UNIVERSIDADE DE LISBOA
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MOLECULAR EPIDEMIOLOGY OF *STREPTOCOCCUS PNEUMONIAE*: IMPACT OF PCV7 IN THE PNEUMOCOCCAL POPULATION RESPONSIBLE FOR INVASIVE PEDIATRIC INFECTIONS

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SUMMARY

Keywords: *Streptococcus pneumoniae*, PCV7, clones, pili

Streptococcus pneumoniae is considered to be one of the major pathogens worldwide in the pediatric care setting. A variety of antimicrobial agents is used to treat pneumococcal infections, but the recent increase and worldwide dissemination of strains resistant to the main antimicrobials used, namely β-lactams and macrolides, is difficulting the treatment of infections. With the purpose of reducing the burden of invasive pneumococcal disease (IPD) and the dissemination of resistant strains, a seven-valent conjugate vaccine (PCV7), containing the polysaccharides of the seven most prevalent serotypes in the USA, was licensed first in USA and then in other countries. The efficacy of this vaccine has been demonstrated in the USA, but due to significant variations in terms of prevalence of infection, serotype distribution and antimicrobial resistance, the usefulness of this vaccine in other countries is compromised.

The aim of this work was to evaluate the potential impact of PCV7 in Portugal, which has been administered since late 2001 through the private sector. In order to achieve this, a collection of 475 *S. pneumoniae* isolates recovered between 2003 and 2008, from invasive infection in children and adolescents (<18 yrs), was characterized by serotyping, antimicrobial susceptibility testing, pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). The data was then compared with previous studies in order to infer the possible changes in the pneumococcal population after vaccine availability.

The results obtained in this study revealed a significant reduction of the overall proportion of IPD caused by serotypes covered by PCV7. Yet this decrease was accompanied by an increase of non-vaccine serotypes such as serotypes 1, 19A and 7F. Due to the considerable variation in terms of genetic lineages within the same serotype it was important to determine which clones were associated with the expansion of these non-vaccine types. Furthermore, it was essential to establish if the lineages identified in the post-vaccine period were genetically related to the pre-vaccine clones. Overall, the molecular typing of the invasive isolates demonstrated that the clones identified in the emerging serotypes were already in circulation before vaccine availability.

One of the main serotypes associated with IPD and antimicrobial resistance after PCV7 availability was serotype 19A. This serotype became increasingly important in several countries but the different studies performed in several geographic regions indicated that the genetic lineages associated with its expansion were unrelated. While in Portugal, the main lineage in expansion was represented by the clone Denmark^{14}-ST230, the one found increasing in the USA was represented by ST320 and ST199. To determine if the clone increasing in Portugal was restricted to the invasive

population we performed a study concerning all serotype 19A strains recovered from IPD, non-invasive infections and asymptomatic carriers between 2001 and 2006 in Portugal. Indeed, certain lineages were found to be more associated with carriage (ST1151 and ST416) while others were associated with invasive disease (ST193) but the main lineage associated with 19A expansion, represented by ST230, was found in all three populations.

PCV7, along with the more recent vaccines PCV10 and PCV13, cover a restricted number of serotypes, and taking into consideration that the introduction of the first pneumococcal conjugate vaccine led to the increase of non-vaccine serotypes, the need for new, non-serotype based, vaccines persists. Several proteins have already been proposed but none of them seem to improve the coverage of the already available vaccines. Nevertheless, a recently discovered set of proteins, which compose pilus-like structures, were suggested to be used in a new vaccine formulation. To evaluate the potential use of pili in vaccine and to determine their possible role as virulence factors in *S. pneumoniae*, we determined the prevalence of the two known pili (PI-1 and PI-2) in a collection of invasive isolates recovered from children and adolescents (<18 years) between 1999 and 2008.

Overall, only 17% of the invasive pneumococcal strains presented PI-1 while PI-2 was found in 33% of the population. An association between pili and serotype and PFGE clone was identified yet, all piliated strains presented one of the capsular types covered by the recent PCV13. Since only a small proportion of invasive pneumococcal strains presented pili and because all piliated pneumococci harbored capsular types covered by the most recent conjugate vaccines we consider that pili-based vaccines might not be the appropriate choice for prevention of pneumococcal infection.

The results obtained in this study indicate that the pneumococcal population has an extraordinary ability to adapt to stressful situations, such as the introduction of a vaccine, due to the extensive diversity of capsular types and genetic lineages found worldwide. Furthermore, taking into consideration the changes observed after PCV7, the new conjugate vaccines will most probably face the same problem which enhances the need for continuous surveillance of IPD.

RESUMO

Palavras chave: *Streptococcus pneumoniae*, PCV7, clones, pili

Streptococcus pneumoniae é considerado um dos principais agentes patogénicos em infecções pediátricas a nível mundial. Apesar de existir uma variedade de antibióticos para o tratamento das infecções pneumocócicas, o recente aumento dos níveis de resistência e a disseminação internacional de estirpes resistentes aos principais antibióticos utilizados na terapêutica, nomeadamente beta-lactâmicos e macrólidos, têm dificultado o tratamento destas infecções. Com o intuito de reduzir a incidência da infecção pneumocócica invasiva e de estirpes resistentes aos antibióticos, foi licenciada uma vacina pneumocócica conjugada (PCV7). Esta vacina, inicialmente introduzida nos Estados Unidos da América (EUA) e posteriormente em outros países, encontra-se direccionada para os sete serotipos mais prevalentes em infecção invasiva e com os níveis mais elevados de resistência aos antimicrobianos, nos EUA. A eficácia desta vacina foi já comprovada nos EUA, porém variações da distribuição e prevalência dos serotipos em termos geográficos têm questionado a sua eficácia noutros países.

O objectivo do presente trabalho consistiu na aferição do potencial impacto da PCV7 em Portugal, um país onde a vacina, apesar de não se encontrar no plano nacional de vacinação, está a ser prescrita pelo sector privado desde o ano 2001. Para tal, procedeu-se à caracterização de 475 estirpes isoladas de infecção invasiva em crianças e adolescentes (<18 anos) entre 2003 e 2008, recorrendo a métodos de serotipagem, determinação da susceptibilidade aos antimicrobianos e tipagem molecular. Os dados obtidos neste estudo foram comparados com resultados anteriores de modo a inferir possíveis alterações na população pneumocócica após disponibilização da vacina.

Os resultados obtidos demonstraram uma redução significativa dos serotipos incluídos na vacina PCV7 em infecção invasiva. No entanto, este decréscimo foi acompanhado por um aumento de serotipos não vacinais na população, nomeadamente o 1, 19A e 7F. Dado que o pneumococo pode apresentar diversas linhagens genéticas dentro do mesmo serotipo era importante não só determinar quais os clones associados aos serotipos em expansão como também averiguar a existência de proximidade genética dos clones encontrados nos períodos pré e pós-vacinais. A tipagem molecular das estirpes isoladas de infecção permitiu verificar que os clones associados aos serotipos em expansão já tinham sido identificados no período pré-vacinal. Consequentemente, o aumento da prevalência de alguns serotipos resultou da disseminação de clones em circulação em Portugal, não se tendo verificado situações de transformação capsular nos serotipos mais prevalentes.

Um dos serotipos mais prevalentes em infecção e associado à resistência aos antibióticos no período pós-vacinal foi o serotipo 19A. Diversos estudos demonstraram a sua importância em infecção invasiva apesar de se ter verificado diferenças significativas entre os clones reportados em Portugal e os clones descritos nos EUA. Enquanto que em Portugal a principal linhagem genética em circulação era a representada pelo clone Denmark^{14}-ST230, nos EUA a expansão do 19A resultou do aumento do clone composto pelos ST320 e ST199. Com o intuito de averiguar se o clone associado ao aumento do serotipo 19A em Portugal estava restricto à população pneumocócica invasiva, procedeu-se à caracterização de todas as estirpes de serotipo 19A isoladas de infecção (invasiva e não invasiva) e de portadores assintomáticos, entre 2001 e 2006. Foram identificadas algumas linhagens associadas à colonização (ST1151 e ST416) e outras associadas à infecção invasiva (ST193). Porém, o principal clone deste serotipo foi encontrado nas três populações em estudo e está associado à expansão do serotipo 19A.

Um dos principais problemas da vacina PCV7, assim como das vacinas recentemente introduzidas PCV10 e PCV13, é o número restricto de serotipos que esta vacina protege. Isto, em conjunto com o aumento de serotipos não incluídos na PCV7 após a introdução desta, tem levado a uma necessidade de novas estratégias vacinais para a prevenção da infecção pneumocócica.

Foram identificadas recentemente proteínas na superfície do pneumococo que compõem estruturas do tipo pili. Uma vez que estas proteínas são bastante imunogénicas foi proposta a sua utilização na formulação de uma nova vacina anti-pneumocócica. Porém, a distribuição destas estruturas na população pneumocócica não está totalmente definida. Com o intuito de averiguar a potencial utilização destas proteínas numa vacina, procedemos à determinação da prevalência dos dois pili até agora identificados em *S. pneumoniae*, numa colecção de estirpes invasivas isoladas de crianças e adolescentes (<18 anos) entre 1999 e 2008 em Portugal.

De uma forma geral, verificou-se que 17% da população pneumocócica incluída neste estudo era portadora da ilha de patogenicidade do pili tipo 1 (PI-1) enquanto que o pili tipo 2 (PI-2) foi identificado em 33% das estirpes. Foi encontrada uma associação entre pili, serotipo e clone tendo-se verificado que todas as estirpes portadoras de pili apresentavam um dos tipos capsulares existente na vacina PCV13. Consequentemente, tendo em conta que apenas uma pequena fracção da população pneumocócica é portadora das ilhas de patogenicidade que codificam os pili e que todas as estirpes portadoras de pili se encontravam abrangidas pela vacina PCV13, não consideramos que os pili sejam uma escolha adequada para a formulação de uma nova vacina pneumocócica.

Os resultados obtidos neste estudo demonstraram que a população pneumocócica associada à infecção invasiva possui uma capacidade extraordinária de adaptação a situações de stress, como é o caso da introdução de uma nova vacina, devido à elevada diversidade de tipos capsulares e clones existente nesta espécie.

THESIS OUTLINE

The main purpose of this work was to determine the effect of the PCV7 in the pneumococcal population associated with infection in children in Portugal. To achieve this, phenotypic and genotypic typing methods were used among a collection of clinical isolates and the results obtained were compared with previous work.

The subject of this thesis is presented in 6 chapters organized as follows:

Chapter 1: General Introduction – This chapter provides a review of the epidemiology of *S. pneumoniae*. The significance of this bacterium in infection, the diversity of the serotypes and genetic lineages along with the importance of surveillance are the main issues addressed.

Chapter 2 and 3: Changes in pneumococcal serotypes and clones after PCV7 – A description of the effect of PCV7 in serotypes, levels of antimicrobial resistance and genetic lineages among an invasive pneumococcal population recovered in Portugal is performed in these two chapters.

Chapter 4: Serotype 19A lineages – In this chapter the emergence of serotype 19A as an important serotype both in infection and carriage is discussed. Overall three pneumococcal populations are analyzed (invasive, non-invasive and carriage) in terms of PFGE and MLST with the aim of identifying associations between the populations and certain genetic lineages

Chapter 5: Pneumococcal pili – A review of the pneumococcal proteins proposed for new pneumococcal vaccines is performed in this chapter with emphasis given to the recently discover pili. The importance of pili and its distribution among a collection of invasive isolates are the main issues addressed.

Chapter 6: Concluding remarks – Discusses the major findings obtained in the previous chapters and highlights issues that could be addressed in future studies.

This thesis is based on the following papers:

Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M. 2008. Changes in *Streptococcus pneumoniae* serotypes causing invasive disease with non-universal vaccination coverage of the seven-valent conjugate vaccine. Clin Microbiol Infect.14(9):835-43.

Aguiar SI, Brito MJ, Gonçalo-Marques J, Melo-Cristino J, Ramirez M. 2010. Serotypes 1, 7F and 19A became the leading causes of pediatric invasive pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. Vaccine.19;28(32):5167-73.

Aguiar SI, Pinto FR, Nunes S, Serrano I, Melo-Cristino J, Sá-Leão R, Ramirez M, de Lencastre H. J. 2009. Increase of Denmark14-230 clone as a cause of pneumococcal infection in Portugal within a background of diverse serotype 19A lineages. Clin. Microbiol. 48(1):101-108.

Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M. 2008. The presence of the pilus locus is a clonal property among pneumococcal invasive isolates. BMC Microbiol. 28; 8:41.

ABBREVIATIONS

ACIP- Advisory committee on immunization practices
AOM- Acute Otitis Media
CLI- Clindamycin
CHL-Chloramphenicol
CLSI- Clinical laboratory standards institute
CPS- capsular polysaccharide
CRM – cross-reactive material
CTX- Cefotaxime
EPNSP- Erythromycin and penicillin non-susceptible pneumococci
ERP- Erythromycin resistant pneumococci
ERY- Erythromycin
Hib- *Haemophilus influenzae* type b
IPD- Invasive Pneumococcal Disease
MDR- Multi-drug resistant
MIC - Minimum inhibitory concentration
MLEE- Multi-locus enzyme electrophoresis
MLST- Multi-locus sequence typing
NTHi - Non-typable *Haemophilus influenzae*
NVP - National vaccination plan
NVT – Non-vaccine type
PBPs – Penicillin Binding Proteins
PCV7 – 7-valent pneumococcal conjugate vaccine
PCV10 -10-valent pneumococcal conjugate vaccine
PCV13 – 13-valent pneumococcal conjugate vaccine
PEN- Penicillin
PFGE- Pulsed field gel electrophoresis
PNSP- Penicillin non-susceptible pneumococci
PPV23 – 23-valent polysaccharide vaccine
ST- Sequence type
SXT- Co-trimoxazole
TET- Tetracycline

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

SUMMARY iii

RESUMO v

THESIS OUTLINE vii

ABBREVIATIONS ix

CHAPTER 1: General Introduction 15

1.The importance of *Streptococcus pneumoniae* 3

2.Historical overview 3

3.Characteristics of *Streptococcus pneumoniae* 4

3.1 Identification of *S. pneumoniae* 5

4.Host-Pneumococcal interaction 8

4.1 Pneumococcal colonization 8

4.2 Epidemiology of pneumococcal infection 9

4.3 The pneumococcal serotypes 12

5.Pneumococcal vaccines 13

5.1 Available pneumococcal vaccines 13

5.2 New pneumococcal conjugate vaccines 15

5.3 Serotype replacement: Threat to vaccination success 16

6.Antimicrobial resistance in *S. pneumoniae* 17

6.1 Definition of resistance 18

6.2 Mechanisms of antimicrobial resistance 19

6.3 Epidemiology of *S. pneumoniae* resistance 22

6.4. Effect of PCV7 vaccine on drug resistant *S. pneumoniae* 23

7.Molecular epidemiology 24

7.1 Molecular typing systems 25

7.2 goeBURST 28

7.3 Molecular epidemiology of *S. pneumoniae* 29

7.3.1 Association between serotype, antimicrobial resistance, PFGE and MLST 30
7.4 Global antibiotic resistant clones 31
CHAPTER 2: Changes in invasive *Streptococcus pneumoniae* serotypes with non-universal vaccination coverage of the seven-valent conjugate vaccine. 43
SUMMARY 45
INTRODUCTION 46
MATERIALS AND METHODS 49
Bacterial isolates 49
Serotyping 49
Antimicrobial susceptibility testing 50
Statistical analysis 51
RESULTS 52
Isolate collection 52
Serotype distribution 52
Antimicrobial resistance 56
DISCUSSION 59
ACKNOWLEDGEMENTS 63
CHAPTER 3: Post-Vaccine genetic lineages of invasive pneumococcal serotypes recovered from pediatric patients in Portugal. 69
SUMMARY 71
INTRODUCTION 72
MATERIALS AND METHODS 73
Isolates 73
PFGE 73
MLST 73
Statistical analysis 74
RESULTS 75
New MLST alleles and allelic profiles 75
PMEN clones 78

Genetic lineages of PCV7 serotypes 78
Lineage distribution of emerging serotypes 83
Minor serotypes not included in any conjugate vaccine formulation 87
Correlation between the different typing methods 87
DISCUSSION 89
ACKNOWLEDGMENTS 93
CHAPTER 4: Denmark^{14}-230 Clone as an Increasing Cause of Pneumococcal Infection in Portugal within a Background of Diverse Serotype 19A Lineages 97
SUMMARY 99
INTRODUCTION 100
MATERIALS AND METHODS 101
Bacterial isolates 101
Isolates causing infections 101
Carriage isolates 101
Serotyping and antimicrobial susceptibility testing 101
PFGE and MLST 102
Statistical analysis 102
RESULTS 103
Proportion of isolates expressing serotype 19A 103
Antimicrobial susceptibility 103
Genotypic analysis and evaluation of invasive disease potential 104
Temporal fluctuations in PFGE clusters 106
PFGE cluster and antibiotic resistance 106
DISCUSSION 108
ACKNOWLEDGMENTS 113
CHAPTER 5: Pneumococcal vaccine candidates: Are pili an appropriate alternative? 119
SUMMARY 121
INTRODUCTION 123
MATERIALS AND METHODS 130

Bacterial isolates 130
Detection of the PI-1 islet 130
Identification of the *rlr*A pilus clade type 131
Characterization of the chromosomal region where the *rlrA* islet is usually found in non-piliated strains 131
Detection of the PI-2 islet 132
Statistical analysis 133
RESULTS 134
Distribution of the PI-1 islet among invasive pneumococci 134
Association between the presence of pilus islet and resistance to antimicrobials 136
Presence of the pilus islet is a clonal property 137
DISCUSSION 146
ACKNOWLEDGEMENTS 151
BIBLIOGRAPHY 152
CHAPTER 6: Concluding remarks 157
Changes in pneumococcal serotypes 159
Pneumococcal genetic lineages 160
Protein vaccines: the pneumococcal pili 162

CHAPTER 1

General Introduction

1. The importance of *Streptococcus pneumoniae*

Streptococcus pneumoniae, commonly referred as the pneumococcus, is one of the most common bacterial respiratory pathogens, being in fact the most frequent cause of community acquired pneumonia (59). It is also a major cause of meningitis, bacteremia, sinusitis, and otitis media and it occasionally infects tissues at other sites (such as the eye) (109). Although the main interest in the pneumococcus is primarily due to its importance as a pathogen, the main biological niche of this organism is the nasopharynx of healthy individuals, where it colonizes asymptomatically the mucosal surfaces (108). Disease is thought to occur when colonizing pneumococci from the upper respiratory tract gain access to normally sterile sites such as the middle ear, lung or bloodstream and therefore, colonization is considered to be the initial step in the pathogenesis of all pneumococcal infections (108).

The importance of the pneumococcus resides on the fact that it is the leading cause of illness and death in young children, in individuals with underlying medical conditions and in the elderly (86). In fact, more people die in the United States of America (USA) of pneumococcal pneumonia than of AIDS (www.preventpneumo.org). Furthermore, significant differences regarding the molecular epidemiology of the pneumococcus are found in terms of age group and geographic region, among other factors, which make it difficult to develop a universal policy to prevent pneumococcal infections.

Several strategies have been implemented and are being evaluated in order to reduce the incidence of invasive pneumococcal disease (IPD). With the increase of antimicrobial resistance, an urgent need to prevent IPD led to the development and use of vaccines, first the 23-valent polysaccharide vaccine (PPV23) and more recently the 7-valent pneumococcal conjugate vaccine (PCV7). Yet, as observed with the emergence of resistant *S. pneumoniae* strains following antimicrobial use, an increase of infections due to strains not covered by the vaccines is questioning the effectiveness of the most recent conjugate vaccines.

2. Historical overview

Streptococcus pneumoniae was first isolated in 1881 simultaneously by two microbiologists, George M. Sternberg in the USA and Louis Pasteur in France. Independently, the two researchers observed lancet shaped pairs of coccoid bacteria in the human saliva and demonstrated its pathogenicity by injecting the saliva in rabbits (107). Pasteur named these cocci as *Microbe Septicemique du salive*, while Stenberg referred them as *Micrococcus pasteuri* (107).

The association between the pneumococcus and pneumonia was first described by Carl Friedlander and Charles Talamon in 1883, but pneumococcal pneumonia was only confirmed upon the discovery of the Gram stain in 1884 (107). In 1886, due to the propensity of these bacteria to cause pulmonary disease, Albert Fraenkel designated this organism as "*pneumokokkus"*. Later, in 1920, it was renamed *Diplococcus pneumoniae*, as referring to pairs of cocci causing pneumonia and in 1974 the pneumococcus obtained its final designation as *Streptococcus pneumoniae*, primarily due to its characteristic growth in chains of cocci in liquid media (107).

The extensive studies performed in the pneumococcus have provided numerous contributions to the scientific knowledge. In fact the pneumococcus was at the basis of the discovery by Frederick Griffith in 1928 of the transforming principle which converted non-infective rough (unencapsulated) colony-forming diplococci to virulent smooth (capsulated) forms, later identified by Oswald Avery, Colin MacLeod and Maclyn McCarthy as DNA (39). Other studies based on the pneumococcus also contributed to the understanding of the mechanisms of bacterial pathogenesis, host immune response to infection and also development of anti-bacterial vaccines (77). Yet, even after 100 years of intense research, the pneumococcus persists as a leading cause of infection, particularly among young children and the elderly (77).

3. Characteristics of *Streptococcus pneumoniae*

Streptococcus pneumoniae belongs to the viridans group of streptococci which comprise gram-positive cocci that colonize the oropharynx, gastrointestinal tract and genitourinary tract. Viridans species are nutritionally fastidious requiring complex media supplemented with blood products and, frequently, incubation in an atmosphere enriched with 5% to 10% of CO_2 (93).

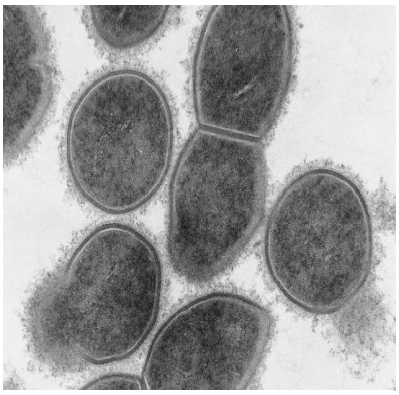

Figure 1.1: Morphology of *S. pneumoniae*. Reproduced from Alonsodevelasco *et al* (4).

Pneumococci are lanceolate shaped diplococci, normally arranged in short, straight chains and often encapsulated (Figure 1.1). Colonies of encapsulated strains are generally large (1 to 3 mm in diameter on blood agar), round and sometimes mucoid. Colonies of non-encapsulated strains are smaller and appear flat. On blood agar, colonies are alpha-hemolytic resulting from the production of pneumolysin, a protein that mediates erythrocyte lysis leading to the appearance of the greenish halo (67, 93).

One of the main features of *S. pneumoniae* is its capsule, which forms the outermost layer of this bacterium. Capsule production is indispensable for pneumococcal virulence, being in fact considered the primary virulence factor, and is strongly anti-phagocytic in non-immune hosts (59).

Although non-encapsulated strains have been associated with superficial infections such as conjunctivitis, clinical isolates from normally sterile sites are usually encapsulated and spontaneous non-encapsulated derivates of these strains have already been shown to be largely avirulent (59).

The bacterial capsule is also used as the primary typing system. The method traditionally used is the capsular reaction test, named Quellung or Neufeld reaction, using the Danish system of nomenclature (101). Currently, 93 antigenically distinct pneumococcal polysaccharides have been described. Some types are structurally and antigenically related to each other and consequently are classified in the same group - for example, 9A, 9L, 9N and 9V belong to serogroup 9 - while types without a close antigenic relationship to other types are given numbers only - for example, types 1, 2, 3, 4, 5 (60). Hence, the 93 serotypes are organized into 21 numbered serogroups, each of which contains 2 to 5 related serotypes (56).

3.1 Identification of *S. pneumoniae*

Isolation of *S. pneumoniae* from culturing a normally sterile body fluid (such as blood or pleural effusion, synovial, ascitic, or cerebrospinal fluid) provides a conclusive evidence of invasive pneumococcal disease (IPD) (109). Despite its importance, IPD can be difficult to confirm microbiologically and conclusive evidence of pneumococcal infection is achieved for only a minority of cases of IPD (109). On the other hand, the isolation of the pneumococcus from sputum, for example in the case of non-invasive pneumococcal pneumonia, may provide a falsely positive indication of pneumococcal etiology due to the uncertainty regarding the differentiation of infection from colonization (92).

Laboratory identification of pneumococci rely on the recognition of typical features such as the alpha-hemolysis on blood agar and the central depression of the colonies (93). Nevertheless further tests are necessary for pneumococcal confirmation and differentiation from other viridans species. Two key tests are used for this distinction: the optochin susceptibility and bile solubility (109).

Optochin (ethylhydrocupreine) is an antibacterial agent not employed in therapeutics but used in screening and identification of the pneumococcus. This test was introduced five decades ago and pneumococcal resistance to this antibiotic was not report during the first 30 years of its use (84). However, in 1987 the first resistant strains (isolated from blood and middle ear fluid) were described in Finland (63).

The bile solubility test is based on the autolysis of *S. pneumoniae* in the presence of the surfactant sodium deoxycholate. Pneumococci produce enzymes called autolysins that digest peptidoglycan. The bile salts such as deoxycholic acid, activate these autolysins in most strains and

consequently *S. pneumoniae* strains lyse rapidly when exposed to a bile solution (73, 109). *S. pneumoniae* isolates are typically susceptible to optochin (inhibition zones ≥ 14 mm) and bile soluble whereas other viridians streptococci are typically resistant and bile insoluble (109).

The identification of *S. pneumoniae* has recently become more complicated due to the description of *Streptococcus pseudopneumoniae*, a species that is phenotypically and genetically distinct from pneumococci but that has the potential to be incorrectly identified as pneumococci (7). *S. pseudopneumoniae* is characterized by resistance or intermediate susceptibility to optochin when incubated in 5% CO_2 but susceptible to optochin when incubated in ambient air, insolubility in bile and the absence of a pneumococcal capsule (2).

Other tests that are useful in the identification of this species include the detection of a pneumococcal capsule (Quellung reaction), as well as biochemical tests found in a number of generic commercial identification systems relying on batteries of phenotypic tests like the API 20 STREP (bioMérieux, Marcy l'Etoile, France), Rapid ID32 Strept kit (bioMérieux) and Vitek2 (bioMérieux). Various currently available commercial rapid tests are based on the detection of pneumococcal specific properties such as immunochromatographic detection of the C-polysaccharide cell wall antigen (NOW binax, Portland, ME) (32) or a DNA probe targeting specific regions of the 16S rRNA gene (AccuProbe Probe, San Diego, CA) (31). In addition to the well-established DNA-DNA reassociation studies, a number of gene amplification methods were also proposed for presumptive identification of pneumococci based upon the detection of pneumococcal virulence factors (7, 22, 106).

Overall, the European Commission has defined a case of IPD if one of the following three criteria's is met (47):

- Isolation of *S. pneumoniae* from a normally sterile site
- Detection of *S. pneumoniae* nucleic acid from a normally sterile site
- Detection of *S. pneumoniae* antigen from a normally sterile site

3.1.1 Importance of optochin resistance

As described previously there are several methods for pneumococcal identification but most of them are not available in clinical laboratories and optochin susceptibility continues to be widely used for the screening and identification of *S. pneumoniae* (2). Consequently it is important to monitor the levels of resistance to this antibiotic.

Resistance to optochin is rare among *S. pneumoniae* isolates with a few sporadic cases documented in the literature (12, 63, 65, 71, 83, 84). Some studies reported fully resistant isolates (12, 63, 65, 83, 84), while others described strains with mixed sub-populations of optochin-

susceptible and optochin-resistant organisms (71, 84). As in most clinical microbiology laboratories, in Portugal presumptive identification of *S. pneumoniae* is based on optochin susceptibility. A few years ago we reported an increase in the isolation of optochin-resistant *S. pneumoniae* in our country from 0.3% in 2004 to 3.2% in 2005. Resistant isolates consisted of mixed populations of optochin-susceptible and optochin-resistant isolates (2). Our first judgment when we observed a strain presenting colonies within the halo of inhibition (Figure 1.2-A) was that the sample corresponded to a mixture of pneumococci and other viridians streptococci. We therefore decided to perform the bile solubility test in both optochin-resistant and optochin-susceptible isolates of the same strain and both were positive. Further tests were performed, including serotyping, antimicrobial susceptibility profiling and pulsed-field gel electrophoresis (PFGE), which confirmed that the optochin-resistant and optochin-susceptible isolates represented two subpopulations of the same pneumococcal strain. This was the case for all 32 optochin-resistant isolates described in the study, all recovered from invasive and non-invasive pneumococcal infections (2).

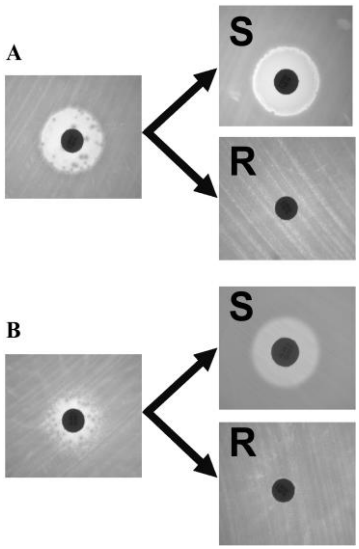

Figure 1.2: Optochin susceptibility test of representative strains of *S. pneumoniae.* (Left) Original strain with optochin-resistant colonies in the inhibition zone around the optochin disk. (Right) Subpopulation of uniformly optochin-resistant bacteria obtained after subculture of a colony picked from within the inhibition zone (R) and subpopulation of uniformly optochin-susceptible bacteria obtained after subculture of a colony picked from the quadrant away from the optochin disk (S). (A) Strain 2005V0786S. (B) Strain 2004V0319S.

The findings reported in this study highlighted the importance of routine use of additional sensitive tests to accurately identify *S. pneumoniae.* We recommended that at least the bile solubility test should be routinely performed in cases of suspected pneumococcal etiology even if the isolates are optochin-resistant.

4. Host-Pneumococcal interaction

The upper respiratory tract is the ecological niche for many bacterial species. In children the nasopharyngeal flora becomes established during the first months of life and usually comprises a broad variety of microorganisms including potential pathogenic bacteria such as *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (10). Nasopharyngeal colonization by *S. pneumoniae* is quite common: probably all humans are colonized with this organism at least once early in life. In circumstances of crowding, as in day care-centers, nursing homes, hospitals and jails, the risk of colonization with pneumococci is particularly high (10). Colonization is not usually followed by disease, since this is prevented by the innate and adaptive immune responses. However, disturbance of the homeostasis between host and pathogen, for example through viral infections, malnutrition or local damage of the mucosa, can be associated with the development of pneumococcal infection (11, 93).

4.1 Pneumococcal colonization

Pneumococcal carriage rates are highest in the first two years of life and may exceed 50%, particularly in children, but decline to less than 10% in adult populations (75, 108). As a consequence of their high and prolonged carriage rate, along with the mode of transmission (respiratory droplets), young children play an important role in the dissemination of pneumococcal strains in crowded settings such as day care centers and households (79). The rates of bacterial acquisition and carriage depend on a variety of factors such as age, geographic area, genetic background, and socio-economic conditions (10). Moreover, the rates of carriage also vary widely among the 93 known pneumococcal capsular serotypes. The factors that are responsible for these differences, and the variations in serotype distribution between the regions and over time, are not entirely understood (59).

Pneumococcal dissemination is thought to occur through direct contact with the secretions of colonized individuals (Figure 1.3). Once acquired, an individual strain can be carried for weeks to months before its eventual clearance (59). In most situations, transient nasopharyngeal colonization rather than disease is the normal outcome of exposure to pneumococci. Yet, in some circumstances, probably multifactorial and still poorly understood, bacteria can spread to normally sterile sites resulting in infection (76, 114).

But the importance of colonization is not limited to being the first step for infection. The human nasopharynx is possibly the privileged site for pneumococcal evolution. The pneumococcus is a naturally transformable bacterium, which means that genetic material may be exchanged

between different strains. Since the human pharynx is a highly populated microbial environment it provides exogenous DNA from closely related oral streptococcal species and other colonizing pneumococcal strains (59). These nucleic acids can be taken up with high efficiency by *S. pneumoniae* and result in significant changes of the recipient strains, such as the acquisition of a new capsule or of resistance to antimicrobials, such as β-lactams, which is now a common problem in pneumococcal infections (59).

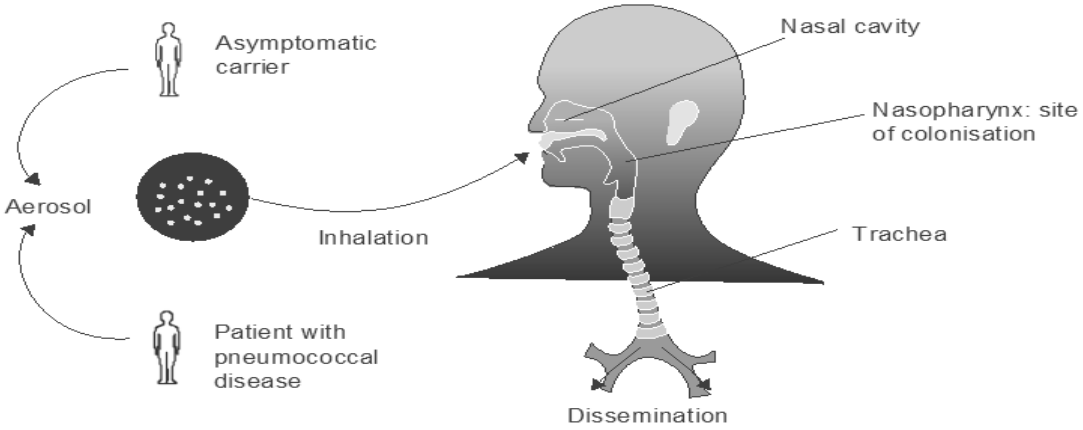

Figure 1.3: Pneumococcal transmission and interaction with the human host. Adapted from Musher *et al* (74).

Intensive surveillance, which includes monitorization of the serotypes, antibiotypes and molecular types, has been performed extensively for pneumococcal strains recovered from the nasopharynx of preschool children attending day-care centers, in several countries. In Portugal, in the pre-vaccine era, 47% of the children attending day care centers harbored *S. pneumoniae* in their nasopharynx, 24% of which were resistant to penicillin (30). In the post-vaccine era the global levels of carriage maintained constant and no reduction in the number of antimicrobial resistant strains was reported (41).

4.2 Epidemiology of pneumococcal infection

Infections caused by pneumococci are a major cause of morbidity and mortality all over the world (114). Although all age groups may be affected, the highest rate of pneumococcal disease occurs in young children (<5 years) (Figure 1.4) and in the elderly population (≥60 years). Additionally, persons suffering from a wide range of chronic conditions and immune deficiencies (including HIV infection, leukemia and lymphoma) are at increased risk (52, 88, 114). However, the risk factors for invasive pneumococcal disease (IPD) are very diverse and include age, ethnicity,

geographic location, concomitant chronic illnesses, and attendance of care facilities (Table 1.1) (20, 79).

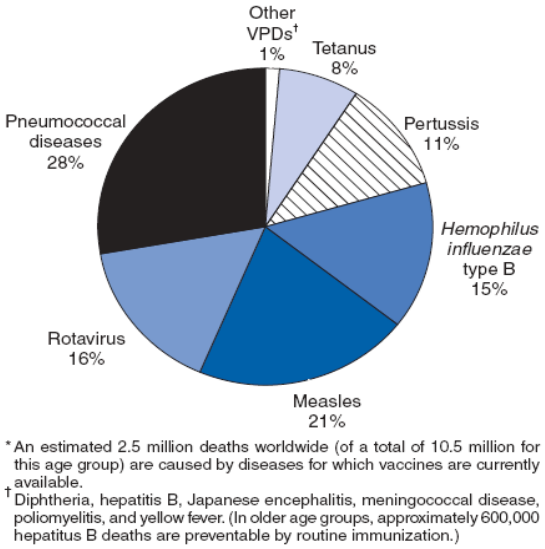

Figure 1.4: Percentage of deaths from vaccine-preventable diseases among children aged <5 years, worldwide. Figure reproduced from Adams *et al* (20).

Table 1.1: Basis for increased risk of paediatric pneumococcal disease according to risk group. Table adapted from O'Brien *et al* (79).

