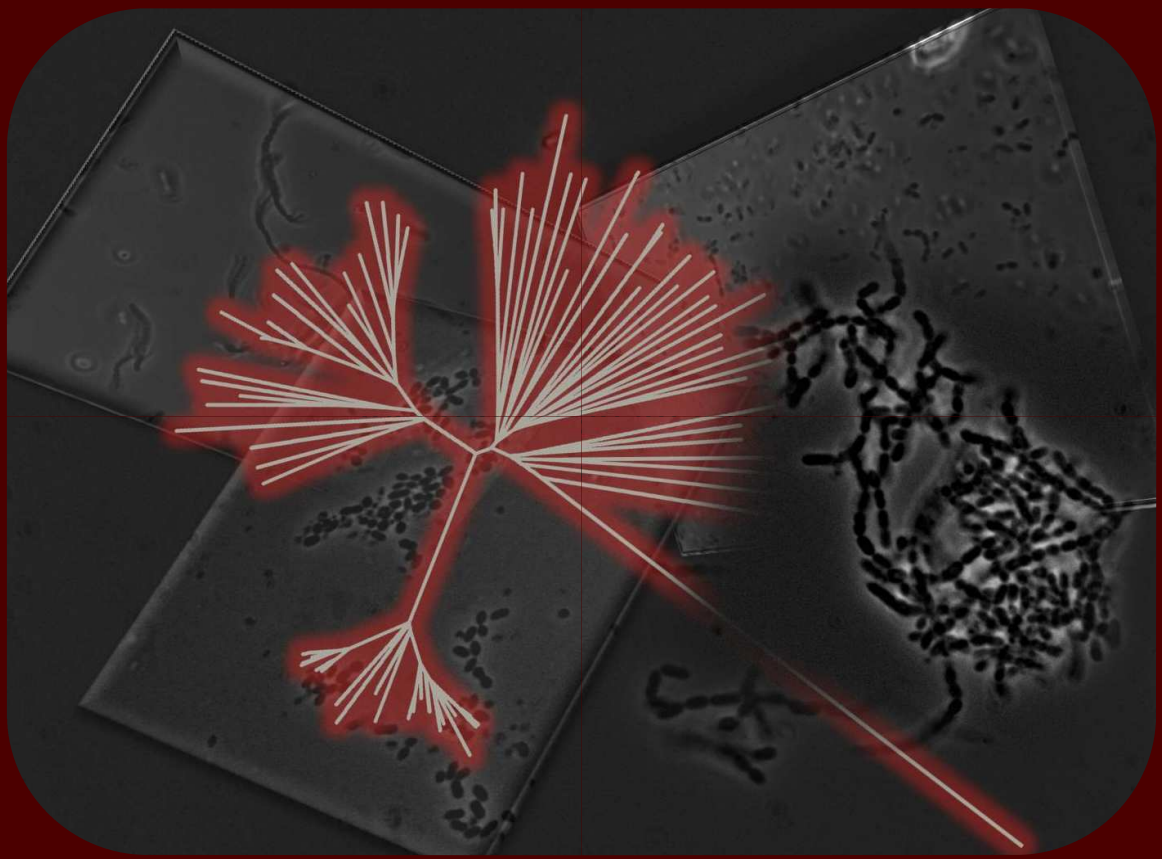
Insights into the epidemiology of drug-resistant *Streptococcus pneumoniae* and closely-related streptococci in the era of conjugate vaccines

Alexandra Sofia Simões



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

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

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ABSTRACT

In Portugal, the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) has led to significant changes in the population structure of *Streptococcus pneumoniae*. However, the levels of antimicrobial resistance have not decreased and have been a matter of concern.

In this thesis, two studies were performed aiming to understand the reasons for maintenance of antimicrobial resistance.

The first one resulted from an unexpected observation. While performing surveillance studies aimed to describe the impact of PCV7 on colonization in children attending day care centers (DCC), an increase from 0.7% to 5% in the prevalence of high level penicillin resistance (MIC 2-6 µg/ml) among presumptively pneumococci was observed. Fifteen of the twenty two penicillin-resistant isolates were optochin-resistant, but were bile soluble and thus considered to be pneumococci. In order to clarify the nature of these isolates, a combination of phenotypic and genotypic approaches was performed which includes multilocus sequence analysis (MLSA) and comparative genomic hybridization (CGH). The results obtained led to the classification of these isolates as “streptococci of the mitis group” (SMG) that however were distinct from typical *S. pneumoniae* or *Streptococcus mitis*. CGH further indicated that several pneumococcal virulence genes, and around 70% of the pneumococcal core genome, were present in these atypical isolates. These results highlighted the importance of correct identification of atypical isolates. After the correct identification of these isolates, it

became clear that high level penicillin resistance had remained stable among colonizing pneumococci.

In the second study, it was found that, despite the extensive pneumococcal serotype replacement, observed among children in DCC due to PCV7 introduction, no significant changes were observed in the rates of non-susceptibility to penicillin resistance, to macrolides, or multidrug resistance in general. In order to investigate the mechanisms leading to the maintenance of antimicrobial resistance rates, we compared through molecular typing antibiotic resistant pneumococci recovered from young carriers in 2006 and 2007 (era of high-PCV7 uptake) with collections of isolates from 2002-2003 (low-PCV7 uptake era) and 1996-2001 (pre-PCV7 era). We observed that the group of clones that accounted for antimicrobial resistance since 1996 was essentially the same as the one identified in the PCV7 era. However, the relative proportions of such clones had evolved substantially overtime. The expansion of clones expressing non-PCV7 capsular variants of the original strains was the major mechanism leading to the maintenance of antimicrobial resistance rates. Emergence of novel clones and *de novo* acquisition of resistance contributed little to the observed scenario. No evidence of capsular switch events occurring after PCV7 introduction was found.

In a third study we focused on non-typeable pneumococci (NTPn), atypical *S. pneumoniae* isolates that do not react with any available capsular serum and that appear to lack a capsule, a major virulence factor routinely used to identify and type pneumococci. NTPn are difficult to identify as their differentiation from closely related species, such as, *Streptococcus pseudopneumoniae* and SMG is not always straightforward. We aimed to develop a method to easily identify

these isolates and estimate their true prevalence in colonization samples.

We developed a low cost and easy assay to detect and quantify NTPn in primary samples obtained from nasopharyngeal swabs. The strategy was based on a multiplex PCR targeting *lytA* (a virulence factor ubiquitous in pneumococci that is often used as an identification marker of this species), *cpsA* (a conserved pneumococcal capsular gene), *aliB*-like ORF2 (a gene described as present in the capsular region of non-typeable pneumococci) and *16S rDNA* (used as a positive internal control) genes, plus a restriction fragment length polymorphism (RFLP) assay to differentiate typical from atypical *lytA.* The application of this new methodology found that the prevalence of NTPn in colonization was three-fold higher than estimated by routine methods.

Altogether, these three studies have improved our knowledge on the population structure of antimicrobial resistant pneumococci in the era of PCV7, and highlighted the importance of correct identification of atypical strains.

RESUMO

Em Portugal, a introdução da vacina pneumocócica conjugada sete valente (PCV7) levou a mudanças significativas na estrutura populacional de *Streptococcus pneumoniae*. Contudo, os níveis de resistência aos antimicrobianos em isolados de colonização não diminuíram o que é motivo de grande preocupação a nível nacional.

Realizámos dois estudos com o objectivo de perceber quais as razões que levaram à manutenção dos níveis elevados de resistência aos antimicrobianos.

O primeiro estudo resultou de uma observação inesperada. Durante um estudo de vigilância epidemiológica em que se pretendia descrever o impacto da PCV7 na colonização em crianças que frequentam jardins-de-infância, observou-se um aumento de 0,7% para 5% na prevalência de pneumococos com alta resistência à penicilina (MIC 2-6 µg/ml). Observámos que quinze, dos vinte e dois isolados resistentes à penicilina, eram resistentes à optoquina mas solúveis em sais de bile, sendo assim considerados pneumococos. A fim de esclarecer a natureza destes isolados, foi realizada uma combinação de testes fenotípicos e genotípicos, incluindo “multilocus sequence analysis” (MLSA) e “comparative genomic hybridization” (CGH). Os resultados obtidos levaram à classificação destes isolados como “*Streptococcus* do grupo mitis" (SMG), embora distintos dos *S. pneumoniae* ou *Streptococcus mitis* típicos. A aplicação do método de CGH indicou ainda que vários genes de virulência e 70% do genoma de pneumococos estão presentes nestes isolados atípicos. Estes resultados realçaram a importância da correcta identificação dos isolados atípicos. Após a correcta identificação destes isolados,

verificou-se que a prevalência de alta resistência à penicilina tinha-se mantido estável.

No segundo estudo observou-se que após a introdução da PCV7, ocorreu uma substituição de serótipos em crianças portadoras de pneumococos. Contudo, não foram observadas alterações significativas nas taxas de não-susceptibilidade à penicilina, resistência aos macrólidos ou multirresistência em geral. A fim de investigar os mecanismos que conduziram à manutenção da resistência aos antimicrobianos, foram comparados, pneumococos resistentes aos antimicrobianos isolados de colonização em 2006-2007 (período de alto consumo de PCV7) com colecções de isolados de 2002-2003 (período de baixo consumo de PCV7) e isolados de 1996-2001 (pré-PCV7). A caracterização por tipagem molecular permitiu-nos observar que o grupo de clones que apresentam resistência antimicrobiana, é essencialmente o mesmo na era pré-PCV7 e na era da PCV7. No entanto, as proporções relativas dos respectivos clones, sofreram alterações substanciais ao longo dos anos. A expansão de clones a expressar variantes capsulares (não incluídos na PCV7) das estirpes originais, foi o principal mecanismo que levou à manutenção da resistência. O aparecimento de novos clones resistentes e a aquisição de resistência em clones já existentes pouco contribuiu para o cenário observado. Não se encontraram exemplos de transformação capsular devido à introdução da PCV7.

Num terceiro estudo, focámo-nos nos pneumococos não-tipáveis (PnNT), isolados atípicos de S. *pneumoniae* que não reagem com qualquer um dos soros capsulares disponíveis comercialmente e que parecem não ter cápsula, o principal factor de virulência que é

normalmente usado na identificação e tipagem dos pneumococos. Os PnNT são difíceis de identificar e a diferenciação de espécies estritamente relacionadas, tais como *Streptococcus pseudopneumoniae* e SMG nem sempre é clara.

O nosso objectivo foi desenvolver um método que facilmente identifique estes isolados e calcular a sua prevalência real em amostras de colonização. Neste estudo, desenvolveu-se um método fácil e de baixo custo que permite detectar e quantificar PnNT em amostras primárias obtidas a partir de zaragatoas da nasofaringe. A estratégia foi baseada num PCR para detecção simultânea do *lytA* (um factor de virulência ubíquo em pneumococos e que é frequentemente usado para identificação da espécie), *cpsA* (gene capsular conservado em pneumococos), *aliB*-like ORF2 (gene presente na região capsular nos PnNT) e *16S rDNA* (usado como controlo interno positivo), seguido de um ensaio de RFLP ("Restriction Fragment Length Polymorphisms" - Análise do polimorfismo dos fragmentos de restrição do DNA) para diferenciar *lytA* típicos de *lytA* atípicos. A aplicação desta nova metodologia permitiu determinar uma prevalência de PnNT em colonização três vezes maior do que o estimado pelos métodos de rotina.

Em conjunto, estes três estudos melhoraram o nosso conhecimento sobre a estrutura populacional de pneumococos resistentes a antibióticos na era da PCV7 e alertaram para a necessidade da identificação correcta das estirpes atípicas.

THESIS OUTLINE

The purpose of the work presented in this thesis was to gain insights into the epidemiology of *Streptococcus pneumoniae* and closely-related streptococci in the era of pneumococcal conjugate vaccines.

Chapter I is a general introduction where important aspects of *S. pneumoniae* epidemiology are reviewed in the context of the results described in the thesis. Topics such as epidemiology, identification and typing methods, resistance to antimicrobial agents, and vaccines are discussed. The major recent findings about non-typeable *S. pneumoniae* and pneumococcus-like streptococci are also addressed.

Chapter II describes a detailed analysis of a collection of penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in the era of pneumococcal conjugate vaccines. The analyses include several phenotypic and genotypic assays complemented by multilocus sequence analysis and comparative genomic hybridization.

Chapter III describes the mechanisms that led to the maintenance of antibiotic resistance rates among colonizing pneumococcus in Portugal, besides a significant shift in serotypes following the introduction of the seven-valent pneumococcal conjugate vaccine.

Chapter IV describes a low cost and easy strategy to rapidly identify non-capsulated *S. pneumoniae* in primary culture samples isolated from nasopharyngeal swabs, which may contain multiple species. In addition, the method allows detection of co-colonization and enables

a more accurate estimation of the prevalence of non-capsulated pneumococci.

In **Chapter V**, the results obtained in chapters II to IV are discussed as a whole and future perspectives are presented.

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

Chapter II - Simões, A. S., R. Sá-Leão, M. J. Eleveld, D. A. Tavares, J. A. Carriço, H. J. Bootsma, and P. W. Hermans. 2010. Highly penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in Oeiras, Portugal: genomic characteristics and implications for surveillance. J Clin Microbiol **48:**238-246.

Chapter III - Simões, A. S., L. Pereira, S. Nunes, A. Brito-Avô, H. de Lencastre, and R. Sá-Leão. 2011. Clonal evolution leading to maintenance of antibiotic resistance rates among colonizing pneumococci in the PCV7 era in Portugal. J Clin Microbiol **49:** 2810-2817.

Chapter IV - Simões, A. S., C. Valente, H. de Lencastre, and R. Sá-Leão. 2011. Rapid identification of non-capsulated *Streptococcus pneumoniae* in nasopharyngeal samples allowing detection of co-colonization and re-evaluation of prevalence. Diagn Microbiol Infect Dis. **71:**208–216.

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Chapter I

General Introduction

STREPTOCOCCUS PNEUMONIAE - HISTORICAL BACKGROUND

Streptococcus pneumoniae or pneumococcus was one of the first bacterial pathogens to be isolated and characterized. It was first described in 1881 by Louis Pasteur and Georg Sternberg while working independently, although the first to recognize pneumococci was Edwin Klebs in 1875 when he observed infected sputum and lung tissue (reviewed in (50)). Since then, *S. pneumoniae* has played an important role in the development of microbiology, molecular biology, bacteriology, genetics and vaccines. The use of polysaccharide antigens as vaccines (13), the ability of polysaccharides to induce antibodies (66), the mechanism of bacterial gene transfer (51), the identification of DNA as the genetic material (12), the therapeutic efficacy of penicillin (126), the role of bacterial capsule in resistance to phagocytosis (43), and the first bacterial quorum sensing factor (127), were firstly described in pneumococci.

Following its first description, its name has changed several times. “Microbe septicémique du saliva” and *Micrococcus pasteuri* were the first names given by Pasteur and Sternberg, respectively. In 1883, Mátray used the term “pneumoniekokken” and in 1886 Albert Fraenkel used the word “pneumokokkus”. In the same year, the name *Diplococcus pneumoniae* became officially recognized due to its appearance when observed under the microscope, and, in 1974, it was reclassified as *S. pneumoniae* based on its growth in chains in liquid media (50).

As of today pneumococcus remains a major pathogen. It has been estimated that 14.5 million episodes of serious pneumococcal disease and 826.000 deaths/year occur in children aged 1–59 months (98).

PNEUMOCOCCUS AND CLOSELY RELATED SPECIES

S. pneumoniae is part of the bacterial streptococcus group. This is a heterogeneous group of gram positive, catalase-negative, spherical or ovoid bacteria comprising species that colonize and infect humans and animals (139). Streptococci can be classified according to the hemolytic pattern (partial, complete or no blood hemolysis when grown in blood agar plates); Lancefield group (serological reaction against the major cell-wall carbohydrate antigen); or species (according to their metabolic reaction in various culture media) (139). Figure 1 shows the phylogenetic relationships among 34 species of the genus *Streptococcus*.

S. pneumoniae is part of the mitis group. Streptococci of the mitis group are part of the commensal flora of the upper respiratory tract of humans. This group includes not only *S. pneumoniae* but also *S. pseudopneumoniae*, *S. mitis, S. oralis, S. gordoni, S. sanguis* and *S. parasanguis* (Figure 1) (10, 16).

The closest relatives of *S. pneumoniae* are *S. mitis, S. oralis* and the recently described *S. pseudopneumoniae* (10). *S. oralis* and *S. mitis* are usually considered to be commensals of the human oral cavity (in spite of being able to cause bacterial endocarditis and bacteremia, especially, in immunocompromised patients (17, 38)); *S. pseudopneumoniae* although apparently having low incidence in clinical samples, has been associated with chronic obstructive pulmonary disease (75). *S. pneumoniae* is the most important species causing human disease in this group, since it can cause a wide range of diseases such as otitis media, pneumonia, bacteremia and meningitis (18).

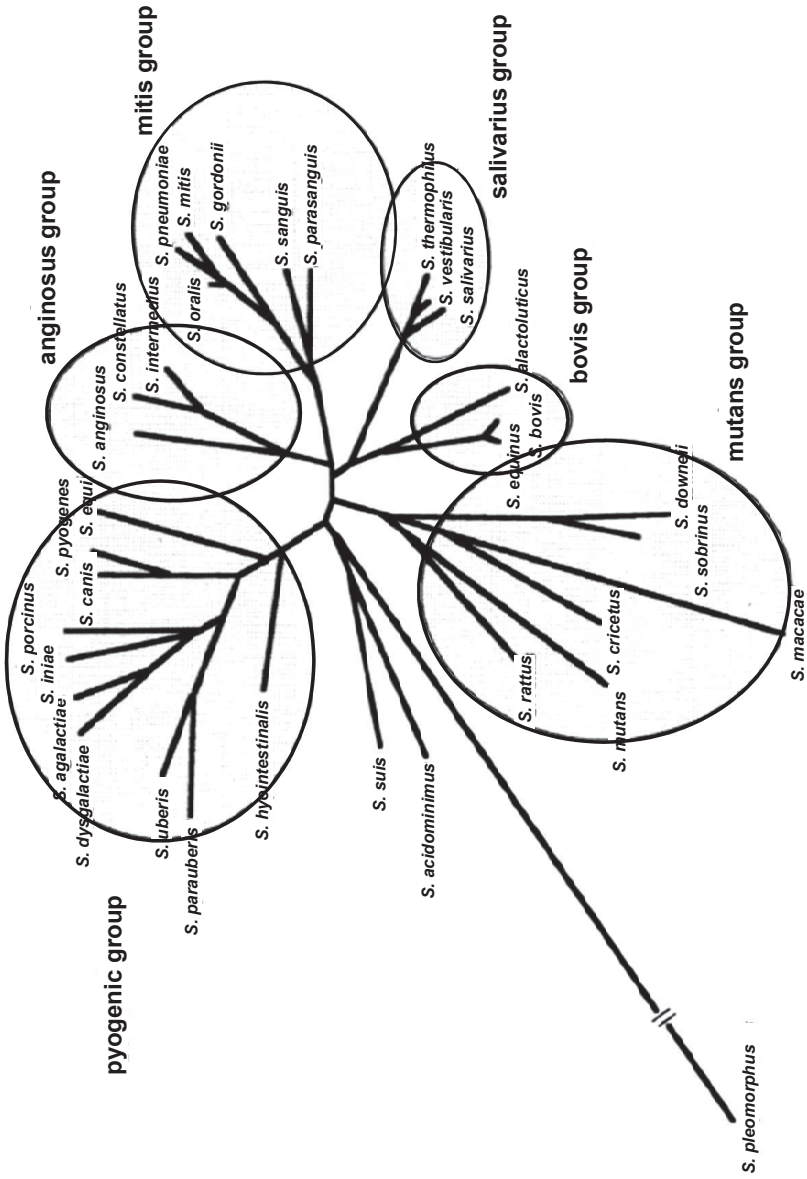

Figure 1. Phylogenetic relationships among 34 *Streptococcus* species (73).

Bacteria are promiscuous (46) and, in the nasopharynx, pneumococci co-habit with several other bacterial species, including its closest relatives: *S. pseudopneumoniae*, *S. mitis*, and *S. oralis*. Genetic

exchange with related species sharing the same ecological niche is one of the mechanisms of *S. pneumoniae* evolution. Indeed, *S. mitis* has been described as the main reservoir of genetic diversity of *S. pneumoniae* (37).

The exchange of genetic elements between species can lead to difficulties in species identification. The mitis group has caused considerable confusion for both clinical microbiologists and taxonomists (42). The correct differentiation between *S. pneumoniae* and closely related species is essential, not only for clinical diagnostics, but also for colonization studies. For example, the incorrect identification of these isolates could falsely increase the rates of pneumococci non-susceptible to antimicrobials (136).

Since the correct identification of pneumococci is one of the major subjects of this thesis, the following sections will describe *S. pneumoniae* and closely related species and the methods used to distinguish them.

S. pneumoniae

S. pneumoniae is a gram-positive bacterium, lancet-shaped, typically less than 2µm in diameter, which can grow in pairs or short chains. It is catalase negative, facultative anaerobic, usually susceptible to optochin, soluble in bile (deoxycholate) salts and shows α-hemolysis in blood agar plates. It is a fastidious organism that requires the presence of blood or serum in the medium for growth, and also the presence of 5% of CO_2 (121). Most of the isolates gave a positive result in the Quellung reaction (based on an immunological reaction between polysaccharide capsular antibodies and specific antisera).

More detailed information about *S. pneumoniae* will be described later in this chapter.

S. pseudopneumoniae

S. pseudopneumoniae, first described in 2004 (10), is a gram-positive bacterium, similar to pneumococcus when grown in agar media, is not soluble in bile, and is resistant to optochin when incubated under an atmosphere of increased CO_2, although it is susceptible to optochin when incubated in ambient atmosphere. It has a negative result in the Quellung reaction (10).

S. pseudopneumoniae has low incidence in clinical samples but it has been associated with chronic obstructive pulmonary disease (75) and it has been demonstrated to have disease potential. In a study by Harf-Monteil *et al*, it was demonstrated that were able to kill 100% of infected animals (immunocompetent 6-week-old Swiss mice) in 36 hours in a peritonitis/sepsis model (60).

In two studies, in New Zealand, that described *S. pseudopneumoniae* isolates recovered from respiratory samples between 2000 and 2007, high rates of resistance to erythromycin (c.a. 60%) and tetracycline (c.a. 70%) have been observed. No resistance to any β-lactam agent has been observed, although about one-third of the isolates showed reduced susceptibility to penicillin and ampicillin (74, 75).

S. mitis* and *S. oralis

S. mitis and *S. oralis* are commensal species of the human oral cavity that belongs to the mitis group of streptococci. They are gram-positive cocci arranged in chains, catalase negative, and require 6.5% of NaCl to grow. They differ from pneumococcus because they are not soluble

in bile, and are resistant to optochin. Similarly to *S. pseudopneumoniae* they give a negative result in the Quellung reaction (reviewed in (35)).

Although commensals, these streptococci are isolated from a variety of infections but most significantly from patients with subacute bacterial endocarditis and from neutropenic patients with cancer (17, 38).

Regarding antimicrobial resistance, several studies have indicated that *S. mitis* and *S. oralis* strains are commonly found in blood cultures of cancer patients and are commonly resistant to β-lactam antimicrobials (reviewed in (42)). In fact, high rates of resistance to penicillin and high MICs (minimum inhibitory concentration) are frequent among *S. mitis* isolates (69).

Pneumococcus-like streptococci

Atypical pneumococcal isolates that are putatively identified as pneumococci but that give negative results in one or more of the classical assays have been described (87, 88, 138). These isolates were considered to be pneumococcus-like streptococci and the denomination “streptococci of the mitis group” (SMG) has been proposed (87).

