Ricardo Pedro Moreira Dias

Genetic diversity and epidemiology of antimicrobial resistant *Streptococcus pneumoniae* isolates

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Genetic diversity and epidemiology of antimicrobial resistant *Streptococcus pneumoniae* isolates

Dissertation presented to obtain a Ph.D. degree in Biology, speciality in Microbiology, by Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia.

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SUMMARY OF THESIS

Infection with *Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide. The main purpose of the studies in this thesis was to clarify the spread of antimicrobial resistant *S. pneumoniae* isolates recovered in Portugal.

First we analysed antimicrobial resistance rates of invasive *S. pneumoniae* isolated from the Portuguese population. An increasing trend of *S. pneumoniae* isolates nonsusceptible to penicillin was observed until 2000. In the following years, the decline in β-lactam nonsusceptibility was thought to be due to the introduction of the heptavalent pneumococcal conjugate vaccine (PCV-7) in Portugal in 2001.

To shed some light on the impact of the introduction of the PCV-7 on the susceptibility of invasive *S. pneumoniae* to antimicrobial agents, we evaluated the association between IPD, serotypes and antimicrobial susceptibility in the paediatric population between 1999 and 2004. Our results suggest an increase in multidrug resistance after 2002, caused by a replacement of vaccine serotypes by nonvaccine serotypes among nonsusceptible isolates after the introduction of PCV-7.

To provide useful information about the main factors involved in the selection of penicillin- and erythromycin-nonsusceptible invasive isolates, we evaluated the role of antimicrobial and vaccine use. The results obtained suggest that the use of macrolides was the main factor associated with an increase of penicillin- and erythromycin-nonsusceptible isolates. Furthermore, the heptavalent vaccine is failing to reduce antimicrobial resistance, as was expected, possibly because of the increased consumption of azithromycin.

To a better understand of the emergence of erythromycin-nonsusceptible invasive *S. pneumoniae* isolates, we studied their genetic structure. We noted that the prevalence of macrolide-nonsusceptible *S. pneumoniae* isolates has increased in Portugal, from two routes: i) expansion of pre-existing nonsusceptible clones, and ii) importation of nonsusceptible clones mainly present in other European countries and their local diversification.

Our results suggested that, in the Portuguese penicillin-nonsusceptible *S. pneumoniae* isolates that are closely related to the major Spanish clones, Spain^{9V}-3 and Spain^{23F}-1, the penicillin nonsusceptibility had spread by horizontal transfer of the *pbp1A*, *penA* and *pbpX* genes. We found higher divergence among the amino acid sequences of PBP2B, PBP2X and PBP1A from a collection of penicillin-intermediate invasive isolates than among those from highly resistant isolates.

Finally, we demonstrate that serine/threonine kinase StkP of *S. pneumoniae* is involved in the bacterial response to penicillin and suggests that the activity of StkP allows the bacteria to bypass cell wall injury due to penicillin, while being independent from the *pbp* genes.

RESUMO DA TESE

Infecções por *Streptococcus pneumoniae* são uma importante causa de morbilidade e mortalidade a nível mundial. Os estudos incluídos na presente tese teveram como principal objectivo clarificar a propagação da resistência aos antibióticos de isolados de *S. pneumoniae* em Portugal.

Inicialmente foi estudada a resistência aos antibióticos em *S. pneumoniae* invasivos, tendo sido observado que, até ao ano 2000, houve um aumento crescente de isolados não susceptíveis à penicilina. Foi verificado existir uma diminuição de isolados não susceptíveis aos antibióticos beta-lactâmicos, em anos posteriores, sugerindo estar a mesma associada à introdução da vacina anti-pneumocócica conjugada (PCV-7), em Portugal, em 2001.

Para uma melhor compreensão sobre o impacto da introdução da PCV-7 na susceptibilidade de *S. pneumoniae* invasivos aos agentes antimicrobianos, foi avaliada a associação entre doença pneumocócica invasiva, serótipos e susceptibilidade aos antibióticos relacionados com isolados colectados na população pediátrica entre 1999 e 2004. Os resultados obtidos sugerem um aumento de isolados multirresistentes após 2002, devido à substituição dos serótipos vacinais por serótipos não-vacinais em isolados com resistência aos antibióticos, após a introdução da vacina.

Para conhecer os principais factores envolvidos na selecção de isolados invasivos não susceptíveis à penicilina e eritromicina, foi avaliado o contributo do consumo de antibióticos e da utilização de vacina pneumocócica. Os resultados obtidos sugerem uma associação entre o consumo de macrólidos, sendo este o principal factor, e o aumento de isolados com resistência à penicilina e eritromicina; indicam ainda que a vacina heptavalente não tem diminuido a resistência aos antibióticos, como esperado, possivelmente devido ao aumento do consumo de azitromicina.

Para esclarecer sobre a emergência de isolados de *S. pneumoniae* invasivos não susceptíveis à eritromicina a estrutura genética destes foi estudada. Foi assinalado um aumento da prevalência de *S. pneumoniae* não susceptíveis aos macrólidos em Portugal devida à expansão de clones não susceptíveis aos macrólidos, quer pré-existentes quer importados principalmente de outros países europeus, quer ainda devido a diversificação destes últimos.

Os resultados apresentados demonstram que, entre *S. pneumoniae* isolados em Portugal não susceptíveis à penicilina, relacionados com os clones espanhóis Spain^{9V}-3 e Spain^{23F}-1, a resistência à penicilina se disseminou por transferência horizontal dos genes *pbp1A*, *penA* e *pbpX*. A divergência assinalada nas sequências aminoacídicas de PBP2B, PBP2X e PBP1A, relativas a uma colecção de isolados invasivos, era superior nos isolados

que expressavam susceptibilidade intermédia à penicilina do que nos isolados com alta resistência.

Finalmente, foi demonstrado que a serina/treonina cinase StkP, presente no *S. pneumoniae*, se encontra envolvida na resposta da bactéria à penicilina, sugerindo que a actividade de StkP lhe permite contornar as alterações na parede bcteriana causadas pela penicilina independentemente da acção dos genes *pbp*.

THESIS OUTLINE

Infection with *Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide. Recently, the Strategic Advisory Group of Experts from WHO ranked the prevention of pneumococcal disease as “very high priority”, on a global scale. The spread of antimicrobial- and multidrug-resistant pneumococci has become a worldwide problem in the last decades, difficulting the management of pneumococcal disease. The main purpose of the studies presented in this Thesis is to shed some light regarding the spread of antimicrobial resistant *S. pneumoniae* isolates in Portugal, where we can observe a complex context of high usage of antimicrobial agents and where a vaccine against pneumococcal disease has been recently introduced.

The order of presentation of the chapters in this Thesis dissertation does not necessarily reflect a chronological order, since some of the studies described below were done simultaneously and the results obtained during one particular study influenced the progress of other studies. Furthermore, the time it takes from the submission of an article to its publication largely depends on the journal, and on possibly necessary revisions.

Chapter I comprises a general overview about the current knowledge of *S. pneumoniae.* In this chapter, special attention was given to the adaptive power of *S. pneumoniae*, the mode of action of β-lactams and macrolides, their targets, and strategies developed by the bacteria to acquire resistance to these antimicrobial agents. A review concerning the epidemiology of antimicrobial resistance in *S. pneumoniae,* and current prophylactic strategies to prevent the disease caused by this pathogen, are also addressed. The issues focused on this chapter were put into context to emphasize the relevance of the studies presented.

In **Chapters II to IV**, we characterized the antimicrobial susceptibility of the *S. pneumoniae* isolates recovered from cases of invasive disease in several regions of Portugal between 1994 and 2004, and analyzed the impact of the introduction of the heptavalent pneumococcal conjugate vaccine (PCV-7) and the consumption of antimicrobial agents on the trends of antimicrobial susceptibility in this pathogen.

In Chapter II, we characterized the susceptibility to several classes of antimicrobial agents of *S. pneumoniae* isolates over an 11-year period (1994 to 2004).

Chapter III studies the impact of PCV-7 on the antimicrobial nonsusceptibility rates of pneumococci recovered from the paediatric population. The rates of invasive pneumococcal disease, serotype distribution and antimicrobial susceptibility, prior to and after the introduction of the heptavalent pneumococcal conjugate vaccine in Portuguese children, were evaluated.

In Chapter IV, we begin by evaluating the consequences of antimicrobial and vaccine use in the resistance to penicillin and erythromycin among the *S. pneumoniae* isolates in Portugal, by using linear regression models. This study allowed us to understand the relation between the use of antimicrobial agents, as well as their relation with the efficiency of PVC-7 in reducing nonsusceptibility rates in the Portuguese population.

Chapters V and VI describe the characterization of invasive macrolide-nonsusceptible *S. pneumoniae* clones from a collection of isolates recovered from invasive disease in Portugal using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

In Chapter V, the phenotype of invasive *S. pneumoniae* strains isolated between 1999 and the first 6 months of 2002 were characterized using serotyping and antimicrobial susceptibility testing. The phylogenetic relatedness of erythromycin-nonsusceptible strains from serotype 14, the largest contributor to the emergence of resistance to macrolides, was assessed by applying the PFGE and MLST techniques. The correlation between antimicrobial consumption of macrolides and the rates of nonsusceptibility was also evaluated.

Chapter VI reported the genetic structure of a population of macrolide-nonsusceptible *S. pneumoniae* strains recovered from cases of invasive disease between 1994 and 2004 by PFGE and MLST. Subsequent comparison of our MLST results with those from isolates of other countries allowed us to observe the genetic relatedness of the Portuguese isolates in a worldwide context, as well as to identify the geographic dissemination of macrolide nonsusceptibility. Moreover, we noted the main routes responsible for the increased prevalence of macrolide nonsusceptibility in Portuguese pneumococci.

Chapters VII to IX characterized the mechanism of spread of penicillin nonsusceptibility in Portuguese clinical isolates as well the genetic diversity of *pbp* genes among those isolates. Furthermore, the role of StkP in penicillin susceptibility was evaluated.

In Chapter VII, we studied the genetic relatedness of Portuguese penicillin-nonsusceptible 9V and 23F *S. pneumoniae* of clinical origin, using PFGE and Restriction Fragment Length Polymorphism (RFLP) analysis of the *pbp1A*, *penA* and *pbpX* genes, to evaluate the spread in Portugal of the genes encoding penicillin resistance between strains closely related to the international Spain^{9V}-3 and Spain^{23F}-1 clones.

Chapter VIII explores the genetic diversity of the *penA, pbpX* and *pbp1A* genes of clinical penicillin-intermediate and -resistant *S. pneumoniae* strains collected in Portugal.

In Chapter IX, we assess the role of the serine/threonine kinase StkP of *S. pneumoniae* in susceptibility to β-lactams by mutational analysis, and it's relation with the *pbp* genes. Furthermore, this study analysed the genetic conservation of StkP among clinical isolates and their relation with the MIC values of those strains.

Finally, in **Chapter X**, a general discussion is presented, where we highlight and discuss the major findings of this Thesis. New directions of investigation are also proposed, including projections on how to proceed in following up the observations produced in this Thesis' work.

Chapters II to IX may be read separately. They transcribe the contents of the following publications:

Chapter II - Dias R, Louro D & Caniça M (2006). *Antimicrob Agents Chemother* **50:** 2098-2105.

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Chapter IX - Dias R, Felix D, Caniça M & Trombe MC (2008) (unpublished).

LIST OF ABBREVIATIONS

Ala, alanine

ANOVA, analysis of variance

Arg, arginine

ARSIP, Antimicrobial Resistance Surveillance Program in Portugal

Asn, asparagine

Asp, aspartic acid

ARU, Antibiotic Resistance Unit

ATC, Anatomic Therapeutic Chemical

ATCC, American Type Culture Collection

CC, clonal complex

CLSI, Clinical and Laboratory Standards Institute

CSP, competence-stimulating peptide

Cys, cysteine

d, estimate of the mean evolutionary diversity

DCC, day care centres

DDD, defined daily doses

DID, defined daily doses units per 1,000 inhabitants daily

DLV, double-locus variant

DNA, deoxyribonucleic acid

EARSS, European Antimicrobial Resistance Surveillance System

Glu, glutamic acid

Gln, glutamine

Gly, glycine

His, histidine

HIV, human immunodeficiency virus

Ile, isoleucine

IPD, invasive pneumococcal disease

Leu, leucine

Lys, lysine

M, resistance phenotype to macrolides

MDR, multidrug-resistance

MEGA, macrolide efflux genetic assembly

Met, methionine

MIC, minimal inhibitory concentration

ML, resistance phenotype to macrolide and lincosamide

MLS_B, resistance phenotype to macrolides, lincosamides, and streptogramin B

MLST, Multilocus Sequence Typing

MS_B, resistance phenotype to macrolide and streptogramin B

NCCLS, National Committee for Clinical Laboratory Standards

NIH, National Institute of Health

NT, non-typeable

PASTA, penicillin-binding protein and serine/threonine kinase-associated domain

PBP, penicillin-binding protein

PCR, Polymerase Chain Reaction

PCV-7, heptavalent pneumococcal conjugate vaccine

PFGE, Pulsed-Field Gel Electrophoresis

Phe, phenylalanine

PMEN, Pneumococcal Molecular Epidemiology Network

Pro, proline

RFLP, Restriction Fragment Length Polymorphism

RNA, ribonucleic acid

rRNA, ribossomic ribonucleic acid

S_D, Dice band-based similarity coefficient

Ser, serine

SLV, single-locus variant

ST, sequence type

StkP, pneumococcal serine-threonine kinase

Thr, threonine

tRNA, transfer ribonucleic acid

TSB, triptycase soy broth

Tyr, tyrosine

Val, valine

WHO, World Health Organization

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CHAPTER I

General introduction

1.1 - A brief history of the *Streptococcus pneumoniae* in biomedical research

The pneumococcus was one of the first pathogenic bacteria to be identified. This bacterium was isolated for the first time in 1881 by Pasteur and Sternberg (Pasteur & Chamberland, 1881; Sternberg, 1881). Initially it was named "Microbe septicemique du salive" by Pasteur and *Micrococcus pasteuri* by Sternberg (Pasteur & Chamberland, 1881; Sternberg, 1885). In 1886 this organism was being referred to as Pneumococcus by Fraenkel due to its tendency to cause pulmonary infections (Watson *et al.*, 1993). This organism had been associated to human lobar pneumonia since the early 1880s (Watson *et al.*, 1993); also in the same decade, the pneumococcus was clearly demonstrated to be a cause of meningitis (Netter, 1887). In the early 1900s, it was renamed to *Diplococcus pneumoniae* (Winslow *et al.*, 1920). However, only in 1974 was it given its present name, *Streptococcus pneumoniae*, based in its characteristic growth as chains of cocci in liquid media (Deibel & Seeley, 1974).

Pneumococcus was used since their description as object of many investigations that led to important scientific discoveries. One of the most relevant, was the "transforming principle", described by Griffith (1928). He observed that when heat-killed encapsulated pneumococci and live strains lacking a capsule were concomitantly injected into mice, the nonencapsulated could be changed into encapsulated pneumococci with the same capsular type as the heat killed strain (Griffith, 1928). Two decades later, in 1944, the cause of this genetic transformation was shown by Avery *et al*. (1944) to be DNA.

Since then, research in the fields of genetics, microbial physiology and resistance to antibiotics increased outstandingly our knowledge about this pathogen. Even so, nowadays pneumococcus is one of the leading causes of death, mainly in developing countries.

1.2 - Public health impact

Infection with pneumococcus is a major cause of morbidity and mortality worldwide. Diseases caused by *S. pneumoniae* are a major global public health problem. Pneumonia, pneumonia with empyema and/or bacteraemia, febrile bacteraemia and meningitis constitute the commonest manifestations of invasive pneumococcal disease (IPD) (Tuomanen, 1999). Middle-ear infections, sinusitis and bronchitis represent non-invasive and less severe manifestations of pneumococcal infection, but they are considerably more common.

Children aged <2 years and elderly people carry the major burden of the disease. In Europe and the United States, *S. pneumoniae* is the most common cause of community-acquired bacterial pneumonia in adults. In these regions, the annual incidence of IPD ranges from 10 to 100 cases per 100 000 inhabitants (Hausdorff *et al.*, 2001; Reinert, 2004). Deaths from pneumococcal disease occur primarily among elderly people, in whom bacteraemic pneumonia is associated with case–fatality rates of 10–20% and pneumococcal bacteraemia with a rate of up to 60% (World Health Organization, 2007a).

According to recent estimates from the World Health Organization (WHO), 1.6 million deaths are caused by this agent annually all over the world; this estimate includes deaths of 0.7-1 million children aged <5 years. In developing countries, most of these deaths occur in children aged <2 years mainly due to non-bacteraemic pneumonia. Also in developing countries the magnitude of the burden of pneumococcal disease among elderly people is undefined at the present (World Health Organization, 2007a). In those countries, the incidence of IPD in children aged <5 years is several times higher than it is in industrialized countries (Bogaert *et al.*, 2004a; World Health Organization, 2007a). Recently, the Strategic Advisory Group of Experts from the WHO ranked the prevention of pneumococcal disease as "very high priority", on a global scale (World Health Organization, 2008).

Healthy children and adults carry *S. pneumoniae* in the respiratory tract. It was observed that 70% of children attending day care centres (DCC) in Portugal are colonized with pneumococcus in the upper respiratory tract (Frazão *et al.*, 2005), which represent higher values than the observed in the DCC in United States (20 - 40%) (Bogaert *et al.*, 2004a). Pneumococci are transmitted by direct contact with respiratory secretions from patients and healthy carriers. Transient nasopharyngeal colonization is the normal outcome of exposure to pneumococci. Rarely, pneumococcal invasive infections are primary infections. Indeed, several factors predispose the colonized host to infection such as damage of the respiratory tract, fatigue, malfunctioning of the immune system, viral diseases, indoor pollutants, tobacco smoke, premature weaning, attending day care centres (Gray & Dillon, 1989; Madhi & Klugman, 2004; McCullers, 2006). Groups at high-risk of pneumococcal infection include elderly people, infants (especially under the age of 2), patients with chronic

medical conditions such as immunosuppressive conditions [e.g., congenital immunodeficiency, human immunodeficiency virus (HIV) infection, leukemia, lymphoma, multiple myeloma, Hodgkins disease, or generalized malignancy], organ or bone marrow transplantation, chronic renal failure or nephrotic syndrome, functional or anatomic asplenia, (e.g., sickle-cell disease), chronic cardiovascular diseases (e.g., congestive heart failure or cardiomyopathy), chronic pulmonary diseases (e.g., chronic obstructive pulmonary disease or emphysema) and chronic liver diseases (e.g., cirrhosis) (Aavitsland *et al.*, 1994; Bogaert *et al.*, 2004a; Centers for Disease Control and Prevention, 1997; Neto *et al.*, 2003; World Health Organization, 2007a). Therapies with alkylating agents, antimetabolites, or systemic corticosteroids, and nutritional deficiencies are also known to be risk factors for pneumococcal infections (Ball, 1999).

1.3 - Adaptive power of pneumococcus

1.3.1 - Recombination

Natural transformation is a widespread mechanism for genetic exchange in bacteria; it has repercussions in the evolution of the genetic structure of the chromosome of pneumococci and also to their population structure.

Genetic plasticity plays a central role in the biology of the human pathogen *S. pneumoniae*. This is illustrated by the existence of at least 90 different capsular types, as well as by the rapid emergence of penicillin-nonsusceptible pneumococcal isolates. Natural genetic transformation is believed to be essential for this genetic plasticity; capsular types can be switched by intraspecies transformation, whereas interspecies transformation is responsible for the appearance, in the penicillin-nonsusceptible isolates, of mosaic *pbp* genes, which encode proteins with reduced affinity for penicillin. Association between transformation and changes in environmental conditions are related to the adaptative "strategy" deduced for *S. pneumoniae* (Claverys *et al.*, 2000).

1.3.2 - Natural transformation in *S. pneumoniae*

The term "natural genetic transformation" has been used to distinguish it from other *in vitro* procedures used to introduce DNA molecules into bacterial cells (Lorenz & Wackernagel, 1994).

The molecular explanation for the bacterial transformation obtained by Fred Griffith was later provided by Avery *et al.* (1944); they purified the active fraction of cell-free extracts and showed it to consist of DNA.

Competence for transformation in streptococci is not constitutive, as it is in *Neisseria* spp., but is regulated by a quorum-sensing system encoded by two genetic loci, *comCDE* and *comAB* (Lee & Morrison, 1999; Whatmore *et al.*, 1999). The *com* operon contains three genes *comC*, *comD* and *comE*, encoding a competence-stimulating peptide (CSP), a histidine kinase, and a response regulator, respectively (Lee & Morrison, 1999; Whatmore *et al.*, 1999). Two genes located elsewhere on the chromosome, *comA* and *comB*, code for proteins responsible for the export of CSP from the cell (Whatmore *et al.*, 1999). CSP induces competence when a critical extracellular concentration is reached. The *comD*-encoded transmembrane histidine kinase is believed to be a receptor for CSP and to phosphorylate a *comE*-encoded transcription regulator, producing an active form that up regulates both the *comCDE* operon and a number of other genes involved in competence development (Lee & Morrison, 1999; Whatmore *et al.*, 1999).

1.3.3 - Common factors affecting *S. pneumoniae* natural transformation

Tomasz (1965) demonstrated that cell density was inverse to the function of frequency of competence in a growing culture. Several decades later, competence had been demonstrated in *S. pneumoniae* at cell densities ranging from $10^6 – 10^8$ C.F.U. (Pearce *et al.*, 1995). It has been suggested that in the nasopharynx this could be important, because if the cell density critical for competence is close to that which triggers host defenses, competence could then serve as an adaptation of *S. pneumoniae* to host defense-generated stress (Claverys *et al.*, 2000). There seem to be several factors affecting development of competence, such as environmental and cellular factors, which also appear to vary from strain to strain.

Competence seems to be optimal at a slightly basic pH (7.4 to 8.0) and no competence was detected at pH less than 7.0, the reason being that the CSP is more active at alkaline than neutral pH values (Lorenz & Wackernagel, 1994).

It has been suggested that induction of competence is a *S. pneumoniae* response to stress produced by high concentrations of calcium (1 mM), which may prevail in the body fluids. However, calcium is also essential for growth and autolysis of pneumococcus (Lorenz & Wackernagel, 1994; Lunsford, 1998).

It was established by Tomasz (1965) that serum albumin was required for *S. pneumoniae* natural transformation. It has been suggested that albumin may function to stabilize or limit the absorption of certain competence factors to *in vitro* surfaces (Lunsford, 1998). Also oxygen availability is a major determinant for competence development in exponentially growing cultures of *S. pneumoniae* (Chapuy-Regaud *et al.*, 2001).

The presence of the polysaccharide capsule abolishes competence for natural genetic transformation (Lorenz & Wackernagel, 1994). Several surveys conducted with a collection of clinical virulent encapsulated *S. pneumoniae* isolates showed them all to be nontransformable (Lorenz & Wackernagel, 1994; Pozzi *et al.*, 1996). However, some of these isolates developed competence when induced by exogenously adding the competence factor of highly transformable laboratory strains. The presence of the capsule may constitute a physical barrier for the excretion and penetration of the produced competence factor. Several other reasons have been suggested for nontransformability of natural isolates: the presence of a defect in the production or excretion of the competence factor in some strains, and a defect in the DNA-processing machinery in strains which were nontransformable even with the exogenously added competence factor (Lorenz & Wackernagel, 1994).

1.3.4 - Genome

At the present time, 11 strains of *S. pneumoniae* have been described as finished or undergoing genome sequencing according to the NCBI genome database. The first genome to be published was from the TIGR4 strain in 2001 (Tettelin *et al.*, 2001) (Table 1). Also there are other publicly available genomes (Dopazo *et al.*, 2001; Hoskins *et al.*, 2001; Lanie *et al.*, 2007), namely: a 19F strain sequenced by Glaxo-SmithKline and strain R6 by Eli Lilly. Strain R6 is a descendant of the type 2 capsule strain D39 used by Avery to demonstrate the genetic function of DNA, and it is used worldwide as a standard laboratory strain (Lanie *et al.*, 2007).

The genome of the R6 strain was reported to be 2,038,615 base pairs long and have a 40% G + C content. It contains 2,043 predicted protein coding regions and 73 noncoding RNA genes (Hoskins *et al.*, 2001). As a consequence of the capacity of *S. pneumoniae* to take up DNA, its genome is littered with genes that are apparently derived from other bacteria. There are 40 ORFs that are similar to genes in Gram-negative bacteria and that have not been found in other Gram-positive genome sequences (Hoskins *et al.*, 2001). This is not surprising, because *S. pneumoniae* occupies the same niche in the human respiratory system as several Gram-negative species such as *Haemophilus influenzae* and *Branhamella catarrhalis*. Additionally, at least 2% of the genes were found to be significantly truncated relative to orthologous genes characterized in other bacteria. Transporter-encoding genes are the most frequently truncated and it was shown that there are five ORFs that are similar to genes encoding drug efflux pumps (Hoskins *et al.*, 2001).

Table 1.1 - Summary of the first three available *S. pneumoniae* genome data

Strain	ATCC Number	Sequencing Company	Genome Size (bp)	ORFs	Reference
R6	BAA-255	Eli Lilly	2,038,615	2,043	Hoskins et al., 2001
TIGR4	BAA-334	TIGR	2,160,837	2,236	Tettelin et al., 2001
G45	-	Geneva Biomedical Research Institute and Glaxo SmithKline	2,074,072	2,047	Dopazo et al., 2001

The virulent isolate sequenced by TIGR was shown to have 2,160,837 base pairs with G + C content of 39.7%. It contains 2236 predicated coding regions; of these, 1140 (64%) were assigned a biological role. Similarly to the R6 strain, a significant percentage of aproximately 5% of the genome is composed of insertion sequences that may contribute to genome rearrangements through uptake of foreign DNA (Tettelin *et al.*, 2001). Glaxo-

SmithKline presented the sequence and functional annotations for the 2.1 Mbp of a 19F strain which was shown to have 2,046 open reading frames (Dopazo *et al.*, 2001).

1.3.5 - Genetic plasticity

The sequences of the genome of eight nasopharyngeal strains of *S. pneumoniae* isolated from paediatric patients with upper respiratory symptoms plus nine publicly available pneumococcal strains revealed, by quantitative genomic analysis that 46% of the gene clusters were conserved among all 17 strains (NCBI). The majority (54%) of the gene clusters were not found in all strains suggesting that each strain's genome contained 21-32 % of non-core genes. These extensive genic diversity data support the distributed genome hypothesis, which states that pathogenic bacteria possess a supragenome that is much larger than the genome of any single bacterium, and that these pathogens utilize genetic recombination and a large non-core set of genes as a means of generating diversity. The presence of the supragenome for *Streptococcus agalactiae*, *H. influenzae* and *Staphylococcus aureus* has also been demonstrated; it appears that the possession of a distributed genome is a common host-interaction strategy (Hiller *et al.*, 2007; Holden *et al.*, 2004). The differential distribution of large mobile elements carrying virulence and drug-resistance determinants may be responsible for the clinically important phenotypic differences in these strains (Holden *et al.*, 2004).

Recent work has shown that among sets of prokaryotic genomes in which most homologous genes show extremely low sequence divergence, gene content can vary enormously, implying that those genes that are variably present or absent are frequently horizontally transferred (Gogarten & Townsend, 2005). According to Nakamura *et al.* (2004) an average of 14% of open reading frames in 116 prokaryotic genomes were subjected to recent horizontal transfer, ranging from 0.5% to 25%. Based on the analysis of the *S. pneumoniae* TIGR4 strain, the authors predicted that 17.9% of the 2.066 open reading frames in this strain suffered recently horizontal transfer. The differences in the proportions are possibly due to the evolutionary processes of these species. Except for mobile element genes, the analyses of the biological functions of horizontally transferred genes belonged to three categories: cell surface, DNA binding, and pathogenicity related functions. Many genes belonging to the "cell envelope" category were classified under "surface structure" or "biosynthesis and degradation of surface polysaccharides and lipopolysaccharides". Cell surface genes may also be involved in the pathogenicity-related functions, because cell surface genes might have contributed to defense against immunological responses from infected hosts; in addition, some horizontally transferred genes in the "surface structures" subgroup, related to pilus structure, might be involved in virulence, as they enable microbes

to attach to the host cells. Thus, the transferability of genes seems to depend heavily on their functions.

This has consequences at several levels, allowing for the pneumococci to have an adaptive response to the host's environment, the microbial composition of their ecosystem, as well as to selective pressure such as the use of antimicrobial agents.

1.3.6 - Population structure

Traditionally, it is assumed that successful horizontal gene transfer provides a selective advantage to either the bacteria or the gene itself, but Frasier *et al.* (2005) has suggested that horizontally transferred genes obtained through genetic transformation can be neutral or nearly neutral. It was shown for three important human pathogens, *S. pneumoniae*, *Neisseria meningitidis*, and *S. aureus*, that the evolution of their genetic structure can be explained by using an evolutionary model based on neutral mutational drift, caused by mutation and localized recombination. This drift is influenced by epidemic transmission in local populations as well as by molecular processes (Fraser *et al.*, 2005). Due to the natural transformation of pneumococci, like in other natural recombinant species, it was suggested that the boundaries between streptococcal species are fuzzy and, therefore, the principles of population genetics must be broadened, and applied to higher taxonomic categories. (Gogarten & Townsend, 2005; Fraser *et al.*, 2007).

The population structures of bacterial species are complex and often controversial. To a large extent, this is due to uncertainty about the frequency and impact of recombination in bacteria. The existence of clonal lineages in the bacterial populations and linkage disequilibrium between alleles at different loci is often cited as evidence for low rates of recombination. However, clones and linkage disequilibrium are almost inevitable in species that divide by binary fission and can be present in populations where recombination is frequent (Spratt *et al.*, 2001). In recent years, it has become possible to directly compare rates of recombination in different species. Among pathogenic bacteria a wide range of ratio recombination/mutation events is observed with natural consequence to their population's genetic structure. It is estimated that recombination has generated new alleles at a frequency 10-fold higher than mutation in pneumococci (Feil *et al.*, 2000b). However, in other species well adapted to humans, such as *S. aureus*, point mutation give rise to new alleles at least 15-fold more frequently than recombination (Feil *et al.*, 2003). In species where recombination is more frequent than mutation, the long-term evolution of the population is dominated by recombination; however, this does not occur at a sufficiently high frequency to prevent the emergence of adaptive clones, although these are relatively short-lived and rapidly diversified (Feil & Spratt, 2001).

1.3.7 - Intra- and Inter-species relationship envolving *S. pneumoniae*

Recently, it has been shown that *S. pneumoniae* exhibited genetic transformation in response to antibiotic stress, suggesting that transformation can be a stress response mechanism. This induction requires the *com* operon, encoding the competence regulatory cascade, to be functional. This had been shown for aminoglycoside and fluoroquinolone antibiotics, as well as mitomycin C, a DNA-damaging agent. However, for β-lactams and erythromycin no induction effect was observed (Prudhomme *et al.*, 2006).

Several commonly used antibiotics induce the SOS response, potentially hastening genetic changes, inducing horizontal transfer of antibiotic resistance (Beaber *et al.*, 2004; Ubeda *et al.*, 2005). As in many temperate bacteriophages, integrating conjugative elements recruit the SOS DNA damage response to mobilize themselves from the bacterial chromosome and infect other cells. This process transfers resistance to multiple antibiotics.

The microbial communities of humans are characteristic and complex mixtures of microorganisms that have co-evolved with their human hosts. The species that make up these communities vary between hosts as a result of restricted migration of microorganisms between hosts and strong ecological interactions within hosts, as well as host variability in terms of diet, genotype and colonization history. The shared evolutionary fate of humans and their symbiotic bacteria has selected the mutualistic interactions that are essential for human health, and ecological or genetic changes that uncouple this shared fate, which can result in disease (Dethlefsen *et al.*, 2007).

Since the mucosal surfaces may be simultaneously colonized by multiple species, such as pneumococci and other *Streptococcus*, *H. influenzae*, several *Neisseria* and *B. catarrhalis*, these species have the abbility to compete with their co-inhabitants. Thus, colonization by pathogenic bacteria in the presence of the commensal flora requires strategies for the *S. pneumoniae* to successfully compete for nutricional and special resources and dislocate other commensal bacteria from the microbial niche.

Lysenko *et al.* (2005) observed in a murine model, that co-colonization of *H. influenzae* and *S. pneumoniae* resulted in the clearance of *S. pneumoniae* from the upper respiratory tract, associated with an increase of neutrophils in the paranasal spaces, indicating that pneumococci clearance was due to enhanced opsonophagocytic killing. It was also suggested by the authors that the components of *H. influenzae*, such as those from the cell wall, stimulated the complement-dependent phagocytic killing of *S. pneumoniae* (Lysenko *et al.*, 2005). This shows that innate immune responses also have an important role in the competitive interactions between the species colonizing the nasopharynx, like pneumococci. However, it has been described that *S. pneumoniae* is able to desialylate the cell surface lipopolysaccharides of both *N. meningitidis* and *H. influenzae*. These structures

mimicked host components in their normal state, being used to avoid recognition by the innate immunity of the host. By altering these structures, pneumococci expose those bacteria to the innate immunity of the host gaining a competitive advantage (Shakhnovich *et al.*, 2002).

1.3.8 - Invasion of immune recognition by mucosal surfaces

Commensal bacteria are harmless and even beneficial under normal circumstances, but may cause local or systematic inflammatory diseases when the integrity of the host surface tissues is compromised. The success of several bacteria species relies on their ability to avoid, resist and counteract host defence mechanisms. Thus, sometimes bacteria provoke activation of the immune system, which leads to disruption of the epithelial barrier and consequent bacterial invasion. The strategies usually used by bacteria include recognition of the surface immune receptors, secretion of antimicrobial effector molecules, internalization and degradation by phagocytes and activation of the humoral as well as the cellular immune systems (Hornef *et al.*, 2002).

The integrity of the mucosal membrane is protected by the active removal of bacteria, for exemple by the ciliate movement in the upper respiratory tract. Thus, the attachment factors are essential for the colonization and invasiness of pneumococci to overcome this mechanism of the innate immune system. The *S. pneumoniae* is well adapted to live in its natural ecosystem – the human nasopharynx. For instance, in *S. pneumoniae*, the presence of pili, long organelles able to extend beyond the polysaccharide capsule, enhances the bacterial adhesion and the subsequent ability to cause invasive disease (Barocchi *et al.*, 2006).

The mechanical barriers are also covered by an array of opsonising factors, such as the antibodies, usually IgA class. These will immobilize and remove bacteria from the mucosal membranes. Pneumococci, as also other bacteria colonizing the upper respiratory tract, produce proteolytic degradation of the antibody by IgA1 protease to avoid opsonisation (AlonsoDeVelasco *et al.*, 1995).

1.3.9 - Capsule and serotypes (nomenclature, classification and distribution)

The surface structures of pathogens like pneumococci, called pathogen-associated molecular patterns, which includes lipopolysaccharides, peptidoglycan and bacterial DNA, are important antigens for the epithelial cell of the host to identify the microbial organism (Boneca, 2005). In fact, the capsule has been widely described as one of the major virulence

factors of the pneumococci (Hava *et al.*, 2003; Paton *et al.*, 1993). This structure is quite important to avoid phagocytosis, which is mediated by the professional phagocytic cells resident in the subepithelial space (AlonsoDeVelasco *et al.*, 1995; Chudwin *et al.*, 1985; Holzer *et al.*, 1984). For a successful invasion, it is critical for pneumococci to overcome this step of the invasive process. If pneumococci successfully escape phagocytosis, this will lead to systemic spread in the host, through the blood and lymph circulatory systems. However, the limited supply of essential nutrients, such as iron, requires the pneumococci to adapt to this environment (Brown *et al.*, 2001). Additionally, pneumococci will have to contour the humural defences. Soluble factors, such as the C-reactive protein and other acute phase proteins are important to the induction of the complement cascade as well as opsonin factors. To prevail over these defence mechanisms, pneumococci possess surface structures that bind complement regulatory proteins, inhibiting the depositation of C3b on the bacterial surface, therefore inhibiting the formation of the membrane attack complex (Brown *et al.*, 1983; Horstmann *et al.*, 1988).

Encapsulated species typically exhibit antigenic variation and express one of a number of immunochemically distinct capsular polysaccharides that define serotypes. As mentioned before, the capsule is particularly involved in the virulence of *S. pneumoniae*, with at least 90 different types encoded by the *cps* locus in these bacteria (AlonsoDeVelasco *et al.*, 1995; Bentley *et al.*, 2006). Expression of a polysaccharide capsule is essential for survival in the blood, but it is also a target for host antibodies and the basis for effective vaccines.

The capsular polysaccharides are antigenic distinct, being used in the serological classification of pneumococcal strains (Bentley *et al.*, 2006). Two systems of classification of the pneumococcal serotypes are available. The American nomenclature assigns the numbers according to order of discovery. Nowadays, however, the Danish nomenclature is used worldwide as the standard classification. This nomenclature classifies serotypes according to structural and antigenic characteristics; for example, serotypes 6A and 6B are structure and antigenic related (Baker, 1990).

The distribution of serotypes recovered from invasive disease in a population is quite complex, depending also on factors such as: gender, race, age, geographic localization of the population, site of infection and previous antimicrobial use (Feikin & Klugman, 2002; Hausdorff *et al.*, 2000a; Hausdorff *et al.*, 2001; Scott *et al.*, 1996). Even from year to year significant variation in the serotype distribution of one population is observed (van Dam *et al.*, 1990). The genetic background of the circulating clones is also related to the distribution of the serotypes in a given geographic area (Sandgren *et al.*, 2004).

The serotypes circulating among adults or among children differ greatly. According to Hausdroff *et al.* (2000a), the serogroups 4, 6, 9, 14, 18, 19 and 23 are the cause of 70% to 80% of the IPD observed in children of the North America, Australia, Africa and Europe, and 65% of the infection in South America and Asia. In these two last regions serotypes 1 and 5 are responsible for 7% to 31% of the infections among children. In Asia, the serogroups 2, 12 and 15 were also responsible for 4% of the invasive infections among older children and adults (Hausdorff *et al.*, 2000a). In Portugal, during the period between 1989 and 1993, the serogroups 23, 19, 3, 6 were prevalent mainly among adults (Vaz Pato *et al.*, 1995).

The prevalence of serotypes which are present into the formulation of the heptavalent conjugate vaccine (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) increased along the XX century. In the United States those serotypes increased from 15% to 64% among adult and from 53% to 87% in paediatric population, respectively. However, the serotypes 1, 2, 3 and 5, called epidemic (as related to outbreaks), had decreased until 2001 (Feikin & Klugman, 2002).

1.4 - Mechanisms of action of antimicrobial agents

The treatment and management of *S. pneumoniae* infections is currently focused on the use of several antimicrobial agents from different groups (Table 1.2).

Table 1.2 - Classification of antimicrobial agents by mechanism of action (adapted from Neu, 1992).