Risk group	Likely pathophysiology
Children <2 years of age	Lack of serotype-specific protective antibody
Children in day care	Increased exposure to pneumococcus
Ethnicity (American Indian, Alaska Native, African American)	Multifactorial
Cerebrospinal fluid leak	Disruption of the central nervous system barrier
Functional asplenia (e.g., sickle cell disease) or anatomic asplenia (e.g., splenectomy)	Reduced bacterial clearance from blood and reduced production of immunoglobulin M
Human immunodeficiency virus infection	Multifactorial, including lack of functional serotype-specific antibody
Congenital immunodeficiency	Defective serotype-specific antibody formation
Neoplastic: leukemia, lymphoma, myeloma, Hodgkin's disease	Multifactorial
Bone marrow transplantation	Lack of serotype-specific protective antibody
Nephrotic syndrome	Lack of sufficient amounts of protective antibody because of antibody loss in urine

In the case of children, attendance of day-care center is an important predisposing factor for infection, particularly with drug-resistant pneumococcal strains. The close interactions between

children at day-care centers allow a more efficient transmission of pneumococci from the nasopharynx of one child to another. Increased rates of disease in children result in increased use of antibiotics, selecting for nonsusceptible pneumococci in the nasopharynx (79).

Besides the age associations, prevalence of pneumococcal infection is also geographically dependent (Figure 1.5). Greenwood and collaborators reported that the incidence of IPD in children under 2 years of age may vary from less than 100 cases/100,000 children in Finland to more than 1,000/100,000 children in Australian Aboriginal children (43). There is no complete explanation for the differences in the incidence of infection found among geographic regions even though certain ethnicities have a higher risk of pneumococcal infection. For example, in the USA, higher rates of invasive pneumococcal disease occur among African-American individuals, Alaskan natives, and specific American-Indian populations compared with individuals of European origin (18). Whether this is due to a genetic background more prone to infection, or due to other socio-demographic reasons is still not quite understood.

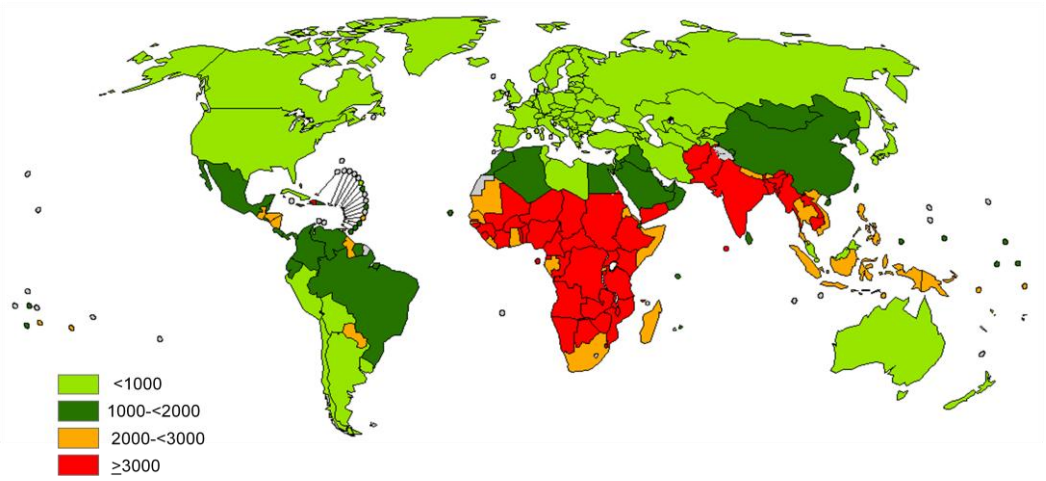

Figure 1.5: Pneumococcal incidence rate in children < 5 years (incidence in children per 100.000 children). Figure reproduced from www.who.int.

Despite the importance of pneumococcal disease, there is a scarcity of information regarding the disease burden, particularly from developing countries. In 2007, the World Health Organization (WHO) estimated that 1.6 million people die of pneumococcal disease every year. This estimate includes the deaths of 0.8-1 million children aged <5 years, most of whom live in developing countries (113). Still, in the developed world, children aged <2 years and elderly people also carry the major burden of disease. In Europe and the United States, pneumococcal pneumonia is the most common community-acquired bacterial pneumonia, estimated to affect approximately 100 per 100.000 adults each year. Compared with invasive disease, the non-invasive manifestations, such as otitis media, are usually less severe, but considerably more common (88, 114).

4.3 The pneumococcal serotypes

Several studies have been undertaken regarding the distribution of pneumococcal serotypes in different parts of the world, including some developing countries. The different pneumococcal serotypes vary in prevalence and virulence, depending on a multitude of factors, including host age, region and country (60, 96). Moreover, even within the same region temporal fluctuations have been observed (50). Although there are 93 different capsular types and the relative importance of pneumococci expressing individual serotypes is variable, a relatively small number of serotypes, about ten serotypes, are usually responsible for the majority of IPD (Figure 1.6) (43, 50).

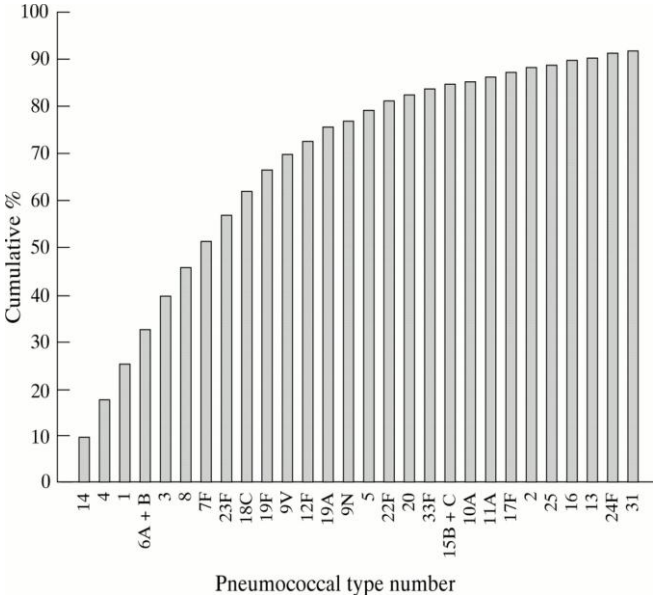

Figure 1.6 : Pneumococcal serotypes responsible for invasive disease in several countries. Reproduced from Kalin, M (60).

In IPD one of the most important associations identified so far is the correlation between age group and serotype distribution. For unknown reasons, in young children only a few types are responsible for a large proportion of pneumococcal disease and before vaccine implementation, the most important paediatric serotypes were PCV7 serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, which accounted for almost 60% of the infections worldwide, with the exception of Asia (4, 16, 50, 60). Moreover significant group age-related associations are observed in some serotypes, e.g. serotypes 1 and 3. While serotype 1 is quite frequent in young children (< 6 years), serotype 3 has been described to be common across all age groups except children younger than 6 years of age even though it has been associated with infection in the neonates (54, 96).

Besides the geographical, age and temporal differences, some authors have proposed that the site of isolation of the bacteria also influences the types most likely to be found. Indeed, serotype distribution of isolates obtained from normally sterile sites differs from that obtained from carriers, suggesting that some serotypes have an enhanced propensity to cause invasive disease. Furthermore, some serotypes are more frequently isolated from certain biological products suggesting a higher propensity to cause specific types of infection (43, 49). For example, serotype 1 has been increasingly associated with post-pneumonic empyema, a severe complication of bacterial pneumonia particularly common in children (17).

A few serotypes also account for the majority of nasopharyngeal carriage isolates recovered from children. These include serotypes commonly found in invasive infection such as 14, 6B, 19F and 23F, which compose the PCV7, as well as serotypes 6A, 19A, 3 and 7F (50). Conversely highly invasive serotypes such as serotype 1 and 5 are rarely detected in the nasopharyngeal carriage samples (48). The inability to culture specific pneumococcal serotypes from the nasopharynx is presumably a function of their density and duration of colonization, since all invasive serotypes are assumed to be carried, at least transiently, before causing disease (51).

In a study conducted by Brueggemann and colleagues, the differences in the ability of pneumococcal serogroups to cause invasive disease was measured (15). The study led to the conclusion that pneumococcal serotypes and serogroups differ in invasiveness and that there is a statistically significant inverse relationship between invasiveness and prevalence in carriage. The most invasive serogroups were the least commonly carried while the most prevalent serogroups in carriage were the least likely to cause invasive infections (15).

It is very important to determine which serotypes are commonly found in infection since the capsule is the target for protective immunity induced by conjugate vaccines. Hence the selection of the serotypes to be included in these vaccines depends on the rank order of serotypes recovered from invasive infection, assuming that the most frequent serotypes are also the most invasive (15).

5. Pneumococcal vaccines

5.1 Available pneumococcal vaccines

Once the significance of capsular serotypes was understood, vaccine development focused on vaccines based on the most prevalent serotypes (78). Current pneumococcal vaccines are exclusively targeting the capsular polysaccharide (CPS) of *S. pneumoniae* and these vaccines provide a strictly serotype-specific protection. The first pneumococcal polysaccharide vaccines were released in the US in 1946, but were soon withdrawn from the market when penicillin and sulfa

drugs became widely available. Pneumococcal vaccines were re-introduced in the USA in 1977, when a 14-valent polysaccharide pneumococcal vaccine was released, only to be replaced by a 23-valent vaccine in 1983, the PPV23. This vaccine contains 23 purified capsular polysaccharide antigens, namely serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F, together accounting for 85-90% of bacteraemic pneumococcal disease (88).

In the USA the advisory committee on immunization practices (ACIP) recommends the administration of PPV23 to persons 2 years of age or older who have any of several underlying medical conditions, and to all persons 65 years of age or older (86). In the European community, PPV23, has been widely recommended in some countries for groups perceived to be at higher risk of invasive disease (patients with splenic dysfunction, diabetes mellitus, immunodeficiency, among other) (81). PPV23 has been shown to be effective in reducing invasive pneumococcal disease (IPD) among adults and the elderly but due to the fact that CPS are T-cell independent antigens, and are therefore poorly immunogenic, this vaccine is less effective in immunocompromised individuals and younger children (59). T-independent antigens stimulate mature B-lymphocytes but not T-lymphocytes. This type of antigen induces an immune response that is neither long-lasting nor characterized by an anamnestic response upon subsequent challenge with native polysaccharides (18).

The poor immunogenicity of CPS antigens has been overcome by conjugation to protein carriers. By complexing a protein carrier to the pneumococcal polysaccharide, the antigenic properties are altered to those of T-cell dependent antigens inducing immunologic memory (1, 18). This approach was used for *Haemophilus influenzae* type b (Hib) and its conjugate vaccine became a major success in eliminating Hib among immunized children (1).

Unlike the case with Hib vaccine, a major limitation of the pneumococcal conjugate vaccines, is the limited number of polysaccharides which can be conjugated to the carrier protein (1, 88). Concerns include a reduced vaccine effectiveness due to antigenic competition caused by an excess number of serotypes in the vaccine and possible induction of immune tolerance, or even immune suppression, to epitopes associated with a carrier protein following repeated immunization with conjugate vaccines using the same carrier (88). In addition there is ongoing discussion regarding which serotypes to include in potential conjugate vaccines. Epidemiological data suggest different vaccine formulations based on geography, age and type of disease (88).

In February 2000 a seven-valent pneumococcal polysaccharide-protein conjugate vaccine (PCV7) was licensed in the USA for use in infants and young children. The PCV7 includes seven purified capsular polysaccharides of *S. pneumoniae*, each coupled to a diphtheria toxoid (cross-reactive material, CRM). The vaccine contains approximately 2μg of each capsular polysaccharide from serotypes 4, 9V, 14, 19F and 23F, and an oligosaccharide from 18C, 4μg of serotype 6B and

20μg of the carrier protein CRM197 (78). The vaccine is recommended for all children under two years of age and for children two to four years of age who have certain chronic illnesses or other high-risk conditions (18). The primary series of PCV7 consists of 3 intramuscular doses administered to infants at intervals of at least 4 weeks, starting at the age of 6 weeks or later (18). Controlled clinical trials have shown that the vaccine, when given as a four-dose regimen to infants, is highly efficacious against invasive disease and also affords some protection against otitis media (111).

The serotypes included in the PCV7 vaccine were chosen based on their importance in invasive infection in the USA. Overall these serotypes were responsible for 70-89% of IPD in that country. Because antibiotic non-susceptibility was largely concentrated in these serotypes, the vaccine would also cover a significant fraction of resistant strains (46).

Following PCV7 introduction dramatic declines in invasive pneumococcal disease were reported as early as 2001 (112). The Northern California Kaiser Permanente study revealed that the efficacy of the vaccine was 97% for IPD caused by PCV7 types and 89% for all serotypes. From 1998 through 2007, the Active Bacterial core surveillance of the Centers for Disease Control and Prevention (CDC) showed that the US burden of invasive pneumococcal disease decreased around 45% in all age groups and 76% in children <5 years. PCV7-type disease declined by 78% among children younger than 2 years of age compared with pre-vaccine rates in 1998-1999. Yet, the efficacy of PCV7 against all acute otitis media (AOM) episodes was 6%-7% while against AOM caused by serotypes included in PCV7 was 57% in children < 2 years (35, 85, 86, 111).

The use of PCV7 has also reduced transmission of vaccine-serotype pneumococci (i.e herd effect). Declines in the incidence of PCV7-type invasive disease among adults were observed first in 2001 and have continued through 2004, reducing the incidence to 64%-77% (86). Much of the changes observed in adults may be due to decreased transmission of pneumococci from children (111).

5.2 New pneumococcal conjugate vaccines

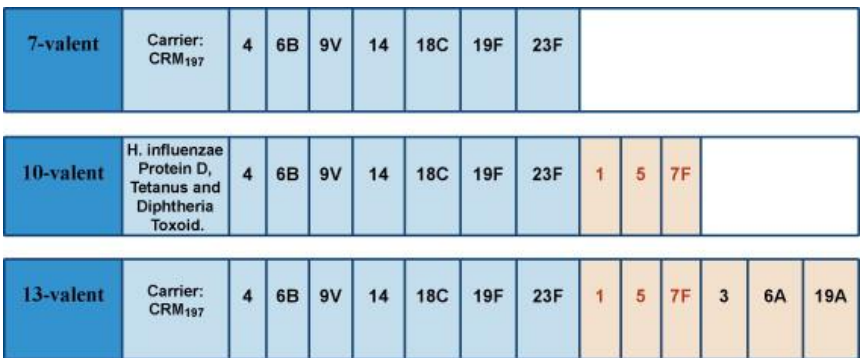

Figure 1.7: Pneumococcal serotypes included in PCV7 and PCV10 and PCV13. Reproduced from Isaacman *et al* (55).

Two conjugate vaccines have recently become commercially available, the 10-valent (PCV10) and the 13-valent (PCV13) conjugate vaccine. PCV10 includes, in addition to the PCV7 serotypes, serotype 1, 5 and 7F, while PCV13 includes all PCV10 serotypes plus serotype 3, 19A and 6A (Figure 1.6).

The new 10-valent pneumococcal non-typeable *Haemophilus influenzae* (NTHi) protein D-conjugate vaccine uses a recombinant version of protein D, a nonlipidated form of a highly conserved cell surface lipoprotein of non-typeable *H. influenzae*, as a carrier protein for 8 of the 10 vaccine serotypes. For the other 2 serotypes, diphtheria toxoid and tetanus toxoid are the carrier proteins (28). It is estimated that PCV10 will cover more than two thirds of all invasive isolates from children <5 years in virtually all countries studied and ≥80% of isolates from North America, Western Europe and parts of South America.

PCV13 will substitute PCV7 and will increase coverage of IPD in young children to over 80% in many other parts of the world as well. Serotypes 1, 5 and 7F, included in both PCV10 and PCV13, are necessary for a global vaccine formulation. Only PCV13 contains serotype 19A, a serotype of increasing importance in some countries, including Portugal. Both vaccines will protect against the bulk of pneumococcal disease. A potential added benefit of PCV10 is its suggested ability to protect against NTHi, an important pathogen in respiratory tract infections (28).

5.3 Serotype replacement: Threat to vaccination success

There are reasons to believe that pneumococcal conjugate vaccines may not be the ultimate answer for the control of pneumococcal disease. A major limitation of protein conjugation of pneumococcal polysaccharide is the number of serotypes that can be included in a vaccine preparation due to manufacturing considerations and cost of production. Thus, vaccine composition will be determined by the rank of pneumococcal serotypes in a given region, and the target group. Furthermore, the current unit cost of glycoprotein conjugate vaccine is likely to be unaffordable to use in several developing countries, where the burden of disease is the heaviest (77).

In addition, studies of nasopharyngeal colonization with *S. pneumoniae* showed that although carriage of vaccine serotypes was reduced, the vacated niche was promptly occupied by non-vaccine pneumococcal serotypes that are potentially capable of causing disease, in a process referred to as serotype replacement (41). Similar trends were observed in pneumococcal infection after the general introduction of the conjugate vaccine in the United States (112). Additionally, Singleton and colleagues have reported increases in invasive pneumococcal disease in Alaskan native children despite high PCV7 coverage. According to this study, following PCV7 introduction (2001-2003), the rates of invasive pneumococcal infection decreased by 67%, from 403.2/100000

children to 134/100000 children. However, Alaskan native children experienced a significant increase in invasive pneumococcal disease more recently (2004-2006) to 244.6/100000 children (100). Thus in the long term, the widespread introduction of PCVs may merely alter the serotype distribution of invasive pneumococcal disease, rather than reducing its overall burden (59).

The increase among non-vaccine type (NVT) strains may be occurring in several ways: clones that were present before vaccination could now be expanding into the vacated niche left by the vaccine serotypes. Alternatively, due to the high frequency of recombination in this species, clones of vaccine serotypes may have acquired a new capsule through genetic exchange of the capsular locus, a process known as serotype switching (46). Current data suggest that capsule switching is a rare event, although definite instances of its occurrence have been already documented. However, careful analysis of the clonality of strains carried in vaccinated children is needed to monitor this possibility (41, 61).

To date, it is unknown whether non-vaccine serotypes exhibit the same fitness and virulence as vaccine serotypes. The much higher frequency of vaccine serotypes in the pre-PCV7 period among colonizing and pathogenic pneumococcal isolates suggests that these serotypes have advantages compared with non-vaccine serotypes. These advantages could hypothetically consist of faster replication, decreased immunogenicity, increased virulence, enhanced airborne spread, resistance to environmental factors or even direct inhibition of a competing pneumococcal strain in the natural habitat, i.e the human nasopharynx (87).

Clearly there is an urgent need to develop alternative pneumococcal vaccines that do not suffer from these shortcomings. The most promising approach to date is to develop vaccines that are based on pneumococcal proteins that contribute to virulence and are common to all serotypes. Such vaccines need to be highly immunogenic and elicit immunological memory even in infants, who have already been shown to respond well to T-cell-dependent protein antigens. Several candidate protein vaccine antigens have been identified, including non-toxic derivates of pneumolysin, choline-binding proteins, such as PspA and CbpA, metal-binding lipoproteins, such as PsaA and PiaA and the neuraminidase NanA (59). The pneumococcal protein vaccine candidates will be discussed in more detail in chapter 5.

6. Antimicrobial resistance in *S. pneumoniae*

Resistance to a wide range of antibacterial agents used in therapy of pneumococcal infections has emerged in the last four decades, with vancomycin being the only classical antibacterial agent to which no resistance has yet been reported. Substantial global diversity exists

in the proportion of resistant strains to the two common classes of drugs used to treat pneumococcal infections, namely β-lactams and macrolides, but different surveillance studies have revealed an increase of resistance among pneumococcal isolates (19, 55).

High antibiotic use is considered to be one of the most important risk factors for the selection of resistant pneumococci. Resistance to both β-lactams and macrolides is low in countries where antibiotic prescribing is low, as in the case of the Netherlands and Switzerland (6), whereas the highest global resistance is in countries with high antibiotic consumption and where overcrowded institutions, can amplify the selected resistant strains (29). Consequently, due to the attendance of day-care centers, along with the fact that children are the main antibiotic consumption age-group (56), higher antibiotic resistance rates are commonly found among invasive pneumococcal isolates recovered from children.

6.1 Definition of resistance

The efficacy of antimicrobial agents against specific pathogens can be determined based on the interpretation of Minimum Inhibitory Concentration (MIC) values in Etest strips or zone diameter measures of disk diffusion susceptibility testing, based on breakpoints established by the Clinical and Laboratory Standards Institute (CLSI) (25). MIC values reflect the *in vitro* potency of an antibiotic but provide only limited information about the *in vivo* activity of the agent (57). MICs are often reported as MIC_{50} and MIC_{90} values, which describe the MICs at which 50% and 90%, respectively, of the strains in a sample are inhibited (57). Microorganisms may be reported as being susceptible, as showing intermediate resistance or as resistant to a particular antibiotic based on the MIC values obtained for each antibiotic (Table 1.2).

Table 1.2: CLSI breakpoints for *Streptococcus pneumoniae.* MIC values in μg/mL[a] (25).

Antibiotic	Susceptible	Intermediate	Resistant
Penicillin[a]	≤0.06	0.12-1.0	≥2.0
Amoxicillin	≤2.0	4.0	≥8.0
Cefotaxime or Ceftriaxone (meningitis)	≤0.5	1	≥2
Cefotaxime or Ceftriaxone (non-meningitis)	≤1.0	2.0	≥4.0
Macrolides	≤0.25	0.5	≥1.0
Tetracycline	≤2.0	4.0	≥8.0
Levofloxacin	≤2.0	4.0	≥8.0
Co-trimoxazole	≤0.06	...	≥2.0

[a] 2007 breakpoints

Table 1.3: Penicillin breakpoints for *Streptococcus pneumoniae.* MIC values in μg/mL[a] (26).

Antibiotic	Susceptible	Intermediate	Resistant
Parenteral, non-meningitis	≤2.0	4.0	≥8.0
Parenteral, meningitis	≤0.06	...	≥0.12
Oral (Penicillin V)	≤0.06	0.12-1.0	≥2.0
Oral amoxicillin	≤2.0	4.0	≥8.0

[a] 2010 breakpoints

6.2 Mechanisms of antimicrobial resistance

The major targets for the main classes of antibiotics include cell wall biosynthesis enzymes and substrates (beta-lactams and glycopeptides), bacterial membrane structure (daptomycin), bacterial protein synthesis (macrolides, clindamycin, chloramphenicol, linezolid and tetracyclines), bacterial nucleic acid replication and repair system (quinolones) and metabolic pathways (co-trimoxazole). Both fluoroquinolone and co-trimoxazole resistance result from mutations within bacterial genes, whereas resistance to other antibacterial classes usually results from the acquisition of foreign genetic material (104).

6.2.1 Resistance to β-lactam antibiotics

Penicillins remain the mainstay of therapy for pneumococcal pneumonia (92). The first penicillin non-susceptible pneumococci, were described in Australia and Papua New Guinea in the late 1960s. Pneumococci with higher levels of resistance were subsequently reported in 1977 in South Africa and by the 1980s, a high prevalence of antibiotic resistance among pneumococci was being reported in other countries, leading to serious therapeutic problems, mainly in the treatment of pneumococcal meningitis. The emergence and rapid dissemination of antibiotic-resistant pneumococcal clones in areas of Southern and Eastern Europe, North America and Asia in 1990s was associated with an increase in antibiotic consumption (66).

The mechanism of action of β-lactams is based on the binding of the antibiotic to cell wall synthesizing enzymes, the penicillin binding proteins (PBPs), thereby interfering with the biosynthesis and remodeling of the bacterial peptidoglycan (23). Penicillin resistance involves chromosomal gene changes that alter the structure of one or more of the PBPs, thereby reducing the affinity of these enzymes for the whole class of β-lactam antibiotics (27, 57). Higher concentrations of the antibiotic are required for inhibition of these altered PBPs and thus for *in vivo* activity of the drug.

Point mutations that result in decreased affinity to β-lactams, and confer resistance to the drugs, are generally located within the penicillin-binding module of essential PBPs and can easily be selected in the laboratory. In contrast, low affinity PBPs from clinical isolates of *S. pneumoniae* are encoded by mosaic genes with regions diverging up to 25% of the sequences found in penicillin-sensitive strains. These mosaic genes are assembled by homologous recombination between pneumococcal PBPs and PBP genes from closely related species of viridans streptococci (21, 44).

Previous studies have demonstrated that penicillin non-susceptible *Streptococcus pneumoniae* (PNSP) (MIC≥0.12 μg/mL) can be associated with treatment failure in patients with meningitis, because the levels of penicillin reached in the cerebrospinal fluid or the standard dosage of third-generation cephalosporins are insufficient to eradicate the infecting organism (66). The classical CLSI, formerly NCCLS, breakpoints for penicillin (susceptible ≤0.06 μg/L; intermediate, 0.12-1 μg/mL and resistant ≥2 μg/mL) (Table 1.2) were established in the late 1970s in order to prevent failures in patients with pneumococcal meningitis caused by PNSP. In contrast, *S. pneumoniae* strains with penicillin MICs of 0.12-2 μg/mL had little effect on outcome in patients with pneumonia and other non-meningeal systemic pneumococcal infections that were treated with parenteral penicillin, amoxicillin, cefotaxime, or ceftriaxone. According to these observations, the CLSI breakpoints for ceftriaxone and cefotaxime were modified in 2002, distinguishing between meningeal infections and non-meningeal infections. However penicillin breakpoints were not modified until 2008 when the site of infection and route of administration were considered. The current penicillin breakpoints for non-meningeal infections are presented in Table 1.3 (66).

6.2.2 Resistance to Macrolides and lincosamides

Macrolides are composed of 14 (erythromycin and clarithromycin), 15 (azithromycin) or 16 membered lactones to which amino and/or neutral sugars are attached via glycosidic bonds (91). Macrolides inhibit protein synthesis by stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation. This results in chain termination and a reversible stoppage of protein synthesis (91).

Two mechanisms for erythromycin resistance in pneumococci have been described. The first mechanism involves posttranslational modification of the 23S rRNA by a methylase and has been designated MLS_B phenotype. This enzyme adds one or two methyl groups to a single adenine in the 23S rRNA moiety. Genes encoding these methylases have been designated *erm* (erythromycin ribosome methylation). The binding site in the 50S ribosomal subunit for erythromycin overlaps the binding site of the newer macrolides, as well as the structural unrelated lincosamides and

streptogramin B antibiotics (MLS_B) (91). Consequently, MLS_B resistance results in simultaneous resistance to macrolides, lincosamides (such as clindamycin) and streptogramin B.

The second mechanism confers resistance only to 14-membered (erythromycin and clarithromycin) and 15-membered (azithromycin) macrolides, and not to the 16-membered macrolides, lincosamides (clindamycin) and streptogramins, resulting in the so-called M (Macrolide) phenotype (115). The M resistance phenotype is conferred by a mechanism of efflux of the antibiotic from the cell. The gene *mef* (macrolide efflux) usually carried on a conjugative transposon, encodes a transmembrane hydrophobic protein that constitutes an efflux pump (23).

Epidemiological studies have shown differences in the geographic distribution of macrolide resistance, which frequently correlates with antimicrobial consumption, but also differences in the distribution of the macrolide resistance phenotype. For example, the M phenotype predominates in the US whereas the MLS_B phenotype is more prevalent in the rest of the world (56).

6.2.3 Resistance to other antibiotics

Fluoroquinolones: are a broad-spectrum antibiotics that mainly target DNA gyrase (GyrA) and topoisomerase (ParC) (115). Quinolones primarily cause arrest of DNA replication by interfering with DNA supercoiling and relaxation (115). In general, quinolones have shown poor activity against gram-positive bacteria, particularly streptococci. However, increasing penicillin resistance in pneumococci and the development of novel compounds has led to the use of quinolones for pneumococcal infections. Resistance to fluoroquinolones has been attributed to either efflux of the antibiotic from the cell, or more commonly, to mutations in the so-called quinolone resistance determining region QRDR, of GyrA and/or ParC (115).

Chloramphenicol: inhibits bacterial protein synthesis by targeting the enzyme responsible for the catalysis of the peptide bond formation during translation, the peptidyl transferase (23). Resistance to this antibiotic is achieved by the production of an enzyme, the chloramphenicol acetyl transferase which converts this antibiotic to its derivatives. These derivatives are unable to bind to the 50S subunit of bacterial ribosomes and therefore are incapable of inactivating the peptidyl transferase (23).

Tetracycline: binds to the 30S subunit of the ribosome, preventing binding of the tRNA. Ribosomal protection mediated by the genes *tet*(M) and *tet*(O) is the only resistance mechanism that has been described thus far in pneumococci (6, 23). Resistance to tetracycline is a common characteristic of the MLS_B phenotype because the *erm*(B) and *tet*(M) genes can be found in the

same transposon, Tn1545. In contrast, the *mef*(A) gene, responsible for the M phenotype, is not known to be linked to tetracycline resistance (5).

Co-trimoxazole: The combination of trimethoprim with sulfamethoxazole (co-trimoxazole) has been used extensively for the treatment of lower respiratory tract infections in developing countries due to its attractive cost and effectiveness. They both interfere with the biosynthesis of folic acid (23). Resistance among *S. pneumoniae* to co-trimoxazole is attributed to resistance to the trimethoprim component. Specifically, mutations in the dihydrofolate reductase gene lead to reduced affinity of trimethoprim for its target enzyme, the dihydrofolate reductase (6).

Multidrug-resistance (MDR): Pneumococci resistant to more than three different classes of antibiotics are considered to be multiply resistant. The reasons behind the development of resistance to several antimicrobials is still not clear although some of the genetic determinants of resistance can be carried together in the same transposon (6).

6.3 Epidemiology of *S. pneumoniae* resistance

Streptococcus pneumoniae is one of the most common pathogenic bacteria in young children and an increase and dissemination of resistance to the normally administrated antibiotics, such as de β-lactams and macrolides, has become a worldwide problem (70). The emergence and spread of antimicrobial resistance in pathogenic bacteria takes many forms, but always represents a process of adaptation in response to selective antimicrobial pressure generated by the use of antimicrobial agents (90). Because the largest number of antibiotic prescriptions is for respiratory infections, there is considerable selective pressure on respiratory pathogens. This generally occurs during carriage of these bacteria in the nasopharynx, but can also take place at the site of infection, such as the lung, sinus or middle ear. In addition the other colonizing flora, such as the viridians group streptococci, are also exposed to these agents and often develop resistance, which in turn can be transferred to common pathogens, such as *S. pneumoniae* (56). Moreover, since antibiotic consumption is particularly common in young children, antimicrobial resistance levels are usually higher in children than in the adult population (56).

Resistance to tetracycline and erythromycin first appeared in the 1960s, followed by resistance to chloramphenicol, co-trimoxazole, and more recently, to the new fluoroquinolones. One interesting feature of pneumococci is that the proportion of resistance to the various classes of antimicrobials vary substantially between different geographic areas (68) and over time (94). To

monitor the evolution of antimicrobial resistance, various surveillance studies have been performed and revealed the spread of resistance among pneumococcal isolates in many countries.

One example is the Sentry program, initiated in 1997, it was designed to monitor the predominant pathogens and antimicrobial resistance of both nosocomial and community-acquired infections (8). In one of the studies, which included pneumococcal strains recovered between 1997 and 1999, the data showed a high degree of resistance to penicillin, cefuroxime, erythromycin, tetracycline and co-trimoxazole. Moreover, resistance to penicillin ranged from 6.8% in Canada to 17.8% in the Asian-Pacific region and particularly high levels of resistance to tetracycline and erythromycin (around 40%) were reported in the Asian-Pacific area (53).

The Alexander project has also been monitoring resistance in respiratory pathogens, including *S. pneumoniae,* for several years and the data reported has shown an overall increase in the levels of resistance to penicillin and to the macrolides (38). One of the studies performed, which included strains recovered between 1998 and 2000 in several European countries, showed that resistance to penicillin ranged from over 50% in France to less than 5% in the Netherlands. Similarly, resistance to macrolides ranged from over 50% in France but below 10% in Germany, The Netherlands and the Czech Republic. Some of these differences may be explained by differing policies of drug usage (58). More recent data (2001-2003) has shown resistance to be particularly high in France and Spain where, 46.7% and 61.9%, respectively, of the strains were found to be non-susceptible to penicillin. In Portugal the levels of penicillin non-susceptibility (23%) are lower than those found in Spain, but significantly higher than the reported in Italy (13%) (89, 98). Concerning macrolide resistance, Portugal ranks among the countries with lower proportion of resistant isolates studies discussed above.

Despite these alarming penicillin resistance levels, pneumococci with penicillin or cefotaxime/ceftriaxone MICs ≥4 μg/mL are rarely described worldwide. If the revised CLSI breakpoints for parenteral penicillin (Table 1.3) are applied to pneumococci isolated from non-meningeal infections more than 95% of invasive pneumococcal isolates collected worldwide are currently susceptible to penicillin and third generation cephalosporins (38). The findings reported by these studies support the need for a continuous surveillance within each geographic location (94).

6.4. Effect of PCV7 vaccine on drug resistant *S. pneumoniae*

Due to the prolonged nature of carriage (by contrast with the relative transience of episodes of invasive disease) it is in the nasopharynx where strains are likely to be exposed to antibiotics and to other commensal species with the ability to pass on antibiotic-resistance genes. Indeed, the frequency with which certain serotypes are isolated from the nasopharynx seems to roughly

correlate with their likelihood to become resistant to antibiotics. These serotypes are largely represented in PCV7, in particular 6B, 9V, 14, 19F, 23F, and the vaccine related types 6A and 19A. Conversely serotype 1, rarely identified in colonization studies, remains highly susceptible to antibiotics (51, 64).

Because of the close relationship of resistance with serotype, use of pneumococcal vaccines has the potential to reduce the number of drug-resistant pneumococcal infections that occur (110). Indeed, after vaccine implementation, several studies reported not only a large decrease in invasive disease, but also a reduction in the number of infections caused by resistant strains (64, 103, 112). Nevertheless, the effect of PCV7 on antibiotic resistance was quite variable. While a decrease in penicillin non-susceptibility was noted in all countries among isolates responsible for paediatric IPD in the post-PCV7 period (3, 64, 72, 82), such a decline was not apparent in adults in Canada, Portugal and Spain (3, 9, 82). These variations can be due to differences in the impact of PCV7 in colonization since it was shown that in Portugal vaccination with PCV7 was not associated with diminished colonization with antibiotic resistant isolates (95).

One of the main questions raised upon vaccine implementation was whether vaccine use would induce the emergence of resistant serotypes that were typically not resistant, due to either serotype replacement or capsular switching. Indeed, serotype replacement in invasive disease among children and adults has been observed in the US after vaccine use, and invasive pneumococcal disease among children <5 years is now predominantly due to resistant serotype 19A (14). Continuous monitoring of serotype distribution and antimicrobial resistance, as well as genetic characterization of pneumococcal strains, is thus necessary to understand how the bacterial population will respond to the vaccine pressure.

7. Molecular epidemiology

Molecular epidemiology is the result of the integration of molecular biology into traditional epidemiology research. In clinical microbiology, molecular epidemiology focuses on the identification of specific pathways, molecules and genes that influence the risk of developing an infection (40).

The identification of the infection determinants is achieved by using comparing typing systems. These techniques provide a means of distinguishing different subpopulations within the same species and also allow the temporal and spacial tracking of a certain group of strains. The information gathered are essential to the understanding of how particular subgroups of species are associated with different clinical manifestations and to the identification of the factors behind the introduction and spread of an organism in a community (45).

7.1 Molecular typing systems

Before introducing the methods for molecular typing it is important to define relevant concepts which are commonly used in epidemiological studies. The following definitions were proposed by Tenover et al (105) and are relevant for this thesis:

Isolate: Term used for a pure culture of bacteria obtained by subculturing a single colony recovered from an isolation plate. No information is available besides the genus and species category.

Strain: Is a descriptive subdivision of a species. It consists of an isolate or a group of isolates that are distinguished from other isolates by phenotypic and genotypic characteristics.

Clone: Defines the progeny of a common precursor strain by asexual reproduction that share phenotypic and genotypic traits obtained by one or more typing methods.

Historically microbial typing has been performed by using phenotypic methods such as serotyping, bacteriophage and bacteriocin typing and antimicrobial resistance profiling. With the development of DNA based techniques a series of new genotypic methods were implemented which have been fundamental for the comparison of populations in terms of genetic features (99). For the purpose of this thesis two main DNA based assays will be presented, the pulsed-field gel electrophoresis (PFGE) and a PCR based method which is designated Multilocus sequence typing (MLST).

7.1.1 Pulsed-field gel electrophoresis

PFGE is one of the most commonly used molecular typing systems. It involves the digestion of total genomic DNA with an infrequent cutting restriction endonuclease (SmaI in most cases), followed by separation of the DNA fragments in an alternating electric field. Each isolate originates a digestion pattern which is used to create comparisons with the remaining population making it possible to infer their genetic relationships. Figure 1.8 presents a typical PFGE gel of *S. pneumoniae*.