The origin of these species has been investigated. In 2008, Killian and co-workers studied the evolution of *S. pneumoniae* and its close commensal relatives and proposed that *S. mitis* lineages evolved by genome reduction from a pathogenic population of an ancestral pneumococcus-like bacterium which had all the properties associated with virulence. The genes which nowadays differentiate *S. mitis* from

S. pneumoniae would have been acquired more recently, presumably by individual *S. mitis* lineages (77). In addition, most genes associated with pathogenicity which are shared by *S. pneumoniae* strains, can also be present in *S. mitis*, *S. oralis,* and *S. infantis* (37).

Like pneumococci, most members of the mitis group are naturally competent for genetic transformation, i.e., they are able to take up DNA fragments and incorporate them into their genome (65, 93). Another theory for the origin of these bacteria is that horizontal gene transfer between naturally competent streptococci that share the same ecological niche contributes to the attenuation of putative barriers between these species (10, 55, 56). In fact, the group of Regine Hakenbeck described the genome of *S. mitis* B6 (a high level penicillin and multiple antibiotic resistant isolate) and suggested that the genome of this strain is an example of genome modification by the acquisition of genes and gene clusters from other sources such as *S. pneumoniae*, since both species share a core genome of over 900 genes (43% of total *S. mitis* genome) (34). The same group described an identical mosaic structure of *pbp2x* gene in *S. pneumoniae*, *S. mitis* and *S. oralis*, suggesting inter- and intraspecies recombination events (28).

Still, several attempts to correctly distinguish pneumococci from closely relatives have been made, as detailed in the following section.

PNEUMOCOCCAL IDENTIFICATION METHODS AND DIFFERENTIATION FROM OTHER STREPTOCOCCI

There is not a single gold standard method to identify pneumococci. Routine identification of *S. pneumoniae* is based on the following characteristics: typical colony morphology on blood agar plates

(colonies with a depression in the center showing alpha-hemolysis), susceptibility to optochin when incubated in CO_2 atmosphere, solubility in 1% of sodium deoxycholate, and assignment of a capsular type through serotyping (76). For most pneumococci, which are capsulated, its identification is straightforward. However some isolates give an atypical result in one or more of these assays (80, 87, 138).

Susceptibility to optochin has been described as a method with 90% to 100% of sensitivity and 99% to 100% of specificity (76). However, optochin-resistant pneumococcal isolates have been described (4, 94, 97, 104) and optochin-susceptible non-pneumococcal isolates have been observed (76).

Solubility in 1% of sodium deoxycholate (or bile salts) is considered by some authors the standard method to identify pneumococci since it has a sensibility of 98% and a specificity of 100% (112). However, some strains can harbor a mutation in the *lytA* gene (a deletion of two amino acids responsible for cell wall anchoring in the carboxy-terminal domain) that confers a deoxycholate-insoluble phenotype (100). Additionally, some streptococcus of the mitis group were described as bile soluble (87).

As already mentioned in this thesis, the Quellung reaction (or serotyping) is applied in *S. pneumoniae* to distinguish between more than 90 different capsular types described to date and is based on an immunological reaction between polysaccharide capsular antibodies and specific antisera. However, isolates with non-specific agglutination with any serum have been described (88), as well as,

non-pneumococcal streptococci with positive cross-reactions with antipneumococcal polysaccharide capsular antibodies (67).

In addition, in recent years several molecular methods have been proposed to differentiate pneumococcus from closely related species. For example, amplification of ubiquitous pneumococcal genes such as autolysin (*lytA*), surface antigen A (*psaA*) and pneumolysin (*ply*) have been proposed to identify pneumococci (92).

In 2004, Messmer *et al* compared the specificity of these three genes for the correct identification of *S. pneumoniae* and concluded that genes *lytA* and *psaA* had a specificity of 100% and 99%, respectively (92). However, for the *ply* gene, the specificity was 50%, since it was found in eight of the 16 atypical isolates tested (92). In addition, in 2007, Jefferies *et al* described a homologue of the *ply* gene named mitilysin (*mly*) that could be found in some *S. mitis* isolates (71). Furthermore, homologues of *lytA, psaA* and *ply* have been detected in strains of closely related streptococcal species (70, 95, 111, 138).

More recently, detection of the pneumococcal species specific gene *piaA* (which encodes for a lipoprotein component of two iron ABC transporters) has been proposed as a diagnostic tool (137). However, this gene is not ubiquitous in pneumococci (137).

Some authors also described *sodA* and *16S rDNA* as good targets for identification of *S. pneumoniae* although *sodA* does not distinguish *S. pneumoniae* from *S. pseudopneumoniae* (10, 68, 77) and, on the basis of *16S rDNA* sequence, *S. oralis, S. mitis and S. pneumoniae*, share over 99% of sequence identity (73).

The DNA fragment Spn9802 has also been described as a specific target for *S. pneumoniae* (1, 125) but some authors have described its presence in *S. pseudopneumoniae* and *S. mitis* (40, 101).

A 181-bp *S. pneumoniae*-specific fragment, located between the gene Spn23F20600 (from nucleotide 2,002,250 to nucleotide 2,000,793), and gene Spn23F20590 (from nucleotide 2,000,770 to nucleotide 2,000,489), in *S. pneumoniae* ATCC700669, was described as being able to discriminate *S. pneumoniae* from other streptococci (106).

The sequence of the intergenic spacer (ITS) region and part of *gdh* gene was also proposed but ITS region cannot be used for the species-level identification of the mitis group (only at the group level) and sequence analysis of the partial *gdh* gene is apparently able to differentiate *S. mitis, S. oralis*, and *S. pneumoniae*, but is not able to differentiate *S. mitis* from *S. pseudopneumoniae* (96). In addition, this method cannot be easily applied in a clinical microbiology laboratory that has not sequencing facilities (96).

A *recA* PCR assay was described as useful to differentiate pneumococcus from other viridans streptococci (142) but do not correctly differentiate *S. pneumoniae* from *S. mitis* and *S. pseudopneumoniae* (142).

Analysis of bacterial colonies by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry has been proposed as a rapid, accurate method for identifying bacteria (118). However, it should not be used to identify *S. pneumoniae* since it misidentifies *S. mitis* and *S. oralis*, as *S. pneumoniae* (110, 124).

In addition, multilocus sequence typing (MLST) has been described as an important tool to define pneumococcus (55) and the construction of phylogenetic trees using concatenated sequences of all MLST loci except *ddl* (an approach denominated multilocus sequence analysis (MLSA)), has been proposed as a good alternative molecular technique to differentiate pneumococci from other closely related streptococci (55). However, the average sequence divergence between *S. mitis* and *S. pneumoniae* clusters is 5.8%, only slightly greater than within the *S. mitis* cluster itself (54).

Overall, methods based on *lytA* gene appear to be the most reliable (24, 87). In particular, in 2006, Llull and co-workers described a RFLP method to differentiate pneumococcus from closely related species based on signatures characteristic of typical pneumococcal *lytA* or atypical *lytA* (87). More recently, a *lytA* based asymmetric PCR for the specific detection of pneumococcus was developed and it has been claimed that it has no cross reactions with *S. mitis* or *S. pseudopneumoniae* (24).

A simple and low cost method that can be easily applied in a microbiology laboratory is essential to correctly identify pneumococci and differentiate them from closely related species.

In summary, differentiation between atypical *S. pneumoniae* and closely related species remains a challenge since they share several phenotypic and genotypic characteristics.

EPIDEMIOLOGY OF *S. PNEUMONIAE*

S. pneumoniae is a commensal bacterium from the upper respiratory tract and its ecological niche is the nasopharynx. The colonization is

asymptomatic and it is common in children under five years old. The colonization occurs soon after birth (9) and it peaks around the age of three (18). After that, there is a steady decline until the age of ten and remains low in adulthood (18).

The duration of carriage is variable (typically ranging between one to seventeen months (49)) and depends on several factors such as the host's age and the serotype of the colonizing strain (30). The transmission is either person-to-person by direct contact, or by aerosols (134). Recently, it was described that pneumococci are able to survive long-term desiccation, suggesting that environmental surfaces may also serve as sources of pneumococcal infection (132).

The risk factors associated with pneumococcal carriage include age (children less than two years old, and adults older than 65 years old), frequent contact with children, crowded environments (day care center attendance, military training camps, prisons, nursing homes), prior respiratory infection, cigarette smoking, asthma and other chronic respiratory diseases (82).

The levels of carriage also vary from country to country. For example, in Norway, in a study conducted between 2006 and 2008 aimed to evaluate the impact of pneumococcal conjugate vaccine on colonization, the overall pneumococcal carriage rates in children attending day care were 80% (130). In the Netherlands, in 1999, also in children attending day care, it was around 59% (20) and in Sweden, in 1995, the rates varied between 21 and 50%, depending on the age of the children (29). In Portugal, rates of carriage in children up to six years old attending day care centers were 68% in 2006 (114).

Colonization precedes pneumococcal disease (18). As a pathogen, *S. pneumoniae* can cause a wide range of diseases from otitis media, to pneumonia, bacteremia, and meningitis. Worldwide, the morbidity and mortality associated with pneumococcal infections remain very high (98).

Most *S. pneumoniae* strains are covered by a polysaccharide capsule that constitutes the main virulence factor of this pathogen. The capsule is 200 to 400nm thick (120) and plays a major role in colonization and disease by promoting bacterial interaction with the epithelium and provide protection against complement-mediated opsonophagocytosis (141).

There are more than 90 different capsules described, however, only a restricted number of serotypes are responsible for disease. Differences in virulence between pneumococcal serotypes have been observed and the association serotype disease depends on several factors, such as, the age of the host (different serotypes between children and adults), geographic location, and disease manifestation (62).

As mentioned above, the prevalence of serotypes varies depending on the host's age. Serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were the most common serotypes isolated from invasive disease in children in United States of America before the introduction of the pneumococcal conjugate vaccine (103) and serotypes 1, 3, 5 and 7F were frequently detected in newborns (62). In adults, the serotypes present in the 23 valent polysaccharide vaccine (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F

and 33F) were associated with more than 80% of the pneumococcal infections (19).

Regarding disease manifestation some studies described serogroups 6, 14, 19 and 23 associated with otitis media (11, 78, 131), serotypes 1, 4 as 14 isolated from blood (63) and serotypes 1 and 3 associated with pneumonia (62). In addition, some serotypes appear to have higher invasive disease potential than others. In a Finnish study, serotypes 6B, 14, 18C and 19A were more related with invasive disease and serotypes 6A, 35F and 11A were associated with carriage (57). In Portugal, in a study to estimate the invasive disease potential of serotypes and clones circulating in Portugal before extensive use of the seven-valent pneumococcal conjugate vaccine it was found that serotypes 1, 3, 4, 5, 7F, 8, 9N, 9L, 12B, 14, 18C, and 20 had an enhanced propensity to cause invasive disease, while serotypes 6A, 6B, 11A, 15B/C, 16F, 19F, 23F, 34, 35F, and 37 were associated with carriage (115).

The susceptibility to antimicrobial agents appears to be associated with some serotypes. For instance, penicillin resistance and multiple resistance appears to be restricted to few serotypes such as 6A, 6B, 9V, 14, 19A, 19F and 23F (32). In the same way, strains isolated from the nasopharynx were frequently more resistant to antimicrobial agents than those isolated from infections (62).

PNEUMOCOCCAL RESISTANCE TO ANTIMICROBIAL AGENTS

The history of pneumococcal resistance to antimicrobial agents goes back to 1912 when the first optochin resistant pneumococcus was documented following optochin therapy of experimentally infected mice. Acquired pneumococcal resistance to optochin during therapy

of patients was documented in 1917 (reviewed in (79)). However, the first *S. pneumoniae* clinical strain resistant to an antimicrobial agent (penicillin) was described in 1967 from a 25-year-old woman (reviewed in (79)). Since then, emergence of resistant strains to several classes of antimicrobials such as β-lactam and macrolides, has been observed (79).

Nowadays, the overall rates of antimicrobial resistant isolates vary from country to country being high in several geographical areas. In Europe, the prevalence of penicillin-non susceptible *S. pneumoniae* varies between 1-5% in the Netherlands, Norway, Germany, the United Kingdom, Sweden, and Denmark; and 20% in Ireland, Spain, and France. Resistance levels to macrolides also vary significantly: 4-10% in Sweden, the United Kingdom, Denmark, the Netherlands, Norway and Germany; and 20-30% in Spain, Belgium, France, and Finland (39). In the United States the rates of non-susceptible *S. pneumoniae* are of approximately 10% for penicillin and 25% for macrolides (25).

In Portugal, in 2006 the antimicrobial resistance rates were comparable between carriage and invasive isolates from children up to six years old. Non-susceptibility to penicillin was 22% for carriage and 23% for invasive disease isolates, macrolide resistance was observed in 23% of colonizing isolates and 27% of invasive isolates, and resistance to tetracycline was found for 18% of carriage and 22% of invasive disease isolates (3, 114).

A positive correlation between antimicrobial resistance and antibiotic consumption has been described (48, 109). High rates of resistance are generally observed in countries where antibiotic consumption is

moderate to high, such as southern and eastern European countries (48) and remain low in northern European countries where antibiotic consumption is low (109).

PNEUMOCOCCAL VACCINES

As in other diseases, prevention of pneumococcal disease is the first step to control it. A 23-valent pneumococcal polysaccharide vaccine (PPV23) has been available since 1983, and contains the 23 most common serotypes causing disease in adults from the United States of America (USA) at the time it was launched. This vaccine is not effective in children less than two years old as it elicits a T-cell-independent immune response, which is underdeveloped in young children (19, 123). PPV23 is used nowadays to vaccinate high risk adults and older children, and the elderly.

In 2000, a seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in the USA universal immunization program. This vaccine targets the seven most common serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) that caused invasive pneumococcal disease (IPD) in children in the USA (27). More recently, two other pneumococcal conjugate vaccines were licensed - a ten-valent pneumococcal conjugate vaccine (PCV10) which includes the PCV7 serotypes plus serotypes 1, 5 and 7F, as well as, an antigen that stimulates antibody production against non-typeable *Haemophilus influenzae* (www.gsk.com/products/vaccines/synflorix); and a 13-valent pneumococcal conjugate vaccine (PCV13) that includes the PCV10 serotypes plus serotypes 3, 6A and 19A (www.prevnar13.com).

In Portugal, PCV7 became available in June 2001 and was replaced by PCV13 in January 2010. Since 2009, PCV10 has also been

available. Despite widespread vaccination, none of these vaccines is part of the National Vaccination Program nor is reimbursed by the state. However, available data indicates that around 60% of children up to 5 years old were vaccinated in 2006 (107, 114,Pfizer Portugal data).

Vaccine impact on pneumococcal population structure

In areas where there has been a massive use of PCV7, the pneumococcal population has changed substantially: a significant reduction in the incidence of pneumococcal disease and a replacement of serotypes in circulation has been observed; non-vaccine types have increased among asymptomatic carriers and as causes of invasive pneumococcal disease - for a review see (133).

In the USA, where the vaccination with PCV7 is universal since 2000, it has been observed, that the incidence of invasive pneumococcal disease due to vaccine serotypes decreased by 94%, between 1998 and 2003, among children less than five years old (26). The same was observed in the European countries. In a study that compared data from 1996 and 2006 obtained in Spain, Belgium and France from children less than five years old, it has been observed that the incidence of invasive pneumococcal disease caused by PCV7 serotypes decreased shortly after PCV7 introduction, by 58%, 22% and 52%, respectively. In these three countries, the proportion of children <2 years of age who had received an average of three PCV7 doses was 33%, 48% and 42%, respectively (58). In Portugal, between 2006 and 2008, serotypes included in PCV7 accounted for 17% only of IPD pediatric cases (3). The decrease in the proportion of PCV7 serotypes in IPD cases was observed not only in vaccinated children, but also in the whole population, due to herd immunity (86).

In colonization, in studies conducted in Portugal and Norway (five and two years after PCV7 was commercially available, respectively), carriage by vaccine serotypes decreased; however, the overall prevalence of pneumococcal carriage among vaccinated and unvaccinated children remained the same (114, 130). The maintenance of rates of carriage was due to serotype replacement. In Norway, an increase of serotypes 9N, 16F, 24F, 35F and 35B (130) was observed. In Portugal, rates of non-vaccine types 1, 6C, 7F, 15A, 16F, 21, 23A, 29 and non-typeable strains increased significantly (114).

Serotype replacement has also been observed in disease. In the USA serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were the most common serotypes isolated from invasive disease in children before the introduction of PCV7 (103). After vaccine introduction (2000), the percentage of otitis media cases due to penicillin-nonsusceptible non PCV7 serotypes increased from 12% in 1999 to 23% in 2002 and was mainly associated with the emergence of serogroups 3, 11, 15 and non-typeable strains (90). The increase of IPD cases associated with non-vaccine serotypes 19A and 35B has been documented in the USA (108). In Europe, in a study conducted in 2006, in four European countries (France, Spain, Belgium, and United Kingdom), to monitor the impact of PCV7 among children <15 years of age, it was found that the incidence of serotypes 1, 7F and 19A increased (58). In Portugal, in isolates responsible for pediatric IPD between 2006 and 2008 (after vaccine introduction), serotypes 1, 7F and 19A represented 61% of non vaccine types (3). However, when analyzing studies that evaluate temporal trends of pneumococcal serotypes it is also important to bear in mind that serotype prevalence is dynamic and serotype distribution can fluctuate substantially in the absence of

vaccination. In fact, several studies described changes in the pneumococcal population prior to the introduction of PCV7 (8, 45, 59, 72).

In contrast with what had been anticipated by some authors (83) the proportion of antimicrobial resistant pneumococci did not decrease in all countries that introduced PCV7 (6, 108, 114). In Portugal, in colonization isolates, rates of intermediate resistance to penicillin and resistance to macrolides, tetracycline and multidrug resistance remained unchanged due to a balance between reduction of antimicrobial resistant vaccine types and an increase among non–vaccine types (114). Regarding invasive isolates, there was not an overall reduction in the proportion of infections caused by resistant pneumococci in children and adults (6). In the USA, in invasive and non invasive infections isolates from all age groups, the prevalence of isolates with intermediate penicillin resistance and resistant to erythromycin has decreased, but the levels of multidrug resistance have not changed (108).

The maintenance of antimicrobial resistance levels, despite extensive serotype replacement, can be due to mechanisms such as *de novo* acquisition of resistance, introduction of new clones, expansion of existing clones and capsular switch. In 2007, in a study involving children from Massachusetts, a rapid outgrowth of non vaccine clones already present in the population was observed (53). Clone Netherlands^{15B}ST199 (mainly associated with serotypes 15B/C and 19A) was the major clone in the PCV7 era. The same behavior was observed in other studies, but with different clones: in 2004-2005 Taiwan^{19F}ST236 (serotype 19A variant) and Utah^{35B}ST377 were the

dominant clones in the USA (108). In Norway, the expansion of Portugal^{19F}ST177 and the emergence of Taiwan^{19F}ST236 were observed (130). Regarding capsular switch events, isolates with genotypes that previously were only associated with vaccine serotypes, but now express non-vaccine serotypes due to recombination at the capsular locus, have been well documented (22). In Portugal, no information about the mechanisms that led to the maintenance of antimicrobial resistance levels in colonization, despite serotype replacement among carriage isolates had been unavailable until recently (see Chapter III).

The introduction of PCV10 and PCV13 will also have an impact in pneumococcal invasive disease (IPD) and carriage. A study conducted in 2010 in the USA predicted that vaccination with PCV13 would reduce the incidence of IPD and acute otitis media by approximately 106 thousand and 16.3 million cases over a ten-year period (113). In a study from the United Kingdom, the serotype coverage of IPD in children under five years of age for PCV10 and PCV13 was less in 2007/2008 than in 2005/2006 which suggests that the potential coverage of PCV10 and PCV13 will be less than expected due to the routine use of PCV7 and the associated serotype replacement (47).

PNEUMOCOCCAL TYPING METHODS

Typing pneumococcus is very important since it contributes to our understanding of the natural history and epidemiology of the species. Typing methods are of high importance in epidemiological studies, for example to understand the impact of pneumococcal vaccines available and to clarify whether specially formulated vaccines are needed at different times, for different age groups, for different parts

of the world, or for protection against different kinds of pneumococcal infections. Methods for bacterial typing have also had a high importance in studies of outbreaks cases (129).

Pneumococcus can be typed by phenotypic and genotypic methods. Genotypic methods have generally higher discriminatory power than phenotypic methods.

The most common phenotypic methods are serotyping and antimicrobial susceptibility testing. Serotyping was already described here, and consists on the determination of capsular type using antipneumococcal polysaccharide capsule antibodies (81). In the antimicrobial susceptibility testing, the isolates are tested by diffusion or dilution methods against a panel of antimicrobial agents and an antibiotype is obtained.

The most common molecular typing methods are pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PFGE 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 that switches the direction of current according to a predetermined pattern. The different orientation of the electric pulses during electrophoresis allows the separation of large DNA fragments. In *S. pneumoniae*, typically, 10 to 19 bands of 20 to 300 kb are obtained when de DNA is digested with *Sma*I (85).

Multilocus sequence typing (MLST) is a nucleotide sequence based approach for the unambiguous characterization of isolates. MLST

consists on the amplification of approximately 450bp of the internal fragments of seven housekeeping genes – *aroE*, *gdh*, *gk*i*, recP*, *spi*, *xpt*, and *ddl* (41). The sequences are submitted to an international database available online (available at www.mlst.net) and a number is attributed to each allele. The seven alleles defined the sequence type (ST).

As described early in this chapter, multilocus sequence analysis (MLSA) use concatenated sequences from the multiple house-keeping loci to construct a dendrogram and observe the patterns of clustering of genotypes (54) and has also been proposed as a good alternative molecular technique to differentiate pneumococci from other closely related streptococci (55).

Comparative genomic hybridization (CGH) is based on the hybridization of DNA samples, labeled with fluorescent dyes, to microchips containing multiple oligonucleotides spotted on the chip surface. The fluorescence pattern is recorded by a scanner, quantified and analyzed. In pneumococcus, CGH has been used to compare the genomic content between clinical isolates (99, 119) and differentiate/compare pneumococcus from closely related species (84).

NON-TYPEABLE *S. PNEUMONIAE*

Most *S. pneumoniae* are surrounded by a polysaccharide capsule that is the major virulence factor. The pneumococcal capsule is used to type these bacteria through the Quellung reaction (see typing methods section). However, some isolates do not react with any commercially available serum and, when grown in agar medium, the

colonies are generally rough (12). These atypical isolates are called non-typeable *S. pneumoniae* (NTPn).

The mechanisms leading to non-typeability by the Quellung reaction may be at least four: (i) the expression of capsule may be severely down-regulated; (ii) the *cps* region, which contains the genes encoding the enzymes responsible for capsule biosynthesis, may have been disrupted; (iii) a novel capsular type not described yet for which there is no specific antisera may be expressed; and (iv) the capsular operon may be absent.

In 2003, Hathaway analyzed 27 non-capsulated pneumococci (seven from invasive disease and 20 from colonization) and divided them into two groups (61). Group I strains were closely related to capsulated strains since they had typical capsule genes and their genetic backgrounds, as determined by MLST, were characteristic of typeable strains. Group II strains were less closely related to capsulated pneumococci by MLST but had a clonal population structure among themselves and a genetic background associated with two geographically widespread clones able to cause epidemic conjunctivitis (89). Group II strains lacked typical capsule genes, but instead possessed two ORFs, which exhibited 61% to 64.5% homology to the ATP-binding cassette (ABC) transporter gene *aliB* and were designated *aliB*-like ORF 1 and *aliB*-like ORF 2 (61).

In 2006, Sá-Leão *et al* described a collection of 213 non-capsulated isolates recovered from the nasopharynx of young children that lacked the *cpsA* gene and had diverse genetic backgrounds which were distinct from the ones found among serotypeable strains (116).