Mechanism of action	Family	Example of antimicrobial agents
Inhibition of synthesis or damage to cell wall	β-Lactams	Penicillins Cephalosporins
	Glycopeptide	Vancomycin Teicoplanin
Inhibition of synthesis or metabolism of nucleic acids	Quinolones	Ciprofloxacin
	Rifamycins	Rifampicin
Inhibition of protein biosynthesis	Aminoglycosides	Amikacin
	Chloramphenicol	Chloramphenicol
	Macrolides	Erythromycin Azithromycin
	Lincosamide	Lincomycin Clindamycin
	Tetracyclines	Tetracycline
	Spectinomycin	Spectinomycin
Modification of energy metabolism		Sulfonamides Trimethoprim

1.4.1 - β-Lactams: action in the penicillin-binding proteins

The β-lactams are the most widely used antibiotics. The current choice of treatment of pneumococcal infections using β-lactams is the use of amoxicillin or cephalosporins, such as cefotaxime and ceftriaxone.

It has been shown that penicillin kills susceptible bacteria by specifically inhibiting the transpeptidases that catalyze the final step in cell wall biosynthesis, the cross-linking of peptidoglycan (Tipper & Strominger, 1965; Wise & Park, 1965). There has been considerable investigation and controversy described in the literature regarding the molecular details of this inhibition. In the external layer of the citoplasmatic membrane, the proteins which are target for penicillin are present – penicillin binding proteins (PBPs). In *Escherichia coli*, PBPs 1, 2 and 3 showed transpeptidase and transglycosylase activity, while PBPs 4, 5 and 6 act as D,D- carboxypeptidases. The penetration of β-lactams is easier in Gram-positive bacteria due to the absence of the external membrane which is present in Gram-negative bacteria.

Usually the bacterial peptidoglycan (murein) layer is composed of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid, and a small group of amino acids (Figure 1.1). The peptide moiety of the peptidoglycan precursor consists of the pentapeptide L-Ala-γ-D-Glu-Xaa-D-Ala-D-Ala, where Xaa can change according to bacterial species. Synthesis of peptidoglycan during cell growth involves controlled cutting of bonds connecting small areas of preexisting peptidoglycan, by the action of autolysin, with the simultaneous insertion of new pieces of peptidoglycan. The final step in cell wall synthesis is the formation of the peptide cross-links between adjacent glycan chains. The transpeptidation crosslink between the pentapeptides is undertaken by PBPs.

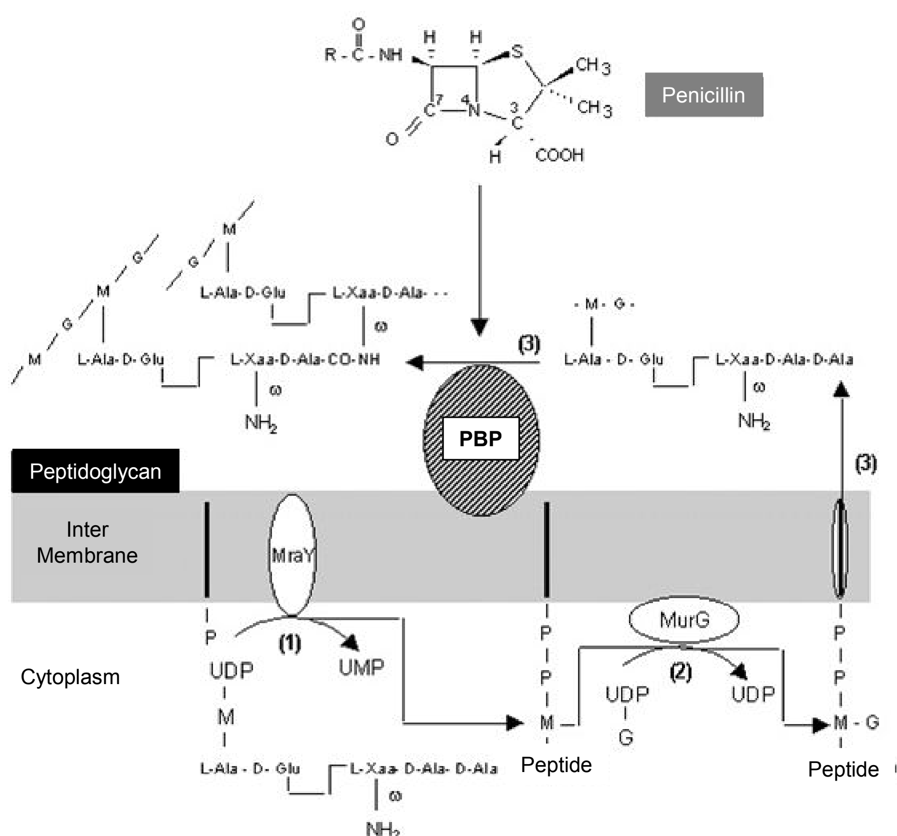

Figure 1.1 - Schematic section of bacterial peptidogylcan layer. (1) MraY catalyses the transfer of phospho-N-acetylmuramyl-pentapeptide (P-MurNAc-pentapeptide) or L-Ala-D-Glu-L-Xaa tripeptide from UDP to the lipid carrier (represented by a thick vertical line). (2) MurG catalyses the transfer of N-acetylglucosamine (GlcNAc) from UDP to the lipid-linked MurNAc-pentapeptide (or tripeptide). (3) Cell-wall-peptidoglycan assembly. The point at which the components cross the plasma membrane is not known. Assembly is catalysed by the penicillin-binding proteins (PBP). The structure of penicillin is also shown. G, N-acetylglucosamine; M, N-acetilmuramic acid; Xaa, lisine residue on *S. aureus* or mesodiaminopimelic acid residue on *E. coli*; UDP, difosfate of uridine; UMP, monofosfate of uridine; MurG and MraY, transpetidases. Adapted from Ghuysen (1994).

The β-lactams form covalent complexes with PBPs, due to the structural similarities of the β-lactamic ring and the natural substrate of these enzymes (the moiety D-Ala-D-Ala)

(Figure 1.2). The carboxyl group of the β-lactams and the serine residue from the active site of the transpeptidase domain form one covalent complex causing the irreversible inhibition of the transpeptidase.

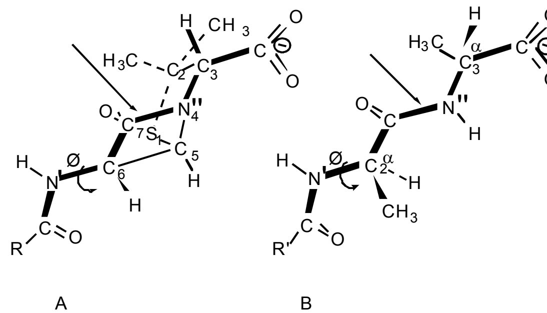

Figure 1.2 - Structure of penicillin (A) and acyl-D-alanyl-D-alanine (B). Adapted from Tipper & Strominger (1965).

The persistence of the complex β-lactam-PBP determines the efficiency of the antibiotic. Greater stability of the complex causes greater effect of the antibiotic, consequently inhibiting the PBP by competitive inhibition (Spratt, 1994).

The inhibition of the transpeptidation step by β-lactam antibiotics thus leads to the formation of weakened peptidoglycan which eventually leads to lysis of the bacterial cell (Zhao *et al.*, 1997). However, the mechanism by which inhibition of wall synthesis is coupled to the "suicidal" activity of this enzyme was not clear for a long time. Tomaz and Waks (1975), observed that during penicillin treatment of autolysin defective pneumococcal mutants, cultures are inhibited by the same concentration of penicillin that induces lysis in the wild type, as well as the mutant strains treated with the minimum growth inhibitory concentration of penicillin lyse upon the addition of wild-type autolysin to the growth medium; this suggests an irreversible inhibition of cell wall synthesis by penicillin, triggering bacterial hydrolases and autolytic enzymes, such as LytA, which will destabilize the endogenous complex that inhibits the autolysin action (lipoteichoic acid) (Tomasz & Waks, 1975).

1.4.2 - Macrolides: action in the bacterial protein synthesis

The first macrolide, the natural product erythromycin A, was initially isolated by Eli Lilly in 1949 from the metabolic products of a strain of *Saccharopolyspora erythraea* (later *Streptomyces erythreus*) found in soil samples, and was introduced in clinic use in the early 1950s (Mims *et al.*, 2004).

Macrolides have a common structure formed by a large lactone ring. Most of the 14-member-ring macrolides, which include erythromycin and its related compounds, have three structural components: the lactone ring, the desosamine sugar, and the cladinose sugar. They are agents that reversibly inhibit bacterial protein synthesis by binding to the 23S ribosomal RNA of the 50S ribosomal subunit, which causes premature dissociation of the peptide during translation (Leclercq & Courvalin, 2002). The reactive groups of the desosamine sugar and the lactone ring of erythromycin and its second generation derivatives, clarithromycin and roxithromycin, mediate all the hydrogen-bond links with the segment of 23S ribosomal RNA at the peptidyl transferase cavity (Schlunzen *et al.*, 2001). The macrolide binds to the entrance of the tunnel in the peptidyl transferase cavity, allowing the formation of six to eight peptide bonds before the forming protein chain reaches the bound macrolide. When bound, macrolides reduce the diameter of the tunnel entrance disabling the syntheses of peptides greater than eight residues. These antibiotics could also inhibit the peptidyl transferase by interfering with the proper positioning and movement of the tRNAs at the enzymatic cavity (Yonath *et al.*, 1987). In addition, antibiotic binding may physically link regions known to be essential for the proper positioning of the aminoacyl- and peptidyl-tRNAs, and thus prevent the conformational requirements needed for protein biosynthesis (Nissen *et al.*, 2000; Schlunzen *et al.*, 2001).

1.5 – Mechanisms of resistance of *S. pneumoniae* to antimicrobial agents

Thanks to the discovery of penicillin, attributed to Alexander Fleming in 1929, the management of infectious diseases dramatically changed since the 1940s. However, in 1967, the first pneumococci nonsusceptible to penicillin were reported in Australia and New Guinea (Hansman *et al.*, 1971; Hansman *et al.*, 1974). Ten years later, five cases of infection by pneumococci were observed, all in the same hospital in South Africa, which resulted in three infant deaths (Austrian, 1999). The resistance pattern of those strains showed to be highly-resistant to penicillin and also resistant to macrolides, tetracycline and chloramphenicol (Jacobs *et al.*, 1978). Since then, strains of *S. pneumoniae* highly resistant to antibiotics started to be reported worldwide (Klugman & Koornhof, 1989). Unquestionably, resistance to the antimicrobial agents is one of the major concerns in term of public health worldwide.

1.5.1 - Resistance to β-lactam agents

The resistance mechanism of election among β-lactam resistant pneumococci is the assemblage of functional low-affinity PBPs (Tomasz, 1986). Evidence from mutagenic studies on pneumococcal laboratory isolates and on the genetic transformation of resistance has indicated that penicillin resistance is a multigenic property acquired in a stepwise fashion (Hakenbeck *et al.*, 1994; Handwerger & Tomasz, 1986; Laible & Hakenbeck, 1991; Laible *et al.*, 1991)

1.5.1.1 - Altered Penicillin-Binding Proteins

Early studies using radioactively labelled penicillins incubated with isolated bacterial membranes showed that *S. pneumoniae* had three major groups of penicillin-binding proteins, namely PBPs 1, 2, and 3 (Chalkley & Koornhof, 1988; Percheson & Bryan, 1980) (Table 1.3). Evaluation of susceptible and resistant strains revealed that resistant bacteria retain a normal set of PBPs; however, at least one of these groups has lower binding capacity for β-lactams in strains resistant to those agents (Percheson & Bryan, 1980; Zighelboim & Tomasz, 1981). Immunological studies pointed out that resistant and susceptible strains had similar PBPs, but with different binding affinity to β-lactams (Hakenbeck *et al.*, 1986; Severin *et al.*, 1992).

Until now it has been recognized that *S. pneumoniae* contains six PBPs, which are classified according to their molecular sizes (Zhao *et al.*, 1997) (Table 1.3). The first group consists of the low-molecular-weight PBP3 (43 kDa). This protein was considered to be

unessential in pneumococci, since the penicillin affinity of PBP3 is not altered in penicillin-resistant strains (Laible & Hakenbeck, 1987; Zhao *et al.*, 1997). The second group of PBPs consists of the high-molecular-weight enzymes. Unlike the low-molecular-weight PBPs, these are essential. The transpeptidase activity of these PBPs is inhibited by β-lactam antibiotics.

Table 1.3 - Penicillin-Binding Proteins of *S. pneumoniae* (adapted from Hakenbeck *et al.*, 1986; Hakenbeck *et al.*, 1999; Hoskins *et al.*, 1999; Laible & Hakenbeck, 1987; Laible & Hakenbeck, 1991).

PBP Group	PBP	Gene[a]	Molecular Mass[b]	Class	Importance in Resistance Development
1	PBP1A	*pbp1A*	98 kDa (HMW)	class A	Required for high level β-lactam resistance
	PBP1B	*pbp1B*	89 kDa (HMW)	class A	Role not established
	PBP2X	*pbpX*	82 kDa (HMW)	class B	Primary resistance determinant; confer low-level β-lactam resistance. Required for high resistance
2	PBP2A	*pbp2A*	81 kDa (HMW)	class A	Some low affinity forms have been found
	PBP2B	*penA*	77 kDa (HMW)	class B	Primary resistance determinants; confer low-level β-lactam resistance
3	PBP3	*pbp3*	43 kDa (LMW)	-	Does not participate in resistance development

[a] According to nomenclature given to TIGR4 strain (Tettelin *et al.*, 2001).
[b] HMW, High molecular weight; LMW, Low molecular weight.

High-molecular-weight PBPs are subdivided into class A and class B, differing mainly in the sequences of their N-terminal regions (Hoskins *et al.*, 1999)(Table 1.3). The PBPs of class B are primary resistance determinants, being essential for the development of resistance. These PBPs have all the essential transpeptidase function located at the C-terminal domain. The function of the N-terminal domain of class B PBPs is not well established; however, for class A PBPs, that's where the transglycosylase activity is observed (Hoskins *et al.*, 1999; Zhao *et al.*, 1997).

The X-ray structure of *S. pneumoniae* PBP2X, determined by X-ray crystallography, reveals that PBP2X consists of three domains: an N-terminal domain with the appearance of a "sugar-tongue" that is in close association with a second, a central penicillin-binding domain, and a C-terminal domain, that is connected via 28-residue-long flexible loop (Dessen *et al.*, 2001; Pares *et al.*, 1996). The transpeptidase domain contains three conserved amino acid motifs that are in a close spatial relationship as part of the active site of the enzyme (Hakenbeck *et al.*, 1999). The PBPs interact with β-lactams by forming a relatively stable covalent complex via the active site serine. Eventually, deacylation of this

complex takes place, resulting in a biologically inactive β-lactam derivative (Gordon *et al.*, 2000).

Mutations that contribute to penicillin resistance in *S. pneumoniae* strains are located in the transpeptidase-penicillin binding domain near the active site and they reduce the affinity to β-lactams rather than affect the deacylation step (Hakenbeck *et al.*, 1999). Several mutations in one PBP are required to cause a significant decrease in penicillin-binding affinity, and more than one PBP needs to change into low-affinity variants in order to achieve high resistance levels. In *S. pneumoniae*, alterations in the high-molecular-weight class B PBPs (PBP2X and PBP2B) confer low resistance and are the prerequisite for high resistance. To achieve high levels of resistance, alterations in PBP1A (high-molecular-weight class A PBP) are also needed (Contreras-Martel *et al.*, 2006; Dessen *et al.*, 2001; Goffin & Ghuysen, 1998; Grebe & Hakenbeck, 1996).

Extensive research has been done on *S. pneumoniae* PBPs, however, the mechanism of PBPs underlying penicillin resistance is not fully understood. For instance, the role of some of the high-molecular-weight PBPs, such as PBP1B and PBP2A, is not clearly understood.

1.5.1.2 - The role of horizontal gene transfer in the development of altered penicillin-binding proteins

Transference of genetic material among bacteria may occur by either of three processes: conjugation, which requires cell-to-cell contact, transduction, mediated by a bacteriophage, and transformation, the process in which a recipient cell acquires plasmids or extracellular free genetic material from its surroundings, and subsequently incorporates such fragments into its chromosomal DNA.

The naturally transformable *S. pneumoniae* have complete access to a wide pool of genetic diversity from other strains of the same species as well as closely related species. The shuffle of advantageous genes along time has resulted in resistance of these organisms to antibiotics in a period of less than 30 years. The best examples of mosaic genes related to antibiotic resistance that have arisen from interspecies recombination are the altered PBPs in *Streptococcus* and *Neisseria* (Bowler *et al.*, 1994; Maiden, 1998). It is thought that the use of antibiotics has created a selection pressure that has promoted the spread of mosaic genes encoding proteins with decreased affinity for the β-lactams.

Genetic analysis of genes encoding PBPs concluded that these mosaic genes were the result of localized recombination events between *S. pneumoniae* and the homologous genes from *Streptococcus mitis* and *Streptococcus oralis* (Chi *et al.*, 2007; Laible *et al.*,

1991). These organisms are commensal members of the oral flora coexisting in the oral cavity, facilitating the interspecies gene transfer.

It has been shown that to be highly resistant to penicillins, *S. pneumoniae* must acquire low-affinity variants of three PBPs (PBPs 1A, 2B, and 2X). The accumulation of three separate penicillin-resistant PBPs encoded by mosaic genes seems at first glance to be unlikely, but analysis of nucleotide sequence data show that this occurred frequently. This lead to the widespread penicillin resistance observed in the pneumococcus. Moreover, in *S. pneumoniae*, the *pbp1A* and *pbpX* genes are close to each other in the chromosome, leading to cotransformation of these genes. Thus, β-lactam resistance has the potential for rapid spread among strains (Maiden, 1998).

Genetic studies comparing sequences of *pbpX* genes in susceptible and resistant strains strongly suggests that the altered *pbpX* genes have arisen by localized interspecies recombinational events. Laible *et al*. (1991) showed that, in a 2kb region, clinical susceptible *S. pneumoniae* isolates differed at only 0.4% nucleotide sites and had one single amino acid difference in PBP2X. However, the sequences of the *pbpX* genes from the resistant isolates differed overall from those of the susceptible isolates between 7 and 18% of nucleotide sites and resulted in between 27 and 86 amino acid substitutions in PBP2X. The presence of highly diverged regions within the *pbpX* genes of the resistant isolates contrasted with the uniformity of the *pbpX* genes in the susceptible isolates. In addition, the emergence of multiresistant *S. pneumoniae* variants can also arise by natural transformation involving recombinational replacements, within and around the capsule biosynthesis (*cps*) locus, which is as large as 25 kb (Coffey *et al.*, 1999). The *cps* locus of *S. pneumoniae* is flanked by the *pbpX* and *pbp1A* genes and can be transformed in a single event. Hence, resistance to β-lactams can emerge from susceptible strains combined with unusual serotypes. In addition, the same authors also showed that transfer of the *cps* locus can occur at an elevated rate in β-lactam-selected transformants (Trzcinski *et al.*, 2004).

1.5.1.3 - Other genes involved in resistance to β-lactams

Recently, isolates resistant to β-lactams with non-compatible *pbp* nucleotide sequences have been reported, suggesting the role of other non-PBP mechanisms of resistance to β-lactams. Also these findings indicate that the development of high-level β-lactam resistance is a more complex process than thought before (Chesnel *et al.*, 2005; Du Plessis *et al.*, 2002; Smith & Klugman, 2000).

In the last 10 years, a third level of resistance mechanisms has been identified in laboratory mutants and, in some cases, among clinical strains. These mechanisms are

characterized by mutated non-*pbp* genes and development of resistance was accompanied by deficiency in genetic transformation (Hakenbeck *et al.*, 1999). Two such non-*pbp* genes have been described by Hakenbeck *et al.* (1999) as a putative glycosyltransferase, CpoA, and a histidine protein kinase, CiaH. It was proposed that these non-*pbp* genes are involved in the biosynthesis of cell wall components at a step prior to the biosynthetic functions of PBPs. It was also proposed that mutations in those genes were selected during β-lactam treatment, counteracting the effects caused by the low affinity penicillin-binding proteins (Hakenbeck *et al.*, 1999).

S. pneumoniae ciaH mutants are associated with a decrease of genetic competence. However, the same is not observed for the disruption of the *ciaR* gene, encoding a CiaH associated response regulator. The competence is repressed when the *cia* operon is activated. Competence is dramatically reduced when cells go into the stationary phase. Mutations in the *cia* operon have a higher effect on the regulation of competence than on the entrance and incorporation of foreign DNA, and they are complemented by the addition of CSP. This suggests that the *cia* operon interferes directly with the expression of *comC* (Hakenbeck *et al.*, 1999).

Mutations in the *cpoA* gene seem associated with the regulation of the main autolysins. There is also some evidence of the *cpoA* involvement in the biosynthesis of teichoic acids. It was demonstrated that *cpoA* is an essential gene, associated with a decrease of the production of PBP 1A on *cpoA* mutants. However, the mechanism underlying that regulation is not well established.

Filipe *Filipe and Tomasz (2000)* has also described that the inactivation of the *murMN* operon in resistant strains caused a “reversion” of penicillin resistance. The *murM* and *murN* genes encode enzymes involved in the biosynthesis of branched structured cell wall muropeptides. These observations indicate that the ability to produce branched cell wall precursors plays a critical role in the expression of penicillin resistance in *S. pneumoniae* (Filipe & Tomasz, 2000).

For decades it was thought that penicillin resistance was exclusively due to alterations in the genes encoding the high-molecular-weight PBPs; however, recent advances in the knowledge of the biosynthesis of the cell wall components have shown that resistance to β-lactam antibiotics is a broad and complex subject. This is expected specifically in murein biosynthesis, since the β-lactams act in several targets of the final steps of the biosynthesis of peptidoglican. Thus, any operon that regulates the production of the cell wall precursors will inevitably affect the level of susceptibility of *S. pneumoniae* to β-lactams. A putative example is the pneumococcal serine threonine kinase (StkP). This enzyme has been described to regulate by phosphorylation the activity of the

phosphoglucosamine mutase GlmM (Novakova *et al.*, 2005), which is envolved in one of the first steps of cell wall biosynthesis (Mengin-Lecreulx & van Heijenoort, 1996). The same function has also been reported in *E. coli* (Jolly *et al.*, 1999).

1.5.2 - Resistance to macrolides

The macrolides are another major classe of antimicrobial agents used against *S. pneumoniae* infections. The extensive clinical use of macrolides has resulted in the increasing emergence of macrolide resistance in Gram-positive cocci, in particular *S. pneumoniae*. The literature shows that the first isolates resistant to erythromycin and clindamycin associated to multiresistance were found in the late 70s in South Africa (Jacobs *et al.*, 1978).

1.5.2.1 – Major phenotypes

The resistance mechanisms to macrolides are expressed in two major phenotypes:

- MLS_B – This phenotype is due to the resistance to macrolides (e.g. erythromycin), lincosamides (e.g. clidamycin), and streptogramin B (e.g. pristinamycin), and it is conferred by methylation of the 23S rRNA at Ala2058 by ribosomal methylases encoded by *erm* (erythromycin ribosomal methylase) genes, which blocks the binding of macrolides. Expression of *erm* genes can be constitutive ($cMLS_B$), resulting in high-level resistance, or inducible ($iMLS_B$), resulting in low-level resistance.
- M – This phenotype is due to the resistance to macrolides, but not lincosamides or streptogramin B; it is caused by the reduction of intracellular accumulation of the macrolide due to the activity of efflux pumps, encoded by *mef* (macrolide efflux pump) genes in streptococci (Leclercq & Courvalin, 2002). The Mef(A) pump belongs to the major facilitator superfamily class. It contains 12 transmembrane domains spanning the cytoplasmic membrane, and efflux is driven by the proton motive force (Clancy *et al.*, 1996).

More recently, two novel mechanisms of macrolide resistance in pneumococci have been described, which are related to two novel resistance phenotypes: resistance to macrolide and lincosamide (ML) and to macrolide and streptogramin B (MS_B). The ML phenotype is related to mutations in Domain V of the 23S rRNA, and the MS_B phenotype to mutations in ribosomal protein L4 and in ribosomal protein L22 that constitute part of the assembled 50S rRNA (Tait-Kamradt *et al.*, 2000a). The mutations in the domain V of 23S

rRNA are situated at very close positions (Ala2058Gly, A2059Gly, or Ala2611Gly) or at the same location as the adenine that gets methylated by *erm* (Ala2058). Curiously these mutations are substitutions of adenine to guanine. This region of domain V is at the active translation site (peptidyl transferase region) of the 50S rRNA subunit (Ban *et al.*, 2000). In pneumococci, there are four copies of rRNA, and it is suggested that the degree of resistance probably depends on whether the mutations are in two, three, or all four rRNAs. The mutations described in ribosomal protein L4 or L22 showed a diversity of changes, including substitutions, deletions, and insertions (Tait-Kamradt *et al.*, 2000a; Tait-Kamradt *et al.*, 2000b).

These two novel mechanisms complicate the inference of the genetic mechanism of resistance based on the phenotype. ML and MS_B phenotypes can be confused with the MLS_B and M phenotypes, respectively. However, the new phenotypes can be differentiated from the M and MLS_B phenotypes by the analysis of the susceptibility to streptogramin B and clindamycin (Tait-Kamradt *et al.*, 2000a).

- **Erythromycin ribosomal methylase genes (*erm*)**

As stated previously, the main gene classes that encode for macrolide resistant phenotypes are the *erm* and *mef* genes. Multiple *erm* gene types are recognized, and the nomenclature is quite confusing (Table 1.4). However, Marilyn Roberts *et al.* (1999) proposed a nomenclature system with 21 different classes based on their genetic structure. The *erm* genes found in pneumococci are mainly associated to genes from class B, namely the *ermB* gene (Bozdogan *et al.*, 2004). Nevertheless, pneumococcal strains carrying genes from class A are sporadically found, probably acquired from other streptococci, being frequent among the *Streptococcus pyogenes* (Camilli *et al.*, 2008).

Table 1.4 - rRNA methylase genes implicated in MLS_B resistance (adapted from Roberts *et al.*, 1999).

Class	Genes
A	*erm(A), erm(TR)*
B	*erm(AM), erm(B), erm(AMR), erm(BC), erm(P), erm(BP), erm(IP), erm(Z), erm, erm(2)*

The *erm* genetic regulation is complex and may involve several mechanisms. It is known that the regulatory region of *erm* genes consists of two leader peptides containing a

number of inverted repeats that can act as attenuator regulation. This type of genetic regulation was first found in *S. aureus* (Murphy, 1985). Among strains of *Streptococcus intermedius*, *S. pyogenes*, and *S. pneumoniae*, genetic alterations had been also observed including deletions, different tandem duplications, and point mutation, as well as insertions (Doktor & Shortridge, 2005; Werckenthin & Schwarz, 2000; Wolter *et al.*, 2008). All the alterations either completely prevented the formation of RNA secondary structures in the *erm* regulatory region or favoured the formation of those mRNA secondary structures that allowed translation of the *erm* transcripts, causing the increase of resistance levels to macrolides.

- **Macrolide efflux pump genes (*mef*)**

A large number of different antibiotic resistance genes encoding for efflux transport proteins is described in the literature. Three different efflux systems which confer resistance have been described for Gram-positive cocci: *msr*(A), encoding for macrolide and streptogramin B resistance; *mef*(A) and *mef*(E), macrolide efflux; and *vga*, virginiamycin factor.

Among streptococci strains the most common genes found are the *mef(A)* and *mef(E)*, the former described in *S. pyogenes* and the latter in *S. pneumoniae*. These two genes are 90% homologous, and it has been proposed that both are referred to as *mef(A)* (Leclercq & Courvalin, 2002). The *mef*(A) gene can be transferable among pneumococci being carried by large transposable elements (Gay & Stephens, 2001; Del Grosso *et al.*, 2002; Santagati *et al.*, 2000). Frequently, the genetically closely related *mel* gene (*msrA* homolog from *S. aureus*), which encodes a proton motive force pump and a putative ATP-binding cassette transporter homolog, can be found near the *mef*(*A/E*) genes. The *mef*(*A/E*) and *mel* genes are transcribed as an operon inducible by the presence of macrolides (Ambrose *et al.*, 2005). The *mef*(*A*) gene alone is sufficient to confer resistance, although one cannot be excluded the possibility that the pump interacts with other proteins (Clancy *et al.*, 1996). Indeed, the requirement of both Mef and Mel proteins for full efflux-mediated macrolide resistance in *S. pneumoniae* and other Gram-positive bacteria was shown (Ambrose *et al.*, 2005).

1.5.2.2 - Genetic structures contributing to the dissemination of macrolide resistance

In *S. pneumoniae*, the *ermB* gene is usually part of conjugative transposons related to Tn*1545*, Tn*1545*-like elements, or a Tn*917*-like element that is part of a larger composite

transposon, the Tn*3872* (Leclercq & Courvalin, 2002). *S. pneumoniae* strains have also been described to have *erm*(B) as part of Tn*3872* or a modified form of Tn*916* and Tn*1545* (Poyart-Salmeron *et al.*, 1991). Transposition occurs from chromosome to chromosome in strains of *S. pneumoniae*, causing the horizontal spread of macrolide resistance. Nevertheless, both clonal spread and horizontal spread of genetic elements have been pointed out to account for the readily transfer of the *erm*(*B*) gene across species and genus barriers (Roberts *et al.*, 1999).

The *mef* genes responsible for the M phenotype are carried by three highly conserved chromosomal genetic elements:

- Macrolide Efflux Genetic Assembly (MEGA), which is 5.5 kb in lenght, and is typical of *S. pneumoniae*. It carries *mef(E)* and can be inserted at different sites in the bacterial chromosome and in other genetic elements, such as in Tn*916* (Del Grosso *et al.*, 2004). MEGA does not have genes encoding putative recombinases or transposases, and it is not conjugative (Gay & Stephens, 2001).

- Tn*1207.3*, has 7Kb, and is typical of *S. pyogenes*; it carries *mef(A)* and integrates at a single specific site in the bacterial chromosome. This transposon has three ORFs encoding for putative recombinases, and it is transferred by conjugation among different streptococcal species, including *S. pneumoniae* (Santagati *et al.*, 2000).

- Tn*1207.1*, has 7.2 kb, and is a defective form of Tn*1207.3* found in a clonal population of *S. pneumoniae*; this transposon carries *mef(A)*, it integrates at a single specific site in the bacterial chromosome (the same of Tn*1207.3*), and is not conjugative (Pozzi *et al.*, 2004).

Indeed, the resistance related to the macrolide M phenotype can be easily disseminated on a population. In addition, the *mef* and *erm*(B) genes can both be found in the same strains (Klugman & Lonks, 2005).

1.6 - Epidemiology of antimicrobial resistance of *Streptococcus pneumoniae*: contemporaneous scenario

1.6.1 - Selection pressure: consequences

Some decades ago infections caused by multiresistant isolates have often been detected (Vaz Pato *et al.*, 1995). The increase of the prevalence of those isolates is related to the misuse of the antimicrobial agents with consequence on the efficiency, duration and cost of the therapy (Goossens *et al.*, 2005; Vincent, 2003).

The evolution of antimicrobial resistance in pneumococcus has led to a reappraisal of the treatment of the infections caused by this pathogen. This implies an increased effort to detect resistance in clinical pneumococcal isolates, a greater emphasis on routine susceptibility testing of that pathogen, and the use of the recently developed protein-polysaccharide conjugate vaccine to prevente pneumococci infections (Liu, 1999; Whitney *et al.*, 2003).

1.6.2 - Penicillin-nonsusceptibility

Ten years after the first infections by *S. pneumoniae* nonsusceptible to penicillin reported in Australia, pneumococcal strains highly resistant to penicillin were isolated from South Africa (Hansman *et al.*, 1971; Jacobs *et al.*, 1978). In the late 70s, the number of strains highly resistant to penicillin had been reported across Europe. In the United States, Whitney *et al.* (2000) had described an increase of nonsusceptibility to this antimicrobial agent from 21% to 25% between 1995 and 1998. In southern European countries consistently higher proportions of penicillin resistance were observed (Linares *et al.*, 1992). In the period of 1998-2000, France was the European country with the highest rate of nonsusceptibility to penicillin (57.8%), followed by Spain (40.6%) (Jacobs *et al.*, 2003). The highest rate of nonsusceptibility to penicillin in this country was observed in 1997 (16.6% and 34.8% of intermediate and highly resistant strains to penicillin, respectively) (Felmingham & Gruneberg, 2000). According to the European Antimicrobial Resistance Surveillance System (EARSS), 9% of the *S. pneumoniae* isolates in 2006 were nonsusceptible to penicillin in the 30 countries engaged in the network (European Antimicrobial Resistance Surveillance System, 2007). However, penicillin nonsusceptibility showed a heterogeneous picture in Europe; most northern countries had levels of nonsusceptibility below 5%. In the last years some of these northern countries, such as Belgium, Finland and Ireland, reported increased levels of resistance (Figure 1.3). On the other hand, some southern European countries, such as Bulgaria, Italy and Malta, have reported decreased levels of penicillin nonsusceptibility in 2006. Cyprus, France, Israel, Romania and Spain were the countries with

the highest proportions of pneumococci nonsusceptible to penicillin in that year (European Antimicrobial Resistance Surveillance System, 2007). Recently (1999-2006), Finland, Italy, Slovenia and Sweden reported increasing levels of penicillin nonsusceptibility. However, other countries such as France, Spain and the UK have shown decreasing trends of penicillin nonsusceptibility (European Antimicrobial Resistance Surveillance System, 2007).

In Portugal, in 1989, only 4.6% of *S. pneumoniae* isolates were nonsusceptible to β-lactams; however, in 1993 this value increased to 17.9% (Vaz Pato *et al.*, 1995). Among isolates recovered from invasive infections, this value increased to 29% in 2000, stabilizing at 17% during the period 2005 -2006 (European Antimicrobial Resistance Surveillance System, 2007).

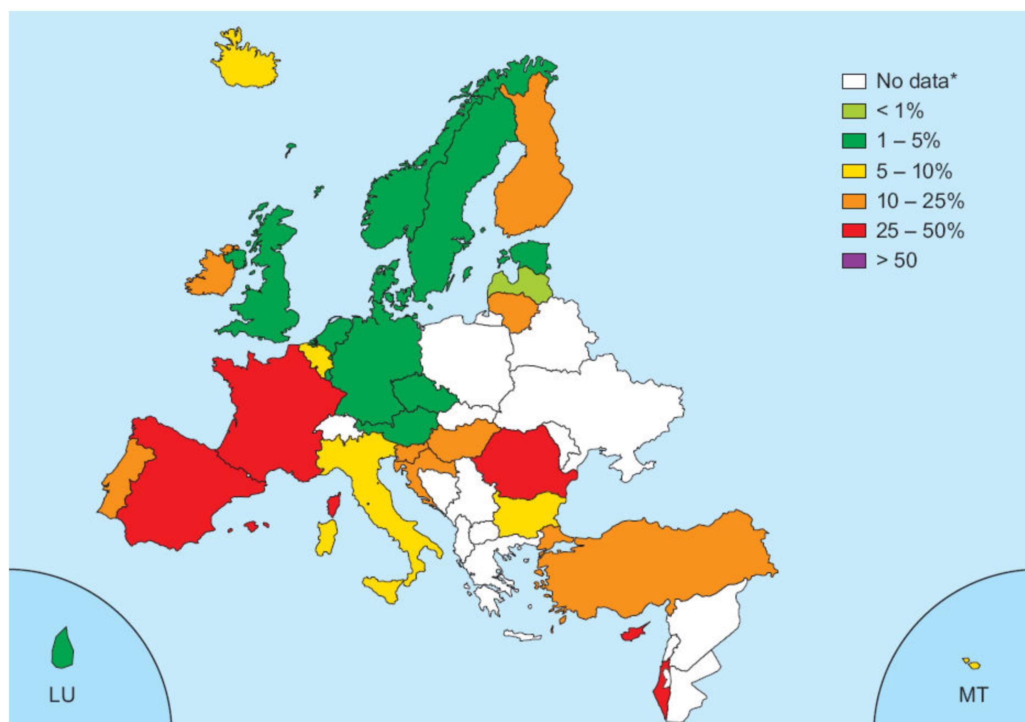

Figure 1.3 – *S. pneumoniae*: proportion of invasive isolates nonsusceptible to penicillin in 2006. * These countries did not report any data or reported less than 10 isolates. Adapted from European Antimicrobial Resistance Surveillance System (2007).

1.6.3 - Erythromycin-nonsusceptibility

In the European countries participating in the EARSS, erythromycin-nonsusceptible isolates account for 16% of all *S. pneumoniae* isolated in 2006; most countries had proportions between 10 and 25% in the last years. Five of those countries showed a significant increase in erythromycin resistance, mainly Finland (1999, 6%; 2006, 24%) and Turkey (2003, 7%; 2006, 16%). However, the proportion of isolates nonsusceptible to erythromycin from Spain (1999, 31%; 2006, 21.6%) and France (2001, 49%; 2006, 36%) have shown a significant decrease. Still, Belgium (31%), Cyprus (31%), France (36%), Luxemburg (36%), Italy (32%) and Malta (47%) reported over 30% erythromycin resistance in *S. pneumoniae* isolates in 2006 (European Antimicrobial Resistance Surveillance System, 2007) (Figure 1.4).

According to EARSS data, dual resistance to penicillin and erythromycin during 2006 remained below 5% for 16 countries, between 5-10% for 18 countries and between 10-20% for four countries. The highest percentage of coresistance was found in France (26%) and Cyprus (23%) (European Antimicrobial Resistance Surveillance System, 2007) (Figure 1.4).

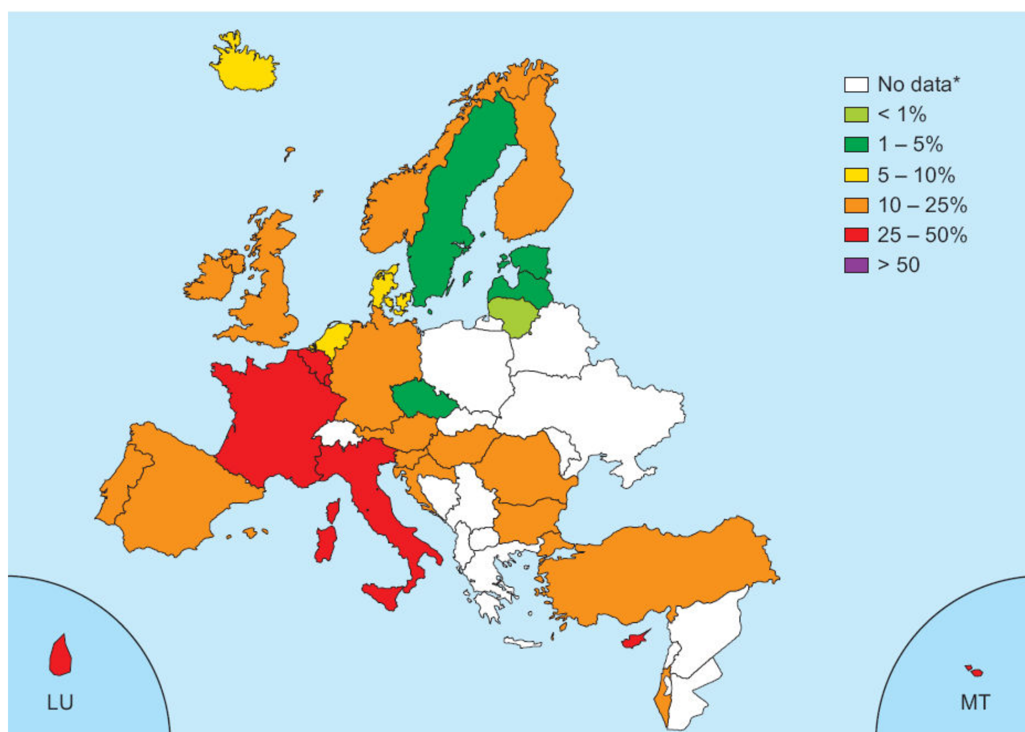

Figure 1.4 – *S. pneumoniae*: proportion of invasive isolates nonsusceptible to erithromycin in 2006. * These countries did not report any data or reported less than 10 isolates. Adapted from European Antimicrobial Resistance Surveillance System (2007).

