated with vaccination status, the distribution of serotypes among vaccinated and unvaccinated children was different (Table 3). While prevalence of NVT (46.5%) and VT (43.7%) was similar among unvaccinated, NVT were clearly predominant (78.2%) among children with ≥1 doses of PCV7 (P<0.0001).The proportions of VT and NVT were also associated with age group (P<0.0001) as the proportion of NVT which increased to maximum levels among age groups ">12-24" and ">24-36" months of age and then decreased gradually to the minimum among the eldest (Figure 2).

Antibiotic resistance

The antibiotic resistance profile of serotyped pneumococci is show on Table 4. Of all isolates, 14.4% strains had a penicillin MIC ranging from 0.12 to 1 μg/ml and 1.3% had a MIC of 2 μg/ml; 7.2% of the strains had a cefotaxime MIC of 1 μg/ml and 0.7 had a MIC of 2 μg/ml (Table 4). Other rates of resistance were 20.5% to erythromycin, 17.4% to clindamycin, 15.0% to tetracycline, 10.2% to SXT, and 1.3% to chloramphenicol. All pneumococci were susceptible to levofloxacin and vancomycin. For any studied antibiotic on Table 4, the proportions of resistant strains were significantly higher among VT (p <0.0001).

Significantly higher proportions of decreased susceptibility to penicillin, and resistance to tetracycline were observed among strains from unvaccinated, when compared to vaccinated children (Table 4). Nevertheless, when the confounding effect by VT strain was controlled through stratification of vaccination status, the apparent association between vaccination status and resistance to antibiotics was no longer observed.

Table 4. Resistant *Streptococcus pneumoniae* according to serotype and vaccination status

Antibiotic	All isolates n=305 no. (%)	Type				Vaccination status		
		Non-VT n=216 no. (%)	Non-typeable n=26 no. (%)	VT n=63 no. (%)	p-value	Unvaccinated n= 71 no. (%)	Vaccinated* n = 234 no. (%)	p-value
Oral penicillin V								
Penicillin^{I}	44 (14.4)	17 (7.9)	3 (11.5)	24 (38.1)	<0.0001	16 (22.5)	28 (12.0)	0.026
Penicillin^{R}	4 (1.3)	0	0	4 (6.3)	n.d.	2 (2.8)	2 (0.9)	n.d.
Penicillin parenteral (meningitis)	48 (15.7)	17 (7.9)	3 (11.5)	28 (44.4)	<0.0001	18 (25.4)	30 (12.8)	0.011
Penicillin parenteral (nonmeningitis)	0	0	0	0	-	0	0	-
Cefotaxime (meningitis)								
Cefotaxime^{I}	22 (7.2)	5 (2.3)	1 (3.8)	16 (25.4)	<0.0001	10 (14.1)	12 (5.1)	0.011
Cefotaxime^{R}	2 (0.7)	0	0	2 (3.2)	n.d.	2 (2.8)	0	n.d.
Cefotaxime^{I} (nonmeningitis)	2 (0.7)	0	0	2 (3.2)	n.d.	2 (2.8)	0	n.d.
SXT	31 (10.2)	12 (5.5)	5 (19.2)	14 (22.2)	<0.0001	13 (18.3)	18 (7.7)	0.035
Chloramphenicol	4 (1.3)	4 (1.9)	0	0	n.d.	0	4 (1.7)	n.d.
Erythromycin	48 (20.5)	25 (11.6)	5 (19.2)	37 (58.7)	<0.0001	19 (26.8)	50 (21.3)	0.341
Clindamycin	40 (17.4)	20 (9.3)	2 (7.7)	35 (55.6)	<0.0001	17 (23.6)	41 (17.4)	0.227
Tetracycline	35 (15.0)	15 (6.9)	3 (11.5)	35 (55.6)	<0.0001	18 (25.4)	36 (15.3)	0.054

*≥1doses of Prevenar; Oral penicillin V: Penicillin^{I} 0.12≤MIC<1 µg/mL, Penicillin^{R} MIC≥2 µg/mL; Penicillin parenteral meningitis: Penicillin^{R} MIC≥0.12 µg/mL; Penicillin parenteral nonmeningitis: Penicillin^{I} MIC=4 µg/mL; Cefotaxime (meningitis): Cefotaxime^{I} MIC=1.0µg/mL, Cefotaxime^{R} MIC≥2.0µg/mL; Cefotaxime (nonmeningitis): Cefotaxime^{I} MIC=2.0µg/mL; SXT, sulfamethoxazole-trimethoprim; n.d., not done (small number of isolates); MIC, minimum inhibitory concentration.

Of note, serotype 19F, a VT, was the second most frequently isolated serotype (being 23A the most frequent one). Six out of 32 children with 19F were <2 years of age, 22/32 (69%) had received at least one dose of PCV7 and 18/32 (56%) were age-appropriately vaccinated. Of 32 serotype 19F isolates, 46.9% had decreased susceptibility to penicillin (intermediate and high-level penicillin resistance were found in 15.7% and 31.2% of the strains, respectively). The rates of resistance to other antimicrobial agents were: 40.6% to cefotaxime, 90.6% to erythromycin, clindamycin and tetracycline. All were susceptible to chloramphenicol, levofloxacin and vancomycin.

Among NVT, penicillin non-susceptibility and resistance to macrolides was more frequent among serotypes 19A, 15A and 6A. For serotype 19A (n=18), penicillin non-susceptibility was 33%, resistance to macrolides and to clindamycin was 55.6% and to cefotaxime was 27.8%.

Clonal analysis of drug-resistant strains

DNA fingerprinting through PFGE was performed for all 86 drug-resistant isolates which included 13 serotypes and 7 NT strains (Table 5). Twenty-four clones were identified, six associated with specific VT, 15 associated with specific NVT, and three associated with both VT and NVT. Among the latter, two clones had isolates of serotypes 19A and 19F and one had isolates expressing serotypes 14, 15A, 19A, and 19F. Fifteen clones were represented by unique isolates and the remaining were represented by two to 31 isolates. The largest clone, 19F/19A-1, included 26 of the 30 drug resistant serotype 19F isolates and five of the 13 drug-resistant 19A isolates. This clone was isolated from 6 day-care centers.

Clonal diversity was higher among drug resistant NVT strains than among drug-resistant VT strains: SID for NVT was 0.92 (95% CI, 0.89-0.96) compared to 0.68 (95% CI, 0.54-0.82) for VT. There was a low association between drug-resistant clones and individual DCCs as the AR was only 0.17 for NVT strains and 0.13 for VT strains.

Table 5. Clonal distribution of all antibiotic-resistant strains

Serotype	No. of isolates with PFGE done[a]	PFGE clones[b]	Resistance profile	Day-care centers (no. of isolates)
Vaccine types				
6B	4/19	6B-1 (1)	^{I}P, Ery, Da, Tet	A (1)
		6B-2 (3)	^{I}P, SXT	C (3)
14	11/11	14-1 (3)	P (1), ^{I}P, SXT	A (2); H (1)
		14-2 (3)	Ery (2); Tet, SXT	E (1); F (2)
		15A/14/19F/19A-1 (5)	^{I}P, Tet (4), Ery, Da, SXT (4)	B (3); C (1); F (1)
19F	30/32	19F/19A-1 (26)	^{I}P(1), P(2),Tet, Ery, Da	A (9); B (10); C (3); D (2); E (2)
		15A/14/19F/19A-1 (2)	^{I}P, Tet, Ery, Da	B (2)
		19F/19A-2 (1)	^{I}P, Tet, Ery, Da, SXT	H (1)
		19F-3 (1)	P, SXT	F (1)
23F	1/1	23F-1 (1)	^{I}P	C (1)
Nonvaccine types				
6A	4/12	6A-1 (1)	^{I}P, Tet, Ery, Da	H (1)
		6A-2 (3)	^{I}P, Tet, Ery, Da	H (3)
11A	5/12	11A-1 (3)	^{I}P (1), Ery, Da (2), ^{I}SXT (1)	A (1); B (2)
		11A-2 (2)	Ery, SXT	A (2)
15A	5/6	15A/14/19F/19A-1 (5)	^{I}P, Ery, Da	A (3); C (1); D (1)
15B/C	1/4	15B/C-1 (1)	Ery, SXT	A (1)
17F	1/16	17F-1 (1)	Tet	C (1)
18A	1/1	18A-1 (1)	^{I}P	B (1)
19A	13/18	19F/19A-1 (5)	Ery, Da, Tet, Chl	B (2); G (3)
		15A/14/19F/19A-1 (1)	P, Tet, Ery, Da	A (1)
		19F/19A-2 (5)	^{I}P, Tet, Ery, Da, ^{I}SXT	A (4); H (1)
		19A-3 (2)	^{I}SXT, ^{R}SXT	B (1); C (1)
21	1/17	21-1 (1)	SXT	G (1)
29	1/4	29-1 (1)	Ery	E (1)
NT	7/26	NT-1 (3)	Tet (2), Ery, Da (2), SXT(1)	D (1); H (2)
		NT-2 (1)	P	C (1)
		NT-3 (1)	^{I}P, SXT	C (1)
		NT-4 (1)	Ery, Da	C (1)
		NT-5 (1)	^{I}P, Tet, Ery, SXT	C (1)

Ery – erythromycin; Da – clindamycin; Tet – tetracycline; Chl – chloramphenicol; SXT - sulfamethoxazole-trimethoprim; Penicillin^{I} 0.12≤MIC<1 µg/mL; Penicillin^{R} MIC≥2 µg/mL; [a]PFGE was done for antimicrobial-resistant isolates; [b]PFGE clones were named by using a combination of serotype(s) found within the clone followed by an arbitrary sequential number.

DISCUSSION

This is the first study of NP pneumococcal carriage undertaken among healthy children attending DCC in the central region of Portugal. Although similar studies have been conducted for over a decade in the Oeiras and Lisbon area, the epidemiology of colonization in other regions of the country has not been studied. Thus it is unclear whether features consistently found in Oeiras and Lisbon are representative of the country.

Several parallels can be drawn between the findings of this study and those conducted in Oeiras (i) the high prevalence (61.7%) of *S. pneumoniae* NP carriage was similar to that reported in Oeiras (14, 29, 36), but above those observed in other countries after the introduction of PCV7, such as Greece (18) and Canada (22); (ii) relatively high rates of drug resistance were found, with 29% of the isolates being resistant to at least one antibiotic, a finding comparable to what has been found in Oeiras (26, 29, 40); (iii) in this study we found very high PCV7 uptake among children attending DCC in Coimbra (76.7% had received at least one dose), consistent with reports from Oeiras (37).

Of interest, colonization proportions were found to vary with the number of children attending each DCC and with age but not with vaccination status or other studied factors. The association between *S. pneumoniae* colonization and the number of children in each DCC found in our study is consistent with the finding that DCC play a key role in the pneumococcal carriage rate of its attendees and even on the levels of carriage in the community where they are located (19). The association between colonization and age group has also been reported (3). In our study, colonization was not associated with level of the mother's education, number of siblings below six years of age, living with a smoker or previous use of antibiotics, factors which have been associated with increased carriage of pneumococci (13, 22).

Consistent with other studies NP carriage was not associated with PCV7 vaccination status. However, while prevalence of NVT and VT was similar among unvaccinated, NVT were clearly predominant among vaccinated children ($P<0.0001$), that had significantly lower prevalence of VT. Although we have no pre-vaccination data to compare with, our findings are consistent with those reported by others (14, 15, 18, 20, 27, 28, 32), with reduction of VT strains and increase in NVT (serotype replacement) among vaccinated children.

In our study, VT serotypes 4, 9V and 18C were not identified and the most prevalent VT serotype were 6B, 14 and 19F, which accounted for approximately 20% of the isolates. Of note, despite widespread use of PCV7, 19F was the second most frequently isolated serotype. Grivea *et al.* (18) on their observational study about the impact of PCV7 in DCCs in Central Greece reported a substantial reduction of colonization for VT 6B, 14 and 23F; serotype 19F decreased but the decline did not reach a statistically significant level. Of interest, a recent study aimed to estimate the vaccine efficacy against acquisition of pneumococcal carriage using previously published trial data found no statistically significant efficacy of PCV7 against vaccine serotypes 19F and 14 (35a). In the present study, 9.1% of the pneumococci isolates were NT. In other studies, including the ones conducted in Oeiras, comparable proportions were found (18, 38).

In this study the clonal diversity among drug-resistant NVT strains was significantly higher than among drug-resistant VT strains a finding that warrants continuous surveillance as NVT are increasingly abundant. Three clones were identified that expressed more than one serotype. The same clones expressing these various serotypes were detected in a previous study conducted in Oeiras between 2001-2003 an observation that suggests that capsular switch events that led to the circulation of these isolates sharing a common molecular type but expressing different capsular types occurred at least a few years ago (26).

In conclusion, rates of vaccination and NP carriage are high in DCCs in the central region of Portugal and most children are colonized with NVT. While prevalence of NVT and VT was similar among unvaccinated, NVT were clearly predominant among vaccinated children. Although we have no pre-vaccination data from this region of Portugal to compare with, our findings are consistent with those reported by others showing reduction of colonization by VT after vaccination and replacement by NVT. The proportion of resistant pneumococci was significantly higher among unvaccinated children and among VT. Relatively high rates of drug resistance were found. This study is a platform for future surveillance activities. Ongoing surveillance is critical, because changes in the resistance patterns among NVT can occur with time.

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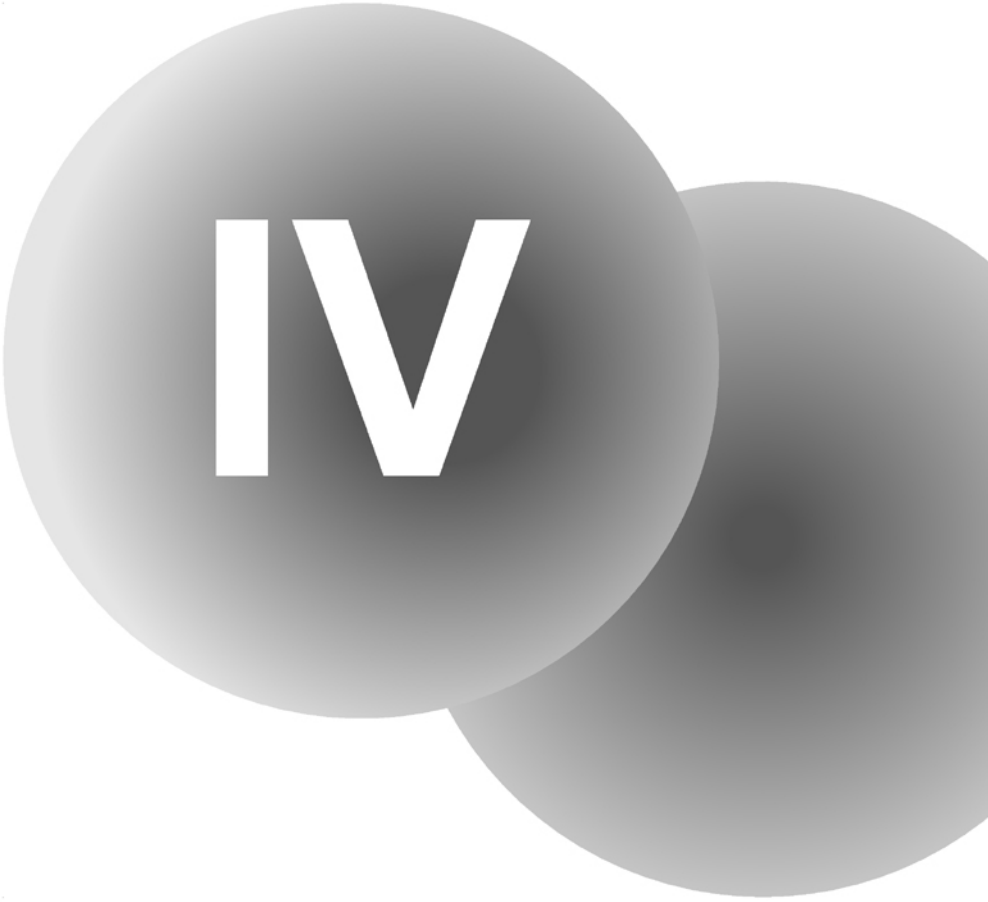
Antibiotic consumption remains a main driving force of antimicrobial resistance in the era of pneumococcal conjugate vaccines



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The characterization of pneumococci was responsibility of S. Nunes. The statistical analysis was performed by A.C. Paulo.