Hanage and colleagues used MLST to characterize a set of 121 non-typeable presumptive pneumococci from Finland and identified three classes: (i) NTPn isolates with STs identical to capsulated pneumococci (suggesting pneumococci in which capsular expression had been down regulated or lost), (ii) isolates that clustered with capsulated pneumococci in a phylogenetic tree based on MLSA but had STs that differed from those of capsulated pneumococci in the MLST database (these isolates seemed to lack the capsular operon); and (iii) isolates that did not have typical pneumococci MLST alleles and did not cluster with capsulated pneumococci (all seven MLST alleles were 5% to 10% divergent from those of pneumococci) (56).

Based in these and other studies, non-capsulated strains are often referred to as non-typeable strains (in this thesis the two names are often used as synonyms).

Non-capsulated strains are less virulent than capsulated strains but have been associated to conjunctivitis outbreaks in rates that range from 23% to 51% (14, 15, 91). They have also been described as otopathogens (140). Regarding invasive disease, these isolates represented 2% of the cases in some studies (21, 44, 52) and in carriage, these rates were up to 10% (7, 116, 117, 130). In a study performed in Israel, NTPn was the most common pneumococcal type in acute conjunctivitis cases (14.1%), it was the seventh among carriage isolates (5.1%), was rarely found in acute otitis media (0.7%), and was not found in the invasive pneumococcal disease group (105).

The importance of NTPn in the context of pneumococcal ecology is not well studied. However, the lack of capsule has been

demonstrated to increase the adherence to the epithelial tissue (2, 31, 135), biofilm formation (36), and facilitates transformation events (102, 134).

Furthermore, it has been suggested that NTPn may act as privileged vectors to horizontal gene transfer: non-capsulated pneumococci may acquire DNA fragments from commensal streptococci and act as a shuttle vector of resistance genes for capsulated pneumococci. Specifically, it has been shown that a serotype 19F ST444 has evolved from low level to high level penicillin resistance by uptaking penicillin binding proteins gene fragments from a NTPn isolate (64).

In addition non-typeable pneumococci were detected often in co-colonized samples (i.e., samples that contain more than one pneumococcal isolate) (23, 116) which may lead to increased opportunities for horizontal gene transfer between capsulated and non-capsulated pneumococci.

Population structure: clones and antimicrobial resistance

As documented in the MLST database (www.mlst.net), NTPn have been isolated in various countries and from a variety of clinical sources including sterile sites. In spite of the highly diverse genetically backgrounds (more than 280 STs in the MLST database), a few lineages represent the majority of NTPn strains (Figure 2). In Portugal, in a study performed with more 200 NTPn isolated from carriage, a dominant clonal complex - CC344 (accounting for 66% of the isolates) - was identified (116). NTPn strains recovered in Spain between 1997 and 2002 belonged mainly to two clonal lineages: CC 941 and a lineage represented by the same CC detected in Portugal (15). Other lineages such as ST448 and ST449 are also

internationally spread and have been described in acute otitis media, conjunctivitis and carriage (56, 116, 140).

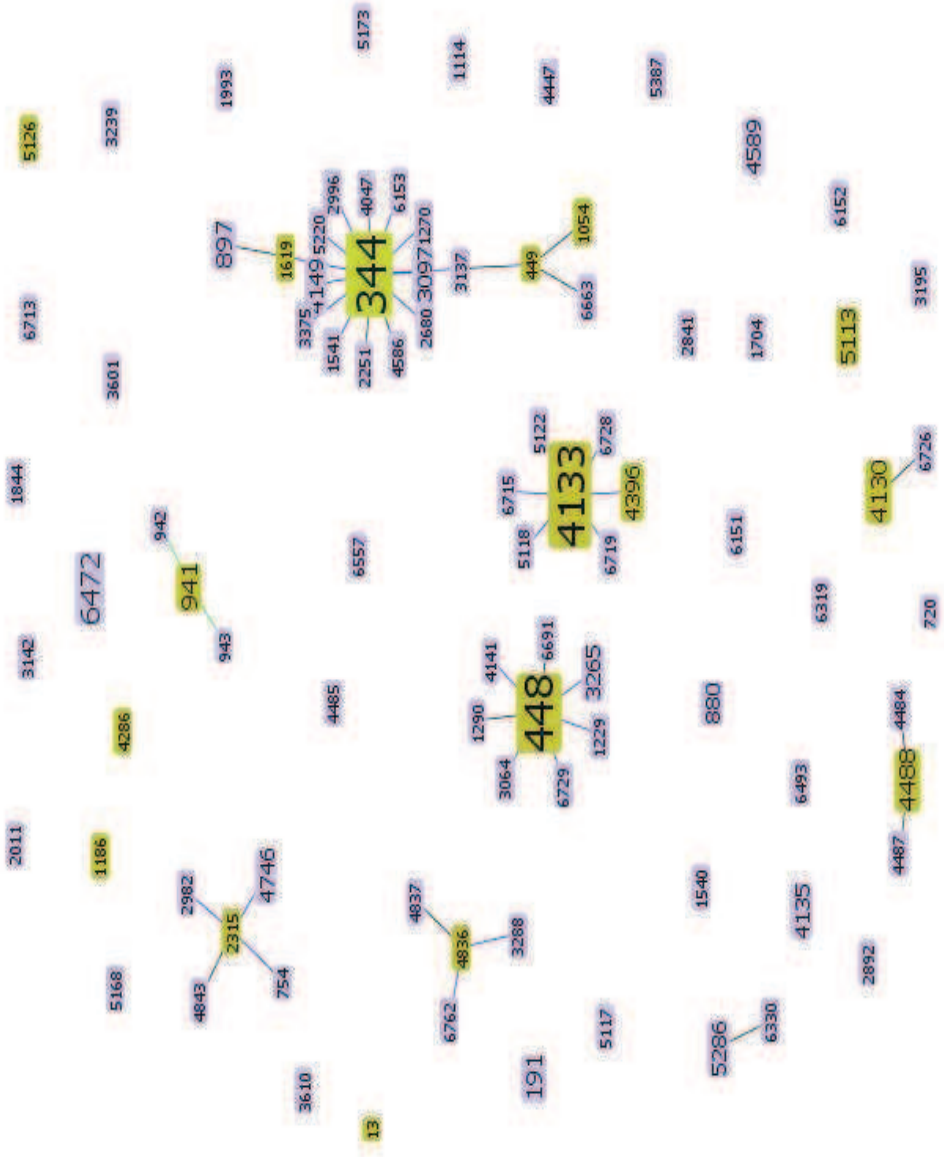

Figure 2. Go-eburst of the NTPn isolates available in the MLST database. The numbers represent the sequence types (ST). The lines between STs connect single locus variants. The size of the number is proportional to the amount of isolates of the ST in the MLST database (www.mlst.net).

NTPn are often multidrug resistant (116). The international clone Norway^{NT}ST344 has a characteristic pattern of multiresistance to penicillin, erythromycin, clindamycin, tetracycline and sulfamethoxazole-trimethoprim (122). However, it seems that NTPn isolates recovered from nasopharynx are more resistant than those recovered from other sites. In a study performed in Israel, 82% of NTPn isolates recovered from carriage were non-susceptibility to penicillin in contrast with rates of 65% and 53% found for acute conjunctivitis and acute otitis media respectively (105). In a Spanish collection ST941, ST942, and ST943 (isolated from the conjunctiva) were fully antibiotic susceptible (15) and the same happened with ST448 described as othopathogen (140) and associated with conjunctivitis outbreaks (89, 105).

These high rates of antibiotic resistance among NTPn (especially the ones from colonization) could possibly be explained by a combination of two factors: (i) increased exposure to transforming DNA due to high transformability rates (NTPn have transformability rates up to 1,075-fold higher than capsulated strains (102)) and, (ii) greater selective antibiotic pressure during nasopharyngeal colonization (33).

Identification of non-typeable pneumococci

NTPn exhibit rough and small colonies and, in most surveillance studies where a single pneumococcal colony is picked for further characterization NTPn tend to be neglected when smooth and bigger colonies typical of capsulated pneumococci are also present (23, 116). In addition, non-typeable pneumococci are often difficult to distinguish from closely related species as other streptococci of the mitis group (138) (as described early in this chapter). This distinction

is of clinical importance, since a misidentification of the causative agent can influence the diagnosis and treatment of disease (138).

Non-agglutination with any available capsular anti-serum is the method that *per se* defines NTPn. However, this may be tricky. For example, in a study by Sá-Leão *et al*, the presumptive identification of NTPn based on colony morphology, optochin susceptibility, and a negative capsular serotype reaction led to the misclassification of 19% NTPn isolates (116). Molecular methods are useful for NTPn identification and have been largely used (55, 116, 128). Sá-Leão and colleagues suggested the simultaneous detection of *lytA* and *psaA*, since the odds of having a non-pneumococcal isolate carrying these both pneumococcal genes is smaller (116). Hanage and co-workers described MLSA as an important tool to identify NTPn isolates (55). A serotype microarray that can detect NTPn in co-colonization has also been proposed (128).

AIM OF THE WORK

Since the introduction of pneumococcal conjugate vaccines, changes have been observed in the ecology of nasopharynx. In Portugal, the maintenance of antimicrobial resistance rates among disease and carriage isolates (5, 114) and an increase of rates of non-typeable strains among carriage isolates (114) was observed.

The aim of this thesis was to gain insights into: (i) the epidemiology of drug-resistant *S. pneumoniae* and closely-related streptococci in the era of conjugate vaccines and (ii) the true prevalence and molecular nature of non-typeable *S. pneumoniae.*

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Chapter II

Highly penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in Oeiras, Portugal: genomic characteristics and implications for surveillance

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A. S. Simões was responsible by all experimental work except the phylogenetic tree (that results from the multilocus sequence analysis) that was performed by J. Carriço; Comparative genomic hybridization performed by R. Sá-Leão, M. J. Eleveld and H. J. Bootsma and analysis of *comC* allelic variation performed by D. A. Tavares.

SUMMARY

While performing surveillance studies in Oeiras, Portugal, aimed to describe the impact of pneumococcal conjugate vaccine on colonization, we observed an increase from 0.7% in 2003 to 5% in 2006 in the prevalence of penicillin resistance (MIC 2-6 mg/L) among presumptively identified pneumococci. Although 15 of the 22 penicillin-resistant isolates obtained in 2006 were optochin-resistant, they were bile soluble and thus considered to be *bona fide* pneumococci. This study aimed to clarify the nature of these isolates by using a combination of phenotypic and genotypic approaches that included routine strategies for pneumococcal identification, multilocus sequence analysis (MLSA), and comparative genomic hybridization (CGH). By MLSA all isolates were classified as "streptococci of the mitis group" that, however, were distinct from typical *S. pneumoniae* or *S. mitis*. A single isolate was identified as *S. pseudopneumoniae*. CGH confirmed these findings and further indicated that a considerable part of the proposed pneumococcal core genome is conserved in these isolates, including several pneumococcal virulence genes (e.g. *pavA*, *spxB*, *cbpE*, and *cbpD*). These results suggest that among pneumococci and closely related streptococci, universal unique phenotypic and genetic properties that could aid on species identification are virtually impossible to attain. In pneumococcal colonization studies, when atypical strains are found, MLSA and CGH are informative tools that can be used to complement routine tests. In our study, after correct identification of the penicillin-resistant true pneumococci, we found that penicillin resistance levels among pneumococci remained stable from 2003 to 2006.

INTRODUCTION

Streptococcus pneumoniae is a bacterial pathogen that frequently colonizes the nasopharynx of humans, particularly young children of

preschool age. Colonization is mostly asymptomatic and only rarely results in disease (3). However, when disease does occur it may range from a mild infection such as otitis media to severe septicemia or meningitis. Globally, the morbidity and mortality associated with pneumococcal infections is extremely high. A recent report from WHO estimated that 0.7-1.0 million deaths occur annually among children aged <5 years due to pneumococcal infections (50).

Four phenotypic characteristics are classically used in the diagnostic laboratory for the presumptive identification of *S. pneumoniae*: colony morphology (colonies with a depression in the center showing alpha-hemolysis on sheep blood agar), optochin susceptibility, deoxycholate solubility (commonly referred to as bile solubility), and a positive reaction with antipneumococcal polysaccharide capsule antibodies (20). In particular, optochin susceptibility and deoxycholate solubility have been associated with a high sensitivity and specificity (between 98% and 100%). However, a number of studies have reported on sporadic optochin-resistant *S. pneumoniae* isolates (28, 34) and rare deoxycholate-insoluble strains (32).

On the other hand, some non-pneumococcal oral streptococci (such as *Streptococcus mitis*) may have a colony morphology similar to pneumococci, but are classically optochin resistant, bile insoluble, and do not react with antipneumococcal polysaccharide capsule antibodies (25). Still, optochin-susceptible non-pneumococcal isolates have been described occasionally (20), as well as isolates with positive cross-reactions with antipneumococcal polysaccharide capsule antibodies (11). Recently, a new species – *Streptococcus pseudopneumoniae* – was described (1). *S. pseudopneumoniae* isolates were found to be resistant to optochin when incubated in an atmosphere enriched in CO_2 but were optochin-susceptible

when incubated in ambient atmosphere. As a result, *S. pneumoniae* isolates may be difficult to distinguish from closely related species such as *S. pseudopneumoniae* and *S. mitis*.

Since the biochemical tests commonly used are not always sufficient to distinguish *S. pneumoniae* from other closely related upper respiratory streptococci, molecular approaches based on amplification of ubiquitous pneumococcal genes, such as pneumolysin (*ply*) and autolysin (*lytA*) have been proposed (27). However, homologues of *lytA* and *ply* genes have been detected in strains of closely related streptococcal species (15, 29, 37, 49). Recently, detection of *piaA* (which encodes for a lipoprotein component of two iron ABC transporters) has been proposed as a diagnostic tool for pneumococci as it was suggested to be specific for this species (47). Some authors also described 16S rRNA and *sodA* as good targets for identification of *S. pneumoniae* although *sodA* does not distinguish *S. pneumoniae* from *S. pseudopneumoniae* (1, 12, 21). The construction of phylogenetic trees from the concatenated sequences of the genes used for multilocus sequence typing (MLST) – an approach commonly designated as multilocus sequence analysis (MLSA) - has also been proposed as a good alternative molecular technique to differentiate pneumococci from other closely related streptococci (9, 18).

In recent years, we have been studying atypical pneumococci recovered from colonization samples collected from children attending day-care centers in Portugal. We first described a collection of over 200 non-serotypeable pneumococci which were mostly multidrug resistant and displayed low-level resistance to penicillin. All isolates were found to be true pneumococci that lacked a capsular operon. The isolates were genetically diverse although close to half belonged to a single lineage (39). Overall, these isolates were relatively abundant in asymptomatic carriers.

The second group of atypical pneumococci that we described consisted of isolates resistant to optochin. Again, all strains were found to be true pneumococci, genetically diverse and, in this case, most expressed a pneumococcal capsular type (30).

In the current study, we describe a third set of presumptively identified atypical pneumococcal strains. These isolates were all obtained in 2006 during a cross-sectional study aimed to describe the impact of the seven-valent pneumococcal conjugate vaccine in colonization. Strikingly, 5% (22 of 441) of the presumptively identified pneumococcal isolates were found to have penicillin MICs ranging from 2 to 6 mg/L, a value that between 2001 and 2003 never exceeded 1.7% and was 0.7% in 2003. Of note, 15 of these isolates, although resistant to optochin, were bile soluble and thus, according to routinely accepted criteria, were considered to be *bona fide* pneumococci. Since such high MICs of penicillin are extremely rare among this population, we initiated a detailed characterization of these isolates consisting of classical strategies for pneumococcal identification, MLSA, and comparative genomic hybridization (CGH). Ten other optochin-resistant, bile-soluble isolates with penicillin MICs ranging from 0.064 to 0.75 mg/L were also identified and further characterized.

We describe here that these isolates are not true pneumococci but have phenotypic properties and genomic determinants that are frequently associated with *S. pneumoniae*, challenging their correct identification by several currently accepted assays.

MATERIALS AND METHODS

Strain collection. Strains used in this study were isolated during January and February of 2006 from the nasopharynx of preschool children attending day-care centers (DCC) in Oeiras, Portugal. Swabs were inoculated in

tryptic soy agar (TSA) containing 5% defibrinated sheep blood supplemented with 5mg/L gentamicin and incubated overnight in anaerobic jars at 37°C to select and identify *S. pneumoniae*. As controls, we used *S. pneumoniae* strains R6 and TIGR4, and *S. pseudopneumoniae* type strain ATCC BAA-960 (kindly supplied by Maria de Gloria Carvalho, Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, USA). In addition, pneumococcal strains D39, G54, 23F, OXC14, INC104B, and INV200, and *S. mitis* NCTC 12261 were used in the comparative genomic hybridization experiments described below.

Antimicrobial susceptibility testing. MICs of penicillin, ceftriaxone, erythromycin, clindamycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim were determined with the Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. Results were interpreted following the recommendations and definitions of the Clinical and Laboratory Standards Institute (CLSI) (5).

Detection of antimicrobial resistance genes. Macrolide (*ermB*, *mefA/E*) and tetracycline (*tet*M) resistance genes were screened by PCR using primers and conditions previously described (26).

DNA fingerprinting by PFGE. Preparation of chromosomal DNA, digestion with SmaI endonucleases, separation of DNA fragments by PFGE and interpretation of results were carried out as previously described (40).

Optochin susceptibility. The optochin susceptibility was tested by disk diffusion, using commercially available optochin discs (5 µg; 6 mm; Oxoid, Hampshire, England) applied to blood agar plates (trypticase soy agar supplemented with 5% sheep blood) inoculated with colonies from overnight cultures. Plates were incubated overnight at 37℃ in 5% CO_2

atmosphere. A similar assay was carried out in ambient atmosphere as described by Arbique *et al.* (1). Isolates were considered to be resistant to optochin if they displayed inhibition zones smaller than 14 mm.

Capsular typing. Capsular serotyping was performed using the chessboard system (42) with specific antisera from the Statens Serum Institute (SSI, Copenhagen). Omniserum (SSI, Copenhagen), a serum that contains antibodies to all known pneumococcal serotypes, was used to confirm non-typeability.

Detection of *cpsA* (a conserved pneumococcal capsular gene present in 89 of the 91 capsular operons described to date) (2) was done by PCR using primers cpsAF2 (5'-AGCAGTTTGTTGGACTGACC-3') and cpsAR2 (5'-GTGTGAATGGACGAATCAAC-3').

***lytA* PCR detection, RFLP signatures, and Southern blotting.** PCR was used to screen for the presence of the gene encoding the major autolysin (*lytA*) in *S. pneumoniae*, a virulence factor ubiquitous in pneumococci that is often used as an identification marker of this species, using the primers described by Obregon *et al.* (32) - LA5_Ext (5'-AAGCTTTTTAGTCTGGGGTG-3') and LA3_Ext (5'-AAGCTTTTTCAAGACCTAATAATATG-3') - which yield a PCR product of ca. 1,200 bp encompassing the entire *lytA* gene. RFLP signatures characteristic of typical pneumococcal *lytA* or atypical *lytA* were determined as described before by digesting the PCR product with BsaAI and separating the fragments by agarose gel electrophoresis (24).

For Southern blotting, DNA fragments separated by PFGE were transferred to nylon membranes (41) and hybridized with a *lytA* probe obtained by PCR

amplification of TIGR4 DNA with primers LA5_Ext and LA3_Ext described above.

Deoxycholate (Doc) solubility assays. Doc solubility was initially performed according to standard procedures (38): colonies from an overnight culture were suspended in 1 ml of a 0.85% NaCl (w/v) solution to a turbidity equal to 0.5-1 McFarland standard. This suspension was distributed into two tubes, and three to four drops of a 10% Doc solution were added to one tube while the other served as control. Both tubes were incubated at 37ºC for up to 2h. A sample was considered positive when clearing of the turbidity occurred in the tube with Doc but not in the control.

For a more detailed characterization of the isolates, Doc solubility was re-assayed in a slightly different way as described by Llull *et al*, 2006 (24): 1 ml of exponentially growing cultures received 100 µl of 1 M potassium phosphate buffer (pH 8.0) and 100 µl of lise solution (Doc at a final concentration of 1% or 0.1%). The mixtures were incubated for 15 min at 37℃. Turbidities of the solutions were read at 620 nm using an Ultrospec III (Pharmacia LKB, Cambridge, UK). When the turbidity of the cell suspension decreased more than 50% from the initial value, the assay was considered positive. The experiments were repeated three times on different days.

***ply* and *mly* PCR detection and BsaI-RFLP signatures.** The presence of *ply* (encoding for pneumolysin, a cholesterol-dependent citolysin) or *mly* (a *ply* homologue recently identified in some *S. mitis* isolates that has been named mitilysin) (16) was detected by PCR. The primers used for detection of both genes were *plyF* (5'-TTCTGTAACAGCTACCAACG -3') or *plyF2* (5'-CGATGAGTTTGTTGTTATCG-3') and *plyR* (5'-ACCTTATCYTCTACCTGAGG-3'), yielding an internal fragment of 1,223

bp with primer *plyF* and 1,283 bp with primer *plyF2*. Alignment of the sequences of *ply* and *mly* alleles available at NCBI (accession no. EF413923-EF413926, EF413929-EF413931, EF413933, EF413934, EF413936, EF413937, EF413939, EF413943 for *ply* alleles and EF066514-EF066520 for *mly* alleles) showed that the PCR products obtained for these genes could be distinguished by differential restriction sites for BsaAI: BsaAI cuts the *ply* PCR product once, resulting in two fragments of 830 bp and 393 bp (or 890 bp and 393 bp), while the *mly* PCR product does not contain a restriction site for BsaAI. Thus, after purification of the PCR products, the fragments were digested with BsaAI, separated by agarose gel electrophoresis, and the presence of *ply* or *mly* was inferred from the pattern obtained.

Screening for pneumococcal-specific lipoprotein *piaA*. Detection of *piaA,* a gene encoding the lipoprotein component of the pneumococcal iron ABC transporter Pia and described as 100% conserved and uniquely found in pneumococci (47) was done by PCR. The primers used were those previously described: *piaAFor*- 5'-AGAGCATGCGCCTGATAAAAT-3' and *piaARev*- 5'-CATGAGGCTGCTAACGGTGTAT-3' (47).

Multilocus sequence typing (MLST). Amplification of internal fragments of seven housekeeping genes – *aroE*, *gdh*, *gk*i*, recP*, *spi*, *xpt*, and *ddl* - was done according to the MLST scheme developed by Enright and Spratt for *S. pneumoniae* (6). Sequencing reactions were conducted at Macrogen, Inc. (Seoul, Korea). Sequencing analysis was done with DNAStar (Lasergene). Tentative allele number assignment was done at the international MLST database for *S. pneumoniae* (www.mlst.net).

Multilocus sequence analysis (MLSA). Phylogenetic analysis of MLST data was done by concatenating the sequences of all MLST loci except *ddl*

to obtain one single sequence of 2,758 bp (9). One isolate failed to yield an amplification product for a single locus (*gdh* for PT5645a) and, in this case, a shorter contig was constructed. MLST sequences of *Streptococcus* spp. including *S. mitis*, *S. pseudopneumoniae*, and *S. oralis*, previously described by Hanage et al. and Chi et al. (4, 9) were also used. Sequence alignment was performed using ClustalW algorithm included in MEGA version 4 build 4025 (43). A bootstrap consensus tree inferred from 1,050 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method.