1.6.4 - Serotypes/groups of *S. pneumoniae* related to nonsusceptibility

The serotypes among antimicrobial nonsusceptible *S. pneumoniae* strains from invasive disease are quite well conserved worldwide. In fact, in these cases, nonsusceptibility is only observed in a small number of serotypes/groups, namely 23, 6, 19, 9 and 14 (Table 1.5). The majority of the clinical strains with a high level of resistance to β-lactams are actually related to serotypes 6B, 9V, 14, 19F and 23F (Fenoll *et al.*, 2002; Richter *et al.*, 2002). However, the first penicillin- and macrolide-nonsusceptible *S. pneumoniae* isolates, also mutiresistant, found in South Africa were already associated to serotypes 6A, 6B and 19A (Jacobs *et al.*, 1978).

According to the EARSS project, the ranking order of serotypes, as well as the resistance within serotypes varies considerably between European countries. Nevertheless, serotype 14 seemed to be highly prevalent in almost all European countries. This serotype is also associated to the high coresistance to penicillin and erythromycin, representing 46% of serogroup 14 in Belgium (European Antimicrobial Resistance Surveillance System, 2007). A study undertaken in 18 European Countries from 2001 to 2003, has also shown that penicillin-nonsusceptible isolates were mainly from serotypes 14 (18.4%), 23F (13.7%), 6B (13.7%), 19F (12.5%), 19A (8.0%) and 9V (7.7%) (Reinert *et al.*, 2005b). Among erythromycin-nonsusceptible isolates, serogroups 1 and 33 were also found (European Antimicrobial Resistance Surveillance System, 2007).

1.6.5 - Clones of *S. pneumoniae* nonsusceptible to the antimicrobial agents

The emergence of antimicrobial resistant pneumococcal strains is related to the spread of a relative small number of clones (McGee *et al.*, 2001). The Pneumococcal Molecular Epidemiology Network (PMEN), whose main aim is the global surveillance of antibiotic-resistant *S. pneumoniae*, currently considers 43 clones as being globally spread (Table 1.6).

Some of these clones have truly disseminated worldwide, and it is thought that this is related to the import and dissemination of a small number of clones, in association with the selection pressure of antibiotic use (Baquero *et al.*, 2002; McGee *et al.*, 2001; van Bambeke *et al.*, 2007). Clonal diversification associated with *in vivo* selection of local variants with PBPs of low affinity to penicillin can cause the emergence of local clones.

Table 1.5 - Main serotypes among antimicrobial nonsusceptible pneumococcal strains recovered from invasive disease

Country	Period of the study	Nb. of isolates (age of patients)[a]	Nb. of penicillin NS isolates[b] (%)	PCV-7 %[c]	PNS PCV-7 %[d]	Main serotypes/groups in NS isolates[e]					Reference
						1st	2nd	3rd	4th	5th	
Europe											
Sweden	1998-2001	827 (C/A)	22 (2.7)	42	73						Backhaus *et al.*, 2007
Denmark	1995-1999	5452 (C/A)	114 (2)			9V	14	19F	19A	15B	Konradsen & Kaltoft, 2002
Germany	2001-2003	647 (A)	27 (4.2)	47.4							Reinert *et al.*, 2005d
Austria	2001-2003	56 (C)	12 (21.4)	69.6							Rendi-Wagner *et al.*, 2004
Czech Republic	2001-2003	483 (C/A)	25 (5.2)			9V	19F	14			Urbaskova *et al.*, 2004; Zemlickova *et al.*, 2006
Switzerland	2001-2004	2388 (C/A)	313 (13.1)			6B	9V	14,	19F		Kronenberg *et al.*, 2006
Scotland	1999-2002	1741 (C/A)	67 (3.8)		98						Denham & Clarke, 2005
France	2000-2002	257 (C/A)	120 (46.7)			14	19	23	9	6	Decousser *et al.*, 2004
Italia	1997-1999	503 (<5 years)	50 (10)			9	19				Pantosti *et al.*, 2000
Greece	2001-2002	65 (C)	6 (9.2)	69							Zissis *et al.*, 2004
Spain	1997-2001	1165 (C/A)	5310 (47.5)			14	19	6	23	9	Fenoll *et al.*, 2002
Portugal	1992-1994	520 (C/A)	164 (17.5)			23	9	14			Vaz Pato *et al.*, 1995
Portugal	1999-2002	465 (C/A)	107 (23)	63.2[f]							Serrano *et al.*, 2004
America											
Canada	1998-2003	1868 (<16 years)	299 (16)	79	89						Bettinger *et al.*, 2007
US	1996-2004	24825 (C/A)	- (22.7 - 21.7)			14	9V	19A	23F	19F	Kyaw *et al.*, 2006
Porto Rico	2001	177	88 (49.7)			14	19F	6B	6A	9V	Rivera-Matos & Rios-Olivares, 2005
Colombia	1994-2004	2022 (C/A)	602 (29.8)			14	6B	23F	1	5	Agudelo *et al.*, 2006
Brazil	1993-2004	6470 (C/A)	1352 (20.9)	62.0		14	6B	23F	19A	19F	Brandileone *et al.*, 2006
Asia - Oceania											
Kuwait	2004-2005	397 (C/A)	253 (64)			23F	19F	6B	14	9A	Mokaddas *et al.*, 2008
Bangladesh	200-2006	34 (<5 years)	1 (2.9)	61.7%[g]							Brooks *et al.*, 2007
Taiwan	2002-2003	522 (C/A)	353 (67.7)	85.0	90.8	23F	19F	6B	14		Chen *et al.*, 2006
Singapore	1997- 1999	180 (C)	114 (63.3)			19F	23F	14	6B	NT	Soh *et al.*, 2000
Australia	2003	1995 (C/A)		84	85	19F	14	6B			Watson *et al.*, 2004
Africa											
Egypt	1998-2003	205 (C/A)	100 (49)	38		6B	19A	23F	6A		Wasfy *et al.*, 2005
Gambia	1996-2003	531 (≤6 years)	39 (7.3)			1	5	6A	14	19A	Adegbola *et al.*, 2006
Ghana	2002-2005	140 (<5 years)	17 (12)	26		14					Holliman *et al.*, 2007

[a] C, patients less than 18 years of age; A, patients with or higher than 18 years of age.
[b] Number of isolates nonsusceptible to penicillin.
[c] PCV-7 %, Rate of the isolates with serotypes included in the heptavalent pneumococcal conjugate vaccine (4, 6B, 9V, 14, 18C, 19F and 23F).
[d] PNS PCV-7 %, Rate of the penicillin nonsusceptible strains with serotypes included in the heptavalent pneumococcal conjugate vaccine.
[e] Main serotypes/groups among isolates nonsusceptible to penicillin.
[f] Rate of isolates among children less than 2 years of age.
[g] Rate of isolates with serotypes included in the 13-pneumococal conjugate vaccine (PCV-7 serotypes and 1, 3, 5, 7F, 9A and 19A).

The so called multiresistant Spanish clones (Spain^{23F}-1, Spain^{6B}-2 and Spain^{9V}-3) and their variants are the major disseminated clones worldwide. As presented in Table 1.6, Spain^{23F}-1 (ST81) has been detected in more than 38 countries. This clone is described as being nonsusceptible to penicillin, tetracycline and choramphenicol, and is also commonly found as being resistant to macrolides.

The second most internationally disseminated clone, Spain^{9V}-3 (ST156), formally called France^{9V}-3, has been detected in 31 countries. Nowadays, however, this clone, which has two major variants with serotypes 9V and 14, may be the most prevalent international clone. The original 9V variant (ST156) was susceptible to penicillin; however, it seems to have acquired *pbp* genes by horizontal transfer from the Spain^{23F}-1 clone, thus obtaining resistance to β-lactams (Coffey *et al.*, 1991).

Nowadays, the Spain^{9V}-3 variant expressing serotype 14 is widely disseminated, being described as resistant to penicillin, tetracycline, choramphenicol and also to macrolides and/or fluoroquinolones (Ardanuy *et al.*, 2006; De La Campa *et al.*, 2004). This variant has arisen on more than one occasion by large recombinational replacements that extend from the start of the *cps* locus into the *pbp1A* gene (Coffey *et al.*, 1999).

The third most important clone is Spain^{6B}-2, which was found in at least 16 different countries. The major multiresistant phenotype included resistance to penicillin, cephalosporin, tetracycline and choramphenicol. Variants expressing serotypes 14 and 23F were also found for this clone (Aanensen & Spratt, 2005; McGee *et al.*, 2001).

1.6.6 - Capsular Switching

The horizontal transfer of capsular biosynthetic genes in *S. pneumoniae* is called capsular switching, which was described as being related to the hitchhiking effect of *pbp1A* and *ddl* genes, with impact in the β-lactam susceptibility of the recipient strain (Coffey *et al.*, 1991; Coffey *et al.*, 1999). The most frequent serotypes involved in capsular switching on nonsusceptible strains were 23F, 19F, 9V and 14. In a case of a capsular switching from serotype 23F to 14, the donor and the receipt strains had been isolated from the same children (Nesin *et al.*, 1998).

Table 1.6 - Pneumococcal Molecular Epidemiology Network clones as at April 2006 (adapted from Aanensen & Spratt, 2005; McGee, 2008)

Clone	Serotype Variants[a]	Antibiotic resistance phenotype[b]	MIC to Penicillin(mg/L)[c]	ST[d]	Countries[e]
Spain^{23F}-1	23F, 19F, 19A, 9V, 14, NT, 3	PEN TET CHL	2	81	Argentina, Australia, Belgium, Brazil, Bulgaria, Canada, Chile, Colombia, Croatia, Czech Republic, Egypt, Finland, France, Germany, Greece, Hong Kong, Hungary, Iceland, Ireland, Italy, Japan, Korea, Malaysia, Mexico, Oman, Poland, Portugal, Singapore, South Africa, Spain, Sri Lanka, Taiwan, Thailand, The Netherlands, UK, USA, Uruguay, Vietnam
Spain^{6B}-2	6B, 14, 23F	PEN CTX TET CHL	2	90	Australia, Chile, Colombia, Finland, France, Germany, Hong Kong, Iceland, Italy, Poland, Portugal, Spain, Taiwan, The Netherlands, UK, USA
Spain^{9V}-3	9V, 14, 19F, 11A, NT, 9A, 15C, 13	PEN CTX	2	156	Argentina, Brazil, Bulgaria, Canada, Chile, Colombia, Czech Republic, Denmark, France, Germany, Greece, Hungary, Ireland, Israel, Italy, Korea, Lebanon, Mexico, Poland, Portugal, Qatar, Spain, Sweden, Switzerland, Syria, Taiwan, Thailand, The Netherlands, UK, USA, Uruguay.
Tennessee^{23F}-4	23F	PEN CTX ERY	0.12	37	Denmark, Finland, Germany, USA
Spain^{14}-5	14	PEN CTX TET CHL	2	18	Spain
Hungary^{19A}-6	19A	PEN TET ERY CLI CHL	2	268	Czech Republic/Slovakia, Hungary
S. Africa^{19A}-7	19A	PEN	0.5	75	South Africa
S. Africa^{6B}-8	6B	PEN	0.5	185	Australia, South Africa
England^{14}-9	14, 19F	ERY	0.03	9	Argentina, Australia, Belgium, Germany, Greece, Hungary, Italy, Poland, Portugal, Switzerland, UK, USA
CSR^{14}-10	14	PEN CTX TET ERY CLI CHL	8	20	Colombia, Czech R./Slovakia
CSR^{19A}-11	19A	PEN CTX TET ERY CLI CHL	4	175	Czech R./Slovakia
Finland^{6B}-12	6B	PEN TET ERY CLI	1	270	Finland, Portugal
S.Africa^{19A}-13	19A	PEN CTX TET ERY CLI CHL	8	41	South Africa, USA
Taiwan^{19F}-14	19F, 6, 38	PEN CTX TET ERY CHL	2	236	Australia, China, Germany, Greece, Hong Kong, Korea, Malaysia, Oman, South Africa, Taiwan, UK, USA, Vietnam
Taiwan^{23F}-15	23F	PEN CTX TET ERY CLI	1-2	242	Brazil, Germany, Italy, Norway, Taiwan, USA
Poland^{23F}-16	23F	PEN CTX TET ERY CLI	8	173	Poland
Maryland^{6B}-17	6B	PEN CTX ERY	4	384	USA
Tennessee^{14}-18	14, 9L	PEN CTX TET ERY CLI	8	67	Germany, Taiwan, USA
Colombia^{5}-19	5, NT	TET CHL	0.03	289	Brazil, Colombia, Egypt, Germany, Italy, Niger, Poland, Switzerland, UK, USA
Poland^{6B}-20	6B, 23F	PEN TET ERY CLI	0.12	315	Bulgaria, Germany, Italy, Kuwait, Poland, Portugal, Russia, Slovakia, Spain, Sri Lanka, USA
Portugal^{19F}-21	19F	TET ERY CLI CHL	0.06	177	Brazil, Germany, Greece, Iceland, Iceland, Portugal, Spain, USA
Greece^{6B}-22	6B, 6A	TET ERY CLI CHL	0.015	273	Finland, Germany, Greece, Iceland, Israel, Italy, Portugal, Switzerland
N.Carolina^{6A}-23	6A	PEN CTX ERY	1	376	USA
Utah^{35B}-24	35B	PEN CTX	2	377	USA
Sweden^{15A}-25	15A, 23F, 19F, 19A, 14	PEN TET ERY CLI	0.12	63	France, Germany, Italy, Niger, Portugal, Spain, Sweden
Colombia^{23F}-26	23F, 23A	PEN	0.12	338	Brazil, Colombia, France, Iceland, Malaysia, Norway, Portugal

[a] According to the serotypes described in the pneumococcal MLST database at Abril of 2006 (Aanensen & Spratt, 2005); NT, non-typeable.
[b] PEN, penicillin; CTX, cefotaxime; TET, tetracycline; ERY, Erythromycin; CLI, clindamycin; CHL, chloramphenicol.
[c] Minimal inhibitory concentration to penicillin.
[d] ST, Sequence Type
[e] Countries where the respective clone had been reported

Capsular switching has been detected in many other serotypes, such as serotypes 3, 6, 15 and 24F (Gherardi *et al.*, 1999; Gherardi *et al.*, 2000; Gherardi *et al.*, 2007; Jefferies *et al.*, 2004; McEllistrem *et al.*, 2003; Medina *et al.*, 2005; van Selm *et al.*, 2003) (Table 1.6). Nowadays, in the presence of the heptavalent pneumococcal conjugate vaccine, capsular switching has an increased importance due to its role in the replacement effect of the *S. pneumoniae* disease. McEllistrem *et al.* (2003) has reported between 1999 and 2001, in the United States, rates of capsular switching from 26% to 30% in the nonvaccine serogroups.

The magnitude of the consequences of these dynamic exchanges is difficult to predict. Thus, Nesin *et al.*, (1998) has shown that, in a mouse model, multiresistant strains with serotype 3 were more virulent than strains with the same genotype expressing serotype 23F. Indeed, it has been documented that *in vivo*, a single recombinational event can change both serotype and penicillin susceptibility. This can impact the potential to reduce antimicrobial resistance by the heptavalent pneumococcal conjugate vaccine (PCV-7) on a long term (Brueggemann *et al.*, 2007).

1.7 - Vaccines against *Streptococcus pneumoniae*

Antibodies to capsular polysaccharide antigens of *S. pneumoniae* provide serotype-specific protection against pneumococcal infections. In 1999, the WHO had highlighted the importance of vaccination in the management of invasive infections as well as in respiratory infections (World Health Organization, 1999a). In addition, the growing resistance of *S. pneumoniae* to commonly used antibiotics underlines the advice of the WHO for the urgent need of vaccines to control pneumococcal disease (World Health Organization, 1999a). Recently, the World Health Organization (2007) considered that it should became a priority to include the PCV-7 vaccine in national immunization programmes, mainly in countries where mortality among children aged less than 5 years old was >50/1000 live births or where more than 50,000 children die annually. It was also recommended that children with sickle-cell disease or in countries with a high prevalence of HIV should be vaccinated as well (World Health Organization, 2007a).

Nevertheless, the variability of serotypes is considerable when talking the geographic region into account, making it important to obtain knowledge about the rates of resistance to antimicrobial agents before the implementation of a vaccine in a certain location. Factors such as fatality rate and virulence characteristics of each serotype on a population are also important to consider in the choice of a vaccine for each population.

1.7.1 - Pneumococcal capsular polysaccharide vaccine

The polysaccharide vaccine currently in the market includes 23 purified capsular polysaccharide antigens of the more frequent *S. pneumoniae* strains. This vaccine includes serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F (Centers for Disease Control and Prevention, 1997). The pneumococcal polysaccharide vaccine (Pneumovax 23) was licensed in the United States in 1983 and became available in Portugal as Pneumo23 since 2003 (Centers for Disease Control and Prevention, 1997; Índice Nacional Terapêutico, 2003).

However, several problems were associated with these vaccines. The major problem was the absence of imunogical response in children under 2 years of age, one of the most critical age group, associated with elevated rates of meningeal infections caused by pneumococci. (van Dam *et al.*, 1990). It also does not protect against upper respiratory tract infections, such as acute otitis media and sinusitis, and does not produce immunization on asymptomatical carriers (Schrag *et al.*, 2000).

The 23 capsule types in the vaccine represent at least 85-90% of the serotypes that cause invasive *S. pneumoniae* infections among children and adults in the United States and it includes the six serotypes (6B, 9V, 14, 19A, 19F, and 23F) that most frequently cause invasive drug-resistant pneumococcal infection in that country (Centers for Disease Control and Prevention, 1997). Another drawback is the weak imunological response against serotypes 6B, 14, 19F, 23F, which are associated to the majority of strains resistant to β-lactams (Butler, 1997).

Immunization against *S. pneumoniae* disease by the pneumococcal polysaccharide vaccine is recommended for all adults and children with 2 years of age and older, particularly (Pai *et al.*, 2002; World Health Organization, 1999a; World Health Organization, 2007a):

- Adults older than 65, to protect against invasive disease;
- Adults and children with 2 to 64 years of age and chronic illnesses, such as sickle cell disease;
- Those with spleen problems or without spleens;
- Those who are at increased risk for *S. pneumoniae* disease, because of other illnesses (e.g., heart disease, lung disease, diabetes, alcoholism, liver disease, HIV infection, organ or bone marrow transplantations, and cancer);
- Those living in special environments or social settings (e.g., Alaskan Natives and certain American Indian populations);
- Those residents in nursing homes, or other long-term-care facilities.

1.7.2 - Pneumococcal conjugate capsular polysaccharide vaccines

The pneumococcal conjugate vaccines are designed to cover the serotypes most frequently associated with severe pneumococcal disease, as well as the main serotype associated with antibiotic-nonsusceptibility (Hausdorff *et al.*, 2000a). Presently, PCV-7 was registered in more than 70 countries and included in at least 12 national immunization programmes. The PCV-7 is available in Portugal since 2001 and in the United States since 2000 (Centers for Disease Control and Prevention, 2002; Índice Nacional Terapêutico, 2003). In most but not all countries, three doses of vaccine are administered during the first year of life, conferring a high level of protection against invasive pneumococcal disease. In some countries, two doses of PCV-7 are given during the first year, with a third dose offered early in the second year of life (World Health Organization, 2007a). Following primary immunization in infancy, the duration of protection against invasive disease has so far been shown to be at least 2–3 years, but it is expected to last considerably longer.

The PCV-7 protects against infections caused by serotypes 4, 6B, 9, 14, 18C, 19F and 23F, but also produces antibodies with cross reaction against serotype 6A. However, this latter serotype is not functionally reactive in vaccinated children (Vakevainen *et al.*, 2001). According to the work of Hausdorff *et al.* (2000a,b), PCV-7 serotypes account for 65-80% of strains associated with IPD among young children in western industrialized countries. In general, in young children, protection against IPD caused by vaccine serotypes may exceed 90%. However, this coverage varies in different populations and may be lower in many developing countries (Hausdorff *et al.*, 2000a; Hausdorff *et al.*, 2000b). Other pneumococcal conjugate vaccines with wider serotype coverage are in phase III clinical trials: a 10-valent vaccine (PCV-7 and serotypes 1, 5 and 7F) and a 13-valent vaccine (PCV-10 and serotypes 3, 6A and 19A) (U.S. National Institutes of Health, 2008).

PCV-7 is well tolerated and has a good safety profile. It induces a T-cell dependent immune response characterized by immune memory, as well as a booster antibody response on subsequent challenge with the pneumococcal polysaccharides included in the vaccine. PCV-7 is effective against invasive infection, pneumonia, acute otites media as well as immunize asymptomatic carriers (Alpern *et al.*, 2001; Darkes & Plosker, 2002; Pelton & Klein, 2002). However, this vaccine does not significantly reduce the incidence of acute otites media episodes (Straetemans *et al.*, 2002). It also stimulates mucosal immunity, resulting in reduced nasopharyngeal carriage (Jones *et al.*, 2005), and a herd immunity effect it was also observed, most likely as a result of the reduction of transmission of vaccine-type pneumococci in the population (O'Brien *et al.*, 2007). PCV-7 is highly immunogenic in all age groups, but it is currently licensed for use only in children aged <5 years, including infants aged <12 months (Centers for Disease Control and Prevention, 2002). This vaccine is also recommended for patients at high risk of invasive pneumococcal disease, as mentioned for the polysaccharide vaccine (World Health Organization, 2007a).

CHAPTER II

Antimicrobial susceptibility of invasive *Streptococcus pneumoniae* isolates in Portugal over an 11-year period

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2.1 - Summary

This national surveillance study presents the *in vitro* activities of the main antimicrobial agents against 1,331 *Streptococcus pneumoniae* isolates as tested by an agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS). The strains were isolated in several regions of Portugal from cases of invasive disease over an 11-year period (1994 to 2004). This study shows that the percentage of penicillin-nonsusceptible strains increased from 12% in 1994 to 28.5% in 2000. Then the rate declined to 17.7% in 2003 but increased again to 23.2% in 2004. Nevertheless, the rate of highly resistant isolates declined consistently, to 0.9% in 2001 to 2004. Ceftriaxone- and cefotaxime-nonsusceptible isolates became less frequent, from 4% and 8%, respectively, in 1994 to <1% in 2004. The macrolide-lincosamide-streptogramin B phenotype was the predominant macrolide phenotype found. The increase in the percentage of isolates that were only nonsusceptible to erythromycin (3.7% in 1994 to 1998 to 9.1% in 2002 to 2004) was similar to that for isolates with coresistance to penicillin and erythromycin (3.3% in 1994 to 1998 to 9.1% in 2002 to 2004). The nonsusceptibility to ciprofloxacin increased during recent years, from 0.5% in 2002 to 3.5% in 2004. Multiresistance also increased in recent years: from 7.9% in 2002 to 15.6% in 2004. The increasing use of macrolides could be causing the increase in penicillin and multiresistance, due to the coresistance to macrolides. The use of penicillin to treat empirical non-severe invasive pneumococci infections may need to be reconsidered.

2.2 - Introduction

Streptococcus pneumoniae is an important pathogen responsible for serious invasive diseases, including meningitis and septicemia. The spread of multidrug-resistant (MDR) pneumococci has become a worldwide problem, making treatment more difficult (Klugman, 2002). Indeed, in addition to resistance to penicillin, resistance to other antibiotics, including erythromycin, tetracycline and chloramphenicol, has emerged and is spreading (Felmingham *et al.*, 2002).

Since 1989, the National Institute of Health (NIH) Dr. Ricardo Jorge reference laboratory has been continually monitoring the *in vitro* activity of antimicrobial agents against *S. pneumoniae* collected from invasive sources. This Antimicrobial Resistance Surveillance Program in Portugal (ARSIP) provides a unique collection of Portuguese pneumococci isolates. This national surveillance study reported that 4.6% of isolates were penicillin-nonsusceptible in 1989, and this value remained generally stable until 1991 (6.4%) (Pato *et al.*, 1995). Fully penicillin-resistant isolates (minimal inhibitory concentrarion [MIC] of 2 mg/L) were reported for the first time in 1992 (0.8%), and made up 5.5% of isolates in the following year (Pato *et al.*, 1995).

Here, we describe the surveillance of pneumococci by the reference laboratory in Portugal. We report the *in vitro* activities of different antimicrobial agents used against *S. pneumoniae* isolated from invasive sources over 11 years (from 1994 to 2004).

2.3 - Materials and methods

Patients and bacterial isolates. Between 1 January 1994, and 31 December 2004, the ARSIP survey conducted by the Antibiotic Resistance Unit (ARU) from the NIH Dr. Ricardo Jorge constantly monitored pneumococcal isolates from cases of invasive disease in various regions of Portugal. The national laboratory-based surveillance system collected 1,331 invasive pneumococcal strains, which were isolated in 24 bacteriology laboratories in hospitals and public health institutions. In the period 1994 to 1998, 12 hospitals participated in the study, and since 1999, 12 more hospitals have been added to the network. Isolates were included if they were nonrepetitive or consecutive blood, cerebrospinal fluid (CSF) or pleural fluid samples from patients with symptoms compatible with invasive pneumococcal disease. No changes were made to the methods of data collection during the study. Some isolates were from outpatients, but most were from patients hospitalized with community-acquired invasive pneumococci disease. Only one isolate per patient was considered. Patients over 15 years old were considered to be adults.

Identification and serotyping. The isolates were sent at -20ºC by hospital laboratories to the reference laboratory, ARU, in Triptycase soy broth (TSB; Oxoid, Basingstoke, England) containing 20% glycerol. On reception by the ARU, the purity of the pneumococcal isolate was checked using standard methods, and the isolate then stored at -80ºC in TSB containing 20% glycerol. Isolates were serotyped by dot-blotting, the Quellung reaction, or both (Fenoll *et al.*, 1997).

Antimicrobial susceptibility testing. Susceptibility testing was performed by the agar dilution method. MICs of penicillin (Wyeth Lederle Portugal, Algés), cefotaxime (Farma-APS Produtos Farmacêuticos, Lisboa), ceftriaxone (Roche Farmacêutica Química, Amadora), tetracycline (Laboratórios Atral, Carregado), chloramphenicol (Edol, Linda-a-Velha), erythromycin (Abbott Laboratórios, Amadora), clindamycin (Pharmacia Corporation Laboratórios, Carnaxide), ofloxacin (Aventis Pharma, Mem-Martins), ciprofloxacin (Bayer Portugal, Carnaxide) and vancomycin (Lilly Farma, Algés) were determined according to the testing conditions and susceptibility interpretation standards proposed by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) (National Committee for Clinical Laboratory Standards, 2004). Susceptibility to trimethoprim-sulfamethoxazole was performed by a disk diffusion method according to CLSI recommendations (National Committee for Clinical Laboratory Standards, 2004). Isolates with intermediate- or with high-level resistance were classified as nonsusceptible. Isolates that were nonsusceptible to at least three different antibiotic classes were classified as multidrug resistant. Erythromycin-nonsusceptible isolates were classified as having the macrolide (M) or macrolide-lincosamide-streptogramin B (MLS_B) phenotypes. The M phenotype was scored when the isolate was nonsusceptible only to erythromycin. The MLS_B phenotype was scored when the

isolate was nonsusceptible to both erythromycin and clindamycin (Leclercq & Courvalin, 2002). MICs to vancomycin and ciprofloxacin were only determined from 1 January 1999. An isolate with a MIC of ciprofloxacin ≥4 mg/L was considered as nonsusceptible according to the association with mutations in the genes encoding the DNA topoisomerase IV and DNA gyrase A (Richardson *et al.*, 2001).

Statistical analyses. SPSS software, version 13.0 was used for statistical analysis. The chi-square test or Fisher's exact test were used when appropriate. Two-sided *P* values of < 0.05 were considered to be statistically significant. Correlations between antimicrobial nonsusceptibility rates were assessed using the Spearman correlation coefficient.

2.4 - Results

A total of 1,331 pneumococci strains were isolated during an 11-year period in 24 hospitals and public health institutions across Portugal and were included in the study: 73.3% were isolated from blood, 20.4% from CSF, 4.5% from pleural fluid and 1.9% from both blood and CSF (Table 2.1). The age of the patient was known for 1,219 (91.6%) of the isolates. Ninety-seven (8.0%) isolates were from patients <1 year old, 156 (12.8%) were from patients 1 to 9 years old, 77 (6.3%) were from patients 10 to14 years old, 557 (45.7%) were from patients 15 to 64 years old, and 332 (27.2%) from patients ≥ 65 years of age. The ages of patients for the remaining 112 isolates were unknown.

Table 2.1 - Penicillin susceptibility and multidrug resistance according to biological source

Period[a]	Biological source	PEN[b]	No. (%) of isolates with MDR[c]	Total no. of isolates
1994-1998	Blood	22 (10.3/3.0)	9 (5.7)	165
	CSF	20 (18.6/4.7)	5 (5.9)	86
	CSF + blood	0 (0)	0 (0)	1
	Pleural fluid	4 (11.8/11.8)	3 (17.6)	17
1999-2001	Blood	78 (18.4/3.7)	23 (6.6)	354
	CSF	20 (16.2/2.9)	5 (4.8)	105
	CSF + blood	5 (41.7/0.0)	0 (0)	12
	Pleural fluid	6 (21.1/10.5)	2 (11.1)	19
	Pleural fluid + blood	0 (0)	0 (0)	1
2002-2004	Blood	98 (20.9/0.7)	57 (12.9)	455
	CSF	16 (18.8/1.3)	7 (9.0)	80
	CSF + blood	1 (0.0/8.3)	0 (0)	12
	Pleural fluid	4 (16.7/0.0)	1 (4.3)	24

[a] For the period 1994 to 1998, 12 laboratories participated; and for the periods 1999 to 2001 and 2002 to 2004, 24 laboratories participated. The period 2002 to 2004 was after the introduction of pneumococcal conjugate vaccine.
[b] No. of isolates nonsusceptible to penicillin (% of isolates with intermediate / high-level resistance).
[c] Multidrug resistance.

The *in vitro* susceptibility data for 10 antimicrobial agents are presented in Table 2.2. The proportion of penicillin-nonsusceptible isolates was 20.6% (18.0% with intermediate resistance [MIC of 0.1 to 1 mg/L] and 2.6% with high-level resistance [MIC of ≥ 2 mg/L] and the MIC_{90} was 0.5 mg/L (Table 2.2). Isolates with intermediate penicillin resistance became more frequent: from 13.0% in the period 1994 to 1998 and 18.5% in 1999 to 2001 to 20% in 2002 to 2004 (P = 0.047) (Table 2.3). However, the percentage of isolates with high level resistance decreased from 4.1%, to 3.7% and then to 0.9% (P = 0.003) (Table 2.3). The percentage of isolates nonsusceptible only to penicillin decreased in the most recent years to 11.7% (2002 to 2004). Penicillin nonsusceptibility was found associated with macrolide

Table 2.2 - Cumulative MICs of 10 antimicrobial agents for isolates of *S. pneumoniae* collected in Portugal between 1994-2004

Antimicrobial Agent	Cumulative % of isolates inhibited by MIC (mg/L) of:													% of isolates with resistance [a]:		Total no. of isolates
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	I	R	
Penicillin	57.7[b]	76.8	79.4	84.7	86.2	92.3	97.4	100.0[c]						18.0	2.6	1,331
Cefotaxime	5.3[b]	57.1	60.6	62.6	87.8	94.3	98.8	99.9	100.0					2.3	0.2	1,329
Meningeal isolates	5.1[b]	62.8	68.9	71.6	88.5	93.6	99.3	100.0						5.7	0.7	296
Nonmeningeal isolates	5.3[b]	55.5	58.3	60.0	87.6	94.5	98.6	99.9	100.0					1.2	0.1	1,033
Ceftriaxone	4.1[b]	56.6	60.8	62.5	87.4	94.8	99.2	99.9	100.0					1.9	0.2	1,329
Meningeal isolates	2.7[b]	62.2	68.9	71.3	88.5	94.3	99.7	100.0						5.4	0.3	296
Nonmeningeal isolates	4.5[b]	55.0	58.5	60.0	87.1	95.0	99.0	99.9	100.0					0.9	0.1	1,033
Tetracycline			0.7[b]	9.8	50.1	62.4	85.4	87.7	88.5	90.4	94.0	97.2	100.0[c]	0.8	11.5	1,329
Erythromycin		1.3[b]	49.1	85.2	85.6	86.2	89.5	90.4	91.6	93.4	94.1	95.7	100.0[c]	0.5	13.8	1,329
Clindamycin		0.8[b]	61.0	90.1	90.6	90.8	92.6	93.0	93.2	93.5	93.9	94.9	100.0[c]	0.2	9.2	1,322
Choramphenicol						1.2	8.5	74.1	97.3	98.4	100.0			NA	2.7	1,322
Ofloxacin						0.5	29.0	98.0	99.7	99.8	99.8	100.0		1.7	0.3	1,322
Ciprofloxacin				0.1	3.4	22.6	81.4	98.6	99.7	99.8	99.8	99.8	100.0	NA	1.4	1,104
Vancomycin			0.1[b]	6.1	57.4	100.0								0.0	0.0	1,103

[a] I, Intermediate; R, resistant. NA, not applicable.
[b] MIC equal to or less than the given value.
[c] MIC equal to or greater than the given value.

Table 2.3 - Year-to-year changes in nonsusceptible and multidrug-resistant *S. pneumoniae* isolates during 11-year study

Year	% of nonsusceptible isolates (no. intermediate/resistant)[a]									Total no. of isolates	% (no.) of isolates with MDR[b]
	PEN	CRO	CTX	TET	ERY	CLI	CHL	CIP	OFX		
1994	12.0 (2/1)	4.0 (1/0)	8.0 (2/0)	8.0 (1/1)	4.0 (0/1)	4.0(1/0)	4.0 (NA/1)	ND	4.0 (0/1)	25	4.0 (1)
1995	20.0 (13/1)	2.9 (1/1)	2.9 (1/1)	10.0 (0/7)	5.7 (0/4)	2.9 (0/2)	4.3 (NA /3)	ND	4.3 (3/0)	70	2.9 (2)
1996	14.5 (4/5)	4.8 (3/0)	8.1 (5/0)	11.3 (0/7)	4.8 (0/3)	4.8 (0/3)	6.5 (NA/4)	ND	4.8 (3/0)	62	8.2 (5)
1997	18.2 (8/4)	4.5 (3/0)	7.6 (4/1)	9.1 (1/5)	7.6 (0/5)	6.1 (0/4)	0.0 (NA/0)	ND	1.5 (1/0)	66	6.3 (4)
1998	17.4 (8/0)	2.2 (1/0)	0.0 (0/0)	4.3 (0/2)	13.0 (0/6)	6.5 (0/3)	2.2 (NA/1)	ND	2.2 (1/0)	46	11.4 (5)
1999	16.6 (19/10)	2.9 (5/0)	2.3 (5/0)	8.7 (1/14)	12.7 (0/22)	9.2(0/16)	1.2 (NA/2)	1.2 (NA/2)	1.2 (0/2)	175	4.6 (8)
2000	28.5 (35/4)	2.9 (3/1)	4.4 (5/1)	13.1 (2/16)	11.7 (1/15)	10.9(0/15)	2.2 (NA/3)	0.0 (NA/0)	0.0 (0/0)	137	6.6 (9)
2001	22.9 (37/4)	2.2 (4/0)	2.2 (4/0)	11.2 (0/20)	16.8 (0/30)	11.7 (0/21)	2.2 (NA/4)	0.6 (NA/1)	1.1 (2/0)	179	7.3 (13)
2002	21.1 (42/2)	1.4 (3/0)	1.4 (3/0)	10.0 (1/20)	12.0 (0/25)	7.7 (0/16)	2.9 (NA/6)	0.5 (NA/1)	0.0 (0/0)	209	7.9 (16)
2003	17.7 (28/1)	0.0 (0/0)	0.0 (0/0)	17.7 (3/26)	20.1 (2/31)	13.0 (2/19)	3.1 (NA/5)	1.8 (NA/3)	1.2 (2/0)	164	11.8 (19)
2004	23.2 (44/2)	0.5 (1/0)	0.5 (1/0)	18.2 (1/35)	23.2 (4/42)	11.4 (0/22)	3.6 (NA/7)	3.5 (NA/7)	5.7 (10/1)	198	15.6 (30)
Total	20.6 (240/34)	2.0 (25/2)	2.4 (30/3)	12.3 (10/153)	14.4 (7/184)	9.4 (3/121)	2.7 (NA/36)	1.4 (NA/15)	2.0 (22/4)	1331	8.6 (112)

[a] PEN, penicillin; CRO, ceftriaxone; CTX, cefotaxime; TET, tetracycline; ERY, Erythromycin; CLI, clindamycin; CHL, chloramphenicol; CIP, ciprofloxacin; OFX, ofloxacin. NA, not applicable; ND, not determined.
[b] MDR, Multidrug resistance

nonsusceptibility (*P* = 0.002), trimethoprim-sulfamethoxazole resistance (*P* = 0.017), and multidrug resistance (*P* = 0.001) among adults. The isolates with intermediate penicillin resistance from adults were also associated with isolates nonsusceptible to penicillin plus erythromycin isolates (*P* = 0.006). Similar proportions of isolates had intermediate and high-level resistance in blood (18.1%, and 2.4%, respectively) and CSF (17.9% and 3.0%, respectively) (*P* = 0.84) (Table 2.1). Thus, the proportions of isolates nonsusceptible to penicillin were similar for the blood (20.5%) and CSF (20.9%) isolate subgroups (*P* = 0.87) (Table 2.1). Isolates with intermediate resistance were more common in children between 1 and 9 years of age (36.5%) (Figure 2.1). The frequencies of both isolates with intermediate resistance (23.0%) and those with high-level resistance (6.0%) were higher in children than in adults (15.8%; 1.0%, respectively) (*P* < 0.001) (Figure 2.1). Serotype 14 was consistently the most frequent of the nonsusceptible (both intermediate and high-level resistance) isolates. Serogroup 19 increased approximately fivefold between the periods 1999 to 2001 and 2002 to 2004. Between the same periods, serogroups 23, 9 and 6 decreased (Table 2.4).