Abstract

We compared colonization patterns between two areas: Montemor-o-Novo, a rural area, and Oeiras, an urban area. Between January-March of 2009 and 2010, 1,088 and 756 nasopharyngeal samples were obtained from children attending day care centers in the urban and in the rural area, respectively. Socio-demographic and clinical data were also obtained. Pneumococci were isolated, serotyped and antibiotyped. We used multiple logistic regression to evaluate association between carriage, socio-demographic and clinical variables. Carriage (c.a. 61%) and PCV7 use (complete schedule, c.a. 70%) were comparable in both areas. In the urban area there were higher rates of antimicrobial resistance (32.4% vs 21.6%, p<0.001) and higher rates of antimicrobial consumption in the month preceding sampling (16.6% vs 11.6%, p=0.004). Antimicrobial consumption during the last month (OR=2.58, 95%CI: 1.61-4.17), being colonized with serotype 19A (OR=3.52, 95%CI: 1.79-6.91) or a NT strain (OR=11.33, 95%CI: 5.36-23.96) and attending day care in the urban area and simultaneously being colonized with 19A (OR=2.49, 95%CI: 1.07-5.81) were the variables that better explained the differences in antibiotic resistance between the two areas. This study shows that despite the widespread use of PCV in Portugal, antibiotic consumption remains a driving force for maintenance of antimicrobial resistant pneumococci in the community.

Introduction

Streptococcus pneumoniae is one of the major pathogens implicated in pediatric infections that can range from acute otitis media to more severe conditions such as bacteremia and meningitis. *S. pneumoniae* is also a frequent colonizer of the human nasopharynx. Since nasopharyngeal carriage is particularly common in

pre-school children (1) and colonization precedes disease (8) it is very important to monitor the epidemiology of pneumococci in this age group.

Our group carried out pneumococcal colonization studies since 1996 in day care centers (DCC) in the Lisbon area (2, 6, 11, 13, 20). Recently, we described the impact of the 7-pneumococcal conjugate vaccine (PCV7) in pneumococcal colonization in this particular population (18, 21) and also in Coimbra, a city in the central region of the country (17).

The study presented here was conducted in a rural area, in the southern part of Portugal, Montemor-o-Novo. The results were compared to the ones obtained in Oeiras (a suburb of Lisbon).

Methods

Study population

Nasopharyngeal samples were collected from DCC attendees up to 6 years old living in Oeiras or Montemor-o-Novo. Oeiras is a village and a municipality in western Lisbon metropolitan area, with 168,475 inhabitants. It is one of the most populous municipalities in Portugal, with a density of 3,687/km^2 distributed through ten civil parishes in a total area of 45.7km^2. Montemor-o-Novo is a city and municipality in the southern region and is one of the largest municipalities of Portugal with a total area of 1232.10km^2, but with a low population density of about 15/km^2. The majority of the population (12,000 of 18,540 inhabitants) lives in the city that includes 2 out of 10 civil parishes. In Oeiras we invited 9 DCC to participate in the study, while in Montemor-o-Novo all DCC were invited (n=16) in order to have a comparable number of children in the two regions. The number of attendees in each DCC varied between 25 and 174 in Oeiras and between 7 and 104 in Montemor-o-Novo. At time of this study, PCV7 and

PCV10 were not included in the National Vaccination Program neither subsided by the state but were available in the Portuguese market since June 2001 and April 2009, respectively. PCV13 replaced PCV7 in January of 2010.

Questionnaire and pneumococcal isolation

In the winter months of January - March of 2009 and 2010 one nasopharyngeal swab was taken from each child, whose parents or guardians had signed informed consent, as described in our previous studies (19). Additionally, information about age, gender, antibiotic consumption, recent disease, and pneumococcal and flu vaccinations was obtained through questionnaires. Pneumococcal identification was done by routine procedures (20), based on colony morphology on blood agar plates, optochin susceptibility and bile solubility. All strains were stored at -80ºC. In both regions the same specialized nurses did sampling; pneumococcal isolation was performed in our laboratory.

Interpretation of vaccination status

The classification “complete”, “complete to age” and “incomplete” was done as previously described by Sá-Leão (18). Vaccination was considered “complete” if, at the time of sampling, it was done according to one of the following schemes: 4 doses, if starting vaccination at 2 months of age; 3 doses, if starting vaccination at 7-11 months of age; 2 doses, if starting vaccination at 12-24 months of age and 1 dose if starting vaccination at ≥ 24 months. Vaccination was considered “complete to age” if at the time of sampling the child had received all recommended doses, according to the age at initiation of vaccination. Vaccination was considered “incomplete” if at time of sampling the child had not received all recommended doses according to the age at initiation of vaccination.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer technique, according to the Clinical and Laboratory Standards Institute (CLSI) recommendations and definitions (4). The antimicrobial agents tested were chloramphenicol, erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim (SXT). Antibiotic disks were purchased from Oxoid (Hampshire, England). MICs for penicillin, ceftriaxone and amoxicillin were performed by E-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations. In the interpretation of decreased penicillin susceptibility, isolates were divided into intermediately resistant (0.1 µg/ml ≤ MIC < 1.5 µg/ml) and resistant (MIC ≥ 1.5 µg/ml), according to CLSI guidelines for oral penicillin V (4).

Capsular typing

Serotyping was carried out for all pneumococcal isolates by a combination of multiplex PCRs using the primers previously described by Pai *et al*. (14) or by CDC (www.cdc.gov). The isolates that could not be typed by this method were serotyped by Quellung reaction using specific antisera (Statens Serum Institute, Copenhagen, Denmark) (23). Non-typeable strains (NT) were defined by the absence of *cpsA* gene and a negative reaction with Omniserum (Statens Serum Institute, Copenhagen, Denmark).

Statistical analysis

Differences between Montemor-o-Novo and Oeiras were tested using a two tailed t-test or a two tailed Wilcoxon test to compare means and the Fisher exact test to compare percentages (25). A p-value of 0.05 was used as a threshold to decide which variables were significantly associated with differences between the urban (Oeiras) and the rural (Montemor-o-Novo) regions. A modified Simpson's index of diversity was used to analyze serotype diversity (3). For the multiple regression logistic analysis a backward stepwise regression was used

to choose the most parsimonious model, that is, the one with the lowest *Akaike information criterion* (AIC) and the lowest number of independent variables. The model was further inspected for multicollinearity and the goodness of fit was checked (7). For each variable odds ratio (OR) were estimated as well as the corresponding confidence interval at 95%. A variable whose OR confidence interval did not contain 1 was considered statistically significant. Accordingly, an OR lower than 1 was considered a protective factor and an OR higher than 1 was considered a risk factor.

Results

Population characterization

A total of 1,848 children were enrolled in this study, 1,092 from Oeiras and 756 from Montemor-o-Novo (Table 1). About 53% of these children were enrolled during 2009 and 48% were female. The mean number of children in each DCC in the two regions was significantly different (p-value <0.001). The mean age of the children enrolled was lower in Oeiras (3.6 vs 3.2, p<0.001).

During this study 79.5% of the children had taken at least one dose of pneumococcal conjugate vaccine, however only 1.1% had taken PCV10 and none had taken the PCV13. A high percentage of children in Montemor-o-Novo had taken at least one dose of pneumococcal vaccine (83.2% vs 76.4%) (Table 1). There was, nonetheless, a difference in the vaccination status in the two regions. The proportion of unvaccinated children in Oeiras (23.1%) was significantly higher (p=0.001) than in Montemor-o-Novo (16.7%). On the other hand, the number of incomplete doses was higher among children in Montemor-o-Novo (Table 1). No difference between the regions was noticed regarding the vaccination against seasonal influenza and H1N1 (Table 1).

Table 1 – Demographic data

	Total	Urban area	Rural area	p-value
Population (< 7 years)	1848	1092	756	
Average number of children		94.4±50.7	36.1±30.7	**<0.001**
Mean age (years)		3.2±1.5	3.6±1.5	**<0.001**
Gender (female)	958	48.3% (525/1088)	47.7% (361/756)	0.84
Pneumococcal vaccine[a]		N=1069	N=720	
unvaccinated	367	23.1% (247)	16.7% (120)	**<0.001**
at least one dose	1422	76.9% (822)	83.2% (600)	**0.001**
4 doses	986	52.1% (557)	59.6% (429)	**0.002**
complete to age[b]	1227	67.7% (724)	69.9% (503)	0.35
incomplete	195	9.2% (98)	13.5% (97)	**0.004**
NA	59	23	36	
Flu vaccine[c]	45	7.2% (15/422)	4.6% (30/331)	0.164
H1N1[d]	102	10.2% (49/481)	14.1% (53/376)	0.109
Antibiotic consumption				
at sampling	62	3.8% (41/1065)	2.8% (21/743)	0.293
during last month	255	16.6% (172/1034)	11.6% (83/713)	**0.004**
6 months before (≥3 courses)	546	41.3% (406/983)	19.6% (140/713)	**<0.001**

Bold, statistically significant; NA, not available; [a]the majority of children took only PCV7. Nine children (5 in Oeiras and 4 in Montemor-o-Novo) took also one dose of PCV10 and 4 children in each region took only PCV10; [b]in this group children with 4 doses of vaccine are included; [c]only asked in 2010; [d]only available in 2010

The consumption of antibiotics among children in the two regions was not statistically different at the time of sampling, but it was significantly higher in Oeiras during the preceding month and this difference was even more clear with recurrent use of antibiotics (three or more courses in the last 6 months) (Table 1). Amoxicillin and the association between amoxicillin and clavulanic acid were the antibiotics more frequently used in these populations and otitis and throat infections were the major causes for antibiotherapy (data not shown).

Pneumococcal carriage and serotype distribution

The prevalence of colonization was not different in the two regions, being c. a. 60% (Table 2). A significant difference in the prevalence of antibiotic resistant strains was observed between the urban and rural region, with a higher prevalence in Oeiras (32.4% vs 21.6%) (Table 2). The highest rate of resistance was found for macrolides 21.6%; 19.8% of the isolates were resistant to three or more antimicrobial agents.

Tabela 2 - Pneumococcal colonization and antibiotic resistance

Characteristic	Urban area	Rural area	p-value
Colonization (yes)	61.8% (675/1091)	60.0% (454/756)	0.467
Antibiotic resistance (yes)	32.4% (219)	21.6% (98)	**<0.001**
Macrolides	25.9% (175)	15.1% (69)	**<0.001**
Penicillin (R)	2.4% (16)	1.5% (7)	0.395
Penicillin (I)	19.4% (131)	10.1% (46)	**<0.001**
MDR	24.1% (163)	13.2% (60)	**<0.001**

Penicillin I, 0.1≤MIC<2µg/mL; Penicillin R, MIC≥2µg/mL; MDR, multidrug resistant-resistant to 3 or more antimicrobial agents; bold, statistically significant

A total of 1,129 pneumococcal isolates were serotyped and 46 serotypes plus non-typeable strains were recovered. In particular, the 454 pneumococcal isolates from Montemor-o-Novo belonged to 34 serotypes, and the 675 pneumococcal isolates from Oeiras belonged to 39 serotypes (Figure 1). Diversity, as estimated by the modified Simpson's index of diversity, was higher in Montemor-o-Novo (0.95, CI 95% 0.942-0.955) than in Oeiras (0.928, CI 95% 0.92-0.934). Furthermore, the rank of the serotypes was also different in both regions. In Montemor-o-Novo, the most prevalent serotypes were 19A (11%),

11A (8.8%) and NT (8.6%) whereas in Oeiras the most prevalent serotypes were 19A (13.9%), 6C (12.4%) and 3 (9.9%) (Figure 1). Potential coverage of the PCV10 and PCV13 were 9% and 35%, respectively, in Oeiras. In Montemor-o-Novo corresponding figures were 9% and 27%.

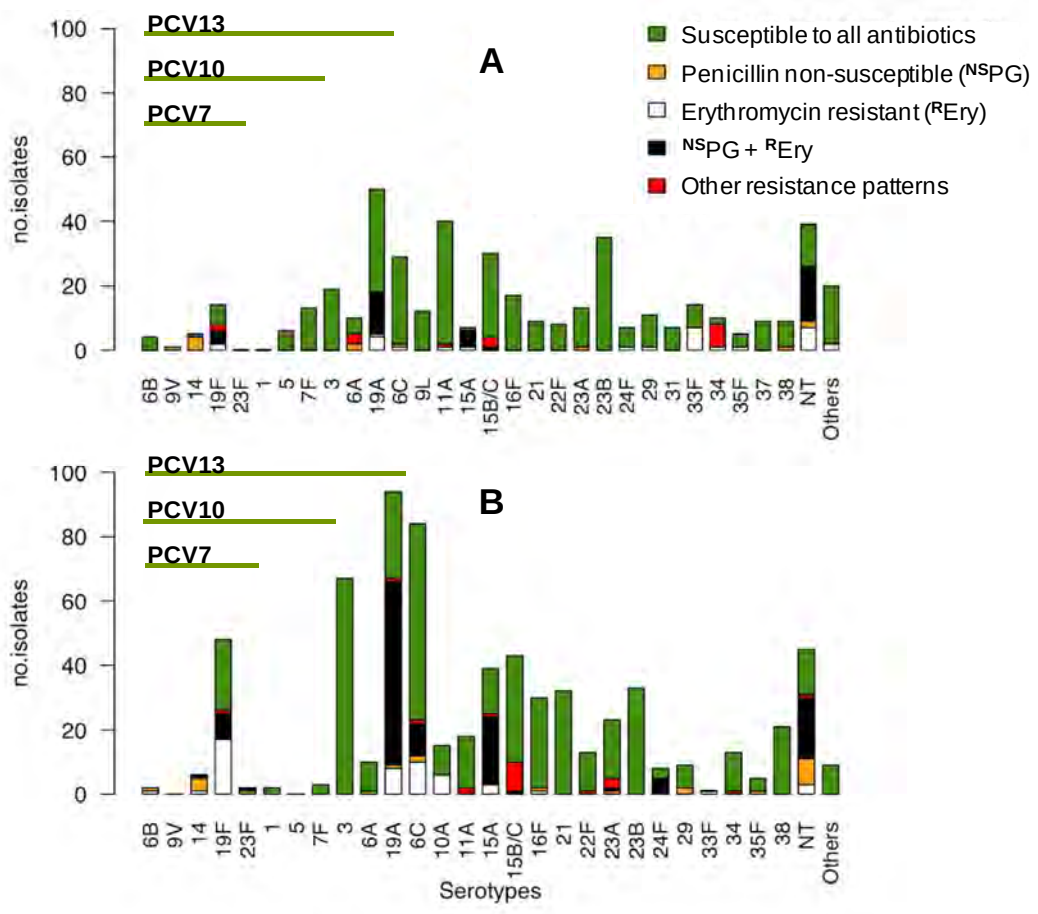

Figure 1 – Serotype and antibiotype distribution in Montemor-o-Novo (A) and in Oeiras (B).

Antibiotic resistance and serotypes

Figure 1 shows the distribution of the major serotypes in the two regions. In the majority of cases resistance to β-lactam and macrolides was associated with the

same serotypes in both regions (19A, 15A and NT strains), with the exception of serotype 6C, that was associated with this type of resistance in Oeiras, only.

Multiple logistic regression: pneumococcal colonization

Based on the differences found in the previous exploratory analysis we adjusted a model to explain pneumococcal colonization considering the number of children in each DCC, pneumococcal vaccination status, geographic region, and antibiotic consumption as independent variables. The final model included the variables in which we observed a significant difference in the univariate analysis: region, age, antibiotic consumption during the last month and the recurrent use of antibiotics in the last six months. Additionally, we tested for one interaction term between children age and antibiotic consumption during the last month. Only age and antibiotic consumption during the last month were statistically associated with pneumococcal colonization (Table 3). Children that took antibiotic during the last month had 73% (OR=0.27, 95%CI: 0.13-0.52) less probability of being colonized than children that did not. Being older decreased the odds to be colonized with pneumococci, in average the decreased was about 1.8% in prevalence for each year of age.