Comparative Genomic Hybridization (CGH). Microarrays used in this study have been described in detail before and contain PCR amplicons representing 2,087 ORFs of *S. pneumoniae* TIGR4, all spotted in duplicate (10). Chromosomal DNA for CGH was isolated from bacterial cultures by cetyltrimethylammonium bromide extraction as described previously (44). In all cases, TIGR4 genomic DNA was used as the reference sample. For CGH, 400 ng DNA from each strain was fluorescently labeled using the BioPrime Array CGH Genomic Labeling System (Invitrogen). The concentrations of nonfluorescent nucleotides in the 50-μl reaction mixtures were 0.2 mM each dATP, dCTP, and dGTP and 0.1 mM dTTP, and fluorescent nucleotide analogs (Cy5-dUTP or Cy3-dUTP; Amersham Biosciences) were added to a final concentration of 0.1 mM. The reaction was incubated overnight at 37℃ and stopped by addition of 5 μl of 0.5 M EDTA (pH 8.0). Reactions were cleaned up using the purification module of the BioPrime kit, after which yields and incorporation of dye were verified using a Nanodrop ND-1000 (Nanodrop Technologies). Labeled sample and

reference DNA were combined and precipitated with ethanol in the presence of 0.3M NaAc (pH 5.2). Dried samples were resuspended in 65 µl of Slidehyb buffer 1 (Ambion) and applied to the microarrays underneath a lifterslip (Erie Scientific Co.). Following overnight hybridization at 45℃, arrays were washed with 2xSSC (Invitrogen) containing 0.25% SDS for 5 min, followed by 2 washes in 1xSSC and 0.5xSSC for 5 min each. Finally, slides were dipped into H_2O and dried by centrifugation. Two replicate hybridizations (dye swap) were performed for all strains.

CGH data acquisition and analysis. Dual channel array images were acquired on a GenePix 4000AL microarray scanner and analyzed with GenePix Pro software (Axon Instruments, Union City, CA, USA). Automatically flagged spots, spots with low background-subtracted signal intensities (sum of median Cy3 and Cy5 net signals, <1,000), and spots with >40% saturated pixels were filtered out of all data sets prior to analysis. Slides used for CGH of pneumococcal strains were normalized by an array-based Lowess transformation. Due to the highly skewed distribution of log ratios with the atypical strains, array-based Lowess transformation could not be performed with these arrays. Instead, we selected a subset of genes (list available on request) that gave consistent hybridization signals for all strains, and calculated a normalization factor so that the mean ratio across this gene set was 1. Next, average normalized log ratios were calculated for genes with at least three measurements per strain in the dye swap pair. As a final selection criterion, only genes with valid data for 15 out of the 25 atypical strains (1,838 total) were used. Designation of genes in each strain as present, divergent, or absent was performed using the graded assignment categorization option of the GACK software (22). GACK uses the ratio distribution per strain to calculate an estimated probability of presence (EPP) value for each gene without the need for arbitrarily defined cut-offs. A calculated EPP of 100% indicates a

gene is present (assigned '0.5'), an EPP of 0% indicates a gene is absent (assigned '-0.5'), and intermediate EPP values indicate a gene is divergent (assigned a value between -0.5 and 0.5 on a linear scale, where a value of '0" indicates that a gene has a 50% change of being divergent or present). Clustering was based on the GACK scores and was done with the Euclidian distance metric and average linkage, using TIGR Multiexperiment Viewer (MeV; http://www.tm4.org/mev.html).

Analysis of *comC* allelic variation. Chromosomal DNA from each strain was obtained using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instructions. *comC* PCR products of 337 bp were obtained as previously described by Whatmore and co-workers (48) using the primers comC_f (5'-TGACAGTTGAGAGAATCTT-3') and comC_r (5'-CTTTTCTATTTATTTGACCT-3'). Sequencing was conducted at Macrogen, Inc. (Seoul, Korea), and subsequent analysis of obtained sequences was done with DNAstar (Lasergene).

RESULTS

Presumptive identification of atypical streptococci. The twenty-five strains described in this study were all isolated from selective agar media (containing gentamicin) routinely used to recover pneumococci, while inhibiting the growth of other bacterial species that colonize the nasopharynx. All strains displayed a pneumococcus-like colony morphology and although they were resistant to optochin, they were bile soluble. Therefore, they were presumptively identified as optochin-resistant pneumococci.

Abnormally high levels and prevalence of penicillin and multidrug resistance. Antimicrobial susceptibility testing showed that all but one of

the 25 strains were non-susceptible to penicillin (MIC higher than 0.06 mg/L), and 64% were multidrug resistant (defined as non-susceptibility to three or more classes of antimicrobial agents) (Table 1). Among the 19 isolates resistant to macrolides, 3 contained *ermB*, 14 contained *mefA/E*, and two contained both genes; the seven isolates resistant to tetracycline contained *tetM* (Table 1). Furthermore, penicillin MICs of 15 strains were high (ranging from 2 to 6 mg/L), values that are extremely rare in Portuguese colonization samples and could have important implications. These results prompted a detailed characterization to clarify whether the 25 strains were true pneumococci.

Phenotypic characterization. No pneumococcal capsule could be detected in any of the strains by the Quellung reaction: eight isolates showed no positive reaction and the remaining autoagglutinated. In addition, *cpsA* could not be detected by PCR in any of the strains, suggesting its absence.

To determine if the isolates showed the CO_2-dependent optochin phenotype described for *S. pseudopneumoniae* (1), we examined optochin susceptibility in 5% CO_2 and ambient atmosphere in parallel. Twenty isolates were fully resistant to optochin in both environments (no halos were obtained). Four isolates (PT5295b, PT5557b, PT5645, and PT5729) had increased susceptibility in ambient atmosphere but were still considered resistant according to the proposed interpretation criteria. One isolate (PT5479) had results identical to those described for *S. pseudopneumoniae*, i.e., it was resistant to optochin when incubated in 5% CO_2 but susceptible when incubated in ambient atmosphere.

Deoxycholate solubility assays (Doc) at 1% and 0.1% final concentration following the protocol proposed by Obregon et al. (32) were carried out.

These authors reported that streptococci of the mitis group harboring an atypical *lytA* did not lyse with 1% Doc solution but lysed at 0.1%, i.e., in the latter case the turbidity of the cell suspension decreased at least 50% after 15 min at 37ºC. In our study, all but one strain lysed in the presence of both concentrations of Doc, a result reminiscent of typical pneumococci and similar to control strains R6 and TIGR4. The only exception found (PT5479) was the strain that, based on optochin susceptibility, was tentatively classified as *S. pseudopneumoniae.* The same behavior was observed for *S. pseudopneumoniae* control strain ATCC BAA960.

Screening for pneumococcus-specific genes. Screening of *lytA* by PCR yielded a product with the expected size in 18 isolates (Table 1). By BsaI-RFLPs, a signature identical to the atypical *lytA* found in some “streptococci of the mitis group” was obtained for 17 isolates (data not shown) (24). For one strain (PT5534b) a restriction pattern could not be obtained (Table 1). In the remaining seven isolates no PCR product could be obtained despite several repeated attempts in different experimental conditions. However, by Southern blotting of SmaI-PFGE patterns, in all but one strain (PT5479), at least one fragment hybridized with a *lytA*-specific probe suggesting that some form of this gene was present (Table 1).

PCR screening for *ply* or *mly* indicated that all 25 strains harbored one of these allelic variants. Subsequent BsaAI-RFLP analysis indicated that five isolates harbored pneumolysin and 20 harbored mitilysin (Table 1). PCR screening for the *piaA* gene suggested that it was absent in all 25 strains characterized in this study.

Table 1. Properties of strains characterized in this study.

Strain ID	DCC Code	Child's age	Bile solubility	MIC (mg/L)[1]							Presence of resistance genes	PFGE pattern	*lytA*[2]		Presence of *ply* or *mly*	MLST allele assignment[3]						
				PenG	Tx	Ery	Cc	Tet	Chl	SXT			RFLP	Southern		*aroE*	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
PT5274	16	5	pos	**0.75**	0.25	**>256**	0.125	**8**	3	**3**	*ermB, tetM*	1	atypical	pos	*mly*	P	O	A	Q	S	G	G
PT5283	16	4	pos	**6**	**0.75**	**8**	0.094	0.25	2	**1**	*mefA/E*	2	na	pos	*mly*	N	T	181	N	T	T	A
PT5295b	32	4	pos	**2**	0.38	**24**	0.094	0.38	2	**2**	*mefA/E*	3	na	pos	*mly*	R	I	G	O	U	U	K
PT5346b	32	5	pos	**0.5**	0.19	**24**	0.094	**32**	2	**0.75**	*mefA/E, tetM*	4	atypical	pos	*mly*	U	A	F	E	K	V	I
PT5479	20	5	neg	**0.38**	0.25	0.094	0.094	0.19	2	0.064		5	atypical	neg	*mly*	X	J	O	B	D	C	6
PT5526b	35	3	pos	**4**	0.5	**16**	0.064	0.38	2	**1.5**	*mefA/E*	6	na	pos	*mly*	V	T	P	F	V	N	A
PT5525c	35	3	pos	**0.094**	0.094	**16**	0.064	0.38	3	**3**	*mefA/E*	7	atypical	pos	*mly*	A	B	D	C	L	I	S
PT5532	35	4	pos	**0.25**	0.25	**>256**	**>256**	**32**	3	**6**	*ermB, tetM*	8	atypical	pos	*mly*	L	R	D	J	A	D	S
PT5534b	35	5	pos	**4**	**2**	**12**	0.094	0.25	2	0.5	*mefA/E*	9	a)	pos	*ply*	Q	K	L	K	E	E	H
PT5557b	35	5	pos	**3**	0.38	**24**	0.064	0.38	2	**1**	*mefA/E*	6	na	pos	*mly*	F	T	181	F	I	N	A
PT5590	19	4	pos	**3**	**0.75**	**16**	0.094	0.38	2	0.19	*mefA/E*	10	atypical	pos	*ply*	J	E	A	S	W	W	G
PT5590b	19	4	pos	**6**	0.5	**48**	**>256**	1	3	**3**	*ermB, tetM*	11	na	pos	*mly*	T	P	S	H	G	R	L
PT5645	21	4	pos	**6**	0.38	**16**	0.094	0.38	2	**1.5**	*mefA/E*	12	na	pos	*mly*	M	na	M	I	P	L	D
PT5645b	21	4	pos	0.064	0.064	0.064	0.094	0.25	3	0.094		13	atypical	pos	*ply*	K	S	J	G	Q	S	102
PT5714	21	3	pos	**0.25**	0.064	**16**	0.094	0.38	2	0.125	*mefA/E*	14	atypical	pos	*mly*	D	Q	H	G	F	A	J
PT5729	21	3	pos	**0.5**	0.125	**12**	0.094	**24**	2	**1.5**	*mefA/E, tetM*	15	na	pos	*mly*	E	C	R	F	N	F	R
PT5736b	21	3	pos	**0.38**	0.04	**32**	0.094	**32**	2	0.125	*mefA/E, tetM*	16	atypical	pos	*mly*	C	94	K	R	O	B	B
PT5779	22	3	pos	**0.25**	0.094	**16**	0.094	0.38	3	**6**	*mefA/E*	17	atypical	pos	*ply*	I	D	E	H	C	H	F
PT5787b	22	2	pos	**3**	**0.75**	0.064	0.064	0.25	2	**6**		18	atypical	pos	*mly*	W	H	A	A	M	Q	M
PT5790b	22	0	pos	**3**	**0.75**	0.094	0.094	0.38	2	**6**		18	atypical	pos	*mly*	S	H	A	A	M	P	M
PT5793b	22	0	pos	**3**	0.5	**>256**	**>256**	**32**	4	**3**	*ermB, mefA/E, tetM*	19	atypical	pos	*mly*	B	F	I	P	J	O	O
PT5794b	22	0	pos	**2**	**0.75**	0.064	0.064	0.25	3	**3**		20	atypical	pos	*mly*	W	H	A	A	M	22	P
PT5796b	22	2	pos	**6**	**0.75**	0.094	0.094	0.25	3	**3**		21	atypical	pos	*mly*	1	L	B	D	B	K	C
PT5798b	22	2	pos	**3**	**1**	**16**	0.125	0.25	2	**2**	*mefA/E*	22	atypical	pos	*mly*	O	N	N	M	161	J	Q
PT5804	22	0	pos	**2**	0.38	**>256**	**>256**	**24**	3	**1**	*ermB, mefA/E, tetM*	23	atypical	pos	*ply*	G	G	Q	L	R	P	C

Legend of Table 1.[1] In bold are indicated MICs corresponding to non-susceptibility (intermediate and resistant); PenG, penicillin; Tx, ceftriaxone; Ery, erythromycin; Cc, clindamycin; Tet, tetracycline; SXT, sulfamethoxazole-trimethoprim; Chl, chloramphenicol; (I), intermediate resistance; neg, negative; pos, positive; [2] a), no RFLP could be obtained although a PCR product with expected size was amplified; na, PCR product could not be obtained. [3]MLST alleles were assigned capital letters when they were not described in the MLST pneumococcal database.

Genotyping by PFGE and MLST/MLSA. PFGE analysis showed a high level of diversity between the 25 atypical strains, as twenty-three distinct PFGE patterns were identified, and only two pairs of strains displayed the same profile (Table 1).

Sequence analysis of the seven housekeeping genes included in the *S. pneumoniae* MLST scheme showed that among the 174 alleles obtained only seven had been previously deposited in the pneumococcal database (PT5283 and PT5557b-*gki*-181; PT5479-*ddl*-6; PT5645b-*ddl*-102; PT5736b-*gdh*-94; PT5794b-*xpt*-22; PT5796b-*aro*-1; PT5798b-*spi*-161) (Table 1). All other alleles diverged up to 9% from the closest available match. The simultaneous occurrence of 6-7 novel alleles in each strain suggested they were not authentic pneumococci (9).

The phylogenetic tree resulting from the MLSA clearly placed the strains described in this study apart from *S. pneumoniae* and *S. oralis* (Figure 1). In particular, all strains but one clustered together with the isolates of the “Streptococcus of the mitis group” (SMG) described by Chi et al. (4). However, among the SMG, the strains described here formed a separate group that diversified close to the root of the tree. One strain, PT5479, clustered together with several *S. pseudopneumoniae* isolates described by Hanage *et al.* (9).

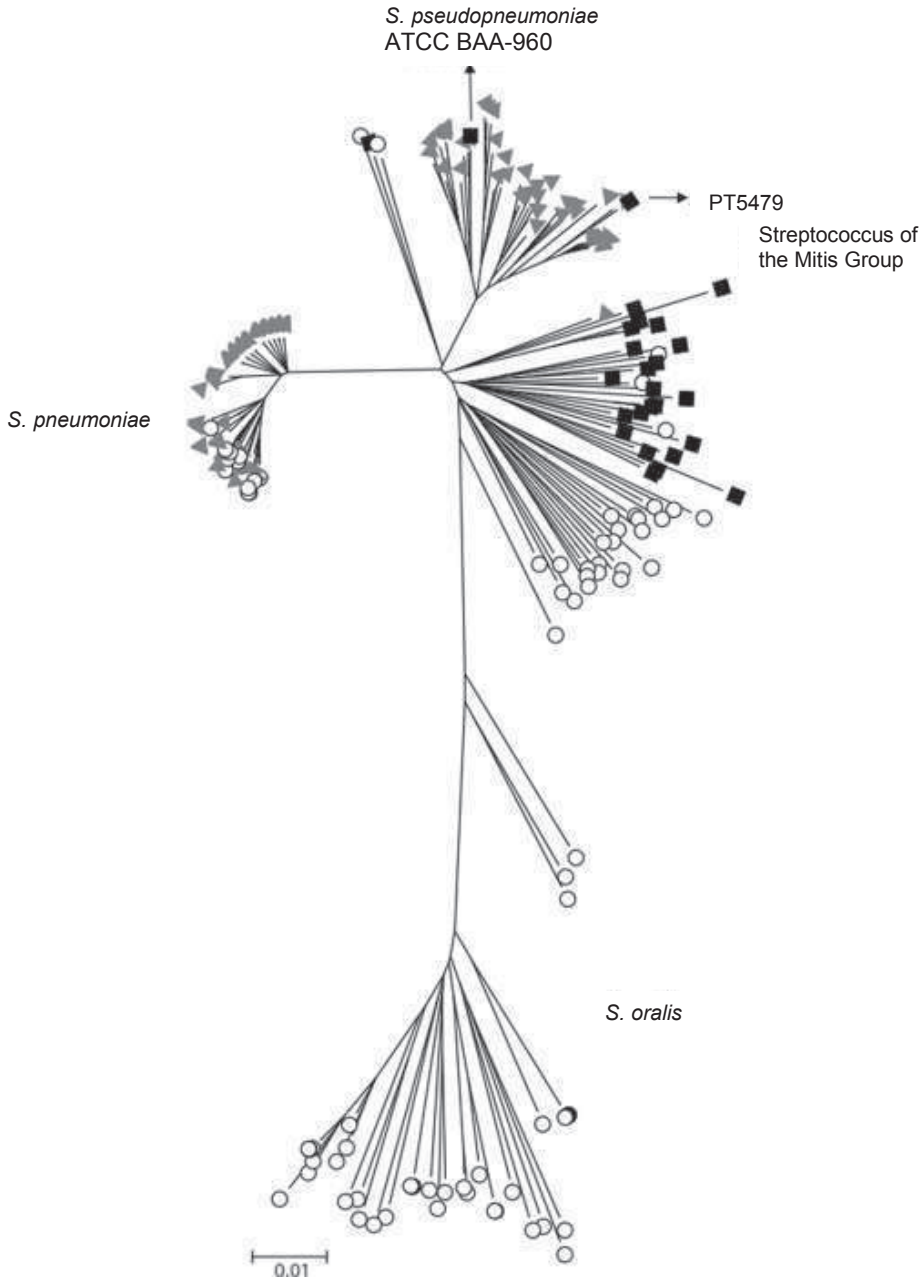

Figure 1. Genetic relationships of the strains determined by MLSA. The symbols represent: black square, strains described in this study, grey triangle, strains described by W. Hanage (9); white circle, strains described by F.Chi (4) (details in text).

Genomic characterization by CGH. To obtain insights into the variation of gene content between the isolates described in this study and *S. pneumoniae*, we performed CGH using *S. pneumoniae* TIGR4 as reference strain. In addition to the 25 atypical isolates, we also used seven true pneumococcal strains (six clinical isolates and strain R6), *S. mitis* NCTC 12261, and *S. pseudopneumoniae* ATCC BAA960. Clustering of strains based on the GACK absent/present/divergent gene scores for the 1,838 TIGR4 genes that passed our selection criteria showed that all 25 atypical strains clustered together, and that they appeared to be more similar to streptococcus of the mitis group than to the cluster of true pneumococcal strains, consistent with the MLSA findings. CGH also confirmed the close relationship of the two strain pairs with identical PFGE pattern, PT5525b/PT5557b, and PT5787b/PT5790b. A few gene clusters appeared to be absent or divergent in all strains examined, including the pneumococcal strains such as the TIGR4-specific capsule locus, the *rlrA* pathogenicity islet, a cluster of hypothetical ORFs, and a fructose phosphotransferase system (PTS).

Of the 1,838 genes used for the phylogenetic analysis, 1,365 belonged to the pneumococcal core genome proposed by Obert *et al.* (31). CGH analysis indicated that 394 pneumococcal core genes were divergent or absent in at least one of the 25 atypical isolates, with an average of 157 per strain. In total, 53 core genes were divergent or absent in all 25 isolates. In *S. pseudopneumoniae* and *S. mitis*, respectively, 135 and 318 core genes were absent or divergent. Interestingly, most of the absent or divergent core genes were grouped into 12 TIGR4 chromosomal regions of diversity (Supplementary Table 1). These genes are predicted to encode proteins with a variety of functions, but most prominent classes are involved in transport (*e.g.*, ABC transporters, PTS systems) and energy metabolism.

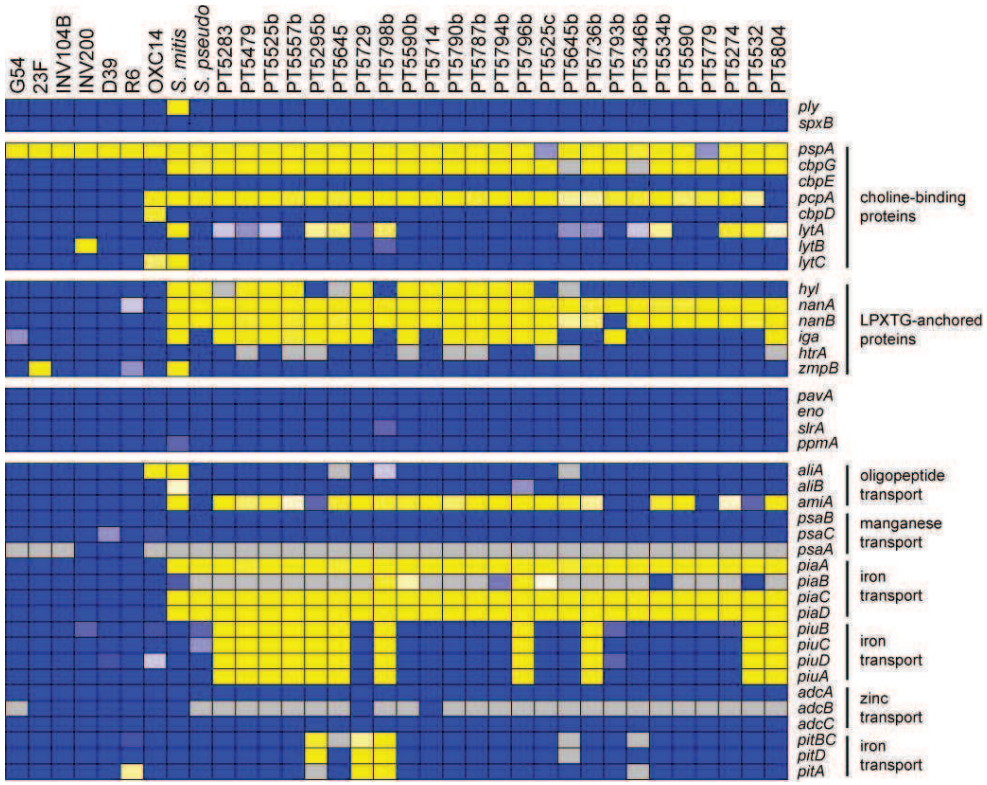

Figure 2. Gene content variation in pneumococcal virulence factors. CGH data for selected genes encoding major pneumococcal virulence factors are shown. The colors indicate the status of the ORFS, as follows: blue, present; yellow, absent; gray, no data.

To assess whether the atypical strains contained factors known to be important for infection potential of *S. pneumoniae*, we focused on the CGH results for some of the major pneumococcal virulence genes (Figure 2). As already demonstrated by the PCR-RFLP analysis described above, *ply* or *mly* (*ply*-like) sequences could be detected in all of the atypical strains. The *lytA* gene was found to be present in 11 of the 25 strains, while it was classified as absent or divergent in the remainder, including five of the seven strains that failed to yield a *lytA*-PCR product. Major differences between *S. pneumoniae* and the atypical strains appeared to reside in the PiaA iron ABC transporter system (as was observed by PCR), and in genes encoding the choline-binding proteins surface proteins CbpG and PcpA,

and the neuraminidases NanA and NanB (Figure 2). By contrast, other genes involved in virulence in *S. pneumoniae*, such as *pavA*, *spxB*, *cbpE*, and *cbpD*, were found to be present in the atypical isolates as well.

Allelic variation of competence stimulating peptide (*comC*). Most members of the mitis group are naturally competent for genetic transformation, and CGH indicated that the known competence genes were indeed present in our isolates. To examine potential sequence diversity of *comC* in more detail, we sequenced a *comC*-PCR product from 23 strains. A total of 14 different alleles based on amino acid sequence of ComC were identified. Of these, only four have been previously described (Table 2) (21, 35).

Table 2. *comC* alleles of strains characterized in this study.