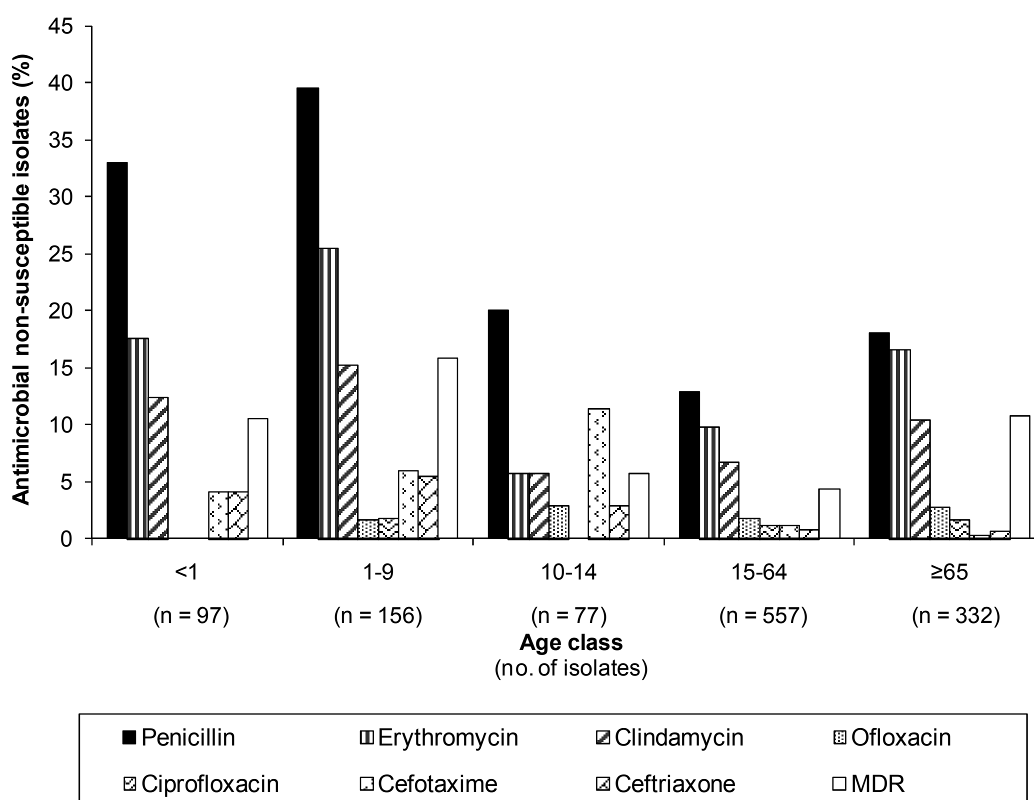

Figure 2.1 - Distribution by age class of *S. pneumoniae* isolates that were nonsusceptible to antimicrobial agents.

S. pneumoniae isolates that were nonsusceptible to either cefotaxime (*P* = 0.001) and ceftriaxone (*P* = 0.007) became rarer over the time, from 5.2% and 3.7% (1994 to 1998), respectively, to 0.7 (2002 to 2004) (Table 2.3). *S. pneumoniae* isolates were classified for susceptibility to cephalosporins according to two sets of CLSI criteria: those in use before 2002 and those in use after 2002 (Table 2.5). Nonsusceptibility to celphalosporins appeared to be less frequent after 2002. According to the 2002 guidelines, isolates nonsusceptible to cefotaxime (*P* < 0.001) and ceftriaxone (*P* < 0.001) were more frequently recovered from CSF (6.4% and 5.7%, respectively) than blood (1.3% and 1.0%, respectively). Using the CLSI guidelines in force before 2002, no such association was found for either cefotaxime (6.4% *versus* 5.5%; *P* = 0.57) or ceftriaxone (5.7% *versus* 5.0%; *P* = 0.66). Cefotaxime-nonsusceptible isolates were more prevalent in the 10- to 14-year-old age group (11.4%; *P* = 0.005), whereas isolates that were nonsusceptible to ceftriaxone were most common in the 1- to 9-year-old age group (5.4%; *P* < 0.001) (Figure 2.1). The main serotype and serogroup among these isolates were serotype 14 (51.9% of isolates nonsusceptible to ceftriaxone and 56.3% of those nonsusceptible to cefotaxime) and serogroup 9 (25.9% for ceftriaxone, and 25.0% for cefotaxime) (Table 2.4).

Among the 191 erythromycin-nonsusceptible (MIC, ≥ 0.5 mg/L) isolates recovered in this study (14.4% of all isolates; Table 2.2), 67.4% were also clindamycin-nonsusceptible (MLS_B) and 32.6% had the M phenotype. Erythromycin nonsusceptibility increased from 7.1% (1994 to 1998) to 18.2% (2002 to 2004) among pneumococci (*P* < 0.001) (Table 2.3). The frequency of MLS_B phenotype was 68.4% in the period from 1994 to 1998, increased to 76.5% in 1999 to 2001 and then decreased to 60.8% in 2002 to 2004 (*P* < 0.001). Isolates nonsusceptible only to macrolides increased from 3.7% (1994 to 1998) to 8.4% (1999 to 2001) and then to 9.1% (2002 to 2004). Erythromycin nonsusceptibility was associated with multidrug resistance (*P* = 0.017) and with nonsusceptibility to penicillin plus erythromycin (*P* < 0.001) among children. In isolates from adults, erythromycin nonsusceptibility was also associated with tetracycline (*P* < 0.001), chloramphenicol (*P* = 0.017) and ciprofloxacin (*P* = 0.012) nonsusceptibility. Erythromycin- and clindamycin-nonsusceptible isolates were most frequent among the isolates from children 1 to 9 years of age (25.5% and 15.2% respectively), children <1 year of age (17.5% and 12.4%, respectively), and adults ≥65 years of age (16.6% and 10.4%, respectively) (Figure 2.1). Many erythromycin-nonsusceptible isolates were of serogroups 19 and 15, and serotype 33 emerged after the period 1999-2001. Overall, however, serotype frequencies did not change through time (*P* = 0.068) (Table 2.4).

During the period of the study, the frequencies of ciprofloxacin- and ofloxacin-nonsusceptible isolates reached 1.4% and 2.0%, respectively; the MIC_{90} of both antibiotics was 2 mg/L (Table 2.2). Isolates nonsusceptible to ciprofloxacin increased from 0.6% (1999-

2001) to 1.8% (2003) and to 3.5% (2004) (P = 0.04) (Table 2.3). The percentage of pneumococci nonsusceptible to ofloxacin decreased from 3.3% (1994 to 1998) to 0.8% (1999 to 2001) and then increased to 2.3% (2002 to 2004) (P = 0.04) (Table 2.3). The proportion of isolates from children that were nonsusceptible to ciprofloxacin (1.3%) was not significantly different from that observed for isolates from adults (2.1%) (P = 0.47) (Figure 2.1). Ciprofloxacin-nonsusceptible isolates were from serotypes 1, 6, 11, 22, 33 and mainly from serotype 14 (26.7%), which appeared only since the period 1999 to 2001 (Table 2.4). All isolates were susceptible to vancomycin, with a MIC_{90} of 0.5 mg/L (Table 2.2).

The frequency of multidrug-resistant pneumococci were 6.5% (1994 to 1998) and 6.2% (1999 to 2001), and then increased to 11.7% in 2002 to 2004 (P = 0.002) (Table 2.3). The proportions of multidrug-resistant isolates among blood (9.3%) and CSF (6.3%) isolates were similar (P = 0.28) (Table 2.1). Isolates with multidrug resistance were most frequent in children of the 1- to 9-year-old age group and in adults ≥65 years old (Figure 2.1). Overall, the multidrug resistance of pneumococci isolated from children (13.1%) was higher than that from adults (6.7%) (P < 0.001). In children, the rate of multidrug-resistant isolates was mainly associated with macrolides (P = 0.017), and in adults this rate was mainly associated with resistance to penicillin (P = 0.001), tetracycline (P = 0.005), macrolides (P > 0.001), chloramphenicol (P = 0.037), and ciprofloxacin (P = 0.03). Among multidrug-resistant isolates, serogroup 6 and serotype 14 decreased greatly between the periods 1999 to 2001 and 2002 to 2004, and the serotype 23 decreased from 1994 to 1998 to 7.7% in the period 2002 to 2004. In contrast, serogroup 19 was not found in 1994 to 1998 but made up 43.1% of the isolates in 2002 to 2004 (P < 0.001) (Table 2.4). The 29 multidrug-resistant phenotypes observed during the 11 years of the study are shown in Table 2.6. The predominant phenotype of multidrug resistance was nonsusceptibility to penicillin, tetracycline, erythromycin, and clindamycin. Strains with this phenotype were mostly serotypes 6, 14, 15, 19 and 24. The second most frequent phenotype was nonsusceptibility to penicillin, erythromycin and trimethoprim-sulfamethoxazole and all isolates with this phenotype were serotype 14 or serotype 1. The multidrug-resistant phenotype involving nonsusceptibility to penicillin, tetracycline and erythromycin (the third most frequent) was only found in 2002 to 2004 and only among isolates of serotype 19. Only 6 of the 29 phenotypes did not include isolates with nonsusceptibility to macrolides. Isolates with coresistance to penicillin and erythromycin became increasingly frequent, rising from 3.3% (1994 to 1998) to 5.5% (1999 to 2001) and then 9.1% (2002 to 2004) (P = 0.001).

Table 2.4 - Serotype distribution of *S. pneumoniae* isolates that were nonsusceptible to different antimicrobial agents from 1994 to 2004

Antimicrobial agent and period	No. (%) of isolates with the pneumococcal serogroup/type:														Total
	1	3	6	7	9	14	15	17	19	23	24	33	Others[a]	ND[b]	
Penicillin															
1994-1998	0		3 (6.5)	0	7 (15.2)	20 (43.5)	1 (2.2)	0	3 (6.5)	10 (21.7)	0		2 (4.3)	0	46 (100)
1999-2001	0		7 (6.4)	1 (0.9)	17 (15.6)	58 (53.2)	1 (0.9)	0	5 (4.6)	19 (17.4)	0		1 (0.9)	0	109 (100)
2002-2004	1 (0.8)		4 (3.4)	0	10 (8.4)	37 (31.1)	5 (4.2)	1 (0.8)	26 (21.8)	16 (13.4)	2 (1.7)		0	17 (14.3)	119 (100)
Total	1 (0.4)		14 (5.1)	1 (0.4)	34 (12.4)	115 (42.0)	7 (2.6)	1 (0.4)	34 (12.4)	45 (16.4)	2 (0.7)		3 (1.1)	17 (6.2)	274 (100)
Cefotaxime															
1994-1998			0		4 (28.6)	6 (42.9)			0	4 (28.6)					14 (100)
1999-2001			1 (7.1)		4 (28.6)	9 (64.3)			0	0					14 (100)
2002-2004			0		0	3 (75)			1 (25)	0					4 (100)
Total			1 (3.1)		8 (25)	18 (56.3)			1 (3.1)	4 (12.5)					32 (100)
Ceftriaxone															
1994-1998			0		3 (30)	3 (30)			1 (10)	3 (30)					10 (100)
1999-2001			1 (7.7)		4 (30.8)	8 (61.5)			0	0					13 (100)
2002-2004			0		0	3 (75)			1 (25)	0					4 (100)
Total			1 (3.7)		7 (25.9)	14 (51.9)			2 (7.4)	3 (11.1)					27 (100)
Erythromycin															
1994-1998	0	0	7 (36.8)	0	1 (5.3)	6 (31.6)	1 (5.3)	0	2 (10.5)	1 (5.3)	0	0	1 (5.3)	0	19 (100)
1999-2001	0	2 (2.9)	20 (29.4)	2 (2.9)	3 (4.4)	21 (30.9)	3 (4.4)	0	12 (17.6)	3 (4.4)	0	1 (1.5)	1 (1.5)	0	68 (100)
2002-2004	1 (1.0)	0	10 (9.6)	0	3 (2.9)	21 (20.2)	5 (4.8)	1 (1.0)	28 (26.9)	2 (1.9)	2 (1.9)	4 (3.8)	1 (1.0)	26 (25.0)	104 (100)
Total	1 (0.5)	2 (1.0)	37 (19.4)	2 (1.0)	7 (3.7)	48 (25.1)	9 (3.1)	1 (0.5)	42 (22.0)	6 (3.1)	2 (1.0)	5 (2.6)	3 (1.6)	26 (13.6)	191 (100)
Clindamycin															
1994-1998		0	7 (53.8)	0	0	1 (7.7)	1 (7.7)	0	2 (15.4)	1 (7.7)	0	0	1 (7.7)	0	13 (100)
1999-2001		2 (3.8)	17 (32.7)	1 (1.9)	0	14 (26.9)	2 (3.8)	0	12 (23.1)	3 (5.8)	0	1 (1.9)	0 (0.0)	0	52 (100)
2002-2004		0	9 (15.3)	0	2 (3.4)	4 (6.8)	5 (8.5)	1 (1.7)	17 (28.8)	2 (3.4)	2 (3.4)	4 (6.8)	1 (1.7)	12 (20.3)	59 (100)
Total		2 (1.6)	33 (26.6)	1 (1.8)	2 (1.6)	19 (15.3)	8 (6.5)	1 (0.8)	31 (25.0)	6 (4.8)	2 (1.6)	5 (4.0)	2 (1.6)	12 (9.7)	124 (100)
Ciprofloxacin															
1994-1998		0	0			0						0	1 (100)	0	1 (100)
1999-2001		1 (33.3)	0			2 (66.7)						0	0 (33.3)	0	3 (100)
2002-2004		1 (9.1)	1 (9.1)			2 (18.2)						1 (9.1)	1 (9.1)	5 (45.5)	11 (100)
Total		2 (13.3)	1 (6.7)			4 (26.7)						1 (6.7)	2 (13.3)	5 (33.3)	15 (100)

[a] Include serogroups/types 4 (n=1), 8 (n=1), 10 (n=1), 11 (n=1); 22 (n=1) and 34 (n=1).
[b] Not determined.

Table 2.5 - Frequencies of susceptibility to cefotaxime and ceftriaxone among 1329 *S. pneumoniae* isolates according to CLSI guidelines published before and after 2002

Antimicrobial agent and CLSI guideline	No. (%) of nonsusceptible isolates						
	1994-1998	1999	2000	2001	2002	2003	2004
Cefotaxime							
Before 2002	20 (7.4)	16 (9.2)	11 (8.0)	10 (5.6)	7 (3.3)	6 (3.7)	6 (3.0)
After 2002	14 (5.2)	4 (2.3)	6 (4.4)	4 (2.2)	3 (1.4)	0 (0.0)	1 (0.5)
Ceftriaxone							
Before 2002	16 (5.9)	17 (9.8)	8 (5.8)	10 (5.6)	7 (3.3)	7 (4.3)	4 (2.0)
After 2002	10 (3.7)	5 (2.9)	4 (2.9)	4 (2.2)	3 (1.4)	0 (0.0)	1 (0.5)
Total no. of isolates	269	173	137	179	209	164	198

Table 2.6 - Distribution of 112 multidrug-resistant *S. pneumoniae* isolates by serogroup/type in 1994 to 1998, 1999 to 2001, and 2002 to 2004

Multidrug resistance phenotype[a]	No. of MDR isolates/period[b]			Total no. of isolates	Serogroup/type (%)
	1994-1998	1999-2001	2002-2004		
PEN TET ERY CLI	1	9	22	32	19 (31.3), 14 (25.0), 6 (15.6), 15 (15.6), 24 (6.3), 17 (3.1), 23 (3.1)
PEN ERY SXT	2	1	7	10	14 (90.0), 1 (10.0)
PEN TET ERY			9	9	19 (100)
TET ERY CLI SXT	2	5	1	8	6 (75.0), 14 (35.0)
TET ERY CLI CHL SXT	2	2	3	7	6 (71.4), 19 (28.6)
PEN TET ERY CLI SXT	1	2	4	7	6 (57.1), 9 (28.6), 19 (14.3)
PEN TET ERY CLI CHL SXT		2	4	6	23 (50.0), 6 (16.7), 14 (16.7), 19 (16.7)
PEN TET CHL SXT	1		4	5	23 (80.0), 19 (20.0)
TET ERY CLI CHL			3	3	19 (66.7), 6 (33.3)
PEN TET CHL CTX CRO SXT	2		1	3	23 (66.7), 19 (33.3)
ERY CLI CHL SXT		2		2	6 (100)
PEN ERY CLI SXT	1	1		2	14 (50.0), 15 (50.0)
PEN TET ERY CLI CHL CTX CRO SXT	1	1		2	6 (50.0), 23 (50.0)
TET CHL SXT	1			1	6 (100)
TET ERY CHL			1	1	19 (100)
TET ERY CHL SXT			1	1	6 (100)
PEN OFL CIP SXT	1			1	11 (100)
PEN ERY CTX CRO SXT		1		1	14 (100)
PEN ERY OFL CIP SXT			1	1	14 (100)
PEN TET SXT			1	1	14 (100)
PEN TET CHL CTX SXT	1			1	23 (100)
PEN TET ERY SXT			1	1	19 (100)
PEN TET ERY OFL CIP SXT		1		1	14 (100)
PEN TET ERY CHL SXT			1	1	14 (100)
PEN TET ERY CLI CTX SXT		1		1	14 (100)
PEN TET ERY CLI CTX CRO	1			1	14 (100)
PEN TET ERY CLI OFL CIP			1	1	6 (100)
PEN TET ERY CLI OFL CIP SXT		1		1	14 (100)
PEN TET ERY CLI CHL		1		1	23 (100)
Total	17	30	65	112	

[a] PEN, penicillin; CRO, ceftriaxone; CTX, cefotaxime; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; CIP, ciprofloxacin; OFX, ofloxacin; SXT, trimethoprim-sulfamethoxazole.
[b] MDR, multidrug-resistant.

2.5 - Discussion

Between 1994 and 2004 the ARU at the NIH Dr. Ricardo Jorge in Lisbon recovered 1331 invasive *S. pneumoniae* strains isolated in 24 hospitals covering several regions of Portugal.

The first pneumococci isolates nonsusceptible to penicillin to be described in Portugal were isolated in 1989 (4.6%) as part of a collection of strains from invasive and non-invasive diseases (Vaz Pato *et al.*, 1995). Such strains became more widespread over the years until they made up 9% (1992 to 1994) of isolates collected from invasive disease (Carvalho *et al.*, 1996a). We report that this trend continued, and these strains accounted for 28.5% of isolates in the year 2000. Then the rate declined to 17.7% in 2003 and in 2004 increased again to 23.2%. Overall, the proportion of isolates that was nonsusceptible to penicillin increased until the period 1999 to 2001 (22.2%), and then stabilized in the period 2001 to 2004 (20.8%).

The frequency of penicillin-nonsusceptible pneumococci isolates in our collection is lower than that observed among invasive isolates in other countries, such as Spain (35.6% in 2001 to 2003) (Oteo *et al.*, 2004), France (47.5% in 2000 to 2002) (Decousser *et al.*, 2004), and Israel (37.3% in 2004) (European Antimicrobial Resistance Surveillance System, 2005). It was, however similar to those of several eastern European countries (European Antimicrobial Resistance Surveillance System, 2005) and higher than those in Italy (12.1% in 1999 to 2000) (Moro *et al.*, 2002), Belgium (17.7% in 2000) (Flamaing *et al.*, 2002), Luxembourg (11.2% in 2004). and the majority of the northern European countries (European Antimicrobial Resistance Surveillance System, 2005). Differences in rates of pneumococcal penicillin resistance between countries have been shown to be associated with levels of antimicrobial consumption (Cars *et al.*, 2001; Bronzwaer *et al.*, 2002; Goossens *et al.*, 2005).

We found that penicillin nonsusceptibility was associated with macrolides ($P = 0.002$), trimethoprim-sulfamethoxazole ($P = 0.017$) and multidrug resistance ($P = 0.001$). Similar associations have been reported elsewhere in the world (Klugman, 2002; McCormick *et al.*, 2003; McEllistrem *et al.*, 2005). Previous studies in Portugal gave similar findings, except for macrolides (Vaz Pato *et al.*, 1995; Carvalho *et al.*, 1996a); however, the resistance to macrolides was very low when these studies were conducted.

Despite nonsusceptibility being relatively prevalent in Portugal, the frequency of high-level resistance declined consistently, to 0.9% in 2001 to 2004. Work in animal models (Azoulay-Dupuis *et al.*, 2000; Rieux *et al.*, 2001) and humans (Winston *et al.*, 1999; Metlay *et al.*, 2000) suggests that high-level penicillin resistance is associated with decreased virulence of pneumococci. Several studies reported that nonmeningeal pneumococcal

infections caused by isolates with a penicillin MIC of ≤2 mg/L can be treated successfully with penicillin (Pallares *et al.*, 1987; Yu *et al.*, 2003). However, in severely ill patients with pneumococcal bacteraemia, combination antibiotic therapy reduces mortality (Baddour *et al.*, 2004). Several studies have suggested that initial monotherapy with β-lactams for severe pneumococcal bacteraemia may be suboptimal, and the use of combined therapy involving a macrolide or quinolone may improve the outcome of the disease (Baddour *et al.*, 2004; Waterer, 2005). Empirical combination antibiotherapy avoids discordant therapy in the context of multidrug-resistant isolates, but also causes a selection pressure on those isolates.

Using the 2004 CLSI guidelines (National Committee for Clinical Laboratory Standards, 2004), we found that ceftriaxone- and cefotaxime-nonsusceptible isolates became less prevalent over the time and made up ≤1% of the isolates in 2004. Nevertheless, according to previous CLSI guidelines (National Committee for Clinical Laboratory Standards, 2000), the frequency of isolates nonsusceptible to cephalosporin was stable until 2001 and then only decreased to 3% in 2002 to 2004. These apparent differences are due to the changes in the CLSI susceptibility interpretation according to the source of isolation. Indeed, the number of isolates recovered from blood or CSF in the study influences the total frequency of nonsusceptible isolates. This fact can cause misleading interpretations of the frequency trends of cephalosporin-nonsusceptible isolates. Earlier studies indicated that cephalosporin-nonsusceptible isolates started to increase after 1993 (Carvalho *et al.*, 1996a), and our study suggests that their prevalence started to decline after the introduction of the pneumococcal conjugate vaccine in 2001. These findings support the recommendations for the use of cephalosporins, alone or combination with vancomycin as initial empiric therapy for treatment of bacterial meningitis (Infectious Diseases and Immunization Committee, 2001; World Health Organization, 1998). In Portugal, the antibiotics most used for meningitis have been ceftriaxone (87%) and cefotaxime (10%) (Caniça *et al.*, 2004).

The rates of resistance to macrolides have increased worldwide (Klugman & Lonks, 2005), consistent with our findings in Portugal. This is mainly due to the widespread use of macrolides, mostly azithromycin (Dias & Caniça, 2004; Klugman & Lonks, 2005). In Portugal, the predominant macrolide phenotype was MLS_B, as shown in this study and among isolates recovered from respiratory tract infections (Melo-Cristino *et al.*, 2003). The majority of southern European countries also have a high prevalence of the MLS_B phenotype (Reinert *et al.*, 2005c). In contrast, the M phenotype is predominant in the United States. (McEllistrem *et al.*, 2005).

The rate of isolates nonsusceptible only to erythromycin (3.7% in 1994 to 1998 to 9.1% in 2002 to 2004), showed the same increasing trend as the rate of isolates with coresistance to penicillin and erythromycin (3.3% in 1994 to 1998 to 9.1% in 2002 to 2004).

The increasing use of macrolides could be causing the increase of penicillin and multidrug resistances, due to the coresistance to macrolides. Fatal cases due to macrolide resistance have been described following azithromycin monotherapy (Waterer *et al.*, 2000). The new ketolide, telithromycin, is a potential alternative to the currently used macrolides. However, isolates resistant to telithromycin have already been reported all over the world (Hsueh *et al.*, 2003; Reinert *et al.*, 2005a). It seems that telithromycin may be of limited therapeutic value in the long term due to the associated resistance mechanism (Hisanaga *et al.*, 2005).

We found a significant frequency of isolates resistant to tetracycline and chloramphenicol. These antibiotics are not commonly used in Portugal (Goossens *et al.*, 2005). The high prevalence of such strains can be explained by coresistance, mainly between tetracycline and macrolides. This coresistance is commonly associated with the presence of several transposons which carry the genetic determinants encoding resistance to both antibiotics (Leclercq & Courvalin, 2002; Montanari *et al.*, 2003).

Nonsusceptibility to ciprofloxacin increased in recent years from 0.5% in 2002 to 3.5% in 2004. This was associated with multidrug resistance, mainly among adults. In other studies, an association between ciprofloxacin resistance and individuals ≥65 years old has been observed (Jones *et al.*, 2003; Sahm *et al.*, 2000). This antibiotic is mostly used in elderly individuals.

Multidrug resistance increased in the last years of our study: from 7.9% in 2002 to 15.6% in 2004. In children, this mainly involved resistance to macrolides; in adults, it was mostly nonsusceptibility to penicillin, macrolides, and ciprofloxacin. The increasing resistance may be associated with the increased use of these antibiotics in recent years (Dias & Caniça, 2004; Goossens *et al.*, 2005).

In view of the antibiotic resistance patterns among pneumococci in Portugal, penicillin should be used to treat uncomplicated nonmeningeal pneumococci infections. The use of cephalosporins to treat meningeal pneumococci infections and its use combined with a macrolide or quilonone to treat severe nonmeningeal pneumococci infections seem also to be reasonable choices.

CHAPTER III

Invasive pneumococcal disease in Portugal prior and after the introduction of pneumococcal heptavalent conjugate vaccine

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3.1 - Summary

The rates of invasive pneumococcal disease (IPD), serotype distribution and antimicrobial susceptibility prior to and after the introduction of the heptavalent pneumococcal conjugate vaccine in Portuguese children were evaluated. The changes in incidence of IPD in children under 1 year old between the two periods of the study was not significant (*P* = 0.53), despite the 21% decline. In children under 18 years old there was a 27.7% decrease in vaccine serotypes. All nonvaccine serotypes increased 71.4%. The decrease in vaccine serotypes was more impressive during the first year of life (-54.8%) than for children between 1 and 5 years of age (-19.1%). Among children under 1 year old, penicillin-nonsusceptible isolates declined between the two periods of the study (47.2% versus. 25.0%) (*P* = 0.03), as did those of cefotaxime and ceftriaxone-nonsusceptible isolates. No changes were observed for isolates nonsusceptible to tetracycline and macrolides. The serotypes of these nonsusceptible isolates differed after the introduction of vaccine (*P* = 0.01). Multiresistance increased 57.1% after the introduction of vaccine. Multiresistant isolates with vaccine serotype declined 42.9% (*P* < 0.001), and nonvaccine serotypes appeared during the vaccination period (*P* < 0.001). These findings suggest a replacement of vaccine serotypes by nonvaccine serotypes, mainly among nonsusceptible isolates.

3.2 - Introduction

In Portugal, the heptavalent pneumococcal conjugate vaccine was licensed in February 2001, and has been widely distributed in the paediatric population since 2002, on a voluntary basis. The vaccine is currently recommended by the Portuguese Society of Paediatrics for all children under 2 years old and in other well known populations at risk of invasive pneumococcal disease (IPD) (Sociedade Portuguesa de Pediatria, 2003). The vaccine is not included in the the National Vaccination Program.

Recent data suggest that in 2004, 65% of the Portuguese population under 6 months of age was vaccinated with an average of 2.5 doses of conjugate vaccine (Rocha, 2005). In the first year where the vaccine was available, 54.5% of the children born after 1999 in north of Portugal were vaccinated (De Queirós *et al.*, 2004).

The seven-valent conjugate vaccine reduces nasopharyngeal carriage of serotypes included in the vaccine (Dagan *et al.*, 1996), preventing further transmission of *S. pneumoniae*. Several studies have evaluated the impact of pneumococcal conjugate vaccine on nasopharyngeal carriage (Dagan *et al.*, 1996; Frazão *et al.*, 2005; Pelton *et al.*, 2004), but few studies, mostly in the United States, have assessed the impact of pneumococcal vaccine on IPD (Black *et al.*, 2001; Byington *et al.*, 2005; Whitney *et al.*, 2003).

The introduction of the seven-valent conjugate vaccine may result in the replacement of the vaccine serotypes by nonvaccine-related serotypes among both colonization and disease (Edwards & Griffin, 2003; Pelton *et al.*, 2004; Spratt & Greenwood, 2000). The effects of such selection are still unclear, but may well include the prevalence of antimicrobial resistant pneumococci (Spratt & Greenwood, 2000).

The propose of this study was to evaluate the changes on the incidence of IPD, serotype distribution and antimicrobial susceptibility of isolates from a Portuguese paediatric population, after the introduction pneumococcal conjugate vaccine.

3.3 - Material and methods

Population studied. The ARU at the NIH Dr. Ricardo Jorge, in Lisbon, constantly monitored invasive pneumococcal infections confirmed by the clinical microbiology laboratories of the ARSIP in four of the five Portuguese Regions on the mainland. We ran a population- and laboratory-based surveillance study with 295 pneumococci isolates collected between January 1, 1999, and December 31, 2004, from the paediatric population attending 18 hospitals (university and tertiary care hospitals, general and district hospitals and others). The total Portuguese paediatric population under surveillance in 2002 was 1503,062 individuals under 18 years old (72% of the paediatric population), of which 168,796 were children under 2 years of age. ARSIP participating hospitals submitted pneumococci isolates to NIH. Every month the ARU collect samples and laboratory records from all laboratories in ARSIP. Patient data were collected through a standardized data collection form with specific information on the bacterial isolate, patient, hospital, clinical diagnosis, disease outcomes and risk factors. The methods of data collection were unchanged throughout the study. The year 1999 to 2001 (n = 139) was defined as the period prior the introduction of vaccine and the year 2002 to 2004 (n = 156) was the vaccination period.

Case definitions. A case of invasive pneumococcal disease was defined as a resident of the surveillance area for who *S. pneumoniae* was isolated from a sample of normally sterile body fluid. Inclusion criteria for laboratory diagnosis were: one isolate for patient isolated from blood, CSF and pleural fluid samples collected consecutively from patients under 18 years old with symptoms compatible with invasive pneumococcal disease.

Bacteriology. The isolates were sent to the reference laboratory, ARU, at -20ºC in TSB (Oxoid, Basingstoke, England) containing 20% glycerol. At ARU the purity of the isolates was checked using standard methods and the samples stored at -80ºC in TSB, 20% glycerol.

Serotyping. Two hundred and ninety-five isolates were serotyped by dot-blot or the Quellung reaction or both (Sorensen, 1993; Fenoll *et al.*, 1997). Pneumococci serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were defined as vaccine serotypes. Pneumococcal serotype 6A was defined as a vaccine-related serotype. All isolates with other serotypes including non-typeable strains were considered to have nonvaccine serotypes; according to a growing consensus, serotype 19A was considered as nonvaccine serotype (Hanage *et al.*, 2007).

Antimicrobial susceptibility testing. Antibiotic susceptibility of each isolate was tested by an agar dilution method. Testing conditions and susceptibility interpretation standards were those proposed by CLSI, formerly NCCLS (National Committee for Clinical Laboratory Standards, 2004). Intermediate and resistant strains were classified as

nonsusceptible. Isolates nonsusceptible to at least three different antibiotic classes were classified as multiresistant.

Statistical analysis. Annual incidence was calculated from 1999 to 2004 on the basis of the yearly population estimates from the National Institute of Statistic (census 2001), and is reported as cases per 100,000 individuals. To calculate serotype-age class disease rates, we used an imputation method to impute missing age data, assuming that the distribution of age classes for cases with missing data (5.4%) was the same as that for cases of known age. The serotype distribution and antimicrobial susceptibility among cases with missing data was similar to the distribution among cases with age data available. Fisher's exact test was used to compare the incidence of IPD in the years after the introduction of vaccination (2002 to 2004) with the base-line incidence (1999 to 2001). Changes in IPD incidence after the introduction of vaccination were then measured. Fisher's exact test was used to compare the number of vaccine and nonvaccine serotypes between the vaccine period and the prevaccine period. For Two-sided *P* values the significance level was set at 5% (0.05). SPSS software version 13.0 was used for statistical analysis.

3.4 - Results

Incidence of invasive pneumococcal disease. The population- and laboratory-based surveillance study recorded 295 strains between 1999 and 2004 in a Portuguese paediatric population (214 recovered from blood, 55 from CSF, 13 from CSF and blood, 12 from pleural fluid, and 1 from blood and pleural fluid). The incidence of IPD among children under 18 years old was 2.8 per 100,000 inhabitants in 1999, 2.7 in 2000, 3.7 in 2001, 3.6, in 2002, 3.1 in 2003, and 3.7 in 2004. The average incidence (1999 to 2001) was 3.1 cases per 100,000 inhabitants prior the introduction of the vaccine and 3.5 during the vaccination period (2002 to 2004): the incidence did not change significantly.

The incidence of IPD in children under 1 year old was stable from 1999 to 2002 (mean 23.0±1.0 cases per 100,000), then declined 33% (mean 15.3±2.0 cases per 100,000) in 2003 to 2004 (P = 0.26) (Figure 3.1). The changes between the average incidence of the period prior the introduction of the vaccine and the vaccination period in this age group were not significant (22.5 cases per 100,000 versus. 17.9; P = 0.53), despite the 21% decline. However, the incidence among 1-year-olds before vaccine introduction was 11.5 cases per 100,000 and during the vaccination period 14.3 (P = 0.70); among children aged 2-3 years the incidence rate was 5.4 cases and 5.6 per 100,000, respectively (P = 1) (Figure 3.1). Among 4-5 year old children, the rate declined from 3.1 cases per 100,000 inhabitants before the introduction of the vaccine to 2.6 during the vaccination period (P = 1). Among older children (6 to <18 years of age), the average incidence increased from 0.6 to 1.2 cases per 100 000 between pre-vaccine period and vaccination period (P = 1).

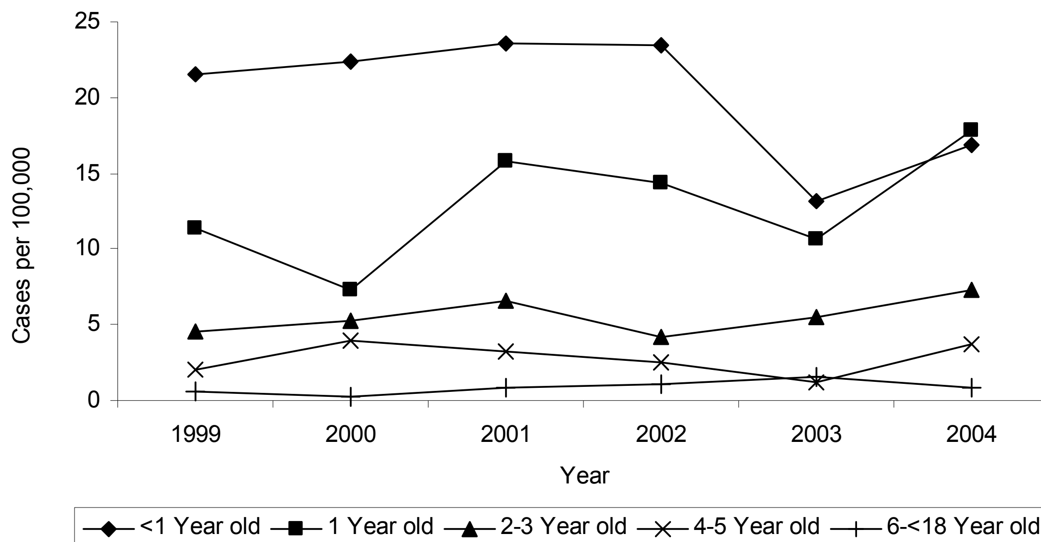

Figure 3.1 - Incidence of laboratory-confirmed invasive pneumococcal disease in Portugal according to age group and year, from 1999 to 2004.

Serotypes. Overall, there was a 27.7% decrease in vaccine serotypes (from 83 isolates in 1999 to 2001 to 60 in 2002 to 2004) (Table 3.1). All nonvaccine serotypes (including serotype 6A) increased during the same period from 56 to 96 (71.4%). The decline of vaccine serotypes was more evident for children under 6 year of age (*P* = 0.01) than for the other age groups. However, the decrease in vaccine serotypes was more impressive during the first year of life (-54.8%) than for children between 1 and 5 years of age (-19.1%). No such decrease was found in children between 6 and <18 years (Table 3.1). Inversely, the number of nonvaccine serotypes (including serotype 6A) increased 29.2%, 90.0% and 125.0% for the age groups <1, 1-5, and 6 to <18 years, respectively.

The nonvaccine serotypes identified during the study were (in descending order) 1, 19A, 7F, 3, 33F, 9N, 10A, 5, 15A, 16F, 22F, 23A, 15C, 17F, 35F, 15B, 18A, 8, 18F, 37, 23B, 19C, 20, 11A, 34, 7C and non-typeable (NT). Of these, 19A, 7F and 33F increased from 10 isolates (1999 to 2001) to 35 isolates after the introduction of the conjugate vaccine (2002 to 2004), mainly in children under 6 years of age (*P* = 0.04). Serotype 33F emerged, mainly among children under 1 year old, in the same period.

Table 3.1 - Changes in rates of invasive pneumococcal disease in Portugal according to age group, year and serotype, from 1999 to 2004.

Age Group	Serotype	No. of cases (%)		Change in the no. of cases (%)	*P* Value[c]
		1999-2001[a]	2002-2004[b]		
<1 year old					
	Vaccine serotypes	31 (56.4)	14 (31.1)	-17 (-54.8)	0.01
	6A[d]	2 (3.6)	2 (4.4)	0.0	1
	Nonvaccine serotypes[e]	22 (40.0)	29 (64.4)	7 (+31.8)	0.40
	19A	3 (5.5)	8 (17.8)	5 (+166.7)	0.15
	Total	55 (100)	45 (100)	-10 (-18.2)	0.27
1-5 year old					
	Vaccine serotypes	47 (70.1)	38 (50.0)	-9 (-19.1)	0.28
	6A[d]	0	2 (2.6)	2 (not defined)	0.50
	Nonvaccine serotypes[e]	20 (29.9)	36 (47.4)	16 (+80.0)	0.04
	19A	0	10 (13.2)	10 (not defined)	0.002
	Total	67 (100)	76 (100)	9 (+13.4)	0.56
6-<18 year old					
	Vaccine serotypes	5 (29.4)	8 (22.9)	3 (+60.0)	0.42
	6A[d]	0	3 (8.6)	3 (not defined)	0.12
	Nonvaccine serotypes[e]	12 (70.6)	24 (68.6)	12 (+100.0)	0.04
	19A	0	2 (5.7)	2 (not defined)	0.50
	Total	17 (100)	35 (100)	18 (+105.9)	0.01

[a] Period prior the introduction of the vaccine (base-line).
[b] Period after the introduction of the vaccine.
[c] *P* value was calculated by Fisher's exact test.
[d] Serotype 6A is considered to be vaccine-related serotype.
[e] Nonvaccine serotypes has serotype 19A included.

The related-vaccine serotype 6A became more common in all age groups, but not significantly so (between $P = 0.12$ and $P = 1$).

Antibiotic Susceptibility and Multiresistance. The proportion of isolates that were penicillin-nonsusceptible declined significantly in the 2 years following the introduction of vaccine (Figure 3.2). Among children under 18 years old, the nonsusceptibility rate to penicillin was 56.1% (23/41) in 2000, 38.2% (21/55) in 2001 (the year in which the vaccine started to be used), and 21.3% (10/47) in 2003; it approximately doubled, to 40% (22/55) in 2004 ($P = 0.01$) (Figure 3.2). For children under 1 year old, the proportion of penicillin-nonsusceptible isolates declined from 47.2% (26/55) (1999 to 2001) before to 25.0% (11/45) (2002 to 2004) after the introduction of the vaccine ($P = 0.03$).