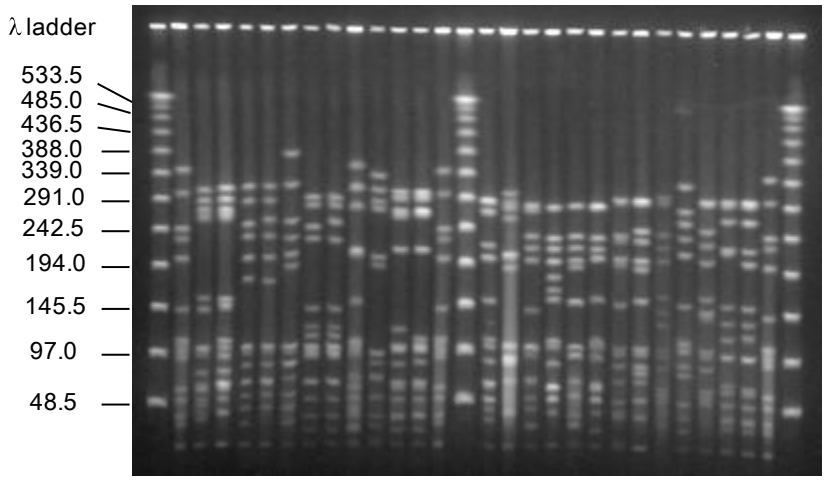

Figure 1.8: Pulsed-field gel electrophoresis (PFGE) profiles generated following SmaI digestion of total DNA from *S. pneumoniae*. Fragment sizes (Kb) of the Lambda (λ) PFGE marker (New England Biolabs, Berverly, EUA) are presented.

Criteria for analysis of the restriction patterns generated by PFGE were proposed by Tenover and colleagues (105). According to these guidelines a banding pattern difference of three fragments could have occurred due to a single point mutation and consequently these isolates are classified as highly related. Differences of four to six fragments are likely due to two independent genetic events and the strains are considered possibly related. Finally isolates with more than 7 fragment differences, which are due to three or more independent genetic events are considered unrelated (105).

Nowadays analysis of the restriction patterns is achieved through specialized software programs such as the BioNumerics (Applied Maths, Kortnijk, Belgium) which significantly facilitate the analysis and comparison of PFGE patterns. Furthermore, these applications allow normalization of PFGE gels and the simultaneous comparison of a significant number of strains which would be manually undoable. Inference of genetic relationships is achieved by the use of algorithms that allow phylogenetic analysis of the strains. In general, strains are considered identical if they show 100% similarity and clonally related if they show more than 80% similarity (which is comparable to a three-fragment difference of the Tenover criteria) (105). The typical output is the dendrogram which provides a visual representation of strain lineages and of genetic similarities and differences between groups (99).

As with any technique PFGE, has its limitations. It is quite laborious and time consuming and requires specialized equipment. Moreover, interpretation of the restriction patterns, even with the help of appropriate software, is, in some circumstances, not straightforward.

PFGE is quite useful for short term epidemiological studies such as local outbreak studies and local epidemiology since the genetic variation indexed by this method appears to accumulate relatively rapidly (33). Nevertheless, this can also be a disadvantage for answering epidemiological

questions such as whether epidemics of a certain pathogen in different countries are caused by the same clone. Longer term or global epidemiology require a method that retains the ability to distinguish a vast number of genotypes but uses genetic variation that accumulates at a slower pace (33).

7.1.2 Multilocus sequence typing

MLST provides a mean of characterizing isolates for global epidemiology that is far superior in several aspects to the majority of molecular typing methods that rely on comparison of DNA fragment patterns (37). MLST is based on the sequencing of polymorphic fragments of seven housekeeping genes. For each strain nucleotide sequences of approximately 400- to 500-bp are compared to an online database (www.mlst.net) and assigned an allele number. Each strain is thus defined by the combination of the alleles at the different loci (the allelic profile) and each allelic profile (or genotype) is assigned a sequence type (ST) (Figure 1.9) (36).

MLST is a highly discriminatory technique, compared to its predecessor, multilocus enzyme electrophoresis (MLEE) since it detects all the nucleotide polymorphisms within a gene rather than just those non-synonymous changes that alter the electrophoretic mobility of the protein product (37). Nevertheless, the cost and technically demanding nature of MLST are part of its limitations. Furthermore MLST is intolerant of DNA sequencing errors which can result in mistyping since a single nucleotide error may originate a new allele and a new ST. Finally, unlike PFGE, MLST is the combination of the results of seven separate experiments and hence any pipetting, sequencer gel tracking, or other error can lead to the generation of new recombinant STs (80).

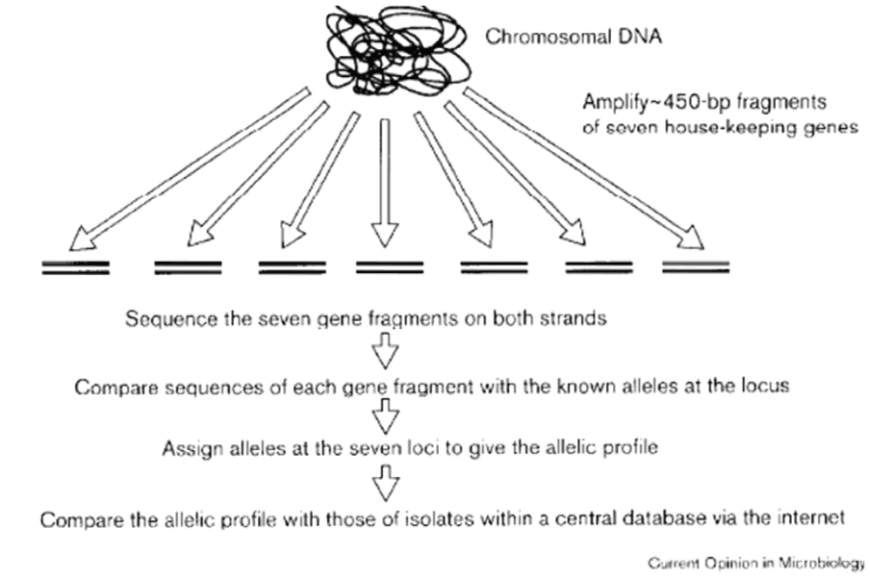

Figure 1.9: Multilocus sequence typing (MLST) scheme. The method for the allocation of the allelic profile, or sequence type, of a bacterial isolate is shown. Clustering of related isolates can be visualized as a dendrogram, constructed from the matrix of pairwise differences between the allelic profiles of the isolates. Reproduced from Spratt *et al* (102).

The major success of MLST relies on the fact that it provides molecular typing data that, in contrast to PFGE, can be easily compared within the same or different laboratories. Moreover, since this method involves housekeeping genes, the genetic variation is expected to accumulate relatively slowly, allowing a better estimate of the extent of recombination within a bacterial species (34).

7.2 goeBURST

The relationships between isolates of bacterial species or a population characterized by MLST is currently achieved by using an algorithm called goeBURST. This algorithm defines clonal complexes, ancestral genotypes and clonal variants from the allelic profiles obtained by MLST.

goeBURST is based on the BURST rules proposed by Feil and colleagues (36) that use a simple model of bacterial evolution in which an ancestral genotype increases in frequency in the population and starts to diversify. The goeBURST algorithm first identifies mutually exclusive groups of related genotypes in the population and attempts to identify the founding genotype of each group. The putative ancestral allelic profile within each goeBURST group can therefore be assigned as that which has the highest number of descendents that differ at one of the seven loci (single locus variants – SLVs) (Figure 1.10) (37). If two STs have the same number of associated SLVs, the one with the greatest number of double-locus variants (DLVs) is selected as the founding ST. Eventually, STs will diversify further, to produce variants that differ at two of the seven loci (DLVs),

at three of the loci (triple-locus variants [TLVs]) and so on. This cluster of related genotypes is often referred to as “clonal complex” (CC) (36).

In the goeBURST algorithm the user can group the population based on different degrees of relatedness. The most commonly used group definition is the default goeBURST settings which identifies groups of related STs using the most stringent definition: all members assigned to the same group share identical alleles at 6 of the 7 loci with at least one other member of the group. Using this definition (6/7 shared alleles) isolates in the group defined by goeBURST will be considered to belong to a single clonal complex. A less stringent approach is to define the groups by the sharing of alleles at least 5 of the 7 loci (36). In this case a goeBURST group cannot be assumed to belong to a single clonal complex.

Clonal complexes are normally represented by CC followed by the number of the ST founder identified by the goeBURST (e.g CC156 is the clonal complex in which ST156 was identified as founder). A software implementation is available at http://goeBURST.phyloviz.net.

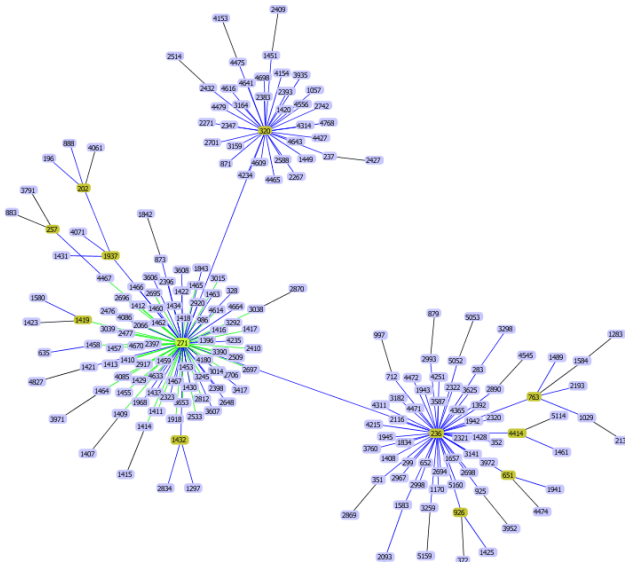

Figure 1.10: Representative diagram of a goeBURST group.

7.3 Molecular epidemiology of *S. pneumoniae*

Serotyping and antimicrobial resistance profiling are the main methods for pneumococcal characterization. To discriminate the clones associated with IPD other methodologies are being used, namely PFGE and MLST (13, 24, 42). As focused previously, PFGE allows rapid clustering of genetically related isolates and MLST can be used to easily assign, comparable and portable clone identifiers to clusters of similar isolates (97).

The general scheme for the pneumococcal characterization performed in most laboratories, including the Instituto de Microbiologia da Faculdade de Medicina de Lisboa, is represented in Figure 1.11.

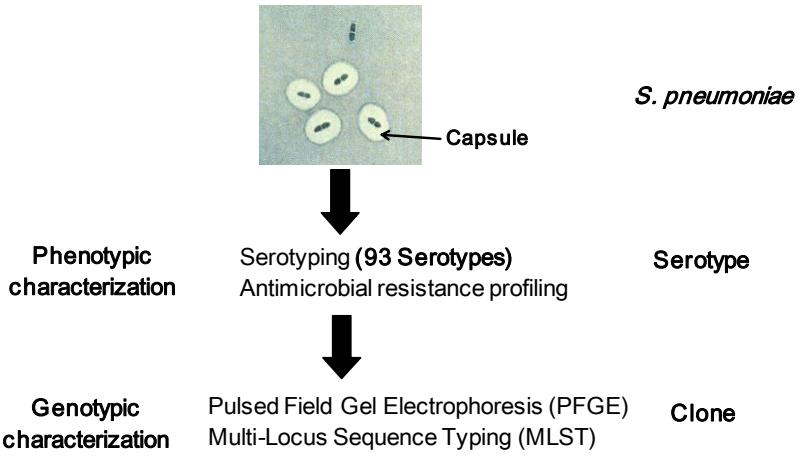

Figure 1.11: Schematic representation of the molecular characterization of *S. pneumoniae*.

PFGE is usually carried out to all pneumococcal isolates and involves total DNA restriction with SmaI followed by electrophoretic separation. For MLST analysis we select isolates representative of PFGE clones due to its high cost. For the pneumococcus in particular, sequencing of polymorphic fragments of the following seven genes is performed: *aro*E (shikimate dehydrogenase), *ddl* (D-alanine-D-alanine ligase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *rec*P (transketolase), *spi* (signal peptidase I) and *xpt* (xanthine phosphoribosyltransferase). The different sequences are then compared to an online database (http://spneumoniae.mlst.net/), assigned different alleles numbers and each allelic profile (or genotype) is assigned a sequence type (ST) (36).

7.3.1 Association between serotype, antimicrobial resistance, PFGE and MLST

Several studies from different countries have been performed concerning the molecular epidemiology of *S. pneumoniae* associated both with disease and with asymptomatic colonization. Though there are significant differences regarding the distribution of serotypes, level of antimicrobial resistance and genetic lineages associated with serotypes, what has been constantly observed in all of these studies is that there is a strong correlation between these three features. For example, antimicrobial resistance is consistently found in specific serotypes, such as serotype 14, 6B, 19A and 19F, and even within serotypes in specific clones, such as ST156. Moreover, a small number of highly successful clones dominate the population of antibiotic-resistant pneumococci (62). On the

other hand, major invasive serotypes, such as serotype 3 and 1, and their genetic lineages, are rarely associated with resistance to any antibiotics (89, 90).

In addition, PFGE and MLST analysis have shown that the diversification of genetic lineages is quite variable within serotype. One can identified serotypes with several unrelated clones, such as serotype 6B, and serotypes where only one genetic lineage is identified, such as serotype 7F (89). Factors behind these different evolutionary paths have not been completely clarified. Nevertheless, frequent carriage and/or prolonged residence in the nasopharynx may provide ample opportunities for strains, such as serotypes 6B, 14 and 23F, to undergo genetic exchanges with other pneumococcal clones and also with other bacterial commensal species. Such exchanges could lead to increased genetic diversification, which may be reflected in the diversity of STs identified among strains with these serotypes. One may suggest that the relative genetic homogeneity of the serotypes 1, 5 and 7F isolates reflects the relatively infrequent identification of these serotypes among colonizing pneumococci (117).

We have discussed previously that serotypes are differentially represented in carriage and infection. Similar observations have been made for clones, that is, certain pneumococcal genetic lineages are more common in invasive disease than others. The most invasive clones are also frequently expressing the most invasive serotypes (13). In addition significant differences in serotype-ST associations have been found in different geographic locations. Even within the most prevalent serotypes found in IPD, different genetic lineages are consistently identified in geographically dispersed countries (13, 42). What leads to this differential evolution of strains belonging to the same serotype is still unknown but is possibly a multi-factorial process (34).

7.4 Global antibiotic resistant clones

A network of researchers established a nomenclature for global pneumococcal clones (PMEN - Pneumococcal Molecular Epidemiology Network - http://www.sph.emory.edu/PMEN/index.html). The Network was established with the aim of global surveillance of antibiotic resistant *S. pneumoniae* and the standardization of nomenclature of resistant clones. The criteria for inclusion in the Network are:

a) The clone should have a wide geographic distribution (isolated on at least 2 continents; not just within a single city or country);
b) The clone can be resistant to one or more antibiotics that are in wide clinical use; or a global susceptible clone known to be important in disease;

c) Data on the clone needs to be published or in press before ratification by the network;

d) New clones for consideration into the network need to be proposed at annual PMEN meeting;

e) The clone must be made available to the network members for typing analyses and confirmation before acceptance into the network;

f) The clone must be made available for deposit into the ATCC collection of clones;

g) The clones will be also available through the Streptococcus Reference Laboratory in Germany and Emory University in the USA

In summary each of the strains included in the network is characterized by frequently being resistant to at least one antimicrobial in common use, by persistence of that clone over time, and by its wide geographic diversity (69). The suggested nomenclature of pneumococcal clones includes the country in which the clone was first isolated, the serotype first identified, and the strains sequence type (ST), for example, Spain^{23F}-ST81 (62). At least 43 resistant pneumococcal clones have been formally recognized, with the validity of each confirmed by MLST, PFGE and by PCR-RFLP analysis of the pbp1a, pbp2b and pbp2x genes (116).

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CHAPTER 2

Changes in invasive *Streptococcus pneumoniae* serotypes with non-universal vaccination coverage of the seven-valent conjugate vaccine.

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SUMMARY

The seven-valent conjugate vaccine (PCV7) is not part of the National Vaccination Plan (NVP) in Portugal but has been administered since late 2001 through the private sector. To evaluate the impact of PCV7 use, the serotypes and antimicrobial susceptibility of pneumococci causing invasive disease in Portugal during 2003–2008 were determined and compared with previous data for the period 1999–2002.

Overall, serotypes included in PCV7 accounted for 21% of IPD. Comparing with previous data from Portugal (44), this study showed a decline of PCV7 serotypes after vaccine availability. Between 2003 and 2008, serotypes 1 (n=110, 23%), 19A (n=94, 20%) and 7F (n=48, 10%) were the most frequent causes of IPD with 19A as the most frequent serotype among erythromycin and penicillin non-susceptible isolates. Serotype 1 was associated with older children and empyemas, while serotype 19A was associated with IPD in younger (<2 years) children. The higher valency vaccines PCV10 and PCV13 have a potentially superior coverage, 56% and 83% respectively, but non-vaccine serotypes are emerging as important causes of IPD since 17% of the invasive strains still presented serotypes not included in any conjugate vaccine formulation.

INTRODUCTION

Studies in the USA, following vaccine introduction, showed a dramatic reduction in the proportion of cases of invasive infections caused by the serotypes targeted by the vaccine (1, 3, 32, 39, 47). Because asymptomatic colonization of children constitutes the main reservoir of pneumococci and PCV7 also prevents colonization with the seven serotypes included in the vaccine (41), the effects of vaccination were propagated to older age groups, including the elderly, who have a high burden of pneumococcal disease, as a consequence of herd immunity (1, 3, 8, 32, 39). The swiftness and magnitude of the decline seen in older age groups was unexpected and constitute a significant fraction of the number of cases of invasive pneumococcal disease (IPD) prevented by PCV7 (39). In the USA this group effect is responsible for most of the overall reduction in IPD (8).

Results from other studies indicate that the vaccine also had an impact on non-invasive infections by reducing the cases of otitis media due to isolates of vaccine serotypes (15) and by reducing the cases of radiologically confirmed pneumonia (34). The use of PCV7 has also been implicated in the reduction of the proportion of resistant isolates observed in the last years in the USA since at the time of its introduction, five of the seven serotypes in PCV7 accounted for a large fraction of penicillin non-susceptible isolates (13, 24).

In the European Union, the vaccine was licensed in February 2001, and actual availability in individual countries possibly required approval by the national regulatory agencies (25). In contrast to the USA, availability was not followed shortly after by recommendations for universal vaccination of infants and children under 2 years of age (47). By 2003, most European countries had issued vaccination recommendations for risk groups only, although the definition of these groups varied among countries and could be widely targeted or include large portions of the pediatric population (36). This situation started changing in 2005, and by January 2007, ten countries, including the UK, France and Germany, had adopted universal vaccination plans (25).

It has been argued that IPD differs between western Europe and the USA in its incidence, which seems to be lower in Europe (22), although this finding has been questioned because of different cultural practices (42). The prevalence of the serotypes found among invasive infections also varies between the two continents, which consequently affects the potential coverage of PCV7, due to the limited number of serotypes included in the vaccine. Estimation of vaccine coverage for invasive pneumococcal infections in children <5 years of age varies from 53.8% in a Spanish study to 85% in Denmark (28), but is mostly below that estimated for the USA (87%) (5). It remains to be shown whether the consequences of vaccination documented in the USA are due to the particular structure of the circulating pneumococcal population and whether they will be replicated in the European countries where universal vaccination was recently adopted.

The situation in countries where the vaccine is available but not universally administered, such as Italy (25), Spain (http://www.msc.es/ciudadanos/ proteccionSalud/infancia/docs/neumo.pdf) and Portugal, is even more complex. In spite of the unquestioned efficacy of PCV7 in preventing invasive infections by vaccine serotypes among vaccinees (34), depending on the fraction of vaccinated children, one might expect a variable magnitude of reduction of the infections caused by vaccine serotypes in the overall pediatric population, and also the existence and scope of indirect effects on infections in adults. In Portugal, PCV7 has been available since June 2001, and although it is not currently included in the National Vaccination Plan, it has been administered to children through the private sector without government funding. By 2002, about 33% of the children up to 3 years of age had received at least one dose of PCV7, mostly after 23 months of age (14). More recently, a survey was conducted using a methodology that involves direct evaluation of vaccination records and that was previously shown to provide highly reliable vaccination estimates (18). In this study, the vaccination records of approximately one-third of all children born in Portugal between 2001 and 2005 were evaluated, taking into account the currently recommended PCV7 vaccination schedule in Portugal of three doses and a booster administered at 2, 4, 6 and 18 months or at 3, 5, 7 and 18 months. The proportion of children with three doses at 12 months of age increased steadily from 23.7% in the cohort of birth in 2001 to 51.2% in the 2005 cohort, as did the proportion of children with four doses at 24 months, which increased from 20.2% in the 2001 cohort to 43.1% in the 2004 cohort (data from the 2005 cohort were not yet available) (Queirós et al., 'Cobertura pela vacina pneumocócica conjugada heptavalente nas coortes de nascimento de 2001 a 2005 na região norte', available at: http://www.dgs.pt).

In spite of the remarkable initial benefits of PCV7, it was always recognized that the serotypes not included in the vaccine could emerge to replace the decline of the PCV7 serotypes. Such a replacement has indeed been observed, although its magnitude was highly variable - being modest in North America (3, 39) and much more significant in Spain (32, 38). Even within North America specific populations showed different behaviors, for instance among the Alaskan native children, the effects of PCV7 in overall IPD have been dramatically eroded with time (45). One of the main emerging serotype is 19A, which is being found as an increasingly cause of invasive infections in all age groups in several countries (35). Yet, other emerging non-vaccine serotypes differ among the various geographic locations and also between age groups (1, 3, 39).

Recognizing that historically important serotypes were not included in PCV7, some of which increased in importance since PCV7 was introduced, two new conjugate vaccine formulations were developed and have recently become commercially available. PCV10, which includes PCV7 serotypes plus serotypes 1, 5 and 7F, received approval from the European Medicines Agency (EMA) in March 2009. PCV13, which includes all PCV10 serotypes plus serotypes 3, 19A and 6A, received EMA approval in December 2009 and from the Food and Drugs Administration (FDA) in

February 2010. The introduction of these vaccines into clinical practice has the potential to once again change the characteristics of IPD, eventually blunting or even reversing the rise of some of the most successful serotypes that emerged since the introduction of PCV7. However, the potential effects of these novel vaccines will probably also be conditioned by the different characteristics of the pneumococcal population causing IPD in each region.

Continuous studies of the epidemiology of *S. pneumoniae* infection are thus needed to understand the response of the pneumococcal population to the vaccine introduction. It was previously shown that the distribution of serotypes of pneumococci causing invasive infections were essentially stable until 2002 (43, 44), establishing a baseline against which the impact of vaccination could be evaluated. The present study aims to determine changes on serotype distribution and antimicrobial resistance subsequent to vaccination in different pediatric groups and evaluating the potential impact that the availability of PCV10 and PCV13 may have in pediatric IPD in Portugal.

MATERIALS AND METHODS

Bacterial isolates

Since 1999, the Portuguese Surveillance Group for the Study of Respiratory Pathogens has monitored pneumococci causing invasive infections in Portugal. This is a laboratory-based surveillance system, in which 30 microbiology laboratories throughout Portugal are asked to identify all isolates responsible for invasive pneumococcal infections and to send them to a central laboratory for characterization.

A case of invasive disease is defined by an isolate of *S. pneumoniae* recovered from a normally sterile body site and does not include isolates recovered from the middle ear. Although the laboratories were contacted periodically to submit the isolates to the central laboratory, no audit was performed to ensure compliance, which may be highly variable in this type of study. Only one isolate from each patient was considered and whenever isolates from blood and cerebrospinal fluid (CSF) were available, only the CSF isolate was considered.

Isolates recovered from pediatric invasive infections, i.e. recovered from patients <18 yrs, between 2003 and 2008 were characterized. All strains were identified as *S. pneumoniae* by colony morphology and hemolysis on blood agar plates, optochin susceptibility and bile solubility.

Serotyping

Serotyping was performed by the standard capsular reaction test using the chessboard system (46) and specific sera (Statens Seruminstitut, Copenhagen, Denmark). Serotypes were classified into vaccine serotypes, i.e., those included in PCV13 (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A and 23F) and that comprise all the serotypes found in the lower valency vaccines (PCV10 and PCV7), and non-vaccine serotypes (NVT).

Serotype confirmation of serogroup 6 strains, and the identification of serotype 6C, was performed by multiplex PCR. The multiplex PCR reaction included primers specific for serogroup 6 capsule (6Bwzy-f and 6Bwzy-r), serotype 6A (cpsS6A-d cpsS6A-r) and serotype 6C (cpsS6C-d cpsS6C-r). Template DNA was prepared by diluting 9 μl of an overnight culture in 441 μl of water and boiling this mixture for 2 minutes. The PCR reactions were performed in a 25 μl volume containing 10 μl of template solution, 1X reaction buffer (Promega, Madison, USA), 2mM of $MgCl_2$ (Promega, Madison, USA), 5mM dNTPs (Fermentas, Vilnius, Lithuania), 0.75U of Gotaq polymerase (Promega, Madison, USA), 7.5 pmol of primers 6Bwzy-f and 6Bwzy-r and 10 pmol of each of the remaining primers. The PCR program consisted of 30 cycles of 95ºC for 45s, 54ºC for 45s and 72ºC for 45s followed by 10 min of incubation at 72ºC. Serotype 6B, 6A, and 6C were

differentiated according to the size and number of amplified products (Figure 2.1). 6B isolates originate one fragment of 250 bp, 6A isolates produce two fragments, one of 160 bp and another of 250 bp and finally serotype 6C isolates are identified by the presence of 3 fragments 160 bp, 250 bp and 727 bp. All serogroup 6 isolates were tested by this multiplex PCR reaction.

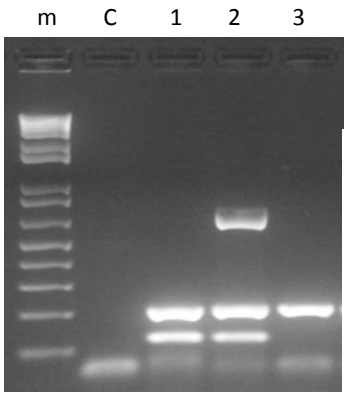

Figure 2.1: Agarose gel of PCR products resulting from the amplification of serotype 6A, 6B and 6C capsular loci. m- DNA marker (1 kb plus, Invitrogen, Carlsbab, CA); C- negative control (no template); Lane 1 – serotype 6A strain; Lane 2- serotype 6C strain; Lane 3 – serotype 6B strain.

Antimicrobial susceptibility testing

Etest strips (AB Biodisk, Solna, Sweden) were used to determine the MICs for penicillin, cefotaxime, ceftriaxone and levofloxacin, as previously described (29) and the 2007 CLSI-recommended breakpoints were used to interpret MIC value (Table 1.2) (10). Previous susceptibility breakpoints for penicillin (minimal inhibitory concentration MIC ≥0.06 μg/mL) were compared with new 2008 breakpoints (MIC ≥2.0 μg/mL) as established by CLSI (Table 1.2 and 1.3) (11).

Isolates were further characterized by determining their susceptibility to erythromycin, clindamycin, vancomycin, linezolid, tetracycline, co-trimoxazole and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria (11).

Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, accordingly to a previously published procedure (30). Simultaneous resistance to erythromycin and clindamycin characterizes de MLS_B phenotype (resistance to macrolides, lincosamides and streptogramins B) while non-susceptibility only to erythromycin indicates the M phenotype (resistance to macrolides only). Identification of macrolide resistance genes was performed by multiplex PCR reaction, which included primers that allowed amplification of *erm*(B) gene, *erm*(A) gene and *mef*(A)/*mef*(E) gene. No distinction was done between *mef*(A) and *mef*(E). The assay was executed as described previously (16).

Statistical analysis

Unless otherwise stated, analyses were performed using the information available for the entire period 2003–2008, taking into consideration three age groups: children <2 years (n=207); children 2 to 5 years of age (n=145) and the group of individuals aged 6–17 years (n=123). The two former groups relate to current recommendations suggesting vaccination of children aged <5 years who attend day-care centers (9) and most children in Portugal in this age group are indeed attending day-care centers. The proportions of IPD cases caused by serotypes included in PCV7 and in the two new conjugate vaccines, PCV10 and PCV13, were determined.

Wallace coefficients (W) provide an estimate of, given a typing method, how much new information is obtained from another typing method. A high value of Wallace's coefficient indicates that partitions defined by a given method could have been predicted from the results of another method, suggesting that the use of both methodologies is redundant. Wallace coefficients were used to compare two sets of partitions (7, 40).

Simpson's index of diversity (SID) measures the discriminator ability of typing systems. The index indicates the probability of two strains sampled randomly from a population belonging to two different types. Simpson's index of diversity was used to measure the population diversity (7).

Differences in proportion were evaluated by calculating the 95% confidence intervals (CI95%) or by the Fisher exact test (2). Linear regressions models were used to test trends in time. The Friedman's test and the Dunn's post test were used to compare resistance in the various age groups. A $p<0.05$ was considered significant for all tests.

A global comparison of the yearly serotype distributions during 1999-2005 was performed through the estimation of mutual information. The value of the mutual information is a measure of dependency (similarity) between two probability distributions, expressing how much information is common to the two distributions. The absolute minimal value of mutual information is 0 and implies that the two distributions become more similar, it becomes easier to predict one distribution by knowing the other, and this corresponds to an increase in mutual information (12). This technique was used to identify a breakpoint delimiting two periods when the serotype distribution among invasive isolates changed most significantly.

RESULTS

Isolate collection

A total of 475 strains recovered from invasive pediatric infection between 2003 and 2008 were included in the present study. An increase in the total number of isolates recovered each year was noticed (n=29 in 2003, n=42 in 2004, n=51 in 2005, n=93 in 2006, n=145 in 2007 and n=115 in 2008).

The mean age of the patients was 3.92 years. 43.6% (n=207) of the strains were obtained from children below 2 years of age, 30.5% (n=145) from children 2-5 years of age and 25.9% (n=123) were recovered from patients 6-17 years of age. 285 strains were recovered from male patients and no information of the patient sex was provided for 2 strains. Regarding the biological sample, the majority of the isolates (n=389, 81.9%) were recovered from blood, 59 isolates (12.4%) were recovered from CSF, 19 isolates (4.0%) from pleural fluid and 8 (1.7%) from other normally sterile sites.

Serotype distribution

Among the 475 strains, 43 different capsular types were identified. The most frequent serotypes were 1 (n=110; 23.2%), 19A (n=94, 19.8%), 7F (n=48, 10.1%), 14 (n=30, 6.3%) and 3 (n=24, 5.0%). Together these five serotypes accounted for 64.4% of the invasive pediatric infections between 2003 and 2008.

Serotypes covered by the PCV7 vaccine constituted only 20.6% of all isolates, though an overall tendency to decrease was observed during the period of the study (Figure 2.2). On the other hand, PCV10 and PCV13 vaccine serotypes represented 55.6% and 82.7%, respectively. However, even when considering the highest valency of the conjugate vaccines available, 17.3% of the isolates still presented serotypes not included in any of the conjugate vaccines formulations.

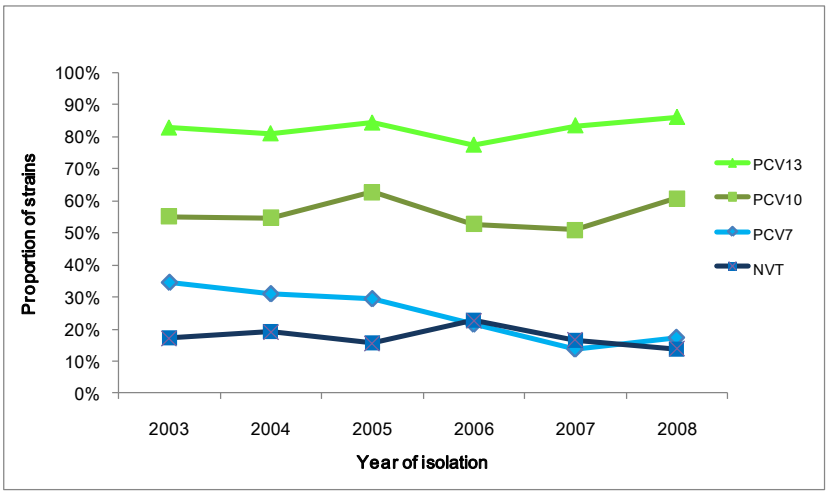

Figure 2.2: Proportion of strains presenting serotypes included in PCV7, PCV10 and PCV13 formulations, and proportion of strains presenting non-vaccine serotype, in each year of the study.

The 10 most frequent serotypes identified between 2003 and 2008, when considering the entire pneumococcal population was, in decreasing rank order: 1, 19A, 7F, 14, 3, 23F, 19F, 6B, 18C and 6A, which altogether account for almost 80% of invasive infections. Yet, not all serotypes presented the same degree of importance when we considered the serotype distribution in terms of age group. The results of serotyping of the isolates recovered between 2003 and 2008 for each age group considered are summarized in Figure 2.3.

While serotype 1 was found in 3.9% (n=8) of the isolates causing infections in children <2 years, it was the most prevalent serotype of the two other age groups considered, namely 2 to 5 yrs (n=49, 33.8%) and 6 to 17 yrs (n=53, 43.1%), where it accounted for more than a third of the isolates. The difference of 9.3% (CI95% -20.7% to 2.3%) between the two groups of older children is not significant. In contrast the proportion of serotype 1 isolates in children <2 years was less 29.9% (CI95: -38.2% to -21.9%) of that found among 2-5 year old and 39.2% (CI95%: -48.2% to -30.1%) less than that of the older age group (6 to 17 yrs), both significant. Serotype 19A, on the other hand was the most prevalent capsular type in the <2 yrs group accounting for 30.4% (n=63) of the invasive infections, while in isolates recovered from children 2 to 5 yrs it was only found in 17.2% (n=25) of the isolates. The 13.2% (CI95%: -21.6% to -4.1%) difference was statistically significant. The proportion of 19A in older children and teenagers was 4.9% (n=6), a significant drop of 25.5% (CI95%: -32.6% to -17.6%) in relation to the <2 yrs old. The relative contribution of the serotypes not included in any vaccine formulations to IPD in young children was more important than in older groups (21.2% in <2 years versus 12.4% in 2 to 5 yrs and 16.2% in 6 to 17 years).

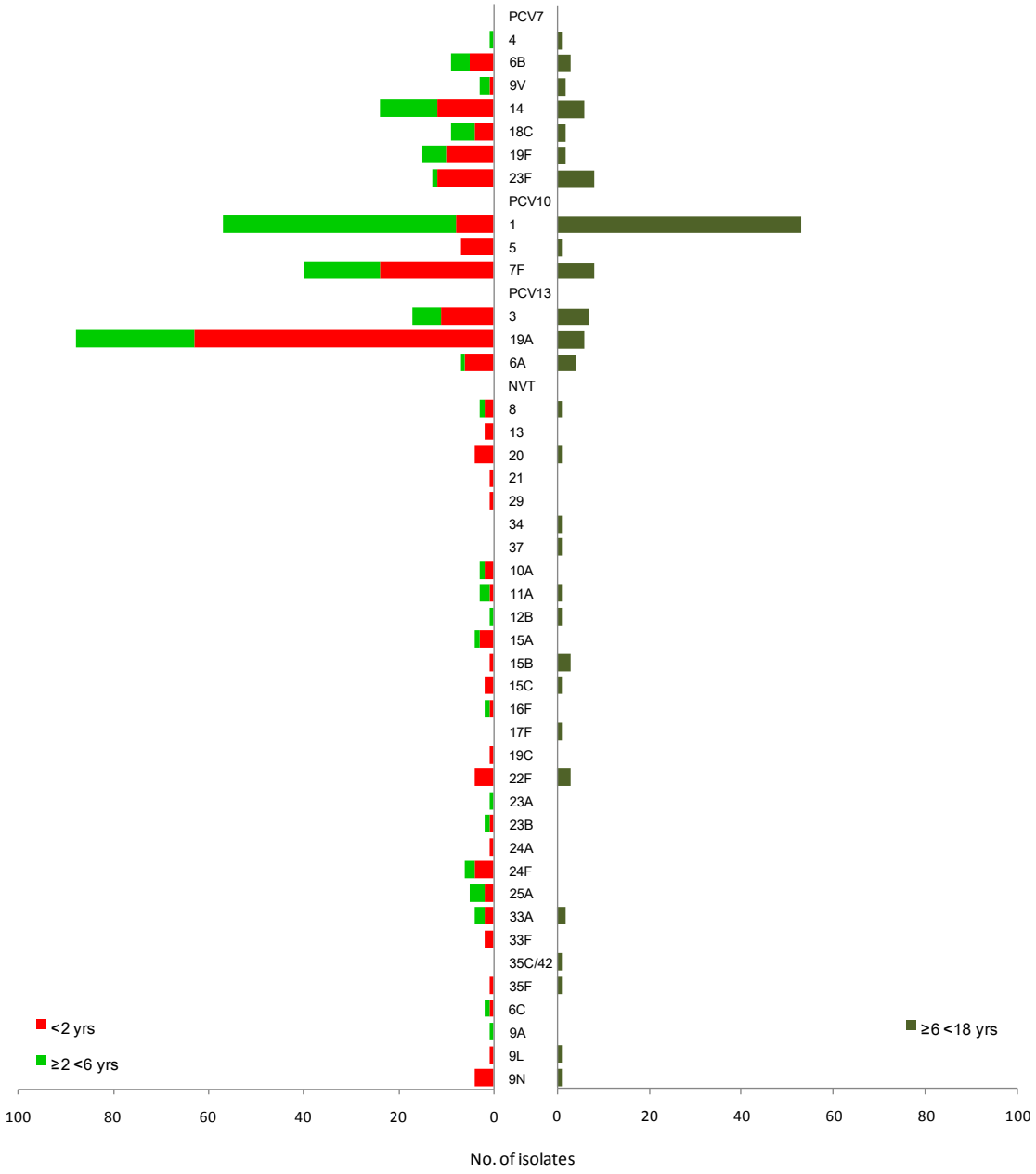

Figure 2.3: Cases of invasive pneumococcal disease according to serotype and age group in Portugal (2003–2008). Serotypes are categorized as belonging to one of the three conjugate vaccines available (PCV7, PCV10, and PCV13) or as non-vaccine types (NVT).