Multiple logistic regression: pneumococcal resistant strains

Concerning the proportion of resistant isolates and taking into account the results obtained after data exploration we adjusted a multiple logistic model to explain the proportion of resistant isolates colonizing children, considering the region, antibiotic consumption, class of antibiotic, age, vaccine status and the pneumococcal serotypes in which the observed number of resistant isolates was significantly different from the one estimated - 19A and NT. We also tested two interactions terms region and serotype, and serotype and antibiotic consumption during last month. The final model included region, serotype, antibiotic consumption during the last month and an interaction term between region and serotype. In the model, only serotype, antibiotic consumption during

the last month and the interaction term between region and serotypes explained the differences in the prevalence of resistance between the two regions (Table 4). Antibiotic consumption during the last month increased the odds to be colonized by resistant pneumococcal strains in 2.5 fold (OR=2.58, 95%CI: 1.61-4.17). Colonization by serotype 19A and NT strains increased the odds to be colonized by resistant pneumococcal strain in 3.5 (OR=3.52, 95%CI: 1.79-6.91) and 11.3 (OR=11.33, 95%CI: 5.36-23.96), respectively. The odds of a child to be colonized with a resistant strain of 19A serotype was 2.5 higher (OR=2.49, 95%CI: 1.07-5.81) in children attending DCC in Oeiras compare to Montemor-o-Novo.

Table 3 - Odds ratio and confidence intervals estimated from logistic model to explain pneumococcal colonization

VARIABLE	unadjusted OR	adjusted OR	CI 95%	p-value
Region				
Montemor-o-Novo	1	1		
Oeiras	1.02	1.18	0.95 – 1.45	0.131
Antibiotic consumption:				
during last month (yes)	0.54	0.27	0.13 – 0.52	**<0.001**
6 months before sampling (yes)	0.89	0.81	0.64 – 1.01	0.066
Age	0.95	0.92	0.86 – 0.99	**0.045**
Age x antibiotic consumption during the last month	0.99	1.01	0.83 – 1.23	0.900

Bold, statistically significant

Table 4 - Odds ratio and confidence intervals estimated from logistic model to explain the colonization with resistant pneumococcal strains

VARIABLE	unadjusted OR	adjusted OR	CI 95%	p-value
Region				
Montemor-o-Novo (rural)		1		
Oeiras (urban)	1.50	1.81	0.85 – 2.10	0.208
Antibiotic consumption:				
during last month (yes)		2.58	1.61 – 4.17	**<0.001**
Serotype				
Other	1	1		
19A	2.56	3.52	1.79 – 6.91	**<0.001**
NT	2.79	11.33	5.36 – 23.96	**<0.001**
Serotype x Region				
19A x Montemor-o-Novo	1	1		
19A x Oeiras	1.97	2.49		
NT x Montemor-o-Novo	1	1	1.07 – 5.81	**0.034**
NT x Oeiras	1.03	0.66	0.24 – 1.82	0.429

Bold, statistically significant; NT, nontypeable

Discussion

Several papers have described risk factors for pneumococcal colonization (1, 5, 9, 10, 12, 16, 22), and the majority are consensual in the fact that major risk factors can change from country to country or from region to region depending on several factors (10, 24).

In this study we compared the prevalence of pneumococcal colonization and the prevalence of antibiotic resistant pneumococcal strains in urban and rural area of Portugal.

We observed that although the pneumococcal carriage was similar, the serotype diversity and the prevalence of resistant strains were different. We tried to investigate the variables that could account for this difference. We accessed for differences among the demographic data in the two areas and we observed that the mean age of children from Montemor-o-Novo was significantly higher. Also, on average, the number of children per DCC unit was higher in the urban area. Antibiotic consumption during the last month and the recurrent use of antibiotics were also significantly higher in Oeiras. The proportion of unvaccinated children in Oeiras was also significantly higher; however the proportion of children with incomplete vaccination was higher in Montemor-o-Novo. However, all these variables were not significant in the multiple logistic regression models that were constructed to explain pneumococcal colonization in general and carriage of resistant strains, in particular.

The colonization model showed that only previous antibiotic consumption and being older were protective factors for pneumococcal colonization. In a previous study conducted in Italy, where the colonization rates were compared between a rural and urban area, the authors mention that living in a rural area significantly decreased the odds of carrying nasopharyngeal respiratory pathogens (15). This difference in the conclusions reached by the two studies could be attributed to the differences between regions.

When we performed the multivariable analysis to explain the difference in the resistance rates in the two regions, we observed that (i) antibiotic consumption during the last month, (ii) colonization with pneumococcal serotype 19A or a NT

strain, and (iii) attending DCC in Oeiras and being colonized by a resistant 19A strain increased the odds of being colonized by pneumococcal resistant strains.

The results obtained in this study led us to conclude that the most important factor influencing carriage of resistant pneumococci is antibiotic consumption. These results alert, that in the era of widespread use of PCVs antibiotic consumption remains a main driving force for maintenance of pneumococcal resistant strains in the community. Judicious use of antibiotics remains a priority in public health.

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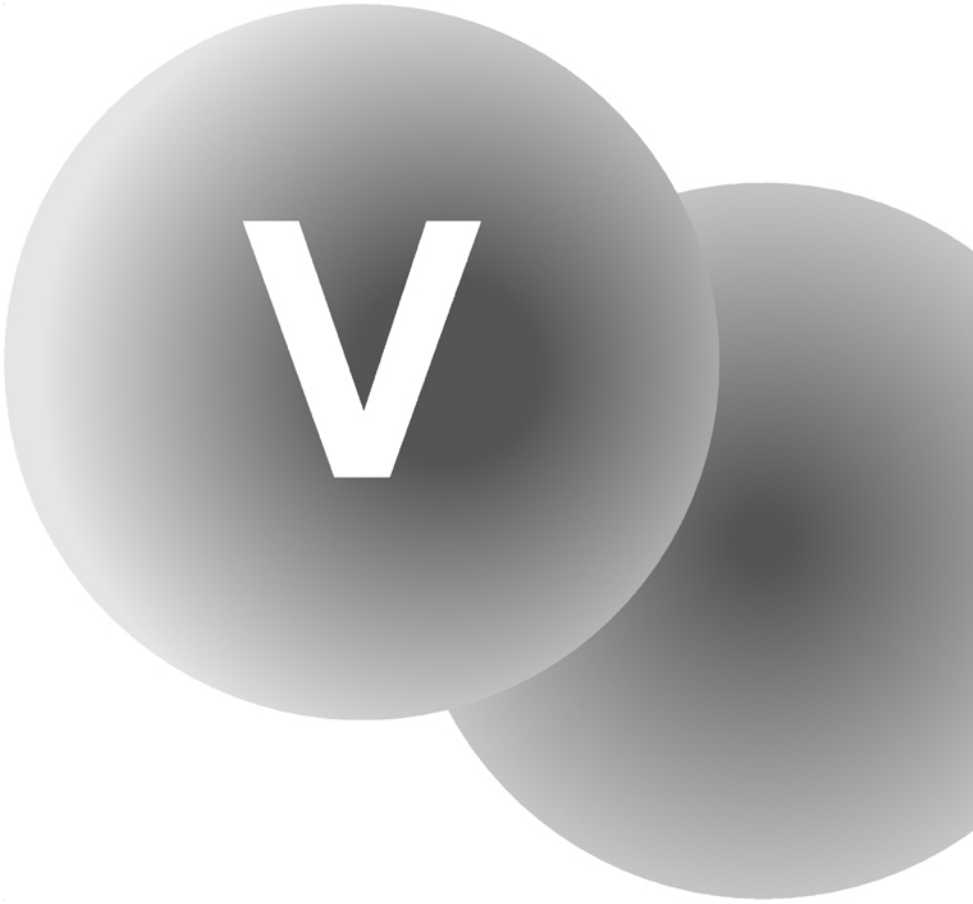
Low pneumococcal carriage and high serotype diversity among elderly living in Portugal.



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ABSTRACT

Background: While numerous studies have looked at colonization among young children, much less is known among the elderly, the other age group for which the incidence of pneumococcal disease is high. This study, pioneer in Portugal, aimed to evaluate pneumococcal carriage in adults aged over 60 years.

Methods: Between April and November of 2010, nasopharyngeal and oropharyngeal swabs of adults (>60 years), living in Oeiras (n= 651), an urban area, or in Montemor-o-Novo (n=647), a rural area, were obtained. Pneumococci were identified by standard procedures and by PCR detection of genes *cpsA* and *lytA*. Isolates were characterized by antibiotype, serotype and MLST. Associations between pneumococcal carriage, socio-demographic and clinical factors were evaluated using a multiple logistic regression.

Results: A total of 1,298 adults were enrolled. Only 4.1% were smokers. Fifty-nine percent of the participants were vaccinated with the seasonal influenza vaccine and only 4.3% with the 23-valent pneumococcal polysaccharide vaccine. Twenty-nine (2.2%) adults carried *bona fide* capsulated pneumococci. There were more adults carrying pneumococci in the rural area (3.4%) than in the urban area (1.1%) (p=0.004). One adult carried two different pneumococcal strains in the nasopharynx. The 30 strains expressed 18 capsular types and 22 MLST types. Sixteen isolates showed antimicrobial resistance. Smoking increased seven-fold the odds for carriage (OR=7.4, CI 95%: 2.56-21.27) whereas living in an urban area decreased the odds to colonization by 72% (OR=0.28, CI 95%: 0.11-0.68).

Conclusion: The prevalence of pneumococcal carriage in the elderly is low and serotype and genotype diversity is high. Smoking is an important risk factor for colonization whereas living in Oeiras seems to be a protective factor.

INTRODUCTION

Streptococcus pneumoniae or pneumococcus remains a leading cause of infectious diseases worldwide. The incidence of invasive pneumococcal disease is age-dependent being highest at the extremes of age. While the burden of disease is substantial, pneumococcus is a commensal that colonizes asymptomatically the nasopharynx of humans. Carriage is important as it is a fundamental step for infection and for transmission (reviewed in (3)). While, several pneumococcal carriage studies have been conducted among young children much less is known about the carrier state among the elderly, the other age group where the incidence of invasive pneumococcal disease is highest.

One of the main virulence factors of pneumococcus is the capsule and until now, more than 90 serotypes have been described (4, 5, 13, 25). Vaccination proved to be the best way to decrease infections and in 1983 a pneumococcal polysaccharide vaccine, Pneumovax23, was licensed. This polysaccharide vaccine was designed preferentially for adults and for children older than 2 years old, but its efficacy remains controversial (8, 22). In 2000 another pneumococcal vaccine, the seven-valent conjugate vaccine (PCV7) targeting the seven serotypes that frequently caused invasive disease in children in USA, was introduced in the market. The efficacy of this vaccine on the decline of invasive pneumococcal infection (IPD) caused by serotypes covered by PCV7 was reported in several countries (2, 9, 11, 14-16). In the USA, the effect of PCV7 was also observed in all populations, with a concomitant reduced incidence of IPD in adults (26, 28). Recently, we published a study that described the changes that occurred in colonization among Portuguese children when c.a. 60% of them had received at least one dose of PCV7 (29). In that study we observed a decline of PCV serotypes, but carriage and antimicrobial resistance was maintained. In 2010, a new pneumococcal conjugate vaccine (PCV13) appeared in the market, potentially covering for six additional serotypes: 1, 3, 5, 7F, 6A, and 19A. The effect of this

vaccine in IPD and in pneumococcal colonization has been studied for the past two years. In February of 2012 this vaccine was also licensed for adults.

In the current study, conducted in 2010, we aimed to gain insights on pneumococcal colonization patterns among elderly and establish a baseline before the introduction of the conjugate vaccine in adults with more than 50 years of age.

METHODS

Study design. Between April and November of 2010, adults older than 60 years of age, living in Oeiras, an urban area, or in Montemor-o-Novo, a rural area of Portugal, were invited to participate in the study. At the time of the study the 10-valent and 13-valent pneumococcal conjugate vaccines were commercially available in the country although they were not part of the national immunization plan nor were subsidized by the state. Still the commercial use of the vaccine was high with an estimated national coverage of PCV13 in infants in 2010 of 58% (Pfizer, personal communication). The municipality of Oeiras has a population of over 160,000 inhabitants of which 15% are over 65 years of age, and a population density of 3,537/km^2. By contrast, the municipality of Montemor-o-Novo has a population of 18,500 inhabitants of which 26% are over 65 years of age, and a population density of 13/km^2. In both areas individuals were enrolled in the local health care center***** and, in addition, in Montemor-o-Novo retirement homes were also visited. Samples were obtained by specialized laboratory technicians (in Oeiras) or nurses (in Montemor-o-Novo). The study was registered and approved at each health care center. Study participants provided informed written consent prior to enrollment. For each adult one nasopharyngeal sample and one oropharyngeal sample were obtained. Information about socio-demographic data and medical history were obtained through a questionnaire. All samples and information were coded upon collection and processed anonymously afterwards.

*****Health care centers are part of the National Health Service and provide primary care, with consultations are on general practice and routines; they serve the entire Portuguese population.

Sampling and isolation. Nasopharyngeal and oropharyngeal samples were collected as previously described (30). Swabs were streaked on gentamycin blood agar and suspected pneumococcal colonies were isolated and identified following routine standard procedures based on optochin susceptibility and bile solubility (19, 30). Additionally, isolates showing one or more atypical results in the latter assays but displaying a characteristic pneumococcal colony morphology were further characterized by the identification of *lytA* pneumococcal-specific RFLP signatures as described by Lull *et al.* (17). The swabs and the pure cultures were frozen and stored at -80ºC in STGG (20).

Capsular typing. Isolates were capsular typed by multiplex PCR using primers previously described (23) and www.cdc.gov and/or by the Quellung reaction using commercially available pneumococcal antisera (Statens Serum Institute, Copenhagen, Denmark) (31).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using the Kirby-Bauer technique, according to the CLSI recommendations and definitions (7). The antimicrobial agents tested were chloramphenicol, erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim (SXT). Antibiotic disks were purchased from Oxoid (Hampshire, England). Interpretation of results followed the CLSI guidelines (7). Isolates were also screened for MICs to penicillin and ciprofloxacin with the E-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations. In the interpretation of decreased penicillin susceptibility epidemiological breakpoints were used: isolates were considered intermediately resistant if the MIC was ≥0.1 µg/ml and <1.5 µg/ml and were considered resistant if the MIC was ≥1.5 µg/ml. For ciprofloxacin, strains with MIC≥2 µg/ml were considered resistant.

Multilocus sequence typing (MLST). MLST was done essentially as previously described (10) using primers with universal M13 tails. Sequencing was conducted at Macrogen, Inc. (Seoul, Korea).

Statistical analysis. Socio-demographic and clinical variables were described using the mean and standard deviation or percentages as appropriate. Differences for each variable, between Oeiras and Montemor-o-Novo, were tested using the Student's t-test or the Mann-Whitney U-test for continuous variables and the Fisher's exact test to compare proportions (32). Univariate and multiple logistic regression analysis were used to assess the goodness of fit of the socio-demographic and clinical variables to the dependent variable, to be colonized or not and, to calculate non- and adjusted odds ratios (OR). A conservative p-value <0.1 was used to select the variables in the univariate model to be included in the multiple logistic model. For all statistical tests, differences were considered to be statistically significant if the p-value < 0.05 or when the adjusted 95% CI odds ratio did not include 1.0. Statistical analyses were carried out using R.