The main changes in vaccine serotypes of penicillin-nonsusceptible isolates between prevaccine (94.6%; 53/56) and vaccination periods (70.2%; 33/47) involved a decline (-37.7%) mainly of serotypes 14, 6B and 9V ($P = 0.001$). A fourfold increase of the number of isolates with nonvaccine serotypes was observed between the same periods (5.4%; 3/56 versus. 29.8%; 14/47, respectively) ($P = 0.001$), mainly associated to serotype 19A (from 1 to 11 isolates, respectively) (Table 3.2).

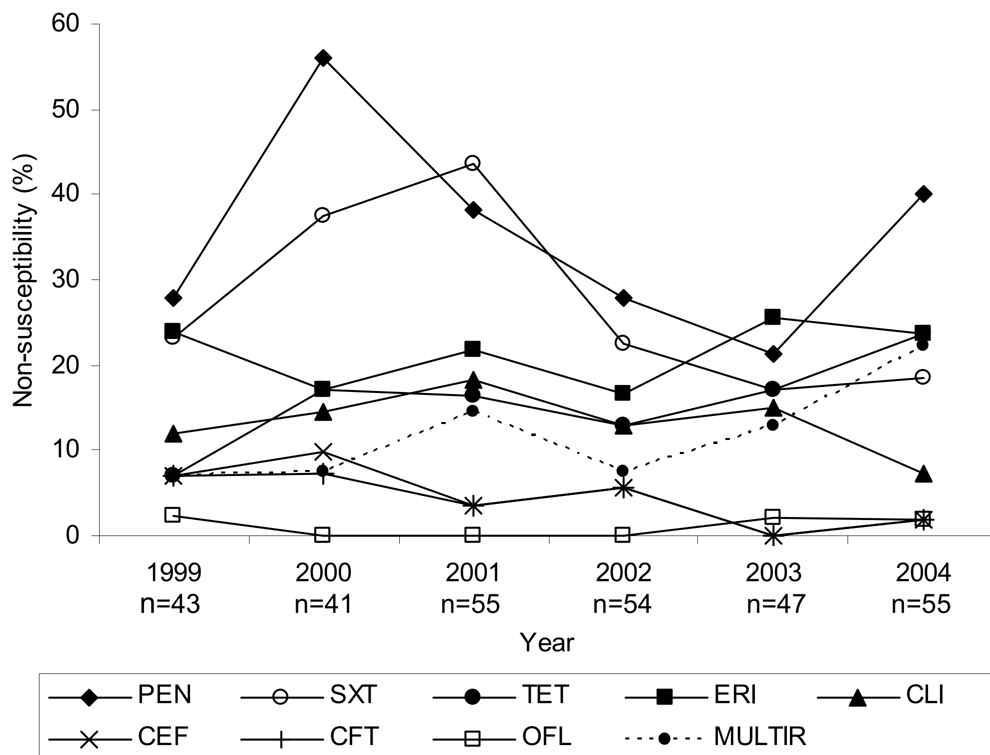

Figure 3.2 - Rates of antibiotic nonsusceptible isolates collected from invasive pneumococcal disease among Portuguese children (<18 years old), according to year. PEN, penicillin; SXT, trimethoprim-sulphamethoxazole; TET, tetracycline; ERI, erythromycin; CLI, clindamycin; CEF, cefotaxime; CFT, ceftriaxone; OFL, ofloxacin; MULTIR, multiresistance.

The proportion of cefotaxime and ceftriaxone-nonsusceptible isolates also declined following the introduction of the vaccine period from 8.6% (12/139) to 2.6% (4/156) and from 7.2% (10/139) to 2.6% (4/156) (*P* = 0.05; *P* = 0.10), respectively (Figure 3.2). The declines were significant among children under 1 year old (9.0%; 5/55 versus 0.0%; 0/45) (*P* = 0.04). There was no significant change in the serotypes of cefotaxime and ceftriaxone isolates between the 2 periods (*P* = 0.49 and *P* = 0.40, respectively).

The proportion of trimethoprim-sulphamethoxazole-nonsusceptible isolates also declined significantly after the introduction of vaccine, from 35.5% (49/138) (1999 to2001) to 19.5% (30/154) (2002 to 2004) (*P* = 0.002), mainly among children under 1 year of age (38.9%; 21/54 versus 9.1%; 4/44) (*P* = 0.001). No significant changes were observed in the proportion of ofloxacin (1/138 versus 2/156; *P* = 1), tetracycline (19/138 versus 28/156; *P* = 0.34), erythromycin (29/138 versus 34/156; *P* = 0.88) or clindamycin (21/138 versus 18/156; *P* = 0.39) nonsusceptible isolates (Figure 3.2). However, the serotypes of erythromycin (*P* = 0.01) (Table 3.2), clindamycin (*P* = 0.03) and tetracycline (*P* = 0.01) nonsusceptible isolates changed significantly, mainly due to the increase of serotypes 19A, 15A and 33F and the decline of some vaccine serotypes.

Multiresistant isolates increased 57.1% after the introduction of vaccine. The vaccine serotypes fell 42.9% (*P* < 0.001) and nonvaccine serotypes emerged (*P* < 0.001) (Table 2). This emergence was mainly due to serotype 19A and 15A. The rate of isolates nonsusceptible to only 3 different antibiotic classes increased significantly between the 2 periods of the study (6.6%; 9/137 versus 11.0%; 17/154) in opposite to isolates nonsusceptible to 2 different antibiotic classes, which declined significantly after the introduction of the vaccine (34.3%; 47/137 versus 16.9%; 26/154) (*P* = 0.03). This was most significant among children under 1 year old (*P* = 0.006).

Table 3.2 - Changes in nonsusceptibility to penicillin and erythromycin and multiresistance rates of invasive pneumococcal disease among Portuguese children (< 18 years old) between the prevaccine (1999 to 2001) and vaccine (2002 to 2004) period, according to serotype

Antibiotic and Serotype[a]	No. of cases (%)		Change in the no. of cases (%)	*P* value[d]
	1999-2001[b]	2002-2004[c]		
Penicillin				
Vaccine Serotypes	53 (94.6)	33 (70.2)	-20 (-37.7)	0.001
6B	3 (5.4)	1 (2.1)	-2 (-66.7)	
9V	8 (14.3)	4 (8.5)	-4 (-50.0)	
14	32 (57.1)	16 (34.0)	-16 (-50.0)	
19F	1 (1.8)	2 (4.3)	1 (+100.0)	
23F	9 (16.1)	10 (21.3)	1 (+11.1)	
Nonvaccine serotypes	3 (5.4)	14 (29.8)	11 (+366.7)	0.001
7F	1 (1.8)	0 (0.0)	-1 (-100.0)	
15A	0 (0.0)	2 (4.3)	2 (Not defined)	
19A	1 (1.8)	11 (23.4)	10 (+1000.0)	
19C	0 (0.0)	1 (2.1)	1 (Not defined)	
34	1 (1.8)	0 (0.0)	-1 (-100.0)	
Total	56 (100.0)	47 (100.0)	-9 (-16.1)	0.09
Erythromycin				
Vaccine serotypes	24 (82.8)	18 (52.9)	-6 (-25.0)	0.02
4	0 (0.0)	1 (2.9)	1 (Not defined)	
6B	11 (37.9)	4 (11.8)	-7 (-63.6)	
9V	1 (3.4)	0 (0.0)	-1 (-100.0)	
14	8 (27.6)	11 (32.4)	3 (+27.3)	
19F	3 (10.3)	2 (5.9)	-1 (-33.3)	
23F	1 (3.4)	0 (0.0)	-1 (-100.0)	
Nonvaccine serotypes	5 (17.2)	16 (47.1)	11 (+220.0)	0.02
7F	1 (3.4)	0 (0.0)	-1 (-100.0)	
15A	0 (0.0)	2 (5.9)	2 (Not defined)	
15C	2 (6.9)	0 (0.0)	-2 (-100.0)	
19A	0 (0.0)	11 (32.4)	11 (Not defined)	
33F	1 (3.4)	3 (8.8)	2 (+200.0)	
34	1 (3.4)	0 (0.0)	-1 (-100.0)	
Total	29 (100.0)	34 (100.0)	5 (+17.2)	0.88
Multiresistance				
Vaccine serotypes	14 (100.0)	8 (36.4)	-6 (-42.9)	< 0.001
6B	8 (57.1)	2 (9.1)	-6 (-75.0)	
14	5 (35.7)	5 (22.7)	0 (0.0)	
19F	0 (0.0)	1 (4.5)	1 (Not defined)	
23F	1 (7.1)	0 (0.0)	-1 (-100.0)	
Nonvaccine serotypes	0 (0.0)	14 (63.6)	14 (Not defined)	< 0.001
15A	0 (0.0)	2 (9.1)	2 (Not defined)	
19A	0 (0.0)	11 (50.0)	11 (Not defined)	
19C	0 (0.0)	1 (4.5)	1 (Not defined)	
Total	14 (100.0)	22 (100.0)	8 (+57.1)	0.37

[a] Vaccine serotypes are all cases with serotype 4, 6B, 9V, 14,18C, 19F or 23F (covered by the conjugate vaccine). Nonvaccine serotypes are the sum of cases with serotypes not covered by the conjugate vaccine.
[b] Period prior the introduction of the vaccine.
[c] Period after the introduction of the vaccine.
[d] *P* value for total of vaccine serotypes, nonvaccine serotypes and antibiotic nonsusceptibility was calculated by Fisher's exact test.

3.5 - Discussion

In children under 1 year old the percentage of IPD in Portugal, prior the introduction of vaccine, caused by vaccine serotypes was 56.4%. The introduction of the conjugate vaccine was expected to reduce the incidence of IPD and the proportion of pneumococcal isolates with antimicrobial nonsusceptibility. However, there was no significant decline of the overall incidence of laboratory-confirmed IPD in the paediatric population in Portugal after the introduction of the heptavalent pneumococal conjugate vaccine. The incidence of IPD observed was consistent with that reported previously in Portugal (Brito & Grupo de Estudo da Doença Pneumocócica, 2005). There was a decline in the incidence of IPD mainly among younger children, up to 1 year old (-21%), followed, in 2004, by an increase among children up to 6 years old. In the United States, a decline of 69% of IPD was reported after the introduction of the vaccine in children up to 2 years old (Whitney *et al.*, 2003), and other studies showed the same trend (Black *et al.*, 2001; Kaplan *et al.*, 2004). The incidence we observed in Portugal are lower than those in the United States and similar to those elsewhere in Europe (Reinert, 2004). This difference may be due to differences in blood culturing practices between the United States and Europe (Hausdorff *et al.*, 2001).

IPD caused by vaccine serotypes declined among Portuguese children under 1 year old (-54.8%) and in the 1-5 years-old group (-19.1%) after introduction of the vaccine. However, the number of nonvaccine serotypes increased among children under 1 year old (31.8%), and also in the 1-5 years-old group (80.0%); in this last age group are included the first children vaccinated in 2001 (De Queirós *et al.*, 2004). In the 6 to <18 years old group an increase of IPD between the period 1999 to 2001 and 2002 to 2004 (105.9%) with the contribution of vaccine and nonvaccine serotypes was observed. This age group was not usually vaccinated in Portugal (De Queirós *et al.*, 2004). Overall, these results suggest a replacement effect: vaccine serotypes replaced by nonvaccine serotypes, mainly serotypes 19A, 7F and 33F. In this study, serotypes 19A and 7F were mostly found in the 1-5 years-old group; this age group includes the children at risk because of attending day-care centres (DCC). Although serotype 19A is part of the same serogroup as 19F, other authors clarified that vaccine produces limited functional antibody response to the serotype 19A and doubtful efficacy against 19A carriage (Hanage *et al.*, 2007). Some other possible emerging serotypes are particularly dangerous: serotype 3 is associated with an increased risk of death, and a mortality rate of 50% for serotype 19A has been observed (Martens *et al.*, 2004). Serotype 1 is associated with invasive disease (Brueggemann *et al.*, 2003). Serotypes 1, 2 and 5 are also associated with outbreaks among adults in closed facilities (Hausdorff *et al.*, 2005), as is serotype 12F (Centers for Disease Control and Prevention, 2005). Recently in the United States, a replacement of vaccine serotypes by nonvaccine serotypes associated to an increase of incidence of empyema and severe IPD (Byington *et al.*, 2005), has also been described. Vaccinated children remain susceptible to colonization by other

serotypes (Hausdorff *et al.*, 2005), or even by other species (Bogaert *et al.*, 2004c; Regev-Yochay *et al.*, 2004), thus emergence of these or other serotypes is a serious threat for the future.

Several studies report that conjugate pneumococcal vaccine did not reduce overall pneumococcal carriage. However, there was a replacement effect among carriers: the proportions of vaccine serotypes were significantly reduced and those of nonvaccine serotypes increased (Bogaert *et al.*, 2004a; Frazão *et al.*, 2005; Pelton *et al.*, 2004). A disease replacement effect has also been observed in a clinical trial of pneumococal conjugate vaccine for otitis media: there was a reduction in the vaccine-type otitis media and an increase of nonvaccine-type otitis (Eskola *et al.*, 2001).

It has been suggested that the selection pressure of antibiotics enhances the replacement effect, such that in countries with high antibiotic consumption the effects of vaccination will be limited (Temime *et al.*, 2005). This may explain the effect in Portugal, as it is one of the European countries with the highest antibiotic consumption, particularly macrolides (Dias & Caniça, 2004; Goossens *et al.*, 2005). In this study, the number of *S. pneumoniae* isolates nonsusceptible to penicillin declined initially, and then doubled in 2004. Nonsusceptibility to macrolides and tetracycline, and multiresistance did not decrease following the introduction of the vaccine. The increase in the number of isolates nonsusceptible to 3 different antibiotic classes and the decline of isolates nonsusceptible to 2 or one antibiotic classes were compatible with an increase of multi-antibiotic selection pressure. Nevertheless, the serotype distribution of isolates nonsusceptible to those antibiotics changed. Other studies, mainly in the United States, reported a different trend (Klugman *et al.*, 2003; Stephens *et al.*, 2005; Whitney *et al.*, 2003).

Among pneumococci with nonvaccine serotypes, an increase of susceptible isolates to penicillin (P = 0.03) and erythromycin (P = 0.04) after the introduction of the vaccine (data not shown) was also observed. This suggests that the multi-antibiotic selection pressure is not the only factor enhancing the replacement effect.

Seventy per cent of children attending DCC in Portugal are colonized with pneumococcus (Frazão *et al.*, 2005), which is a higher value than the observed in DCC in the United States (20-40%) (Bogaert *et al.*, 2004a). A high prevalence of colonization implies frequent transmission and, consequently, a large number of circulating clones and serotypes. Therefore, under the selection pressure of the vaccine, the replacement effect could be enhanced. Probably as a consequence, the serotype coverage in Portugal by the vaccine (63.8%) is much lower than that in the United States (83%) (Dias *et al.*, 2004; Whitney *et al.*, 2003). We also suggest that the high rates of colonization observed in Portugal might cause a more broad and regular immunization into the population, giving more protection against IPD and, consequently, lower IPD rates than in the United States.

Rarer serotypes, like 33F, 19A and 15A, emerged after the introduction of the vaccine. It is not clear whether such emergence is limited to a small number of serotypes. A study to determine the invasiveness and virulence of nonvaccine serotypes among vaccinated children should be undertaken to clarify this issue.

This study suggests a replacement effect of IPD in a European country, mainly among antimicrobial nonsusceptible isolates. Mathematical modelling studies predicted the occurrence of this effect in a long term (Lipsitch, 1997; Temime *et al.*, 2004), however, it is shown here that the replacement was rapid, which may be due to high rate of capsular switching phenomena, of dissemination of several genetic lineages (Pai *et al.*, 2005), of coresistance or/and of other factors (Long, 2005). Further studies are needed to answer about the dynamic of replacement in invasive pneumococcal disease.

CHAPTER IV

Trends in resistance to penicillin and erythromycin of invasive pneumococci in Portugal

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Dias R & Caniça M (2008). *Epidemiol Infect* **136:** 928-939

4.1 - Summary

Antimicrobial resistance of pneumococci is influenced by serotypes, antimicrobial consumption and vaccine use. Serotyping of 697 out of 1,331 pneumococcal isolates, recovered in Portugal from 1994 to 2004, showed that the theoretical rate of heptavalent conjugate vaccine coverage was 91.7% and 63.6% for penicillin and erythromycin-nonsusceptible strains, respectively, in children up to 1 year old. The use of amoxicillin and erythromycin decreased in the vaccine period 2001 to 2004 ($P = 0.04$ and $P < 0.01$, respectively) but azithromycin usage increased in the same period ($P < 0.01$). By using linear regression models, we evaluated the role of antimicrobial and vaccine use in the trends of resistance to penicillin and erythromycin among the isolates. The models suggest that the use of macrolides was the main factor associated with an increase of penicillin and erythromycin-nonsusceptible isolates from adults ($P < 0.01$) and erythromycin-nonsusceptible isolates among children ($P = 0.006$). These models also suggest that heptavalent vaccine is failing to reduce antimicrobial resistance as expected, possibly due to the increased consumption of azithromycin ($P = 0.04$). The efficient use of new antibiotics may reverse the present trends of antimicrobial resistance.

4.2 - Introduction

The spread of penicillin- and multidrug-resistant *Streptococcus pneumoniae* has become a worldwide problem in recent years, making it more difficult to treat IPD (Klugman, 2002). The risk of morbidity and mortality caused by IPD is particularly high for young children, the elderly, and immunocompromised individuals (Klein, 1981). This risk has been increasing worldwide due to the development of resistance to antibiotics in addition to penicillin, such as erythromycin, tetracycline and chloramphenicol (Felmingham *et al.*, 2002). The nonsusceptibility of bacterial isolates to antibiotics is considered to be strongly associated with their use in the patient community (Goossens *et al.*, 2005).

In Europe, the prevalence of resistance to penicillin and macrolides is increasing mainly in the southern countries (Bruinsma *et al.*, 2004), and the increase is associated with a small number of serotypes. A national surveillance study in Portugal indicated that penicillin-nonsusceptible strains of *S. pneumoniae* were responsible for 17.1% of cases of IPD in 1994 to 1998 (Dias *et al.*, 2006). The most prevalent serogroups found then were 14, 23 and 9 (Dias *et al.*, 2006), but an increase in erythromycin-resistant isolates, predominantly serotype 14, was also documented in Portugal (Dias & Caniça, 2004). The 7-valent pneumococcal conjugate vaccine protects against disease, and reduces the asymptomatic carriage of *S. pneumoniae*. Its generalised use also reduces the percentage of antibiotic-resistant strains with the serotypes most often implicated in invasive disease (Whitney *et al.*, 2003). This vaccine has been licensed for use in children <2 years of age in Portugal since 2001 and has been used voluntarily since then. We set out to evaluate the impact of antibiotic use and vaccination on antimicrobial resistance rates and explore the relationships between penicillin and erythromycin-nonsusceptible isolates and their serotype.

4.3 - Materials and methods

Patients and bacterial isolates. The national laboratory surveillance study of pneumococcal infections in Portugal recovered 1,331 *S. pneumoniae* isolates of invasive origin, between January 1994 and December 2004 (269 for the period 1994 to 1998, 491 for the period 1999 to 2001 and 571 for the period 2002 to 2004). A case of IPD was defined as disease in which a pneumococcus was isolated by culture from normally sterile sites (blood, CSF or pleural fluid) in a resident of the surveillance area. The isolates were collected in 24 hospitals throughout the country and one public health institution. The population covered by these centres in 2001 was 8203,515 (79% of the Portuguese population), including 1214,796 children (75% of Portuguese child population). There were 974 isolates from blood, 271 from CSF, 25 from both blood and CSF, 60 from pleural fluid, and 1 from both pleural fluid and blood. These were transported in TSB (Oxoid, Basingstoke, England) with 20% glycerol at -20ºC to the ARU at the NIH Dr. Ricardo Jorge in Lisbon. At ARU the identification and purity of strains were checked using standard microbiological methods and the strains stored at -80ºC. The inclusion criteria for laboratory diagnosis were: non-repetitive and consecutive blood and/or CSF and/or pleural fluid sampling in individuals having symptoms compatible with IPD. Patients up to 18 years old were included in the paediatric population. Isolates from children aged <5 years accounted for 72% (240/332) of those from the paediatric population. The period up to the year 2000 was defined as the prevaccine period and between 2001 and 2004 was defined as the vaccine period.

Antimicrobial susceptibility testing. MICs of penicillin (Wyeth Lederle Portugal, Algés), cefotaxime (Hoechst Marion Roussel, Mem Martins, Portugal), ceftriaxone (Roche Pharmaceuticals, Amadora, Portugal) and erythromycin (Abbott Laboratórios, Amadora) were determined by an agar dilution method according to the conditions and interpretative criteria proposed by CLSI, formerly NCCLS (Clinical and Laboratory Standards Institute, 2007). Intermediate- and high-level resistant strains to penicillin were analysed as a single group due to the low number (34/1331) of high-level resistant strains and were classified as nonsusceptible.

Serotyping. Isolates were serotyped by dot-blot and/or the Quellung reaction using type-specific antiserum (Fenoll *et al.*, 1997).

Antimicrobial and vaccine use data. National outpatient sale data were obtained, from 1994 to 2004, for the commonly used β-lactams and macrolides (amoxicillin, J01CA04; amoxicillin plus clavulanic acid, J01CR02; flucloxacillin, J01CF05; cefatrizine, J01DB07; cefuroxime, J01DC02; cefaclor, J01DC04; cefixime, J01DD08; clarithromycin, J01FA09; erythromycin, J01FA01; azithromycin, J01FA10; and telithromycin, J01FA15). Data on anti-pneumococcal vaccine were obtained from International Medical Statistics (IMS Health).

Antimicrobial usage data were grouped according to the Anatomic Therapeutic Chemical (ATC) classification and expressed as defined daily dose (DDD) units per 1,000 inhabitants daily (DID), as proposed by WHO (World Health Organization, 2005). Anti-pneumococcal vaccine use was expressed as annual units of pneumococcal heptavalent conjugate vaccine sold in Portugal. Linear associations between antimicrobial consumption, heptavalent vaccine use and rates of nonsusceptibility were assessed by the Pearson correlation coefficient using a time lag of 0 or 1 year. The Mann-Whitney *U* test was performed to compare antimicrobial use between the period prior to (1994 to 2000) and after introduction of the vaccine (2001 to 2004).

Antimicrobial consumption, vaccination and antimicrobial resistance. The impact of antimicrobial and vaccine use on penicillin and erythromycin resistance rates was assessed using a linear regression method on data for isolates from children and adults for the period 1994 to 2004. Four models were adjusted for the following dependent variables: erythromycin nonsusceptibility rate among children (EC); erythromycin nonsusceptibility rate among adults (EA); penicillin nonsusceptibility rate among children (PC) and penicillin nonsusceptibility rate among adults (PA). To obtain the best-fitted set of independent variables for inclusion in each model, we performed a backward selection method on variables that reflected the use of antibiotics and vaccine which correlated positively with antimicrobial resistance rates (determined previously by the Pearson coefficient); these variables were also associated to the respective age of the population (Falcão *et al.*, 2003). When none or a single correlation was found to be associated with the dependent variables, the main antibiotics used were added to the input of the backward selection method as well as the anti-pneumococcal vaccine consumption. The reduced number of years and the correlation between the independent variables were the major limitations on the use of the multiple regression models in the study. The fit diagnosis was assessed by adjusted R^2 statistic and *F*-ANOVA test. In each model, the regression coefficients associated with each independent variable were estimated by the ordinary least squares method (Belsley *et al.*, 1980), and *P* value for their significance and standard error were determined.

Preliminary analyses with the PC model suggested an association between the penicillin nonsusceptibility rate among children and the following independent variables: amoxicillin, flucloxacillin, azithromycin and vaccine use. The latter two factors were removed from the equation corresponding to the PC model and new estimations of the values of the dependent variable were made based on the new equations obtained. All statistical procedures were undertaken with SPSS software, version 13.0 (SPSS Inc, Chicago, IL, USA). Two-sided *P* values <0.05 were considered as significant.

4.4 - Results

In total, 697 isolates of *S. pneumoniae* of invasive origin were recovered between January 1999 and December 2002 comprising 177 from children, of which 105 were aged <2 years; 469 from adults of whom 182 were ≥ 65 years; and 51 of unknown age.

Forty-five different serotypes were identified. Serotypes 14, 1, 23F, 6B, 7F, 19F, 9V, 3 and 6A (in descending order) accounted for 80% of the paediatric isolates comprising a total of 27 serotypes. Among adults, serotypes 3, 1, 14, 8, 4, 9V, 23F, 7F, 19F, 9N, 6B, 18C, 6A, 19A, 12F, 16F and 17F (in descending order) were responsible for 80% of infections.

Antimicrobial susceptibility and serotypes. The frequency of penicillin-nonsusceptible pneumococci from children aged <18 years was 36.4% (64/476) and from adults 15.8% (74/467). For children aged <2 years old, 41% (43/105) yielded penicillin-nonsusceptible isolates of serotypes 14 (67.4%), 23F (18.6%), 6B (9.3%), 19A (2.3%) and 34 (2.3%); serotypes 9V and 19F were only isolated from children between 2 and 18 years old (8/21) (Figure 4.1A). In the elderly penicillin-nonsusceptible isolates (32/182) were predominantly serotype 14 (48.4%), 9V (22.6%) and 23F (9.7%) (Figure 4.1B).

A small number (8/105) of pneumococcal isolates from children aged <2 years were not susceptible to cefotaxime and ceftriaxone and only 2 isolates from the elderly were not susceptible to these agents; these isolates were also not susceptible to penicillin and were of serotypes 14, 9V and 6B. The frequency of erythromycin-nonsusceptible isolates from children aged <18 years old was 19.2% (34/177) and among adults 10.7% (50/467); in the <2 years age group it accounted for 21% (22/105) and comprised serotypes 6B (40.9%), 14 (22.7%), 33F (13.6%), 15C (9.1%), 19F (4.5%), 23F (4.5%), and 34 (4.5%); serotypes 9V and 19A were only isolated from children aged between 2 and 18 years old (Figure 4.1C). Among cases aged ≥65 years, erythromycin-nonsusceptible isolates accounted for 12.6% (23/182) and were mostly serotype 14 and 6B (Figure 4.1D).

Vaccine coverage of drug-susceptible and resistant strains. The three conjugated vaccines (7-, 9-, 11-valent) have theoretical rates of coverage between 91.7% and 100% for penicillin-nonsusceptible isolates from children; a similarly high coverage is offered by the polysaccharide vaccine (95.8% to 100%). In the elderly, however, the theoretical rate was 93.5% for all conjugated vaccines, and 96.8% for the polysaccharide vaccine (Figure 4.2A). For erythromycin-nonsusceptible isolates coverage of the conjugate vaccines fell to 63.6% (100% for children) and the highest coverage in the ≥65-years age group was 81.8% for conjugated vaccines (the 11-valent formulation), and 96.8% for the polysaccharide vaccine (Figure 4.2B). All vaccine types offered 100% coverage for cefotaxime and ceftriaxone-nonsusceptible isolates in both children and adults.

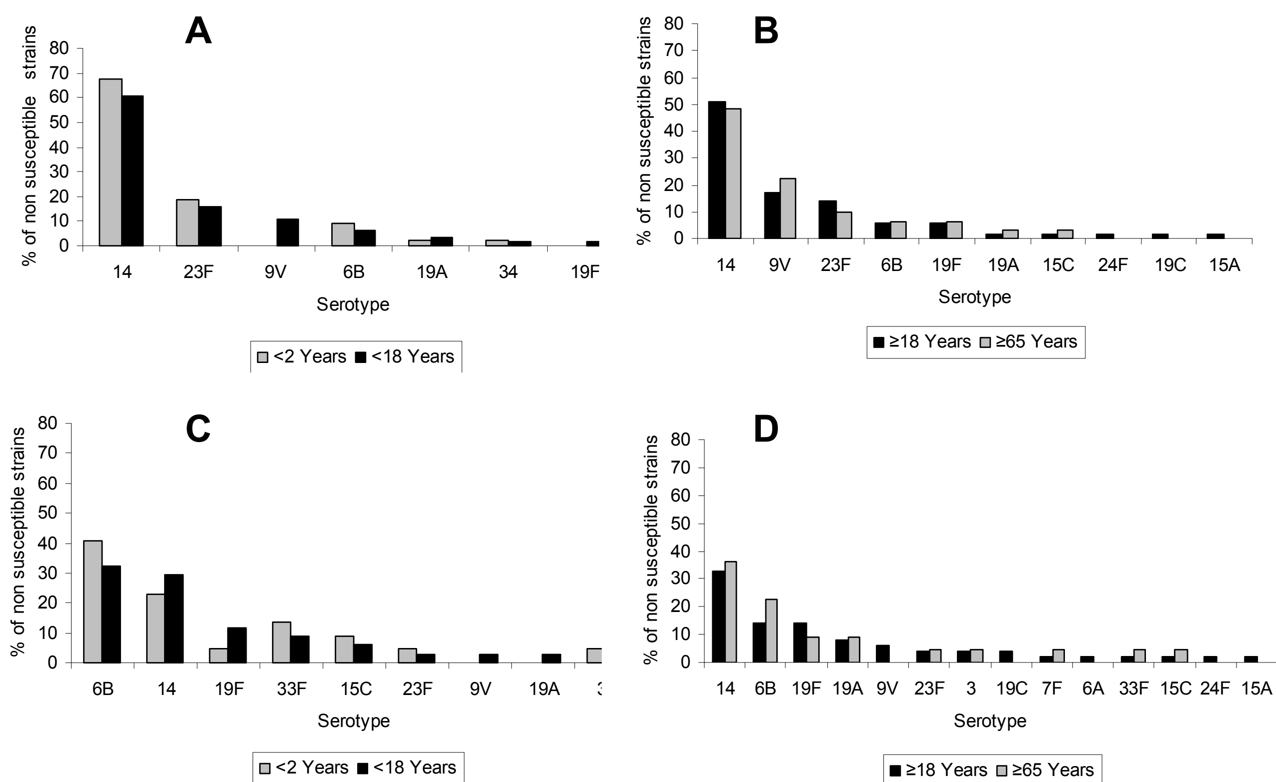

Figure 4.1 - Distribution of nonsusceptible strains to penicillin and erythromycin by pneumococci serotypes isolated from invasive disease in Portugal between years 1999 and 2002. (A) Distribution of nonsusceptible strains to penicillin by serotypes isolated from children aged <2 years and <18 years. (B) Distribution of nonsusceptible strains to penicillin by serotypes isolated from adults aged ≥18 years and ≥65 years. (C) Distribution of nonsusceptible strains to erythromycin by serotypes isolated from children aged <2 years and <18 years. (D) Distribution of nonsusceptible strains to erythromycin by serotypes isolated from adults aged ≥18 years and ≥65 years.

Seven-, 9-, 11-valent vaccines gave theoretical coverage rates of 55.9%, 70.6% and 81.9% respectively for all *S. pneumoniae* isolated from children and the corresponding rates for the <2 years age group were 60.0%, 68.6% and 80.0% (Figure 4.2C). The theoretical coverage of all formulations represented was higher for the 2-4-years age group, with the exception of the 7-valent vaccine. Children of 10-17 years had the lowest coverage by the 7- (16.7%), 9- (41.7%) and 11-valent (50.0%) vaccines of all the subpopulations. Among adults the 7-, 9-, 11-valent vaccines gave a theoretical coverage of 39.9%, 52.2% and 66.7%, respectively (Figure 4.2C) with the 11-valent vaccine showing the highest rate, particularly for those aged >50 years, due to the higher frequency of serotype 3 in this group. Strains with serotypes included in the polysaccharide vaccine formulation made up 85.4% of the isolates from adults.

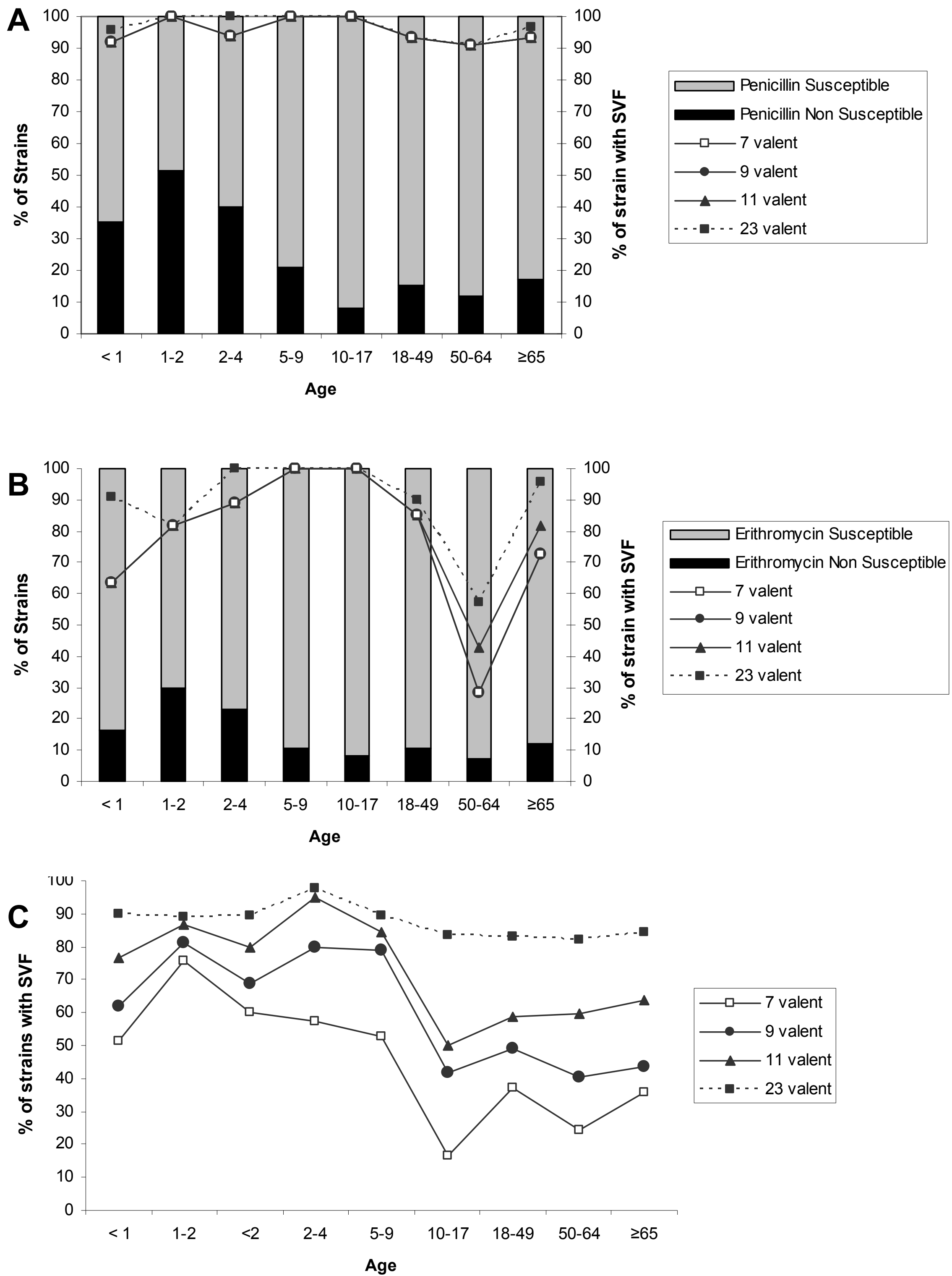

Figure 4.2 - Serotype vaccine coverage according to age group for penicillin- and erythromycin-nonsusceptible strains. (A) Percentage of strains with serotypes included in the vaccine formulations (SVF), according to age group for penicillin-nonsusceptible strains. (B) Percentage of strains with SVF, according to age group for erythromycin-nonsusceptible strains. (C) Percentage of strains with SVF, according to age group for susceptible and nonsusceptible strains.

Antimicrobial use and resistance. Outpatient use of penicillin decreased between the period before and after the vaccine was introduced ($P = 0.04$). The use of macrolides did not change significantly between the two periods ($P = 0.65$). Amoxicillin and erythromycin were the main antibiotics with a decreased use in the vaccine period ($P = 0.04$ and $P < 0.01$, respectively). In contrast azithromycin use increased significantly ($P < 0.01$), and clarithromycin use remained static ($P = 0.07$) (Table 4.1). Telithromycin was only sold in Portugal since 2003 and its use increased in 2004.

The rate of nonsusceptibility to erythromycin of pneumococcal isolates from children was found to be associated with macrolides use, mainly clarithromycin and azithromycin, as well as cephalosporins and amoxicillin plus clavulanic acid (Table 4.2). In adults, the nonsusceptibility rate to erythromycin was positively associated with prescribing of clarithromycin, azithromycin and cefuroxime, all with a time lag of 1 year; a positive association with the use of heptavalent conjugate vaccine was also found (Table 4.2). Penicillin nonsusceptibility in children was linked only with amoxicillin use with a time lag of 1 year. Among adults, this rate was found to be positively associated to amoxicillin plus clavulanic acid with a time lag of 1 year, and to cephalosporins, clarithromycin and azithromycin use (Table 4.2).

Table 4.1 - Antimicrobial and vaccine use in Portugal during 1994 to 2000 and 2001 to 2004

Consumption	Mean of DID values 1994-2000[a]	Mean of DID values 2001-2004 [a]	*P* Value[b]
J01C (Penicillins)	20.2	18.2	0.04
Amoxicillin	19.4	17.3	0.04
Amoxicillin and clavulanic acid	8.9	10.0	0.41
Flucloxacillin	0.9	0.9	0.79
J01D (Cephalosporins)	3.6	4.0	0.16
Cefatrizine	0.8	0.9	0.53
Cefuroxime	0.2	0.3	0.006
Cefaclor	1.4	1.8	0.11
Cefixime	1.1	1.0	0.11
J01F (macrolides, lincosamides, streptogramins)	5.5	5.7	0.65
Clarithromycin	2.0	2.9	0.07
Erythromycin	2.5	0.9	0.006
Azithromycin	0.5	1.5	0.006
Vaccine[c]	0.0	224.9	0.006

[a] DID, daily dose (DDD) units/1,000 inhabitants daily.
[b] Mann-Whitney *U* test.
[c] Mean of 1,000 pneumococcal heptavalent conjugate vaccine units sold.

Table 4.2 - Correlation between rates of susceptibility to penicillin and erythromycin in children and adults and antibiotic use

Susceptibility[a]	Use[b]	Pearson correlation coefficient	*P* value
PNS for children			
	Amoxicillin[c]	0.682	0.021
PNS for adults			
	Vaccine[d]	0.730	0.003
	Cefuroxime[e]	0.794	0.003
	Azithromycin[e]	0.857	0.001
	Cephalosporins (J01D)[e]	0.785	0.014
	Amoxicillin plus clavulanic acid[f]	0.797	0.004
	Clarithromycin[c]	0.937	0.003
ENS for children			
	Amoxicillin + clavulanic acid[e]	0.781	0.008
	Cefaclor[e]	0.787	0.007
	Clarithromycin[e]	0.865	0.001
	Azithromycin[e]	0.796	0.006
	Cefuroxime[f]	0.707	0.02
ENS for adults			
	Vaccine[d]	0.790	0.003
	Clarithromycin[c]	0.800	0.001
	Cefuroxime[c]	0.868	0.001
	Azithromycin[c]	0.905	< 0.001

[a] PNS, penicillin nonsusceptibility; ENS, erythromycin nonsusceptibility.
[b] Only antibiotics which were associated with nonsusceptibility are present.
[c] Exponential of antibiotic use with a time lag of 1 year.
[d] Heptavalent pneumococcal conjugate vaccine use.
[e] Antibiotic use.
[f] Antibiotic use with a time lag of 1 year.