Strain ID	Amino acid sequence of *comC* (sequence of mature CSP is given in bold capital letters)	Ref	Allele/ strain
PT5714	mkntvkleqfvalkekdlqnikgg**ESRISDILLDFLFQRKK**	35	CSP8
PT5590	mkntvkleqfvalkekdlqnikgg**ESRISDILLGFLFQRKK**		
PT5479, PT5779	mkntvkleqfvalkekdlqkikgg**EMRLPKILRDFIFPRKK**	35	CSP6.1
PT5274, PT5525c, PT5736b, PT5787b, PT5790b, PT5794b	mkntvkleqfvalkekdlqkikgg**ESRLSRLLRDFIFQIKQ**	21	SK612
PT5645	mkntvkleqfvtlkekdlqkiqgg**ESRLSRLLRDFIFQIKQ**		
PT5798b	mkntvkleqfvalkekdlqkiqgg**ESRLSRLLRDFIFQIKQ**		
PT5283	mkntvkleqfvalkekdlqkikgg**ESRMPKILRDFIFPRKK**		
PT5295b, PT5525b, PT5557b	mkntvkleqfvtlkekdlqkikgg**ESRMPKILRDFIFPRKK**		
PT5804	mkntvkleqfvtlkekdlqeirgg**ESRMSKFLLDFLFQRKK**		
PT5346b	mkntvkleqfvalkekdlqeirgg**ESRVSRIILDFLFLRKK**	21	SK675
PT5729	mkntvkleqfvalkekdlqeiqgg**ESRLSKLLRDFILQRKK**		
PT5590b	mkntvkleqfvtlkekdlqeiqgg**EMRKKIESFPGIFNFFRRR**		
PT5532, PT5796b	mkntvkleqfvalkekdlqniqgg**EMRKMNEKSFNIFNFFRRR**		
PT5645b	mknavkleqfvslkekdlqkikgg**DMRKKIESFPGLFNFFRRR**		

DISCUSSION

This study describes several phenotypic and genotypic properties of a collection of streptococcal isolates obtained in a single cross-sectional study aimed to describe the impact of PCV7 in colonization. Based on routinely used assays such as colony morphology, bile solubility, and optochin susceptibility, these isolates were initially classified as optochin-resistant, bile soluble pneumococci. However, they did not agglutinate with anti-pneumococcal capsular antibodies, and several isolates displayed unusually high MICs to penicillin (2-6 mg/L), indicating a sudden increase in the prevalence of penicillin resistance from 0.7% to 5% over a period of three years.

Further attempts to characterize these isolates by PCR screening for *lytA* and *ply* were not conclusive, as most strains appeared to have some form of *lytA* and *ply*. Additional analyses showed that the isolates contained atypical *lytA* genes, as defined by Llull et al. (24), five had pneumolysin and the others had the recently described mitilysin (16); and all lacked *cpsA* and *piaA*. However, the latter two genes are known to be absent in some non-capsulated true pneumococci (47) and thus, their usefulness in defining the species is limited when they are absent. DNA fingerprinting by PFGE indicated that the strains were genetically diverse. Epidemiological data also suggested that they were mostly unrelated as they came from seven different day-care centers.

Isolates distinct from pneumococci but that cannot be resolved from them by optochin susceptibility, bile solubility, or the presence of genes such as *lytA* and *ply,* have been described in the literature (9, 14, 23, 32, 45, 49).

To establish the relationship between our strains and true pneumococci, we characterized them by MLSA and CGH and compared results with those

obtained for control strains of *S. pneumoniae*, *S. pseudopneumoniae, S. mitis*, and *S. oralis*. On the basis of MLSA, the isolates were identified as "Streptococcus of the mitis group (SMG)", a classification that has been proposed for a group of evolutionarily related streptococcal isolates for which species classification challenges currently accepted criteria (32). In the SMG group, one cluster included several *S. pseudopneumoniae* isolates described by Hanage et al. and strain PT5479 isolated in this study (9). The latter was the single strain that had been identified as *S. pseudopneumoniae* based on optochin susceptibility assays. CGH also indicated that the atypical strains are indeed distinct from true pneumococci. However, it showed that a considerable part of the proposed pneumococcal core genome (88% on average) is conserved in these isolates. Absent regions corresponded mainly to loci encoding proteins involved in nutrient uptake and metabolism. Interestingly, many of the known pneumococcal virulence factors were detected by CGH, with a few notable exceptions: CbpG, a surface-protein that functions as an adhesin to eukaryotic cells, the neuraminidases NanA and NanB, and the Pia iron-uptake system.

Why species definition is sometimes difficult (if not impossible) for some non-hemolytic streptococcal isolates is a matter of debate. Killian et al. (21) have recently proposed that the ancestor of the pneumoniae-mitis-pseudopneumoniae group was a pneumococcus-like bacterium with all the properties associated with virulence. The commensal streptococci subsequently evolved from this pathogen by genome reduction, thus explaining the random (but syntenic) presence of pneumococcal virulence genes in representatives of this group. Others have suggested that horizontal gene transfer between naturally competent streptococci that share the same ecological niche significantly contributes to attenuation of putative barriers between these species, resulting not only in mosaic genes

but also in mosaic genomes (1, 4, 7, 8). While our results based on CGH do not enable us to favor any of these theories, they do enhance our perception of how closely related these species are.

Indeed, known competence genes required for natural transformation were detected in the atypical isolates by CGH. Furthermore, several different *comC* alleles, coding for the competence stimulating peptide (CSP), were found by sequence analysis, most of which appeared to be novel. Cross-communication between isolates requires recognition of the CSP by a ComD receptor. Cross-talk between different alleles due to promiscuous ComD has been described (17). Whether that is the case for the isolates described in this study has not been investigated, but could potentially contribute to the ambiguous nature of these strains.

High rates of resistance to penicillin and high MICs are frequent among *S. mitis* (13, 32, 33). Therefore, misidentification of SMG such as those described here can result in over-reported levels of penicillin resistance, a problem previously identified (36, 46). In our study, after ruling out the fifteen SMG isolates, penicillin resistance levels among pneumococci were found to have remained stable over time.

It is unclear why we have identified these SMG isolates only now, as we have been conducting surveillance studies since 1996. Most (15 of 25) were picked from the primary selective blood agar plate as secondary isolates that displayed colony morphologies distinct from the majority of the sample, but still resembled pneumococci. Our recent focus on non-capsulated pneumococci and optochin resistance, as well as an interest in multiple colonization, most likely contributed to an increased propensity to pick such colonies. Alternatively (and not mutually exclusively) it could be that changes in the nasopharynx ecosystem due to introduction of the

seven-valent pneumococcal conjugate vaccine facilitated colonization by SMG, thereby increasing its abundance in the population. The prevalence of these “confounding” organisms remains unknown and their contribution to pneumococcal evolution remains to be ascertained.

In conclusion, in the era of multivalent pneumococcal conjugate vaccines where surveillance is very important, researchers need to be aware that routinely used tests to identify pneumococci may not be sufficient when atypical isolates are found. This is critical in colonization studies since closely related streptococci inhabit the same niche and can be highly resistant to antibiotics. On the other hand, although *S. pseudopneumoniae* has been implicated in chronic obstructive pulmonary disease (COPD) (19), it is not clear whether the atypical “confounding” organisms described in this study may cause disease. This will be the subject of future research, aiming at increasing the insight into the ecological and clinical role of these strains.

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SUPPLEMENTARY MATERIAL

Table 1. Pneumococcal core genes not found in the atypical strains

Region of diversity	TIGR4 locus ID	TIGR annotation	TIGR main role
RD1	SP_0136	glycosyl transferase, family 2	Cell envelope
	SP_0137	ABC transporter, ATP-binding protein	Transport and binding proteins
	SP_0138	hypothetical protein	Hypothetical proteins
	SP_0139	conserved domain protein	Hypothetical proteins-Domain
	SP_0140	UDP-glucose 6-dehydrogenase, authentic frameshift	Cell envelope
RD2	SP_0506	integrase/recombinase, phage integrase family	DNA metabolism
	SP_0507	type I restriction-modification system, S subunit, putative	DNA metabolism
	SP_0508	type I restriction-modification system, S subunit	DNA metabolism
	SP_0509	type I restriction-modification system, M subunit	DNA metabolism
	SP_0510	type I restriction-modification system, R subunit	DNA metabolism
RD3	SP_0704	hypothetical protein	Hypothetical proteins
	SP_0705	hypothetical protein	Hypothetical proteins
	SP_0706	hypothetical protein	Hypothetical proteins
	SP_0707	ABC transporter, ATP-binding protein	Transport and binding proteins
	SP_0709	amino acid ABC transporter, ATP-binding protein	Transport and binding proteins
	SP_0710	amino acid ABC transporter, permease protein	Transport and binding proteins
RD4	SP_1160	lipoate-protein ligase, putative	Protein fate
	SP_1161	acetoin dehydrogenase complex, E3 component, dihydrolipoamide dehydrogenase, putative	Energy metabolism
	SP_1162	acetoin dehydrogenase complex, E2 component, dihydrolipoamide acetyltransferase, putative	Energy metabolism
	SP_1163	acetoin dehydrogenase, E1 component, beta subunit, putative	Energy metabolism
	SP_1164	acetoin dehydrogenase, E1 component, alpha subunit, putative	Energy metabolism
RD5	SP_1395	phosphate transport system regulatory protein PhoU, putative	Regulatory functions
	SP_1396	phosphate ABC transporter, ATP-binding protein, putative	Transport and binding proteins
	SP_1397	phosphate ABC transporter, ATP-binding protein, putative	Transport and binding proteins

Table 1. Continued.

Region of diversity	TIGR4 locus ID	TIGR annotation	TIGR main role
RD5	SP_1398	phosphate ABC transporter, permease protein, putative	Transport and binding proteins
	SP_1399	phosphate ABC transporter, permease protein, putative	Transport and binding proteins
	SP_1400	phosphate ABC transporter, phosphate-binding protein, putative	Transport and binding proteins
RD6	SP_1855	alcohol dehydrogenase, zinc-containing	Energy metabolism
	SP_1856	transcriptional regulator, MerR family	Regulatory functions
	SP_1857	cation efflux system protein	Transport and binding proteins
	SP_1859	conserved domain protein	Transport and binding proteins
RD7	SP_1883	dextran glucosidase DexS, putative	Energy metabolism
	SP_1884	PTS system, IIABC components	Transport and binding proteins
	SP_1885	trehalose operon transcriptional repressor	Regulatory functions
RD8	SP_1895	sugar ABC transporter, permease protein	Transport and binding proteins
	SP_1896	sugar ABC transporter, permease protein	Transport and binding proteins
	SP_1897	sugar ABC transporter, sugar-binding protein	Transport and binding proteins
RD9	SP_2016	nicotinate-nucleotide pyrophosphorylase	Biosynthesis of cofactors, prosthetic groups, and carriers
	SP_2017	membrane protein	Cell envelope
	SP_2020	transcriptional regulator, GntR family	Regulatory functions
	SP_2021	glycosyl hydrolase, family 1	Energy metabolism
	SP_2022	PTS system, IIC component	Transport and binding proteins
	SP_2023	PTS system, IIB component	Transport and binding proteins
	SP_2024	PTS system, IIA component	Transport and binding proteins

Table 1. Continued.

Region of diversity	TIGR4 locus ID	TIGR annotation	TIGR main role
RD10	SP_2031	conserved hypothetical protein	Hypothetical proteins-Conserved
	SP_2032	transcriptional regulator, BglG family	Regulatory functions
	SP_2033	L-ribulose 5-phosphate 4-epimerase AraD, putative	Energy metabolism
	SP_2034	hexulose-6-phosphate isomerase, putative	Energy metabolism
	SP_2035	hexulose-6-phosphate synthase, putative	Energy metabolism
	SP_2036	PTS system, IIA component	Transport and binding proteins
	SP_2037	PTS system, IIB component	Transport and binding proteins
	SP_2038	PTS system, membrane component, putative	Transport and binding proteins
RD11	SP_2081	conserved hypothetical protein	Hypothetical proteins-Conserved
	SP_2082	response regulator	Signal transduction
	SP_2083	sensor histidine kinase PnpS	Signal transduction
	SP_2084	phosphate ABC transporter, phosphate-binding protein	Transport and binding proteins
	SP_2085	phosphate ABC transporter, permease protein	Transport and binding proteins
	SP_2086	phosphate ABC transporter, permease protein	Transport and binding proteins
	SP_2087	phosphate ABC transporter, ATP-binding protein	Transport and binding proteins
	SP_2088	phosphate transport system regulatory protein PhoU	Regulatory functions
	SP_2089	transposase, IS1380-Spn1 related, truncation	Mobile and extrachromosomal element functions
RD12	SP_2148	arginine deiminase	Energy metabolism
	SP_2150	ornithine carbamoyltransferase	Energy metabolism
	SP_2151	carbamate kinase	Energy metabolism
	SP_2152	conserved hypothetical protein	Hypothetical proteins-Conserved
	SP_2153	peptidase, M20/M25/M40 family	Protein fate

Chapter III

Clonal evolution leading to maintenance of antibiotic resistance rates among colonizing pneumococci in the PCV7 era in Portugal

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A. S. Simões was responsible for the characterization of the 2007 collection. The 2006 collection was characterized by L. Pereira and S. Nunes.

SUMMARY

The introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) in Portugal led to extensive serotype replacement among carriers of pneumococci with a marked decrease of PCV7-types. Although antimicrobial resistance was traditionally associated with PCV7-types, no significant changes were observed in the rates of non-susceptibility to penicillin, resistance to macrolides or multidrug resistance. This study aimed to investigate the mechanisms leading to maintenance of antimicrobial resistance despite marked serotype replacement. We compared, through molecular typing, 252 antibiotic resistant pneumococci recovered from young carriers in 2006 and 2007 (era of high-PCV7 uptake) with collections of isolates from 2002-2003 (n=374, low-PCV7 uptake era) and 1996-2001 (n=805, pre-PCV7 era). We observed that the group of clones that account for antimicrobial resistance since 1996 is essentially the same as the one identified in the PCV7 era. The relative proportions of such clones have, however, evolved substantially overtime. Notably, widespread use of PCV7 led to an expansion of two PMEN clones expressing non-PCV7 capsular variants of the original strains: Sweden^{15A}ST63 (serotypes 15A and 19A) and Denmark^{14}ST230 (serotypes 19A and 24F). These variants were already in circulation in the pre-PCV7 era, although they have now become increasingly abundant. Emergence of novel clones and *de novo* acquisition of resistance contributed little to the observed scenario. No evidence of capsular switch events occurring after PCV7 introduction was found. In the era of PCVs antimicrobial resistance remains a problem among the carried pneumococci. Continuous surveillance is warranted to evaluate serotype and clonal shifts leading to maintenance of antimicrobial resistance.

INTRODUCTION

Streptococcus pneumoniae is a gram positive bacterium that frequently colonizes asymptomatically the nasopharynx of young children. However, it is also an important human pathogen that can cause a wide range of diseases from otitis media to pneumonia and meningitis. Worldwide, it has been estimated that 14.5 million episodes of serious pneumococcal disease and 826,000 deaths per year occur in children aged less than five years old (23).

In 2000, a seven-valent pneumococcal conjugate vaccine (PCV7) targeting the seven most common serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) causing invasive disease among young children in the USA became available. Since then, in countries where the vaccine has been introduced in the universal vaccination plan, a dramatic reduction in the incidence of invasive disease caused by PCV7 serotypes has been observed in all age groups (11, 17, 35). This extended phenomenon, beyond the target group, known as herd immunity, has been attributed to decreased transmission of pneumococci from children to other age groups. Indeed, the nasopharynx of children is the main reservoir of pneumococci and a decrease in carriage of PCV7 serotypes has been observed in vaccinated children (5). In addition to that, a replacement of PCV7 serotypes by non-PCV7 serotypes has been observed in carriage and disease (20, 25, 27, 38).

PCV7 became commercially available in Portugal in June 2001 but has not been introduced in the National Vaccination Program and is not reimbursed by the state. However, available data indicate that vaccination of children through the private sector has been increasing steadily reaching c.a. 70% of the target group by 2007 (this study; Pfizer, Portugal).

We have been studying pneumococcal colonization in Portugal since 1996. In the pre-PCV7 era, we documented that most drug-resistant isolates colonizing young children were associated with PCV7 serotypes (32, 33). Between 2001 and 2003, we conducted a pilot study to evaluate the impact of PCV7 on nasopharyngeal carriage (12). More recently, we described that since PCV7 became available in Portugal, extensive serotype replacement has occurred among vaccinated and unvaccinated children. In addition, the proportion of colonizing isolates resistant to at least one antibiotic did not change (30).

In this study, we aimed to identify the mechanisms responsible for maintenance of antimicrobial resistance levels despite extensive serotype replacement. We characterized a collection of drug resistant pneumococcal colonizing isolates obtained from young children in 2006 and 2007 (high-PCV7 uptake era) and compared it to two collections obtained years before: one before the introduction of PCV7 in Portugal (1996-2001, pre-PCV7 era) and other recovered in the two years after PCV7 introduction (2002-2003, low-PCV7 uptake era).

MATERIALS AND METHODS

Study design and study collection. Nasopharyngeal samples were obtained from children aged up to six years old attending day-care centers in Oeiras and/or Lisbon, two contiguous urban areas of Portugal. All samples were collected between the winter months of January-March. Day-care centers were selected to include different social backgrounds. In each year one sample was obtained from each child. Information regarding age, gender, recent antimicrobial consumption, and PCV7 vaccination was obtained. Approval for the study was obtained from the Ministry of Health and directors of day-care centers. Signed informed consent was obtained from the parents or guardians of participating children. This study design for

sampling has been maintained for several years allowing direct comparisons overtime (19, 21, 33).

To evaluate clonal changes among antibiotic-resistant pneumococci colonizing healthy children following introduction of PCV7, three time periods were considered: (i) 1996-2001 – pre-PCV7 era; (ii) 2002-2003 – low-PCV7 uptake era when 17.5% of the children enrolled in our studies had received at least one dose of the vaccine; and (iii) 2006-2007 - high-PCV7 uptake era with 67.1% of the children having received at least one PCV7 dose. In this latter period 30.7% had received four doses and 20.0% were appropriately vaccinated for age but had not yet reached the four doses.

For each time period, drug-resistant pneumococcal (DRPn) isolates were selected originating the following collections: (i) 805 DRPn isolated during the pre-PCV7 era (out of 2,152 isolates, recovered from 3,370 children), (ii) 374 DRPn isolated during the low-PCV7 uptake era (out of 1,116 isolates, recovered from 1,600 children); and (iii) 272 DRPn isolated during the high-PCV7 uptake era (out of 720 isolates, recovered from 1,109 children). Antibiotic-resistant isolates obtained between 1996 and 2003 were characterized before (19, 21, 33, 37). Isolates obtained in 2006 were partially (serotypes) described before (29) and isolates obtained in 2007 are first described in this study.

Nasopharyngeal swab and isolation of *S. pneumoniae*. Nasopharyngeal sampling, transport, pneumococcal isolation, identification, and preparation of frozen stocks were done as previously described (29, 32).

Antimicrobial susceptibility testing. MICs to penicillin were determined with the Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's

instructions. The interpretation criteria used were ≤0.06µg/ml, 0.1-1µg/ml, and ≥2µg/ml for classification of susceptibility, intermediate resistance, and resistance to penicillin, respectively. Testing of susceptibility to erythromycin, clindamycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim (SXT) was performed by disk diffusion, according to the recommendations and definitions of the Clinical and Laboratory Standards Institute (6). Multidrug resistance was defined as resistance to three or more classes of antimicrobial agents.

Capsular typing. Capsular assignment was performed by a combination of multiplex PCRs targeting serotypes 1, 3, 6A, 6B, 6C, 7F, 9N/L, 9V, 10A, 11A, 14, 15A, 15B/C, 16F, 17F, 18A/B/C/F, 19A, 19F, 22F, 23A, 23F, 31, 33F/33A/37, 34, 35F, and 38 using primers previously described (2, 24); www.cdc.gov). Strains that could not be typed by this method were serotyped by the Quellung reaction using specific antisera (Statens Serum Institute, Copenhagen, Denmark) (36). Non-typeable strains were defined by the absence of *cpsA* gene (screened by PCR using primers previously described (2)) and a negative reaction with omniserum (Statens Serum Institute, Copenhagen, Denmark).

DNA fingerprinting by pulsed-field gel electrophoresis (PFGE). Preparation of total DNA, digestion with SmaI endonuclease, and separation of DNA fragments by PFGE were carried out as previously described (33). PFGE patterns were analyzed with Bionumerics software (version 5, Applied Maths, Gent, Belgium) and a dendrogram was generated using the Dice similarity coefficient with an optimization of 1.0% and a tolerance of 1.5%. PFGE clusters were defined by a similarity of 80% or higher on the dendrogram (4, 37). Representatives of PMEN clones were also used for comparison of molecular types (www.sph.emory.edu/PMEN).

Multilocus sequence typing (MLST). MLST was undertaken essentially as described by amplifying internal fragments of seven housekeeping genes – *aroE*, *gdh*, *gk*i, *recP*, *spi*, *xpt*, and *ddl* (9). Sequencing reactions were conducted at Macrogen, Inc. (Seoul, Korea). Sequencing analysis was done with DNAStar (Lasergene). Allele number and sequence type assignments were done at the MLST database for *S. pneumoniae* (www.mlst.net).

Selection of isolates to MLST was based on analysis of the dendrogram generated by clustering of PFGE patterns: for each PFGE cluster with five or fewer isolates at least one isolate was selected for MLST; for larger clusters, at least one fifth of the strains were selected for MLST. Within a cluster, strains were selected to cover the diversity of PFGE patterns obtained.

Comparison between typing methods. Simpson index of diversity (SID) was used to measure the diversity of the populations. Sets of partitions were compared by using the adjusted Rand and Wallace values as described previously using the online tool for quantitative assessment of classification agreement available at www.comparingpartitions.info (4).

RESULTS

Resistance patterns, serotypes and clonal types among antibiotic-resistant pneumococci recovered in 2006-2007 – high-PCV7 uptake. Of the 272 antibiotic resistant pneumococcal isolates available for characterization, close to one third were associated with PCV7 serotypes 14 (11.8%), 23F (8.1%), 19F (7.7%), and 6B (1.1%). The non-PCV7 serotypes associated with antibiotic resistance were, in decreasing order of prevalence, 19A (23.2%), 15A (12.1%), 6C (9.6%), 6A (7.4%), 11A (4.4%),

24F (2.6%), 33F (2.6%), 23B (1.1%), 3 (0.3%), 22F (0.3%), 34 (0.3%); 7.4% of the isolates were non-typeable (NT).

The antibiotic resistance patterns and associated serotypes are summarized in Table 1. Multidrug resistance was detected in vaccine types 6B, 19F and 23F and non-vaccine types 6A, 6C, 15A, 19A, 24F, 33F and NT. In particular, non-susceptibility to penicillin associated with resistance to erythromycin, clindamycin, and tetracycline was the most common antibiotype being detected in one-third of the drug-resistant isolates. High-level resistance to penicillin (MIC≥2 µg/ml) was identified in isolates of serotypes 14 (n=20), 15A (n=1), 19A (n=3), and NT (n=1).

For molecular analysis, twenty isolates exhibiting resistance to SXT only (of serotypes 6A (n=14), 23B (n=3), 19A (n=2), and 34 (n=1)) were excluded. Isolates with resistance to SXT only from previous study periods were not systematically characterized and, thus, their inclusion would hinder comparisons between the three time periods. All remaining isolates (n=252) were characterized by PFGE, and close to one third (n=80) were also characterized by MLST. The PFGE profiles of PMEN representative strains (clones 1 to 26) were determined and used in the clustering analysis.

Thirty-six PFGE clusters and 37 STs were identified (Table 2). An excellent correlation between PFGE and related-STs (defined as MLST profiles that were identical or single locus variants of each other) was obtained as indicated by the Wallace coefficient of 1.000. In other words, for any two strains classified within the same PFGE cluster, the STs obtained were either identical or SLVs. These observations supported that our results based on PFGE/MLST analysis were suitable for comparison with MLST data from other studies. Of note, MLST could not discriminate between two

PFGE clusters of serotype 6C (both with ST 3396), two PFGE clusters of serotype 23F (ST338), and six PFGE clusters of NT isolates (ST344).