RESULTS

Characteristics of the study population. A total of 651 adults over 60 yrs of age living in the urban area of Oeiras and 647 living in the rural area of Montemor-o-Novo participated in the study. Women were slightly overrepresented in both populations. The two populations differed significantly in several socio-demographic and medical characteristics (detailed in Table 1). On average, participants from the rural area were older and had less years of formal education. Although most participants were retirees, the proportion of those still working was significantly lower in the rural area. The household size was comparable in both areas but participants from the rural area had significantly less contact with young children. On the other hand, in the rural area, participants engaged more

TABLE 1. Socio-demographic characteristics and medical history of participants

	Urban area	Rural area	p-value
Population (> 60 years)	651	647	
Mean age (yrs)	73.07±7.54	75.45±8.19	<0.001
Gender (female)	349 (53.6%)	381 (58.8%)	0.057
Years of school education			
0	59 (9.1%)	282 (43.6%)	<0.001
1-4	266 (41.0%)	332 (51.3%)	<0.001
≥ 5	324 (49.9%)	33 (5.1%)	<0.001
Retirees	566 (86.9%)	609 (94.1%)	<0.001
Housing			
family home	649 (99.7%)	532 (82.2%)	
retirement home	0 (0.0%)	114 (17.6%)	<0.001[a]
other	2 (0.3%)	1 (0.2%)	
Household size[b]	1.44±0.36	1.43±0.39	0.553
Contact with children ≤ 6 yrs			
daily at home	26 (4.0%)	13 (2.0%)	0.007
at least once a week	221 (33.9%)	127 (19.6%)	<0.001
Recreational activities			
at least one activity	119 (18.3%)	340 (52.6%)	<0.001
club	69 (10.6%)	88 (13.6%)	<0.001
day center	12 (1.8%)	125 (19.3%)	<0.001
senior university	16 (2.5%)	3 (0.5%)	<0.001
other	19 (2.9%)	10 (1.5%)	<0.001
Smoker	38 (5.9%)	15 (2.3%)	0.002
Chronic disease	515 (79.1%)	543 (83.9%)	0.032
COPD	59 (11.6%)	140 (25.8%)	<0.001
asthma	18 (3.5%)	55 (10.1%)	<0.001
hepatic disease	3 (0.6%)	23 (4.2%)	<0.001
renal disease	7 (1.4%)	35 (6.4%)	<0.001
Respiratory infection in previous year	219 (33.6%)	365 (56.3%)	<0.001
Hospitalization in previous year	95 (14.6%)	93(14.4%)	0.937
Symptoms of respiratory disease at sampling[c]	212 (32.6%)	273 (42.2%)	<0.001
Vaccination			
seasonal flu	363 (55.8%)	397 (61.4%)	0.043
H1N1	77 (11.8%)	36 (5.6%)	<0.001
PPV23[d]	26 (3.9%)	20 (3.1%)	0.454
Antibiotic consumption			
at sampling	17 (2.6%)	10 (1.5%)	0.249
month before	40 (6.1%)	32 (4.9%)	0.396
previous 3 months	70 (10.8%)	44 (6.8%)	0.014

[a]test between levels family household and retirement home; [b]among those living at family home; [c]symptoms included runny nose, shortness of breath, sore throat, sputum, cough, fever and others; [d]23-valent pneumococcal polysaccharide vaccine; COPD, chronic obstructive pulmonary disease.

frequently in regular recreational activities. Although smoking was not common in these populations, the proportion was higher in the urban area (Table 1) with no differences (among smokers from the two regions) in the number of smoking years and cigarettes per day (data not shown).

Regarding medical history, participants from the rural area were globally less healthy. A high prevalence of chronic diseases was reported in both populations being higher in the rural area. When individual diseases were analyzed, chronic obstructive pulmonary disease (COPD), asthma, hepatic and renal diseases remained significantly higher in the rural area. The proportion of participants reporting a respiratory infection in the previous year, and current respiratory infection symptoms was also higher in the rural area.

Regarding vaccination over half of the participants had received the seasonal flu vaccine but significantly less had been vaccinated against H1N1. A tiny proportion (<4%) had received PPV23. Antibiotic consumption was low in both regions although higher in the urban area. Urinary infections were reported as the main reason for taking antibiotics.

Pneumococcal carriage. Of the 1,298 adults, 29 (2.2%) were pneumococcal carriers. One adult carried two different isolates. To explore which factors contributed to pneumococcal carriage, univariate and multiple logistic regression analyses were performed. There were significantly more carriers in the rural area than in the urban area (3.4% vs 1.1%, respectively, p=0.004). Carriage was also more frequent among smokers (9.4% vs 1.9%, p=0.005), and those with COPD (4.5% vs 1.8%, p=0.032). Other variables did not yield statistical significant differences.

The best multiple logistic regression model included the region, smoking and having COPD. In the final model, elderly living in the urban area had a lower risk of carrying pneumococci, while smokers had an increased risk (Table 2).

TABLE 2. Socio-demographic characteristics and medical history of participants

Variable		Unadjusted OR	Adjusted OR (95% CI)	p-value
Region				
	rural		1	
	urban	0.31	**0.28 (0.11 – 0.68)**	0.004
Smoking (yes)		5.29	**7.38 (2.56 – 21.27)**	0.001
COPD (yes)		2.55	2.00 (0.88 – 4.54)	0.111

COPD, chronic obstructive pulmonary disease.

Characteristics of pneumococcal isolates. Among the 30 pneumococcal strains a high diversity of serotypes and genotypes was detected as 18 serotypes and 22 sequence types were identified (Table 3). Overall, 23% and 43% of the isolates belonged to serotypes included in PCV13 and PPV23, respectively. By MLST, two novel sequence types were identified (STs 6987 and 6988 associated with serotypes 18A and 23B, respectively). No major lineages were detected and the largest group of strains sharing an ST contained three isolates only. Close to half of the isolates (46.7%) were resistant to at least one antimicrobial agent; only two isolates were multidrug resistant (i.e. resistant to three or more classes of antimicrobials) (Table 3).

TABLE 3. Characterization of the pneumococcal strains

Serotype	MLST	Resistance pattern	Origin	No. of isolates
3	180	susceptible	rural	2
5	1223	Cip	rural	1
6A	1714	Tet, Cip	urban	1
6A	460	susceptible	urban	1
9L	66	Cip	rural	1
11A	62	SXT	urban	1
11A	62	Cip	urban	1
11A	408	Cip	rural	2
15A	63	[I]PG, Tet	urban	1
15B	3097	[I]PG, Ery, Clin, Tet	rural	1
16A	30	susceptible	urban	1
18A	6987	susceptible	urban	1
19A	276	[I]PG, Ery, Clin, Tet, Cip	rural	1
19A	1201	susceptible	rural	1
21	1877	Cip	rural	1
22F	433	susceptible	rural	3
23A	42	Cip	rural	1
23B	6988	susceptible	rural	1
23B	439	susceptible	rural	2
31	1766	susceptible	rural	1
35F	1635	Ery	rural	1
35F	1368	susceptible	rural	1
36	1635	Ery, Cip	rural	1
37	447	Cip	rural	2

[I]PG, intermediately resistant to penicillin; Ery, erythromycin; Clin, clindamycin; Tet, tetracycline; SXT sulfamethoxazole-trimethoprim; Cip, ciprofloxacin

DISCUSSION

This is the first study conducted in Portugal aiming to evaluate pneumococcal carriage in the elderly population. Previous studies from other countries such as Belgium, Israel, and Kenya, described low rates of colonization (typically up to 5%) among adults (1, 12, 27). In our study, the pneumococcal carriage rates were also low, being higher in the rural area when compared to the urban area (3.4% vs 1.1%, p=0.004).

Although several differences were noticed among socio-demographic characteristics and medical history of the two populations studied, statistical analysis identified only two factors that were associated with pneumococcal carriage: living in the urban area and being a smoker. While living in Oeiras seemed to be a protective effect, decreasing the risk of colonization by 3.5 fold, smoking increased the risk of being colonized by seven-fold. Smoking has been previously associated with pneumococcal carriage (18). Why living in the urban area reduced the risk of carriage is not self-evident. One may hypothesize that other important variables to explain this observation were not studied. Alternatively, it is conceivable that due to the small number of carriers identified in the samples resulted in low statistical power to identify additional variables as significant.

Characterization of pneumococcal isolates revealed a high serotype and genotype diversity. Only one serotype (11A) was common to both areas; however, the genotypes and antibiotypes associated with it were distinct.

This study has some limitations. Although the size of the population enrolled was high (c.a. 650 adults enrolled in each area) the low number of pneumococcal isolates identified hindered the statistical analysis. Secondly, we used culture-based only methods to detect pneumococci, which may have resulted in a lower

carriage rate estimate. Although we used a selective medium to prevent overgrowth of undesirable bacteria, and easily identify pneumococci, we may have been unable to detect low-density carriers. In fact, Ogami *et al.* reported that real-time PCR increased significantly detection of carriage in healthy children when compared to culture alone (21). The same is plausible for detection of carriage in adults (24). More recently, Chien *et al.* reported that at carriage density of $\leq 10^5$ CFU/ml detection of pneumococci by culture was significantly less sensitive than PCR (6). These limitations are being further explored in ongoing initiatives carried by this group. Our study has also some strength. It generated for the first time data on pneumococcal colonization patterns in the elderly in Portugal. In addition, pneumococcal isolates were characterized in detail and a wealth of epidemiological data was obtained.

In conclusion, this study showed that pneumococcal colonization in the elderly in Portugal is low (2.2%). Colonizing isolates are very diverse in terms of genotype and serotype. Smoking increases the risk of being colonized with pneumococci by 7-fold while living in an urban area seems to be a protective factor for colonization. This study will contribute to establish a baseline that may be used in future studies to monitor how novel pneumococcal vaccines impact on colonization in adults.

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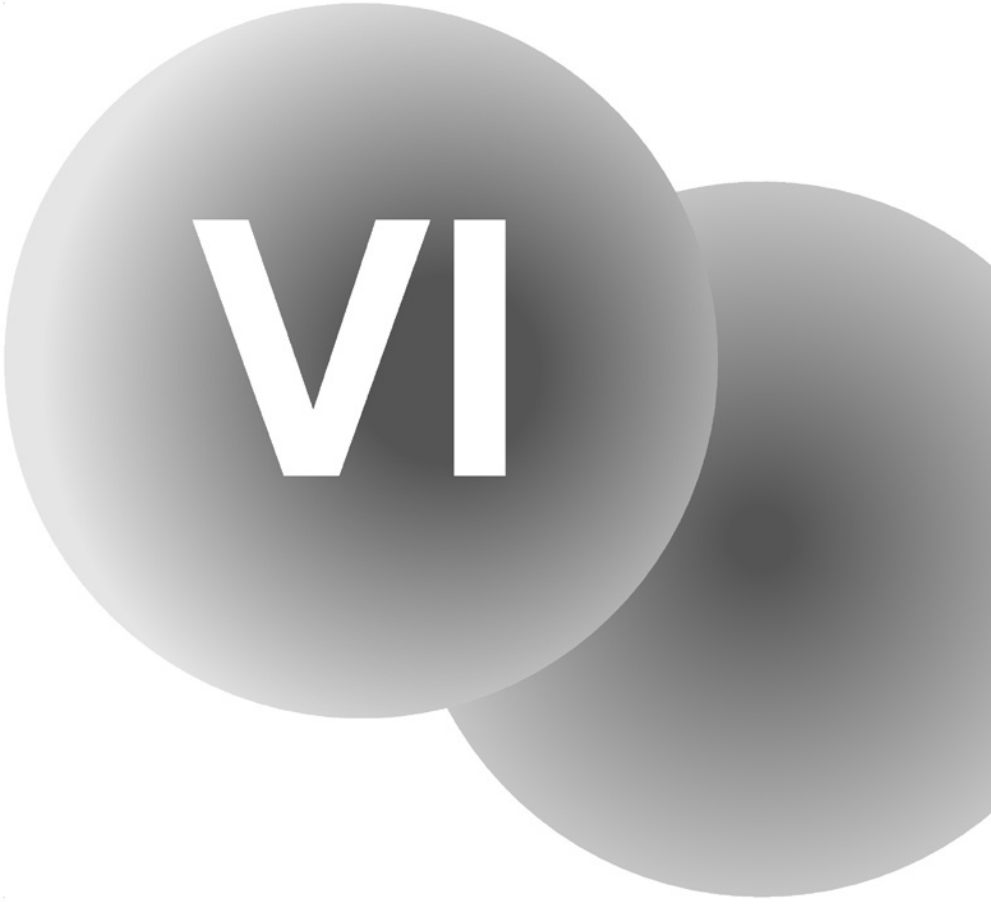
Multiple-Locus Variable number tandem repeat Analysis for Streptococcus pneumoniae*: comparison with PFGE and MLST*



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S. Nunes was responsible for MLVA.

Abstract

In the era of pneumococcal conjugate vaccines, surveillance of pneumococcal disease and carriage remains of utmost importance as important changes may occur in the population. To monitor these alterations reliable genotyping methods are required for large-scale applications. We introduced a high throughput multiple-locus variable number tandem repeat analysis (MLVA) and compared this method with pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

The MLVA described here is based on 8 BOX loci that are amplified in two multiplex PCRs. The labeled PCR products are sized on an automated DNA sequencer to accurately determine the number of tandem repeats. The composite of the number of repeats of the BOX loci makes up a numerical profile that is used for identification and clustering. In this study, MLVA was performed on 263 carriage isolates that were previously characterized by MLST and PFGE.

MLVA, MLST and PFGE (cut-off of 80%) yielded 164, 120, and 87 types, respectively. The three typing methods had Simpson's diversity indices of 98.5% or higher. Congruence between MLST and MLVA was high. The Wallace of MLVA to MLST was 0.874, meaning that if two strains had the same MLVA type they had 88% chance of having the same MLST type. Furthermore, the Wallace of MLVA to clonal complex of MLST was even higher: 99.5%. For some isolates belonging to a single MLST clonal complex although displaying different serotypes, MLVA was more discriminatory generating groups according to serotype or serogroup.

Overall, MLVA is a promising genotyping method that is easy to perform and a relatively cheap alternative to PFGE and MLST. In the companion paper published simultaneously in this issue we applied the MLVA to assess the pneumococcal population structure of isolates causing invasive disease in the Netherlands before the introduction of the 7-valent conjugate vaccine.

Introduction

Streptococcus pneumoniae is an important human pathogen causing diseases like otitis, pneumonia, sepsis, and meningitis. The major virulence factor of the pneumococcus is the capsule (1, 17). Currently, over 90 different capsules have been identified (3, 5, 16, 31). Such high antigenic diversity has long been recognized and serotyping using Quellung reaction has been used for pneumococcal typing for several decades. In recent years, as the DNA sequences of the capsular biosynthetic loci became available (2, 31), alternative strategies for capsular typing based on genotyping methods have been developed (4, 22, 30) (Elberse et al. companion paper, PLoS One, this issue).

For the past two decades, a number of genotyping methods aimed to assess the genetic diversity of pneumococcal isolates have been used. Currently, pulsed-field gel electrophoresis (PFGE) (15, 23) and multilocus sequence typing (MLST) (10) are the gold standards for genotyping of pneumococci.

In PFGE, total DNA is digested with a rare cutter endonuclease (such as *Sma*I or *Apa*I) that yields a limited number of fragments of high molecular weight. The fragments are separated by a variant of gel electrophoresis in which the orientations of the electric field change periodically, enabling megabase size DNA fragments to be effectively separated by size (39). The DNA banding patterns are then compared between isolates and clonal relationships are inferred (23). Although the interpretation criteria may vary depending on the size of the collection and on the goal of the research being conducted, there are general criteria, both visual and computer-assisted that seem to work well (6, 42). Advantages of PFGE are that it has good typeability (the percentage of isolates that can be assigned a type), reproducibility, and resolving power (26, 42). In addition, the costs for materials and equipment are relatively low and handling of the equipment is easy. However, it is laborious and time consuming

and may yield ambiguous results if not performed by a well trained technician. PFGE is quite useful for local epidemiology and it has also been used for global epidemiology once standardized (27, 34). Portability between laboratories is not straightforward, but seems to be attainable provided protocol harmonization is achieved (42).

Multilocus sequence typing (MLST) is a DNA sequence-based method that relies on PCR amplification and sequencing of internal fragments of 7 housekeeping genes (10, 24). For allele assignment, each sequence is compared to all known alleles which are available at an online database (www.mlst.net). Different sequences (even single nucleotide difference) are assigned different allelic numbers. The 7 assigned allele numbers form an allelic profile or sequence type (ST). MLST is expensive if in-house sequencing facilities are not available and, therefore, many laboratories cannot afford to use it routinely. However, it has the advantages of being reproducible, unambiguous, portable allowing intra-laboratory comparisons and suited to create international databases. For *S. pneumoniae*, MLST has a good resolving power being useful for local and global epidemiology. Furthermore, in contrast to PFGE, MLST does not always require a culture and can sometimes be directly performed on samples containing bacterial DNA such as cerebrospinal fluid (9).