Trends of penicillin and erythromycin resistance. The observed rates of penicillin-nonsusceptible pneumococci from children increased between 1994 (33.3%) and 2000 (56.8%), except for a decreased rate in 1998 and 1999 (prevaccine period). This rate decreased further to 21.3% in 2003 but increased to 40.0% in 2004 (Figure 4.3A). No significant difference was found for observed rates of penicillin-nonsusceptible pneumococci between prevaccine (40.2 %) and vaccine periods (31.7 %) ($P = 0.125$). The observed rate consistently increased in adults between 1994 (0.0%) and 2004 (17.0%) (Figure 4.3B), and this was statistically significant for rates between the period prior to (11.0%) and after (17.0%) vaccine introduction ($P = 0.012$).

Among children, erythromycin-nonsusceptible strains only emerged in 1997 and increased more consistently since 2000 (Figure 4.3C); the rates for these strains did not show a significant increase before (16.7%) and during (22.0%) the vaccine period ($P = 0.260$). However, in adults, the rates of erythromycin-nonsusceptible isolates increased from

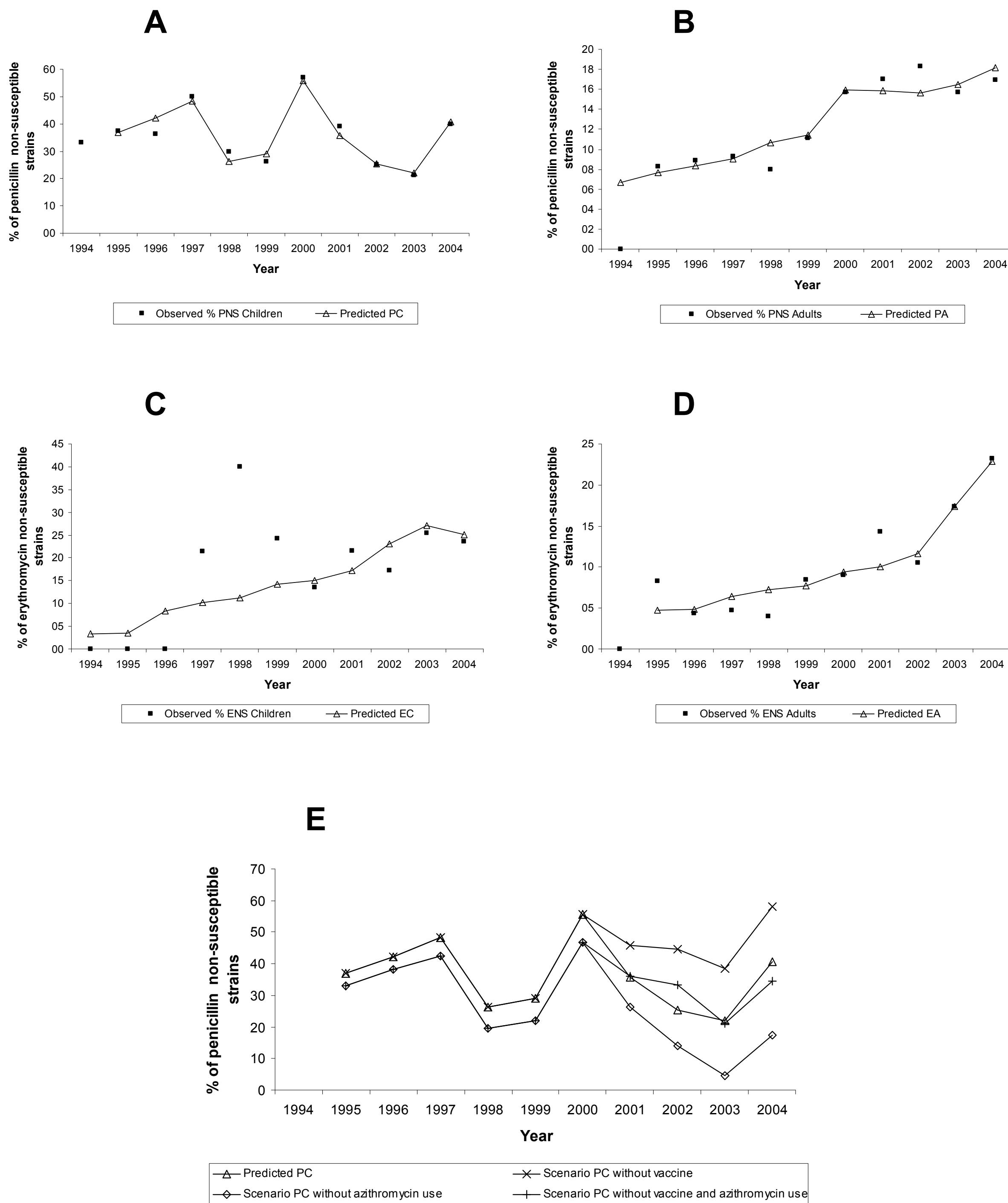

Figure 4.3 - Observed annual rates (1994-2004) of antimicrobial nonsusceptible pneumococci isolated from invasive disease in Portugal and respective linear regression models. (A) Observed and predicted annual rates of penicillin-nonsusceptible strains for children (PC). (B) Observed and predicted annual rates of penicillin-nonsusceptible strains for adults (PA). (C) Observed and predicted annual rates of erythromycin-nonsusceptible strains for children (EC). (D) Observed and predicted annual rates of erythromycin-nonsusceptible strains for adults (EA). (E) Scenarios for annual rates of penicillin-nonsusceptible strains for children; predicted, without vaccine, without azithromycin use, without vaccine and azithromycin use.

none in 1994 to 23.2% in 2004 (Figure 4.3D) and showed a significant increase between in the vaccine period (*P* < 0.001).

Model diagnosis. All models used in this study showed good adjustment with the observed data (Table 4.3). The irregularities observed in the PC model are probably associated with fluctuations in the consumption of several antibiotics. The EC model presented a lower adjustment ($r = 0.796 \pm 0.069$) than other models which could be due to the limited number of years that data were available. The year 1998 was excluded from analysis due to the unexpected high rate of nonsusceptible isolates obtained that year. Isolates nonsusceptible to erythromycin were first observed in Portugal in 1997, but showed only a defined trend since the year 2000. PA and EA models, showed good adjustments to the observed resistance rates ($r = 0.931 \pm 0.024$ and $r = 0.939 \pm 0.015$, respectively) (Table 4.3).

Table 4.3 - Summary of statistical analyses of the four linear regression models used to describe nonsusceptibility rates

Model[a]	Variables	Coefficient estimated	Standard error	*P* Value[b]	R^2	Standard error of regression
PC[c]					0.968	0.038
	Constant	3.064	4.786×10^{-1}	0.001		
	Amoxicillin[d]	2.001×10^{-10}	$< 1.000 \times 10^{-3}$	0.007		
	Flucloxacillin[e]	- 3.202	5.502×10^{-1}	0.002		
	Azithromycin[d]	3.998×10^{-2}	1.455×10^{-2}	0.04		
	Vaccine[f]	$- 6.996 \times 10^{-7}$	$< 1.000 \times 10^{-3}$	0.02		
PA					0.939	0.015
	Constant	6.694×10^{-2}	9.392×10^{-3}	< 0.01		
	Macrolides[g]	4.584×10^{-3}	$< 1.000 \times 10^{-3}$	< 0.01		
EC[h]					0.796	0.069
	Constant	3.316×10^{-2}	3.759×10^{-2}	0.40		
	Azithromycin[e]	1.344×10^{-1}	3.612×10^{-2}	0.006		
EA					0.931	0.024
	Constant	1.239×10^{-2}	1.486×10^{-2}	0.43		
	Azithromycin[d]	3.734×10^{-2}	5.186×10^{-3}	< 0.01		

[a] PC, Rates of penicillin-nonsusceptible strains for children; PA, rates of penicillin-nonsusceptible strains for adults; EC, rates of erythromycin-nonsusceptible strains for children; EA, rates of erythromycin-nonsusceptible strains for adults.
[b] *t* test for coefficient significance.
[c] Variables included in the model for backward selection: amoxicillin plus the main used antibiotics (clavulanic acid, flucloxacillin, clarithromycin, azithromycin) and vaccine consumption (see Material and methods).
[d] Exponential of the antibiotic use with a time lag of 1 year.
[e] Antibiotic use.
[f] Heptavalent pneumococcal conjugate vaccine use.
[g] Exponential of clarithromycin use with time lag of 1 year plus azithromycin use.
[h] Data for 1998 was excluded from this model due to the atypical values.

Parameters estimation and analysis. The EC and EA models suggest that the use of azithromycin was highly associated with macrolide resistance independent of the age group (Tables 4.2 and 4.4). The PC model suggests a link between the nonsusceptibility of isolates and the use of amoxicillin, flucloxacillin and azithromycin. The detection of the effect of the introduction of the heptavalent vaccine in penicillin nonsusceptibility among children is also of note (Table 4.3 and Figure 4.3E). The PA model suggests that the use of clarithromycin and azithromycin were highly associated with penicillin nonsusceptibility among adults with IPD (Table 4.3). Several scenarios were constructed based on the PC model. In the absence of vaccine introduction, nonsusceptibility to penicillin was expected to achieve values greater than 55% in 2004 (Figure 4.3E). In the presence of the pneumococcal conjugate vaccine and in the absence of azithromycin use, the penicillin nonsusceptibility rate was expected to be reduced to 17% against the 40% observed in 2004. If no vaccine and azithromycin were used, the penicillin nonsusceptibility rate was expected to be similar to that actually observed (with vaccine and azithromycin use) (Figure 4.3E).

4.5 - Discussion

The introduction of the heptavalent pneumococcal conjugate vaccine was expected to reduce the burden caused by IPD and reduce the rates of antimicrobial nonsusceptible isolates among pneumococcal strains. This vaccine was licensed in Portugal in 2001 and is currently recommended on a voluntary basis because it is not included in the national vaccination program. The Portuguese Society of Paediatrics recommends the use of the conjugate vaccine in all children aged <2 years old and in the population at risk for IPD (Sociedade Portuguesa de Pediatria, 2003). In the north of Portugal, in 2001 to 2002, 54.5% of the children born after 1999 were vaccinated against pneumococcal infection (De Queirós *et al.*, 2004), and the use of this vaccine has been increasing from 2002 to 2005; it was estimated that vaccine coverage in 2004 was 65% among children aged <6 months who had received an average of 2.5 doses (data not shown).

Here, among children, we demonstrated a decrease (63%) of the rate of penicillin-nonsusceptible *S. pneumoniae* strains from 2000 (57%) to 2003 (21%), followed by an increase (40% in 2004). We were unable to show a significant difference in the rates of penicillin-nonsusceptible pneumococci strains between the prevaccine and vaccine period ($P = 0.125$), although the observed early decrease may have been associated with vaccination. However, a decreased use of the main β-lactam agents was also evident in the same period (Table 4.1). This is consistent with the finding of Whitney *et al.* (2003) who also observed a reduction (35%) of penicillin-nonsusceptible strains from IPD in the United States, 2 years after the introduction of the vaccine.

The PC model indicated that the nonsusceptibility of isolates to penicillin among children correlated positively with the use of amoxicillin and azithromycin, and was negatively associated with the use of pneumococcal conjugate vaccine and flucloxacillin (Table 4.3). These antibiotics were the main β-lactams and macrolide used in Portugal, as found in this study and others (Dias & Caniça, 2004; Falcão *et al.*, 2003; Goossens *et al.*, 2005). The PC model also suggests that if the vaccine had not been introduced in Portugal, nonsusceptibility to penicillin was expected to achieve values greater than 55% in 2004 (Figure 4.3E). This might mean that the pneumococcal conjugate vaccine had potentially reduced by more than 15% the rate of penicillin nonsusceptibility. However, our results also suggest that azithromycin use in the studied population limited the efficiency of the pneumococcal conjugate vaccine. Indeed, if pneumococcal conjugant vaccine was used and azithromycin was not used, the penicillin nonsusceptibility was predicted to be reduced to 17% in 2004 (Figure 4.3E), a similar reduction was experienced by Whitney after the introduction of anti-pneumococcal conjugate vaccine in the United States (Whitney *et al.*, 2003). Conversely if

no vaccine or azithromycin were used, the penicillin nonsusceptibility rate was predicted to be similar to that observed (Figure 4.3E).

The increase of penicillin nonsusceptibility among isolates was due to cross-resistance to macrolides and not to changes in the consumption of β-lactams (Table 4.1). Several studies have shown cross-resistance to be responsible for the emergence of resistance to other antibiotics. Amoxicillin use significantly increased the number of amoxicillin plus erythromycin resistant isolates (Ready *et al.*, 2004). However, other antibiotics (e.g. cephalosporins, macrolides and co-trimoxazole) can be equally or more important than aminopenicillins in promoting the selection of penicillin nonsusceptible pneumococci (Dancer, 2001), and selection by cephalosporins can occur at higher frequencies than selection by amoxicillin (Canet & Garau, 2002). The use of macrolides has been reported to be a more effective selector of strains nonsusceptible to macrolides plus β-lactams than the use of aminopenicillins (Arason *et al.*, 1996; Baquero *et al.*, 2002), and azithromycin use has been linked to macrolide and other resistances in pneumococci (Barkai *et al.*, 2005; Dias & Caniça, 2004). As observed recently in Portugal (Dias *et al.*, 2006), there has also been an increase of penicillin resistance associated with macrolide resistance elsewhere (Barkai *et al.*, 2005; Bronzwaer *et al.*, 2002). These results are consistent with the PC and PA models (Figure 4.3 and Table 4.3). Indeed, the high potential of azithromycin to select resistant, multiresistant, and coresistant strains, may be due to its long half-life (<72 h) (Nightingale, 1997), and its very slow elimination resulting in subinhibitory concentrations in the tissues (Kastner & Guggenbichler, 2001).

In this study amoxicillin plus clavulanic acid use was found to be associated with nonsusceptibility to penicillin and erythromycin (Table 4.1), however our models showed that their impact is reduced in penicillin-nonsusceptible rates (Table 4.3). Amoxicillin plus clavulanic acid has been described to eradicate or suppress all pneumococci susceptible to penicillin and has good activity against strains with intermediate and/or full resistance to penicillin (Ghaffar *et al.*, 2000). The latter study also reported that azithromycin (in children with acute otitis media) was able to clear 33% of susceptible pneumococcal strains, but none of the azithromycin-nonsusceptible strains tested was eliminated (Ghaffar *et al.*, 2000). Azithromycin has also been associated with the selection of a higher number of resistant clones than amoxicillin-clavulanate (Kosowska-Shick *et al.*, 2006). These findings are consistent with the suggested impact of these antibiotics by the models used.

The largest groups of *S. pneumoniae* strains not susceptible to penicillin and erythromycin, in our population, were of serotypes covered by the 7-valent vaccine. Similar to previous studies (Whitney *et al.*, 2003), our results suggest that the vaccine covers almost all nonsusceptible serotypes. However, resistance was also found in serotypes 15C, 19A, and 34, which are absent from the vaccine. This may be a concern after vaccination, as Lipsitch

(1997) pointed out, because serotype replacement is expected in an ecosystem where many serotypes are present. Studies of *S. pneumoniae* isolated in DCC indicate that the 7-valent vaccine can reduce the carriage of resistant isolates (Dagan *et al.*, 2001). A Portuguese study showed a decrease in vaccine serotypes, but an increase of nonvaccine serotypes among drug-resistant *S. pneumoniae* isolated from children attending DCCs (Frazão *et al.*, 2005), and this is supported by Temime et al. (Temime *et al.*, 2004) underlining that serotype replacement constitutes a real threat. Indeed, the replacement effect involving penicillin-nonsusceptible strains usually isolated from carriers might accelerate in the coming years among strains isolated from meningitis, as the nasopharynx is an important reservoir of such strains (Bogaert *et al.*, 2004a) and the selection pressure caused by antibiotics is continuous (Cars *et al.*, 2001).

We found similar rates of vaccine coverage of penicillin-nonsusceptible strains to those reported in other European countries and in the United States (Reinert, 2004; Whitney *et al.*, 2003). The theoretical overall coverage conferred by the pneumococcal conjugate vaccine in Portugal is similar to that in some other European countries (Reinert, 2004), but lower than the coverage shown in the United States (Whitney *et al.*, 2003). As noted previously, we found penicillin-nonsusceptible strains to also be nonsusceptible to cefotaxime and ceftriaxone (Caniça *et al.*, 2002), but specifically among children aged <2 years there are high rates of resistance (7.6%) to these agents, which are the most widely used for empirical treatment of meningitis caused by *S. pneumoniae* (World Health Organization, 1998). Thus, the replacement effect to nonvaccine serotypes that occurs among penicillin-nonsusceptible strains can also contribute to a decrease in the vaccine coverage of cefotaxime- and ceftriaxone-nonsusceptible strains in the future.

Since the introduction of azithromycin in Portugal in 1996 extensive changes among circulating clones of pneumococci have been observed (Dias & Caniça, 2004). As a consequence, erythromycin-nonsusceptible rates among isolates from children between 1996 and 1999 were atypical, as they were higher than the observed trend in the following years; thus the EC model did not fit so well. However, outside of this period, we did not observe marked differences in trends of erythromycin-nonsusceptible strains during the prevaccine and vaccine periods, as suggested by the EC model. This indicates that the introduction of the conjugate vaccine into Portugal did not significantly decrease the incidence of erythromycin-nonsusceptible strains due to the increased use of azithromycin (Figure 4.3, Table 4.1 and 4.3).

Our results illustrate that the use of macrolides class has been stable in the last years, but there has been a substitution of older macrolides mainly by azithromycin. No cross-resistance between macrolides (erythromycin) and telithromycin has been shown (Walsh *et al.*, 2004), but recently the first telithromycin resistant and otherwise multiresistant

isolate of serotype 15A (nonvaccine serotype) was reported from Germany (Reinert *et al.*, 2005a). In Portugal, extensive use of telithromycin in a population who have received the vaccine could lead to the emergence of this clone, with serious implications for resistance levels to penicillin and macrolides due to cross-resistance.

The changes in penicillin susceptibility in *S. pneumoniae* are mainly clonal (Caniça *et al.*, 2003), but for erythromycin susceptibility the selective pressure causes greater oscillations and adaptations within the bacterial population, due to the nature of the resistance and its dissemination (Pozzi *et al.*, 2004). Hence, there is a greater diversity of serotypes among erythromycin-nonsusceptible pneumococci than among penicillin-nonsusceptible strains which cannot be explained solely by drug use. Indeed, the greater diversity of nonsusceptible strains in addition to the selective pressure caused by vaccine and antibiotic consumption could enhance horizontal transfer of resistance elements among nonvaccine serotypes and/or capsular switching among nonsusceptible vaccine serotypes. Thus, replacement among erythromycin-nonsusceptible strains seems to involve a rapid turnover, and this may have serious consequences for public health.

In conclusion, linear regression models were used to assess the role of antimicrobial and vaccine use in the rates of penicillin and erythromycin resistance among pneumococci. The models did not allow a mechanistic interpretation of the antibiotic resistance phenomena, but they provided very useful information on main factors involved in the selection of penicillin- and erythromycin-resistant strains: a complex context where multiple antimicrobial and vaccine use are present. Moreover, the suggestions obtained by the models fitted well with phenotypic observations. It is theoretically possible that the observed trends of antibiotic resistance in Portugal may be reversed by the adequate use of existing and new antibiotics, contributing to control of invasive pneumococcal disease. The reduced number of years and the correlation between the independent variables were the major limitations on the use of the multiple regression models in the study.

CHAPTER V

Emergence of invasive erythromycin-resistant *Streptococcus pneumoniae* strains in Portugal: contribution and phylogenetic relatedness of serotype 14

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5.1 - Summary

To study the phenotype and phylogenetic relatedness of invasive *Streptococcus pneumoniae* strains isolated in Portugal. A total of 614 invasive *S. pneumoniae* strains, isolated in Portugal between 1999 and the first 6 months of 2002, were characterized using serotyping and antimicrobial susceptibility testing. National outpatient sale data for macrolides was compared with erythromycin resistance. We investigated the main clonal lineages from erythromycin-nonsusceptible strains of serotype 14 by Pulsed-Field Gel Electrophoresis (PFGE) and multilocus sequence typing (MLST). The emergence of erythromycin-nonsusceptible strains correlated well with the usage of azithromycin in Portugal during the period of the study (r = 0.900, P = 0.001). Serotype 14 made the largest contribution to this emergence. We found two clonal complexes (CC) among erythromycin-nonsusceptible strains: CC1 was formed by three sequence types (ST), ST156, ST143 and ST1042, and CC2 by ST15 and ST9. All STs described, except ST1042, are putative founders of clonal groups or sub-groups from serotype 14 as defined in the pneumococcal MLST database. This study suggests that macrolides usage is an important factor enhacing the spread of invasive erythromycin-nonsusceptible *S. pneumoniae* clones in Portugal. The study also makes a contribution to the understanding of spread of erythromycin-nonsusceptible clones in an international context.

5.2 - Introduction

The emergence of drug resistant *Streptococcus pneumoniae* is a growing problem worldwide (Appelbaum, 1996). In Europe, the prevalence of macrolide resistance is increasing in non-invasive strains mainly in southern countries (Jacobs *et al.*, 2003). In Portugal, the prevalence of erythromycin resistance of *S. pneumoniae* was stable during 1989 to 1993 (Vaz Pato *et al.*, 1995). To our knowledge, no further data have been puclished since. Molecular typing analyses show that only a small number of dominant clones are responsible for the spread of antibiotic resistance worldwide (McGee *et al.*, 2001).

We analysed the macrolide usage and the genetic background of invasive *S. pneumoniae* clones of serotype 14 responsible for the increase of erythromycin resistance in Portugal. We also evaluated their relationships with the drug-resistant clones in other countries.

5.3 - Materials and methods

Clinical isolates. Between January 1999 and June of 2002, 614 consecutive and non-repetitive invasive *S. pneumoniae* strains were collected in the ARU at NIH Dr. Ricardo Jorge in Lisbon, in a multicenter study with 24 participating hospitals. The strains were isolated from blood, CSF and pleural liquid from clinically ill patients.

Phenotypic characterization. For all isolates, MICs of penicillin (Wyeth Lederle Portugal, Algés, Portugal), cefotaxime and ceftriaxone (Roche Pharmaceuticals, Amadora, Portugal), tetracycline and chloramphenicol (Atral, Carregado, Portugal), erythromycin (Abbot Laboratórios, Amadora, Portugal), clindamycin (Laboratórios Upjohn Internacional, Lisboa, Portugal), ofloxacin (Farmoz, Prior Velho, Portugal) and ciprofloxacin (Bayer Portugal, Carnaxide, Portugal) were determined by an agar dilution method. MICs were interpreted according to NCCLS (National Committee for Clinical Laboratory Standards, 2000), excepting for ciprofloxacin (resistance was defined as a MIC value ≥4 mg/L). The MLS_B phenotype was scored when simultaneous resistance to erythromycin and clindamycin was observed and the M phenotype when resistance to erythromycin only was observed. The M phenotype was confirmed by the induction test described previously (Seppälä *et al.*, 1993). Serotypes were performed by dot-blot and Quellung reaction using type-specific antiserum (Statens Serum Institut, Copenhagen, Denmark) (Fenoll *et al.*, 1997).

Antimicrobal Use data. National outpatient sale data [taken from International Medical Statistics (IMS Health, Portela, Portugal)] were used for macrolides/lincosamides (J01F) (azithromycin, clarithromycin, clindamycin, erythromycin, lincomycin, midecamycin, roxithromycin, spiramycin, telithromycin) for the period 1994 to 2002. The data were normalized to the ATC system proposed by WHO (World Health Organization, 1999b). The number of packages consumed annually per 1,000 inhabitants was used to enable the comparison of macrolide use over time in the same geographic area. The correlation between antimicrobial consumption and rates of macrolides resistance was assessed by the Spearman correlation coefficient applying the natural logarithm to the antimicrobial resistance, in order to have a linear relation. The null hypothesis was rejected for *P* values of <0.01.

Genotypic characterization. All erythromycin-resistant strains of serotype 14 (n=25) were investigated by PFGE and multi locus sequence typing (MLST) (Caniça *et al.*, 2003; Enright & Spratt, 1998). PFGE DNA fingerprints and MLST data were combined and analysed using Bionumerics software (version 3.0) (Applied Maths, St-Martens-Latem, Belgium). e-BURST is an algorithm for displaying the relationships between closely-related isolates of a bacterial species or population. This analysis was used to determine the main CCs and the order of Sequence Types (ST) within CCs from MLST data (Aanensen & Spratt,

2005). The algorithm predicts the descent from the founding genotype to the other genotypes in the group, displaying the output as a radial diagram, centred on the predicted founding genotypes. All strains of serotype 14 (n=274) in the pneumococcal MLST database were included in the analysis (Aanensen & Spratt, 2005), to establish phylogenetic relationships between the erythromycin-nonsusceptible strains of this study and the principal CCs of serotype 14 already described.

5.4 - Results and discussion

Phenotypic characterization. The MICs of 614 strains isolated in Portugal in 1999, 2000, 2001 and the first 6 months of 2002 were determined. The MICs of erythromycin were in the range 0.5 and ≥64 mg/L and for clindamycin in the range 2 and ≥ 64 mg/L. Overall, 13.6% and 10.1% of strains were nonsusceptible to erythromycin (MIC ≥ 0.5 mg/L) and clindamycin (MIC ≥ 0.5 mg/L), respectively. We showed an emerging macrolide resistance compared with the period 1989 to 1993 (Vaz Pato *et al.*, 1995): when 1.9% and 1.4% of strains were nonsusceptible to erythromycin and clindamycin, respectively. This observation is consistent with trends in other countries (Jacobs *et al.*, 2003). In our study, the majority of erythromycin-nonsusceptible strains (74%) were also nonsusceptible to clindamycin and so had the MLS_B phenotype, as in the period 1989 to 1993 (76%).

Most erythromycin-susceptible strains were in one of three serotypes: 14 (31.0%), 6B (26.2%) and 19F (14.3%). The most common multidrug resistance phenotypes were: (i) penicillin, tetracycline, erythromycin and clindamycin, mainly for serotypes 6B, 19F, 14 (in descending order); (ii) tetracycline, erythromycin, clindamycin and chloramphenicol, for serotypes 6B and 19F; and (iii) penicillin, ceftriaxone, cefotaxime, tetracycline, erythromycin and clindamycin only for serotype 14.

Antimicrobial consumption and resistance. Outpatient sales of macrolides varied between 149.2 packages/1,000 inhabitants/year in 1994 to 220.4 in 2002 (Figure 5.1A). Clarithromycin use was increased from 21.3 packages/1,000 inhabitants/year in 1994 to 75.2 in 1999, and decreased until 2002 (58.8 packages/1,000 inhabitants/year) (Figure 5.1A). Azithromycin has only been sold in Portugal since 1995 (0.85 packages/1,000 inhabitants/year). Azithromycin use had a significant increase from 32.2 packages/1,000 inhabitants/year in 1996 to 107.5 in 2002 (Figure 5.1A).

A strong correlation was found between the resistance to erythromycin and the use of azithromycin (r = 0.900, P = 0.001) (Figure 5.1B). These results suggest that the increase of azithromycin use is associated to the increase of macrolide resistance in *S. pneumoniae*.

Genotypic characterization. Genotypic characterization of the largest serotype contributor (serotype 14) showed that the increase of erythromycin resistance is due to clones included in two CCs, formed by five STs (Figure 5.2 and 5.3). The CC1 was formed by ST156, ST143 and ST1042 (Figure 5.2). MLST showed that strains of ST143 are double loci variants (DLV) from ST156 (Figure 5.3A). Isolate 3569 is a previously undescribed single locus variants (SLV) of ST156, and was assigned to ST1042 (Figure 5.3A). CC2 consisted of ST15 and ST9 (Figure 5.2). ST9 is a SLV from ST15 (Figure 5.3B). e-BURST analysis showed that CC1 and CC2 found in this study (Figure 5.2) are part of the lineage 3 (Figure

5.3A) and 2 (Figure 5.3B), respectively. These lineages were found among 121 STs (grouped into 10 lineages) from all serotype 14 strains in the pneumococcal MLST database.

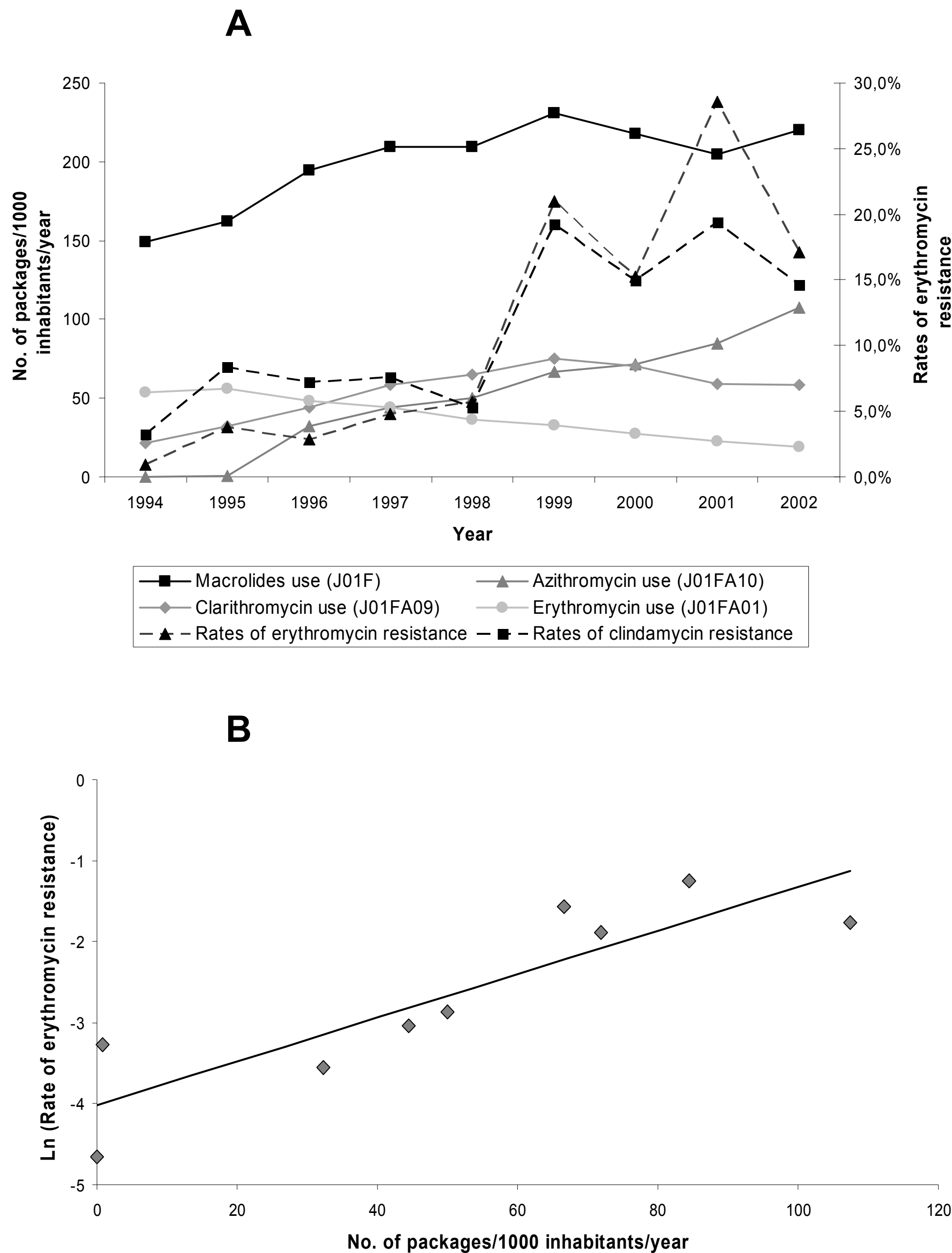
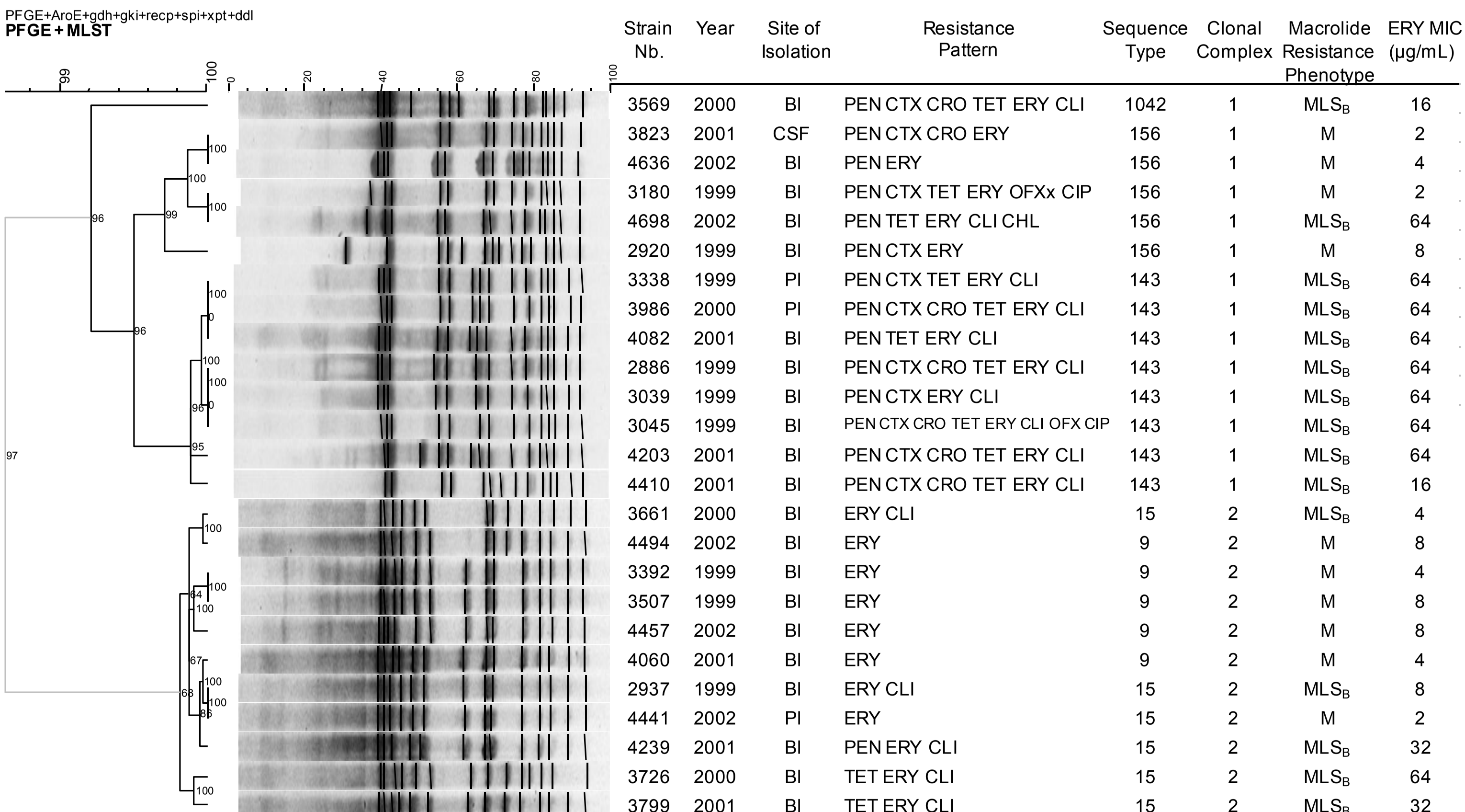


Figure 5.1 – *S. pneumoniae* macrolide resistance and antimicrobial consumption. (A) Antimicrobial usage and resistance. (B) Correlation between azithromycin use and rates of erythromycin resistance, during 1994 to 2002, using Spearman's correlation coefficient (r = 0.900, P = 0.001). Source: rates of antimicrobial resistance (M. Caniça).



Strain Nb.	Year	Site of Isolation	Resistance Pattern	Sequence Type	Clonal Complex	Macrolide Resistance Phenotype	ERY MIC (µg/mL)
3569	2000	BI	PEN CTX CRO TET ERY CLI	1042	1	MLS_B	16
3823	2001	CSF	PEN CTX CRO ERY	156	1	M	2
4636	2002	BI	PEN ERY	156	1	M	4
3180	1999	BI	PEN CTX TET ERY OFXx CIP	156	1	M	2
4698	2002	BI	PEN TET ERY CLI CHL	156	1	MLS_B	64
2920	1999	BI	PEN CTX ERY	156	1	M	8
3338	1999	PI	PEN CTX TET ERY CLI	143	1	MLS_B	64
3986	2000	PI	PEN CTX CRO TET ERY CLI	143	1	MLS_B	64
4082	2001	BI	PEN TET ERY CLI	143	1	MLS_B	64
2886	1999	BI	PEN CTX CRO TET ERY CLI	143	1	MLS_B	64
3039	1999	BI	PEN CTX ERY CLI	143	1	MLS_B	64
3045	1999	BI	PEN CTX CRO TET ERY CLI OFX CIP	143	1	MLS_B	64
4203	2001	BI	PEN CTX CRO TET ERY CLI	143	1	MLS_B	64
4410	2001	BI	PEN CTX CRO TET ERY CLI	143	1	MLS_B	16
3661	2000	BI	ERY CLI	15	2	MLS_B	4
4494	2002	BI	ERY	9	2	M	8
3392	1999	BI	ERY	9	2	M	4
3507	1999	BI	ERY	9	2	M	8
4457	2002	BI	ERY	9	2	M	8
4060	2001	BI	ERY	9	2	M	4
2937	1999	BI	ERY CLI	15	2	MLS_B	8
4441	2002	PI	ERY	15	2	M	2
4239	2001	BI	PEN ERY CLI	15	2	MLS_B	32
3726	2000	BI	TET ERY CLI	15	2	MLS_B	64
3799	2001	BI	TET ERY CLI	15	2	MLS_B	32

Figure 5.2 - Proprieties and phylogenetic relationships based on MLST and PFGE data from 25 erythromycin-nonsusceptible strains with serotype 14. The Dice band-based similarity coefficient was used for PFGE pattern analysis, with a band position tolerance of 1% and an optimization of 1%. PFGE DNA fingerprints and MLST data were combined and analysed using Bionumerics software. Dendrogram was constructed by the unweighted pair-group method with average linkages using the composite data set of MLST and the PFGE patterns. BI, blood; PI, pleural liquid; CSF, Cerebrospinal fluid; PEN, Penicillin; CTX, Cefotaxime; CRO, Ceftriaxone; TET, Tetracycline; ERY, Erythromycin; CLI, Clindamycin; CHL, Chloramphenicol; OFX, Ofloxacin; CIP, Ciprofloxacin.