To analyze serotype diversity within each age group, the Simpson's indices of diversity were determined. The serotypes of the isolates causing IPD in all age groups considered were similarly diverse with overlapping confidence intervals (children <2 yrs [SID:79.95 CI95%: 72.90-87.01]; children 2 to 5 yrs [SID: 87.92 CI95% 84.52-91.32]; and individuals 6 to 17 yrs [SID: 83.62 CI95% 79.28-87.96]. A similar analysis was performed for determining the serotype diversity in each study

year. The SIDs of all years presented overlapping confidence intervals, indicating that there was no major change in serotype diversity between 2003 and 2008 (Table 2.1).

Table 2.1. Simpson's indices of diversity and 95% confidence intervals (CI95%) for serotype distribution in each year of the study.

Year	No. of isolates	SID CI95%
2003	29	91.87 [87.52-96.23]
2004	42	88.73 [82.78-94.69]
2005	51	90.35 [85.88-94.83]
2006	93	90.53 [87.55-93.51]
2007	145	87.66 [84.75-90.57]
2008	115	85.46 [80.92-90.00]

Concerning temporal variations of serotype prevalence during the study period, significant changes were only observed for serotypes 18C and 23F, which declined continuously. The largest increase was seen among serotype 1 isolates that rose from 17.2% in 2003 to 31.3% in 2008, however linear regression models showed that the differences in proportion with time were not significant (p=0.09).

It had previously been shown that the pneumococcal population causing invasive infections in Portugal was stable from 1999 to 2002 (43, 44) in spite of the availability of PCV7 since June 2001. In order to evaluate the potential impact of vaccination, the breakpoint in time that maximizes the differences in serotype distribution was estimated by determining the mutual information of the distributions recovered between 1999 and 2005 for children <6 yrs (Figure 2.4). According to this analysis the breakpoint for children <6 years occurs between 2002 and 2003. For this reason we consider as being the pre-vaccine all the strains recovered before 2002 and the post-vaccine period all the isolates recovered after 2003.

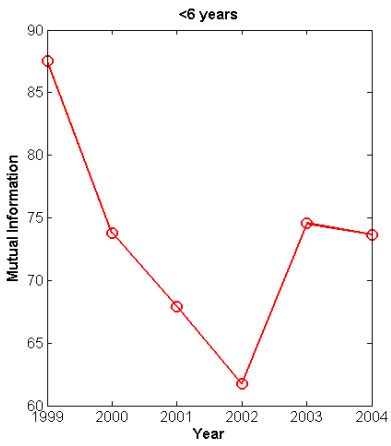

Figure 2.4: Changes in serotype distribution (1999-2005) in children <6 years. Minima in mutual information graphs indicate the years dividing the study in two periods with maximally divergent serotype distributions.

In terms of serotype associations with isolate source, a significant correlation was observed between serotype 1 and pleural fluid. Among the 19 pleural fluid samples obtained, serotype 1 pneumococci was found in 12 (p=0.0002, two tailed Fisher's exact test).

Antimicrobial resistance

Resistance to the antimicrobials tested is presented in Table 2.2. Overall, 95 isolates presented non-susceptibility to penicillin (PNSP), of which 88 (18.6%) expressed low level resistance and 7 (1.5%) expressed high level resistance, according to the 2007 CLSI breakpoints (Table 1.2). A total of 100 strains (21%) were found to be erythromycin resistant (ERP), mostly expressing the MLS_B phenotype (86%, n=86). Among these 13 different serotypes were identified, although 19A was found in 50% of the MLS_B strains. The remaining 14 isolates (14%) presented the M phenotype and mainly belonged to serotype 14 (n=12).

Concerning the macrolide resistance genes, there was a strong correlation between phenotype and resistance gene. Among the 86 strains presenting the MLS_B phenotype, 85 (99%) presented the *erm*(B) gene, while in one isolate neither *erm*(B), *erm*(A) or *mef*(A) was identified. This isolated expressed serotype 19A capsule. Similarly, all 14 isolates showing an M phenotype carried the *mef*(A) gene.

A significant fraction of the isolates (11.8%) were simultaneously erythromycin resistant and penicillin non-susceptible (EPNSP). All isolates were susceptible to vancomycin, levofloxacin and linezolid.

Table 2.2: Antimicrobial susceptibility of isolates responsible for invasive infections (2003–2008).

Antibiotic[a]	No. of resistant isolates (%)		
	<2 years (n=207)	2 to 5 years (n=145)	6 to 17 years (n=123)
PEN	56 (27.1%)	26 (17.9%)	13 (10.6%)
MIC_{90}	0.75	0.75	0.094
MIC_{50}	0.023	0.016	0.016
CTX	8 (3.9%)	4 (2.8%)	3 (2.4%)
MIC_{90}	0.75	0.75	0.094
MIC_{50}	0.032	0.023	0.023
ERY	58 (28.0%)	30 (20.7%)	12 (9.8%)
CLI	52 (25.1%)	25 (17.2%)	9 (7.3%)
CHL	11 (5.2%)	7 (4.8%)	3 (2.4%)
SXT	50 (24.1%)	26 (17.9%)	15 (12.2%)
TET	48 (23.2%)	26 (17.9%)	9 (7.3%)

[a]PEN: Penicillin; CTX: Cefotaxime; ERY: Erythromycin; CLI: Clindamycin; CHL: Chloramphenicol; SXT: Co-trimoxazole; TET: Tetracycline. MIC_{90} and MIC_{50}: minimum inhibitory concentration at which 90% and 50% of the strains are inhibited, respectively.

Temporal fluctuations were observed mainly for penicillin non-susceptibility and macrolide resistance, yet no significant increase was identified during the period of the study. Macrolide resistance varied from 13.8% in 2003, to 31.2% in 2006 and 21.7% in 2008. Similarly, penicillin non-susceptibility ranged from 17.2% in 2003, to 25.8% in 2006 and 19.1% in 2008.

Isolates recovered from patients of the different age groups presented distinct resistance levels (Friedman's test, p=0.009) but the only significant difference was between children <2 yrs and the 6-17 yrs group, with the former consistently showing greater resistance than the latter.

The correlation between serotype and antimicrobial resistance was high (Figure 2.5). The Wallace (W) for serotype and PNSP was 0.802 [CI95%:0.787-0.816], while the W for serotype and erythromycin resistance was 0.783 [CI95%: 0.759-0.808]. The serotype most frequently found in antimicrobial resistant isolates was 19A followed by the PCV7 serotypes 14, 19F, 6B and 23F (Table 2.3). Taken together, serotypes included in the PCV7 vaccine represented 45% of ERP, PNSP and EPNSP, while serotypes included in PCV10 and PCV13 constituted 47% and 86%, respectively, of these resistant isolates. A significant number of resistant isolates was found in a variety of serotypes not included in any of the current conjugate vaccine formulations (6C, 9L, 11A, 15A, 15B, 15C, 16F, 20, 33A and 33F). To determine the diversity of serotypes associated with penicillin non-susceptibility and erythromycin resistance, the Simpson's indices of diversity were determined. For both antibiotics serotype diversity was quite high (PNSP SID: 78.16 [CI95%: 71.99-84.34]; ERP SID: 71.78 [CI95%: 63.23-80.32]. On the other hand, serotypes 1, 3, 7F were significantly associated with susceptibility to most antibiotics studied.

Table 2.3: Serotypes most frequently found among resistant isolates in pediatric IPD (2003–2008).

Serotype	No. of isolates resistant to[a]				
	Fully susceptible	At least one antimicrobial tested	PEN	ERY	MDR
6B	2	10	5	7	3
9V	1	4	3	1	1
14	2	28	16	15	9
15A	0	4	4	4	2
19A	33	61	38	50	45
19F	7	10	5	7	6
23F	5	16	16	1	2
33A	0	6	0	6	0
Other	252	34	8	9	7

[a] PEN: Penicillin; ERY: Erythromycin; MDR: Multi-drug resistant

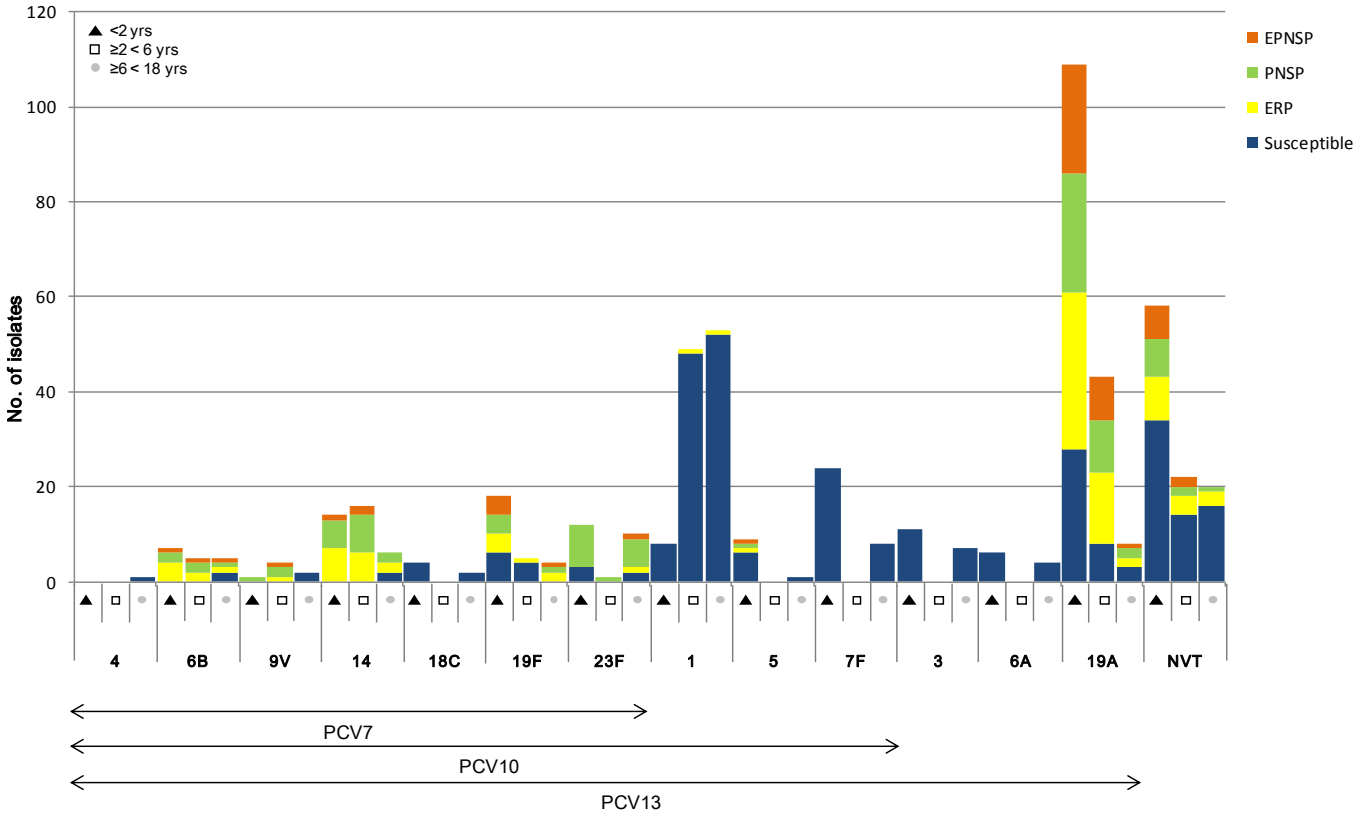

Figure 2.5: Number of isolates expressing serotypes included in conjugate vaccines and associated with invasive infections in Portugal (2003–2008). The number of isolates expressing each serotype in each of the age groups considered is indicated. Isolates recovered from children <2 years are indicated by black triangles. Isolates recovered from children ≥2 and <6 years are indicated by open squares. Isolates recovered from patients ≥6 and ≤17 years are indicated by grey circles. Erythromycin resistant isolates (ERP) are indicated in yellow. Penicillin non-susceptible isolates (PNSP) are indicated in green. Isolates presenting both erythromycin resistance and penicillin non-susceptibility (EPNSP) are represented in red. Isolates susceptible to both penicillin and erythromycin are represented in blue. The serotypes included in each of the conjugate vaccines currently available are indicated by the arrows. NVT non vaccine serotypes, i.e., serotypes not included in any of the currently available conjugate vaccines (PCV7, PCV10 and PCV13). A total of 30 NVT were detected, 7 isolates of serotype 22F, 6 isolates of each of the following serotypes: 33A and 24F, 5 isolates of each of the following serotypes: 20, 25A and 9N, 4 isolates of each of the following serotypes: 8, 11A, 15A, 15B, 3 isolates of each of the following serotypes 10A and 15C, 2 isolates of each of the following serotypes: 13, 12B, 16F, 23B, 33F, 35F, 6C and 9L; and 1 isolate of each of the following serotypes: 21, 29, 34, 37, 17F, 19C, 23A, 24A, 35C/42 and 9A.

DISCUSSION

A total of 475 pneumococcal isolates recovered from invasive infection in pediatric patients between 2003 and 2008 were characterized in terms of serotyping and antimicrobial resistance. This contrast with the 103 isolates recovered between 1999 and 2002 and previously analyzed (44). Although an increase in the number of isolates was apparent, a greater compliance with the surveillance policy and enhanced awareness of IPD due to the availability of PCV7 was possibly a major factor behind this increase. This temporal trend could bias an estimate of the incidence of IPD. Furthermore, being a laboratory based surveillance system and not a population based system, it is not designed to allow the estimation of the incidence of IPD and to quantify the benefits of vaccination that may have resulted from the great reduction in PCV7 serotypes. Therefore the analysis will focus on the relative changes in serotype distribution and antimicrobial resistance phenotypes after the availability of the PCV7 vaccine.

The continued use of PCV7 was associated with a decline in the overall proportion of pediatric IPD caused by the serotypes included in this vaccine, that accounted for 55.9% of infections in 1999-2002 (44) but only 20.6% in 2003-2008. The difference found between the pre-PCV7 and the post-PCV7 periods was significant. Changes in serotype distribution in invasive infections included the decrease in the proportion of vaccine serotypes 4, 6B, 9V, 14 and 23F, which were accompanied by an increase in the proportion of two non-vaccine serotypes 19A and 1, though not statistically significant.

The PCV7 availability did not induce changes only in its primary target, namely pediatric IPD. Similar to the herd effect documented in the USA (8), indirect consequences of vaccination of children were also noted in the serotype distribution of pneumococci causing invasive infections among the adult population in Portugal, although these occurred a year later than the changes in serotypes documented in children (1). In addition, vaccination has already been shown to interfere with asymptomatic nasopharyngeal carriage of *S. pneumoniae* by young children, although in Portugal this did not lead to an overall decrease in the prevalence of colonization (17).

Yet, the decline in the relative importance of the serotypes included in pediatric IPD in Portugal was not so significant as the one documented recently in the USA, where the PCV7 serotypes accounted for only 2.2% of isolates recovered in 2006-2007 in children <5 yrs (39). The universal vaccination program adopted in the USA is in contrast with the administration of PCV7 outside the National Vaccination Plan (NVP) in Portugal that led to a gradual but steady increase in vaccination and may be a major factor accounting for the difference in the reduction of PCV7 serotypes. More importantly, according to the same study (39), the potential coverage of PCV10 in the USA in this age group (14.1%) was clearly below the one described here. This reflects the lower

importance of serotype 1 isolates in the USA when compared to Portugal. Serotype 1 traditionally accounts for a higher proportion of pediatric invasive infections in Europe than in North America (21). In Spain outside the Madrid area, where PCV7 is available but not included in the NVP similarly to Portugal, a study from Barcelona found that serotype 1 increased in pediatric IPD since PCV7 became available and was the leading cause of pediatric IPD in 2006 (32). A study from northern Spain confirmed the increased importance of serotype 1 in IPD but restricted to older children and adults (26). In agreement with the proposed preponderance of serotype 1 in older children (21), we found an association of this serotype with IPD in older children with significant differences also noted between 2-6 yrs old and 7-17 yrs old.

Serotype 1 has not been frequently associated with carriage, suggesting that neither high density nor prolonged colonization is a prerequisite for invasion by this serotype (21). Nevertheless, a study of pneumococcal carriage in Portugal recently described an increase of serotype 1 from 0% in 2001/2002 (pre-PCV7) to 3.1% in 2006, exclusively associated with older children (>2 yrs) (33). This observation suggests that the decline of the PCV7 serotypes in carriage created a niche that was exploited by serotype 1, resulting in increased colonization particularly in older children. This may have resulted in more opportunities to cause invasive infections, contributing to the changes described here. However, cyclic waves of increased serotype 1 IPD have been historically described, in the absence of conjugate vaccine use, (20), weakening a possible casual link between PCV7 introduction and the increase in serotype 1 IPD described here.

Serotype 1, together with serotype 3, are the only serotypes significantly enriched in complicated versus non-complicated pneumonia cases (21) and both serotypes have been associated with increasing rates of empyema in some regions of the USA (6). In our collection serotype 1 was also more frequently recovered from pleural fluid than expected, indicating that empyemas are also associated with this serotype in Portugal. Serotype 1 isolates remain mostly susceptible to all tested antimicrobials as previously described in Portugal and elsewhere (26, 44).

The second most prevalent serotype in our collection was 19A. Like serotype 1, serotype 19A also showed a strong association with age but with an opposite variation to that found among serotype 1. Serotype 19A was overrepresented in infections in children <2 yrs and its relative contribution weakened with increasing age, with the 7-17 years old showing the lowest numbers of 19A infections. Serotype 19A infections increased dramatically in recent years, not only in countries where PCV7 is available or introduced (31, 37) but also in other countries. Similarly to our data, most studies found that serotype 19A pediatric IPD was associated with younger children (23, 31, 32). In contrast to serotype 1 isolates that were almost universally susceptible to all antimicrobials tested, serotype 19A presented a large proportion of resistant isolates, being the most frequent serotype among ERP, PNSP and EPNSP. Due to the increasing importance of serotype 19A, a more detailed analysis will be provided in chapter 4.

Serotype 7F was the third most frequently found serotype and all isolates were found to be susceptible to penicillin and erythromycin, which is in agreement with data described previously in Portugal (44). In contrast to serotype 1 and 19A, no significant association with age was found for serotype 7F. Nevertheless, similarly to serotype 1, serotype 7F isolates have also increased significantly in colonization in Portugal (41).

In spite of the massive reduction in the number of PCV7 serotypes, the five serotypes included in this vaccine that were traditionally associated with resistance (6B, 9V, 14, 19F and 23F), still accounted for 45% of the isolates resistant to either erythromycin, penicillin or both. For example, serotype 14 isolates, formerly the number one serotype in pediatric IPD (44), were mostly resistant to either erythromycin or penicillin or both (28/30, 93%). Yet, one of the beneficial outcomes of vaccination, also observed in the USA, was the reduction of the disease burden associated with antibiotic-resistant pneumococci. The fraction of penicillin-non-susceptible pneumococci causing invasive infections in Portugal decreased in children (<6 years) from 43.9%, before vaccine availability (44) to 20%, considering the CLSI guidelines of 2007. This effect was not observed for macrolide resistance since both periods presented similar levels (approximately 20%).

It has been suggested that local variations in selection pressure are the best predictor of the proportion of pneumococci resistant to β-lactams and macrolides (27). In agreement with this suggestion, the high prevalence of penicillin non-susceptible and erythromycin-resistant isolates observed in Portugal is paralleled by the fact that Portugal is the second largest consumer of β-lactams and the fifth largest consumer of macrolides in Europe (19). The model of McCormick *et al* predicts a decrease in the prevalence of penicillin non-susceptible isolates and an overall increase in erythromycin resistance, accompanied by an increase in the prevalence of isolates that are simultaneously penicillin non-susceptible and erythromycin resistant (27). This model fits the data available from several European countries participating in the European Antimicrobial Resistance Surveillance System (EARSS) project (4). As the data analyzed included isolates up to 2002, they probably do not reflect vaccination, and the changes must be attributed to antimicrobial use. With this in mind, the striking increased importance of serotype 19A among both children could not only have resulted from vaccine pressure but may have been accelerated or compounded by shifting trends in antimicrobial use before vaccination.

In spite of the serotype changes that occurred since PCV7 introduction, higher valency vaccines have the potential to prevent significant fractions of pediatric infections, 56% for PCV10 and 83% for PCV13. However, NVTs now account for an important proportion of pediatric IPD in Portugal. We found that the relative contribution of serotypes not included in any of the conjugate vaccine formulations in IPD in children <2 years was higher than in older age groups, suggesting a higher serotype diversification among isolates causing IPD in this age group. However, this high variability was not supported by the SID values that presented overlapping confidence intervals.

Changes in serotype distribution (serotype replacement) were also reported in a study conducted in Portugal regarding pneumococcal carriage in children. In this study carriage remained high even after vaccination with PCV7 (64.9% vs 68.7%). Interestingly, overall resistance to the different antibiotics also remained approximately constant, indicating that resistant NVTs are replacing the PCV7 serotypes (41). In our study, serotype 19A was the most frequent serotype among ERP, PNSP and EPNSP, although the PCV7 serotypes 6B, 14, 19F and 23F still accounted for an important fraction of resistant isolates.

One limitation of this study was the steady increase of isolates during the years considered. The reasons behind the higher rate of isolate recovery may be multifactorial, and their impact on serotype distribution is difficult to evaluate. On the whole, the data presented here suggests that the use of the conjugate seven-valent vaccine, even substantially below universal coverage, greatly reduced the proportion of invasive infections due to vaccine serotypes and resulted in significant changes on the serotype distribution of pneumococci causing infections in children. In contrast to the USA, but in agreement with previous studies of carriage in Portugal (17), an universal reduction in the proportions of infections caused by resistant pneumococci was not found.

The data presented here highlight the importance of the local dynamics of the serotypes of pneumococci causing invasive infections in determining the momentum and characteristics of the replacement serotypes. Continued surveillance of pneumococcal infections will be necessary to evaluate the benefits of higher valency vaccine use since NVTs may be increasingly selected and rise in importance as causes of pediatric IPD.

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CHAPTER 3

Post-Vaccine genetic lineages of invasive pneumococcal serotypes recovered from pediatric patients in Portugal.

SUMMARY

There is significant information regarding the effect of PCV7 in serotype distribution in different geographic areas. In the previous chapter we determined the effect of the PCV7 in serotype distribution among pediatric IPD in Portugal. Nevertheless, little is known about the effect of this vaccine among the genetic lineages of invasive pneumococci. To better understand the influence of PCV7 among pneumococcal clones, we characterized the same pneumococcal collection previously described, using PFGE and MLST, and compared the results with pre-vaccine data. Overall, the majority of the genetic lineages identified in the post-vaccine period were already in circulation before PCV7. Overall, the clones found among the emerging serotypes had all been described previously (serotype 1-ST306, Serotype 3-ST180 and serotype 7F-ST191) which indicates that the increase of these serotypes was due to the expansion of established clones. Nevertheless, we identified a few clonal lineages in minor serotypes which were related to worldwide disseminated clones (serotype 15B/C-ST411, which is an SLV of Netherlands^{15B}-ST199 clone, and serotype 24F-ST230). Since two new vaccines that cover the emerging serotypes have recently become available it is important to continuously monitor the serotypes and clonal lineages of the pneumococci in circulation.

INTRODUCTION

The prevalence and distribution of serotypes recovered from infection is used to guide the selection of capsular types to be included in conjugate vaccines. Yet, a single serotype may include a number of genetically diverse clones distinguishable by PFGE and MLST techniques. Though studies involving carriage isolates have suggested that the polysaccharide capsule of the pneumococcus may be more important for the virulence of the bacterium than the underlying genotype (2, 8), other studies regarding the effect of serotype and clonal lineages in the invasive capacity of the pneumococcus have postulated that the genetic background may in fact be important for each bacterium invasive potential (9, 17). It is thus important to perform a detailed analysis of the clonal lineages present within each serotype in order to explore the reasons behind the different invasive potentials found within the pneumococcal population.

This need increases in importance with the introduction of the PCV7. As we observed in the previous chapter, PCV7 availability induced significant changes in the serotype distribution of pneumococci responsible for IPD. Given that one serotype can include a variety of genetic lineages it is important to determine the effect of PCV7 within the clones in circulation. Moreover, since pneumococci are naturally transformable bacteria, it can acquire exogenous DNA from the same or different species and incorporate in its genome originating a new phenotype. This ability is particularly relevant when the bacteria are faced with selective forces such as the exposure to an antibiotic or a new vaccine. Taking into consideration this, it is important to determine if the new dominant serotypes share the same genetic background of pre-vaccine serotypes, as a consequence of serotype switching events, or if the increase of non-vaccine serotypes is simply associated with the dissemination of pre-existing clones.

In Portugal there is abundant information regarding the genetic structure of the pneumococcal population found in asymptomatic carriers (4) and also in IPD in the pre-vaccine period (13, 19), but little is known about the genetic lineages in the post-vaccine period. To establish the effect of PCV7 among the genetic lineages of pneumococci associated with IPD and to determine potential genetic relationships between the emerging serotypes and previously identified clones, PFGE and MLST were used to characterize the isolates recovered from IPD in children and adolescents (<18 years) between 2003 and 2008. The data was then compared with previously published obtained in the pre-vaccine period (19).

MATERIALS AND METHODS

Isolates

The strains compromising the pre-vaccine period were recovered between 1999 and 2002 and consist of a total of 102 strains recovered from children and adolescents. The data concerning this population was published previously (19).

The invasive population regarding the post-vaccine period (2003-2008) described in chapter 2, was further characterized in terms of PFGE and MLST.

PFGE

The preparation of chromosomal DNA, digestion with SmaI, separation by PFGE, and analysis of the PFGE patterns were performed as described previously (19). PFGE profiling was performed for all 475 strains.

Analysis of digestion patterns: The BioNumerics software (Applied-Maths, Sint-Martens-Latem, Belgium) was used to make UPGMA (unweighted pair group method with arithmetic means) dendrograms of fragment patterns. The dice similarity coefficient was used with optimization and position tolerance settings of 1.0% and 1.5%, respectively. Dendrograms were constructed for each serotype individually and genetic lineages were defined as isolates with ≥80% genetic relatedness on the dendrogram. DNA fragments smaller than 19 kb were not considered for dendrogram construction.

MLST

MLST was performed as described previously (http://www.mlst.net/). The internal fragments from the *aro*E, *gdh*, *gki*, *rec*P, *spi, xpt* and *ddl* genes were amplified by PCR using the primers described previously (5). Sequencing was performed by a commercial supplier (Macrogen, Seoul, South Korea) and the resulting sequences were edited using the BioNumerics software. Alleles and sequence types (STs) were assigned using the pneumococcal database available at spneumoniae.mlst.net. Representative isolates of each PFGE cluster were selected for MLST analysis according to the following criteria: (i) a minimum of 5 isolates from each PFGE cluster containing 10 or more isolates; (ii) a minimum of 1 isolate from each PFGE cluster containing 3 to 9 isolates. Isolates belonging to PFGE clusters with less than 3 isolates were not systematically selected for characterization by MLST.

MLST alleles and STs were identified by searching the pneumococcal MLST database (spneumoniae.mlst.net). Whenever new alleles were identified, sequence traces of both strands

were submitted electronically to the pneumococcal database curator for approval. Lineage assignment was done by using the goeBURST program (6) and the complete *S. pneumoniae* database available at spneumoniae.mlst.net.

Statistical analysis

Simpson's index of diversity (SID) was used to measure the population diversity (3). Wallace coefficients were used to compare two sets of partitions (3, 15).

The Chi-square statistic was used to evaluate whether there were associations between PFGE clone within each serotype and vaccine period.

RESULTS

The analysis of the clones obtained for each individual serotype will be mainly focused on the comparison of the genetic lineages found in the two vaccine periods. The aim is to determine if the effect of PCV7, which lead to a higher frequency of non-vaccine serotypes, involved the expansion of already existing clones expressing these serotypes, or if previously existent lineages, associated with PCV7 serotypes, are now found in the emerging non-vaccine serotypes due to serotype replacement events. To achieve this, a detailed description of the PFGE clusters obtained for each serotype, containing the pre- and post-vaccine populations, will be performed. The pre-vaccine data was previously published (19).

Table 3.1 and 3.2 summarize the clonal distribution of the invasive pneumococci recovered from children. Table 3.1 reports the clones found among the serotypes present in the three, currently available, conjugate vaccines while Table 3.2 describes the clonal composition of serotypes not covered in any conjugate vaccine formulation. The diversity of the serotypes in terms of PFGE clusters is represented in both tables by the SID value.

New MLST alleles and allelic profiles

Eighteen new STs were identified during this study and submitted to the *S. pneumoniae* MLST database (spneumoniae.mlst.net). Of these, 9 involved new allele sequences, which included two novel alleles in *gdh* (235 and 236) that originated ST4578 and ST4579, respectively, three in *ddl* (247, 347 and 371) which originated ST2633, ST4245 and ST4573, respectively, two in *gki* (115 and 153) which originated ST1654 and ST2402, respectively, one in xpt (313) which originated ST4577 and one in *spi* (207) which originated ST4268. The remaining 9 new STs consisted of new allele combinations and were numbered 1646, 1648, 1650, 1659, 4251, 4253 and 4574 to 4576.

Table 3.1: Clonal distribution of *S. pneumoniae* isolates presenting serotypes included in the three conjugate vaccines.

Serotype (No. of isolates)	PFGE cluster[a]	No. of isolates in PFGE cluster		STs in PFGE cluster (No. of isolates characterized by MLST)	SID [95%CI]
		Pre-vaccine	Post-vaccine		
PCV7					
4 (2)	A_2	0	2	ST1221 (2)	0 [0.0-0.0]
6B (20)	C_2	1	1	ST273(1)+ST315(1)	92.11 [85.40-98.81]
	D_2	0	2	ST138(1)+ST176(1)	
	F_5	5	0	ST273(1)+ST1224(1)	
	G_2	0	2	ST2016(2)	
	A_1; B_1; E_2; H_2; I_1;J_1;K_1	Other (2)	Other (7)	-	
9V (10)	A_7	5	2	ST162(1)+ST557(1)+ST1225(1)	46.67 [23.48-69.85]
	B_3	0	3	ST156(1)+ST2356(1)	
14 (54)	C_{33}	19	14	ST143(1)+ST156(2)+ST557(2)+ST790(1) ST4575(1)+ST4576(1)	53.53 [43.46-63.60]
	D_{17}	4	13	ST9(4)+ST409(2)	
	A_1; B_1; E_2	Other (1)	Other (3)	-	
18C (13)	A_6	2	4	ST113(1)+ST1233(2)	75.64 [58.42-92.86]
	B_3	0	3	ST1650(1)	
	C_2; D_1; E_1	Other (0)	Other(4)	-	
19F (20)	C_3	0	3	ST2307(1)	90.06 [82.61-97.50]
	E_6	1	5	ST179(1)+ST390(1)+ST4245(1)	
	A_3; B_1; D_1; F_2; G_1; H_1; I_1; J_1	Other (2)	Other (9)	-	
23F (37)	A_{28}	12	16	ST277(1)+ST338(7)+ST732(1)	42.32 [22.77-61.92]
	C_2	2	0	ST1371(1)	
	E_3	1	2	ST81(2)	
	B_2; D_2	Other (1)	Other (3)	-	
PCV10					
1 (125)	A_{109}	8	101	ST228(1)+ST306(25)+ST4578(1)+ST4579(1)	22.70 [13.62-31.78]
	C_{15}	6	9	ST304(2)	
	B_1	Other(1)	Other(0)	-	
5 (10)	A_9	2	7	ST289(2)+ST1223(2)	20.00 [0-50.36]
	B_1	0	1	ST30(1)	
7F (52)	A_{51}	3	48	ST191(13)	3.85 [0-11.17]
	B_1	1	0	ST191(1)	
PCV13					
3 (28)	A_{26}	4	22	ST180(4)+ST260(1)+ST505(1)+ST1230(3)+ ST1646(1)	13.76 [0-30.44]
	B_2	0	2	ST260(1)	
6A (13)	A_6	1	5	ST65(1)+ST460(2)+ST1648(1)	67.95 [53.42-82.48]
	B_5	1	4	ST1876(1)+ST4248(1)	
	D_1; E_1	Other (0)	Other (2)	-	
19A (100)	A_4	0	4	ST994(2)	82.20 [77.50-86.90]
	C_{12}	1	11	ST1151(3)+ST2732(2)	
	E_{21}	2	19	ST193(7)+ST416(3)	
	I_{11}	2	9	ST81(2)+ST199(1)+ST276(1)+ST667(1)	
	M_{33}	1	32	ST156(1)+ST230(2)+ST276(10) +ST2307(1)+ST4577(1)	
	B_1; D_4; F_1; G_2; H_1; J_2; K_2; L_4; N_2	Other (0)	Other (19)	-	

[a] PFGE clusters are represented by capital letters and a subscript number indicating the number of isolates included in the cluster.

Table 3.2: Clonal distribution of *S. pneumoniae* isolates presenting serotypes not included in any conjugate vaccine formulation.

Serotype (no. of isolates)	PFGE cluster[a]	no. of isolates in PFGE cluster		STs in PFGE cluster (no. of isolates characterized by MLST)	SID [95%CI]
		Pre-vaccine	Post-vaccine		
6C(2)	A_1	0	1	ST395(1)	100
	B_1	0	1	ST386(1)	
8 (5)	A_4	0	4	ST53(3)	40.00 [0-82.93]
	B_1	1	0	ND	
9A(1)	A_1	0	1	ST280(1)	0.0 [0.0-0.0]
9L(2)	A_2	0	2	ST1654(1)	0.0 [0.0-0.0]
9N(6)	A_2	0	2	ST66(1)	73.33 [53.05-93.62]
	B_3	0	3	ST66(2)	
	C_1	1	0	ND	
10A(5)	A_5	2	3	ST97(1)+ST585(1)	0.0 [0.0-0.0]
11A(4)	A_3	0	3	ST408(1)	50.0 [6.70-93.30]
	B_1	0	1	ST2402(1)	
12B(2)	A_1	0	1	ST989(1)	100
	B_1	0	1	ST218(1)	
13(2)	A_1	0	1	ST923(1)	100
	B_1	0	1	ST4574(1)	
15A(4)	A_4	0	4	ST63(2)+ST4208(1)	0.0 [0.0-0.0]
15B/C (8)	A_7	0	7	ST411(4)	25.0 [0.0-60.89]
	B_1	1	0	ST1706(1)	
16F(2)	A_1	0	1	ST30(1)	100
	B_1	0	1	ST30(1)	
17F (1)	A_1	0	1	ST392(1)	0.0 [0.0-0.0]
19C(1)	A_1	0	1	ST1201(1)	0.0 [0.0-0.0]
20(5)	A_3	0	3	ST1026(1)	70.00 [34.95-100.0]
	B_1	0	1	ST235(1)	
	C_1	0	1	ST1026(1)	
21(1)	A_1	0	1	ST432(1)	0.0 [0.0-0.0]
22F(7)	A_6	0	6	ST433(1)+ST1012(1)	28.57 [0.0-66.36]
	B_1	0	1	ST445(1)	
23A(1)	A_1	0	1	ND	0.0 [0.0-0.0]
23B(2)	A_2	0	2	ST439(1)	0.0 [0.0-0.0]
24A(1)	A_1	0	1	ST4253(1)	0.0 [0.0-0.0]
24F(6)	A_2	0	2	ST72(1)	73.33 [53.05-93.62]
	B_1	0	1	ST72(1)	
	C_3	0	3	ST230(2)	
25A(5)	A_5	0	5	ST393(3)	0.0 [0.0-0.0]
29(1)	A_1	0	1	ST198(1)	0.0 [0.0-0.0]
31(1)	A_1	1	0	ND	0.0 [0.0-0.0]
33A(6)	A_6	0	6	ST717(5)	0.0 [0.0-0.0]
33F(6)	A_2	0	2	ST2633(1)	53.33 [27.67-78.99]
	B_4	4	0	ST717(1)	
34(1)	A_1	0	1	ST1778(1)	0.0 [0.0-0.0]
35C/42(1)	A_1	0	1	ST1955(1)	0.0 [0.0-0.0]
35F(3)	A_2	1	1	ST1368(1)	66.67 [30.38-100.0]
	A_1	0	1	ST446(1)	
37(1)	A_1	0	1	ST447(1)	0.0 [0.0-0.0]

[a] PFGE clusters are represented by capital letters and a subscript number indicating the number of isolates included in the cluster.