In 1992 conserved repeated sequences, named BOX elements, were identified in the genome of the pneumococcus (25). The sequenced genomes of R6 and TIGR4 contain 115 and 127 BOX elements, respectively. BOX elements consist of 3 different subunits, BoxA, BoxB and BoxC. BoxB is the tandem repetitive unit of 45 base pairs flanked by BoxA and BoxC, although elements missing BoxA or missing BoxC have been described (20, 25). The function and origin of BOX elements are unknown; however, it is thought that they may be involved in regulating the expression of virulence-associated genes when they are located

in the promoter regions of genes (20, 25). In the years after the discovery of these elements a number of genotyping methods were introduced based on the variability of these elements. A BOX-PCR described by van Belkum and colleagues showed the usefulness of these repeats for genotyping (44). The primer of this PCR is based on the BoxA sequence and yields PCR products of regions present between BOX loci that are close to each other and in opposite direction. However, the method has the disadvantage of creating banding profiles, which are difficult to interpret and often lack reproducibility. A multiple-locus variable number tandem repeat analysis (MLVA) scheme based on BOX typing was introduced by Koeck et al. in 2005 (21). It analyzes 16 BOX loci that are PCR-amplified in single PCR reactions and products are analyzed by agarose gel electrophoresis. A website (www.mlva.eu) providing a database in which profiles can be compared has been created. At the time of writing this manuscript the MLVA profiles of 1147 isolates had been deposited in the database. The many loci that are analyzed and the choice of agarose gel electrophoresis for sizing makes this method somewhat laborious. This MLVA scheme was successfully applied and compared with MLST and was shown to yield high congruence with MLST, but was more discriminatory than MLST (32, 35).

In this paper we describe a newly developed MLVA typing scheme for pneumococci based on 8 BOX elements. The 8 BOX loci are amplified in 2 multiplex PCRs and the fluorescently labeled PCR products are sized on an automated sequencer yielding high throughput and unambiguous typing results. The generated MLVA results can be compared internationally using the newly available MLVA website www.MLVA.net. On the website the MLVA profiles can be imported to assess the MLVA type (MT). In the current study we assess the validity of this method by comparing MLVA with two well established genotyping methods: PFGE and MLST. In the companion paper published simultaneously in PLoS One, we applied this MLVA scheme to assess the population structure

of pneumococci causing invasive disease in the Netherlands before the introduction of the 7-valent pneumococcal conjugate vaccine.

Materials and methods

Isolate collection.

In this study 263 pneumococcal isolates were used for the comparison of MLVA with PFGE and MLST. The isolates were obtained between 1996 and 2007 from the nasopharynx of children attending several day-care centers in the area of Lisbon, Portugal. The isolates were stored in glycerol broth at -80ºC and were characterized by antibiogram, serotyping, PFGE, and MLST under the scope of previous studies (28, 33, 34). Approval for the study was obtained from the Ministry of Education and from the directors of each day-care center. Written informed consent was obtained from parents or guardians of each child before sampling. All information was stored in an in-house developed online database. Overall, the collection comprised 41 serotypes, 87 PFGE types and 120 STs.

Serotyping, PFGE and MLST.

These techniques were performed as previously described. Briefly, serotyping was performed by the Quellung reaction (41) and/or multiplex PCR (8, 30). For PFGE, total DNA was extracted, restricted with *Sma*I, and DNA fragments were resolved by PFGE (34). MLST was performed using primers and conditions described previously (10). For allele assignment the *S. pneumoniae* MLST database available at www.mlst.net was interrogated. Sequence traces of novel alleles were submitted to the curator for new allele and ST assignment.

Multiple-Locus Variable number tandem repeat Analysis (MLVA).

In the genome sequence of isolate R6 (GenBank number NC_003098), one of the publicly available genomes, 115 BOX tandem repeats were found. Thirteen

randomly chosen BOX loci scattered throughout the R6 genome to be used in the MLVA scheme were tested on a panel of 84 isolates. Eight BOX loci were chosen and primers were designed targeting the flanking regions of the BoxA and BoxC elements (Figure 1, Table 1).

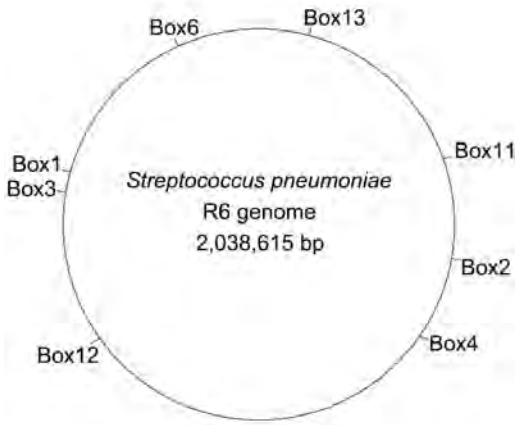


Fig. 1. Schematic representation of pneumococcus R6 genome indicating the location of the 8 BOX loci that are used in the MLVA scheme.

One primer of each primer set was fluorescently labeled with FAM, NED, VIC or PET. For BOX loci 4 and 6 respectively 3 and 4 primers were used to primer set was fluorescently labeled with FAM, NED, VIC or PET. For BOX loci 4 and 6 respectively 3 and 4 primers were used to enhance amplification within all isolates. The 8 loci were amplified in 2 separate multiplex PCRs, creating mixtures each with 4 different fluorescent labels enabling analysis of 4 BOX loci in a single fragment analysis reaction (Table 1)**.** Amplification of the loci was done in Applied Biosystems 9700 PCR machines. The 25 µl PCR reaction mixtures consisted of Qiagen multiplex PCR mix, 10 µM of each primer and 2 µl of 1:10 lysates diluted in sterile water. Lysates were prepared by suspending a loop full of pneumococci in 500 µl TE (10 mM Tris.HCl and 1 mM EDTA, pH 8) followed by heating for 10 minutes by 95ºC. Amplification was performed using the following PCR program: 15 min 95ºC, 25 cycles of 30 sec 95ºC, 1 min 54ºC and 1 min 72ºC followed by a 30 min incubating at 68ºC to ensure complete

addition of the extra 3'adenosin by the terminal transferase activity of the Taq polymerase. Of the PCR product mixtures, 2 µl aliquots, diluted 1:200 in water, were mixed with 10 µl of 1:200 diluted Genescan 1200 LIZ-marker (Applied Biosystems, Foster City, U.S.A.). The product was heated for 5 minutes at 95ºC for denaturation and sized on the AB 3730 DNA sequencer using the Fragment Analysis module.

Determination of the number of repeats in each locus was done using the GeneMarker software (Softgenetics, State College, Pennsylvania, USA). For this purpose, fragment analysis files with the .fsa extension were imported into the software and the number of repeats in each locus was derived from the data. All alleles that yielded PCR product sizes that had not been found before, were analyzed by sequencing using unlabeled versions of the MLVA primers. This included PCR products with aberrant sizes representing loci in which deletions or insertions had occurred in the region flanking the BOX repeats. Thereafter, the loci were assigned the number of BoxB repeats present. For each isolate an allelic profile was generated consisting in a string of 8 integers reflecting the number of repeats in the 8 BOX loci. The allelic profile was assigned an arbitrary sequential MLVA type (MT). The correspondence between MT and allelic profile was deposited at the MLVA database located at www.mlva.net to guarantee that the same nomenclature was maintained for all isolates characterized by this technique regardless of its origin and study.

Data Analysis.

Analysis of PFGE patterns, MLST and MLVA numerical profiles, was performed using Bionumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). PFGE patterns were clustered by UPGMA. A dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 1.0% and a tolerance of 1.5%. PFGE clusters were defined as isolates with a similarity of 80% or higher on the dendrogram (13, 33).

Clustering of MLST and MLVA types were displayed in minimum spanning trees. In this graphic representation, the circles in the tree represent the various types. The size of the circle is proportional to the number of isolates with the same type. Lines linking two types in the tree denote single locus difference between those types. For assignment of MLST or MLVA clonal complex, the entire MLST database (available at www.mlst.net, last accessed on October 20, 2010) or the entire in-house MLVA database (available at www.mlva.net, last accessed on October 20, 2010) were interrogated. MLVA complex assignment was based on a maximum distance of one locus between related types. The minimum number of MLVA types in a MLVA complex was set to 3 with a minimum of 9 entries per MLVA complex, resulting in on average 3 isolates within an MLVA type. These settings resulted in MLVA complexes that were strongly correlated to serotype.

Statistical analysis.

The discriminatory ability of the three typing systems - PFGE, MLST and MLVA - was measured using the Simpson's index of diversity (SID) and 95% confidence intervals were calculated as proposed before (14, 40). The congruence between the typing methods was calculated using the Wallace coefficient (7). All calculations were done using the freely available online tool Comparing Partitions located at www.comparingpartitions.info (7).

Results

Multiple-locus variable number tandem repeat analysis (MLVA)

A combination of highly diverse and less diverse BOX loci were chosen aiming to have a good balance between loci that would evolve faster than others. The 13 randomly chosen BOX loci were tested on a panel of 84 isolates and 8 BOX loci were chosen for the MLVA scheme. The overlap in BOX loci of our MLVA

scheme with the MLVA scheme previously described by Koeck et al. (21) is provided in Table 1. In particular, BOX loci 1, 4, 12 and 13 were rather diverse while BOX locus 11 had less diversity. If BOX loci could not be amplified they were assigned allele number 99. To exclude the possibility that BOX loci could change by laboratory storage and repeated subculture, 4 pneumococcal isolates were subcultured for 29 sequential days. MLVA was performed on samples from days 1, 15, and 29 and no changes in the number of repeats in the 8 BOX loci were observed indicating that the composition of BOX loci remained stable under laboratory conditions (data not shown). In addition, 84 isolates were typed at least twice at different occasions by independent technicians and the same MLVA types (MT) were obtained indicating that the MLVA scheme is reproducible.

Discriminatory power of MLVA

The discriminatory power of MLVA was calculated by using the Simpson's Index of Diversity (SID) applied to the test population. The 263 isolates were resolved in 164 types that contained between 1 and 14 isolates. The SID for MLVA was 0.993, a very high value comparable to the ones obtained for PFGE (0.985) and MLST (0.987) (Table 2). In the Supplemental Table the MLVA, MLST and PFGE data is provided for all isolates that are included in this study (Table S1). The companion paper (Plos One, this issue) highlights its wide application to over 1000 isolates, including 444 MLVA types. Overall, the current MLVA database consists of 2973 isolates, 960 MLVA types and 43 MLVA complexes. The SID for MLVA based on the entire database was 0.986 (95% CI, 0.985-0.988) (www.MLVA.net, last accessed on October 20, 2010).

Comparison of MLVA with serotyping, PFGE, and MLST

To assess the congruence between typing methods the Wallace index was calculated (Table 3). This coefficient indicates the probability that a pair of isolates which is assigned to the same type by one typing method is also typed

as identical by the other method. A very good directional correlation between MLVA and MLST results was found: the probability of two isolates having the same MLVA type (MT) also sharing the same MLST type (ST) was 87.4%. This value was even higher for predicting the MLST clonal complex: 99.5%. Corresponding Wallace indexes between MLVA and other typing methods were: 82.2% for serotyping, and 46.5% for PFGE. By contrast, the chance that two isolates sharing the same ST also shared the same MT was 43.3% and sharing the same MLVA complex was 99.2%. Lower values were obtained for PFGE and serotyping. These differences can be explained by the higher discriminatory power of MLVA compared to the other typing methods.

Table 1. Oligonucleotide primer sequences used in *S. pneumoniae* MLVA scheme.

Assay	Primer	Primer sequence	Accession no.	Coordinates	Koeck et al.
Multiplex reaction 1	BOX_01-Ff[1]	CCAGAGACATTGATGAAGAGA	NC_003098	1611150	Spneu40
	BOX_01-r	CGCTTTGATGAACTTGAGTT	NC_003098	1611477	Spneu40
	BOX_02-Nf[1]	TTGCTTGGTACAGAAAACAAA	NC_003098	571757	Spneu32
	BOX_02-r	CCCCATAAAACCCTCCTTATA	NC_003098	572108	Spneu32
	BOX_03-Vf[1]	TCCAACACGACCTTTATCC	NC_003098	1579329	Spneu15
	BOX_03-r	TTCAGTAAACCCAGCTCGTA	NC_003098	1579997	Spneu15
	BOX_04-f	TGGGTAAAAGTAGACAGGACT	NC_003098	698447	Spneu33
	BOX_04-f2[2]	AGGGGATTTACCCACTACAAA	CP000936	823993	
	BOX_04-Pr[1]	CACTTCTACACTAGTTTGTAAAGCA	NC_003098	698665	Spneu33
Multiplex reaction 2	BOX_06-Nf[1]	GAAAAAGGTCAGGAGTAGGTT	NC_003098	1911454	Spneu38
	BOX_06-r	TCACTTGAGACAATCAGCC	NC_003098	1911736	Spneu38
	BOX_06-Nr2[1,2]	GAAATCTTTGAAAAACTAGGATTT	NC_003098	1911683	Spneu38
	BOX_06-f2[2]	TTATGATTTTTGTCATTTTGCA	NC_003098	1911429	Spneu38
	BOX_11-Vf[1]	TCCAATAATGACAGGTTTTCCTC	NC_003098	411593	
	BOX_11-r	TTCCAATCTACGCCTTTGAAG	NC_003098	412169	
	BOX_12-Pf[1]	TTGCCCTTTTCATCTTCGA	NC_003098	1350183	Spneu37
	BOX_12-r	CAGCAACCATTGAAACGC	NC_003098	1350824	Spneu37
	BOX_13-Ff[1]	TCGCCTTTGCTAATCAAACA	NC_003098	101033	Spneu25
	BOX_13-r	CTGATTATATCGCTCACAAACCC	NC_003098	101490	Spneu25

[1] Primer is fluorescently labeled. F: FAM; N: NED; V: VIC; P: PET.

[2] Additional primer because of polymorphism in primer sites

Relatedness of isolates with similar types when assessed by MLVA or MLST

Although there was considerable concordance between MLST and MLVA, some of the isolates that were grouped by MLST could be further distinguished by MLVA. In Figure 1A a minimum spanning tree is depicted based on the MLST results for the 263 isolates of the test collection. Fifty-six clonal complexes (CC), and 13 singletons were detected and the 7 largest CCs were chosen for further comparisons and are highlighted in color. To visualize the relationship between MLST and MLVA, the same color legend was applied to the minimum spanning tree based on MLVA (Fig. 1B). To create this minimum spanning tree, the entries in the entire MLVA database (n=2973) were used. Subsequently, a subnetwork was generated displaying the branches and complexes of the complete tree but only the nodes representing the isolates used in this study.

Table 2. Simpson's Index of Diversity of the different typing methods

Typing method	SID[1] [95% CI]	No. of types
Serotyping	0.937 [0.926-0.948]	41
PFGE	0.985 [0.983-0.988]	87
MLST	0.987 [0.981-0.992]	120
MLST_CC[2]	0.963 [0.955-0.971]	56
MLVA	0.993 [0.991-0.996]	164
MLVA_MC[3]	0.935 [0.918-0.953]	25

[1] SID, Simpson's Index of Diversity; 95% CI, 95% confidence interval

[2] CC, clonal complex

[3] MC, MLVA complex

MLST CC28 included isolates with serotype 6A and 6C which were also closely related when characterized by MLVA. Similar results were obtained for MLST CC1, CC7 and CC32. CC16 included isolates of serotypes 6A, 6C, and 10A; isolates of serotype 10A were clearly separated by MLVA from the serotype 6A

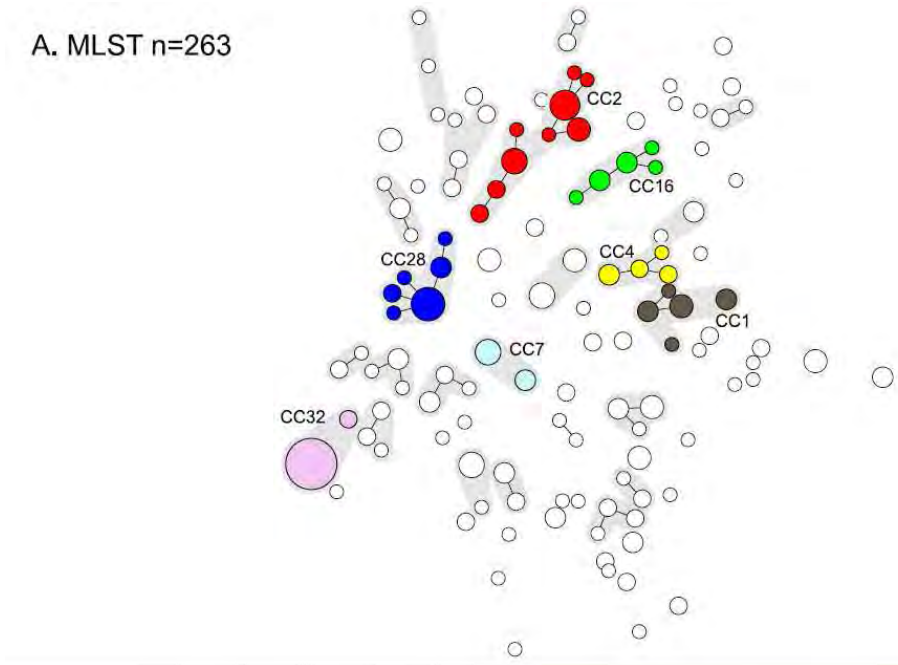
and 6C isolates. CC2 consisted of serotype 6A, 6B, 6C, 15B and 23F isolates. The serotype 6A, 6B and 6C isolates were separated in 2 distinct groups when they were characterized by MLVA. The 15B and 23F isolates were clearly distinct and yielded completely different MTs. This was an example were MLVA distinguished isolates with different serotypes that were grouped within a single CC. The same could be observed for MLST CC4 that comprised serotypes 23A, 23B and 23F that were dispersed into 2 groups using the MLVA.