Erythromycin-nonsusceptible clone ST156 present in Portugal is a serotype 14 variant of the erythromycin-susceptible international clone Spain^{9V}-3 (Spain^{9V}-3-14) (Caniça *et al.*, 2003; Coffey *et al.*, 1999). This variant spread internationally, but most isolates are susceptible to macrolides, as identified in MLST database (Figure 5.4) (Aanensen & Spratt, 2005). ST156 found in this study has been described in different countries, but in Uruguay it was described as being nonsusceptible to erythromycin (Aanensen & Spratt, 2005; Coffey *et al.*, 1999). e-BURST analysis of all strains with serotype 14 in the pneumococcal MLST database may indicate that ST156 was the first in the evolutionary pathway of CC1 (the ancestral type) (Figure 5.3A). The greater variability of PFGE and resistance patterns of ST156 than of ST143 in our sample is consistent with this possibility. ST144, not present among our strains (SLV variant of ST156), is a putative ancestor of ST143.

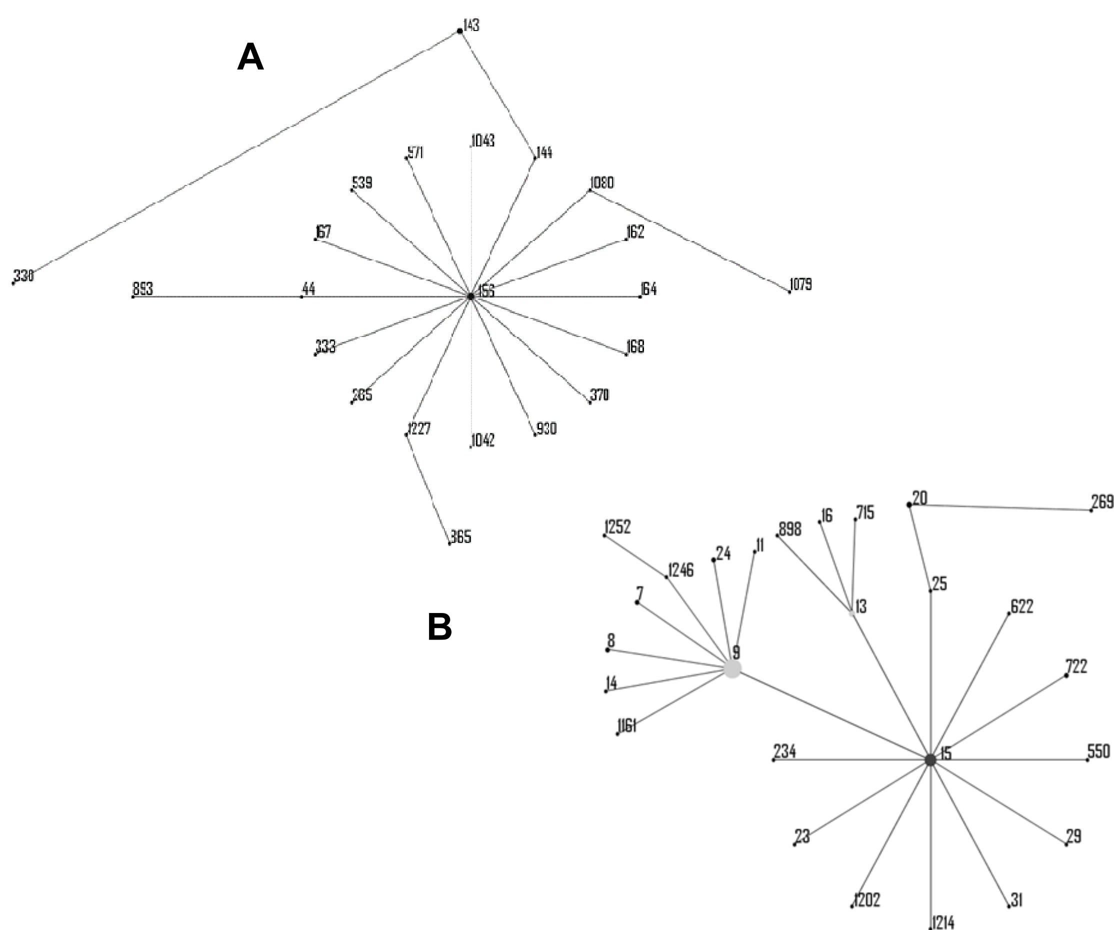


Figure 5.3 - Relationship between 25 erythromycin-nonsusceptible strains of serotype 14 from this study and serotype 14 strains from pneumococcal MLST database by e-BURST analysis. (A) Lineage 3 from eBURST analysis: ST156 is the ancestral type. Clonal complex 1 (ST156, ST143 and ST1042, from this study) is included in this lineage. (B) Lineage 2 from e-BURST analysis: ST15 is the ancestral type. Clonal complex 2 (ST15 and ST9, from this study) is included in this lineage; the black circle in the centre of e-BURST diagrams predicted primary founders; the gray circle defines a subgroup founder; Numbers on diagram correspond to the STs.

ST143 is the biggest clonal lineage in our sample (8 of 25 strains). This lineage has previously been described in Poland: all but one were erythromycin-susceptible isolates (Aanensen & Spratt, 2005). Possibly this Portuguese clone was imported from that country. ST330 is an erythromycin-nonsusceptible clonal lineage, and is SLV of ST143 (Figure 5.3A) (Aanensen & Spratt, 2005). e-BURST analysis suggests that this ST, originally from a subpopulation of ST143 erythromycin-nonsusceptible strains, is probably of the same population as found in our study, and is genetically more distant from ST156. The new multidrug-resistant clonal lineage ST1042 could be a result of a recombinational event at the *ddl* locus.

Our ST9 strains had the same allelic profile and resistance pattern as England^{14}-9, an international erythromycin-nonsusceptible clone (McGee *et al.*, 2001). This clone of serotype 14 has been described in the United Kingdom, Italy, Argentina, Germany (Aanensen & Spratt, 2005), and in carriers in Portugal (Sá-Leão *et al.*, 2001). In Australia, this clone was found in susceptible and nonsusceptible strains, as identified in the updated pneumococcal MLST database (Aanensen & Spratt, 2005). The clonal lineage ST15 we found among Portuguese invasive strains has also been described in the United Kingdom, Taiwan, Italy, Hong Kong, Germany (Aanensen & Spratt, 2005), and in Portuguese carriers (Sá-Leão *et al.*, 2001). Both susceptible and nonsusceptible strains of this ST have been described in Brazil, and susceptible strains have been found in the Netherlands (Aanensen & Spratt, 2005). e-BURST analysis shows that ST15 might be the ancestral type for CC2 (Figure 5.3B). This is consistent with the diversity of ST15 resistance patterns in our study (erythromycin, erythromycin plus clindamycin, penicillin plus erythromycin plus clindamycin, and tetracycline plus erythromycin plus clindamycin) compared with ST9 (only erythromycin resistance pattern). Possibly ST9 diverged from ST15 very recently, differing as attested by a single nucleotide polymorphism in the *xpt* gene sequence. This is reinforced with the PFGE data, which showed that ST15 and ST9 are phylogenetically very close.

Four STs described in this work (ST156, ST143, ST15 and ST9) are putative founders of major clonal groups and subgroups within serotype 14; they probably already existed in Portugal prior to the increase of erythromycin resistance. Both erythromycin-nonsusceptible and susceptible strains from invasive diseases are found in the same clonal lineage in several countries, including Portugal (data not shown). As the STs we describe include members of the major internationally disseminated clones of serotype 14 susceptible to macrolides, this is also suggestive that the resistance mechanisms were acquired locally (Figure 5.4).

To our knowledge, this study is the first report of the genetic relatedness of invasive pneumococcal clones responsible for the emergence of erythromycin resistance in Portugal. This study suggests that azithromycin use is an important selection factor for the spread of these clones in Portugal, which is also associated with the increase in macrolide resistance.

This also provides a contribution to the understanding of the spread of these antibiotic nonsusceptible clones in an international context.

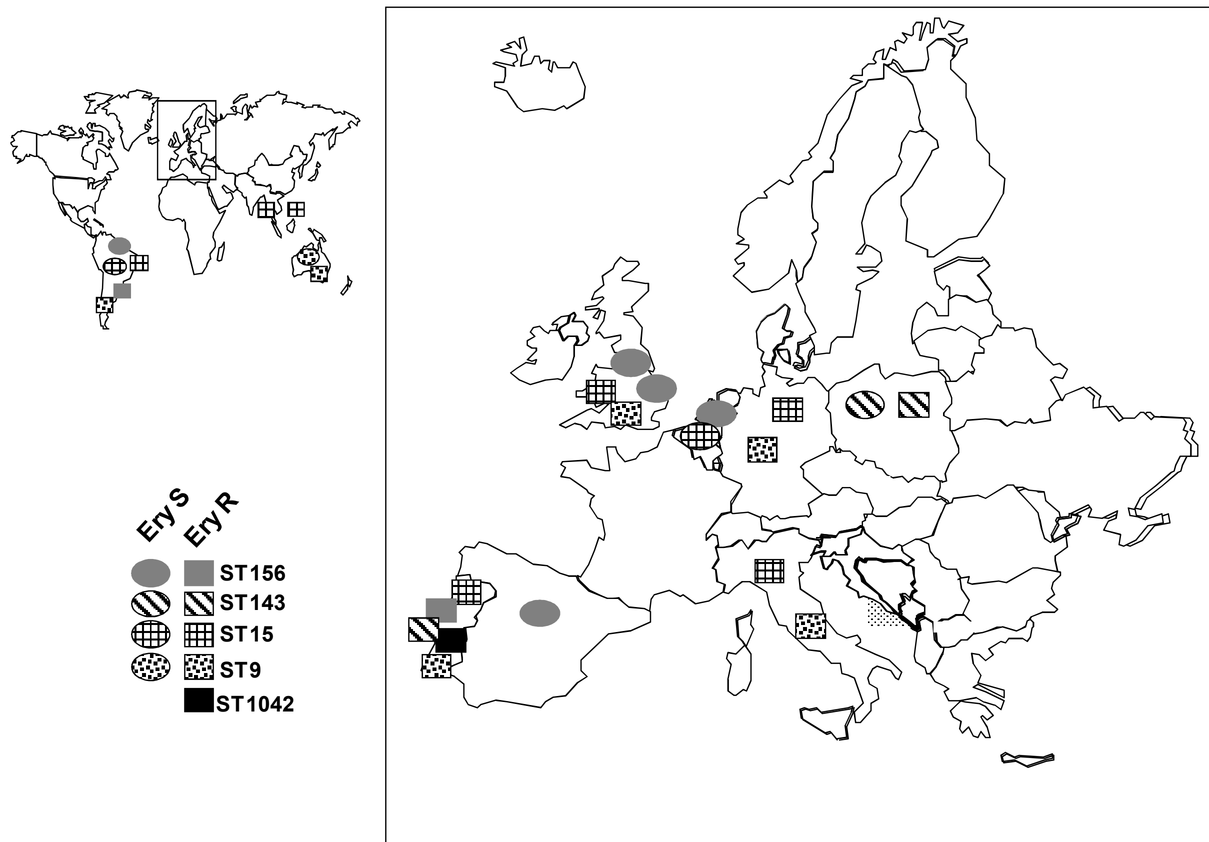


Figure 5.4 - International distribution of pneumococcal clones of serotype 14 found in Portugal. Also shown is a local clone, the ST1042. Ery S, erythromycin-susceptible clones; Ery R, erythromycin-nonsusceptible clones. ST, sequence type. Source: Pneumococcal MLST database (Aanensen & Spratt, 2005).

CHAPTER VI

Characterization of the genetic structure of invasive macrolide-resistant pneumococci in Portugal

Unpublished
Dias R, Felix D, Pissarra C, Ferreira E & Caniça M (2008)

6.1 - Summary

We studied the genetic structure of a population of 186 macrolide-nonsusceptible *Streptococcus pneumoniae* strains recovered from cases of invasive disease in Portugal between 1994 and 2004. Genetic characterization involved PFGE (n=186) and MLST (n=73) followed by linage assignment by e-BURST analysis. We found 41 ST constituting 17 CC and 4 singletons. Most of the STs observed correspond - or are closely related to - internationally disseminated clones. Twenty-three of the STs have been described previously, and 18 have only been noted in Portugal, including 13 STs novel to this study. We note that the prevalence of macrolide resistance in pneumococci has increased in Portugal by two routes: i) expansion of pre-existing nonsusceptible clones, and importation of nonsusceptible clones mainly present in other European countries and their local diversification.

6.2 - Introduction

The spread of macrolide- and multidrug-resistant *Streptococcus pneumoniae* has become a worldwide problem over recent years (Klugman & Lonks, 2005). In Europe, the prevalence of nonsusceptibility to penicillin and macrolides is increasing mainly in the southern countries (Bronzwaer *et al.*, 2002). A national surveillance study in Portugal indicated a significant increase in macrolide-nonsusceptible *S. pneumoniae* infections recovered from invasive disease over the period 1998-2004, mostly associated with MLS_B phenotype (Dias *et al.*, 2006). This trend has been reported to be related to the use of azithromycin (Dias & Caniça, 2004; Dias & Caniça, 2008). The erythromycin nonsusceptibility was also reported to be related to penicillin susceptibility (Dias *et al.*, 2006).

Nonsusceptibility to macrolides can spread through several mechanisms: i) spread of nonsusceptible native clones already present at a low frequency, (clonal expansion); ii) importation and spread of nonsusceptible clones from elsewhere (clonal importation), and iii) the emergence of new resistant clones from local susceptible clones (clonal emergence) (Hanage *et al.*, 2007).

Here we describe a molecular survey with the following aims: (i) to determine the genetic structure of populations of macrolide-nonsusceptible pneumococci in Portugal, (ii) to identify the genetic lineages responsible for the increase of macrolide nonsusceptibility, and (iii) to assess the mechanisms underlying the emergence of the resistance.

6.3 - Materials and methods

Bacterial strains. The 186 pneumococci isolates studied were the viable, erythromycin-nonsusceptible strains from the population of 191 strains described previously isolated from invasive disease in Portugal between January 1, 1994, and December 31, 2004 (Dias *et al.*, 2006).

PFGE. All 186 isolates were studied by PFGE. Chromosomal DNA was prepared and PFGE performed with *Sma*I endonuclease as described previously (Caniça *et al.*, 2003). Gels were stained with ethidium bromide and scanned using the Geldoc 2000 system (Bio-Rad, Hercules, California). The different patterns for each serotype were compared using the Dice coefficient of similarity and cluster analysis with the unweighted pair group method with arithmetic averages. Position tolerance and optimization were set to 1.0 and 1.5, respectively using BioNumerics software version 3.5 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Unique PFGE patterns were assigned capital letter, and the letters were followed by a number for PFGE clusters. A PFGE cluster was defined as 2 or more isolates with ≥80% pattern similarity.

MLST. At least one strain from each major PFGE pattern was randomly chosen for MLST. Two to 7 isolates were used for PFGE clusters containing more than 5 isolates. Thus, 73 strains were subjected to MLST as described by Enright and Spratt (Enright & Spratt, 1998). The *S. pneumoniae* MLST website at http://www.mlst.net (Aanensen & Spratt, 2005) was used to determine allelic profiles, including new alleles, and for ST assignments. e-BURST version 3 with the MLST database updated on May 10th, 2007 (Feil *et al.*, 2004), was used for genetic linage assignment. The following nomenclature was used: CC were labeled with the ST number of the putative founder of the complex and, as appropriate, with the ST number of the founder of the major sub-group; singletons are indicated by the capitals SGT, followed by the number of the corresponding ST. An isolate assigned to a CC if, according to MLST analysis, it had identical alleles with at least one other member of the clonal group at ≥ 6 of the 7 loci (the most restrictive clonal group definition was used). The statistical confidence of the assignment of primary complex founders was determined by 1000 bootstrap re-samplings. Isolate for which only PFGE data was available were assigned to CCs on the basis of PFGE profiles with a similarity greater than 80% to an isolate in that CC genotyped by MLST.

Definition of acquisition of erythromycin resistance. Three periods were considered: the period prior to the emergence of macrolide nonsusceptibility (1994 to 1997) and the period of emergence of macrolide nonsusceptibility split into those before (1998 to 2000) and after (2001 to 2004) the introduction of the pneumococcal heptavalent conjugate vaccine. A nonsusceptible clone was defined as undergoing to clonal expansion if it had

been isolated in Portugal prior to the emergence of macrolide nonsusceptibility (1994 to 1997). An imported clone was defined as an erythromycin-nonsusceptible clone not previously recorded in Portugal but described previously elsewhere. Local acquisition of erythromycin nonsusceptibility was recorded for erythromycin-nonsusceptible variants of susceptible clones previously described in Portugal. An erythromycin-nonsusceptible clone firstly isolated in Portugal was defined as a local clone.

6.4 - Results

Genetic structure of macrolide-nonsusceptible isolates. We used PFGE and MLST to identify the genetic lineages of the 186 viable macrolide-nonsusceptible isolates collected between 1994 and 2004. We found 110 PFGE types, grouped into 14 clusters and 17 unique patterns (Table 6.1). MLST analysis of the 73 PFGE-representative strains revealed 41 STs, with 17 CCs and four SGTs (Table 6.1 and Figure 6.1). Five of the 17 CCs identified were respectively recovered from single isolates associated with unique PFGE types.

Twenty-three of the 41 STs we found have been described previously, mainly in European countries; 18 were only found in Portugal, and 13 of these were described for the first time in this study (Table 6.1). Among the isolates characterized by PFGE, 161 (86%) belonged to clonal complexes related to PMEN clones described elsewhere in the world (McGee *et al.*, 2001); among the 41 STs, 10 were PMEN clones and 21 were SLV or DLV of PMEN clones.

Temporal Trends. The evolution of previously described clonal complexes was assessed. CC156/162, CC15/9, CC146/90, CC315/1093 and CC81 were isolated between 1994 and 1997, and between 1998 and 2000. In contrast, the following clones were only isolated between 1998 and 2000: CC63, CC460/97, CC176/138, CC177/179, CC193/410, CC230/878 and CC717. In the period 2001 to 2004, after the introduction of heptavalent conjugate vaccine, new genetic lineages were isolated, including singletons SGT2357, SGT2358, SGT2359 and SGT2768. Clonal complexes CC88, CC180/505, CC191 and CC306/227 were also found for the first time during this late period of the study (Table 6.2). The rate of penicillin nonsusceptibility among erythromycin-nonsusceptible strains did not changed significantly across time (Chi square test, P = 0.724) (50.0%, period 1994 to 1997; 48.8%, period 1998 to 2000; and 43.5%, period 2001 to 2004).

Serotypes and drug nonsusceptibility related to clonal complexes. The major serotypes of erythromycin-nonsusceptible strains (14, 6B, 19A and 19F, accounting for 70.5% of strains with this phenotype) described previously (Dias *et al.*, 2006), were differently distributed among the clonal complexes we defined (Table 6.1). Penicillin-nonsusceptible strains were related mainly to CC156/162 (82%), CC230/878 (95%), CC63 (78%) and CC315/1093 (70%). However, in other CCs such as CC15/9 (4%), CC146/90 (13%) and CC177/179 (7%) were found low prevalence of penicillin-nonsusceptible strains.

As concerns the major clonal complexes, multidrug resistance was mainly associated with CC81 (100%), CC230/878 (95%), CC315/1093 (70%), CC156/162 (47%) and CC146/90 (39%). None of the strains of CC15/9 or CC717 were multidrug resistant (Table 6.1).

Table 6.1 - Details of the clonal complexes, as defined by PFGE and MLST, in the combined data set of 186 erythromycin-nonsusceptible strains isolated between 1994-2004 in Portugal from invasive disease[a]

CC/SGT	n	ST	PFGE	Serotypes[b]	Macrolide phenotype[b, c]		MDR[b, d] (%)	Antimicrobial resistance pattern[b, e]	International clones[f]	Year of 1st occurrence of the ST in this study	Year of 1st description of the ST in Portugal [S/R] (Source)[g]	The year of 1st description in the MLST database [S/R]	Countries or regions in which the ST was reported before the 1st occurrence in this study [S/R]	Acquisition EryNS[h]
					M	MLS_B								
156/162	38				21	17	47			1995				
		156	C2; C5; C12; E1; Z1	14, 7F	7	1		PEN ERY; PEN TET ERY OFL CIP; PEN TET ERY CHL; PEN TET ERY CLI CHL; PEN CTX CRO ERY	Spain^{9V}-3	1995	2001 [S/R][i] (1, 4)	1988 [S]; 1996[R]	Europe [S]	Nc
		143	C7; C9; C10; C11	14		8		PEN ERY CLI, PEN TET ERY CLI, PEN TET ERY CLI OFL CIP;	DLV Spain^{9V}-3	1999	2001 [R] (1, 5)	1994 [S]; 1996 [R]	Poland [S/R]	Imp
		162	C4	9V	1			ERY	SLV Spain^{9V}-3	2004	2002 [R] (1, 5)	1994 [S]; 1995 [R]	Worldwide [S]; Canada and Portugal [R]	Imp
		1042	C14	14		1		PEN CTX TET ERY CLI	SLV Spain^{9V}-3	2000	Nd[j]	Nd	Nd	Loc
		2351	C16	14	1			PEN ERY	DLV Spain^{9V}-3	1998	Nd	Nd	Nd	Loc
		2354	AE	6B		1		TET ERY CLI	DLV Spain^{9V}-3	2002	Nd	Nd	Nd	Loc
		2356	C15	9V		1		PEN TET ERY CLI	SLV Spain^{9V}-3	2003	Nd	Nd	Nd	Loc
			C1; C2; C3; C5; C6; C8; C12; C13; E1	14, 9V	12	5								
15/9	23				18	5	0			1995				
		9	D1; D7; D8	14	6			ERY	England^{14}-9	1995	1998 [R] (1, 2)	1997 [S]; 1990 [R]	Europe [R]; Australia [S]	Imp
		15	D1; D5; D6; D7; D9	14	1	5		ERY; ERY CLI; TET ERY CLI; PEN ERY CLI	SLV England^{14}-9	1999	1996 [R] (1, 2)	1975 [S]; 1993 [R]	Worldwide [R/S]	Exp
			D1; D2; D3; D4, D7; D10	14	11									
146/90	23				3	20	39			1994				
		273	V9; V15	6B	1	1		TET ERY; TET ERY CLI CHL	Greece^{6B}-22	1994	1998 [R] (1, 2)	1998 [S]; 1994 [R]	Europe [R]; Portugal [R/S]	Nc
		1369	AD	6A		1		TET ERY CLI	DLV Greece^{6B}-22	2001	2001 [R][i] (1, 4)	2001 [R]	Portugal [R]	Loc
		2349	V12	6B		1		TET ERY CLI	SLV Greece^{6B}-22	1995	Nd	Nd	Nd	Loc
		2353	V17	6B		1		TET ERY CLI CHL	SLV Greece^{6B}-22	2000	Nd	Nd	Nd	Loc
			V1; V2; V3; V4; V6; V7; V8; V10; V13; V14; V16; V17; V18; V19; V20	6B, 9V	2	16								
230/878	22				10	12	95			2000				
		230	K1; L	19F, 24F		2		PEN TET ERY CLI	Denmark^{14}-32	2000	2001 [R] (1, 5)	1996 [S]; 1998 [R]	Italy [R/S]; Denmark and Poland [S]	Imp
		276	K1; K2	19A	1	1		PEN TET ERY; PEN TET ERY CLI	SLV Denmark^{14}-32	2003	2001 [R][i] (1, 4, 5)	1997 [S]; 2001 [R]	Netherlands [S]; Portugal [R]	Nc
		2355	K5	17F		1		PEN TET ERY CLI		2003	Nd	Nd	Nd	Loc
			K1; K3; K4; K6	14, 19A, 19F, 24F	9	8								
63	14				1	13	50			1998				
		63	H2	15A		1		PEN TET ERY CLI	Sweden^{15A}-25	1998	1998 [R] (1, 2)	1992 [R]	Europe [R]	Imp
		1621	H4	19C		1		PEN ERY CLI	SLV Sweden^{15A}-25	1999	Nd	2004 [Nd]		Loc
		2097	H4	15C	1			PEN ERY	SLV Sweden^{15A}-25	2001	Nd	2003 [R]		Loc
			H1; H3; H4; H5; H6; H7; H8; H9	19A, 19F, 15A		11								
177/179	14				2	12	21			1999				
		179	F1; F3; F7; J4	19F		4		TET ERY CLI	SLV Portugal^{19F}-21	2000	1998 [R] (1, 2)	1998 [R]	Portugal, Spain, Italy, Germany [R]	Exp
		2352	F4	19F		1		TET ERY CLI	DLV Portugal^{19F}-21	1999	Nd	Nd	Nd	Loc
			F1; F2; F3; F4; F5; Y1	6B, 19A, 19F	2	7								
315/1093	10					10	70			1997				
		315	Z1	6B		1		PEN TET ERY CLI	Poland^{6B}-20	2001	1996 [R] (1, 2)	1992 [R]	Europe [R]	Imp
		887	Z5	6B		1		TET ERY CLI	SLV Poland^{6B}-20	2001	2001 [R][i] (1, 4, 5)	2001 [R]	Portugal [R]	Loc
		1093	Z4	6A		1		PEN TET ERY CLI	DLV Poland^{6B}-20	1997	Nd	1993 [Nd]	Romania [Nd]	Imp
			Z1; Z2; Z3; Z5; Z6; Z7; Z8	6B, 3		7								

(*continued*)

Table 6.1 - *Continued*

CC/SGT	n	ST	PFGE	Serotypes[b]	Macrolide phenotype[b, c]		MDR[b, d] (%)	Antimicrobial resistance pattern[b, e]	International clones[f]	Year of 1st occurrence of the ST in this study	Year of 1st description of the ST in Portugal [S/R] (Source)[g]	The year of 1st description in the MLST database [S/R]	Countries or regions in which the ST was reported before the 1st occurrence in this study [S/R]	Acquisition EryNS[h]
					M	MLS_B								
717	9					9	0			2000				
		717	T1	33F		1		TET ERY CLI		2000	1999 [S] 2001 [R][i] (1, 4, 5)	1999 [S]	Portugal [R/S], UK [R]	AcqLoc
			T1	33F		8								
193/410	6				1	5	33			1999				
		193	J2; J7	19A, 15C		2		ERY CLI, TET ERY CLI CHL	Greece[21]-30	1999	Nd	1997 [S]; 1997 [R]	Europe [S] Vietnam [R]	Nc
		1228	J1	19C		1		TET ERY CLI	SLV Greece[21]-30	1999	1998 [S] (1)	1998 [S]	Portugal [S]	AcqLoc
			J1; J3; J6	19A, 15C	1	2								
81	4					4	100			1995				
		81	M2; M3	23F		2		PEN TET ERY CLI CHL; PEN CTX CRO TET ERY CLI CHL	Spain[23F]-1	1996	2001 [S][i] (1, 4, 5)	1984 [S]; 1987 [R]	Europe [S/R]	Imp
		2350	M4	19F		1		PEN TET ERY CLI CHL	SLV Spain[23F]-1	1995	Nd	Nd	Nd	Loc
			M1	23F		1								
176/138	3				1	2	67			2000				
		338	N1	23F		1		PEN TET ERY CLI CHL	Colombia[23F]-26	2000	1998 [S] (1, 2)	1995 [S]; 1999 [R]	Malaysia [R]; Worldwide [S]	AcqLoc
		469	Y2	6B	1			ERY		2004	2001 [R] (1, 5)	1994 [S]; 2001 [R]	England [S]; Portugal [R]	Exp
			N1	23F		1								
306/227	2				2		0			2004				
		2360	A2	1	1			ERY	DLV Sweden[1]-28	2004	Nd	Nd	Nd	Loc
			A1	1	1									
88	1	88	F6	19F		1	100	PEN TET ERY CLI CHL		2003	Nd	1989 [S]; 1999 [R]	Southern Europe [R/S]	Imp
460/97	1	1282	AB	10F		1	0	ERY CLI		1998	2002 [R] (1, 5)	2002 [R]	Portugal [R]	Loc
180/505	1	180	O	3		1	0	TET ERY CLI		2001	2002 [R][i] (1, 4, 5)	1984 [S]; 1997 [R]	Worldwide; Europe [R]	Imp
191	1	191	AC	7F		1	0	TET ERY CLI	Netherlands[7F]-39	2001	1999-2002 [R][i] (4)	1984 [S]; 2003 [R]	Worldwide [S], Italy [R]	Nc
271/320	1	1584	J5	19F		1	0	TET ERY CLI	DLV Taiwan[19F]-14	2004	Nd	1995 [R]	Germany [R]	Imp
SGT 2357	1	2357	AF	19A		1	0	TET ERY CLI		2003	Nd		Nd	Loc
SGT 2358	1	2358	K1	4		1	0	TET ERY CLI		2004	Nd		Nd	Loc
SGT 2359	1	2359	P	4	1		0	ERY		2004	Nd		Nd	Loc
SGT 2768	1	2768	V5	6B	1		0	ERY		2001	Nd		Nd	Loc
ND	9		B; G; Q, R; S; U; W; X; AA	6B, 19F, 1, 4, 7F, NT, 34	5	4	11	ERY; ERY CIP OFL; ERY CLI; TET ERY CLI; TET ERY CLI CHL; PEN ERY						

[a] n, number of strains; S, susceptible; R, resistant.
[b] Data from reference (Dias *et al.*, 2006).
[c] M, Macrolide resistance phenotype; MLS_B, Macrolide, Lincosamide, and Streptogramin B resistance phenotype.
[d] MDR, Multidrug resistance.
[e] CTX, cefotaxime; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; OFL, ofloxacin; PEN, penicillin; TET, tetracycline.
[f] According to PMEN clone criteria (McGee *et al.*, 2001).
[g] Source: (1) from international MLST database (Aanensen & Spratt, 2005); references: (2) Sá-Leão *et al.*, 2001; (3) Dias & Caniça, 2004; (4) Serrano *et al.*, 2005; (5) Sousa *et al.*, 2005; (6) Sá-Leão *et al.*, 2006.
[h] EryNS, nonsusceptibility to erythromycin; AcqLoc, clones with locally acquired erythromycin nonsusceptibility; Exp, clones undergoing clonal expansion; Imp, imported clones; Loc, STs classified in Portugal as local clones; Nc, clones not classified.
[i] Invasive clones from invasive source.
[j] Nd, Not described.

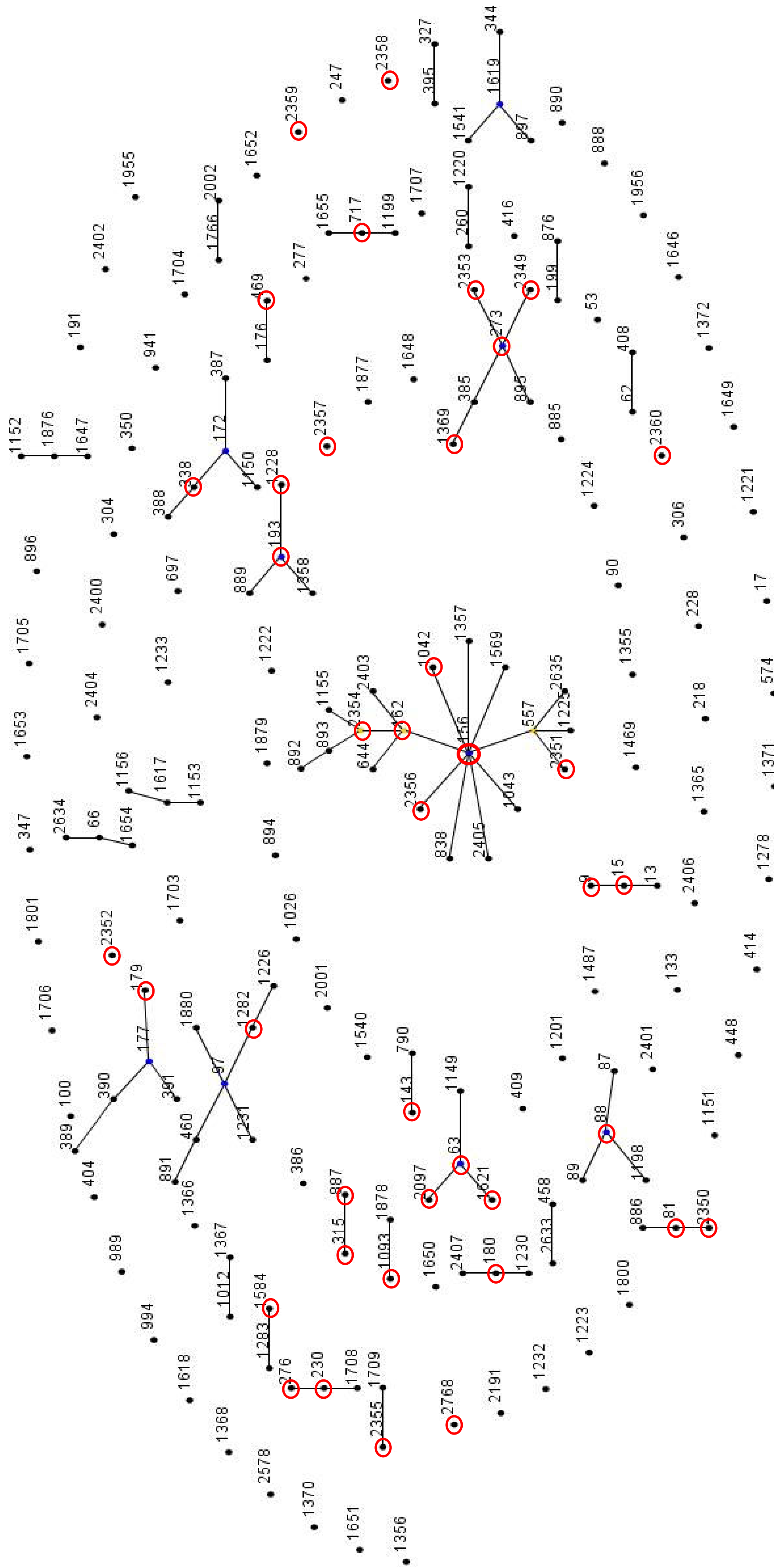


Figure 6.1 - Population snapshot of 192 sequence types (STs) found in Portugal among erythromycin-nonsusceptible strains, between 1994-2004. All STs found in this study in addition to those present in pnemococcal MLST database (Aanensen & Spratt, 2005), and in references (Sá-Leão *et al.*, 2001; Dias & Caniça, 2004; Serrano *et al.*, 2005; Sousa *et al.*, 2005; Sá-Leão *et al.*, 2006) are shown. All STs were combined in a single dataset for this analysis (each ST is represented by a dot). STs differing at a single genetic locus (SLV) are linked by a line. A clonal complex (CC) is a group of STs sharing 6 of 7 alleles with at least 2 members in the group. Those in red circles correspond to erythromycin-nonsusceptible strains found in this study.

Clonal classification according to their emergence in Portugal. The clones of this collection were classified according to their emergence in Portugal as follows (Table 6.1): 19 (46%) were local clones; 11 (27%) imported clones; three (7%) were clones undergoing clonal expansion; three (7%); acquired resistance locally and five were not classified.

Between 1994 and 1997 (12 isolates), we found seven STs in five CCs. Three of these clones were imported, two classified as local clones and two could not be classified. Between 1998 and 2000 (43 isolates), macrolide nonsusceptibility spread, and we found 15 STs with 13 CCs, classified as follows: 4 undergoing clonal expansion (27%), 2 were imported clones (13%), 2 acquired resistance locally (13%), 6 were local clones (40%) and one could not be classified (Table 2). Between 2001 and 2004 (131 isolates), 10 (33%) of the STs were classified as undergoing clonal expansion, 11 (36%) were local clones, one acquired resistance locally (3%), and 6 (20%) were imported clones (Table 6.2).

Local clones. To study the emergence of local clones (*n* = 19; 46%), we used e-BURST to assess their putative founders (SLV and/or DLV STs) against the whole MLST database (Table 6.3). Fourteen of the 19 STs were related to PMEN clones. Sixteen had putative clonal founder STs previously described in Portugal; 11 of these putative clonal founders were of the same serotype, and four of the same serogroup, as the local clones isolated (Table 6.3).

Table 6.2 – Distribution of types of mechanisms of acquisition of erythromycin nonsusceptibility for the STs defined from invasive erythromycin-nonsusceptible isolates in Portugal, between 1994-2004

CC/SGT	ST	ST[a]		
		1994 -1997 (n=12)	1998 – 2000 (n=43)	2001 – 2004 (n =131)
156/162	156	Nc	Exp	Exp
	143		Imp	Exp
	162			Imp
	1042		Loc	
	2351		Loc	
	2354			Loc
	2356			Loc
15/9	9	Imp	Exp	Exp
	15		Exp	Exp
146/90	273	Nc		Exp
	1369			Loc
	2349	Loc		
	2353		Loc	
230/878	230		Imp	Exp
	276			Nc
	2355			Loc
63	63			Imp
	1621		Loc	
	2097			Loc
177/179	179		Exp	Exp
	2352		Loc	
315/1093	315			Imp
	887			Loc
	1093	Imp		
717	717			Acq Loc
193/410	193		Nc	Exp
	1228		Acq Loc	
81	81	Imp		Exp
	2350	Loc		
176/138	338		Acq Loc	
	469			Exp
306/227	2360			Loc
88	88			Imp
460/97	1282		Loc	
180/505	180			Imp
191	191			Nc
271/320	1584			Imp
SGT2357	2357			Loc
SGT 2358	2358			Loc
SGT 2359	2359			Loc
SGT 2768	2768			Loc

[a] Classification of acquisition erythromycin nonsusceptibility related to the STs in this study. AcqLoc, clones with locally acquired erythromycin nonsusceptibility; Exp, clones undergoing clonal expansion; Imp, imported clones; Loc, STs classified in Portugal as local clones; Nc, Clones not classified.

Table 6.3 - Details of the sequence types of 19 local clones identified among 186 erythromycin-nonsusceptible invasive strains, collected in Portugal, between 1994-2004

ST	Serotypes	International Clones[b]	Putative ST ancestries[a]		Year of 1st occurrence of ST in this study
			SLV or DLV[c]	Described in Portugal[d]	
1042	14	SLV Spain^{9V}-3	156, 162, 314, 335, 838, 930, 1184, 1864, 2306, 2335, 2545	**156**, 162	2000
2351	14	DLV Spain^{9V}-3	156, 557	**156**	1998
2354	6B	DLV Spain^{9V}-3	44, 156, 162, 893, 999, 1142, 1155, 1571, 1662	156, 162, 893, 1142, 1155	2002
2356	9V	SLV Spain^{9V}-3	156, 157, 158, 160, 167, 379, 539, 548, 971, 1043, 1925, 2692	**156**, 1043	2003
1369	6A	DLV Greece^{6B}-22	146, 273, 384, 385, 2744	**273**	2001
2349	6B	SLV Greece^{6B}-22	273, 285, 1490, 1831	**273**	1995
2353	6B	SLV Greece^{6B}-22	146, 273, 385, 1566, 1750	**273**	2000
2355	17F	-[e]	1611, 1709, 276, 2674, 2683, 2688, 2691	276, **1709**	2003
1621	19C	SLV Sweden^{15A}-25	63, 1149, 1549, 1830, 2097, 2100, 2447, 2543	*63, 1149,*	1999
2097	15C	SLV Sweden^{15A}-25	63, 1149, 1621, 1830, 2060, 2100, 2447, 2543	*63*, 1149,	2001
2352	19F	DLV Portugal^{19F}-21	177, 179, 1354	**177, 179**	1999
887	6B	SLV Poland^{6B}-20	254, 315, 882, 1032, 1505	**315**	2001
2350	19F	SLV Spain^{23F}-1	81, 714, 932, 1456, 1595, 2032, 2346, 2395	*81*	1995
2360	1	DLV Sweden^{1}-28	306, 1310, 1346	-	2004
1282	10F	-	97, 585, 1226, 65, 98, 460, 481, 529, 1208, 1231, 1497, 1513, 1551, 1713, 1880, 2479	*1226*, *1231*, 1880	1998
2357	19A	-	193, 410, 889, 1145, 1164, 1082, 1267, 1825, 1888, 2110, 2426, 2575	**889**	2003
2358	4	-	247, 2256, 2342, 2389	-	2004
2359	4	-	725, 1652, 2809, 2389	**1652**	2004
2768	6B	-	844, 1969, 2310, 2656	-	2001

[a] Assessed using e-BURST version 3 (Feil *et al.*, 2004).
[b] According to PMEN clone criteria (McGee *et al.*, 2001).
[c] SLV, single locus variant; DLV, double loci variant.
[d] Numbers in bold represent serotype concordance between the putative ancestral and local ST; Numbers in italics represent ST with serogroup concordance between the putative ancestral and local ST.
[e] Unrelated to PMEN clones

6.5 - Discussion

The purpose of this study was to describe the structure of the erythromycin-nonsusceptible pneumococcal population recovered from invasive disease in Portugal. We investigated the evolution and the main factors associated with the dynamics of the population, with particular reference to the increase of erythromycin nonsusceptibility.