Table 1. Antimicrobial-resistant pneumococci isolated in 2006-2007 - high-PCV7 uptake era.

Serotype	No. isolates resistant to				Most common antibiotype
	At least one ATB tested	Pen	Ery	Multidrug	
3	1	0	1	0	Ery, Cli
6A	20	1	6	4	Ery, Cli, SXT
6B	3	2	3	2	---
6C	26	20	23	19	Pen (I), Ery, Cli, Tet
14	32	29	3	0	Pen, SXT
11A	12	0	0	0	Tet
15A	33	33	33	33	Pen (I), Ery, Cli, Tet
19A	63	49	54	54	Pen (I), Ery, Cli, Tet
19F	21	7	21	20	Ery, Cli, Tet
22F	1	1	0	0	Pen (I)
23B	3	0	0	0	SXT
23F	22	22	2	3	Pen (I)
24F	7	7	7	7	Pen (I), Ery, Cli, Tet
33F	7	0	7	2	Ery, Cli
34	1	0	0	0	SXT
NT	20	20	20	20	Pen (I), Ery, Cli, Tet

Pen, penicillin; Ery, erythromycin; Cli, clindamycin; Tet, tetracycline; SXT, sulfamethoxazole-trimethoprim; (I), intermediate resistance to penicillin (MIC ≥0.1µg/ml and ≤1µg/ml); dashed lines; the three 6B isolates had three distinct antibiotypes.

In 2006-2007, the four most frequent drug-resistant clones accounted for over half (52.4%) of the antibiotic resistant isolates and all were PMEN clones: Sweden^{15A}ST63 (expressing serotypes 15A (13.1%) and 19A (7.5%)), Denmark^{14}ST230 (expressing serotypes 19A (9.5%) and 24F (2.8%)), Spain^{9V}ST156 (serotype 14 variant, 11.1%), and Colombia^{23F}ST338 (8.4%).

Overall, 93.7% of the antibiotic-resistant isolates belonged either to PMEN clones or had identical or closely related ST associated with international isolates previously described in the MLST database (Table 2).

Association of drug-resistant clones with use of PCV7 among carriers. As detailed above, in 2006-2007, over two-thirds of the participants had been vaccinated with PCV7. We investigated whether there was an association between carriage of specific drug-resistant clones and vaccination status. We observed that clone Colombia^{23F}ST338 was significantly more prevalent among non-vaccinated children, while clones Sweden^{15A}ST63 (serotype 19A variant), Denmark^{14}ST230 (serotype 24F variant) and clone 33F-27 ST717 were significantly more prevalent among vaccinated children (Table 2).

Table 2. Clonality of antimicrobial-resistant pneumococci isolated in 2006-2007 - high-PCV7 uptake era.

Serotype -PFGE cluster	No. isolates (%)	MLST	PMEN clone	PCV7 use among drug-resistant carriers*		
				0 doses (n=86)	1-4 doses (n=152)	p-value
PCV7 serotypes						
6B-1	1 (0.4)	5217		1	0	0.183
6B-2	1 (0.4)	315	Poland^{6B} ST315	0	1	0.451
6B-3	1 (0.4)	469		1	0	0.183
14-4	28 (11.1)	557(SLV), 5219 (DLV), 4585 (SLV)	Spain^{9V} ST156	8	16	0.763
14-5	3 (1.2)	9	England^{14} ST9	3	0	0.020
14-ND	1 (0.4)	4584		0	1	0.451
19F-6	17 (6.7)	179 (SLV)	Portugal^{19F} ST177	8	9	0.330
19F-7	2 (0.8)	88		1	0	0.183
19F-8	2 (0.8)	5218		1	1	0.682
23F-9	15 (6.0)	338	Colombia^{23F} ST338	13	2	**<0.001**
23F-10	6 (2.4)	338	Colombia^{23F} ST338	3	1	0.102
23F-11	1 (0.4)	81	Spain^{23F} ST81	1	0	0.183

Table 2. Continued.

Serotype -PFGE cluster	No. isolates (%)	MLST	PMEN clone	PCV7 use among drug-resistant carriers*		
				0 doses (n=86)	1-4 doses (n=152)	p-value
Non- PCV7 serotypes						
3-12	1 (0.4)	180	Netherlands^{3} ST180	1	0	0.183
6A-13	5 (2.0)	490		2	3	0.856
6A-14	1 (0.4)	395		0	1	0.451
6C-15	3 (1.2)	2689		1	1	0.682
6C-16	2 (0.8)	2185		1	1	0.682
6C-17	11 (4.4)	3396		6	5	0.193
6C-18	6 (2.4)	3396		1	5	0.315
6C-19	4 (1.6)	3673		2	2	0.560
11A-20	12 (4.8)	4582		5	6	0.510
15A-21	33 (13.1)	2105 (SLV), 63	Sweden^{15A} ST63	7	24	0.092
19A-22	24 (9.5)	276 (SLV), 4267 (SLV)	Denmark^{14}ST230	5	19	0.099
19A-23	11 (4.4)	416 (DLV)	Netherlands^{15B}ST199	3	8	0.531
19A-24	7 (2.8)	4302, 5221		2	5	0.672
19A-21	19 (7.5)	63	Sweden^{15A} ST63	2	15	**0.029**
22F-25	1 (0.4)	433		1	0	0.183
24F-26	7 (2.8)	230, 1708 (SLV)	Denmark^{14} ST230	0	7	**0.043**
33F-27	7 (2.8)	717		0	7	**0.043**
NT-28	9 (3.6)	344, 4586 (SLV)	Norway^{NT} ST344	3	6	0.858
NT-29	2 (0.8)	344	Norway^{NT} ST344	1	1	0.682
NT-30	1 (0.4)	344	Norway^{NT} ST344	0	1	0.451
NT-31	1 (0.4)	344	Norway^{NT} ST344	0	1	0.451
NT-32	1 (0.4)	4583		1	0	0.183
NT-33	2 (0.8)	1156		2	0	0.059
NT-34	1 (0.4)	5220 (SLV)	Norway^{NT} ST344	0	0	---
NT-35	1 (0.4)	344	Norway^{NT} ST344	0	1	0.451
NT-36	2 (0.8)	344	Norway^{NT} ST344	0	2	0.285

PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ND, not determined; SLV, single locus variant of prototype strain of PMEN clone; DLV, double locus variant of prototype strain of PMEN clone; *for 14 carriers, information on PCV7 was not available; bold, significantly different.

Evolution of characteristics of antimicrobial resistant pneumococci. Figure 1 compares antimicrobial resistance rates over the three time periods. The proportion of antimicrobial resistant pneumococci (defined as resistant to at least one antimicrobial tested) decreased significantly from the pre-PCV7 era to the low-PCV7 uptake period (44.3% and 37.5%, respectively, $p<0.001$). This could be attributed to a significant decrease in the rates of pneumococci resistant to SXT and to chloramphenicol. Of note, the levels of high resistance rates to penicillin (MIC ≥ 2 μg/ml) were significantly lower in the low-PCV7 uptake era (1.7%) than in the preceding and following periods (4.1% and 3.8%, respectively). Rates of resistance to macrolides and low-level resistance to penicillin as well as multidrug resistance, did not suffer significant changes overtime.

Clonal diversity measured by the SID, was comparable in all three periods (Table 3). However, changes occurred in the population, as the clonal diversity among drug resistant pneumococci of PCV7 serotypes was lower in the period of high-PCV7 uptake while remaining stable among drug resistant pneumococci of non-PCV7 serotypes. Regarding the major serotypes recovered in 2006-2007 (i.e., types 6A, 6C, 14, 15A, 19A, 19F, 23F, and NT), following introduction of PCV7, a significant decrease in clonal diversity was observed for serotypes 14 and 19F. This effect was not observed for serotype 23F since the clonal diversity decreased in the low-PCV7 uptake but increased again in the high-PCV7 uptake. For non-PCV7 serotypes, the clonal diversity of serotype 6C and non-typeable strains increased. For serotypes 6A and 19A no significant changes in clonal diversity were observed during the three periods. Still, in the case of serotype 6A, the reduced number of isolates obtained in the latter period, may have hindered the observation of a reduction in diversity due to the large confidence interval obtained (Table 3).

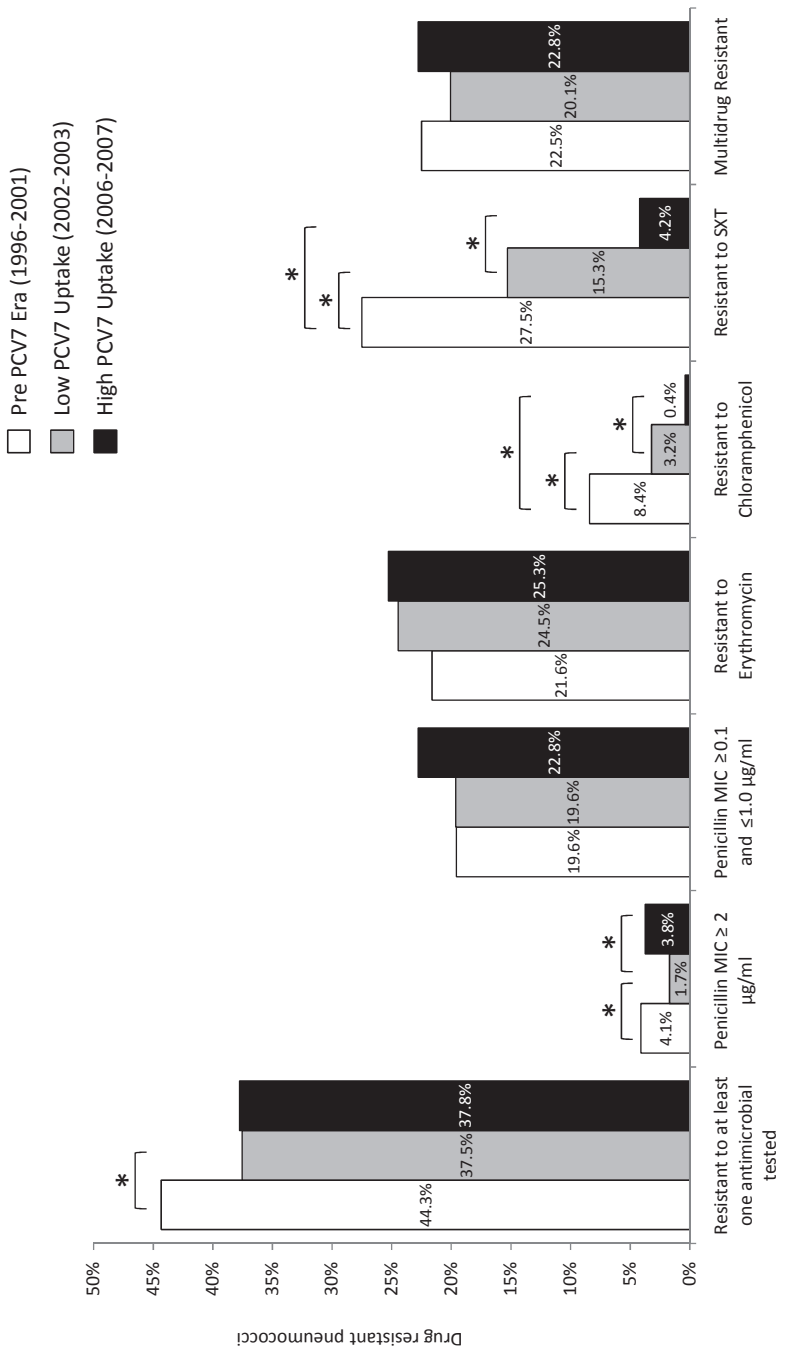

Figure 1. Antimicrobial resistance rates over time. White bars, pre-PCV7 era (1996-2001); grey bars, low-PCV7 uptake (2002-2003); black bars, high-PCV7 uptake (2006-2007); *, significant changes ($p<0.05$) are indicated with asterisk.

Replacement and evolution of antimicrobial-resistant clones. A clear serotype replacement of antimicrobial resistant clones was observed overtime: while in the pre-PCV7 era, 80.0% of the antimicrobial resistant pneumococci were associated with PCV7 serotypes, in the low-PCV7 uptake period and high-PCV7 uptake period corresponding values were, 66.9% and 31.0%, respectively (Figure 2).

Table 3. Clonal diversity of antimicrobial resistant pneumococci.

	No. of clones defined by PFGE, Simpson's ID (95% CI)		
	Pre-PCV7 Era (1996-2001, n=805)	**Low-PCV7 uptake (2002-2003, n=374)**	**High-PCV7 uptake (2006-2007, n=252)**
All serotypes[a]	74, 0.924 (0.917-0.931)	48, 0.936 (0.925-0.947)	36, 0.919 (0.910-0.936)
PCV7 serotypes	49, 0.894 (0.884-0.903)	23, 0.882 (0.860-0.904)	**11, 0.782 (0.727-0.837)**
Non-PCV7 serotypes	34, 0.916 (0.896-0.937)	29, 0.919 (0.898-0.940)	25, 0.873 (0.838-0.907)
Serotype 19A	9, 0.644 (0.493-0.794)	6, 0.641 (0.528-0.754)	4, 0.714 (0.663-0.765)
Serotype 15A	2, 0.173 (-0.027-0.374)	2, 0.500 (0.067-0.933)	1, 0.00 (0.00-0.00)
Serotype 6C	**2, 0.200 (0.104-0.504)**	1, 0.00 (0.00-0.00)	**5, 0.754 (0.649-0.859)**
Serotype 6A	7, 0.818 (0.727-0.909)	5, 0.857 (0.704-1.010)	2, 0.330 (-0.072-0.739)
Serotype 14	6, 0.538 (0.495-0.581)	7, 0.616 (0.510-0.720)	**2, 0.181 (0.094-0.352)**
Serotype 19F	15, 0.743 (0.664-0.822)	7, 0.711 (0.634-0.789)	**3, 0.343 (0.980-0.588)**
Serotype 23F	8, 0.501 (0.440-0.562)	**3, 0.093 (-0.006-0.193)**	3, 0.480 (0.301-0.660)
Non-typeable	3, 0.351 (0.185-0.517)	**5, 0.699 (0.583-0.814)**	9, 0.795 (0.628-0.961)

bold, significantly different

[a]In the pre PVC7 era there were nine PFGE clones that contain both PCV7 serotypes and non-PCV7 serotypes; in the low PVC7 uptake there were four PFGE clones that contain both PCV7 serotypes and non-PCV7 serotypes.

Out of eight major PCV7-type drug-resistant PMEN clones in circulation before PCV7 introduction, five were either not detected in 2006-2007 or were present in low proportions (Figure 2A). These were associated with serotypes 6B (Greece^{6B}ST273, Spain^{6B}ST90, Poland^{6B}ST315), 14 (England^{14}ST9), and 23F (Spain^{23F}ST81). Although variants of Greece^{6B}ST273 associated with non-PCV7 types 6A and 6C had been detected in the pre-PCV7 era, they were not found in the following periods. Similarly, non-PCV7 type variants of Spain^{23F}ST81 detected before PCV7 introduction did not appear to thrive (Figure 2A). Of particular interest, serotype 19A variants of this clone, previously detected, were not found in the period of high-PCV7 uptake.

The three PCV7 clones that remained in circulation in the era of high-PCV7 uptake had different properties: clone Spain^{9V}ST156 (expressing serotype 14) has high resistance to penicillin and is typically susceptible to macrolides. Clone Portugal^{19F}ST177 is typically multiresistant displaying resistance to macrolides, lincosamides, streptogramins, and tetracylines often accompanied by low-level resistance to penicillin. Clone Colombia^{23F}ST338 is typically associated with low resistance to penicillin. Apparently, PCV7 introduction did not affect significantly the prevalence of these clones. The reasons for such are not clear but do not appear to be associated with serotype or a specific antibiotype.

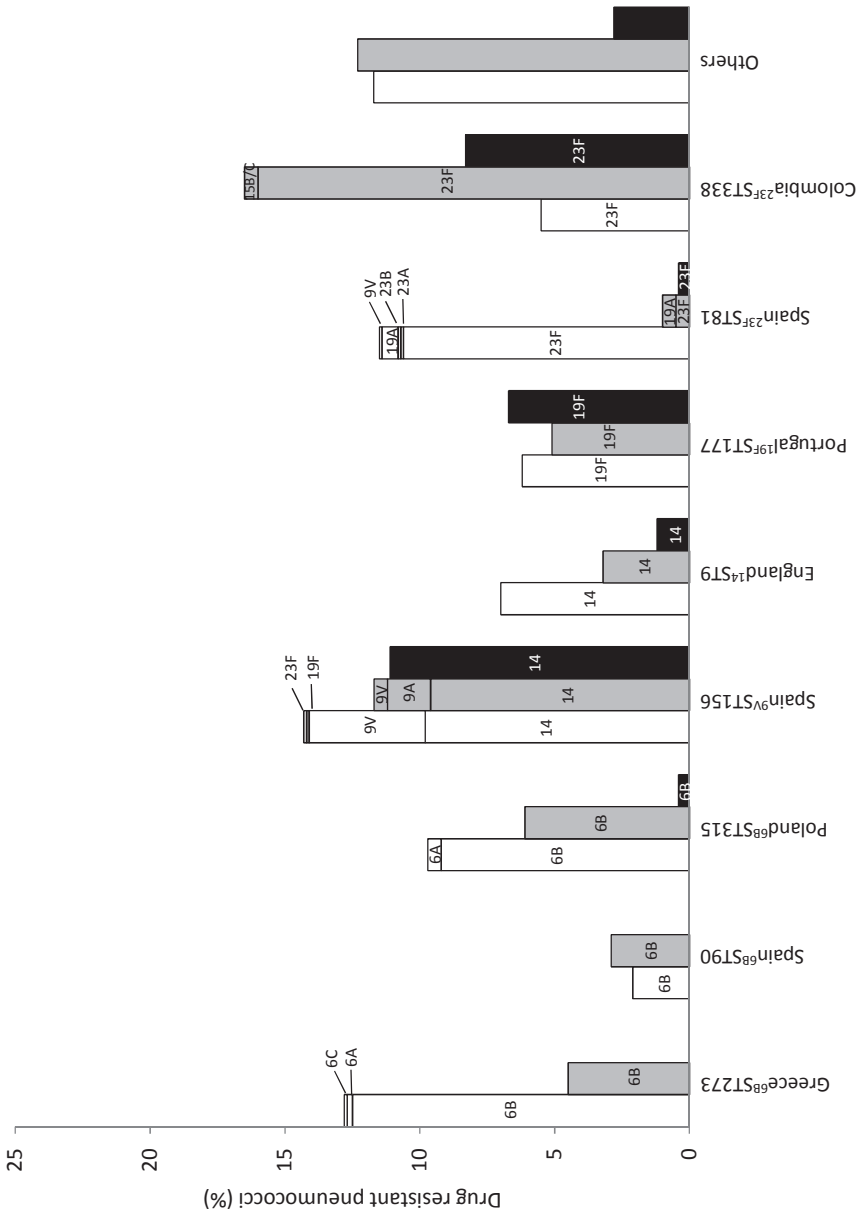

Figure 2A. Prevalence of drug-resistant PCV7 serotype clones over time. White bars, pre-PCV7 era (1996-2001); grey bars, low-PCV7 uptake (2002-2003); black bars, high-PCV7 uptake (2006-2007); the serotypes expressed by each clone are indicated.

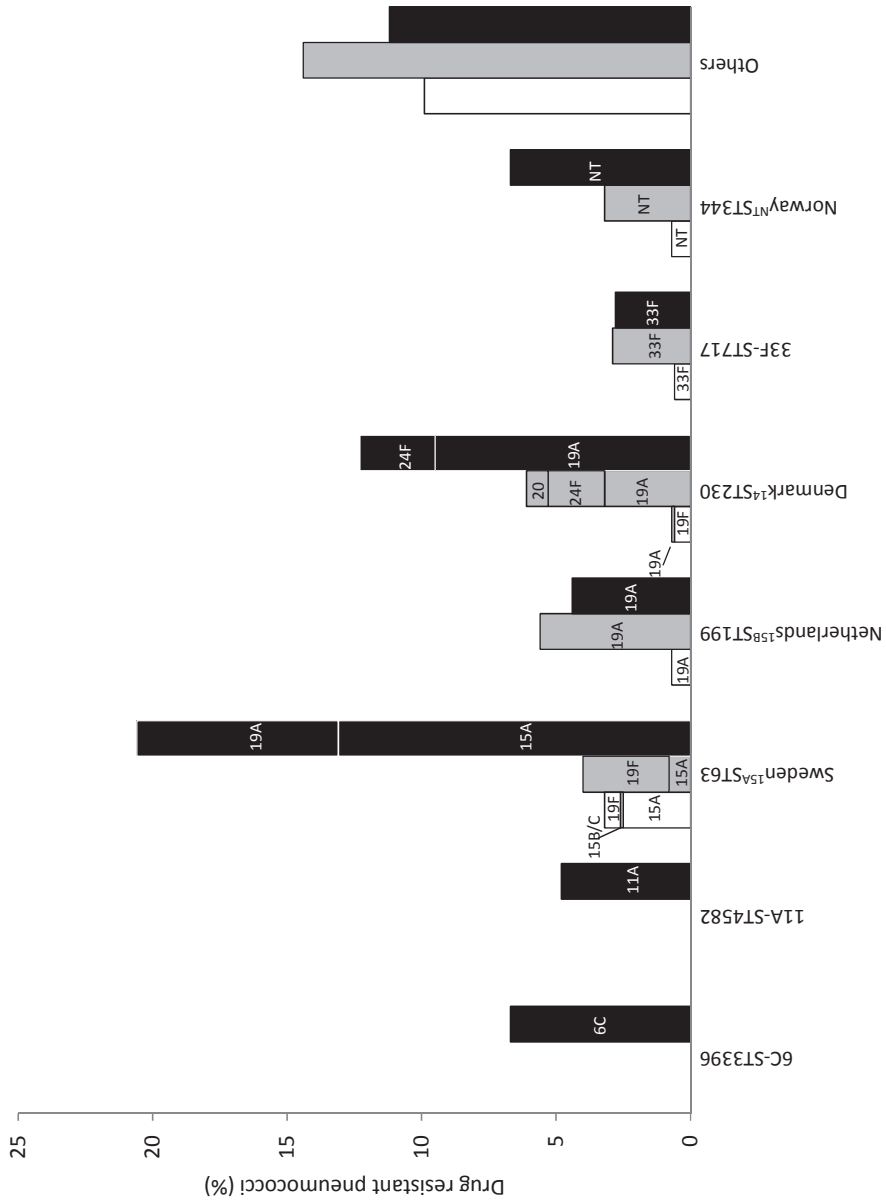

Figure 2B. Prevalence of drug-resistant non-PCV7 serotype clones over time. White bars, pre-PCV7 era (1996-2001); grey bars, low-PCV7 uptake (2002-2003); black bars, high-PCV7 uptake (2006-2007); NT, non-typeable pneumococci; the serotypes expressed by each clone are indicated.

Five of the seven most frequent non-PCV7 serotype clones found in 2006-2007 were already in circulation in the previous periods (Figure 2B). All increased in its prevalence among drug-resistant isolates following PCV7 introduction. In particular, three clones, Netherlands^{15B}ST199 (serotype 19A variant), 33F-ST717, and Norway^{NT}ST344 were always associated with the same serotypes over the three periods (19A, 33F, and NT, respectively). By contrast, for clones Sweden^{15A}ST63 and Denmark^{14}ST230, the serotypes most commonly expressed changed overtime (Figure 2B). In particular, serotype 19A accounted for a significant proportion of isolates from both clones in the era of high-PCV7 uptake. In addition, two new clones emerged in the high-PCV7 uptake period. Clone 6C-ST3396 was associated with serotype 6C and was multiresistant (resistant to macrolides, lincosamides, streptogramins, and tetracycline). Clone 11A-ST4582 was associated with serotype 11A and was resistant to tetracycline only (Figure 2B).