In this test collection there were 9 instances where isolates were characterized by MLST as two different types, whereas by MLVA these isolates yielded the same type. These isolates were all single locus variants, except for a single isolate.

Table 3. Congruence between typing methods expressed by Wallace coefficients

	Wallace coefficient [95% CI] [1]					
Typing method	Serotype	PFGE	MLST	MLST_CC[2]	MLVA	MLVA_MC[3]
Serotype		0.091 [0.064-0.118]	0.187 [0.132-0.242]	0.343 [0.279-0.407]	0.087 [0.053-0.122]	0.631 [0.553-0.710]
PFGE	0.386 [0.329-0.442]		0.325 [0.273-0.376]	0.550 [0.493-0.607]	0.209 [0.157-0.261]	0.718 [0.649-0.786]
MLST	0.875 [0.848-0.902]	0.358 [0.276-0.439]		1.000 [1.000-1.000]	0.433 [0.316-0.550]	0.992 [0.983-1.001]
MLST_CC[2]	0.575 [0.526-0.623]	0.232 [0.180-0.284]	0.419 [0.356-0.481]		0.202 [0.140-0.263]	0.881 [0.849-0.912]
MLVA	0.822 [0.779-0.865]	0.465 [0.396-0.535]	0.874 [0.825-0.923]	0.995 [0.991-1.000]		1.000 [1.000-1.000]
MLVA_MC[3]	0.769 [0.717-0.822]	0.295 [0.224-0.366]	0.582 [0.486-0.679]	0.998 [0.997-1.000]	0.295 [0.199-0.391]	

[1] 95% CI, 95% confidence interval; [2] CC, clonal complex; [3] MC, MLVA complex


A. MLST n=263
CC2
CC16
CC28
CC4
CC1
CC7
CC32

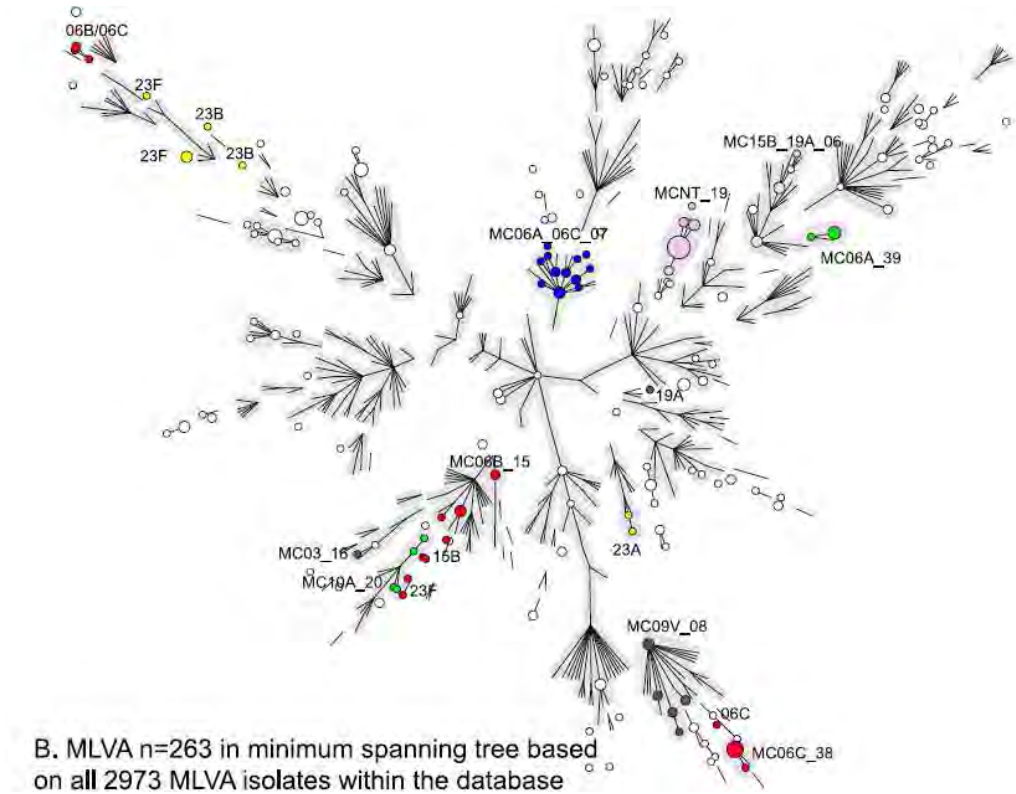

06B/06C
23F
23B
23F
23B
MC15B_19A_06
MCNT_19
MC06A_06C_07
MC06A_39
19A
MC06B_15
MC03_16
15B
MC19A_20
23F
23A
MC09V_08
06C
MC06C_38
B. MLVA n=263 in minimum spanning tree based on all 2973 MLVA isolates within the database

Fig. 2 (left page). High congruence between MLVA and MLST. Minimum spanning tree of the results obtained by MLST (A) and MLVA (B) for the 263 isolates of the test collection. Each circle indicates a genotype. The size of the circle is proportional to the number of isolates with the same type. Lines linking two types denote a single locus difference between those types. In Figure 1A the MLST complexes are indicated by halos. Seven MLST complexes are discussed in the manuscript and these are colored and named according to the nomenclature of *S. pneumoniae* MLST database (www.mlst.net, last accessed on October 20, 2010). The white nodes represent the isolates within this test collection that are not discussed. The same colors were applied to the minimum spanning tree based on MLVA in Figure 1B. This minimum spanning tree is based on the entire MLVA database (last accessed on October 20, 2010), and only the branches are made visible. The MLVA types of the 263 isolates used in this study were depicted as circles. The halos indicate the MLVA complexes.

Discussion

In recent years, typing schemes based on MLVA have been designed and implemented for a number of micro-organisms of public health importance, including *Bacillus anthracis, Staphylococcus aureus, Enteroccus faecium, Haemoplhilus influenza, Bordetella pertussis* and many others (11, 12, 18, 19, 29, 36-38, 43). MLVA-based typing strategies have several appealing characteristics that match the convenience criteria desirable for typing methods (45). For example, once implemented MLVA requires minimal technical expertise and typing results can be obtained in a few hours providing a sequencing facility is readily available. The equipment needed is the same as for MLST, i.e., a thermocycler and a DNA sequencer, equipment that many laboratories performing typing have already access to. It is relatively low cost (cheaper than MLST) and the data are amenable to automatic analysis by computer software (as in MLST). Furthermore, the results are unambiguous numerical profiles, portable, and thus suitable for intra-laboratory comparisons. In addition, PFGE can only be performed on bacterial cultures while MLST can be performed on clinical samples containing bacterial DNA such as cerebrospinal fluid (9). The MLVA has the same potential as MLST, although this has not been described in detail yet.

In this study we assessed the validity of a MLVA strategy developed for *Streptococcus pneumoniae* using as a test collection over 263 pneumococcal isolates previously characterized by the three gold-standard pneumococcal typing methods: serotyping, PFGE, and MLST. The scheme explored polymorphisms scattered throughout the genome of R6 focusing in eight BOX loci for size estimation. The amplification of the 8 loci was combined in two multiplex PCR reactions. Fragment sizing was performed automatically on a DNA sequencer, resulting in a fast, easy and accurate interpretation of results.

The MLVA for the pneumococcus was first described by Koeck et al. (21). In that MLVA scheme, 16 BOX loci were used and analyzed by agarose gel electrophoresis. Remarkably, 7 of the 8 BOX loci used in our MLVA scheme overlap with loci included in the previously published MLVA. We started the selection of the loci for our MLVA already in 2005, before the publication of the MLVA by Koeck et al. and thus the selection was performed independently. Therefore, the overlap is coincidental, but suggests the proper choice of a combination of more and less diverse loci in both MLVA schemes was made. The main advantage of our MLVA is the high throughput that is facilitated by the use of two multiplex assays instead of 16 separate reactions as done by Koeck et al. and the use of capillary electrophoresis for sizing. Furthermore, the electronic data management that we propose makes it easier to analyze the data than images of bands obtained by agarose gel electrophoresis. We restricted our MLVA to 8 VNTR loci to limit the amount of work required to type an isolate. Our results show that this scheme yields a high resolution. The resolution will probably be even higher if more loci were used like in Koeck's MLVA scheme. However, this will depend on the questions that need to be answered. For our studies MLVA should be utilized to determine the genetic background of isolates and relate this to changes in other properties such as capsular composition. In that case MLVA based on 8 loci seems to suffice. Of note, both MLVA schemes showed a high congruence with MLST (32, 35).

Although the test collection included only carriage isolates, we do not consider it a limitation to the validation study for the following reasons: (i) it is a very diverse collection that includes epidemiologically unrelated, as well as closely-related isolates; (ii) it is well known that there are substantial overlaps among isolates being carried and causing disease at a given time and geographic location although the relative frequency in each group may vary significantly (34); (iii) this MLVA scheme has in the mean time been successfully applied to a collection of over 1000 invasive disease isolates from all over the Netherlands

(Elberse et al. companion manuscript, PLoS One, this issue). In addition, in a manuscript in preparation we will use the MLVA to assess temporal changes and vaccine related changes in the genetic background of the pneumococcus.

We found that the MLVA scheme described here met all the performance criteria needed for a good typing method (45): it was stable, typed 100% of the isolates, was reproducible and had a high discriminatory power. Of interest, the congruence between MLVA and MLST was very good suggesting it can be routinely used as an alternative technique to MLST. Furthermore, MLVA was able to discriminate between putative capsular variants sharing the same MLST in a number of occasions suggesting an enhanced epidemiological usefulness. To make this MLVA scheme easily available to the typing community, an electronic pneumococcal MLVA database located at www.MLVA.net has been created. This application contains the detailed protocol and materials needed for implementation of the MLVA scheme, contact information to obtain control samples, an application for MLVA assignment, and a database for international deposition of MLVA types.

In summary, the MLVA scheme for pneumococci proposed in this study has the convenience and performance criteria needed for a good typing method. It has the advantages of being portable as MLST but is faster and has lower costs. The high congruence between MLVA and MLST suggests it can be applied as a general methodology and, if needed, MLST may be performed on selected isolates from clusters sharing related MT. In the era of pneumococcal conjugate vaccines, close surveillance of pneumococcal populations is of crucial importance; this MLVA scheme represents a high-throughput typing technique which combines easiness, speed, low cost, and portability and therefore can be a rather useful tool to achieve such goal.

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Supplementary tables

Table S1. MLVA, MLST and PFGE results for all isolates included in this study (n=263).

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
PT5574	01	261	306	42	2	3	2	4	3	1	7	4	12	8	13	5	16	4	20
PT5037	01	261	306	42	2	3	2	4	3	1	7	4	12	8	13	5	16	4	20
PT5582	01	261	306	42	2	3	2	4	3	1	7	4	12	8	13	5	16	4	20
PT5279	01	256	228	50	2	3	2	4	2	1	7	8	12	8	1	5	17	4	20
PT560	01	210	304	13	2	2	9	4	1	1	4	4	13	8	13	5	17	4	8
PT500	03	339	162	59	3	1	2	2	1	2	99	5	13	11	10	1	6	8	14
WL812	03	423	1230	16	3	3	2	3	2	1	1	3	7	15	2	10	6	1	144
PT4914	03	427	180	16	3	3	2	4	2	1	1	3	7	15	2	10	6	1	22
PT4076	03	427	180	16	3	3	2	4	2	1	1	3	7	15	2	10	6	1	22
WL696	03	251	180	38	2	3	2	3	2	1	1	3	7	15	2	10	6	1	22
WL1135	03	251	1230	63	2	3	2	3	2	1	1	3	7	15	2	10	6	1	144
PT1263	03	340	232	59	3	1	2	2	2	2	99	5	13	9	15	14	10	16	1
PT2537	04	656	1221	32	5	2	16	4	2	2	2	1	7	5	1	5	15	12	14
PT10	06A	382	1876	14	3	2	5	4	2	2	7	2	7	25	4	12	15	20	28
PT457	06A	382	1876	15	3	2	5	4	2	2	7	2	7	25	4	12	15	20	28
PT1696	06A	379	1152	24	3	2	4	4	99	2	7	2	2	13	2	4	9	157	1
PT5659	06A	642	1098	51	5	2	7	4	3	2	7	2	7	25	4	1	15	3	28
PT1583	06A	578	681	13	4	3	12	5	2	1	1	5	2	5	9	1	6	19	14
PT2527	06A	125	1880	26	1	3	7	3	3	1	4	3	5	7	4	1	10	1	27
WL70	06A	66	65	26	1	2	7	3	3	1	4	3	2	7	4	10	10	1	27
PT5401	06A	125	460	26	1	3	7	3	3	1	4	3	5	7	4	10	10	1	27
PT3831	06A	430	1150	5	3	3	4	2	3	2	3	6	7	25	8	6	25	6	8
PT1304	06A	430	1150	5	3	3	4	2	3	2	3	6	7	25	8	6	25	6	8
PT2628	06A	363	176	7	3	2	2	3	1	2	6	6	7	13	8	6	10	6	14
PT1699	06A	412	1878	103	3	3	1	1	2	1	5	1	7	8	1	1	15	178	14
PT2399	06A	412	1878	55	3	3	1	1	2	1	5	1	7	8	1	1	15	178	14
WL613	06A	751	327	81	6	3	12	99	2	1	1	1	1	5	7	12	10	1	14
PT320	06A	689	327	81	5	3	12	99	2	1	1	1	1	5	7	12	10	1	14
PT3651	06A	689	327	81	5	3	12	99	2	1	1	1	1	5	7	12	10	1	14
WL1264	06A	752	1714	83	6	3	12	99	2	1	1	6	1	5	7	12	17	148	14
PT1175	06A	774	273	36	7	2	6	3	1	2	5	2	5	6	1	2	6	1	14
PT4937	06A	388	1879	23	3	2	6	4	2	2	7	4	7	25	54	4	117	20	28
WL215	06A	727	2191	20	6	2	10	6	1	2	1	2	2	2	100	1	15	20	8
PT2407	06A	388	1879	23	3	2	6	4	2	2	7	4	7	25	54	4	117	20	28
PT2703	06B	455	469	17	3	3	11	3	1	2	9	2	7	13	27	6	10	6	14