PMEN clones

Table 3.3 summarizes the PMEN clones identified in our collection. Overall, among the 43 recognized PMEN clones (http://www.sph.emory.edu/ PMEN/index.html) we identified 19 (Table 3.3). As expected, a high correlation between the PMEN clones and antimicrobial resistance was observed. The majority of penicillin non-susceptible (82/95) and macrolide resistant (74/100) strains belonged to a genetic lineage related to one of the widely disseminated PMEN clones. The two main PMEN clones found in resistant strains were the Spain^{9V}-3 ST156 and the Denmark^{14}-32 ST230.

Table 3.3: Serotypes presenting STs of recognized PMEN clones found in our collection.

PMEN Clone	Serotype found in our collection in the post-vaccine period
Spain^{23F}-ST81	19A, 23F
Spain^{9V}-ST156	14,19A, 9V
England^{14}-ST9	14
Colombia^{5}-ST289	5
Poland^{6B}-ST315	6B
Portugal^{19F}-ST177	19F
Denmark^{12F}-ST218	12B
Sweden^{15A}-ST63	15A
Colombia^{23F}-ST338	23F
Sweden^{1}-ST306	1
Greece^{21}-ST193	19A
Netherlands^{3}-ST180	3
Denmark^{14}-ST230	19A, 24F
Netherlands^{8}-ST53	8
Netherlands^{14}-ST124	14
Netherlands^{18C}-ST113	18C
Netherlands^{15B}-ST199	19A
Netherlands^{7F}-ST191	7F
Sweden^{1}-ST304	1

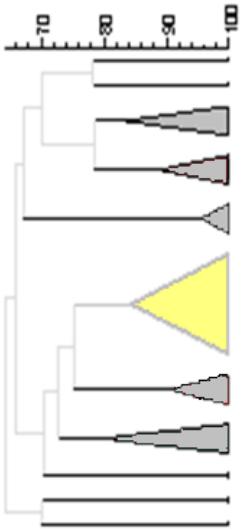
Genetic lineages of PCV7 serotypes

The following paragraphs will describe in detail the clones identified within the serotypes that are covered by PCV7.

(i) Serotype 4: Only two strains presented serotype 4. PFGE analysis demonstrated that they belonged to the same PFGE cluster (A_2) and MLST analysis showed that these two strains presented the same ST, namely ST1221 (CC1221). This serotype, though covered by PCV7 vaccine was not frequently found in invasive infections in younger children in Portugal in the pre-vaccine period. Yet, it was previously found in adults (≥ 18 years) organized in three PFGE clusters representing ST247, ST1221 and ST1222 (19). This means that the two strains of serotype 4 found in this study belonged to a genetic lineage already circulating in Portugal.

(ii) Serotype 6B: Serotype 6B was the most diverse serotype in terms of genetic lineages (Figure 3.1) as it can be observed by the high SID value (Table 3.1). Analysis of the PFGE clusters demonstrates that only two lineages were shared by the two periods considered (C_2 and E_2). The main clone identified in the pre-vaccine period, characterized by Greece^{6B}-ST273 and ST1224 (lineage F_5), was not detected in the post-vaccine years. Conversely a series of new smaller PFGE clusters, characterized by previously unidentified STs, were detected after PCV7. It is unclear whether there was a higher diversification of the 6B genetic lineages associated with pediatric IPD after PCV7, nevertheless the increased number of strains recovered after PCV7 could justify the appearance of previously unidentified genetic lineages.

Serotype 6B (n=20)

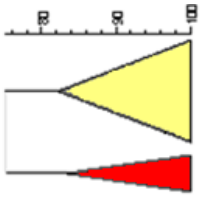


PFGE cluster	No. of isolates Pre-vaccine (n=8)	No. of isolates Post-vaccine (n=12)	Sequence Type (ST)	%PEN^{NS} (No. of isolates)	%ERY^{R} (No. of isolates)
A_1	0	1	ND	0(0)	0(0)
B_1	1	0	ST176	0(0)	0(0)
C_2	1	1	ST273+ST315	50(1)	100(2)
D_2	0	2	ST138+ST176	0(0)	50(1)
E_2	1	1	ND	100(2)	100(2)
F_5	5	0	ST273+ST1224	20(1)	80(4)
G_2	0	2	ST2016	100(2)	0(0)
H_2	0	2	ST386	0(0)	100(2)
I_1	0	1	ND	0(0)	100(1)
J_1	0	1	ST1662	100(1)	100(1)
K_1	0	1	ND	0(0)	0(0)

Figure 3.1: Macrorestriction dendrogram of serotype 6B. The genetic lineages are represented by triangles. The size of each triangle is proportional to the number of strains. PFGE clusters are represented by capital letters and a subscript number indicating the number of isolates included in the cluster. The number of isolates found in each of the vaccine periods considered for each PFGE cluster is presented. PFGE clusters significantly associated with the pre-vaccine period are represented in yellow. PFGE clusters significantly associated with the post-vaccine period are represented in red. PFGE clusters not associated with any vaccine periods are represented in grey. ST information and non-susceptibility to penicillin (PEN^{NS}) and resistance to erythromycin (ERY^{R}) for each cluster is also presented.

(iii) Serotype 9V: Serotype 9V isolates were clustered in two distinct genetic lineages (Figure 3.2), but MLST analysis demonstrated that the ST's obtained for each individual lineage belonged to the same clonal complex (CC). Both lineages are associated with penicillin non-susceptibility and are related to the internationally disseminated Spain^{9V}-ST156 clone (11). In the pre-vaccine period only the genetic lineage represented by ST162, ST557 and ST1225 had been identified in IPD in children and adolescents (<18 yrs).

Serotype 9V (n=10)



PFGE cluster	No. of isolates Pre-vaccine (n=5)	No. of isolates Post-vaccine (n=5)	Sequence Type (ST)	%PEN^{NS} (No. of isolates)	%ERY^{R} (No. of isolates)
A_7	5	2	ST162+ST557+ST1225	57(4)	14(1)
B_3	0	3	ST156+ST2356	100(3)	33(1)

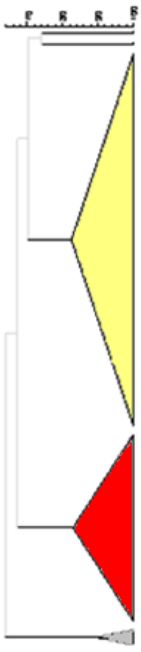
Figure 3.2: Macrorestriction dendrogram of serotype 9V. For details see the legend of Figure 3.1.

(iv) Serotype 14: Serotype 14 was the former most commonly found serotype in invasive infection in children before PCV7 availability and was also highly associated with resistance to penicillin and/or erythromycin (19).

In the present study three distinct genetic lineages, along with two unrelated PFGE patterns, were identified. As it can be observed in Figure 3.3 serotype 14 is strongly associated with antimicrobial resistance. The main clone (C_{33}) was characterized by being fully non-susceptible to penicillin while the second major genetic lineage, presenting ST9 and ST409, was associated with erythromycin resistance.

The two major serotype 14 lineages (C_{33} and D_{17}) were found both before and after vaccine availability. goeBURST analysis indicates that, with the exception of the PFGE cluster presenting ST9 and ST409 which compromises strains related to the England^{14}-ST9 clone, all other genetic lineages identified for serotype 14, for which STs were determined, were related to the widely disseminated Spain^{9V}-ST156 clone (11).

Serotype 14 (n=30)

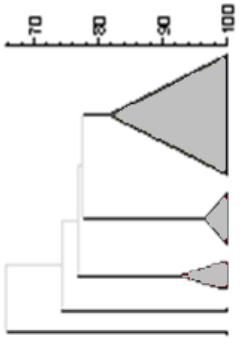


PFGE cluster	No. of isolates Pre-vaccine (n=24)	No. of isolates Post-vaccine (n=30)	Sequence Type (ST)	%PEN^{NS} (No. of isolates)	%ERY^{R} (No. of isolates)
A_1	0	1	ST4573	100(1)	0(0)
B_1	1	0	ND	0(0)	0(0)
C_{33}	19	14	ST143+ST156+ ST557+ST790+ ST4575+ST4576	100(33)	24(8)
D_{17}	4	13	ST9+ST409	6(1)	82(14)
E_2	0	2	ST124	0(0)	0(0)

Figure 3.3: Macrorestriction dendrogram of serotype 14. For details see the legend of Figure 3.1.

(v) Serotype 18C: Serotype 18C has long been recognized as a universally antibiotic susceptible serotype, though significantly prevalent in invasive infection in several countries. In Portugal one main lineage (A_6) was identified in the pre-vaccine era associated with IPD in children. This clone, represented by ST113 and ST1233, was also found in the post-vaccine period. Four additional genetic lineages were identified in the post-vaccine period as it can be observed in Figure 3.4.

Serotype 18C (n=13)

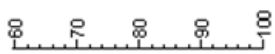


PFGE cluster	No. of isolates Pre-vaccine (n=2)	No. of isolates Post-vaccine (n=11)	Sequence Type (ST)	%PEN^{NS} (No. of isolates)	%ERY^{R} (No. of isolates)
A_6	2	4	ST113+ST1233	0(0)	0(0)
B_1	0	3	ST1650	0(0)	0(0)
C_2	0	2	ND	0(0)	0(0)
D_1	0	1	ST28	0(0)	0(0)
E_1	0	1	ST102	0(0)	0(0)

Figure 3.4: Macrorestriction dendrogram of serotype 18C. For details see the legend of Figure 3.1.

(vi) Serotype 19F: Serotype 19F was the second most genetically diverse serotype identified in our collection (Table 3.1). The largest genetic lineage (E_6), composed by 6 strains and found to be related to the clone Portugal^{19F}-ST177, was recovered in both pre and post-vaccine periods. The second most prevalent cluster (C_3), composed by three strains and represented by a lineage (ST2307) related to the widely disseminated Denmark^{14}-ST230 clone, was only found in the post-vaccine years. Interestingly this lineage was highly associated with antimicrobial resistance and, as it will be shown, was also highly prevalent in serotype 19A.

Serotype 19F (n=20)

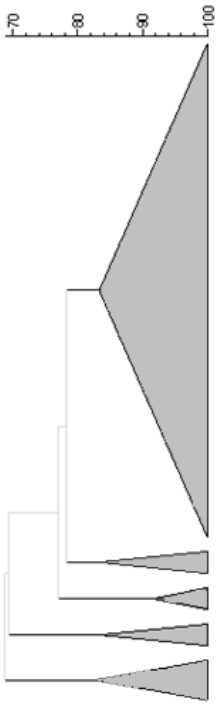


PFGE cluster	No. of isolates Pre-vaccine (n=3)	No. of isolates Post-vaccine (n=17)	Sequence Type (ST)	%PEN^{NS} (No. of isolates)	%ERY^{R} (No. of isolates)
A_3	1	2	ND	33(1)	33(1)
B_1	0	1	ST4251	0(0)	0(0)
C_3	0	3	ST2307	100(3)	100(3)
D_1	0	1	ST177	0(0)	0(0)
E_6	1	5	ST179+ST390+ ST4245	0(0)	33(2)
F_2	0	2	ST476	0(0)	0(0)
G_1	0	1	ST89	100(1)	100(1)
H_1	1	0	ST347	100(1)	0(0)
I_1	0	1	ST72	0(0)	0(0)
J_1	0	1	ST276	100(1)	100(1)

Figure 3.5: Macrorestriction dendrogram of serotype 19F. For details see the legend of Figure 3.1.

(vii) Serotype 23F: The majority of the 23F isolates were clustered in a single lineage (A_{28}), represented by three STs which are related to the Colombia^{23F}-ST338 clone. This lineage was highly associated with non-susceptibility to penicillin. Four additional minor lineages were also identified, one composed by ST338 which belongs to the same lineage as the major clone, and another, composed by the widely disseminated Spain^{23F}-ST81 clone. Overall there were no significant differences in terms of genetic lineages between the two vaccine periods considered.

Serotype 23F (n=37)



PFGE cluster	No. of isolates Pre-vaccine (n=16)	No. of isolates Post-vaccine (n=21)	Sequence Type (ST)	%PEN^{NS} (No. of isolates)	%ERY^{R} (No. of isolates)
A_{28}	12	16	ST277+ST338 +ST732	82(23)	0(0)
B_2	0	2	ST338	100(2)	0(0)
C_2	2	0	ST1371	50(1)	0(0)
D_2	1	1	ST33	0(0)	0(0)
E_3	1	2	ST81	67(2)	33(1)

Figure 3.6: Macrorestriction dendrogram of serotype 23F. For details see the legend of Figure 3.1.

Lineage distribution of emerging serotypes

(i) Serotype 1: Serotype 1 has long been considered an important serotype in invasive infection in Portugal. In the pre-vaccine period it was the second most common serotype among all IPD, including both adults and children, while it was the third most common serotype among invasive infection in children <6 yrs (20). What makes this serotype different, along with two other important serotypes in our collection, namely serotype 3 and 7F, is its relatively low genetic diversity (Figure 3.7). As it can be observed in the dendrogram, among a collection of 125 serotype 1 isolates, only two main lineages were identified. The main lineage, composed by 109 strains, was represented by four STs which belong to the clonal complex CC306 and are related to the widely disseminated Sweden^{1}-ST306 clone. Interestingly, though both lineages were identified in the pre-vaccine period, it seems that the increase in serotype 1 was associated mainly with the expansion of the Sweden^{1}-ST306 lineage. The second lineage, considerably less prevalent that the former and represented by the international Sweden^{1}-ST304 clone, was identified in both vaccine periods. Besides its low genetic diversity (Table 3.1), serotype 1 is also associated with low levels of antimicrobial resistance.

Serotype 1 (n=125)

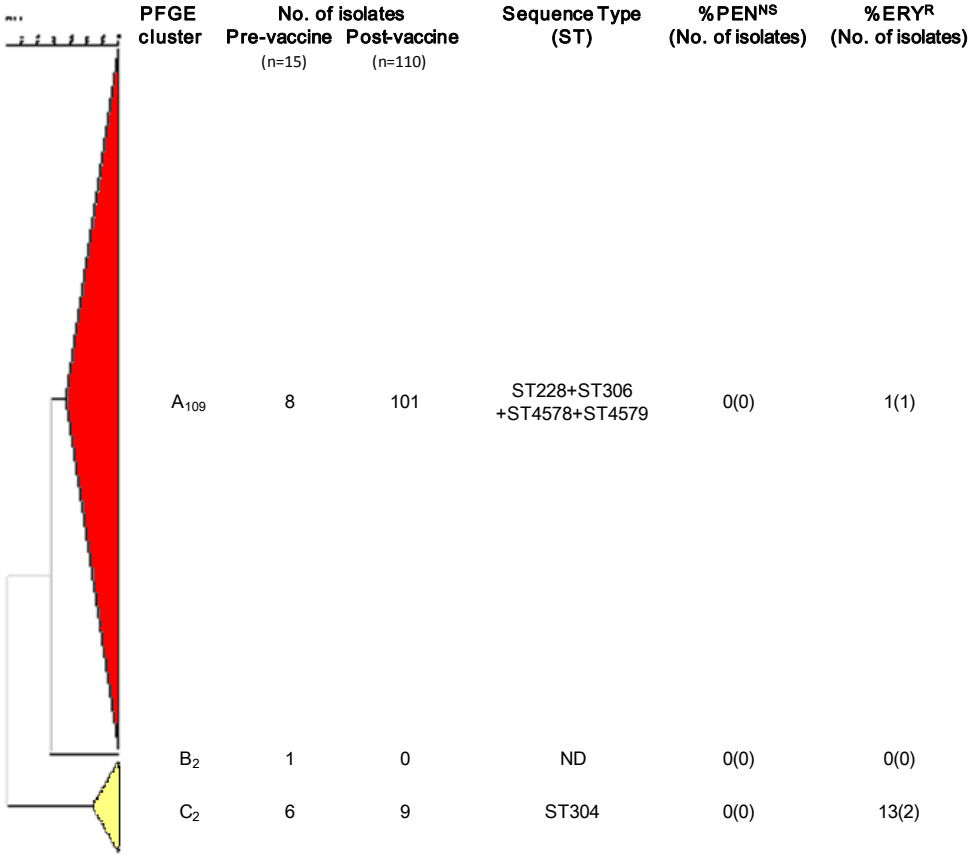
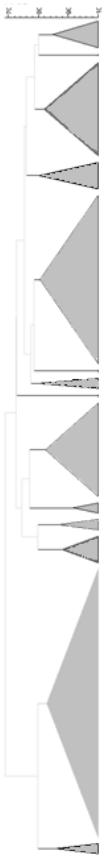


Figure 3.7: Macrorestriction dendrogram of serotype 1. For details see the legend of Figure 3.1.

(ii) Serotype 19A: Serotype 19A was the main emerging serotype associated with antimicrobial resistance. Genetic analysis demonstrated that the dissemination of this serotype is associated with the expansion of particularly one clone. This clone represents ST230 and related STs and is characterized by its high levels of resistance to both penicillin and erythromycin. Due to the importance of this serotype in the post-vaccine period a broader study was conducted, which included strains recovered from infections in adults and strains isolated from the nasopharynx of healthy individuals. The main objective was to determine if this serotype and its genetic lineages were particularly associated with IPD in children. These data will be discussed in the next chapter.

Serotype 19A (n=100)



PFGE cluster	No. of isolates Pre-vaccine (n=6)	No. of isolates Post-vaccine (n=94)	Sequence Type (ST)	%PEN^{NS} (No. of isolates)	%ERY^{R} (No. of isolates)
A_4	0	4	ST994	25(1)	25(1)
B_1	0	1	ND	0(0)	0(0)
C_{12}	1	11	ST1151+ST2732	0(0)	0(0)
D_4	0	4	ST193	0(0)	50(2)
E_{21}	2	19	ST193+ST416	0(0)	66.7(14)
F_1	0	1	ST193	0(0)	100(1)
G_2	0	2	ST193	0(0)	100(2)
H_1	0	1	ND	0(0)	0(0)
I_{11}	2	9	ST81+ST199+ ST276+ ST667	45.4(5)	9.1(1)
J_2	0	2	ST1201	0(0)	0(0)
K_2	0	2	ST994	0(0)	0(0)
L_4	0	4	ST994	0(0)	0(0)
M_{33}	1	32	ST156+ST230 +ST276+ST2307 +ST4577	96.9(32)	93.9(31)
N_2	0	2	ST276	50(1)	50(1)

Figure 3.8: Macrorestriction dendrogram of serotype 19A. For details see the legend of Figure 3.1.

(iii) Serotype 7F: All serotype 7F isolates identified in the pre- and post-vaccine periods belonged mainly to a single genetic lineage representing ST191 and were susceptible to both antibiotics. This clone is the recently proposed Netherlands^{7F}-ST191 clone of PMEN (8, 19).

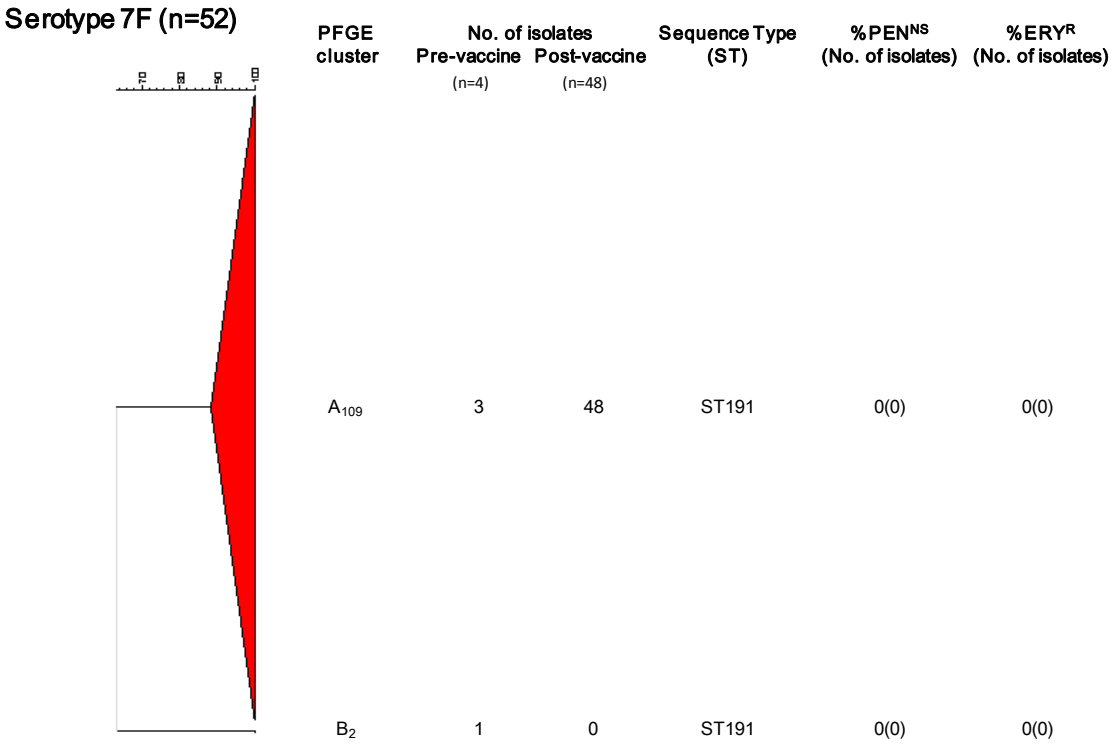


Figure 3.9: Macrorestriction dendrogram of serotype 7F. For details see the legend of Figure 3.1.

(iv) Serotype 3: Serotype 3 presented two PFGE clusters but MLST and goeBURST analysis identified the presence of several unrelated STs in the main lineaged identified. The dissemination of serotype 3 in the post-vaccine period was due to the expansion of the only lineage associated with this serotype, represented by ST180, ST260, ST505, ST1230 and ST1646.

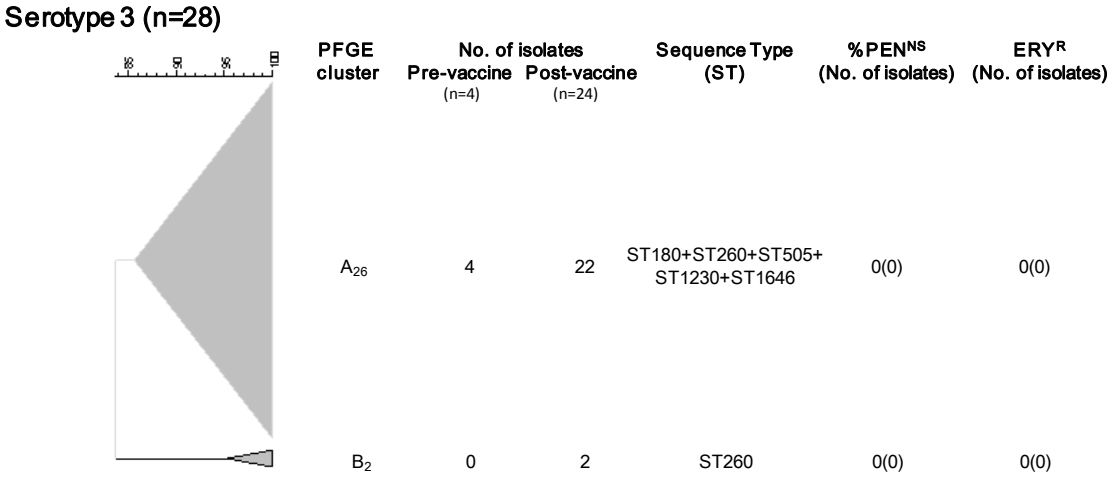


Figure 3.10: Macrorestriction dendrogram of serotype 3. For details see the legend of Figure 3.1.

Minor serotypes not included in any conjugate vaccine formulation

Table 3.2 summarizes the clonal composition of the serotypes for which the dendrogram was not presented. The majority of these serotypes were represented by a small number of strains (less than 10) in both vaccine periods. Nevertheless some serotypes presented particularities that deserve attention. One such case is serotype 24F, which was found in six strains recovered in the post-vaccine period and represented by three genetic lineages. Yet, the main cluster of this serotype was characterized by ST230, the same sequence type highly associated with resistance to penicillin and erythromycin of serotype 19A. Also noteworthy were serotypes 33A, 15B/C and 22F that were identified in six and seven, respectively, post-vaccine strains. Since these are minor serotypes and consequently have little representation in IPD one could minimize their importance. Nevertheless, with the introduction of PCV10 and PCV13, which include all major serotypes identified in this study, it is possible that these particular serotypes may become prevalent in the future.

Correlation between the different typing methods

To determine the association between the different typing methods we calculated the Wallace coefficients. The Wallace coefficient (W) between ST and PFGE cluster was high (W=0.791, 95%CI: 0.730-0.852) but the association between ST and serotype was even higher (W=0.922 95%CI: 0.887-0.958). We had previously seen that the correlation between serotype and antimicrobial resistance was high but genetic analysis demonstrated that this association is elevated when considering the PFGE cluster. The W for PFGE cluster and non-susceptibility to penicillin was 0.939 (95%CI: 0.902-0.977) while the W between PFGE cluster and erythromycin resistance was 0.900 (95%CI: 0.855-0.944).

Though the correlation between serotype and ST was strong, in some occasions it was possible to find STs to which a certain serotype had not been associated with previously. Table 3.4 provides the information regarding the differences found between our data and the MLST database (spneumonaie.mlst.net).

Table 3.4: Differences in associations of ST and serotype found in our study and the MLST database.

Sequence Type (ST)	Serotype in our collection	Serotype in MLST database
ST28	8	18C
ST72	24F	19F
ST30	16F	5
ST447	37	3
ST2307	19F	19A
ST198	35B	29
ST1012	11A	22F
ST445	33F	22F
ST2633	19A	33F

DISCUSSION

The aim of this chapter was to analyze the clonal composition of invasive pneumococci recovered from pediatric infections after the availability of PCV7. We also intended to determine if the emerging serotypes were the result of capsular switching events or a consequence of the expansion of previously established clones. To distinguish between these two types of adaptation we compared the pre- and post-vaccine populations using PFGE and MLST data. Basically, if the new dominant serotypes shared the same genetic background of those found in isolates expressing PCV7 serotypes, this would indicate the occurrence of genetic horizontal transfer events of the capsular locus.

The comparison of the lineages found in the two vaccine periods considerd showed that the majority of the clones associated with the emerging serotypes were already in circulation before PCV7. In some particular cases we identified genetic lineages that had not been described in the previous study (e.g serotype 6B) yet, these clones represented a small fraction of the genetic lineages. Very few possible capsular switching events were detected but none of them represented a significant fraction of the serotypes in expansion, which indicates that they were not a major phenomenon during pneumococcal adaptation to the PCV7. These observations are in agreement with the study performed in the USA by Beall and colleagues, which reported stable clonal structures when comparing pre and post-vaccine clonal data (1).

One of the most interesting findings of this chapter was the differences found between serotypes in terms of clonal diversity. On one side we identified serotypes composed of only two lineages, such as serotype 1 and 7F. Conversely, there were serotypes, such as 6B, 19F and 19A, composed by several unrelated clones, with SID values higher than 80% (Table 3.1). One of the hypotheses for the higher genetic diversification of some serotypes is their increased frequency in the nasopharyngeal niche. Due to the possibility of co-occurrence with other pneumococcal strains and other bacterial species, the pneumococcus can exchange DNA between different pneumococcal clones and also with other commensal bacteria. Indeed, in the pre-vaccine period, serotypes such as 1 and 7F, were rarely found in colonization which could explain the low levels of diversity (7, 13). On the other hand, genetically diverse serotypes such as 6B, 14 and 23F were commonly found in carriage (13, 16). However, after vaccine implementation an increase of less common serotypes, such as serotype 1 and 7F, in carriage was observed (14, 16). Whether this increase in carriage will result in clonal diversification of these serotypes is still unknown. Yet, since the new conjugate vaccine formulations will cover these serotypes their deployment will probably allow little time for these serotypes to diversify.

Another curious finding was the diversity of STs found within the same clonal lineage of a particular serotype. It is frequently assumed that strains belonging to the same PFGE cluster are genetically related and hence share the same or related STs. This is indeed true for most of the cases as it can be observed for serotypes 14 and 19F. Nevertheless, we found certain serotypes, namely serotype 3 and 19A, in which, within the same PFGE cluster, one could find STs that belonged to as much as three different clonal complexes and most probably, according to goeBURST, did not have a common ancestor.

The most extreme case was observed in serotype 3 in which the main PFGE cluster (A_{26}) was represented by STs that belonged to three different clonal complexes (CC180, CC260, CC1646). One possible explanation is the lack of discriminatory power of the PFGE analysis conditions that are being used. Indeed if the threshold for defining PFGE clusters was increased from 80% to 90% similarity on the dendrogram, it would originate five clonal lineages whose sequence types are associated with only one clonal complex. Yet, the use of different PFGE cluster definitions for different serotypes would need careful consideration as to the consequences that this could have on comparisons of genetic diversity between serotypes.

Interestingly, a recent study of invasive serotype 3 isolates recovered in South Africa found a good correlation between PFGE and MLST. In that study, the authors define three main PFGE clusters, namely cluster A represented by ST458, cluster B composed of ST180, ST700 and ST2837 and cluster C represented by ST378. The cluster B found in their collection is equivalent to the cluster A_{26} that we identified in serotype 3 by comparison of the STs and restriction patterns. Though it is not fully specified, apparently the PFGE conditions used in their study are similar to ours which allows us to compare the data of both studies. As observed in our study, the cluster B of the South Africa study also contains STs which belong to two unrelated genetic lineages (12) raising similar questions regarding the congruence between the two typing methods. These specific situations of serotypes 3 and 19A may indicate that the various serotypes have different adaptation and evolution mechanisms.

The analysis of the SID values obtained for the main serotypes (Table 3.1) suggests there might not be a direct correlation between genetic diversity and serotype invasiveness as is illustrated by serotypes 1 and 19A. Both serotypes have been found with increasing prevalence after vaccine availability and yet demonstrated opposite diversity levels. So the main question raised is what do these two, so different, serotypes share that allow them to be major serotypes in pneumococcal infection? Several studies have attempted to determine the main factor behind the success of some strains by evaluating the importance of the genetic background and capsular type in the ability of these bacteria to cause infection. Odds ratios (OR) have been calculated for both serotypes and clones, through the comparison of populations recovered from carriage and invasive disease (2, 9, 18). According to these studies, serotypes 1, 4, 5 and 7F were more likely to cause

invasive disease and were rarely found in the nasopharynx of healthy children. On the other hand, isolates of serotypes 3, 6A, 19F and 23F had low invasive-disease potential and were predominantly found in healthy carriers (2, 18). Yet, even isolates with lower invasive disease potential can be more common in infection than the most invasive ones. This is due to the fact that the ORs are determined as a function of infection versus carriage prevalence. Consequently the most invasive serotypes will be those that are normally found in invasive infection and rarely recovered from carriage.

One of the obstacles for determining which serotypes and clones are more invasive is the geographic diversity of the pneumococcal population. As it was focused in chapter 2 serotype distribution varies considerably between countries. Still, the main serotypes found in pediatric invasive disease have been approximately the same in the majority of the countries which, on the other hand, is not always observed for the genetic lineages. In other words, two different countries may share the same dominant serotype but the predominant genetic lineages can be completely different. This was in fact observed for serotype 19A which will be discussed in the following chapter.

Other minor serotypes were obtained in our collection, some of which presented clonal lineages related to the existent in the pre-vaccine period, such as ST411 in serotype 15B/C which is a SLV of the Netherlands^{15B}-ST199 clone and ST230 in serotype 24F, also found in 19A serotype. Whether this will mean that these serotypes might increase in the population, due to their relatedness to previous disseminated clones, is still unknown. If the genetic lineage influences the strains ability to cause infection, than these serotypes may become important in pneumococcal disease.

Despite the fact that the main genetic lineages found in the post-vaccine period were already in circulation before PCV7 availability, we found a variety of previously unidentified restriction patterns in several serotypes (6B, 18C, 19A, 19F). Similar observations were reported by Beall and colleagues in the USA (1). This increase of diversity among some serotypes might be a consequence of the increased number of strains characterized in the post-vaccine period. Nevertheless, changes in the distribution of clones, as in serotypes, in a relatively short period of time can also occur without the presence of selective pressure such as a vaccine. This has been recently reported in study conducted in Scotland regarding the pneumococcal population recovered before PCV7 implementation (10). Continuous monitoring of these genetic lineages should thus be performed to determine their relevance in future years.

Two new vaccines have recently become available and will cover the new emergent serotypes, such as 1, 19A and 7F. Taking into account what was observed with the introduction of PVC7, it is possible that, after vaccine implementation, a new population will become established. However, it is important to refer that, in our study, these emerging serotypes were already prevalent

in the population in the pre-vaccine period and that the decrease of PCV7 serotypes in the population probably allowed the dissemination of these serotypes. As such, it is of extreme importance to continuously monitor the serotypes and clones associated with disease not only to better understand the dynamics and evolution of the pneumococcal population but also to help predict the consequences of vaccination.

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CHAPTER 4

Denmark^{14}-230 Clone as an Increasing Cause of Pneumococcal Infection in Portugal within a Background of Diverse Serotype 19A Lineages

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SUMMARY

As demonstrated in the two previous chapters, serotype 19A became an important cause of IPD in Portugal after PCV7 availability. This serotype was shown present high levels of antimicrobial resistance and to be particularly associated with infection in children < 2 years. Moreover, expansion of serotype 19A has also been reported in various geographic regions. To determine whether serotype 19A dissemination was mainly occurring in IPD and what were the genetic lineages behind its expansion, we characterized serotype 19A isolates (*n* = 288) found among pneumococci responsible for infections (*n* = 1,925) and pneumococci recovered from asymptomatic carriers (*n* = 1,973) in Portugal between 2001 and 2006. We show that despite the existence of serotype 19A clones that have a greater potential to cause invasive disease or an enhanced colonization capacity, the lineage that is increasing as a cause of infection in Portugal is a multiresistant clone that is competent at both. The expanding Denmark^{14}-230 clone found in Portugal is disseminated in other Mediterranean countries, where it is also increasingly responsible for invasive infections in both children and adults. The lineages driving the rise of serotype 19A infections in Asia and the United States (sequence type 320 [ST320] and ST199) are either absent or account for only a small proportion of isolates in Portugal. These data highlight the importance of locally circulating clones with the ability to compete in the nasopharyngeal niche in the emergence of the serotype 19A lineages which are an increasing cause of infection in various geographic regions.

INTRODUCTION

Although vaccination with PCV7 has frequently been implicated as the cause for the increase in the incidence of serotype 19A infections (7), recent reports documented the same trend in geographic areas where PCV7 was not available (10, 12). Taken together, these observations suggest that vaccination may have simply reinforced or accelerated an ongoing temporal trend. In particular, in South Korea, the rise in the incidence of serotype 19A infections in the absence of PCV7 was tentatively explained by the emergence of a multidrug-resistant lineage of sequence type 320. Antibiotic pressure was likely to be a major factor in selecting for this lineage (10).