Capsular switch. Capsular variants of major DRPn clones have been observed in all three study periods (Figure 2). Although there have been changes in the dominant serotype expressed by a clone, we found no evidence for the emergence of novel capsular variants following introduction of PCV7. Although not shown in Figure 2B, serotype 19A variants of Sweden^{15A}ST63 were detected in 2001-2003 in other study conducted in Portugal (37).

DISCUSSION

In this study we described the variations observed in the population structure of drug-resistant colonizing pneumococci following widespread use of PCV7 in Portugal. The study was triggered by the observation that antimicrobial resistance rates remained essentially unchanged in the years following an increased use of PCV7 in Portugal despite the dramatic

decrease in the proportion of carriers of pneumococci expressing PCV7 types (30). This was, somehow, unexpected as in the pre-vaccine era most antimicrobial resistance was associated with PCV7 serotypes. Furthermore, this observation contrasted with data from other countries where introduction of PCV7 has been associated with a reduction not only in vaccine serotypes but also on antibiotic resistance prevalence (7, 34). Still, such declines have been mostly observed in countries that documented introduction of PCV7 with a concomitant reduction of antibiotic consumption. On the other hand, maintenance of antibiotic resistance levels among carried isolates in the PCV7 era has also been noted in studies from the USA (16, 26).

The mechanisms leading to the maintenance of rates of resistance to antimicrobials despite serotype replacement could be expansion of existing clones, capsular switch, introduction of new clones, and *de novo* acquisition of resistance. To determine which mechanisms were in place and the relative extent of each, molecular typing of pre- and post-PCV7 drug-resistant isolates was required.

We observed that expansion of drug-resistant clones such as Sweden^{15A}ST63 and Denmark^{14}ST230 (mainly expressing non-PCV7 serotypes 15A, 19A and 24F) was an important cause for the maintenance of antimicrobial resistance levels. These clones were already in circulation in Portugal in the years preceding introduction of PCV7 and in 2006-2007 accounted for 32.9% of all drug-resistant pneumococci. Both clones have been found elsewhere in recent years: in 2007, clone Sweden^{15A}ST63 (serotype 15A and 19A variants) was the fourth major clone found among young carriers from Massachusetts (15) and was the major clone (serotype 15A variant) recovered from disease in the United States during 2007 among non-susceptible penicillin isolates of non-PCV7 serotypes other

than 19A and 6A (13). Clone Denmark^{14}ST230 (expressing serotype 19A) has been detected in several countries such as France, Spain, and Israel (7, 8, 34). In Portugal, clone Denmark^{14}ST230 is expanding not only in colonization but also as a major cause of pneumococcal infections (1). Although to a less extent, expansion of clones Netherlands^{15B}ST199 (serotype 19A variant), Norway^{NT}ST344, and 33F-ST717 were also observed.

Even though capsular variants of PMEN drug-resistant clones became increasingly abundant in the PCV7 era, we found no evidence that PCV7 could have triggered or enhanced capsular switch events. Indeed, all capsular variants detected in 2006-2007, had been detected in studies preceding widespread use of PCV7. Of note, 19A variants currently thriving have genetic backgrounds originally associated not only with PCV7 types (as in the case of Denmark^{14}ST230) but also with non-PCV7 types (as in the case of Netherlands^{15B}ST199 and Sweden^{15A}ST63). Our interpretations regarding the time of capsular switch events were only possible due to the large collection of drug-resistant isolates from the pre-PCV7 era (1996-2001) that was available for comparison. On the other hand, one could argue that lack of detection of truly novel capsular switch events in the PCV7 era might be due to limitations in the number of samples analyzed in that period. Still, if that would have been the case those events should not have achieved a large magnitude, as they remained undetected in two consecutive years of study despite the large serotype and clonal diversity identified. Overall, our findings contrast with other studies, which suggested that serotype escape variants might have emerged in the USA following introduction of PCV7 (3, 15).

Introduction of new clones appeared to contribute to a small fraction (11.6%) of the drug-resistant population. In particular, a multidrug resistant

clone of serotype 6C (ST3396) was first detected in 2006-2007. A previous study, on the epidemiology of serotype 6C colonizing isolates in Portugal (22), did not identify this clone nor related susceptible counterparts in earlier collections, suggesting a novel introduction in the population. For the novel detection of a clone of serotype 11A clone (ST4582), resistant to tetracycline only, whether its presence results from a novel introduction in the population or *de novo* acquisition of resistance, is not clear, as the epidemiology of serotype 11A isolates in our population has not been systematically studied. In any case, *de novo* acquisition of resistance appears to have contributed very little to the maintenance of resistance levels in the era of PCV7 use.

Of note, close to one-third (31.0%) of the drug-resistant isolates were still of PCV7 serotypes in contrast with 1.5% among drug-susceptible isolates from the same time period (30), data not shown). This discrepancy is suggestive of a selective pressure that makes some drug-resistant PCV7 clonal types more refractory to extinction than its susceptible counterparts. Even if we have no definitive evidence for the nature of such pressure, which most likely is multifactorial, antibiotic use may play an important role. Although antimicrobial use has been declining in the populations we have been monitoring, it is still high: in 2006-2007, 18.2% of the participants had received antibiotics in the month preceding the sampling and 14.9% had received three or more courses of antibiotics in the previous six months (30), this study). Other studies have shown that outpatient antimicrobial consumption in Portugal has been declining since 2002 even though it is still high (22.61/1000 inhabitants/day in 2008) (http://app.esac.ua.ac.be/public) and a positive correlation between antimicrobial consumption and resistance has been described by several authors (10, 14, 28). However, this does not explain why some PCV7-type clones thrived while others of the same serotype (for instance,

Colombia^{23F}ST338 vs Spain^{23F}ST81) did not. We were also unable to identify a common resistant phenotype among those persistent clones that could explain their maintenance. Clearly, other factors, beyond the capsule, and the resistance determinants, which maybe concealed in the wider genome background of such clones, may play a role. Such factors may be related to increased fitness and transmission, and enhanced capacity to evade the host immune system or escape vaccine pressure (31).

Regarding the diversity of clones in circulation, we observed that it decreased among drug resistant pneumococci of PCV7 serotypes in the period of high-PCV7 uptake and remained stable among drug resistant pneumococci of non-PCV7 serotypes. Our observations are in general agreement with those described by Lipsitch et al. (18) that suggested that PCV7 acts as a "serotype filter" in the sense that the introduction of PCV7 did not change the diversity of the non-PCV7 serotype population.

In conclusion, with this study we were able to describe the mechanisms leading to maintenance of antimicrobial resistance among pneumococcal colonizing isolates in the PCV7 era. We observed that the group of clones that account for antimicrobial resistance since 1996 is essentially the same as the one identified in the PCV7 era. The relative proportions of such clones have, however, evolved substantially overtime. Widespread use of PCV7 led to an expansion of PMEN clones which express non-PCV7 capsular variants of the original strains. These variants were already in circulation in the pre-PCV7 era, although they have now become increasingly abundant. Emergence of novel clones and *de novo* acquisition of resistance seem to contribute little to the observed scenario. In the years to come, following the introduction of novel conjugate vaccines with expanded coverage, continuous surveillance of pneumococcal population is essential to evaluate serotype and clonal shifts that can shed light on the

vaccine's effect, guide future vaccine development, and increase our understanding on the mechanisms of evolution of the pneumococcal population.

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Chapter IV

Rapid identification of non-capsulated *Streptococcus pneumoniae* in nasopharyngeal samples allowing detection of co-colonization and re-evaluation of prevalence

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A. S. Simões was responsible by all experimental work except DNA extraction of the primary samples that was performed by C. Valente.

SUMMARY

Non-capsulated pneumococci are atypical *Streptococcus pneumoniae* that lack a capsule and therefore do not react with any available antisera. These isolates, which are often referred as non-typeable pneumococci (NTPn), 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. We developed a low cost and easy assay to detect and quantify NTPn in primary samples (which may contain multiple species) obtained from nasopharyngeal swabs. 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.* The application of the proposed methodology to over 500 nasopharyngeal samples found that the prevalence of NTPn in colonization was three-fold higher than estimated by routine methods (from 2.9% to 8.6% in the study collection). The international clone Norway^{NT}ST344 was the major clone identified.

INTRODUCTION

Streptococcus pneumoniae is a respiratory pathogen that colonizes asymptomatically the human nasopharynx, and can cause several diseases such as otitis media, sinusitis, pneumonia, septicemia, and meningitis (24).

Most pneumococci are shielded by a polysaccharide capsule that is considered to be the major virulence factor (1). The capsule can be detected by reaction with specific antisera. The capsular genes, in all serotypes but serotype 37, are located in the same region of the chromosome flanked by the *dexB* and *aliA* genes (2, 20). Non-typeable pneumococci (NTPn) are atypical isolates that do not react with any commercially available sera. The mechanisms underlying non-typeability can be diverse such as lack of capsule, mutations in the capsular locus

leading to absence of capsular production, or novel capsules. We have previously described a collection of over 200 NTPn obtained from asymptomatic carriers (29). In our collection, the overwhelming majority of the isolates (over 99%) were found to be non-capsulated. Other studies have also found that the so-called NTPn often lack a capsule (13, 14). For this reason, non-capsulated pneumococci are generally referred to as NTPn (in this paper both terms are used as synonyms). In non-capsulated pneumococci, Hathaway et al. described that a homologue of the *aliB* gene is found in the *dexB-aliA* capsule region (14).

Despite lacking a major virulence factor, non-capsulated pneumococci have been identified as disease causing agents. Non-capsulated pneumococci have been associated with large outbreaks of pneumococcal conjunctivitis (6, 8, 13) and, less frequently, with other disease manifestations including invasive disease (12, 13). Recently, NTPn were clearly identified as otopathogens (36).

The importance of NTPn in the context of pneumococcal ecology is not clear. However, it has been observed that NTPn clones may be in circulation for decades. In particular, a few lineages such as clone Norway^{NT}ST344 and lineages associated with multilocus sequence types (STs) 448 and 449 - are geographically widespread (13, 29). One study suggested NTPn could act as privileged intermediaries for horizontal gene transfer (HGT) between streptococcal species of the oral flora and capsulated pneumococci (15). Indeed, the lack of capsule is known to lead to higher transformation efficiency essential for horizontal gene transference (37). Other studies have shown that non-capsulated pneumococci have phenotypes that contribute to facilitate colonization such as higher adherence to epithelial cells and enhanced capacity for biofilm formation (9).

The identification of NTPn remains difficult as their differentiation from commensal viridans streptococci is not always straightforward and they do not yield a positive Quellung reaction (considered one of the gold standards to identify pneumococci) (35). In addition, isolates that are putatively identified as pneumococci on the basis of some analyses but that give negative results in one or more of the classical assays have been described and the term streptococcus of mitis group (SMG) was proposed to define them (19). The SMG is equivalent to the previously defined “Smit group” which includes not only pneumococci but also several other related species that comprise the *Streptococcus oralis-Streptococcus mitis* group. However, in this paper, we use the SMG designation in a more restricted form that refers to atypical isolates that resemble pneumococci in some of its properties and for which a species designation is difficult to attain. We therefore excluded from this designation all pneumococci and *S. pseudopneumoniae* isolates which could be clearly identified as such.

Several methods have been proposed to differentiate pneumococcus from these closely related species. The presence of *lytA* gene - a virulence factor ubiquitous in pneumococci (23) - and the absence of *cpsA* - a conserved pneumococcal capsular gene have been proposed to identify NTPn. However, *lytA* homologues have been detected in isolates of at least three other streptococcal species (10). More recently, Llull and co-workers described a *lytA*-RFLP strategy to differentiate pneumococcus from closely related species based on signatures characteristic of typical pneumococcal *lytA* or atypical (non-pneumococcal) *lytA* (19). This method correctly distinguished typical from atypical *lytA* present in a group of 17 out of 25 pneumococcus-like strains that harbored a *lytA* gene (33).

As NTPn identification remains often a challenge, these isolates are sometimes disregarded in epidemiological studies and their prevalence is

underestimated. Furthermore, the accurate identification of NTPn is essential not only for a correct diagnosis but also, for colonization studies (35). For example, we recently described a collection of isolates recovered from nasopharyngeal samples that had characteristics similar to NTPn such as colony morphology, bile solubility, lack of capsule and presence of some "typical" pneumococcal virulence factors. However, after a detailed molecular analysis that included multilocus sequence analysis and comparative genomic hybridization, the isolates were identified as SMG (33). The misidentification of these isolates as NTPn would have resulted in reporting a significant increase on the rates of pneumococci non susceptible to antimicrobial agents as SMG isolates were mostly multidrug resistant and exhibited high penicillin MICs. Thus, it is important to develop easily deployable methodological strategies to correctly identify NTPn and distinguish them from closely related species.

In this work, as part of an ongoing effort to understand the role of NTPn in the wider context of pneumococcal ecology, we developed a novel strategy to detect NTPn that can be easily implemented in most diagnostic laboratories. This strategy enables the detection of NTPn in mixed samples that may contain more than one pneumococcal strain as well as other oral streptococci (among other commensals). The application of the proposed methodology to a large collection of nasopharyngeal samples enabled us to estimate more accurately the prevalence of NTPn in the carrier population. Finally, we determined the molecular types associated to NTPn.

MATERIAL AND METHODS

Study samples. The samples used in this study are from collections existing in the laboratory from 2001, 2006, and 2007 (32). Briefly, nasopharyngeal samples were collected from children (aged 18 months to 5 years old) attending day care centers in Oeiras, Portugal, as previously

described (30). The swabs were inoculated in a primary isolation selective plate (hereafter designated “primary plate”) of trypticase soy agar (TSA) containing 5% defibrinated sheep blood and 5 µg/ml gentamicin. Plates were incubated overnight in anaerobic jars at 37℃ to select and identify *S. pneumoniae*. The following day, one presumptive pneumococcal colony was selected for culture and subsequent characterization. Next, all visible bacterial growth was collected from the primary plate and frozen at –80ºC in Mueller-Hinton broth containing 15% (vol/vol) glycerol (hereafter designated “primary sample”). For the current study, 508 primary samples were selected.

DNA preparation. DNA was extracted from 200 µl of the ”primary sample” using a commercial kit (High Pure PCR Template Preparation Kit, Roche, Germany) according to the manufacturer’s instructions.

Multiplex PCR for presumptive identification of NTPn. A multiplex PCR was designed to detect internal fragments of four genes: *cpsA,* a conserved pneumococcal capsular gene (2); *lytA*, a virulence factor ubiquitous in pneumococci that is often used as an identification marker of this species (23); *aliB*-like ORF2, a gene described as present in the capsular region of non-typeable pneumococci (14); and 16S rDNA as a positive internal control. The primer sequences and expected product sizes are shown in Table 1.

The multiplex PCR was done in a 10 µl volume with 1× GoTaq Flexi Buffer (Promega, Madison, USA), 2.5 mM of magnesium chloride solution (Promega, Madison, USA), 0.12 mM of dNTPs Mix (2 mM each) (Bioron, Germany), 1 U/µl of GoTaq DNA Polymerase (Promega, Madison, USA) and 0.5 *p*mol/µl of each primer. As DNA template, 1 µl of DNA extracted from the primary sample was used. Alternatively, when testing pure

cultures, a colony was picked with a yellow tip and directly submerged in the PCR mix. PCR reaction was performed with the following conditions: a pre-denaturation step of 4 min at 94ºC; 35 cycles at 94ºC for 30 s, 52ºC for 45 s, and 72ºC for 1 min; a final extension step of 5 min at 72ºC; and soaking at 16ºC. PCR products were analyzed by electrophoresis on 2% Seakem LE agarose gels (BMA, Rockland, Maine, USA) in 0.5× Tris-borate-EDTA buffer (Biorad, Hercules, Califórnia, USA) at 5.2 V/cm for 2 hours.

Table 1. Primers used in the multiplex PCR for presumptive identification of NTPn.

Gene	Primer Name	Primer Sequence	Size	Ref
aliB-like ORF2	104_FI3.6	AGATGCCAAATGGTTCACGG	290 bp	(14)
	104_b832.10	GAAACTCTTCGTTTACTGGG		
lytA	*lytA* 1145	AATCAAGCCATCTGGCTCTA	395 bp	(23)
	lytA 750	GGCTACTGGTACGTACATTC		
16S rDNA	16S rDNA F2	ACATGCTCCACCGCTTGTG	522 bp	This study
	16S rDNA R2	GCTCTGTTGTAAGAGAAGAACG		
cpsA	*cpsA* F2	AGCAGTTTGTTGGACTGACC	613 bp	This study
	cpsA R2	GTGTGAATGGACGAATCAAC		

Control strains. To evaluate the specificity of the multiplex PCR a panel of 175 pneumococci and several streptococcal species were tested. In particular, we tested 29 NTPn and 146 capsulated strains of the following serotypes: 7A, 8, 12A, 12B, 20, 24B, 33B, and 39 (1 strain each); 1, 4, 6A, 6B, 7F, 9A, 9L, 9N, 9V, 12F, 15A, 15B, 15C, 15F, 17, 18A, 18B, 18C, 18F, 19A, 21, 22F, 23A, 23B, 24F, 29, 31, 33F, 34, 37, and 42 (2 strains each); 10A, 16F and 35F (4 strains each); 3 and 11A (5 strains each); 14, 19F and 23F (6 strains each); and 38 (9 strains). In addition, we tested representative strains of 27 Pneumococcal Molecular Epidemiology Network (PMEN) clones (http://www.sph.emory.edu/PMEN/index.htm): Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, Tennessee^{23F}-4, Spain^{14}-5, Hungary^{19A}-6,

South Africa^{19A}-7, South Africa^{6B}-8, England^{14}-9, CRS^{14}-10, CRS^{19A}-11, Finland^{6B}-12, South Africa^{19A}-13, Taiwan^{19F}-14, Taiwan^{23F}-15, Poland^{23F}-16, Maryland^{6B}-17, Tennessee^{14}-18, Colombia^{5}-19, Poland^{6B}-20, Portugal^{19F}-21, Greece^{6B}-22, N.Carolina^{6A}-23, Utah^{35B}-24, Sweden^{15A}-25, Colombia^{23F}-26, Portugal^{6A}-41. Strains of other streptococcal species are detailed in Table 2. All strains belong to the bacterial collection of Laboratory of Molecular Genetics, Instituto de Tecnologia Quimica e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal. *Streptococcus pseudopneumoniae* type strain (ATCC BAA-960) was kindly supplied by Maria de Gloria Carvalho, Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, USA.

***lytA* RFLP signatures.** *lytA* gene was amplified by PCR using the primers described by Obregon *et al.* (25) - LA5_Ext (5' AAGCTTTTTAGTCTGGGGTG 3') and LA3_Ext (5' AAGCTTTTTCAAGACCTAATAATATG 3') - which yield a PCR product of approximately 1,200 bp encompassing the entire *lytA* gene. RFLP signatures characteristic of typical pneumococcal *lytA* or atypical *lytA* were determined as described before by digesting the PCR product with BsaAI and separating the fragments by agarose gel electrophoresis (19).

Capsular typing. Capsular assignment of pure cultures of pneumococci isolated from single colonies was performed by a combination of multiplex PCRs targeting serotypes 1, 3, 6A, 6B, 6C, 7F, 9N/L, 9V, 10A, 11A, 14, 15A, 15B/C, 16F, 17F, 18A/B/C/F, 19A, 19F, 22F, 23A, 23F, 25A/25F/38, 31, 33F/33A/37, 34, and 35F using primers previously described (4, 27); www.cdc.gov). Strains that could not be typed by this method were serotyped by the Quellung reaction using specific antisera (Statens Serum Institute, Copenhagen, Denmark) (34).

Antimicrobial susceptibility testing. MICs to penicillin were determined by Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. The interpretation criteria used were <0.1 µg/ml, ≥0.1 and ≤1 µg/ml, and ≥2 µg/ml for classification of susceptibility, intermediate resistance, and resistance to penicillin, respectively. Testing of susceptibility to erythromycin, clindamycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim (SXT) was performed by disk diffusion, according to the recommendations and definitions of the Clinical and Laboratory Standards Institute (7).

Multilocus sequence typing (MLST). Amplification of internal fragments of the seven housekeeping genes – *aroE*, *gdh*, *gk*i, *recP*, *spi*, *xpt*, and *ddl* - was done according to the MLST scheme developed by Enright and Spratt for *S. pneumoniae* (11). Sequencing reactions were conducted at Macrogen, Inc. (Seoul, Korea). Sequencing analysis was done with DNAStar (Lasergene). Allele number assignment was done at the international MLST database for *S. pneumoniae* (www.mlst.net).

Table 2. Control strains used to validate the multiplex PCR.

Species tested	No. strains tested	*cpsA*	16S rDNA	*lytA*	*aliB*-like ORF2
Streptococcus pneumoniae					
Representatives of 49 capsules[a]	137	pos	pos	pos	neg
Non-typeable	29	neg	pos	pos	pos
Serotype 38	9	neg	pos	pos	pos
Other streptococcal species					
Streptococcus gordonii (DSM 20568, DSM 6777)	2	neg	pos	neg	neg
Streptococcus infantis (DSM 12492)	1	neg	pos	neg	neg
Streptococcus mitis (DSM 12643)	1	neg	pos	neg	neg
Streptococcus parasanguinis (DSM 6778)	1	neg	pos	neg	neg

Table 2. Continued.

Species tested	No. strains tested	*cpsA*	16S rDNA	*lytA*	*aliB*-like ORF2
Streptococcus peroris (DSM 12493)	1	neg	pos	neg	neg
Streptococcus sanguinis (DSM 20567)	1	neg	pos	neg	neg
Streptococcus sinensis (DSM 14990)	1	neg	pos	neg	neg
Streptococcus vestibularis (DSM 5636)	1	neg	pos	neg	neg
Streptococcus oralis (DSM 20066, DSM 20627, DSM 20397, DSM 20395)	4	neg	pos	neg	neg
Streptococcus cristatus (DSM 8249)	1	neg	pos	neg	neg
Streptococcus pseudopneumoniae (ATCC BAA-960, PT5479)	2	neg	pos	neg	pos
Streptococcus sobrinus (NCTC 10921)	1	neg	pos	neg	neg
Streptococcus equinus (NCTC 10389)	1	neg	pos	neg	neg
Streptococcus intermedius (NCTC 11324)	1	neg	pos	neg	neg
Streptococcus constellatus (NCTC 11325)	1	neg	pos	neg	neg
Streptococcus bovis (DSM 20480)	1	neg	pos	neg	neg
Streptococcus agalactiae (DSM 6784, DSM 2134)	2	neg	pos	neg	neg
Streptococcus anginosus (DSM 20563)	1	neg	pos	neg	neg
Streptococcus canis (DSM 20715)	1	neg	pos	neg	neg
Streptococcus dysgalactiae subsp. *dysgalactiae* (DSM 20662)	1	neg	pos	neg	neg
Streptococcus dysgalactiae subsp. *equisimilis* (DSM 6176)	1	neg	pos	neg	neg
Streptococcus equi subsp. *zooepidemicus* (DSM 20727)	1	neg	pos	neg	neg
Streptococcus mutans (DSM 20523)	1	neg	pos	neg	neg
Streptococcus pyogenes (DSM 20565)	1	neg	pos	neg	neg
Streptococcus salivarius (DSM 20560)	1	neg	pos	neg	neg
Streptococcus of the mitis group (33)	22	21 neg, 1 pos	pos	18 neg, 4 pos	pos

[a]Details on the specific capsules tested are on the material and methods section; pos, positive; neg, negative.