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
PT2743	06B	453	469	17	3	3	10	3	1	2	9	2	7	13	27	6	10	6	14
PT289	06B	521	1078	6	4	2	2	3	1	2	6	6	7	8	8	6	10	6	14
PT4058	06B	521	176	7	4	2	2	3	1	2	6	6	7	13	8	6	10	6	14
PT3572	06B	521	176	7	4	2	2	3	1	2	6	6	7	13	8	6	10	6	14
PT3487	06B	363	176	7	3	2	2	3	1	2	6	6	7	13	8	6	10	6	14
PT211	06B	354	887	103	3	1	8	1	2	1	5	1	20	28	1	1	76	14	14
PT1605	06B	354	887	55	3	1	8	1	2	1	5	1	20	28	1	1	76	14	14
PT3919	06B	352	887	55	3	1	7	1	2	1	5	1	20	28	1	1	76	14	14
PT5054	06B	354	887	55	3	1	8	1	2	1	5	1	20	28	1	1	76	14	14
PT76	06B	776	273	12	7	2	6	3	3	2	1	2	5	6	1	2	6	1	14
DCC2613	06B	776	90	13	7	2	6	3	3	2	1	2	5	6	1	2	6	3	4
PT1430	06B	776	273	36	7	2	6	3	3	2	1	2	5	6	1	2	6	1	14
PT2236	06B	766	90	36	7	2	2	3	3	2	1	2	5	6	1	2	6	3	4
PT3104	06B	814	90	36	8	2	2	3	3	2	1	2	5	6	1	2	6	3	4
PT3536	06B	776	273	36	7	2	6	3	3	2	1	2	5	6	1	2	6	1	14
DCC2623	06B	821	90	36	9	2	6	3	3	2	1	2	5	6	1	2	6	3	4
PT582	06B	776	273	36	7	2	6	3	3	2	1	2	5	6	1	2	6	1	14
DCC2160	06B	401	896	34	3	2	9	3	2	2	1	11	7	62	1	2	36	118	14
PT51b	06B	777	885	8	7	2	6	3	3	2	1	4	1	6	1	2	75	1	14
PTRL11007	06B	401	896	34	3	2	9	3	2	2	1	11	7	62	1	2	36	118	14
PT4036	06C	382	1876	14	3	2	5	4	2	2	7	2	7	25	4	12	15	20	28
PT1769	06C	382	1876	51	3	2	5	4	2	2	7	2	7	25	4	12	15	20	28
PT2100	06C	382	1876	51	3	2	5	4	2	2	7	2	7	25	4	12	15	20	28
PT6257	06C	635	2185	35	5	2	5	1	2	2	4	6	1	10	9	43	5	1	6
PT2030	06C	125	460	45	1	3	7	3	3	1	4	3	5	7	4	10	10	1	27
PT2297	06C	125	460	46	1	3	7	3	3	1	4	3	5	7	4	10	10	1	27
PT5442	06C	454	3673	17	3	3	10	3	1	2	9	3	1	13	27	6	10	6	14
PT6119	06C	454	3673	19	3	3	10	3	1	2	9	3	1	13	27	6	10	6	14
PT4913	06C	738	2689	5	6	3	4	2	3	1	3	6	7	25	8	6	25	28	8
PT4542	06C	430	1150	5	3	3	4	2	3	2	3	6	7	25	8	6	25	6	8
PT4291	06C	430	3672	5	3	3	4	2	3	2	3	6	1	25	8	6	25	6	8
PT3371	06C	430	1150	5	3	3	4	2	3	2	3	6	7	25	8	6	25	6	8
DCC1100	06C	430	1150	5	3	3	4	2	3	2	3	6	7	25	8	6	25	6	8
PT449	06C	430	1150	5	3	3	4	2	3	2	3	6	7	25	8	6	25	6	8
DCC851	06C	431	1150	52	3	3	4	2	99	2	3	6	7	25	8	6	25	6	8

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
PT2642	06C	625	176	6	5	2	2	3	1	2	6	6	7	13	8	6	10	6	14
PT5785	06C	752	1714	42	6	3	12	99	2	1	1	6	1	5	7	12	17	148	14
PT3016	06C	753	395	48	6	3	13	99	2	1	1	6	1	5	7	12	17	1	14
PT2228	06C	735	3218	50	6	3	3	8	2	1	1	6	1	5	7	12	10	1	8
PT2317	06C	690	395	82	5	3	12	99	2	1	1	2	1	5	7	12	17	1	14
DCC1353	06C	693	395	83	5	3	12	99	6	1	2	1	1	5	7	12	17	1	14
DCC1016	06C	691	395	83	5	3	12	99	2	1	1	6	1	5	7	12	17	1	14
PT5019	06C	691	395	84	5	3	12	99	2	1	1	6	1	5	7	12	17	1	14
PT6298	06C	654	1692	84	5	2	12	99	2	1	1	6	1	5	7	12	17	158	14
PT4482	06C	694	395	84	5	3	13	99	2	1	1	6	1	5	7	12	17	1	14
DCC2420	06C	694	395	84	5	3	13	99	2	1	1	6	1	5	7	12	17	1	14
DCC333	06C	696	3711	84	5	3	13	99	2	99	1	6	1	5	7	12	17	1	307
PT4252	06C	695	395	84	5	3	13	99	2	1	1	7	1	5	7	12	17	1	14
PT6262	06C	691	395	85	5	3	12	99	2	1	1	6	1	5	7	12	17	1	14
PT5315	06C	160	3396	59	2	1	8	1	2	1	2	4	32	28	1	1	15	16	14
PT6288	06C	160	3396	60	2	1	8	1	2	1	2	4	32	28	1	1	15	16	14
DCC2182	06C	203	2789	44	2	2	8	2	1	2	2	4	7	60	8	5	6	19	14
DCC2584	06C	728	3671	52	6	2	10	10	2	2	7	1	7	25	4	12	6	1	28
PT3354	07F	468	191	43	3	4	2	1	0	1	1	2	8	9	2	1	6	1	17
WL184	07F	468	191	43	3	4	2	1	0	1	1	2	8	9	2	1	6	1	17
PT5303	07F	468	191	54	3	4	2	1	0	1	1	2	8	9	2	1	6	1	17
PT4216	07F	95	1766	29	1	2	11	3	1	1	2	2	1	5	29	1	46	14	18
PT4217	08	18	53	13	1	2	1	4	1	1	2	1	2	5	1	11	16	3	14
PT2533	09V	817	162	28	8	3	4	1	2	1	3	2	7	11	10	1	6	8	14
PT494	09V	277	162	28	2	3	4	1	2	1	3	3	7	11	10	1	6	8	14
PT4140	09V	277	162	28	2	3	4	1	2	1	3	3	7	11	10	1	6	8	14
PT4737	10A	644	1282	22	5	2	9	5	1	2	5	2	7	7	4	2	10	1	27
WL1395	10A	649	97	22	5	2	10	5	1	2	6	6	5	7	4	2	10	1	27
PT450	10A	648	97	22	5	2	10	5	1	2	5	6	5	7	4	2	10	1	27
PT1345	10A	650	97	22	5	2	11	5	1	2	5	6	5	7	4	2	10	1	27
PT3485	11A	11	408	13	1	2	1	1	1	1	2	1	2	5	6	12	16	3	14
WL1344	11A	11	408	13	1	2	1	1	1	1	2	1	2	5	6	12	16	3	14
PT510	11A	30	62	57	1	2	1	6	1	1	2	1	2	5	29	12	16	3	14
PT476	11A	30	62	57	1	2	1	6	1	1	2	1	2	5	29	12	16	3	14
WL555	11A	205	889	12	2	2	8	6	1	2	1	6	8	10	2	16	1	26	107

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
WL1383	11A	205	889	12	2	2	8	6	1	2	1	6	8	10	2	16	1	26	107
WL586	12B	517	218	14	4	2	1	4	2	2	1	4	10	20	14	1	6	1	29
WL737	12F	517	218	14	4	2	1	4	2	2	1	4	10	20	14	1	6	1	29
PT338	14	790	156	28	7	3	4	1	2	1	3	2	7	11	10	1	6	8	1
PT1570	14	817	156	28	8	3	4	1	2	1	3	2	7	11	10	1	6	8	1
PT3291	14	712	143	28	6	2	4	1	2	1	3	4	7	5	10	18	6	8	1
PT3626	14	771	143	29	7	2	4	1	2	1	3	4	7	5	10	18	6	8	1
PT2737	14	771	143	29	7	2	4	1	2	1	3	4	7	5	10	18	6	8	1
DCC2508	14	790	156	33	7	3	4	1	2	1	3	2	7	11	10	1	6	8	1
PT4034	14	374	17	11	3	2	3	2	2	1	10	5	1	5	4	11	9	3	47
PT2667	14	374	17	11	3	2	3	2	2	1	10	5	1	5	4	11	9	3	47
PT3451	14	425	15	8	3	3	2	3	3	1	9	4	1	5	4	5	5	3	8
PT3438	14	425	15	8	3	3	2	3	3	1	9	4	1	5	4	5	5	3	8
PT952	14	367	9	8	3	2	2	3	2	1	2	5	1	5	4	5	5	1	8
PT356	14	367	9	8	3	2	2	3	2	1	2	5	1	5	4	5	5	1	8
PT5244	14	67	411	8	1	2	7	4	2	1	5	3	2	13	14	4	17	4	14
PT5696	15A	111	2105	40	1	3	4	4	2	1	2	4	2	5	36	12	17	21	4
PT1730	15A	678	63	40	5	3	4	4	2	1	2	4	2	5	36	12	17	21	14
WL1327	15A	35	1956	8	1	2	2	4	2	1	5	3	2	19	2	4	17	4	14
PT2111	15B	208	193	9	2	2	9	3	1	1	1	3	8	10	2	16	1	26	1
PT2901	15B	167	172	3	2	2	2	3	1	2	3	6	7	13	8	6	25	6	8
PT4272	15B	67	411	8	1	2	7	4	2	1	5	3	2	13	14	4	17	4	14
PT5245	15B	67	411	8	1	2	7	4	2	1	5	3	2	13	14	4	17	4	14
WL1313	15C	35	1956	8	1	2	2	4	2	1	5	3	2	19	2	4	17	4	14
WL1254	16F	658	30	54	5	3	1	3	2	1	5	6	1	5	27	20	1	1	1
PT4232	16F	660	30	54	5	3	1	3	3	1	5	6	1	5	27	20	1	1	1
PT2585	16F	660	30	54	5	3	1	3	3	1	5	6	1	5	27	20	1	1	1
PT5686	16F	662	30	61	5	3	1	4	3	1	5	6	1	5	27	20	1	1	1
PT735	17F	147	123	32	1	5	8	5	5	1	5	4	7	2	40	1	10	1	45
WL186	17F	128	123	32	1	3	8	5	4	1	5	4	7	2	40	1	10	1	45
PT2605	18A	737	241	11	6	3	4	2	2	1	1	1	25	31	4	16	32	28	44
PT3401	18A	797	241	21	7	3	4	4	2	1	1	1	25	31	4	16	32	28	44
WL1435	18C	188	1877	12	2	2	5	3	1	1	1	8	10	10	41	16	1	26	1
WL1407	18C	544	1016	23	4	2	7	8	3	1	5	6	5	13	11	4	15	1	19
PT4188	18C	49	113	45	1	2	6	2	2	1	5	1	7	2	1	1	10	1	21

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
PT1348	18C	543	1381	19	4	2	6	4	1	2	6	6	10	11	4	16	15	1	145
WL561	19A	197	847	27	2	2	7	2	2	1	9	6	7	11	4	1	6	112	14
PT4972	19A	281	81	14	2	3	5	2	2	1	1	3	4	4	2	4	4	1	1
PT2574	19A	282	81	14	2	3	5	2	2	1	1	99	4	4	2	4	4	1	1
PT1331	19A	678	63	40	5	3	4	4	2	1	2	4	2	5	36	12	17	21	14
PT2542	19A	678	63	40	5	3	4	4	2	1	2	4	2	5	36	12	17	21	14
PT2679	19A	785	230	47	7	3	2	7	1	1	6	3	12	19	2	17	6	22	14
WL677	19A	768	276	68	7	2	2	6	99	1	6	3	2	19	2	17	6	22	14
PT5360	19A	768	276	68	7	2	2	6	99	1	6	3	2	19	2	17	6	222	14
PT2436	19A	768	276	68	7	2	2	6	99	1	6	3	2	19	2	17	6	22	14
PT5687	19A	767	276	9	7	2	2	6	1	1	6	3	2	19	2	17	6	22	14
WL776	19A	761	1026	102	6	99	9	3	2	1	1	1	10	16	32	1	15	28	31
PT5041	19A	190	193	14	2	2	5	6	1	1	1	6	8	10	2	16	1	26	1
PT3188	19A	707	2732	27	5	4	9	99	1	1	1	8	7	60	9	8	10	3	29
PT4288	19A	700	1151	40	5	4	5	99	2	1	1	8	7	60	9	8	6	3	29
PT3171	19A	756	1151	40	6	4	5	99	2	1	1	9	7	60	9	8	6	3	29
PT2808	19A	289	888	20	2	3	8	7	1	1	1	1	8	74	19	15	6	40	26
PT5094	19A	74	416	43	1	2	7	5	2	2	5	5	1	13	14	4	17	51	14
PT4253	19A	83	416	9	1	2	8	5	2	2	5	5	1	13	14	4	17	51	14
PT2757	19A	69	416	9	1	2	7	4	2	2	5	5	1	13	14	4	17	51	14
PT2752	19A	69	416	9	1	2	7	4	2	2	5	5	1	13	14	4	17	51	14
PT14	19A	69	416	9	1	2	7	4	2	2	5	5	1	13	14	4	17	51	14
PT518	19A	178	994	24	2	2	3	5	2	1	10	1	5	5	62	5	6	11	14
WL290	19A	178	994	24	2	2	3	5	2	1	10	1	5	5	62	5	6	11	14
PT1527	19A	743	1801	24	6	3	5	3	2	1	9	2	2	5	4	10	1	1	1
PT3113	19A	743	1801	19	6	3	5	3	2	1	9	2	2	5	4	10	1	1	1
PT516	19F	678	1149	40	5	3	4	4	2	1	2	4	2	5	36	12	1	21	14
PT464	19F	678	1149	40	5	3	4	4	2	1	2	4	2	5	36	12	1	21	14
PT4815	19F	785	230	48	7	3	2	7	1	1	6	3	12	19	2	17	6	22	14
PT584	19F	228	1228	12	2	2	9	5	1	1	1	6	7	10	2	16	1	26	1
WL101	19F	421	391	14	3	3	2	2	2	2	7	1	7	14	4	12	1	20	14
PT332	19F	440	177	18	3	3	6	99	2	2	7	1	7	14	4	12	1	1	14
PT4099	19F	456	179	30	3	3	11	99	2	2	7	1	7	14	40	12	1	1	14
PT4574	19F	295	179	30	2	3	10	99	2	2	7	1	7	14	40	12	1	1	14
PT1282	19F	297	179	32	2	3	11	99	2	2	7	1	7	14	40	12	1	1	14