A study of the recent increase in the incidence of serotype 19A in the United States identified several lineages currently in circulation, among which the preexisting CCs, CC199 and CC320, were found to be expanding (27). The capsular switch in STs usually associated with vaccine serotypes and the appearance of multiple-drug resistant clonal complexes were also implicated in the rise in the incidence of serotype 19A (25, 27). Detailed analysis of the capsular loci of some of these lineages showed that the concurrent acquisition of the entire capsular locus and the flanking *pbp* genes occurred (7), confirming the emergence of vaccine-escape recombinant strains. On the other hand, a recent study conducted with individuals from the Alaska native population found that the genetic diversity of circulating serotype 19A isolates was reduced. In that setting the increase in the incidence of serotype 19A was only due to the expansion of CC172 (34). In contrast, in Europe, CC230 has consistently been identified as a major serotype 19A lineage causing invasive infections before vaccine introduction. This clonal complex has increased in the era of PCV7 both in children (21) and in adults (4).

In the study described here, we compared the clonal composition of the population expressing serotype 19A responsible for invasive infections in both adults and children to that recovered from children who were asymptomatic carriers and who had non-invasive infections in Portugal.

MATERIALS AND METHODS

Bacterial isolates

Three *S. pneumoniae* collections were examined for the presence of isolates expressing serotype 19A. Each collection contained isolates from different sources: isolates responsible for invasive infections, isolates causing non-invasive infections, and isolates recovered in asymptomatic carriage studies.

Isolates causing infections

On behalf of the surveillance study described in chapter 2, a collection of 1,480 *S. pneumoniae* isolates responsible for invasive pneumococcal infections from 2001 to 2006 was characterized. Among this collection, 122 strains were isolated from children <2 years of age, 102 strains were isolated from children >2 and <6 years of age, 52 strains were obtained from children and adolescents between ≥6 and <18 years of age, and 1,204 strains were obtained from adults (≥18 years of age). The collection of non-invasive pneumococci (mainly recovered from lower respiratory tract specimens and associated with a diagnosis of pneumonia) was composed of 445 isolates, all of which were obtained from children <6 years of age.

Carriage isolates

In 2001, 2002, 2003, and 2006, surveys of pneumococcal carriage were conducted with healthy children (age range, 6 months to 6 years) attending day care centers (DCCs) in the Lisbon, Portugal, area. Between 2001 and 2003, during the months from January to March, 2,314 nasopharyngeal swab specimens were obtained from children attending 13 DCCs. In 2006, during the same months, 571 nasopharyngeal swab specimens were recovered from children attending 11 DCCs. A total of 1,973 pneumococcal isolates were obtained. A description of the resistant strains recovered between 2001 and 2003 was published previously (22, 36), as were the results of serotyping and the antimicrobial resistance of the isolates recovered in 2006 (31).

Serotyping and antimicrobial susceptibility testing

Serotyping was performed by the standard capsular reaction test with a chessboard system (35) and specific sera (Statens Serum Institut, Copenhagen, Denmark). Etest strips (AB Biodisk, Solna, Sweden) were used to determine the MICs for penicillin, as described previously (24), and the CLSI recommended breakpoints (11) were used to interpret the MICs. The isolates were further

characterized by determining their susceptibilities to co-trimoxazole, levofloxacin, erythromycin, clindamycin, tetracycline, and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria (11).

PFGE and MLST

Profiling by pulsed-field gel electrophoresis (PFGE) was performed for all serotype 19A isolates included in this study. The preparation of chromosomal DNA, digestion with SmaI, separation by PFGE, and analysis of the PFGE patterns were performed as described previously (33). Multilocus sequence typing (MLST) analysis (13) was undertaken with a third of the isolates of each PFGE cluster with four or more isolates. The isolates within each PFGE cluster were selected to represent isolates from the three collections analyzed and, as much as possible, isolates collected in all the years studied. Lineage assignment was done by using the goeBURST program (15) and the complete *S. pneumoniae* database available at spneumoniae.mlst.net.

Statistical analysis

Simpson's index of diversity (SID) was used to measure the diversity of the population (8). Clustering comparison coefficients, i.e., the adjusted Rand and Wallace (W) values, were used to compare two sets of partitions (8, 29).

The statistical associations between PFGE clones and population type or antimicrobial resistance were characterized by determination of the odds ratios (ORs) with 95% Wald confidence intervals (CIs) (3). For null ORs, the 95% CIs were computed by the Fisher method implemented in the epitools module for the R language. The significance of the ORs was tested by use of the chi-square statistic. The resulting *P* values were corrected for multiple testing by controlling the false discovery rate (FDR) under or equal to 0.05 through the linear procedure of Benjamini and Hochberg (5).

The ORs for the enhanced invasive potential of the main serotype 19A PFGE clusters identified were calculated by using the number of invasive and carriage isolates in reference to the numbers of all other serotype 19A isolates from these two sources. The ORs measuring the association of particular PFGE clusters with resistance were calculated by reference to the resistance found in all other PFGE clusters expressing serotype 19A identified in the study.

Temporal trends in proportions were evaluated by the Cochran-Armitage test (1). The chi-square statistic or the Fisher exact test was also used to evaluate whether there were associations between population type and antimicrobial resistance. The *P* values were considered significant if they were less than 0.05.

RESULTS

Proportion of isolates expressing serotype 19A

From the collections of pneumococcal isolates responsible for infections, 178 isolates presented serotype 19A. This set was composed of 121 isolates (of which 45 were from children less than 6 years of age) recovered from normally sterile sites and 57 isolates obtained from non-invasive infections (all from children less than 6 years of age). Among the collection of isolates recovered from asymptomatic carriers, 110 isolates presented serotype 19A. Among all pneumococcal isolates responsible for infection, the proportion of isolates expressing serotype 19A increased over time, from 4.1% (n=8) in 2001 to 10.2% (n=48) in 2006 ($P < 10^{-3}$), reflecting an increase in the incidence of serotype 19A isolates among both invasive and non-invasive isolates. Although an increase in the incidence of serotype 19A isolates was also noted among isolates from asymptomatic carriers, from 4.3% (n=20) in 2001 to 6.5% (n=27) in 2006, it did not reach significance ($P = 0.124$).

Antimicrobial susceptibility

The proportions of resistant isolates are summarized in Table 4.1. Although there were differences in the proportions of resistant isolates in each of the three populations analyzed, none were statistically significantly supported. Yet, when the proportion of multidrug-resistant isolates (resistance to three or more different classes of antimicrobials) is considered, it was higher among invasive isolates (49%) than among non-invasive isolates (36%) and carriage isolates (30%) ($P = 0.004$).

Table 4.1: Antibiotic resistance of serotype 19A *S. pneumoniae* isolates (n=288).

Antibiotic	No. of resistant isolates[a] (%)		
	Invasive (n=121)	Non-invasive (n=57)	Carriage (n=110)
Penicillin[b]	56 (46.3)[c]	2 (40.4)	43 (39.1)
Erythromycin[d]	70 (57.9)	23 (40.4)	59 (53.6)
Clindamycin	69 (57.0)	21 (36.8)	58 (52.7)
Tetracycline	71 (58.7)	23 (40.4)	67 (60.9)
Co-trimoxazole	40 (33.1)	20 (35.1)	20 (18.2)
Chloramphenicol	17 (14.0)	3 (5.5)	9 (8.2)
Levofloxacin	0	0	0

[a] A total of 288 isolates were tested. [b] The data for isolates nonsusceptible to penicillin (MIC ≥0.12 µg/ml) are indicated. [c] Five isolates were fully resistant (MIC ≥2 µg/ml). [d] The majority (148/152) of macrolide-resistant isolates presented the MLS_B phenotype, characterized by simultaneous resistance to erythromycin and clindamycin. Only four isolates (2.6%) presented the M phenotype, characterized by resistance to erythromycin only.

Genotypic analysis and evaluation of invasive disease potential

The three populations of serotype 19A were compared genotypically by a combination of PFGE and MLST. All isolates were analyzed by PFGE, and representatives of the PFGE clusters were characterized by MLST (n=95). Overall, serotype 19A strains were distributed throughout 23 different PFGE clusters (Figure 4.1) and 26 distinct STs were identified. The PFGE cluster and the ST demonstrated a good correlation. In fact, the W between the ST and the PFGE cluster was 0.772 (95% CI, 0.678 to 0.865), indicating that there is a high probability that two strains that have the same ST will also belong to the same PFGE cluster. This value is even higher if one considers the clonal complexes defined by goeBURST (W=0.833 and 95% CI=0.744 to 0.922; i.e., four of five pairs of isolates belonging to the same clonal complex will also be classified in the same PFGE cluster). Despite these high Wallace values, a few STs were detected in several PFGE groups, such as ST193 and ST994. Nevertheless, the W indicate that most isolates belonging to the same genetic lineage, as defined by goeBURST analysis, would be classified in the same PFGE cluster, allowing us to compare our data based on the results of PFGE analysis with data in other reports in which only MLST data are available.

The number of invasive isolates of serotype 19A recovered from children (<6 years; n=45) was less than the number of isolates recovered from individuals in other age groups (n=76). This reduced number of isolates could compromise the statistical efficiency of the tests comparing invasive isolates to isolates from other sources. However, if it could be demonstrated that the distribution of clones between children (<6 years) and older individuals was indistinguishable, all invasive isolates could be regarded as representing a single population, greatly enhancing the power of the comparisons. In fact, we could not show a difference in the distribution of the PFGE clusters between the two age groups (Fisher's exact test, P=0.775), indicating that all serotype 19A invasive isolates constitute a homogeneous population independent of the age group considered. We therefore used the entire collection of invasive isolates in the comparisons with the isolates from other sources.

Isolates from all three sources presented a high degree of genetic diversity, as determined by PFGE cluster analysis, with high and undistinguishable SID values (Table 4.2). Analysis of the distribution of isolates between PFGE clusters showed that most clusters included isolates from the three distinct sources. This is highlighted by an adjusted Rand value of 0.044, which indicates a low overall level of congruence between the PFGE cluster and the isolate source. Despite the absence of a strong correlation between the isolate source and the PFGE cluster, some PFGE clusters were not uniformly distributed among the three populations (Table 4.2).

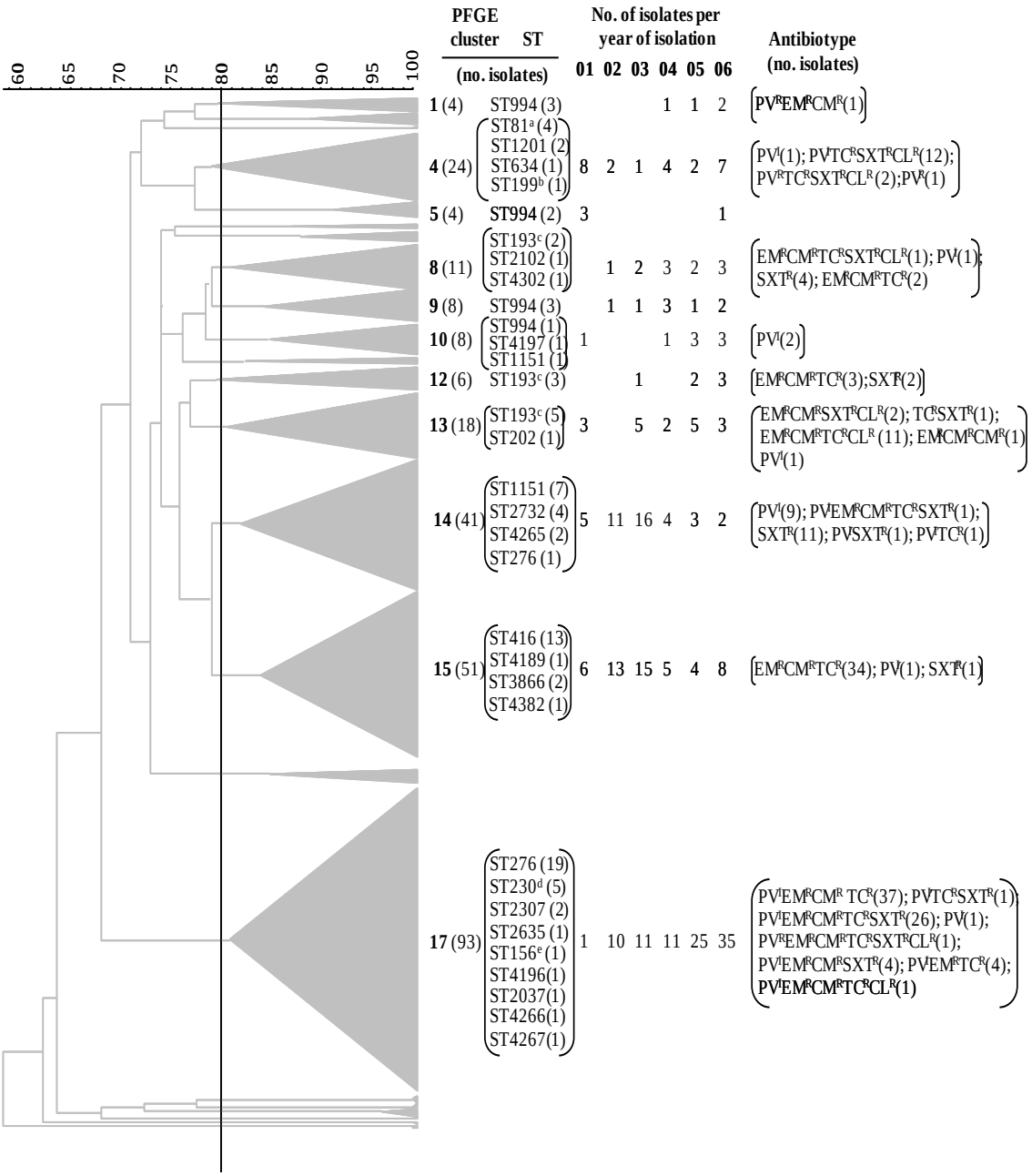

Figure 4.1: Macrorestriction dendrograms and ST information for serotype 19A isolates. Dice coefficient values (percentages) are indicated in the scale above the dendrogram. Whenever two or more isolates had a macrorestriction pattern with a Dice coefficient of ≥80%, a triangle proportional to the number of isolates is indicated in the dendrogram. The number in boldface type beside each triangle indicates the PFGE cluster number, and the number in parentheses indicates the number of isolates grouped in that cluster. All STs determined for isolates of each PFGE cluster are indicated, and the number in parentheses indicates the number of isolates exhibiting that ST. The distribution of the isolates found in each PFGE cluster (n ≥4) over the years of the study is presented. The superscript letters identify STs identifying clones spread internationally, as follows: a, Spain^{23F}-ST81; b, Netherlands^{15B}-ST199; c, Greece^{21}-ST193; d, Denmark^{14}-ST230; e, Spain^{9V}-ST156. Non-susceptibility to various antimicrobial agents is indicated by superscripts, as follows: R, resistant; I, intermediate; CL, chloramphenicol; CM, clindamycin; EM, erythromycin; PV, penicillin; SXT, co-trimoxazole; TC, tetracycline. The number of isolates sharing the same antimicrobial resistance profile is indicated in parentheses.

Table 4.2: Diversity and major PFGE clusters of the three serotype 19A populations analyzed.

Population	%SID (95%CI)	% of isolates in the following PFGE cluster[a]							
		4	**8**	**9**	**10**	**13**	**14**	**15**	**17**
Carriage isolates (n=110)	78.8 (74.5-83.9)	6	1	1	2	2	**21**	**34**	**24**
Invasive isolates (n=121)	82.2 (76.8-87.5)	**12**	5	5	1	**12**	7	7	**37**
Non-invasive isolates (n=57)	81.4 (73.3-89.6)	4	7	2	9	4	**16**	9	**39**

[a] PFGE clusters that included at least 5% of any of the three populations analyzed are represented. When the proportion of the population grouped into one of the PFGE clusters was ≥10%, the value is highlighted in boldface.

To identify the serotype 19A PFGE clusters associated with carriage, the ORs for the main clusters were determined on the basis of the number of isolates of that particular cluster with reference to all other isolates. This approach identified two PFGE clusters associated with carriage: PFGE cluster 14 (OR=0.30 [95% CI=0.12 to 0.73]) and PFGE cluster 15 (OR=0.16 [95% CI=0.06 to 0.36]), both of which are significant after correction for FDR (P=0.030 and $P < 10^{-4}$, respectively). A cluster with enhanced invasive disease potential, PFGE cluster 13 (OR=7.02 [95% CI=1.55 to 65.18], P=0.030), was also identified. Since the MLST analysis of PFGE cluster 13 revealed isolates mostly representing ST193, we repeated the analysis by grouping together all PFGE clusters in which ST193 was found (PFGE clusters 8, 12, and 13), and the result was also significant (OR=8.76 [95% CI=2.54 to 46.80], $P < 10^{-4}$).

Temporal fluctuations in PFGE clusters

To probe for temporal variations in the proportions of serotype 19A isolates in each PFGE cluster, the Cochran-Armitage test was used. This analysis was performed for the major PFGE clusters (n≥10) found among isolates causing infection and also by considering as a single genetic lineage all PFGE clusters associated with ST994 (n=24) and as another single genetic lineage all those associated with ST193 (n=35). The test revealed that among the serotype 19A isolates responsible for infections, the incidence of isolates in PFGE cluster 14 decreased during the study period ($P < 10^{-4}$), while the incidence of isolates in PFGE cluster 17 increased (P=0.002).

PFGE cluster and antibiotic resistance

The relationships between PFGE cluster and antimicrobial resistance were examined by determination of the odds ratios for each antimicrobial and the significant values are presented in Table 4.3. PFGE cluster 17 emerged in the analysis, since it was associated with resistance to all antimicrobials and antimicrobial classes with the exception of chloramphenicol.

Table 4.3 Significant antimicrobial resistance associations with PFGE clones of serotype 19A strains

PFGE Cluster (ST in PFGE cluster) [a]	Penicillin		Erythromycin		Chloramphenicol		Tetracycline		Co-trimoxazole	
	OR [95%CI]	p	OR [95%CI]	p	OR [95%CI]	p	OR [95%CI]	p	OR [95%CI]	p
4 (ST1201, [ST81, 634], ST199)			0 [0.00-0.13]	$<10^{-4}$	**18.32 [7.09-47.31]**	**$<10^{-4}$**			**3.47 [1.48- 8.13]**	**0.003**
9 (ST994) [b]			0 [0.00-0.44]	0.002			0 [0.00-0.44]	0.001		
10 (ST994, ST4197, ST1151) [b]			0 [0.00-0.44]	0.002			0 [0.00-0.44]	0.001		
13 (ST193, ST202)[c]	0.07 [0.01-0.55]	0.001	**4.85 [1.37-17.15]**	**0.007**	**29.76 [9.94-89.07]**	**$<10^{-4}$**				
14 ([ST1151, 2732, 4265], ST276)			0.03 [0.01-0.14]	$<10^{-4}$	0 [0.00-0.74]	0.021	0.06 [0.02-0.18]	$<10^{-4}$		
15 ([ST416, 4189, 3866], ST4382)	0 [0.00-0.07]	$<10^{-4}$			0 [0.00-0.56]	0.008			0.04 [0.01-0.29]	$<10^{-4}$
17 ([ST276, 230, 2037, 2307, 4196, 4266, 4267], [ST156, 2635])[d]	**83.34 [31.46- 220.74]**	**$<10^{-4}$**	**24.01 [10.52-54.82]**	**$<10^{-4}$**	0.14 [0.03-0.59]	0.002	9.70 [4.96-18.96]	$<10^{-4}$	**2.34 [1.37-3.99]**	**0.002**

[a] Only PFGE clusters with a significant association with at least one antimicrobial are indicated. Brackets indicate STs that belong to the same eBURST group.

[b] Only significant values are shown. An OR of >1 (in boldface) indicates a significant association with resistance, whereas an OR of <1 indicates a significant association with susceptibility. [c] If all PFGE clusters exclusively representing ST994 (PFGE clusters 1, 5, 9, and 10) are analyzed together, a significant association with susceptibility to co-trimoxazole (OR = 0 [95% CI = 0.00 to 0.40]; *P* = 0.002) emerges, in addition to those already identified for PFGE clusters 9 and 10. [d] If all PFGE clusters exclusively representing ST193 (PFGE clusters 12 and 13) are analyzed together, no qualitative changes occurred in relation to those identified for PFGE cluster 13. [e] PFGE cluster 17 was the only one presenting significant associations with both multidrug resistance and simultaneous resistance to penicillin and erythromycin.

DISCUSSION

Rates of IPD caused by serotype 19A have increased with this serotype ranking among the most common in all age groups. Importantly, absolute increases in rates of non-PCV7 type IPD remain modest in comparison with the reductions in PCV7 type IPD observed since 2000. However, recent studies reported more prominent increases in non-PCV7 serotypes in some populations, and these increases have partially eroded disease reductions observed after PCV7 introduction (14, 28, 34).

Serotype 19A presented a large proportion of resistant isolates, being the most frequent serotype among ERP, PNSP and EPNSP. Although a high frequency of non-susceptibility to erythromycin and penicillin is a hallmark of currently circulating serotype 19A isolates worldwide, there are differences between those found in North America and Europe. In Europe, PNSP serotype 19A isolates tend to have lower MICs than those circulating in the USA (19, 25), a characteristic that was also noted among our collection and that translates the different properties of the clones found in the two continents (25)

Several studies have demonstrated that after the introduction of a conjugate vaccine, significant changes in the distribution of the serotypes responsible for invasive infections occur. These changes are characterized by a sharp decrease in the number of infections caused by vaccine types and an increase in the number of infections caused by non-vaccine serotypes, even if they are only incremental (chapter 2 and (4, 18, 26, 38)). The pneumococcal serotypes not represented in vaccine formulations are generally considered to be less virulent (37), yet the lower incidence of invasive infections caused by non-vaccine serotypes could be due not to reduced virulence but to a lower transmissibility or to a weaker competitive capacity for nasopharyngeal colonization. If the latter was the case, then removal, by vaccination, of the serotypes commonly found in the nasopharynx would allow the establishment of non-vaccine serotypes that could then emerge as important causes of invasive infections (20). In this scenario, all other things being equal, one could expect that the more virulent clones found among non-vaccine serotypes would increase in prevalence as a cause of invasive infections. Another hypothesis for vaccine selection of non-vaccine serotypes would be the emergence of capsular transformants (30). These would retain the successful genotypes previously found among vaccine serotypes but would express a capsular polysaccharide not targeted by the vaccine (7).

In line with the findings of other studies, an increased proportion of infections caused by serotype 19A isolates has been noted in Portugal, despite non-universal vaccination (chapter 2). If occupation of a vacant colonization niche was the sole driver behind the increase in the incidence of serotype 19A infection, then an increase in the incidence of 19A colonization would be expected. Although we did observe an increase in the incidence of serotype 19A isolates among carriage

isolates, it was not statistically significant and may therefore not offer a full explanation for the rise in the incidence of serotype 19A infections. A possible explanation for the more modest increase in the incidence of serotype 19A carriage isolates may be that isolates of the other serotypes may be equally adapted at exploiting the vacant niche left by the reduction in the incidence of isolates of the vaccine serotypes as colonizers.

Each of the three serotype 19A populations analyzed was highly diverse, and several genetic lineages were identified, most of which were found among all three populations. This finding is in contrast to the findings of other studies that included isolates expressing all serotypes, which found a genetically more diverse population among carriage isolates (17, 32). A higher degree of diversity among carriage isolates is frequently interpreted as supporting the hypothesis that only a few lineages are capable of causing invasive infections. However, this conclusion may be conditioned by the larger number of serotypes found among carriage isolates. Studies of other pneumococcal populations homogeneous for their serotype are needed to clarify if the similar genetic diversity found among serotype 19A isolates causing infections and those carried asymptomatically is an unusual characteristic of serotype 19A or a more general property.

Previous studies have suggested that the capsular serotype may be more important than the genotype in determining the ability of pneumococci to cause invasive disease (6). Of interest, among all serotype 19A lineages identified, two groups established opposing relationships with the human host: one was associated with asymptomatic carriage (PFGE clusters 14 and 15, representing ST1151 and ST416 and closely related STs, respectively), while the other was associated with invasive disease (PFGE cluster 13, mostly representing ST193). The existence of several lineages within serotype 19A with opposing properties and their different prevalences in various geographic regions may explain why some studies identified isolates of this serotype as having enhanced invasive disease potential (32) and others did not (6).

Neither the lineage defined by ST193 (including all PFGE clusters presenting this ST) nor the one defined by ST416 and its single-locus variants increased among the infection isolates over time. Instead, two other genetic lineages did show temporal variations. The incidence of the lineage identified by PFGE cluster 14 (mostly representing isolates of ST1151 and ST2732 that are single-locus variants of each other and STs apparently only detected in Portugal), which was significantly associated with carriage and susceptibility to most antimicrobials, declined as a cause of infection during the study period. On the other hand, the incidence of the lineage characterized by PFGE cluster 17, associated with resistance to most antimicrobials, including β-lactams and macrolides (the antibiotics of choice for the treatment of pneumococcal infections), as a cause of infection increased significantly over time. In fact, this lineage was the most abundant in all collections. A comparison based on the PFGE profiles identified members of cluster 17 as being closely related to clone Denmark^{14}-ST230 (23). MLST analysis of representative isolates of this PFGE cluster

identified ST276 (a single-locus variant of ST230) as the dominant ST and found that most isolates (30 of 32) belonged to CC230, corroborating the findings that isolates in this PFGE cluster mostly represent the Denmark^{14}-ST230 clone.

Studies characterizing invasive isolates in both France (21) and Spain (4) have also identified representatives of clone Denmark^{14}-ST230, in particular, ST276, as major causes of serotype 19A invasive disease in both children and adults. A study from Israel also identified ST276 as an important cause of acute otitis media in recent years (12). The latter is a particularly interesting study, since it associated the presence of ST276 with high levels of antibiotic consumption, a situation that may be mirrored in Portugal.

Studies from two other non-European countries, South Korea and the United States, have also analyzed in great detail the genetic diversity of the pneumococcal population expressing serotype 19A (10, 25). The study from South Korea revealed a serotype 19A population with limited diversity (only 4 STs), while that from the United States revealed a much more diverse population (73 STs); but the major clonal complex found in South Korea (*n* = 53/58), CC320, was also a major lineage in the United States (*n* = 111/528). In contrast, in Portugal, ST320 was not detected, and a single isolate recovered in Portugal was a member of the most frequent lineage in the United States (ST199) and was in a medium-sized PFGE cluster (PFGE cluster 4). Moreover, the most frequent lineage in our study, PFGE cluster 17, representing the Denmark^{14}-ST230 clone (n=93/288, or 32%), was a minor lineage in the United States (accounting for 3% of the serotype 19A isolates) and was not found in South Korea. These data highlight the genetic diversity and geographic differences behind the increase in the incidence of serotype 19A isolates and suggest that the locally circulating clones, as well as other selective pressures, such as antibiotic consumption and vaccination, may play equally important roles in the emergence of serotype 19A as a major cause of pneumococcal infections.

Although this study has limitations, we consider that these do not affect our conclusions. First, isolates causing infections were recovered from centers geographically dispersed in Portugal, whereas pneumococcal carriage isolates were collected only in the Lisbon area. Our previous studies with isolates causing invasive infections did not identify any significant regional asymmetries (2, 33), so we do not expect these to exist in colonization. Second, the three major clones found to be colonizers, which together accounted for close to 80% of the colonization isolates, were each found in six DCCs, indicating that these clones are widely disseminated and do not correspond to local clusters. Third, no colonization isolates were available from 2004 and 2005 because no sampling was performed in those years. This did not allow the analysis of the temporal variations in the clonal composition of the colonization population, but a qualitative evaluation indicated that there was a strong parallel with the changes detected in the infection isolates. The major clones (n ≥10) found among the carriage isolates were present in all years studied, indicating no sudden variations

in the clonal distribution. These two observations reassured us that we were effectively sampling the carriage population. Fourth, we included in our comparison isolates recovered from both adults and children with invasive infections, whereas the carriage isolates were recovered only from children. Our choice was guided by the smaller number of isolates that would have been analyzed if we had excluded those from adults, which would have resulted in a much lower statistical efficiency, and by the observation that there were no differences in the clonal compositions among invasive isolates when they were stratified by age group. In fact, the rate of asymptomatic carriage in adults is low and children are considered the major reservoir of pneumococci (16). Further confirmation of the pivotal role of children in adult infections was obtained from the recent observation that the vaccination of children had a major impact on the incidence of pneumococcal infections in adults (2, 4, 9).

CONCLUSIONS

In summary, we have shown that different genetic lineages expressing serotype 19A preferentially establish colonization or cause infections in the human host. The lineage currently expanding as a cause of infection in Portugal and other southern European countries was not identified as being particularly virulent. The availability of an enlarged nasopharyngeal niche seems to have allowed the expansion of a clone competent at colonization and infection, which drove the rise in the incidence of serotype 19A infections. Similar to what was found elsewhere, this dominant serotype 19A lineage was resistant to most antimicrobials. Together with the decline in the incidence of a successful local clone that was mostly susceptible, this observation suggests that antibiotic use may also be an important factor shaping the pneumococcal population in Portugal and may have been decisive as a cause for the clonal fluctuations among serotype 19A isolates causing infections observed in Portugal and other Mediterranean countries. Nevertheless, the reasons why the successful multiresistant ST320 lineage is not found in Portugal or why the equally resistant ST199 lineage did not expand to dominate the serotype 19A population, as it did in the United States, remain unclear and point to the importance of circulating clones and other local selective forces in driving the expansion of the most successful serotype 19A clones. These data reinforce the importance of continuous surveillance to understand changes in the incidences of particular serotypes in the era of pneumococcal conjugate vaccines and highlight the important differences in the incidences of particular serotypes between Europe and the United States. Such knowledge may allow us to understand the emergence of non-vaccine serotypes and guide the design and use of future pneumococcal vaccines.

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CHAPTER 5

Pneumococcal vaccine candidates: Are pili an appropriate alternative?

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SUMMARY

In the previous chapters we demonstrated the effect of the introduction of PCV7 in a pneumococcal population responsible for IPD in Portugal. The increase of non-vaccine serotypes, such as serotypes 1, 19A and 7F, has raised a number of questions regarding the ultimate efficacy of conjugate vaccines in preventing pneumococcal infection and an urgent need to develop alternative vaccines. There are several candidates for a new pneumococcal vaccine formulation, among which one of the most recently discovered pneumococcal surface proteins, the pili. Pili structures have been recently implicated in the virulence of *S. pneumoniae* and due to their high immunogenicity they have been proposed to be used in a future pneumococcal vaccine. Two pilus-like structures (PI-1 and PI-2) have been identified in pneumococci, however these structures are not universally distributed among pneumococcal strains which questions their applicability in new pneumococcal vaccines (4, 7).

To evaluate the distribution of pili in a pneumococcal population and its possible use in a new vaccine formulation we searched the two pilus islets among a collection of invasive isolates recovered from children and adolescents (<18 years) in Portugal between 1999 and 2008 (n=577) and analyzed its association with capsular serotypes, and clusters defined by PFGE and MLST. We also evaluated the impact of PCV7 in pili distribution by comparing the datasets of the pre-vaccine period (1999-2002) with the post-vaccine period (2003-2008).

Overall, a minority of the isolates were positive for the presence of the PI-1 islet (17%). There was a high correspondence between the serotype and the presence or absence of PI-1 (W = 0.845). In particular, there was an association between the presence of PI-1 and the vaccine serotypes 4, 6B, 9V and 14 whereas the gene was significantly absent from other serotypes, namely 1, 7F, 8, 12B and 23F. In contrast, the PI-2 islet, was found in 33% of the pneumococcal strains included in the study. PI-2 positive strains were found mainly among serotypes 1 and 7F, two serotypes not included in the current PCV7 formulation but covered by the future PCV13.

Even within serotypes, there was variation in the distribution of the pilus islets in terms of PFGE clones and a higher Wallace coefficient (W = 0.979) indicates that carriage of these islets is a clonal property of pneumococci. Moreover analysis of the *rlrA* negative isolates revealed heterogeneity in the genomic region downstream of the *pfl* gene, the region where the islet is found in other isolates, compatible with recent loss of the islet in some lineages.

A decrease in the presence of the *rlrA* islet among invasive pneumococcal strains was observed after more widespread use of PCV7. This change is associated with the decrease of vaccine serotypes since the majority of strains carrying the PI-1 islet expressed vaccine serotypes.

In opposition, the PI-2 islet increased after vaccine availability, mainly resulting from the dominance of serotypes 1 and 7F in the post-PCV-7 period.

INTRODUCTION

Currently, prevention of pneumococcal diseases is achieved through the 23-valent polysaccharide vaccines (PPV23), PCV7 and more recently PCV10 and PCV13. Although polysaccharide vaccines have several disadvantages, of which the most important is its impaired efficacy in children <2 years and immunodeficient patients, they are still useful since they include a significant number of pneumococcal serotypes and are effective for immunocompetent individuals ≥5 years of age who are at risk of pneumococcal infections (43, 44).

The more recently approved conjugate vaccines are highly immunogenic in children <2 years of age, showing better immune responses and immunological memory (12). Unfortunately, the efficacy is limited due to the restricted number of serotypes included, and in the long run their usefulness is threatened by serotype replacement (44). Furthermore due to the geographical variability of pneumococcal serotypes causing infection, the potential coverage of these vaccines may be as low as 50% or less of the strains causing infection in many developing countries (20). Finally, these conjugate vaccines are far too expensive for use in much of the developing world where most of the fatal infections with this pathogen occur (37). Consequently, there is a need to develop cheaper vaccines and with a higher serotype coverage.

Several pneumococcal virulence factors have been identified in last decade. Since these proteins are essential for virulence and pathogenesis, it is reasonable to assume that antibodies raised against them would prevent infection, giving rise to a new generation of pneumococcal vaccines based on protein antigens. Nevertheless, an important limitation of all protein vaccines has been the diversity found among the different pneumococcal serotypes (38).

The ideal vaccine

The ideal vaccine should protect against pneumococcal pneumonia, meningitis, otitis media and bacteremia in young children. Because carriage generally precedes infection, a vaccine that also prevents pneumococcal colonization as well as disease is also aspired in future vaccines (11). An ideally protein antigen should be a surface exposed protein, common to all pneumococcal serotypes with no or limited antigenic diversity. However, this model candidate is yet to be discovered. An important limiting factor in the identification of a single protein candidate has been the existing diversity among many various pneumococcal serotypes (5). On one hand, the existence of variability in a protein suggests that there is immunity to that molecule in the normal host, and variation is a consequence of a selective pressure (11). Consequently, the protein molecules that elicit the best immunity may be those which, like the capsular polysaccharides of pneumococci,

exhibit considerable variation within the population. On the other hand, like in capsule-based vaccines, this diversity hampers the construction of a vaccine with coverage higher than the already available ones. If variable proteins are used as vaccines, it becomes essential to understand the extent of this variation and the degree of cross-protection elicited by different variants of each protein (11).

Potential vaccine antigens

Pneumococcal proteins have been studied thoroughly to evaluate their possible role in a future pneumococcal vaccine. These proteins can either be used as antigens to elicit protection against pneumococcal infection or as the carrier proteins in a conjugate vaccine eliciting protection against the remaining serotypes, thus preventing serotype replacement. Table 5.1 presents some of the proteins already proposed. Among these, those that have been studied most extensively are PspA, pneumolysin, autolysin, neuraminidase, PspC, PsaA, and more recently the pili proteins (RrgA, RrgB, RrgC).

Pneumococcal surface protein A: PspA is a member of a family of structurally related choline-binding surface proteins which interferes with the complement system and also binds to lactoferrin (40). Though is present in all pneumococci one of the concerns regarding the use of PspA as a vaccine is the structural and antigenic variability among different strains since more than 40 different types have been defined (11). Nevertheless, cross-reactive antibodies to heterologous PspA molecules after immunization with a single recombinant PspA preparation have been observed. Moreover, active immunization with PspA in animal models has proven to be protective against invasive infections and nasopharyngeal carriage (10).

Pneumococcal surface protein C: PspC was originally identified by its similarity to PspA. The PspC (also referred to as CbpA, Hic or SpsA) mediates adherence to lung epithelia, the brain microvascular endothelium, possibly contributing to the invasion at these locations, and also has a putative role in adherence to the nasopharynx (5, 39). The *PspC* gene is present in 75%-100% of pneumococcal strains and PspC proteins can be classified into 11 groups (5). Unfortunately, studies have not shown whether vaccination with PspC elicits protection against heterologous PspC type strains though antibodies against this protein show cross-reactivity against PspA. Vaccination with homologous PspC has shown to be protective against sepsis in mice (10).

Table 5.1: Proteins of *S. pneumoniae* involved in vaccine development. Adapted from García-Suarez *et al* (16).