RESULTS

Multiplex PCR for presumptive identification of NTPn. A multiplex PCR was optimized and implemented with the conditions described in the above section. To validate the specificity of the multiplex PCR, we tested 228 isolates that included capsulated pneumococci, non-typeable pneumococci, representatives of 25 other streptococcal species and a collection of 22 SMG recently described by us (33). The results obtained are summarized in Table 2. All isolates were positive for the 16S rDNA gene used as an internal positive control. Capsulated pneumococci were positive for *lytA* and *cpsA* genes. All non-typeable pneumococcal strains were positive for *lytA* and *aliB*-like ORF2 genes and negative for *cpsA* gene. All pneumococci of serotype 38 tested had the same amplification products as non-typeable strains (the same result would be expected if isolates of serotypes 25A/25F would have been tested, (22)). Isolates of other species of streptococci were negative for *lytA*, *cpsA* and a*liB*-like ORF2 genes. The only exception was the two *S. pseudopneumoniae* isolates that were positive for *aliB*-like ORF2. Likewise, the SMG were all positive for *aliB*-like ORF2 and most were negative for *cpsA* (21 out of 22) and for *lytA* (18 out of 22).

The multiplex PCR was then applied to a set of mixed cultures that contained NTPn, capsulated pneumococci, *S. pseudopneumoniae*, and SMG. The multiplex PCR could amplify all expected products in all mixed samples (data not shown).

Thus, the multiplex PCR was useful to presumptively identify NTPn (defined as non-capsulated pneumococci lacking the capsular operon) in pure cultures as the patterns obtained clearly distinguished NTPn from other pneumococci and most other streptococcal species. Still, exceptions were found for pneumococci of serotype 38, *S. pseudopneumoniae*, and

some SMG (Figure 1A). The assay was also useful to detect the potential presence of NTPn in mixed samples (Figure 1B).

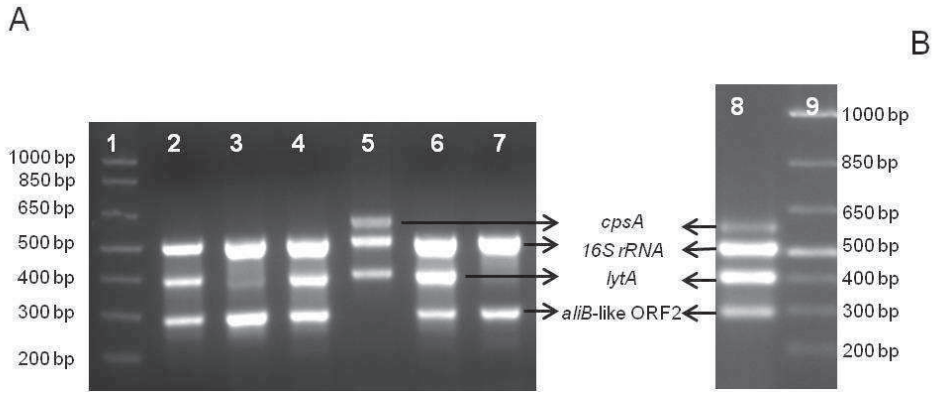

Figure 1. Multiplex PCR to presumptively identify NTPn. A, Multiplex PCR applied to pure cultures. Lanes: 1 - molecular weight ladder, 2 – NTPn, 3 and 4 - streptococcus of the mitis group, 5 – *S. pneumoniae* of serotype 14, 6 – *S. pneumoniae* of serotype 38; 7 - *S. pseudopneumoniae.* B, Multiplex PCR applied to a mixed sample. Lanes: 8, sample containing capsulated pneumococci (serotype 19A) and NTPn; 9, molecular weight ladder.

Identification of NTPn in primary samples. To estimate the prevalence of NTPn in nasopharyngeal samples, DNA was extracted from the 508 primary samples known to contain pneumococci (as a pure culture of pneumococci had been obtained from picking a single colony). The samples were tested with the multiplex PCR described above. Most samples (431, 84.8%) contained only capsulated pneumococci as only three bands corresponding to 16S rDNA, *lytA* and *cpsA* were obtained. The remaining 77 samples yielded at least three bands corresponding to 16S rDNA, *lytA*, and *aliB*-like ORF2 presumptively detecting the presence of NTPn. Of these, 56 also yielded a band corresponding to *cpsA*, indicating the presence of capsulated pneumococci as well (Figure 2).

To exclude samples that might contain the *aliB*-like ORF2 due to the presence of serotypes 25A/25F/38, total DNA was used to detect the *wzy* capsular gene using specific 25A/25F/38 primers previously described (27). Five samples were positive and these were confirmed by the Quellung reaction to correspond to serotype 38.

To distinguish NTPn from closely related streptococcal species such as *S. pseudopneumoniae* and SMG (which may contain *lytA* atypical alleles and *aliB*-like ORF2 genes) in the remaining 72 samples, the *lytA*-RFLP strategy proposed by Llull *et al*. was applied (19). Total DNA was used to amplify a 1200 bp product encompassing the entire *lytA*, and RFLPs were generated as previously described by digesting the amplification product with BsaAI. Three banding patterns were obtained depending on whether the sample contained only pneumococci (RFLP pattern of typical *lytA*), only other streptococci (pattern of atypical *lytA*), or a mix of both (both patterns) (Figures 2 and 3).

A single typical pneumococcal *lytA* pattern was obtained in 44 samples, 29 of which were *cpsA*$^+$ in the multiplex PCR reaction suggesting that these latter group contained capsulated pneumococci in co-colonization with NTPn. The remaining 15 samples seemed to contain only NTPn.

An atypical *lytA* pattern was obtained in one sample suggesting it did not contain pneumococci and contained a closely related species. Finally, a mixed RFLP pattern indicative of the presence of both typical and atypical *lytA* was obtained in 27 samples - all of which were *cpsA*$^+$ in the multiplex – suggesting these samples contained capsulated pneumococci in co-colonization with other closely related species (Figure 2).

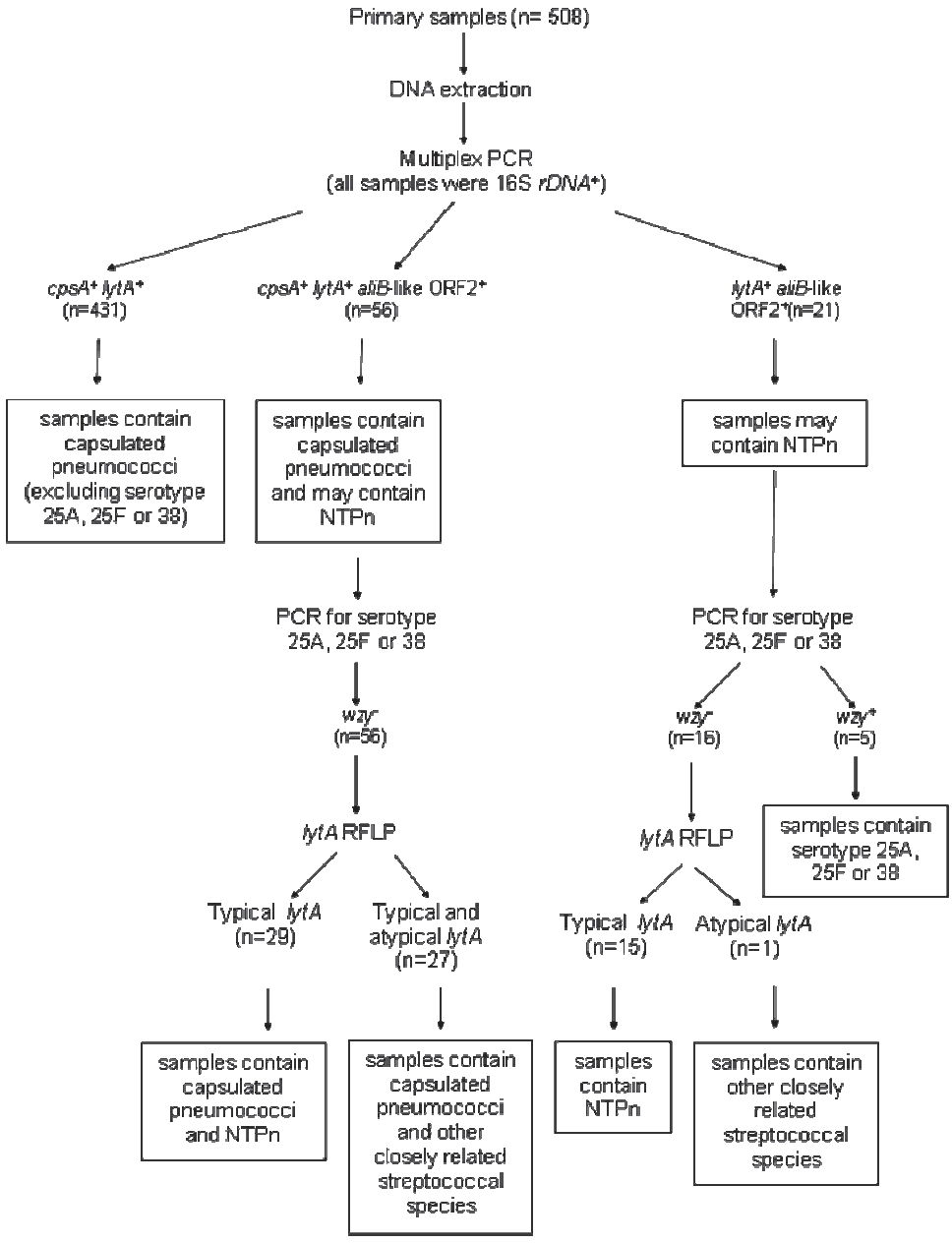

Figure 2. Experimental strategy to identify NTPn in primary nasopharyngeal samples.

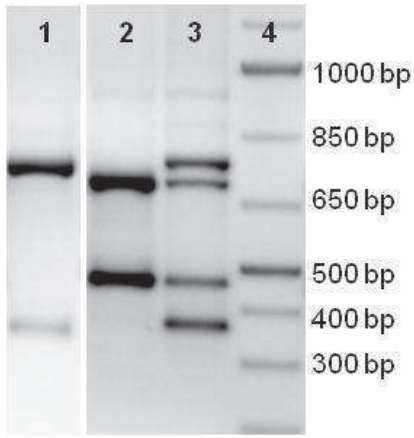

Figure 3. BsaAI-*lytA* RFLPs. Lanes: 1 - atypical *lytA* yields DNA fragments of 362 and 851 bp; 2 - typical *S. pneumoniae lytA* yields DNA fragments of 452 and 761 bp; 3 - sample containing typical and atypical *lytA* alleles; 4 - molecular weight ladder.

Overall, among the 508 primary samples, NTPn were identified in 44 samples (8.7%) of which 29 were in co-colonization with a capsulated strain. The capsulated strains found in co-colonization with a NTPn strain were very diverse: 19A (4 samples), 14 and 19F (3 samples each), 1, 6B, 6C, 9L, 11A, and 35F (2 samples each), and 6A, 15B/C, 16F, 21, 23F, 33, and 37 (1 sample each).

Isolation of NTPn from primary samples. After identification of 44 NTPn in primary samples we aimed to further characterize such strains by molecular typing techniques. For 15 samples a NTPn pure culture had been obtained when a single colony was picked from the primary plate for growth and further characterization. These were the same samples from where a capsulated strain or closely related species could not be identified (Figure 2). For the other 29 samples it was necessary to isolate the NTPn strain from the primary sample that contained a capsulated strain as well.

To isolate the NTPn strain from those samples, aliquots of the primary sample frozen stock were thawed, serial dilutions were prepared and plated in blood agar plates. Individual colonies – at least 96 – were picked and tested with the multiplex PCR described above for the presence of *lytA* and *aliB*-like ORF 2. A NTPn strain could be isolated from nine primary samples. For the remaining 20 samples, no NTPn could be isolated upon testing 96 colonies. Huebner and co-workers, have shown that the number of colonies that need to be examined to have a 95% of chance of detecting a serotype which abundance is 5%, is 59 (18). Similarly, by testing 96 colonies we would have had a 95% chance of detecting a less common serotype if its proportion was 3% or higher. Our results suggest that for the 20 primary samples from which NTPn could not be recovered, the NTPn strain was present in very low abundance. Alternatively, it might be possible that the NTPn strain was no longer viable in the frozen stock but its DNA was still detectable.

Population structure of NTPn isolates. Of the 44 NTPn detected in primary samples, we were able to obtain pure cultures for 24 strains. These were characterized by antimicrobial susceptibility testing and multilocus sequence typing. Table 3 summarizes the results obtained.

Rates of non-susceptibility to penicillin were very high (83.3%); a single isolate had a MIC≥2 µg/ml. Macrolide resistance was detected in 87.5% of the isolates (75.0% had the MLS_B phenotype and 12.5% had the M phenotype). Rates of resistance to other antimicrobial agents were 87.5% to tetracycline and 79.2% to SXT. No resistance to chloramphenicol was detected. Multidrug resistance defined as resistance to three or more classes of antibiotics was detected in 87.5% of the isolates.

Table 3. Population structure of NTPn.

MLST	MLST alleles							Most common resistance pattern[a]	No. of samples
	aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*		
344	8	37	9	29	2	12	53	Pen (I), Ery, Da, Tet, SXT	16
448	8	5	2	27	2	11	71	susceptible	3
888	8	74	19	15	6	40	26	Pen, Ery, Tet, SXT	1
1156	2	13	2	29	91	19	59	Pen (I), Ery, Da, Tet, SXT	1
4583	2	5	2	29	91	19	49	Pen (I), Ery, Da, Tet	2
5220	8	38	9	29	237	12	53	Pen (I), Ery, Tet, SXT	1

[a]Pen, penicillin; Ery, erythromycin; Da, clindamycin; Tet, tetracycline; SXT, sulfamethoxazole-trimethoprim; (I), intermediate resistance to penicillin (MIC >0.1µg/ml and <2µg/ml)

Six STs were obtained by MLST: ST344 (associated with 16 isolates), ST448 (3 isolates), ST4583 (2 isolates). STs 888, 1156 (SLV of ST4583), and ST5220 (SLV of ST344) were associated with a single isolate each. In other words, most strains were representative of PMEN (www.sph.emory.edu/PMEN/) international clone Norway^{NT}ST344 (n=17), and clone USA^{NT}ST448 was also detected (n=3).

DISCUSSION

Conventional culture based techniques are biased to detect the most abundant serotype in a sample. Although co-colonization of pneumococcal strains has long been known to occur (17, 31), in routine surveillance studies, a single pneumococcal colony is picked (and characterized) from the primary culture. Since NTPn are smaller and dryer than capsulated pneumococci, resembling viridans streptococci, they are less prone to be picked from a primary plate and therefore, tend to be underestimated and poorly studied (21, 29). Although an antiserum (anti-R) specific for non-encapsulated *S. pneumoniae* was described and frequently used five decades ago, it is not commercially available (26, 28).

In this paper we have developed a method to detect and quantify non-typeable pneumococci in primary cultures obtained by plating nasopharyngeal samples in a blood agar selective medium containing gentamycin. The multiplex PCR proposed here - targeting *cpsA*, *lytA*, and *aliB-like ORF2* genes - correctly identified capsulated and non-capsulated pneumococci and excluded most streptococcal species. However, due to the high plasticity of pneumococci and closely related species such as *S. pseudopneumoniae* and SMG, the same genes that are present in NTPn were, at times, detected in these related bacteria (19, 33). We used the *lytA*-RFLP strategy to distinguish NTPn (which carry a typical *lytA* allele) from *S. pseudopneumoniae* and SMG. In addition, we distinguished serotypes 25A/25F/38 (that also carry an *aliB*-like ORF2 gene) (2, 22) from NTPn by a simple uniplex PCR with primers specific for *wzy* from capsular types 25A/25F/38 (27).

In our study, we were able to detect NTPn strains in 8.7% of the 508 samples, most of which (n=29) were in co-colonization with a capsulated strain. This represents a three-fold increase (from 2.9% (n=15)) in the prevalence of NTPn compared to what had been estimated by analyzing a single colony from each primary sample. In other words, not only the prevalence of NTPn was found to be significantly higher than routinely estimated ($p<0.001$), but also most NTPn strains were found in co-colonization with a capsulated strain. In line with our observations, other studies have found non-typeable pneumococci often as a co-colonizer (5, 21, 29).

These observations suggest that NTPn, although mostly avirulent, must play a role in the context of pneumococcal ecology. Of note, most strains in our study were multidrug resistant and as co-colonization provides an opportunity for horizontal gene transfer (HGT), NTPn strains can serve as

donors of resistance determinants to capsulated strains. Evidence for this directional HGT was previously described by Hauser *et al*., that described that an international serotype 19F clone had acquired high-level resistance to penicillin by uptake of gene fragments from a NTPn ST344 strain (15). In addition, Hiller *et al.* have recently reported on the characterization of horizontal gene transfer events occurring during a polyclonal pneumococcal infection that led to the identification of a NTPn strain as the most likely donor of DNA to a capsulated strain (16).

A limitation of our study is that we were able to isolate the NTPn strain from only nine of the 29 primary samples from where NTPn were detected. Although we screened at least 96 colonies from each primary sample, we were often unable to isolate the NTPn strain. This could be due to its presence in a low abundance (<3%) (18) or alternatively, NTPn might have lost viability in the sample. Indeed, successive freezing and thawing of samples may alter the composition of the primary sample (that may contain multiple species).

The molecular typing of the NTPn strains isolated revealed essentially the same landscape previously described by us when a collection of over 200 NTPn colonizing samples from Portugal was characterized (29): the majority of the isolates (70.8%) were representatives of clone Norway^{NT}ST344 which has been detected in several other countries (3).

In conclusion, in this paper we describe a simple and low cost strategy to detect and identify NTPn isolates in pure cultures or samples containing multiple species. The application of this method to a large collection of colonization samples revealed that the prevalence of NTPn is significantly higher than previously estimated. In our sample, most NTPn strains belong to the multidrug resistant international clone Norway^{NT}ST344. Further

studies on the ecological role of NTPn are needed especially in the era of multivalent pneumococcal conjugate vaccines which do not target this group of strains.

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Chapter V

Concluding Remarks

Concluding Remarks

The introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) led to a massive shift in the human pneumococcal nasopharyngeal flora. Since 1996, our group has been monitoring the pneumococcal colonization of children attending day care centers in Portugal.

In a previous study, we observed that antimicrobial resistance rates remained essentially unchanged in the years following the introduction of PCV7 in Portugal, despite the dramatic decrease in the proportion of PCV7 serotypes among carriers (16). In order to find the mechanisms that led to the maintenance of antimicrobials resistance rates, we performed the study described in chapter III.

We observed that widespread use of PCV7 led to the expansion of clones which, despite having been described associated with PCV7 serotypes, now express mostly non-PCV7 capsular variants. These variants were previously in circulation in the pre-PCV7 era, although they have become increasingly abundant after several years of use of PCV7. Still, we found no evidence that PCV7 could have triggered or enhanced capsular switch events. Introduction of new clones appeared to have contributed to just a small fraction of the drug-resistant population.

The expansion of existing clones, as the major mechanism for the maintenance of antimicrobial resistance rates, has also been observed in other countries. In 2007, in a study from Massachusetts, a rapid outgrowth of non-vaccine clones previously present in the population was observed (5). Similar behavior was observed in other regions of the United States of America (15) and in Norway (19).

Our continuous interest on the impact of PCV7 in colonization, in the rates of antimicrobial resistance, and in the characterization of non-typeable pneumococci (NTPn) has led to the study described in chapter II. In 2006, we observed a sudden increase in the prevalence of penicillin resistance from 1.7% to 5% over a period of three years with several isolates displaying unusually high MICs to penicillin (2-6 µg/ml). In addition, some of these isolates were optochin-resistant, bile soluble and did not agglutinate with anti-pneumococcal capsular antibodies.

Further attempts to characterize these isolates by PCR, screening for *lytA* and *ply* were not conclusive, as most strains appeared to have some form of *lytA* and *ply*. DNA fingerprinting by pulsed field gel electrophoresis indicated that the strains were genetically diverse. Comparative genome hybridization (CGH) and multi-locus sequence analyses (MLSA) showed that the atypical strains were distinct from true pneumococci. However, CGH also showed that a considerable part of the proposed pneumococcal core genome (70% on average) is conserved in these isolates.

Various studies have been published describing strains distinct from pneumococci but that cannot be easily resolved from them by phenotypic (optochin susceptibility and bile solubility) or molecular methods (presence of *ply*, *psaA*, and *lytA)* (6, 9, 11, 12, 14, 18, 20).

The origin of these atypical isolates has been discussed. Killian *et al*. (10) proposed a pneumococcus-like bacterium with all the properties associated with virulence as the ancestor of the *pneumoniae-mitis-pseudopneumoniae* group. The streptococci currently in circulation would have evolved from this ancestor by genome reduction, which

would explain the presence of pneumococcal virulence genes in atypical strains. The group of Regine Hakenbeck, in Germany, suggested that horizontal gene transfer between naturally competent streptococci that share the same ecological niche significantly contributes to attenuate of putative barriers between these species resulting not only in mosaic genes, but also in mosaic genomes. This is the case of the genome of *S. mitis* B6 (a high level penicillin and multiple antibiotic resistant isolate) that shares a core genome of over 900 genes (43% of the total *S. mitis* genome) with *S. pneumoniae* (3, 4). This latter hypothesis is also advocated by other authors (1, 6, 7).

In our studies, the detection of these isolates in 2006 only, could possibly be explained due to our increased interest in non-typeable strains, which has led to increased isolation and preservation of atypical cultures. In addition, changes in the nasopharyngeal ecosystem due to the introduction of the PCV7 could have facilitated colonization by streptococcus of the mitis group, through increasing its abundance in the population.

Having in mind that routinely used tests to identify pneumococci may not be sufficient when atypical isolates are found, and that it is important to correctly identify pneumococci, in chapter IV we described a method to detect and quantify non-typeable pneumococci (NTPn) in primary cultures based on a multiplex PCR (targeting *cpsA*, *lytA*, and *aliB*-like ORF2 genes) that correctly identified capsulated and non-capsulated pneumococci and excluded closely related streptococcal species.

We observed a three-fold increase in the estimated prevalence of NTPn compared to what had been determined by analyzing single

colonies from primary samples by routine methods. We also observed that most NTPn strains were found in co-colonization with a capsulated strain. In line with our observations, other studies have found non-typeable pneumococci often as co-colonizers (2, 13, 17).

These observations suggest that NTPn, although mostly avirulent, must play a role in the context of pneumococcal ecology. Of note, most NTPn strains in our study were multidrug resistant and, as co-colonization provides an opportunity for horizontal gene transfer, NTPn strains can serve as donors of resistance determinants to capsulated strains as suggested by Hauser *et al* (8).

Overall, the studies performed in this thesis have contributed to a better knowledge of the epidemiology of drug resistant pneumococci in the era of conjugate vaccines, and highlighted not only the importance of the correct identification of pneumococci, but also the existence of pneumococcus-like streptococci in the ecology of the nasopharynx which are difficult to identify using currently accepted assays. Furthermore, the method described in chapter IV is a rapid and efficient assay that allows us to distinguish non-typeable pneumococci from other streptococci of the mitis group and study patterns of co-colonization of these isolates.

Still, several questions remain to be answered in the context of the work developed in this thesis. Further studies should involve:

(i) Continuous surveillance on the impact of novel pneumococcal conjugate vaccines (PCV10 and PCV13), not only in pneumococcal serotypes and levels of antimicrobial resistance, but also in the emergence of closely related species.

(ii) Studies aimed to establish the importance of pneumococcal-like streptococci in the ecology of the nasopharynx and clarify their ability to cause disease. In particular, studies involving transformability rates and ability to uptake DNA from *S. pneumoniae* should be performed. Regarding the ability to cause disease, animal models of infection with pneumococcus-like streptococci should be done.

(iii) Further characterization of NTPn isolates, especially to test the capacity of its genetic background to cause invasive disease (using animal models); and to compare their genomes with capsulated strains (using for example DNA microarray technology).

Finally, the studies described in this thesis increased our knowledge on the ecology of the nasopharynx in the era of pneumococcal conjugate vaccines. In addition, the two studies about non-typeable pneumococci and pneumococcus-like streptococci should be the baseline for future research about how these atypical isolates contribute to the pneumococcal evolution.

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