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
DCC98	19F	275	177	36	2	3	3	99	2	1	7	1	7	14	4	12	1	1	14
PT1809	19F	852	1283	66	99	3	4	2	2	1	5	10	15	16	96	5	6	1	26
WL845	19F	854	236	67	99	3	8	3	1	1	2	1	15	16	19	15	6	20	26
DCC2659	19F	640	87	58	5	2	6	5	3	1	11	2	5	5	7	7	8	5	4
PT4313	19F	636	89	58	5	2	5	5	1	1	10	6	5	5	7	7	8	5	1
PT3815	19F	636	89	58	5	2	5	5	1	1	10	6	5	5	7	7	8	5	1
PT712	19F	359	1487	16	3	2	1	3	2	1	9	2	1	5	4	12	49	3	8
WL1375	19F	556	309	13	4	2	19	3	2	1	10	4	8	10	2	5	9	48	6
PT5137	20	784	230	70	7	3	2	6	99	1	6	3	12	19	2	17	6	22	14
PT505	21	228	1877	12	2	2	9	5	1	1	1	6	10	10	41	16	1	26	1
PT5471	21	230	1877	12	2	2	9	8	1	1	1	7	10	10	41	16	1	26	1
PT4071	22F	302	433	41	2	4	1	1	2	1	1	4	1	1	4	1	18	58	17
WL183	23A	835	42	28	99	2	5	5	2	1	9	3	1	8	9	9	6	4	6
PT5298	23A	836	42	28	99	2	5	5	2	1	9	6	1	8	9	9	6	4	6
PT829	23B	800	439	62	7	3	5	2	2	2	4	2	1	8	9	2	6	4	6
PT380	23B	819	439	9	8	3	5	2	2	2	5	2	1	8	9	2	6	4	6
PT4450	23F	279	81	14	2	3	4	2	2	1	1	99	4	4	2	4	4	1	1
PT1283	23F	279	81	14	2	3	4	2	2	1	1	99	4	4	2	4	4	1	1
PT1309	23F	765	338	26	7	2	2	2	3	2	6	4	7	13	8	6	1	6	8
DCC1587	23F	622	338	3	5	2	2	2	3	2	5	4	7	13	8	6	1	6	8
PT2844	23F	641	338	4	5	2	7	2	3	2	1	6	7	13	8	6	1	6	8
PT5527	23F	710	338	94	6	2	2	2	99	2	5	4	7	13	8	6	1	6	8
PT390	23F	234	242	18	2	2	11	4	4	2	5	4	15	29	4	21	30	1	14
PT239	23F	141	3394	46	1	4	5	10	0	2	4	9	1	13	9	2	6	4	6
PT1592	23F	7	33	49	1	1	5	4	0	2	4	2	1	8	1	2	6	4	6
PT766	23F	7	33	49	1	1	5	4	0	2	4	2	1	8	1	2	6	4	6
PT803	23F	7	33	49	1	1	5	4	0	2	4	2	1	8	1	2	6	4	6
PT2942	24F	780	230	47	7	3	2	2	1	1	6	3	12	19	2	17	6	22	14
PT1721	24F	568	72	25	4	3	7	5	0	1	5	4	2	13	2	4	9	4	1
PT460	29	723	1342	31	6	2	8	1	1	1	3	1	2	12	94	1	6	28	14
PT5412	29	769	198	53	7	2	3	3	0	1	1	1	8	13	4	8	6	22	34
PT5572	31	87	1766	10	1	2	9	3	1	1	2	2	1	5	29	1	46	14	18
PT1287	31	10	533	11	1	1	10	5	1	1	2	1	1	5	29	1	46	1	18
PT1274	31	95	1766	29	1	2	11	3	1	1	2	2	1	5	29	1	46	14	18
PT1581	31	94	2002	9	1	2	11	2	1	1	2	2	1	5	135	1	46	14	18

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
WL386	33F	164	1367	13	2	2	2	1	1	2	1	2	2	16	29	18	42	3	18
DCC2253	33F	173	717	37	2	2	2	6	0	2	2	1	5	35	29	1	45	39	18
PT2655	33F	173	717	37	2	2	2	6	0	2	2	1	5	35	29	1	45	39	18
PT2673	33F	173	717	37	2	2	2	6	0	2	2	1	5	35	29	1	45	39	18
PT5660	33F	173	717	37	2	2	2	6	0	2	2	1	5	35	29	1	45	39	18
PT3341	34	739	1046	27	6	3	4	3	0	2	5	4	15	5	2	1	9	1	18
WL1402	34	92	478	32	1	2	10	1	1	1	1	3	45	13	6	12	9	14	1
PT2552	34	534	2001	30	4	2	6	1	1	1	1	1	5	125	6	1	9	14	14
PT1573	34	534	2001	30	4	2	6	1	1	1	1	1	5	125	6	1	9	14	14
PT5504	34	538	2001	65	4	2	6	1	2	1	1	1	5	125	6	1	9	14	14
PT2398	35B	304	1955	41	2	4	1	3	2	1	1	4	1	118	4	1	18	58	17
PT3419	35F	97	446	41	1	2	15	5	2	1	2	2	5	7	4	19	10	40	27
PT1314	35F	97	446	41	1	2	15	5	2	1	2	2	5	7	4	19	10	40	27
PT5282	35F	316	1368	22	2	4	5	3	1	2	9	3	7	5	4	5	42	92	79
PT3491	35F	316	1368	22	2	4	5	3	1	2	9	3	7	5	4	5	42	92	79
PT3501	37	4	447	2	1	1	2	1	2	2	2	2	29	33	19	1	36	22	31
WL397	38	127	393	27	1	3	7	6	99	2	2	4	10	43	41	18	13	49	6
WL235	38	126	393	27	1	3	7	6	3	2	2	4	10	43	41	18	13	49	6
PT4264	39	156	1126	17	2	1	5	4	2	2	1	8	7	5	92	16	6	1	31
WL1215	42	304	1955	41	2	4	1	3	2	1	1	4	1	118	4	1	18	58	17
PT1683	NT	790	156	28	7	3	4	1	2	1	3	2	7	11	10	1	6	8	1
PT5002	NT	520	1156	1	4	2	2	2	2	1	2	1	2	13	2	29	91	19	59
PT4222	NT	520	1156	1	4	2	2	2	2	1	2	1	2	13	2	29	91	19	59
PT4014	NT	520	1153	52	4	2	2	2	2	1	2	1	2	13	2	29	91	19	141
DCC2648	NT	500	941	109	3	99	2	3	99	1	2	6	8	10	15	27	2	28	71
DCC2787	NT	501	941	109	3	99	2	3	99	1	2	7	8	10	15	27	2	28	71
WL992	NT	812	344	111	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
LgSt142	NT	812	344	112	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
DCC2119	NT	812	344	113	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
DCC689	NT	810	344	113	7	6	2	9	99	1	2	2	8	37	9	29	2	12	53
PT780	NT	812	344	113	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
DCC2489	NT	812	344	113	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
DCC2430	NT	810	344	113	7	6	2	9	99	1	2	2	8	37	9	29	2	12	53
PT5466	NT	823	344	113	9	99	2	2	99	1	4	2	8	37	9	29	2	12	53
DCC2367	NT	335	344	113	2	99	2	9	99	1	2	2	8	37	9	29	2	12	53

					MLVA profiles, BOX locus								MLST profiles						
Key	Serotype	MT	ST	PFGE	1	2	3	4	6	11	12	13	aroe	gdh	gki	recP	spi	xpt	ddl
PT3044	NT	812	344	113	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT806	NT	812	344	113	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT1608	NT	812	344	114	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
WL1210	NT	812	344	114	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT191	NT	812	344	115	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT4671	NT	810	344	116	7	6	2	9	99	1	2	2	8	37	9	29	2	12	53
DCC2362	NT	335	344	116	2	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT944	NT	505	344	117	3	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT998	NT	505	344	117	3	99	2	9	99	1	2	2	8	37	9	29	2	12	53
DCC2870	NT	786	897	118	7	3	2	9	99	1	2	2	8	37	36	29	2	12	14
DCC2879	NT	786	897	118	7	3	2	9	99	1	2	2	8	37	36	29	2	12	14
WL1530	NT	812	344	120	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
WL1514	NT	812	344	120	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT5474	NT	812	344	120	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
PT2987	NT	812	344	47	7	99	2	9	99	1	2	2	8	37	9	29	2	12	53
WL1084	NT	498	448	110	3	99	2	3	3	1	2	6	8	5	2	27	2	11	71
PT4812	NT	520	1278	39	4	2	2	2	2	1	2	1	2	6	4	29	91	19	147
PT673	NT	504	1618	74	3	99	2	4	7	1	1	4	2	98	9	65	107	47	14

MT, MLVA type. ST, Sequence Type.

Conclusions and future perspectives

Concluding Remarks

Since 1996, our research group has been monitoring the pneumococcal population that colonizes children attending day care centers in the Lisbon area, Portugal. Our studies have been providing valuable information on the pneumococcal population, the role of DCC as epidemiological units and the importance of the nasopharynx of children as a reservoir for dissemination into the community of epidemic clones frequently associated with disease.

The introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) in 2001 in Portugal led to a massive shift in pneumococcal nasopharyngeal flora in children attending day care centers in the Lisbon area (22, 23). In those studies it was observed that, despite the dramatic decrease of PCV7 serotypes among carriers, the pneumococcal carriage and the antimicrobial resistance rates remained essentially unchanged in the years following the introduction of PCV7 in Portugal.

To explore to which extent this scenario was mimicked in other regions of Portugal we performed the studies described in Chapters III and IV in two other regions: Coimbra, a city in the central region, and Montemor-o-Novo, a rural area in the south.

In the study conducted in Coimbra, described in Chapter III, nasopharyngeal swabs were taken from children attending day care centers. Around 65% of the children were age-appropriately vaccinated with PCV7 and similar rates of pneumococcal colonization (c.a. 60%), antimicrobial resistance patterns, and genotypes were found when compared to previous studies conducted in the Lisbon area.

In the study, described in Chapter IV, we compared the patterns of pneumococcal colonization among young children attending day care centers in

the rural area of Montemor-o-Novo with those of young children attending day-care in the urban area of Lisbon. In this study around 70% of the children, in both areas, were age-appropriately vaccinated. Although similar rates of colonization were found in the two areas (c.a. 60%), the rates of antimicrobial consumption and pneumococcal antimicrobial resistance were significantly higher in the urban area.

A multivariable logistic regression helped us to identify the factors which contributed for these differences between the two areas and we found that antibiotic consumption in the month preceding sampling, attending day care in the urban area, and being colonized with serotype 19A and non-typeable strains increased the probability of being colonized with pneumococcal antimicrobial resistant strains.

With these studies we were able to conclude that: (i) although the pneumococcal vaccines are not introduced in the Nacional Vaccination Plan of Portugal, at least in these three regions, at the time of these studies, the use of PCV7 was high and comparable, (ii) the rates of pneumococcal colonization were similar in these regions, and (iii) antibiotic consumption remains the main cause for maintenance of antimicrobial resistance among circulating pneumococci.

Pneumococcal colonization is required for the occurrence of pneumococcal disease and also for transmission. For this reason it is essential to study colonization in the two age groups where the rates of pneumococcal infection are higher: children under six years old and adults with more than 60 years of age. To gain insights of pneumococcal carriage patterns in adults living in Portugal, a study was started in 2010 and the first results are described in Chapter V. Low rates of colonization (2.2%) were found and these are in agreement with those reported in other countries (1, 10, 16, 20). Taking into

account the low number of pneumococcal carriers, we were not able to reach robust conclusions in this study regarding factors associated with carriage in the elderly. However, smoking increased in seven fold the risk of being colonized by pneumococci and living in a rural area seems to increase the risk of pneumococcal colonization.

In 2007, Park and co-authors described a new pneumococcal serotype, 6C (17), that was previously indistinguishable from serotype 6A by the Quellung reaction. More recently, other serotypes have been discovered: 6D (3), 11E (previously indistinguishable from serotype 11A) (4), and serogroup 20 was divided in 20A and 20B (5). All these discoveries and the exact determination of the serotypes are particularly important, in the era of conjugate pneumococcal vaccines, to better understand vaccine impact and control for the possibility of cross-reaction with non-vaccine types. Serotype 6C aroused a special interest since PCV7 targets serotype 6B, and it is presumed to induce cross-protection against serotype 6A, but not against 6C (17).

Additionally at the time of the study described in Chapter II, few studies about the epidemiology of this serotype had been published. Nevertheless, one study from South Africa had reported a higher propensity of this serotype to cause meningitis, in HIV^+ adults, than serotype 6A or 6B strains (9). A study from CDC in the USA reported a significant increased of the rate of invasive disease caused by serotype 6C between 1999 and 2006 (7). Another study from Ohio also reported increase of serotype 6C after 2001 (14).

In the retrospective study described in Chapter II, a collection of strains of serogroup 6, recovered from young healthy children, between 1996 and 2007 were serotyped by PCR using the specific primers for serotype 6C described by Park *et al.* (17). Additionally, the serotype 6C strains were characterized by PFGE and MLST. It was observed that this serotype had been circulating in

Portugal at least since 1996. This serotype was genetically diverse, and often antibiotic resistant.

Several studies about serotype 6C have been published in more recent years, from different geographic areas. Some have documented an increase of this serotype in carriage and in disease after PCV7 vaccination (11, 13, 21, 25). In other studies, conducted before the introduction of PCV7, as in Brazil and Israel, serotype 6C has also been found in circulation in colonization and disease (6, 18). PCV13 can potentially impact on this serotype, since this vaccine includes serotype 6A and may provide some cross-protection against serotype 6C (8).

With the introduction of pneumococcal vaccines, surveillance of pneumococcal disease and carriage remains of extreme importance since changes are expected to occur in the pneumococcal population. To monitor these alterations reliable genotyping methods are required for large-scale applications. In Chapter VI a new scheme of multiple-locus variable number tandem repeat analysis (MLVA) for *Streptococcus pneumoniae* is proposed. This technique was compared with the two gold standard methodologies for pneumococcal typing, MLST and PFGE. Since the MLVA methodology proposed is a high-throughput typing technique that can be easily done, in short time, and with low cost (as long as there is access to a sequencer), and has the advantages of portability and access to a web-based database (as MLST), MLVA can be a rather useful tool to achieve such goal.

Overall, the studies performed in this thesis have contributed to a better knowledge on pneumococcal colonization in Portugal in two age groups, children and the elderly. In summary the main conclusions were:

i) the recently described serotype 6C is frequently carried by healthy young children in Portugal, is genetically diverse, and has been circulating in our country at least since 1996;

ii) the patterns of pneumococcal colonization among healthy children living in Coimbra are similar to those living in the Lisbon area;

iii) antibiotic consumption remains a main cause for the maintenance of antimicrobial resistance, in the era of widespread use of pneumococcal conjugate vaccines;

iv) the rates of pneumococcal colonization in the elderly are low and the serotype and genotype diversity are high;

v) MLVA is a promising genotyping method to characterize pneumococci.

Future perspectives

Further studies would be important in the context of the work presented in this thesis and should involve:

I) characterization of serotype 6C after 2007, mainly observing the possible cross-protective effect of PCV13 in this serotype;

II) continuous surveillance on the impact of novel pneumococcal conjugate vaccines (PCV10 and PCV13), not only in pneumococcal serotypes but also in the levels of antimicrobial resistance among pneumococcal colonizing healthy children;

III) continuous surveillance studies on colonization in the elderly in order to increase the data that could help us to take more precise conclusions, mainly in risk factors associated with pneumococcal carriage, the diversity of serotypes and genotypes. In this latter study the use of real time PCR would be a good tool in order to estimate the real rates of pneumococcal colonization;

IV) widespread use of MLVA by other laboratories in other countries in order to use this tool linked to its online database to rapidly, and cost-effectively characterize pneumococci by being able to easily make inter-laboratory comparison.

Additionally, it would be important to design a good follow-up study that could closely measure the variations of the nasopharyngeal flora over time. It would also be interesting to understand the relationships of the entire microbiome in the nasopharynx and verity if “virus colonization” exists as it has been proposed by some authors (19, 24, 26, 27). This notion could be important to complement the idea of a recent paper published by Launes *et al.* (15), which mentions that viruses may facilitate the invasiveness of less invasive pneumococcal serotypes.

Other important aspect, which was mentioned in the introduction, are the rates of pneumococcal vaccine coverage and its impact in on pneumococcal epidemiology. In Portugal pneumococcal vaccines are not part of the National Vaccination plan but in 2010, about 70% of our population in pre-school age was age-appropriately vaccinated. Taking into account the serious economic situation of the country, probably the vaccination rate will decrease and this might change the epidemiology of pneumococci in Portugal. I wonder what will happen. Can the PCV7 serotypes become more prevalent again? Which consequences might this have on the levels of antimicrobial resistance? And in disease, what will happen? The latest reports on IPD in Portugal indicate that serotypes 1, 7F and 19A are the most prevalent in IPD in our country (2, 12). Considering that these serotypes are included in PCV13, which is currently on the market, a decrease in the vaccination uptake might counteract the decreasing tendency in the proportion of IPD in Portugal.

Finally, the studies described are an added value to our knowledge on pneumococcal epidemiology in the nasopharynx, in the era of pneumococcal conjugate vaccines. We described for the first time the pneumococcal colonization patterns in adults aged over 60 years, in Portugal. This can constitute a baseline to monitor the changes that might occur with the introduction of PCV13 for adults. Moreover, the study of serotype 6C was one of

the first to be published after its discovery, reporting important information regarding this serotype. As a final point, in this thesis we validated the application of a promising genotyping method to pneumococci, which, due to its characteristics, might be a good candidate as a gold-standard genotyping method.

From my point of view, epidemiological surveillance studies in carriage as well as in disease will continue to be helpful to show more pieces of the enigmatic pneumococcus that “everyday” lifts the veil and lets us learn a little more about itself.

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