Protein	Bacterial function	Mechanism of virulence	Protection in animal models	Comments	Ref
PspA	Stabilization of capsular charge	Inhibition of complement	Good	Clades and families	(9, 13, 18, 35)
PspC	Adhesion, binding of pIgR, secretory IgA, C3 and factor H	Inhibition of complement	Good	Also known as SpsA, CbpA, and Hic	(39)
FBA and GAPDH	Glycolytic enzymes	Immunogenic in children	Good	Immunoproteomics	(28)
LytA	Cell lysis and growth	Pro inflammatory(?)	Low	Allolysis	(9)
PsaA	Mn^{2+} transporter	Inhibition of complement	Low	Controversial accessibility of antibodies	(9, 19)
PiuA and PiaA	Iron uptake ABC transporters	Mutants had reduced virulence	Good	Protection against intraperitoneal infection	(26)
PpmA	Peptidyl-prolyl cis/trans isomerase	Mutants ppmA-deficient reduced virulence	Low	Controversial accessibility of antibodies	(18, 41)
Hyl	Hyaluronan breakdown	Mutants had no reduced virulence	None	Inhibitors could be useful pharmacological tools	(9)
NanA	Adherence Spreading	Mutants had no reduced virulence	Very low	Good protection in otitis media	(9, 29)
Ply	Cytolysin. Spreading	Cytotoxic. Proinflammatory	Good	Cytoplasmatic.	(9, 39)
Pilus subunits	Adherence, colonization	Mutants had no reduced virulence	Good	Restricted to a subset of strains	(6, 17)

Pneumococcal autolysin A : LytA is, like PspA, CbpA and pneumolysin, a choline binding protein which contributes to virulence by mediating the release of pneumolysin and possibly inflammatory cell wall degradation products (10).

Pneumococcal surface adhesion A: PsaA is a member of the family of metal binding lipoproteins. This protein is part of an ABC transporter complex thought to be involved in the transport of manganese into pneumococci. The first immunization studies showed significant protection against colonization but little to modest protection against invasive disease (10).

Neuraminidases: Pneumococci produce two neuraminidases, NanA and NanB, which cleave N-acetylneuraminic acid from mucin, glycolipids, glycoproteins and oligosaccharides on the host cell surface. Although the precise role in the pathogenesis of *S. pneumoniae* has not yet been identified, neuraminidase is presumed to contribute to adherence to mucosal surfaces by decreasing the viscosity of the mucus layer or through exposing cell surface receptors for pneumococci (10). Immunization with purified NanA confers a limited degree of protection in mice against intranasal challenge with virulent pneumococci (47). In a chinchilla model, immunization with NanA resulted in a significant reduction in nasopharyngeal colonization as well as in the incidence of otitis media with effusion (29).

Pneumolysin: Ply is a thiol-activated hemolysin that uses the cholesterol in cell membranes as a receptor for its cytolitic activity. It is suggested to interfere with host immunity and inflammatory responses by a variety of functions, including complement fixation and inhibition of phagocyte function. It also inhibits ciliary activity in the bronchus and is therefore important in pathogenesis of pulmonary infection (10). This toxin is believed to be located in the cytoplasm and released when cells undergo autolysis.

Initially, the use of pneumolysin as a vaccine antigen was considered due to its sequence conservation and antigenicity among clinical isolates. Nevertheless, it was recently reported by Jefferies and colleagues the existence of at least 18 different pneumolysin variants at the protein level (25). Moreover immunization of pneumolysin was unable to protect mice against a lethal challenge of pneumococcus. Non-toxic forms of pneumolysin have been investigated and have shown promising results, however studies indicate that pneumolysin would be better as a protein carrier for capsular polysaccharides, than as a “stand-alone” vaccine candidate (5).

One of the approaches for vaccine development is the combination of different proteins. Since PsaA and PspA have different functions in virulence, an additive role for these proteins in vaccination has been suggested. Indeed promising results have been found for the combination of PsaA and PspA in the prevention of colonization and otitis media in animal models (10). A major advantage of this combination is its serotype independent protection. In addition this concept will limit the costs due to the relative simple production of these recombinant proteins which would allow the distribution of this vaccine to third world countries where IPD rates are highest (10).

An alternative to proteins are DNA vaccine vectors containing, for example, *psa*A and *psp*A genes. DNA-mediated immunization involves the direct introduction of a plasmid DNA encoding an antigenic protein that is then expressed within the cells of the organism targeted for vaccination. The *in vivo* expression of that protein using expression systems leads to the induction of antigen-

specific immune responses. Because the genes transferred by the plasmids require the host cellular machinery to be expressed, DNA-based immunization resembles a viral infection (53).

DNA vaccines containing *psp*A fragments have been analyzed in terms of cross-reactivity of anti-PspA antibodies (32). Unfortunately, no significant protection was observed between the different PspA familes. Protection was restricted to immunization with a DNA vaccine expressing a complete N-terminal PspA fragment belonging to the same clade as the strain used for challenge (32).

Although several strategies for new vaccine formulations have been proposed none of them seem to improve the coverage of the currently available conjugate vaccines. Another set of proteins, which compose pilus-like structures, were recently identified at the pneumococcal surface and suggested to be used in pneumococcal vaccines. The following paragraphs will describe the studies performed so far regarding these new structures.

The pneumococcal pilus

Non-flagellar polymeric cell-surface organelles designated as pili were initially identified in Gram-negative bacteria. In the last decade, pilus-like surface structures have been described in Gram-positive bacteria like *Corynebacterium* spp., *Actinomyces* spp. and several streptococcal species (50), but only recently were pili identified in the major pathogenic species of the genus: *Streptococcus pyogenes*, Lancefield group A (33); *Streptococcus agalactiae*, Lancefield group B (27) and *Streptococcus pneumoniae* (pneumococcus) (6).

With the idea of eliciting immune responses that counteract the essential biological role of pili, components of pili have been tested as vaccine candidates against different pathogens. Pili-based vaccines have been shown to induce protection in humans against gonococcus and enterotoxigenic *Escherichia coli* and *S. agalactiae*. However, sequence variability has so far been the major obstacle to the development of pilus-based vaccines (30).

In the pneumococcus, two pilus-like structures have been identified at the bacterium surface. Type 1 pili, PI-1, is encoded by the *rlrA* pathogenicity islet, a 14.2 kb region composed of 7 genes encoding a putative transcriptional regulator (*RlrA*), 3 LPXTG surface proteins with weak homology to microbial surface components recognizing adhesive matrix molecules – MSCRAMMs (RrgA, RrgB and RrgC) and 3 sortases (SrtB, SrtC and SrtD). In 2003, the *rlrA* pathogenicity islet was identified in a genome-wide screen by Hava and Camilli. The *rlrA* gene was identified in the original signature tagged mutagenesis study as an essential virulence gene in murine models of infection (21). Later studies confirmed that the RlrA protein acted as a transcription factor recognizing several promoters within the *rlrA* islet and showed it to be essential for wild-type levels of expression of the pili structural genes and associated sortases (22). The product of the *mgrA* gene, located outside

the *rlrA* islet, was shown to act as a transcriptional repressor of the islet genes, including *rlrA*, being responsible for the silencing of the locus in the absence of RlrA (23). It was only recently that the *rlrA* islet was described to encode protein subunits that compose the pneumococcal pili (22).

The presence of the *rlrA* pathogenicity islet was shown to influence pneumococcal capacity to adhere to human lung epithelial cells (6, 23). Mouse models of pneumococcal pneumonia and bacteremia have also suggested a role of pili in virulence and host inflammatory responses (6, 21). More recently, immunization of mice with pilus structural antigens was shown to induce protection against lethal challenge by piliated strains (17). Moreover, these studies indicate that vaccination with the pilus subunits offers the same protection as vaccination with heat killed bacteria, raising the possibility of using pilus antigens in a multivalent pneumococcal vaccine (17).

In spite of these favorable results, early genomic studies indicated that the *rlrA* islet was not present in all pneumococcal isolates, suggesting that it could have been acquired by horizontal gene transfer (51) and raising a cautionary note concerning the use of pilus antigens in any future vaccine. A survey for the distribution of the sortase genes identified the *srtA* gene in all tested bacteria, but the sortases associated with the *rlrA* islet were present in only 17% of the isolates tested (42). A recent study, specifically designed to evaluate the distribution of the *rlrA* islet among pneumococcal isolates associated with colonization and infections in Native Americans, found that only 21% of the isolates were positive for *rrgC*, a gene encoding a pilus structural protein, and that there was an association between the presence of this gene and the serotypes included in PCV7 (7). However, the same study could not show a difference in the presence of *rrgC* between the isolates recovered from the nasopharynx and those recovered from sterile sites. In contrast, the *mgrA* gene encoding the transcriptional repressor of the *rlrA* islet seems to be universally distributed within *S. pneumoniae* (23).

The type 2 pili, PI-2, is a 7 Kb genetic region composed of five genes, two putative sortases (*srtG*1 and *srtG*2), one signal peptidase-related product (*sip*A) and two genes encoding putative LPXTG surface proteins, *pit*B and *pitA*, with *pit*A being a pseudogene due to a stop codon in the N terminus (4). This second pilus type was identified in pneumococci by analysis of the publicly available genomes and was shown to be involved in adherence of pneumococci to epithelial cells. Nevertheless, the effectiveness of attachment to host cells by PI-2 was lower than the observed for PI-1. In addition, like PI-1, not all pneumococci harbour the type 2 pili, and serotype association has been observed (4).

In view of these findings, a better understanding of the distribution of the pili islets in the invasive pneumococcal population would help in the evaluation of their proposed role as virulence factors and as potential vaccine candidates. In the present study, we determined the prevalence of the PI-1 and PI-2 islets in a collection of invasive isolates recovered from patients with < 18 years

with the aim of identifying associations between the presence of the pilus islet and serotype, antimicrobial resistance or clonal types defined by PFGE and MLST.

MATERIALS AND METHODS

Bacterial isolates

For this study, all strains recovered from invasive infection in children and adolescents (<18 yrs) between 1999 and 2008 were considered. To evaluate the possible effect of the PCV7 vaccine in serotype distribution the two vaccine periods were considered.

The reference strains TIGR4 (51) and R6 (24) were used as either positive or negative controls for the presence of the pili islets.

Detection of the PI-1 islet

Identification of isolates carrying the *rlrA* islet was performed by either southern blot hybridization or PCR reaction as follows:

Southern blot: Genomic DNA from *S. pneumoniae* was prepared in agarose plugs as described previously, digested with SmaI and separated by PFGE in a CHEF-DR II apparatus (Bio-Rad Laboratories, USA) (46). Following electrophoresis, the DNA was transferred to nylon membranes (Hybond-N^+, Amersham Biosciences, Bucks, UK) with a VacuGene system (Pharmacia Biotech AB, Uppsala, Sweden) and subjected to Southern hybridization under stringent conditions. The probe was constructed using primers RLRA-up (5'-TCT GAT AGA TGA GAC GCT GTT G-3') and RLRA-dn (5'-CTC CGC TTC TTT CTA CTA CAA G-3'), which allowed the amplification of an 1177 bp internal fragment of the *rlrA* gene using DNA from strain TIGR4 as template. Labeling and hybridization were performed using ECL direct nucleic acid labeling and detection system (GE Healthcare, Buckinghamshire, UK), according to the instructions of the manufacturer. The molecular size of the hybridization signals and the corresponding SmaI fragments were determined.

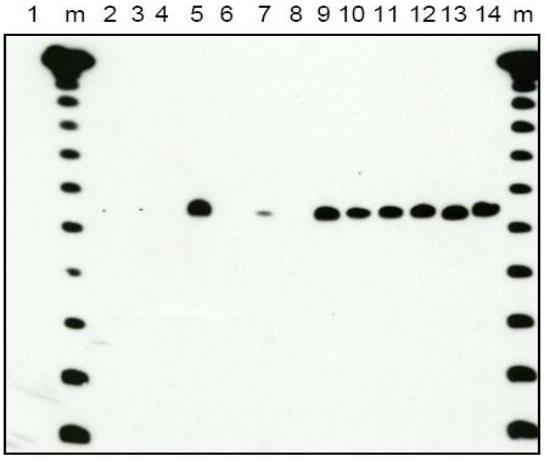

Figure 5.1: Southern Hybridization of a representative set of isolates with *rlrA* gene probe. Lanes m, lambda PFGE marker (New England BioLabs, Beverly, MA). Lane 1, strain R6. Lanes 2-4, 6 and 8, isolates lacking the PI-1 islet. Lanes 5 and 9-14, isolates positive for the presence of rlrA gene. Lane 7, isolate with a weak hybridization signal where the presence of the islet was confirmed by PCR. In all isolates with a negative hybridization result the absence of the pilus islet was confirmed by PCR.

PCR reaction: the *rlrA* gene was detected by PCR, using *RLRA*-up and *RLRA*-dn primers. Template DNA was prepared by diluting 9 µl of an overnight culture in 441 µl of water and boiling of this mixture for 2 min. The PCR reactions were performed in a 50 µl volume containing 20 µl of template solution, 1X reaction buffer (Promega, Madison, USA), 4mM of $MgCl_2$ (Promega, Madison, USA), 10mM dNTPs (Fermentas, Vilnius, Lithuania), 1.5U of Gotaq polymerase (Promega, Madison, USA) and 20 pmol of each of the primers. The PCR program consisted of 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 10 min incubation at 72°C.

Identification of the *rlr*A pilus clade type

Moschioni and colleagues sequenced several *rlrA* islets and multiple alignment of the entire sequences revealed the presence of 3 PI-1 families denominated by clade types I, II and III (34). To determine the clade distribution among the population positive to the *rlr*A islet we used the primers for the clade determination proposed by the authors (34).

PCR reaction: the clade type was detected by PCR, using the primer pairs specific for each clade (Figure 5.3-Panel C) (34). Template DNA was prepared by diluting 9 µl of an overnight culture in 441 µl of water and boiling of this mixture for 2 min. The PCR reactions were performed in a 50 µl volume containing 20 µl of template solution, 1X reaction buffer (Promega, Madison, USA), 4mM of $MgCl_2$ (Promega, Madison, USA), 10mM dNTPs (Fermentas, Vilnius, Lithuania), 1.5U of Gotaq polymerase (Promega, Madison, USA) and 20 pmol of each of the primers. The PCR program consisted of 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 10 min incubation at 72°C.

Characterization of the chromosomal region where the *rlrA* islet is usually found in non-piliated strains

In order to confirm that the *rlrA* negative strains did not carry the pilus pathogenicity islet or parts of it, a set of primers that flanked the *rlrA* pathogenicity islet were developed – PFL-up (5'-ATC TCA TTG ACT ACA CAA GTA TCA CCT C-3') and P-dn (5'-CAA GAG CAT ACT CCA ACT CAT AAA TAT GTG-3') – with the aim of amplifying the entire pilus islet (Figure 5.3 - A). All strains that were negative for the presence of the *rlrA* gene were subject to PCR with primers PFL-up and P-dn, as well as TIGR4 (piliated control), and R6 (non-piliated control). If the pilus islet or parts of it were absent, the PCR product expected using these primers would be similar to that obtained when using R6 DNA as template (pfl region type A - 1310 bp). The PCR reactions were performed in a 50 µl volume containing 20 µl of template solution, 1X reaction buffer (Promega, Madison, USA), 1.5U Gotaq polymerase (Promega, Madison, USA), 10 mM dNTPs (Fermentas, Vilnius, Lithuania) and 20

pmol of each of the primers. The PCR program consisted of 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 3 min, followed by 10 min incubation at 72°C. Whenever no PCR product was obtained using these conditions, and in a subset of the isolates positive for the *rlrA* gene representing the diversity found within the collection, the reaction was performed in conditions allowing the amplification of fragments larger than 10 kb. In this case DNA was extracted using CTAB as described previously (3) and the PCR reaction was performed using the Expand Long Template PCR System (Roche, Mannheim, Germany) according to the manufacturer's instructions. The PCR program consisted of incubation at 94°C for 2 min followed by 10 cycles of 94°C for 10 s, 58°C for 30 s, and 68°C for 13 min, and 20 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 13 min with an incremental increase of 20 s per cycle, followed by 7 min incubation at 68°C.

Strains representative of the various sizes of PCR products obtained were sequenced by primer walking. The sequences were compared using BLAST against the available sequence databases at the NCBI to identify similar sequences and to each other using the MEGA (49) and GATA (36) software programs.

Nucleotide sequence accession numbers

The sequences of isolates representing the various sizes of the PCR products obtained in *rlrA* negative isolates were deposited in GenBank with accession numbers EU126839 and EU126840.

Detection of the PI-2 islet

A similar approach was used for PI-2 detection. In this case PI-2 presence was detected by PCR amplification of *sip*A using the primers sipA-up and sipA-dn (Figure 5.4). A set of primers that flanked the whole PI-2 islet, pepT-up and hem-dn, were used to confirm *sip*A negative strains. All strains negative for the presence of the *sip*A were subject to PCR with primers pepT-up and hemH-dn. If the PI-2 islet was absent than the PCR product expected using these primers would be similar to that obtained when using TIGR4 DNA as template (1049 bp). Again, template DNA was prepared by diluting 9 µl of an overnight culture in 441 µl of water and boiling of this mixture for 2 min. The PCR reactions were performed in a 50 µl volume containing 20 µl of template solution, 1X reaction buffer (Promega, Madison, USA), 4mM of $MgCl_2$ (Promega, Madison, USA), 10mM dNTPs (Fermentas, Vilnius, Lithuania), 1.5U of Gotaq polymerase (Promega, Madison, USA) and 20 pmol of each of the primers. The PCR program consisted of 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 10 min incubation at 72°C.

Statistical analysis

Wallace coefficients (W) were used to compare partitions. Statistical associations between the presence of the *rlrA* islet and serotype, PFGE cluster or antimicrobial resistance profile were characterized by odds ratios (OR) with 95% Wald confidence intervals (CI) (1). For null OR, 95%CI were computed through the Fisher method (15). OR significance or statistical differences between proportions were tested with the chi-square statistic. The resulting p-values were corrected for multiple testing by controlling the False Discovery Rate (FDR) under or equal to 0.05 through the linear procedure of Benjamini and Hochberg (8). The p-values indicated in the text were considered significant if lower than 0.05.

Wallace coefficient analysis was done in a web application (14) and the remaining statistical tests were executed in the R statistical language using functions implemented in the epitools package (2).

RESULTS

Distribution of the PI-1 islet among invasive pneumococci

Among the collection of invasive isolates recovered between 1999 and 2008, only a small portion (16.6%) of our invasive isolates was *rlrA* positive. Nevertheless the prevalence of the PI-1 islet was different when considering the two vaccine periods. In the pre-vaccine period (1999-2002), 32 of the 102 strains (31.4%) were PI-1 positive while in the post-vaccine period (2003-2008) only 13.5% (n=64) harbored these structures.

To confirm the correlation of presence of *rlrA* gene and presence of the entire PI-1 islet a group of representative isolates was subject to long-range PCR. All isolates yielded fragments larger than 14 kb compatible with the presence of the entire pilus locus confirming that the presence of the *rlrA* gene is a good marker for the presence of the entire pilus islet.

Overall, *rlrA* positive isolates were found in 7 serotypes of the 43 serotypes identified in our collection. Isolates expressing serotypes 4, 6B, 9V, 13, 14, 19A, 19F were shown to carry the *rlrA* gene, but 73.9% of the *rlrA* positive isolates expressed PCV7 serotypes (4, 6B, 9V, 14, and 19F). Again, differences between the two vaccine periods were observed. In the pre-vaccine period 96.9% of the PI-1 positive strains presented one of the vaccine serotypes while in the post-vaccine period 62.5% of the *rlr*A positive strains were covered by the vaccine. This decrease was associated with the decline of vaccine serotypes after vaccine availability.

The proportion of isolates carrying the PI-1 islet among isolates of the same serotype was variable (Figure 5.2), but overall serotype was a good predictor of the presence or absence of the pilus islet, as indicated by the high Wallace coefficient (Table 5.2).

Table 5.2: Simpson's index of diversity (SID) and Wallace Coefficients for each of the pilus islets.

	PI-1	**PI-2**
	SID 95% 27.79 [23.65-31.93]	SID 95% 45.49 [43.02-47.96]
Serotype	0.845 [0.818-0.871]	0.911 [0.882-0.939]
PFGE	0.979 [0.972-0.986]	0.976 [0.954-0.999]

In order to identify which serotypes were associated with the presence of the *rlrA* islet, odds ratios were calculated. An OR > 1 implies that the serotype is associated with the presence of *rlrA*, while an OR < 1 indicates that the serotype is significantly depleted of *rlrA* positive isolates. According to this analysis, serotypes 4, 6B, 9V and 14 were highly associated with the presence of

the pilus islet ($p < 0.01$ for all serotypes). On the other hand, the *rlrA* islet was significantly depleted from serotypes 1, 7F and 23F (Table 5.3).

Identification of PI-1 clade type

To identify which of the three subtypes of the *rlrA* islet was more prevalent in our collection and what was the association with serotype and PFGE cluster, the clade type for each PI-1 positive strain was identified (Figure 5.3-Panel C). Overall, 64.6% (n=62) of the *rlrA* positive strains presented clade type I, 16.7% (n=16) the clade type II and finally 18.7% (n=18) belonged to clade type III. Like PI-1, there was a strong correlation between the clade type and the PFGE cluster within each individual serotype (W=0.862, 95%CI: 0.743-0.982) (Table 5.5).

Distribution of PI-2 islet

The type 2 pilus was identified in 33% of the invasive pneumococcal strains. As described with the PI-1 islet, an association of PI-2 islet and serotype was observed (Table 5.3). In this case PI-2 was mainly associated with serotypes 1 and 7F, as it can be observed by the OR presented in Table 5.3, but a small number of strains of serotype 19F (n=4, 20%) and 19A (n=18, 17.8%) were found carrying the PI-2 islet. Interesting, all these strains also harbored the PI-1 islet. Contrary to the PI-1 islet only four PI-2 positive strains, which presented serotype 19F capsule, were covered by the PCV7 vaccine, while 88.9% expressed PCV10 serotypes and 100% of the PI-2 positive strains presented one of the serotypes of PCV13. Differences between the two vaccine periods considered were also observed for PI-2. In this case, contrary to the *rlrA* islet, an increase of PI-2 from 21.6% in the pre-vaccine period to 37.3% in the post vaccine period was observed. This increase is associated with the expansion of serotypes 1 and 7F in invasive disease after PCV7 (Chapter 2).

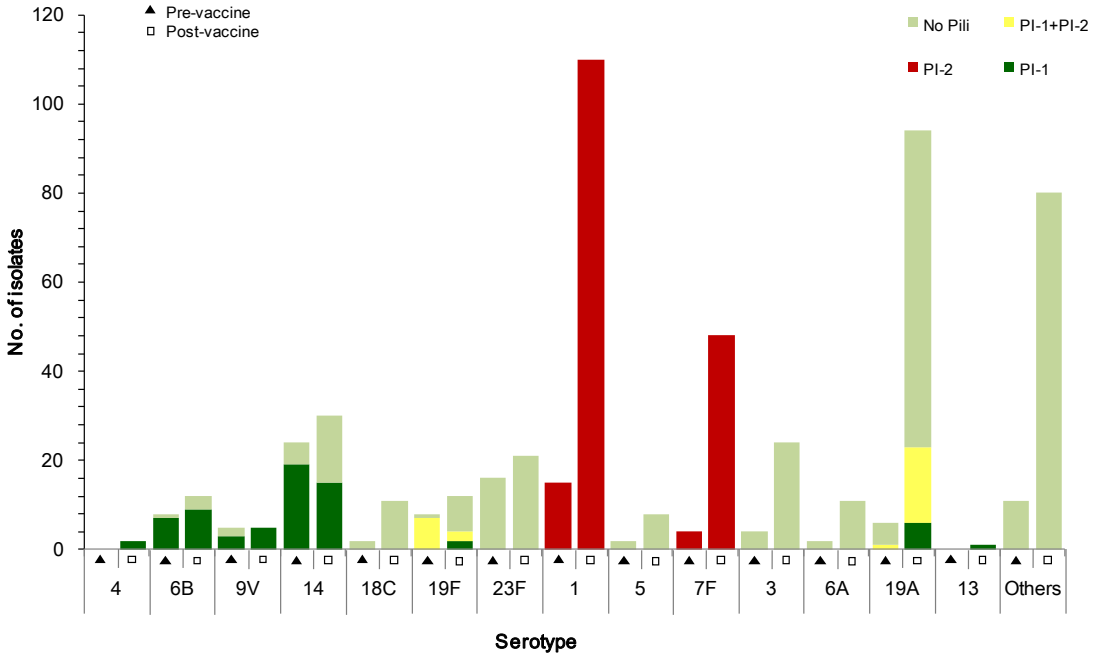

Figure 5.2: Frequency of the PI-1 and PI-2 islets among serotypes. Serotypes included in any of the conjugate vaccines available and non-vaccine serotypes for which one of the pilus was identified are presented. Dark green bars indicate the number or PI-1 positive strains. Light green bars indicate the number of PI-2 positive strains. Green bars indicate the number of serotypes for which the two pili islets were identified. Grey bars indicate the number of PI negative isolates. Isolates recovered in the pre-vaccine period are indicated by black triangles and isolates recovered in the post-vaccine period are indicated by open squares.

Association between the presence of pilus islet and resistance to antimicrobials

A clear correlation between the presence of pili and antimicrobial resistance was observed. Among the 274 piliated strains (PI-1 or PI-2 positive strains) we found that a significant proportion of isolates carrying the pilus islet were resistant to at least one antimicrobial (28.8% (79/274)). Overall, 16.8% (46/274) were non-susceptible to penicillin, 12.0% (33/274) were resistant to erythromycin and 8.4% (23/274) were multi-drug resistant. Nevertheless, differences between the two vaccine periods were observed. In the pre-vaccine period 47% of the piliated strains were non-susceptible to penicillin contrasting the 10% found in the post-vaccine period. Similarly, between 1999 and 2002, 25% of the PI-positive strains were erythromycin resistant while only 9% of the piliated strains recovered after 2003 presented non-susceptibility to this antimicrobial agent.

Yet, the correlation between antimicrobial resistance and pili presence was not uniform for the two pili. While PI-1 was significantly associated with resistance to penicillin and co-trimoxazole, PI-2 was significantly associated with susceptible strains as it can be observed in Table 5.4.

Presence of the pilus islet is a clonal property

Characterization of the genetic lineages of the pneumococcal collection included in the present study had been performed previously [(46) and chapter 3]. Analysis of the isolates by genetic lineage demonstrated that the presence of pili was not only associated with serotype but also with PFGE cluster within each serotype. This association was more obvious for type 1 pilus than for type 2 pilus since, as discussed previously in chapter 3, the lineages associated with PI-2 are more homogeneous. The Wallace coefficient relating the PFGE clusters with the presence or absence of pili was higher than for serotype indicating that these pathogenicity islets are clonally distributed (Table 5.2).

To determine if there were PFGE clusters particularly associated with the presence of pili, odds ratios were determined and the significant values obtained in this analysis are indicated in Table 5.3. In some serotypes identified as associated with the presence of the pilus islet, all the major PFGE clusters also showed a positive association with the presence of pili, such as serotype 4 in the case o PI-1 and serotypes 1 and 7F in the case of PI-2. But in others only some of the genetic lineages were associated with the presence of pili (e.g. serotype 14 in PI-1 and serotypes 19A and 19F in the case of PI-2) (Table 5.3 and 5.8). Note that the OR values for the PFGE clones are more extreme when compared with the values calculated for serotype alone, further supporting the notion that the presence of the pilus islet is a clonal property.

Table 5.3: Association of the *rlrA* islet with serotypes and PFGE clusters.

Serotype	PI-1		PI-2		PFGE cluster[a]	STs in PFGE cluster	PI-1		PI-2	
	OR (95%CI)	p	OR (95%CI)	p			OR (95%CI)	p	OR (95%CI)	p
1	0 [0.0-0.24]	$<10^{-4}$	1277.03 [78.55-20760.16]	$<10^{-4}$	**A_{109}**	ST228+ST306+ ST4578+ST4579	0	$<10^{-4}$	915.60 [56.37-14871.95]	$<10^{-4}$
4	20.47 [0.91-457.45]	0.009	NS	NS	**A_2**	ST1221	20.47 [0.915-457.497]	0.015	NS	NS
14	12.64 [6.85-23.32]	$<10^{-4}$	0 [0.0-0.25]	$<10^{-4}$	**C_{33}**	ST143+ST156+ST557+ ST790+ST4575+ ST4576	503.90 [30.486-8328.89]	$<10^{-4}$	0 [0.0-0.43]	$<10^{-4}$
19F	6.79 [2.73-16.87]	$<10^{-4}$	NS	NS	**E_6**	ST179+ST390+ ST4245	26.32 [3.04-228.40]	$<10^{-4}$	NS	NS
					F_2	ST476	20.47 [0.92-457.49]	0.015		
23F	0 [0.0-0.10]	0.005	0 [0.0-0.78]	$<10^{-4}$	**A_{28}**	ST277+ST338+ ST732	NS	NS	0 [0.0-0.52]	$<10^{-4}$
6B	23.85 [7.77-73.17]	$<10^{-4}$	0 [0.0-0.75]	$<10^{-4}$	**C_2**	ST273+ST315	20.47 [0.92-457.49]	0.0015	NS	NS
					D_2	ST138+ST176	20.47 [0.92-457.49]	0.0015		
					F_5	ST273+ST1224	52.86 [2.86-975.90]	$<10^{-4}$		
					H_2	ST386	20.47 [0.92-457.49]	0.0015		
7F	0 [0.0-0.70]	0.005	267.43 [1.42-16.39]	$<10^{-4}$	**A_{51}**	ST191	0	0.0008	260.51 [15.97-4249.23]	$<10^{-4}$
9V	21.77 [4.55-104.24]	$<10^{-4}$	NS	NS	**A_3**	ST156+ST2356	31.03 [1.54-624.65]	0.0001	NS	NS
					B_7	ST162+ST557+ ST1225	13.16 [2.51-68.87]	$<10^{-4}$		
19A	NS	NS	0.36 [0.21-0.629]	0.0001	**C_{12}**	ST1151+ST2732	27.85 [5.99-129.31]	$<10^{-4}$	9.95 [2.12-45.86]	$<10^{-4}$
					E_{21}	ST193+ST416	3.45 [1.45-8.22]	0.003	NS	NS

[a] PFGE clusters are represented by capital letters and a subscript number indicating the number of isolates included in the cluster.

Table 5.4 Association of the *rlrA* islet with antimicrobial resistance

Antibiotic	no.PI-1 positive strains/ no.resistant isolates (%PI-1 positive strains)	no.PI-1 positive strains/ no.susceptible isolates (%PI-1 positive strains)	OR (95%CI)	p	no.PI-2 positive strains/ no.resistant isolates (%PI-2 positive strains)	no.PI-2 positive strains/ no.susceptible isolates (%PI-2 positive strains)	OR (95%CI)	p
PEN	46/137 (33.6)	50/440(11.4)	3.94 [2.49-6.25]	$<10^{-4}$	2/137 (1.5)	197/440 (44.8)	0.02 [0.01-0.08]	$<10^{-4}$
ERY	29/125 (23.2)	67/452(14.8)	NS	NS	5/125 (3.2)	194/452 (42.9)	0.055 [0.02-0.14]	$<10^{-4}$
TET	21/100 (21.0)	75/477 (15.7)	NS	NS	4/100 (4.0)	195/477 (40.9)	0.06 [0.02-0.17]	$<10^{-4}$
CLO	3/27 (11.1)	93/550 (16.9)	NS	NS	0/27 (0.0)	199/550 (100.0)	0	10^{-4}
SXT	55/124 (44.3)	41/453 (9.05)	7.99 [4.95-12.89]	$<10^{-4}$	12/199 (6.03]	187/453 (41.3)	0.15 [0.08-0.28]	$<10^{-4}$
MDR	23/93 (24.7)	73/484 (15.1)	NS	NS	1/93 (1.1)	198/484 (40.9)	0.015 [0.00-0.11]	$<10^{-4}$

[a] PEN: penicillin; ERY: erythromycin; TET: tetracycline; CLO: chloramphenicol; SXT: Co-trimoxazole; MDR: Multi-drug resistant

In chapter 3 we showed that there was a close correspondence between PFGE cluster and ST. The relationships between sequence types have been explored by various methodologies, including goeBURST that was specially developed to analyze MLST data and is frequently used to define clonal complexes. A goeBURST analysis revealed that all isolates carrying the pilus islet 1 expressing serotypes 9V and 14, which represented 43.8% (42/96) of the total isolates positive for *rlrA*, were clonally related and represented clone Spain^{9V}-ST156 (31). Concerning the STs of the PI-2 positive strains no clonal relationships were identified by goeBURST when comparing the genetic lineages identified in the different serotypes (Table 5.7).

Another association between presence of the pilus islet and PMEN clones was observed in serotype 6B where all five isolates belonging to a genetic lineage represented by ST1224 and ST273, a single isolate in a different PFGE cluster, but also exhibiting ST273, and two other isolates in another cluster characterized by ST138 and ST176, were all positive for the presence of *rlrA* and are related to clone Greece^{6B}-ST273 (52). On the other hand, among serotype 14 isolates related to England^{14}-ST9 clone (PFGE cluster represented by ST9 and ST409), no isolates carrying the pilus islet could be found.

Characterization of PI negative isolates

Over 50% of the isolates were negative for the presence of any of the pili islets (n=303). To explore the possibility that the pili negative isolates still retained parts of the pathogenicity islets, a PCR was performed using a set of primers that flanked the whole islets (Figure 5.3 and 5.4). In the case of the PI-2 islet all *sip*A negative isolates yielded a PCR product compatible with the negative control, strain TIGR4 (Figure 5.4). Regarding the PI-1 islet, the majority of the *rlr*A negative isolates (86.9%, n=418) yielded a product compatible with strain R6 (Figure 5.3-A). The remaining 63 (13.1%) isolates revealed larger amplification products: 60 presented a product designated type C

(Figure 5.3–A, pfl region type C), while the remaining 3 presented a product designated type B (Figure 5.3–A, pfl region type B). In order to determine the genetic differences responsible for the heterogeneity in size of the PCR products, a representative isolate of types B and C was sequenced. The genetic makeup of the various regions is illustrated in Figure 5.3-A.

Both the type B and C regions contain DNA stretches similar to those found in the 3' region of the *rlrA* locus of strain TIGR4. These common DNA elements were found to be similar to the region found upstream of the *rlrA* locus of TIGR4 encoding the IS1167 transposase however, due to mutations and indels, these are no longer capable of producing a full-length functional protein. BLAST searches demonstrated a high number of similarly degraded copies of the IS1167 transposase distributed throughout the available complete genomes of strains of TIGR4, R6 and D39. There is a high identity between the IS1167 degraded transposase sequence found downstream of the *rlrA* locus in TIGR4 and the one found in the type B region (97% identity in the 1277 nt aligned, with only two single nucleotide gaps). The similarity extends to other coding and non-coding regions found between srtD and spr0470 of TIGR4 (Figure 5.3–A–*pfl* region B). This observation is compatible with the hypothesis that region type B was generated by the recent loss of the *rlrA* islet. Further supporting this notion is the observation that this region was found among the few isolates not carrying the pilus islet among large PFGE clusters of *rlrA* positive isolates (Table 5.5).

The DNA sequence corresponding to the degraded copy of the IS1167 transposase in region type C differed more extensively from the two discussed above, namely by a 50 nt insertion and a 16 nt deletion. A comparison of the type C region with the corresponding sequence of strain R6 revealed extensive similarity, including the presence of ORF CDS137 and a downstream repeat region found in strain R6 but absent from the pilus locus in strain TIGR4 (Figure 5.3-A-pfl region C). The type C region is therefore closely related to the one found in strain R6 with the exception of the presence of a degraded copy of the IS1167 transposase, suggesting that the region downstream of the *pfl* gene may constitute a hotspot for recombination events. The type C region was found associated with specific PFGE clusters expressing a limited number of serotypes, but this was not an exclusive association since isolates presenting regions type A and C were frequently grouped in the same PFGE cluster. For instance, the largest PFGE cluster in serotype 3 contains considerable numbers of isolates presenting each of the regions (Tables 5.5 and 5.6)

Table 5.5: Characteristics of the serotypes where the PI-1 islet was identified.

Serotype (No. of isolates)	PFGE cluster[a]	STs in PFGE cluster	% rlrA positive isolates (No. of isolates)	% of type of clade among rlrA positive (No. of isolates)			% of type of sequence among rlrA negative strains (No. of isolates)			% of isolates in PFGE cluster resistant to the antimicrobial (No. Isolates)			
										Penicillin		Erythromycin	
				I	II	III	A	B	C	All strains	rlrA positive strains	All strains	rlrA positive strains
4 (2)	A_2	ST1221	100(2)	0	0	100(2)	0	0	0	0	0	0	0
6B (20)	C_2	ST273+ST315	100(2)	50(1)	0	50(1)	0	0	0	50(1)	50(1)	100(2)	100(2)
	D_2	ST138+ST176	100(2)	0	100(2)	0	0	0	0	0	0	50(1)	50(1)
	F_5	ST273+ST224	100(5)	0	100(5)	0	0	0	0	20(1)	20(1)	80(4)	80(4)
	G_2	ST2016	0	0	0	0	100(2)	0	0	100(2)	0	0	0
	A_1; B_1; E_2; H_2; I_1;J_1;K_1	Other	78(7)	0	71(5)	29(2)	100(2)	0	0	33(3)	29(2)	67(6)	71(5)
14 (54)	C_{33}	ST143+ST156+ ST557+ST790+ ST4575+ST4576	100(33)	94(31)	6(2)	0	0	0	0	100(33)	100(33)	24(8)	24(8)
	D_{17}	ST9+ST409	0	0	0	0	88(15)	0	12(2)	6(1)	0	82(14)	0
	A_1; B_1; E_2	Other	25(1)	100(1)	0	0	100(3)	0	0	25(1)	100(1)	0	0
19F (20)	C_3	ST2307	0	0	0	0	100(3)	0	0	100(3)	0	100(3)	0
	E_6	ST179+ST390 + ST4245	83(5)	0	0	100(5)	100(1)	0	0	0	0	33.3(2)	40(2)
	A_3; B_1; D_1; F_2; G_1; H_1; J_1; K_1	Other	54(6)	50(3)	17(1)	33(2)	80(4)	0	20(1)	36(4)	33(2)	27(3)	16(1)
19A (100)	A_4	ST994	50(2)	100(2)	0	0	0	0	100(2)	25(1)	0	25(1)	0
	C_{12}	ST1151+ST2732	83(10)	100(10)	0	0	0	0	100(2)	0	0	0	0
	E_{21}	ST193+ST416	38(8)	38(3)	12(1)	50(4)	92(12)	8(1)	0	0	0	67(14)	33(3)
	I_{11}	ST81+ST199+ ST276+ST667	0	0	0	0	100(11)	0	0	45(5)	0	9(1)	0
	M_{33}	ST156+ST230+ST276 +ST2307+ ST4577	3(1)	100(1)	0	0	100(32)	0	0	97(32)	100(1)	94(31)	0
	B_1; D_4; F_1; G_2; H_1; J_2; K_2; L_4; N_2	Other	21(4)	75(3)	0	25(1)	40(6)	0	60(9)	5(1)	0	32(6)	25(1)
9V(10)	A_7	ST162+ST557+ ST1225	71(5)	100(5)	0	0	0	100(2)	0	57(4)	40(2)	14(1)	20(1)
	B_3	ST156+ ST2356	100(3)	100(3)	0	0	0	0	0	100(3)	100(3)	33(1)	33(1)
13(2)	A_1	ST923	0	0	0	0	100(1)	0	0	0	0	0	0
	B_1	ST4574	100(1)	0	0	100(1)	0	0	0	0	0	0	0

[a] PFGE clusters are represented by capital letters and a subscript number indicating the number of isolates included in the cluster.

Table 5.6: Characteristics of the serotypes not associated with PI-1 islet.

Serotype (No. of isolates)	PFGE cluster[a]	STs in PFGE cluster[b]	% of type of sequence among *rlrA* negative strains (No. of isolates)			% of isolates in PFGE cluster resistant to the antimicrobial (No. of isolates)	
			A	B	C	Penicillin	Erythromycin
1(125)	A_{109}	ST228+ST306+ ST4578+ST4579	94(103)	0	6(6)	0	1(1)
	C_{15}	ST304	20(3)	0	80(12)	0	13(2)
	B_1	ND	100(1)	0	0	0	0
3(28)	A_{26}	ST180+ST260+ST505+ ST1230+ST1646	61(17)	0	32(9)	0	0
	B_2	ST260	0	0	100(2)	0	0
5(10)	A_9	ST289+ST1223	100(9)	0	0	0	0
	B_1	ST30	100(1)	0	0	100(1)	100(1)
6A(13)	A_6	ST65+ST460+ST1648	100(6)	0	0	0	0
	B_5	ST1876+ST4248	80(4)	0	20(1)	0	0
	D_1; E_1	Other	100(2)	0	0	0	0
6C(2)	A_1	ST395	100(1)	0	0	0	0
	B_1	ST386	0	0	100(1)	100(1)	100(1)
7F(52)	A_{51}	ST191	100(51)	0	0	0	0
	B_1	ST191	100(1)	0	0	0	0
8(5)	A_4	ST53	75(3)	0	25(1)	0	0
	B_1	ND	100(1)	0	0	0	0
9A(1)	A_1	ST280	100(1)	0	0	0	0
9L(2)	A_2	ST1654	100(2)	0	0	0	0
9N(6)	A_2	ST66	100(2)	0	0	0	0
	B_3	ST66	100(3)	0	0	0	0
	C_1	ND	100(1)	0	0	0	0
10A(5)	A_5	ST97+ST585	100(5)	0	0	0	0
11A(4)	A_3	ST408	100(3)	0	0	0	33(1)
	B_1	ST2402	0	0	100(1)	0	0
12B(2)	A_1	ST989	100(1)	0	0	0	0
	B_1	ST218	100(1)	0	0	0	0
15A(4)	A_4	ST63+ST4208	100(4)	0	0	100(4)	100(4)
15B/C(8)	A_7	ST411	100(7)	0	0	0	29(2)
	B_1	ST1706	100(1)	0	0	100(1)	0
16F(2)	A_1	ST30	100(1)	0	0	0	0
	B_1	ST30	100(1)	0	0	0	0
17F(1)	A_1	ST392	100(1)	0	0	0	0
18C(13)	A_6	ST113+ST1233	100(6)	0	0	0	0
	B_3	ST1650	100(3)	0	0	0	0
	C_2; D_1; E_1	Other	50(2)	0	50(2)	0	0
19C(1)	A_1	ST1201	100(1)	0	0	0	0
20(5)	A_3	ST1026	33(1)	0	67(2)	33(1)	33(1)
	B_1	ST235	100(1)	0	0	0	0
	C_1	ST1026	100(1)	0	0	0	0
21(1)	A_1	ST432	100(1)	0	0	0	0
22F(7)	A_6	ST433+ST1012	83(5)	0	17(1)	0	0
	B_1	ST445	0	0	100(1)	0	0
23A(1)	A_1	ND	100(1)	0	0	0	0
23B(2)	A_2	ST439	100(2)	0	0	0	0
23F(37)	A_{28}	ST277+ST338+ST732	100(28)	0	0	82(23)	0
	C_2	ST1371	100(2)	0	0	50(1)	0
	E_3	ST81	100(3)	0	0	67(2)	33(1)
	B_2; D_2	Other	100(4)	0	0	50(2)	0(0)
24A(1)	A_1	ST4253	100(1)	0	0	100(1)	100(1)
24F(6)	A_2	ST72	100(2)	0	0	0	0
	B_1	ST72	100(1)	0	0	0	0
	C_3	ST230	100(3)	0	0	100(3)	100(3)
25A(5)	A_5	ST393	100(5)	0	0	0	0
29(1)	A_1	ST198	100(1)	0	0	0	0
31(1)	A_1	ND	100(1)	0	0	0	0
33A(6)	A_6	ST717	100(6)	0	0	0	100(6)
33F(6)	A_2	ST2633	0	0	100(2)	0	0
	B_4	ST717	100(4)	0	0	0	75(3)
34(1)	A_1	ST1778	0	0	100(1)	0	0
35C/42(1)	A_1	ST1955	100(1)	0	0	0	0
35F(3)	A_2	ST1368	100(2)	0	0	0	0
	A_1	ST446	0	0	100(1)	0	0
37(1)	A_1	ST447	100(1)	0	0	0	0

[a] PFGE clusters are represented by capital letters and a subscript number indicating the number of isolates included in the cluster; [b] ND- Not-determined

Table 5.7: Characteristics of the serotypes associated with PI-2 islet.

Serotype (No. of isolates)	PFGE cluster[a]	STs in PFGE cluster[b]	% of PI-2 positive isolates (No. of isolates)
1(125)	A_{109}	ST228+ST306+ ST4578+ST4579	100(109)
	C_{15}	ST304	100(15)
	B_1	ND	100(1)
7F(52)	A_{51}	ST191	100(51)
	B_1	ST191	100(1)
19A (100)	A_4	ST994	50(2)
	B_1	ND	100(1)
	C_{12}	ST1151+ST2732	83(10)
	D_4	ST193	25(1)
	E_{21}	ST193+ST416	10(4)
	I_{11}	ST81+ST199+ ST276 +ST667	0
	M_{33}	ST156+ST230+ST276 +ST2307+ ST4577	0
	F_1; G_2; H_1; J_2; K_2; L_4; N_2	Other	0
19F(20)	A_3	ND	33(1)
	B_1	ST4251	100(1)
	C_3	ST2307(1)	0
	E_6	ST179+ST390+ST4245	0
	F_2	ST476	50(1)
	H_1	ST347	100(1)
	D_1; G_1; I_1; J_1	Other	0

[a] PFGE clusters are represented by capital letters and a subscript number indicating the number of isolates included in the cluster; [b] ND- Not-determined

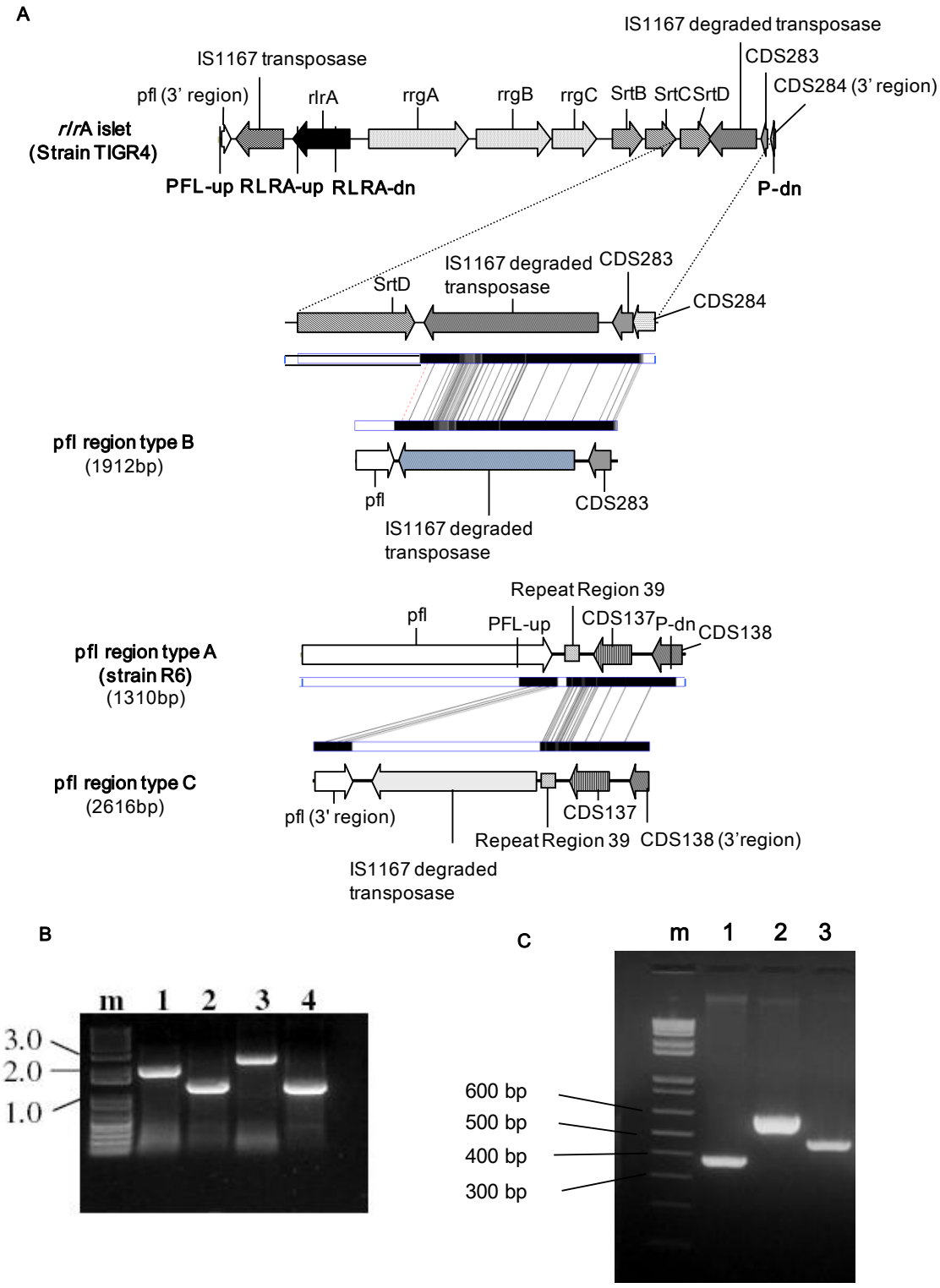

Figure 5.3 Genetic structure PI-1 found among piliated isolates and non-piliated isolates. Panel A: Structure of the region found downstream of the *pfl* gene in four isolates and designated region type B compared to the *rlrA* islet and flanking regions of strain TIGR4 and structure of the region found downstream of the *pfl* gene in 68 isolates and designated region type C compared to the corresponding region of strain R6 (pfl region type A). Genes are indicated by arrows and primers by vertical lines. Genes encoding proteins with similar functions are represented by the same pattern. The fragments with high DNA similarity between the regions compared are represented by black and grey boxes connected by lines (analysis

was performed using the GATA software). Panel B: Agarose gel of the PCR products resulting from the amplification of the region downstream of the pfl gene using primers PFL-up and P-dn. m- DNA ladder (1 Kb plus, Invitrogen, Carlsbab, CA). Lane 1- isolate presenting a region type B. Lane 2- isolate presenting a region type A. Lane 3- isolate presenting a region type C. Lane 4- strain R6. Panel C: Agarose gel of the PCR products for the clade types. m- DNA ladder (1 Kb plus, Invitrogen, Carlsbab, CA). Lane 1- isolate presenting clade type I. Lane 2- isolate presenting clade type II. Lane 3- isolate presenting clade type III.

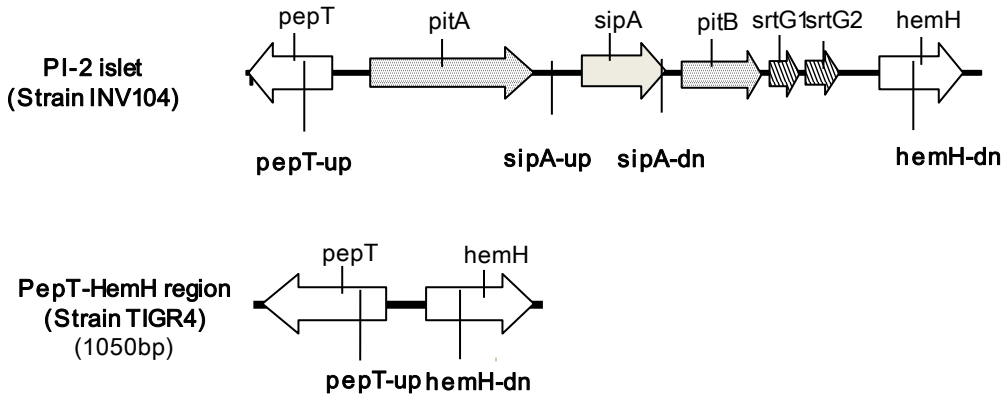

Figure 5.4: Genetic structure of PI-2 islet found among piliated isolates and non-piliated isolates. Structure of the region downstream the *pep*T gene in PI-2 positive (Strain INV104) and PI-2 negative (Strain TIGR4) strains.

DISCUSSION

The long-term efficacy of PCV7 is being challenged by the emergence of infections caused by replacement serotypes not targeted by the vaccine. The difficulty in producing higher valency vaccines and the realization that any polysaccharide based vaccine may be ultimately limited in its efficacy, due to the large number of pneumococcal capsular types, prompted the search for other vaccine targets. Recently, the three structural proteins constituting pneumococcal pili type 1 were shown to protect mice against invasive infections and were proposed as potential vaccine candidates (17). The data presented here argues that the efficacy of such a vaccine would be limited by the small proportion of isolates carrying the PI-1 (17%) and PI-2 (33%) islets and therefore potentially expressing pili, among those causing invasive infections in humans. Moreover, the strong association of the *rlrA* islet with four of the seven serotypes included in PCV7 and of PI-2 islet with serotypes 1 and 7F, both included in PCV10 and PCV13, would further limit the advantages of a pilus based vaccine over the currently available.

Recently, Basset and colleagues explored the distribution of the *rlrA* islet, using *rrgC* as a marker for the presence of the pilus locus (7). The population analyzed by Basset and colleagues differed markedly from the one analyzed here by including a large fraction of isolates associated with colonization. In addition, the distribution of serotypes and, most importantly, the genetic makeup of the pneumococcal population were quite different, with only 21 common STs out of the 236 STs identified overall in the two collections. In spite of these differences, a similar proportion of isolates carrying the pilus islet was found among invasive isolates by these authors (24%, n=123). Taken together the results of the two studies suggest that the PI-1 does not represent an essential virulence factor for invasive disease in humans, in contrast to previous indications from mouse models (6, 21). This suggestion receives further support from the observation that the same fraction of isolates associated with asymptomatic carriage were positive for the pilus islet type 1 (7).

A prior study emphasized the higher prevalence of the pilus islet among the serotypes included in the currently available conjugate vaccine versus all other serotypes (7). Here we expand these findings by identifying which serotypes are particularly enriched or depleted in the presence of the two pilus locus identified so far. Specifically, among the seven serotypes included in the conjugate vaccine, although most serotypes are indeed associated with the presence of the PI-1 locus (4, 6B, 9V and 14), serotype 23F was significantly depleted of the presence of this locus, indicating that an association with the presence of the PI-1 locus is not a property of all vaccine serotypes. Interestingly, while vaccine-serotype 14 was associated with the presence of the PI-1 in the present study, the islet was found in less than 10% of the isolates expressing this serotype among a collection of North American isolates (7). This discrepancy is possibly due to differences in

the genetic structure of this serotype in the two geographic localizations, as discussed below, and highlights the pitfalls of establishing an association between the presence of the pilus locus and serotype without consideration for the clonal structure of the bacterial population expressing a particular serotype.

We could also show an association between the presence of the pilus islet and resistance to several antimicrobials, especially in type 1 pilus (Table 5.3). Such an association was expected since the serotypes where the *rlrA* islet was found also concentrate the majority of resistant isolates. Recently, PI-1 was suggested to play an important role in the dissemination of penicillin non-susceptible isolates in Sweden (48). The clonal lineage implicated in Sweden was represented by ST156 that was also found to be significantly associated with the presence of the *rlrA* islet in our collection, independently of the serotype expressed by the isolates (serotype 9V or 14, Table 5.5).

An analysis of the genetic backgrounds of the isolates showed that the best predictor of the presence of the *rlrA* islet was the PFGE cluster, regardless of the serotype, indicating that this is a clonal property. Particularly interesting, is the fact that different lineages expressing the same serotype can be associated with the presence or absence of the locus. This may confound an analysis of association of the *rlrA* islet with serotype only, disregarding the clonal structure of the population. For instance, among the isolates expressing serotype 14, the dominant lineage characterized by ST156 and related STs was associated with the presence of the pilus locus in a serotype independent analysis (Table 5.5). However, in the second most prevalent lineage expressing serotype 14, characterized by ST15 and related STs, the locus is clearly absent (Table 5.5) A different clonal structure could thus explain the low prevalence (<10%) of the *rlrA* locus in serotype 14 in the report of Basset and colleagues (7) that is in sharp contrast with the high prevalence of the locus in this serotype found in our collection. Importantly, we also show that some genetic lineages are significantly depleted in isolates carrying the PI-1 locus and, since these were the dominant clones among their serotypes, a significant depletion at the serotype level was also evident in most cases (Table 5.6).

The existence of clones expressing the same serotype associated or depleted in the pilus islet raises the possibility that in other geographic areas with a different clonal structure, the association between particular serotypes and the presence or absence of the pilus locus may be different from the one reported here. In fact, a recent study from the USA, conducted by Regev-Yochay and colleagues, reported the re-emergence of type 1 pili after vaccine implementation. This increase was due to the dissemination of serotype 19A, mainly belonging to ST320 and ST695 clones, and serotype 35B (45). In Portugal, as observed in the previous chapters, neither serotype 19A ST320 or serotype 35B are frequently found in the invasive pneumococci recovered from children. Moreover we did not identify any emergence of new serotypes carrying the PI-1 islet. In

contrast, we observed a significant decline of PI-1 islet after PCV7 availability. These observations highlight the importance of locally circulating clones in pneumococcal infection.

A prior study had suggested a strong association between the PMEN clones and the presence of the *rlrA* islet (7). In the present collection, the Spain^{9V}-ST156 clone was found to be strongly associated with the presence of the type 1 islet similarly to what was found previously (7, 48), but other PMEN clones found in our collection were depleted of isolates positive for *rlrA* such as England^{14}-ST9, so that carriage of the *rlrA* locus is not a universal property of widely disseminated lineages.

Basset and colleagues discuss the limitations of using the detection of *rrgC* by PCR as a marker for the presence of the type 1 islet (7). One of the concerns is that this may have been a too broad criterion in that strains that carry the *rrgC* gene may lack other genes essential for pilus expression. Although our study suffers from the same limitation, the long-range PCR performed on a subset of the isolates positive for the *rlrA* gene yielded products whose sizes were compatible with the presence of the entire locus, in agreement with previous studies that indicated that the *rlrA* islet is either present or absent in its entirety (7, 48). However, the primary concern of Basset and colleagues was that theirs could have been a too stringent criterion, since strains not containing the *rrgC* gene or negative by PCR due to variability in this structural gene may still express pili. Our choice of using the presence of *rlrA* to define piliated isolates was designed to avoid these problems. RlrA is a regulatory protein and it is therefore expected to be more conserved than a structural protein. Moreover, the presence of this protein was shown to be essential for wild-type levels of expression of the pilus structural genes and associated sortases (22), indicating that isolates lacking this gene would also be impaired in pilus expression. Finally, the PCR performed using primers targeting conserved DNA regions flanking the *rlrA* locus conclusively showed that all strains lacking the *rlrA* gene also lacked all the sortases and structural genes necessary for pilus biosynthesis. Therefore, the 17% of invasive isolates containing the *rlrA* gene constitute the fraction genetically equipped to express this pilus, although only the availability of specific antibodies targeting the pilus could determine if this genetic potential was fully realized.

The study of the *rlrA* negative isolates revealed different genetic organizations of the region where the pilus islet is usually found, suggesting that this region may be a hotspot for DNA insertions and deletions. Transposons and IS elements have been shown to be a frequent cause of duplications and deletions in bacterial chromosomes through homologous recombination (54). Significantly, the sequence of the IS1167 transposase retained in the 3' region of the pilus locus is severely altered when comparing to the one in the 5' region. (Figure 5.3 - Panel A). This may hinder homologous recombination and stabilize the presence of the pilus locus. Notwithstanding, the distribution and genetic arrangement of region type B, with the presence of CDS283 that was exclusively associated with the pilus locus, suggests that it may have resulted from the recent loss

of the *rlrA* islet in some lineages, possibly through intra-chromosomal homologous recombination between the conserved 3' regions of the flanking IS1167 transposase encoding segments or through an error in excision mediated by a functional IS1167 transposase, such as the one putatively encoded in the 5' region of the locus. A recombination event would be expected to retain most of the 3' copy of the IS1167 transposase and the downstream region as was indeed verified (Figure 5.3 – Panel A). The DNA sequence of the type C region, that differs from the more common type A region found in strain R6 by the presence of a degraded IS1167 transposase encoding segment, offers limited clues as to its origin. However, the presence of an IS1167 sequence could further promote the acquisition of the pilus islet by providing a region of sequence similarity that could facilitate homologous recombination.

PI-2 islet was found in all serotype 1 and 7F strains and in a small number of serotype 19A and 19F strains. The reason underlying the differences in terms of prevalence could be related to the diversity of each individual serotypes. While serotype 1 and 7F are poorly diverse in terms of genetic lineages, normally presenting one or two PFGE clusters, serotypes 19A and 19F are usually defined by several unrelated clusters. Consequently these two later serotypes may accommodate diverse lineages with different proportions of piliated strains. On the other hand, the fact that all serotype 1 and 7F strains possess the PI-2 islet might indicate that this structure may be essential for the virulence of these two serotypes. Unfortunately, little is known about the importance of PI-2 for serotypes 1 and 7F, an issue that deserves further attention.

A recent study described the prevalence of PI-2 in a collection of invasive isolates recovered in Georgia, USA (55). In this study, PI-2 was found in 21% of the strains. Similarly to our study, serotypes 1, 7F, 11A, 19A and 19F were found to be associated with the PI-2 islet (55). The proportion of PI-2 positive strains was slightly higher in our study (33%) which is probably due to the differences in serotype prevalence between the two countries. In the study from the USA, serotype 19A and 7F were the two main serotypes of their collection, while serotype 1 was rarely isolated in the USA (55).

We also found significant differences in terms of the prevalence of PI-2 islet within each individual serotype. While in the study from the Zahner and colleagues, 75% of the 19F strains presented the PI-2 islet (55), in our study only 20% were PI-2 positive. Analysis of the clonal composition of each serotype indicates that these disparities may be explained by differences in the clonal lineages. Indeed, the major cluster found among PI-2 positive serotype 19A and 19F strains were composed of STs related to the CC320 which was not found in our collection. These differences reinforced the importance of the geographic associated genetic lineages which was mentioned in the two previous chapters.

Though PI-2 was more prevalent than PI-1 in our sudy, possibly due to the effect of the introduction of the PCV7, all piliated strains found in our collection presented one of the serotypes

included in the recently available PCV13. Though the possibility of these structures being acquired by non-vaccine serotypes exists we did not see significant changes, in terms of serotype associations, after PCV7 vaccine implementation. Nevertheless it is important to continuously monitor the distribution of pili structures among the pneumococcal population especially when a new selective force is about to alter so profoundly the serotype distribution of the pneumococcus normally found in carriage and in disease.

So far, none of the proteins proposed for new pneumococcal vaccines are considered appropriate to elicit species-wide pneumococcal protection. Therefore a combination of proteins should be considered in future protein vaccine strategies. Which combination of proteins should be chosen remains open for discussion.

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CHAPTER 6

Concluding remarks

Streptococcus pneumoniae (the pneumococcus) is the leading cause of respiratory tract infections, such as pneumonia and otitis media, being particularly prevalent in pediatric invasive infections, such as meningitis and bacteremia. Intensive surveillance of the pneumococcal infections has been performed in different geographic locations and these studies have shown that incidence of infection, serotype distribution, antimicrobial resistance and even genetic lineages vary geographically (6-8).

Ninety three different pneumococcal serotypes have been identified so far, and though apparently all of them are capable of causing infection, only a small fraction is usually associated with invasive disease in children. Whether the invasive potential is only dependent of the capsular polysaccharide is still not yet completely understood, but, since incidence of infection and serotype distribution varies geographically and over time, it may indicate the existence of other unknown and virulence associated factors (4, 5).

To overcome the burden of the pneumococcal infection, vaccines toward the capsular serotype have been and are being developed. In February 2000, the first pneumococcal conjugate vaccine (PCV7) became available for use in infants and young children. This vaccine was developed to cover the seven serotypes most commonly found in invasive infection and also associated with antimicrobial resistance in the United States (9). Consequently PCV7 held the promise of reducing not only invasive infection but also antimicrobial resistance. Vaccine efficacy studies performed following vaccine introduction reported a significant decline of the serotypes targeted by the vaccine not only in disease but also in colonization (3, 14). Moreover the effect of the vaccine was also observed in unvaccinated groups such as the adult population (1). Nevertheless, despite of the benefits of the PCV7 in the USA, the impact of this vaccine in other countries has been questioned due to differences in serotype distribution. Moreover, studies of pneumococcal colonization showed that after vaccine implementation, although the carriage of vaccine serotypes was reduced the available niche was rapidly occupied by non-vaccine serotypes.

In Portugal, studies of the epidemiology of *S. pneumoniae* recovered from both infection (13) and carriage (3, 10) are available but little was known about the invasive pneumococcal population in the post-vaccine period. So the aim of this study was to determine the impact of this vaccine in Portugal and monitor possible changes of invasive pneumococci recovered from children (<18 years) after vaccine availability.

Changes in pneumococcal serotypes

In Portugal PCV7 is not part of the National Vaccination Plan but is being recommended by the Portuguese Pediatric Society. Consequently, though there has been a gradual increase of vaccine uptake, the impact of PCV7 was not expected to be of the same magnitude of the one

observed in the USA. In fact, we observed a decrease of vaccine serotypes from 55.9% in 1999-2002 to 20% in 2003-2008 which contrasts with the 2% of vaccine reported in the USA after 6 years of vaccine use in children < 5 years (11).

Though there was a decrease of vaccine serotypes, we observed an increase of other serotypes not covered by PCV7. In this study the most frequent serotypes were, in decreasing order, serotypes 1, 19A, 7F and 14, whilst in the pre-vaccine era, in children < 6 years, the main serotypes found were 14, 23F, 1 and 6B (13), which means that PCV7 efficacy is being overcome by the increase of non-vaccine serotypes.

A decrease of penicillin non-susceptibility from 44% to 20% was observed after vaccine availability even though macrolide resistance remained constant in the two vaccine periods. The main serotype associated with antimicrobial resistance was the expanding serotype 19A. Yet, despite the decrease of PCV7 serotypes, a significant fraction of antimicrobial resistant strains (44%) still presented one of the PCV7 serotypes.

One of the major findings of this study was the association of particular serotypes with age groups. While serotype 1 was found to be prevalent in children ≥2 years and rarer in infants (<2 years), serotype 19A was the most prevalent capsular type in the <2 years group. Serotype associations with age have been described before, but the reasons underlying them are still poorly understood.

Recently, two other conjugate vaccines became available. These new vaccines have the potential to prevent significant IPD since they include what are now the main serotypes associated with infection. Nevertheless, even these higher valency vaccines still cover only a small fraction of the existent capsular types. Moreover, based on the findings regarding the impact of PCV7, new serotypes may once again occupy the available niche and cause infection. This will obviously depend on the ability of non-vaccine serotypes to cause infection.

Pneumococcal genetic lineages

Several studies have been performed regarding the clonal composition of the pneumococcal population and though the main serotypes associated with IPD in children more or less match in the different geographic regions, significant differences have been found in terms of genetic lineages. This indicates that the pneumococcal genetic evolution is geographically structured and may depend on local factors. Consequently it is extremely important to determine which clones are circulating within each region. The knowledge of the clonal lineages became more imperative with the availability of PCV7. Since pneumococci are natural transformable bacteria the possibility of capsular switch events was raised right after the vaccine became available. So it was crucial to

understand if the emerging non-vaccine serotypes shared the same genetic background of the previously established population.

The aim of chapter 3 was therefore to determine how the vaccine influenced the pneumococcal population clonal composition and to identify the genetic lineages associated with the emerging serotypes. The comparison of the genetic lineages found in this study with the data regarding the pre-vaccine period indicates that the majority of the clones identified, either in the emerging serotypes or in the vaccine serotypes, were already in circulation in the pre-vaccine period. Consequently the increase of the non-vaccine serotypes was basically due to the dissemination of previously existent clones. Moreover we detected very few cases of possible capsular switching events and none of them were associated with the emerging serotypes. Yet, it is important to refer that we also identified some clonal lineages in minor serotypes which were related to some important pre-vaccine clones. Whether this indicates that these serotypes may become prevalent in infection is still unknown but is definitely important to continuously supervise their evolution.

One of the main emerging resistant serotypes in our collection was serotype 19A and molecular analysis showed that this serotype was characterized by a variety of unrelated genetic lineages. Yet, we found one genetic lineage in particular that increased significantly in the post-vaccine period, the CC230 clone, which was characterized by being associated with penicillin non-susceptibility and macrolide resistance. The increase of serotype 19A in IPD was reported in several countries but the interesting feature resided on the fact that different lineages were expanding in different countries. For example while in the USA and Asia the increase of serotype 19A infections was associated with a clone represented by ST320 and ST199, in Portugal this lineage accounted for a small number of isolates.

Due to the increasingly importance of serotype 19A we performed a study which included a collection of invasive, non-invasive and carriage strains recovered between 2001 and 2006. The aim was to identify genetic lineages particularly associated with one of the populations. Overall, PFGE clusters included isolates from the three distinct sources but we identified some clusters with significant associations with one of the isolate sources. To be more precise, we found two clusters, one represented by ST1151 and the other represented by ST416, which were positively associated with carriage. On the other hand, PFGE cluster represented by ST193 was significantly more common among invasive disease. Despite these clonal associations with isolate source, the only clonal lineage identified to be increasing was the CC230 which was highly prevalent in all three collections. In other words, the clone found to be associated with the increase of serotype 19A was also found among non-invasive and carriage populations. The two studies described in chapters 3 and 4 clearly illustrate the importance of monitoring local genetic lineages in order to identify which clones are associated with the circulating serotypes.

The data presented in chapters 2 to 4 describe how the pneumococcal population adapted to the novel selective pressure of PCV7. The use of the conjugate vaccine has undoubtedly resulted in shifts in pneumococcal epidemiology, as evidenced by the recent emergence of invasive disease due to serotype 19A. These effects will in turn, require a reformulation of the vaccines in use. Two new vaccines, with higher valencies, have recently become available and it is possible that they will face the same problem of serotype replacement evidenced after PCV7 use. It is thus important to develop new vaccine strategies to prevent pneumococcal infection.

Protein vaccines: the pneumococcal pili

In chapter 5 the issue of new pneumococcal vaccines was addressed and some of the currently proposed pneumococcal proteins for a new vaccine are reviewed. The most recently suggested proteins are the pili components which were found only a few years ago at the pneumococcal surface. Pili-based proteins have been suggested for other pathogens and were already shown to induce protection. So far, two pilus-like structures have been identified at the surface of this bacterium, the product of PI-1 and PI-2. These structures are genetically encoded in different pathogenicity islets but little was known about its distribution within the pneumococcal population.

We presented a study which aimed to determine the distribution of the pili among the pneumococcal population recovered from invasive infection and its possible associations with serotypes and clones. Our data indicated that pili were serotype and clonally associated. Moreover, with the exception of a few serotype 19A and 19F strains, which presented the two pili locus, the PI-1 and PI-2 were distributed in different serotypes. PI-1 was mainly present in PCV7 serotypes, namely, 6B, 9V and 14, while PI-2 was found largely in serotypes 1 and 7F.

The pneumococcal population included in this study was obtained from a period which included the introduction of PCV7 (12, 13). Since the majority of the isolates positive for the *rlr*A locus expressed vaccine serotypes, a decrease of PI-1 positive strains due to vaccine introduction was observed, similar to the data already reported in the USA (2). However, because some serotypes that emerged due to vaccine pressure, such as 19A, included clones that carried the *rlrA* islet, and the due to diversity of the region associated with the pilus locus, it is possible that loss and acquisition of the islet may be ongoing. PI-2 islet was mainly found in serotypes 1 and 7F and slightly increased in the post-vaccine period which was possibly a consequence of the dissemination of these two serotypes after PCV7. Because the new vaccine PCV13 includes all serotypes in which PI-1 and PI-2 were detected, and taking into account the effect observed with PCV7, it is possible that after PCV13 introduction the prevalence of both pili will be affected. Consequently the potential use of the pili proteins in a new vaccine would offer limited benefits.

The present study revealed the characteristics of an invasive pneumococcal population recovered from pediatric infections after PCV7 availability in Portugal. Our study demonstrated that the pneumococcus has an extraordinary ability to adapt to stressful situations such as the introduction of a vaccine. We saw that even non-universal use of PCV7 led to a decrease of the serotypes covered by the vaccine which was rapidly overcome by the increase of non-vaccine types. Two new vaccines, PCV10 and PCV13 have recently become available and have the potential to prevent significant fractions of pediatric invasive infections, 55% for PCV10 and 83% for PCV13. However, NVTs now account for an important proportion of pediatric IPD in Portugal, particularly among children <2 years. What is the ability of non-vaccine serotypes to cause infection is unknown but understanding how pneumococci adapt would greatly facilitate the development of more efficient strategies to prevent IPD.

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