The combination of PFGE and MLST allowed genetic characterization of most of the erythromycin-nonsusceptible strains. The majority of the STs found in this study are putative founders of clonal clusters or sub-clusters as well as variants of those founders present in Portugal. Many strains of our population (86%) were members of clonal complexes related to PMEN antibiotic nonsusceptible clones. The percentage of such strains was consistent with that previously reported for invasive disease in Portugal (Serrano *et al.*, 2005), and for carriers attending day care centres in the country (Sousa *et al.*, 2005). Most of the 10 PMEN clones found in this study were described, mainly in Europe (Table 6.1) and have not been noted in the United States (Gertz *et al.*, 2003; Hanage *et al.*, 2007). The international clones Spain^{9V}-3, England^{14}-9, Greece^{6B}-22 and Denmark^{14}-32 and their related strains made up 57% of our sample. These clones have also been found to be prevalent among Portuguese carriers (Aanensen & Spratt, 2005; Sá-Leão *et al.*, 2001). Of the 41 STs found in this study, 44% have only been reported in Portugal, and were found at different times (Table 6.1 and 6.2). Overall, these observations suggest that most erythromycin-nonsusceptible strains circulating in Portugal were related to international clones or local variants. Our study also suggests that the clones circulating in Europe and in the United States are different.

Half of the clones that emerged after 1997 (1998-2004) were classified as local clones (44%) or clones which acquired resistance locally (8%). The other clones were classified as imported (21%) or as undergoing to clonal expansion (18%). Seventy-four per cent of the local clones were related to PMEN clones and the putative ancestral STs of most (84%) have been described previously in Portugal, mostly isolated from children attending day care centres (Sá-Leão *et al.*, 2001; Sousa *et al.*, 2005).

Previously, it was described the increase of coresistance to penicillin and macrolides (Dias *et al.*, 2006), due to the selection pressure of the use of macrolides instead of β-lactams (Dias & Caniça, 2008). In our study the rate of penicillin nonsusceptibility, among erythromycin-nonsusceptible strains, is stable along time. The penicillin nonsusceptibility strains are mainly related to imported clones and their local variants, suggesting that the use of macrolides are associated to the importation and local diversification of erythromycin and penicillin coresistant strains

In conclusion, our study suggests that the increased isolation of erythromycin-nonsusceptible pneumococci from invasive disease in Portugal was mostly due to the

importation and local diversification of nonsusceptible international clones but also to clonal expansion and genetic diversification of pre-existing clones.

CHAPTER VII

Two major Spanish clones of penicillin-resistant *Streptococcus pneumoniae* in Portuguese isolates of clinical origin

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7.1 - Summary

We studied the genetic relatedness of 47 Portuguese penicillin-nonsusceptible 9V and 23F *Streptococcus pneumoniae*, of clinical origin, using PFGE and Restriction Fragment Length Polymorphism (RFLP) analysis. PFGE fingerprint showed that 19 isolates from serotype 9V and 24 isolates from serotype 23F are variants of the Spain^{9V}-3 and Spain^{23F}-1 clones, respectively. Polymorphism of *pbp1A*, *penA* and *pbpX* genes showed that all the penicillin-nonsusceptible clones gave similar *Hinf*I restriction-patterns. In this study, serotypes 9V and 23F have different clonal origins and identical PBP genotypes, suggesting a horizontal transfer of resistance. Visual and computer-assisted analysis of PFGE patterns correlated well (r = 0.983).

7.2 - Introduction

The resistance to penicillin as well as antibiotic multiresistance of *Streptococcus pneumoniae* is now widespread and is increasing all over the world. The resistance of *S. pneumoniae* to penicillin is due to altered PBPs encoded by mosaic *pbp* genes. Nonsusceptible isolates carry allelic variants, which differ greatly from *pbp* genes in penicillin-susceptible strains, acquired by horizontal gene transfer from related species (Hakenbeck *et al.*, 1999).

Penicillin resistance among *S. pneumoniae* has also rapidly emerged in Portugal during the last decade to become a major public health problem (Vaz Pato *et al.*, 1995; Carvalho *et al.*, 1999). The proportion of clinical isolates that was penicillin resistant (MIC ≥0.1 mg/L) was 22.4% in 1995, 23.9 % in 1996 and 25.8% in 1997 (Carvalho *et al.*, 1999). Multiresistance is also increasing among penicillin-nonsusceptibility strains: 38.8% in 1995 to 51.6% in 1997 (Carvalho *et al.*, 1999). Many of these clinical strains are serotypes 23F and 9V (Vaz Pato *et al.*, 1995; Carvalho *et al.*, 1999).

We used PFGE and PBP genotyping to study genetic relatedness of penicillin-nonsusceptible isolates of *S. pneumoniae* serotypes 9V and 23F of clinical isolates. Visual comparision and computer-assisted analysis of PFGE fingerprints were also used.

7.3 - Materials and methods

Clinical isolates. We studied 47 penicillin-nonsusceptible pneumococci with reduced susceptibility to at least one other different structurally unrelated antibiotic, randomly selected from serotypes 9V and 23F strains in the ARU collection at the NIH Dr. Ricardo Jorge in Lisbon. One NT penicillin-multiresistant strain and 5 susceptible strains, from the same collection, were also included. The isolates were isolated from Portuguese clinical specimens, obtained from 10 hospitals (Table 7.1). In total 45 strains were isolated from respiractory tract (23 from sputum, 12 from upper respiractory tract and ten from lower respiractory trat), 4 from middle ear, 2 from cerebrospinal fluid, and 2 had unknown origin.

As previously (Vaz Pato *et al.*, 1995), serotyping was performed by latex agglutination with type-specific antiserum (Statens-Serum Institut, Copenhagen, Denmark), MICs of penicillin (Wyeth Lederle Portugal-Farma, Algés, Portugal), ceftriaxone (Roche Farmacêutica Química, Amadora, Portugal), cefotaxime, chloramphenicol (Hoechst Marion Roussel, Mem Martins, Portugal), tetracycline (Laboratórios Atral, Castanheira do Ribatejo, Portugal), ofloxacin (Farmoz, Prior Velho, Portugal), erythromycin (Abbot Laboratórios, Amadora, Portugal), and clindamycin (Upjohn Farmoquímica, Lisboa), were determined by the agar dilution method and susceptibility to trimethoprim/sulfamethoxazole (Oxoid, Hampshire, England) by disk-test.

Analysis of *pbp* genes. PBP genotyping was performed with primers to amplify the genes *pbp1A*, *penA* and *pbpX* by PCR as previously described (Moissenet *et al.*, 1996). Amplified DNA was digested with *Hinf*I (Boehringer Mannheim, Carnaxide, Portugal) and analyzed for RFLP. A number was given to each restriction pattern for each of the three *pbp* genes analyzed, so the PBP genotype has three numbers (example: 1-1-1).

Pulsed-field gel electrophoresis. PFGE was performed as described previously (Carvalho *et al.*, 1996b). *Sma*I-restricted DNA from the penicillin-susceptible unencapsulated strain R6, and from the major Spanish multiresistant serotype 23F clone, Clev1 (both kindly donated by Prof. A. Tomasz, NY), and the Lambda ladder (Biolabs, Beverly, MA) were used as markers for intra-gel normalization and inter-gel comparison. Gels were stained with ethidium bromide and photographed under UV light with a Polaroid system. Photographs were scanned, normalized and saved as TIFF files for computer analysis.

Data analysis. PFGE DNA fingerprints were analyzed by visual comparison (method A) (Tenover *et al.*, 1995). Major patterns (capital letters) differed by more than three fragments, and one to three fragment variations represent subtypes (capital letters with numbers). PFGE patterns were also analyzed by a computer-assisted analysis (method B), using Bionumerics software (version 2.5) (Applied Maths, Kortrijk, Belgium). Clustering was performed using the Dice band-based similarity coefficient (S_D), with a band position tolerance of 2% and an optimization of 1%. A dendrogram was constructed by the

unweighted pair-group method by using average linkages (UPGMA), and a cut-off value of 80% similarity was determined by the cluster cut-off method according to Bionumerics program (Applied Maths). Isolates with a S_D value higher than 80% were considered to belong to the same cluster.

7.4 - Results and discussion

PFGE fingerprint analysis showed that serotypes 9V and 23F do not group together but show similarities (Table 7.1, Figure 7.1). Computer-assisted analysis distinguished subtypes: I-1 to I-6 and II-1 to II-6. Visual comparison showed two major patterns (G and A), with subtypes G1, G2, and A1, A3, A5, A8/9, A12, A14, A16, A17. Penicillin-nonsusceptible 9V isolates from sub-types I-1 to I-3 were closely related and belong to serotype variants of the Spain^{9V}-3 clone (McGee *et al.*, 2001). Penicillin- nonsusceptible 9V isolates from sub-types I-4 to I-6 were possibly related, forming cluster I together with sub-types I-1 to I-3. Penicillin-nonsusceptible 23F isolates were all closely related and belonged to serotype variants of the Spain^{23F}-1 clone (McGee *et al.*, 2001), forming cluster II, but were genetically unrelated to susceptible 23F isolates, which formed clusters III, IV and VI. Clusters I and II were 72% related with a cophenetic correlation of 93% (Figure 7.1). Computer-assisted analysis gave more discriminating subtype patterns than visual comparison, for G2, M and A1. However, sub-type II-6 was closely related to sub-type II-5, as demonstrated by A14 pattern obtained by visual comparison. Visual comparison gave more discriminating subtype patterns for II-1, II-3, II-4 and II-5 (Table 1). The correlation between visual comparison and computer-assisted analysis of PFGE DNA fingerprints was good (r = 0.983).

Resistance to penicillin can emerge independently in closely related clones. We analyzed genetic polymorphism of the penicillin resistance genes *pbp1A*, *penA* and *pbpX*: all the penicillin- nonsusceptible clones of serotype 23F and 9V gave similar *Hinf*I restriction patterns for the three *pbp* genes (PBP genotype 1-1-1) (Table 7.1). The penicillin-susceptible clones, in clusters III, IV, V and VI, had the PBP genotype 2-2-2 (Table 7.1). All isolates (except NT strain 1258) in each cluster had the same PBP genotype and the same serotype, suggesting that penicillin resistance in different lineages of *S. pneumoniae* may result from horizontal transfer of *pbp* genes, as previouly reported by Coffey *et al*., (1991).

S. pneumoniae from cluster I were mostly phenotype penicillin plus trimethoprim-sulfamethoxazole plus cefotaxime plus ceftriaxone, and *S. pneumoniae* from cluster II mostly penicillin plus tetracycline plus chloramphenicol plus trimethoprim-sulfamethoxazole plus cefotaxime or/and ceftriaxone. Cluster II-4 contained a greater diversity of phenotypes than the other clusters/subtypes, possibly a consequence of the presence of insertion sequences specific to the addition of resistance genes, leading to mosaic genes (Hakenbeck *et al.*, 2001). Interestingly, both the single clone with phenotype penicillin plus erythromycin plus clindamycin plus chloramphenicol plus trimethoprim-sulfamethoxazole plus cefotaxime plus ceftriaxone (isolate 1654) and the single clone penicillin plus tetracycline plus trimethoprim-sulfamethoxazole plus cefotaxime plus ceftriaxone (isolate 1258), were in cluster II-4. Isolate 1258, despite having a NT capsular polysaccharide, is closely related to serotype 23F. This isolate appears to be a NT variant of the Spain^{23F}-1 clone, which may have lost the ability to

produce capsule *in vivo*. NT serotype from isolate 1258 can be the result from an occasional recombination event, due to an horizontal transfer of capsular biosynthesis gene, as frequently occur *in vivo* (Coffey *et al.*, 1991; Ramirez & Tomasz, 1999). As a variant of the Spain^{23F}-1 clone it may have lost chloramphenicol resistance but acquired cefotaxime (MIC of 2 mg/L) and ceftriaxone (MIC of 1 mg/L) resistance. The sub-cluster II-4 is the most divergent between the two analysis methods, method A classifying it as three subtypes A1, A8, A12.

We concluded that computer assisted analysis is easier and less time consuming than visual analysis, but the methods are complementary.

In this study, Portuguese serotype 9V and 23F nonsusceptible *S. pneumoniae* showed different genetic patterns by PFGE but identical PBP genotypes, like the Major Spanish clones, Spain^{9V}-3 and Spain^{23F}-1 (McGee *et al.*, 2001), excepting five 9V isolates. These resistant serotypes appear to have a common clonal origin of resistance despite being isolated in different hospitals throughout the country. These findings, reported in Portuguese clinical *S. pneumoniae* isolates, suggest spreading of resistance by clonal dissemination and horizontal transfer. The increasing movements of populations, which contribute to the emergence in antimicrobial resistance worldwide, emphasize the need of vaccination to interrupt the transmission of resistant clones. Further studies are needed to find out contributions of the Portuguese clinical isolates from these and other serotypes to the emergence of penicillin-resistant and multidrug-resistant *S. pneumoniae*.

Table 7.1 - Properties of penicillin-nonsusceptible and penicillin-susceptible *S. pneumoniae* of serotypes 9V and 23F, and one NT isolate

Strain No.	Hospital[a]	Clinical specimen[b]	MIC Penicillin (mg/L)	Resistance phenotype[c]	Serotype	PBP genotype[d]	PFGE pattern[e]	
							method A	method B
1368	A	SP	0.8	PEN SXT	9V	1-1-1	G2	I-1
1567	A	SP	1.0	PEN SXT CRO	9V	1-1-1	G2	I-1
1503	B	-	1.6	PEN SXT CTX CRO	9V	1-1-1	G2	I-2
1843	C	SP	1.6	PEN SXT CRO	9V	1-1-1	G2	I-2
1277	B	LRT	1.6	PEN SXT CTX CRO	9V	1-1-1	G2	I-2
1863	B	SP	1.0	PEN SXT	9V	1-1-1	G1	I-3
1520	C	SP	1.6	PEN SXT CTX CRO	9V	1-1-1	G1	I-3
1553	D	SP	1.0	PEN SXT CTX CRO	9V	1-1-1	G1	I-3
1692	E	SP	0.8	PEN SXT CTX CRO	9V	1-1-1	G1	I-3
1574	F	CSF	1.0	PEN SXT CTX CRO	9V	1-1-1	G1	I-3
1487	E	SP	0.8	PEN SXT CTX	9V	1-1-1	G1	I-3
1528	A	URT	1.6	PEN SXT CTX CRO	9V	1-1-1	G1	I-3
1564	A	LRT	1.6	PEN SXT CTX CRO	9V	1-1-1	G1	I-3
1388	G	URT	0.8	PEN SXT	9V	1-1-1	G1	I-3
1315	E	EAR	1.0	PEN SXT	9V	1-1-1	G1	I-3
1374	H	LRT	1.0	PEN SXT	9V	1-1-1	G1	I-3
1175	G	URT	1.0	PEN SXT	9V	1-1-1	G1	I-3
1534	A	SP	1.6	PEN SXT CTX CRO OFX	9V	1-1-1	G1	I-3
1814	I	LRT	1.6	PEN SXT CTX CRO	9V	1-1-1	G1	I-3
1433	D	LRT	1.6	PEN SXT CTX CRO	9V	1-1-1	F	I-4
1256	B	SP	1.6	PEN SXT CTX CRO	9V	1-1-1	M	I-5
1259	B	SP	0.8	PEN SXT CTX	9V	1-1-1	M	I-5
1665	B	SP	0.8	PEN SXT CTX	9V	1-1-1	M	I-6
1504	B	-	1.6	PEN SXT CTX CRO	9V	1-1-1	M	I-6
1445	D	SP	0.8	PEN TET CHL SXT	23F	1-1-1	A8/9	II-1
1220	J	SP	1.0	PEN TET CHL SXT CTX CRO	23F	1-1-1	A5	II-1
1226	D	SP	0.8	PEN TET CHL SXT	23F	1-1-1	A1	II-2
1399	G	EAR	0.8	PEN TET CHL SXT CTX	23F	1-1-1	A17	II-3
1535	A	SP	0.8	PEN TET CHL SXT CTX CRO	23F	1-1-1	A17	II-3
1598	D	SP	0.8	PEN TET CHL SXT CTX CRO	23F	1-1-1	A1	II-3
1597	D	SP	0.8	PEN TET CHL SXT CTX CRO	23F	1-1-1	A1	II-3
1590	D	SP	0.8	PEN TET CHL SXT	23F	1-1-1	A17	II-3
1849	C	LRT	0.8	PEN TET CHL SXT	23F	1-1-1	A17	II-3
1602	D	LRT	1.0	PEN TET CHL SXT CTX CRO	23F	1-1-1	A1	II-3
1397	G	URT	1.0	PEN TET CHL SXT CTX	23F	1-1-1	A17	II-3
1806	D	SP	0.8	PEN TET CHL SXT	23F	1-1-1	A17	II-3
1455	I	URT	1.0	PEN TET CHL SXT CTX	23F	1-1-1	A3	II-3
1191	G	URT	1.0	PEN TET CHL SXT CTX CRO	23F	1-1-1	A17	II-3
1387	G	URT	1.0	PEN TET CHL SXT CTX	23F	1-1-1	A12	II-4
1654	B	LRT	1.0	PEN ERY CLI CHL SXT CTX CRO	23F	1-1-1	A8	II-4
1258	B	SP	2.0	PEN TET SXT CTX CRO	NT	1-1-1	A1	II-4
1203	G	SP	0.8	PEN TET CHL SXT	23F	1-1-1	A12	II-4
1177	G	URT	1.0	PEN TET CHL SXT CTX	23F	1-1-1	A12	II-4
1392	G	URT	0.8	PEN TET CHL SXT CTX	23F	1-1-1	A12	II-4
1586	D	URT	0.8	PEN TET CHL SXT CTX CRO	23F	1-1-1	A14	II-5
1593	D	LRT	0.8	PEN TET CHL SXT CTX	23F	1-1-1	A14	II-5
1453	I	URT	1.0	PEN TET CHL SXT CTX	23F	1-1-1	A16	II-5
1225	D	EAR	0.8	PEN TET CHL SXT CTX CRO	23F	1-1-1	A14	II-6
913	B	LRT	0.05	S	23F	2-2-2	B1	III-1
977	G	URT	0.025	S	23F	2-2-2	B2	III-2
970	G	EAR	0.025	S	23F	2-2-2	D	IV
605	A	SP	0.025	S	9V	2-2-2	E	V
885	E	CSF	0.025	S	23F	2-2-2	F	VI

[a]Code of hospital where strains were collected.

[b]SP, sputum; CSF, cerebrospinal fluid; LRT, lower respiratory tract; URT, upper respiratory tract; EAR, middle ear; -, unknown.

[c]PEN, penicillin; CTX, cefotaxime; TET, tetracycline; CHL, chloramphenicol; ERY, erythromycin; CLI, clindamycin; SXR, trimethoprim-sulfamethoxazole; CRO, ceftriaxone; OFX, ofloxacin; S, susceptible.

[d]PBP genotypes are represented by a three-number code (e.g. 1-1-1), referring to the RFLP polymorphism patterns of the genes *pbp1A* (pattern 1), *penA* (pattern 1) and *pbpX* (pattern 1), respectively.

[e]PFGE fingerprint patterns obtained by two different methods. Method A: visual comparison of patterns, as described by Tenover *et al.*, (1995). Method B: computer-assisted analysis, using the UPGMA algorithm based on the Dice coefficient (SD).

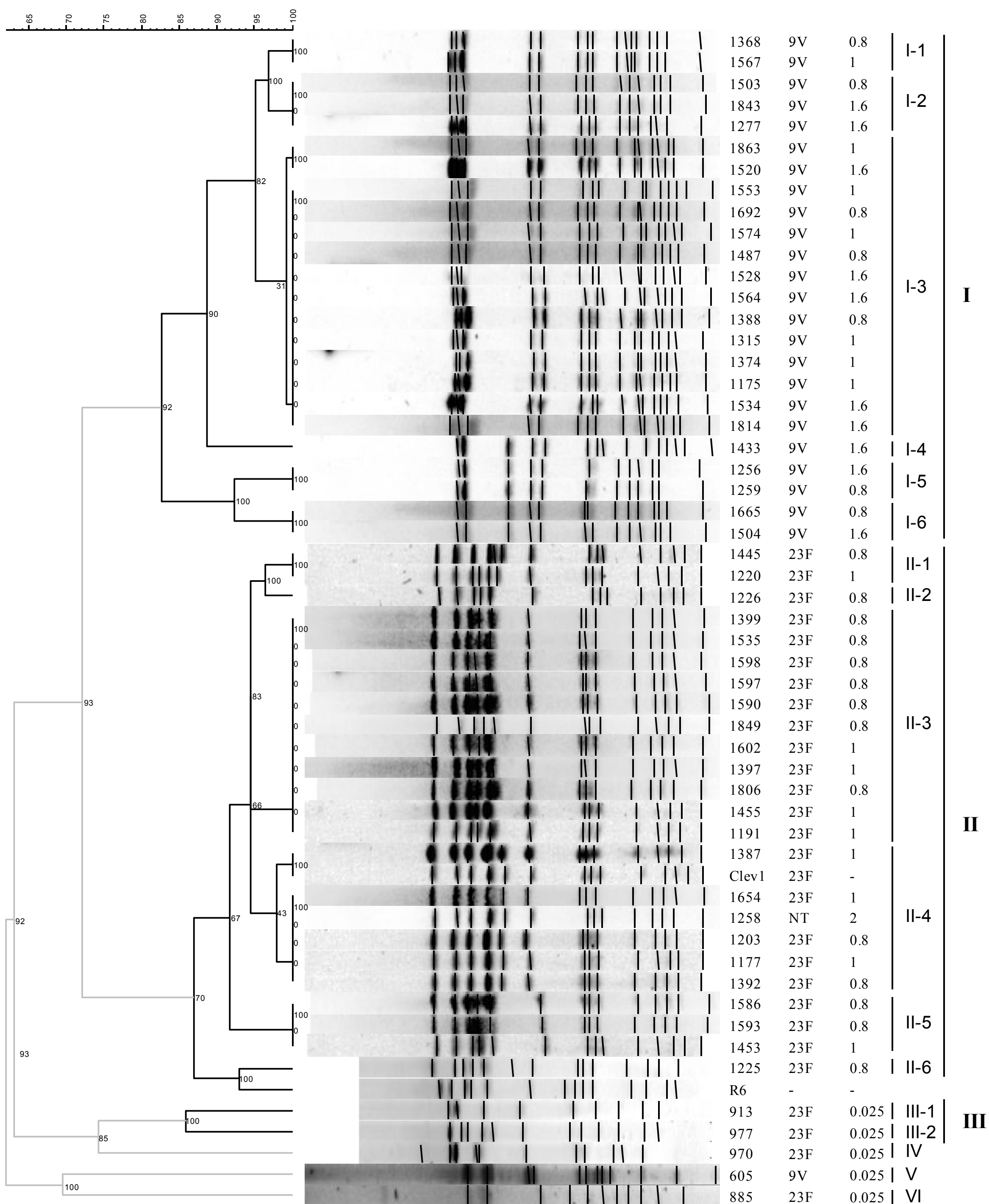

Figure 7.1 - Dendrogram (genetic relatedness) and PFGE fingerprint patterns of 48 nonsusceptible and five susceptible pneumococcal isolates. Strain code number, capsular serotype and MIC of penicillin are given (at right). Clusters **I** and **II**, containing isolates of more than 80% similarity, are indicated by vertical bands. Codes **I**-1 to **I**-6 and **II**-1 to **II**-6 refer to PFGE patterns of *S. pneumoniae* showing up to 98% homology. Cophenetic correlations are shown in each branch of the dendrogram.

CHAPTER VIII

Diversity of penicillin binding proteins among clinical *Streptococcus pneumoniae* strains from Portugal

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8.1 - Introduction

The main mechanism of resistance of pneumococcus to penicillin is the alteration of the affinities of PBPs to β-lactams by heterologous recombination events (Dowson *et al.*, 1989).

8.2 - Material and methods

For the present study, we have investigated the diversity of *penA* (accession numbers AM779386 to AM779409), *pbpX* (AM779338 to AM779361) and *pbp1A* (AM779362 to AM779385) genes of 21 clinical Portuguese *Streptococcus pneumoniae* strains (randomly selected from the strain collection of the ARU at the NIH Dr. Ricardo Jorge in Lisbon) in relation to their penicillin susceptibilities; 4 american type culture collection (ATCC) strains were also added to the sample. Analysed sequences were compared to the R6 amino acid sequence[a]. MICs to penicillin G were determined and interpreted as previously described (Dias *et al.*, 2006).

8.3 - Results and discussion

The amino acid substitutions located in the vicinity of conserved PBP motifs are shown in table 8.1. We found 21, 23, and 20 different alleles in PBP2B, PBP2X and PBP1A, respectively; only 4, 5 and 8 were already present in the DDBJ/EMBL/GenBank databases, respectively. In comparison to the amino acid sequence of strain R6, the average number of amino acid substitutions in strains susceptible (n=8), intermediate (n=10), and resistant (n=7) to penicillin were 3, 32 and 24 for PBP2B, 15, 52, and 23 for PBP2X, 5, 46, and 28 for PBP1A, respectively.

Among nonsusceptible strains, the PBP2B presented short mosaics in Ala-Phe-Ser-Arg-Pro-Met (5/17), Ala-Phe-Ser-Val-Pro-Met (1/17) and Pro-Ala-Phe-Ser-Val-Pro-Thr (1/17) from residues 431 to 437; these mosaics were variants of those found by Dowson and others (Dowson *et al.*, 1989; Sanbongi *et al.*, 2004). The Ala624Gly mutation (4/17) close to the Lys-Thr-Gly motif was previously associated with chromosomal DNA from a *S. mitis* isolate (Hakenbeck *et al.*, 1998). Close to the Ser-Ser-Asn motif of PBP2B, the Thr451Ala (15/17) mutation associated with β-lactam resistance (Grebe & Hakenbeck, 1996), was found associated with the Glu481Gly (16/17) mutation.

[a] The predicted amino acid diversity of PBPs, were assessed by the Poisson correction method using MEGA version 4 software (Tamura *et al.*, 2007). Standard error estimates were obtained by the bootstrap procedure (1000 replicates).

Both these substitutions had also been observed in France, South Africa and Japan (Smith & Klugman, 1995; Kawamura *et al.*, 1999; Sanbongi *et al.*, 2004). These mutations were also associated with a Thr494Ser/Ala (16/17) substitution, making the three the major mutations related to β-lactams resistance in PBP2B (Chesnel *et al.*, 2005). Met400Thr at PBP2X was found in two strains resistant to cefotaxime. These mutations had been described as being implicated in the development of β-lactam resistance (Chesnel *et al.*, 2005; Carapito *et al.*, 2006). The mutation Gln552Glu which participates in an alternative mechanism of resistance (Pernot *et al.*, 2004), was found in 10 strains. The Ile371Thr mutation (10/17) is associated with the conformational change of a loop in the entrance of the active site cavity of PBP2X (Carapito *et al.*, 2006). We also found four insert sequences in PBP1A: 680Ala-Pro-Thr-Thr-Ser681 (1/17), 680Ser-Ser-Ser-Thr-Ser-Gln681 (1/17) and 710Asn-Gln-Asn-Gln711 (2/17).

Amino acid sequences from penicillin intermediate strains showed higher divergence than those from highly resistant strains for PBP2B (d = 0.0573; S.E.:0.0052 and d = 0.0463; S.E.:0.0053, respectively), PBP2X (d = 0.0848; S.E.:0.0067 and d = 0.0435; S.E.:0.0044, respectively) and PBP1A (d = 0.0735; S.E.:0.0059 and d = 0.0527; S.E.:0.0054, respectively); also a greatest diversity of serotypes was found in intermediate resistant isolates (Table 8.1). This might be attributed to the higher clonallity of high-resistant than intermediate resistant clones (Tomasz *et al.*, 1998) or to the recombination events, which may me in origin of the new PBP alleles found in the study (Stanhope *et al.*, 2007).

Table 8.1 - Deduced amino acid substitutions in PBP2B, PBP2X and PBP1A of 25 pneumococcal strains[a]

Strain	Resistance phenotype	Serotype	MIC[b]	PBP2B														
				443	451	481	485	488	494	614	624	629	630	633	635	DM[c]	TP	ORF
R6	S	-	0.0125	Gln	Thr	Glu	Ser	Gly	Thr	Leu	Ala	Ala	Asp	Gln	Thr			
ATCCBAA-334	S	4	0.0125	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0
URA4929	S	9V	0.0125	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0
URA4376	T, E, C	6B	0.0125	.	.	.	.	.	.	.	.	.	.	.	.	1	0	1
URA3537	S	3	0.0125	.	.	.	.	.	.	.	.	.	.	.	.	1	1	2
URA2543	S	NT	0.025	.	.	.	.	.	.	.	.	.	.	.	.	2	0	2
URA4933	E,	14	0.0125	.	.	.	.	.	.	.	.	.	.	.	.	3	0	3
URA3558	P, T, E, C	23	0.2	.	.	.	.	.	.	.	.	.	.	.	.	3	1	4
URA4087	S	19F	0.025	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0
URA4566	P, T, E, C	6B	0.1	.	Ala	Gly	.	.	Ser	.	.	.	Gly	Glu	Asn	0	13	18
URA2542	S	22	0.05	.	Ala	Gly	.	.	Ser	Ser	.	Glu	Gly	Glu	Asn	1	12	17
URA3635	P,	19A	0.1	.	Ala	Gly	.	.	Ser	.	.	.	.	.	.	6	6	13
URA4731	P,	14	0.1	Glu	Ala	Gly	.	Ala	Ala	Ala	.	Glu	Gly	Glu	Asn	1	32	37
URA5391	P,	23F	0.1	Glu	Ala	Gly	.	Ala	Ala	Ser	.	Glu	Gly	Glu	Asn	1	34	40
URA5779	P, T, E, C	15A	0.1	.	Ala	Gly	.	.	Ser	Ala	.	Glu	Gly	Glu	Asn	1	10	16
ATCC700670	P, X, R, T, F	6B	2	Glu	Ala	Gly	.	.	Ser	Ala	.	.	Gly	Glu	Asn	2	11	14
ATCC700673	P, T, E, C	19A	2	.	Ala	Gly	.	.	Ser	.	.	.	.	.	.	2	15	18
URA5805	P, X, R, T, E, C	19A	1.6	Glu	Ala	Gly	Ala	.	Ala	Thr	Gly	.	Gly	Glu	Asn	9	31	50
ATCC51916	P, X, R, E	23F	0.1	.	.	.	.	.	.	.	Gly	.	Gly	Glu	Asn	2	3	14
URA3543	P, X, R	14	2	Glu	Ala	Gly	Ala	.	Ala	Thr	Gly	.	Gly	Glu	Asn	5	21	37
URA6056	P, X, R	ND	3.2	Glu	Ala	Gly	.	.	Ser	Thr	Gly	.	Gly	Glu	Asn	6	33	50
URA3043	P, X, R	14	2	Glu	Ala	Gly	Ala	.	Ala	.	.	.	.	.	.	7	12	20
URA4203	P, X, R, T, E, C	14	1.6	Glu	Ala	Gly	Ala	.	Ala	.	.	.	.	.	.	7	12	20
URA5128	P	14	0.5	Glu	Ala	Gly	Ala	.	Ala	.	.	.	.	.	.	7	12	20
URA3699	P, T, E, C	19F	0.5	Glu	.	Gly	.	Ala	Ala	Ala	.	.	Gly	Lys	Asn	37	33	83
URA5450	P, T, E, C	24F	0.5	.	Ala	Gly	.	.	Ser	.	.	.	.	.	.	4	12	17

(*continued*)

Table 8.1 - *Continued*

Strain	PBP2X																			
	338	339	343	346	347	364	369	371	384	394	400	546	550	552	595	605	DM	TP	PASTA	ORF
R6	Thr	Met	Met	Ala	Ala	Leu	Ala	Ile	Arg	His	Met	Leu	Thr	Gln	Tyr	Asn				
ATCCBAA-334	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	1
URA4929	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	2	0	2
URA4376	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	1
URA3537	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	2
URA2543	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	1
URA4933	.	.	Thr	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	2
URA3558	.	.	Thr	.	.	.	Val	.	.	Leu	.	.	.	.	.	.	16	12	3	37
URA4087	.	.	.	.	.	.	.	.	Gly	.	.	.	.	Glu	.	.	0	13	3	26
URA4566	Ala	.	Thr	.	.	.	Val	.	Gly	.	.	.	.	.	.	.	3	8	1	17
URA2542	.	.	Thr	.	.	.	.	.	Gly	.	.	.	.	Glu	.	.	0	10	0	10
URA3635	Ala	.	Thr	.	Ser	.	Val	.	Gly	.	.	.	.	.	.	.	1	9	4	19
URA4731	.	.	.	.	.	.	.	.	Gly	.	.	.	.	Glu	.	.	2	24	5	44
URA5391	.	.	Thr	.	.	.	.	.	Gly	.	.	.	.	Glu	.	.	4	23	15	54
URA5779	.	.	.	.	.	.	.	.	.	.	.	.	.	Glu	.	.	1	21	2	27
ATCC700670	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	Val	.	.	.	Thr	2	37	1	45
ATCC700673	Ala	.	Thr	Ser	Ser	Ser	Val	Thr	Gly	.	.	.	.	.	.	.	3	28	20	65
URA5805	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	Val	.	.	.	Thr	3	34	18	70
ATCC51916	Ala	Phe	.	Ser	Ser	Phe	.	Thr	Gly	.	Thr	Val	Ala	.	.	Thr	2	37	21	74
URA3543	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	Val	.	.	.	Thr	8	35	21	79
URA6056	Ala	Phe	.	Ser	Ser	Phe	Val	Thr	Gly	.	Thr	Val	.	.	Phe	Thr	3	42	23	82
URA3043	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	Val	.	.	.	Thr	2	37	21	73
URA4203	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	Val	.	.	.	Thr	3	37	21	75
URA5128	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	Val	.	.	.	Thr	3	37	21	75
URA3699	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	.	.	.	.	.	18	32	20	104
URA5450	.	.	.	.	.	.	.	.	Gly	.	.	.	.	Glu	.	.	1	20	3	25

(*continued*)

Table 8.1 - *Continued*

Strain	PBP1A															Total amino acid substitutions[c]
	371	388	432	546	574	575	576	577	606	609	611	612	TG	TP	ORF	
R6	Thr	Glu	Pro	Asn	Thr	Ser	Gln	Phe	Leu	Asn	Leu	Thr				
ATCCBAA-334	.	Asp	.	.	.	.	.	.	.	.	.	.	1	2	8	9
URA4929	.	Asp	.	.	.	.	.	.	.	.	.	.	1	2	7	9
URA4376	.	Asp	.	.	.	.	.	.	.	.	.	.	1	2	8	10
URA3537	.	Asp	.	.	.	.	.	.	.	.	.	.	1	2	7	11
URA2543	.	Asp	.	.	.	.	.	.	.	.	.	.	4	1	10	13
URA4933	.	Asp	.	.	.	.	.	.	.	.	.	.	1	1	9	14
URA3558	.	Asp	.	.	.	.	.	.	.	.	.	.	1	4	56	97
URA4087	.	Asp	.	.	.	.	.	.	.	.	.	.	2	3	11	37
URA4566	.	Asp	.	.	.	.	.	.	.	.	.	.	1	1	7	42
URA2542	.	Asp	.	.	.	.	.	.	.	.	.	.	1	1	7	34
URA3635	.	Asp	.	.	.	.	.	.	.	.	.	.	1	2	8	40
URA4731	.	Asp	.	.	Asn	Thr	Gly	Tyr	.	Asp	.	.	1	11	19	100
URA5391	.	Asp	.	.	.	.	.	.	.	.	.	.	2	2	9	103
URA5779	.	Asp	.	.	.	.	.	.	.	.	.	.	3	28	43	86
ATCC700670	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	2	44	60	119
ATCC700673	.	Asp	.	Gly	Asn	Thr	Gly	Tyr	Val	Asp	.	.	13	44	90	173
URA5805	Ser	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	12	43	80	200
ATCC51916	Ser	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	1	47	60	148
URA3543	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	2	44	52	168
URA6056	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	2	37	52	184
URA3043	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	2	47	55	148
URA4203	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	2	36	43	138
URA5128	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	2	37	52	147
URA3699	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Tyr	1	42	70	257
URA5450	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Tyr	1	42	70	112

[a] The main motifs in the transpeptidase domain in PBP2B are 391SVVK, 448SSN and 619KTG, in PBP2X are 337STMK, 395SSN and 547KSG, and in PBP1A 370STMK, 428SRN and 557KTG. The amino acid positions of R6 strain were used as reference. The positions within or close to conserved motifs in the PBPs are shown. The dots indicate homologous amino acids. DM, dimerization domain; TP, transpeptidase domain; ORF, signifies amino acid substitutions encoded in the open reading frame; PASTA, penicillin-binding protein and serine/threonine kinase-associated domain; TG, transglycosylase domain; S, susceptible; T, tetracycline; E, erythromycin; C, clindamycin; F, chloramphenicol; P, penicillin; X, cefotaxime; R, ceftriaxone; ND, not determined.

[b] MIC values are against penicillin (mg/L); strains were considered penicillin susceptible for MICs ≤0.06 mg/L, intermediate for MICs of 0.1 to 1 mg/L, and resistant for MICs ≥1.6 mg/L.

[c] Total number of amino acid substitutions in PBP2B, PBP2X, and PBP1A.

CHAPTER IX

The highly conserved serine threonine kinase StkP of *Streptococcus pneumoniae* contributes to penicillin susceptibility

Unpublished

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9.1 - Summary

The serine/threonine kinase StkP of *Streptococcus pneumoniae* is a major virulence factor in the mouse model of infection. In laboratory cultures, one target of StkP is the phosphoglucosamine mutase GlmM involved in the first steps of peptidoglycan biosynthesis. In order to further elucidate the importance of StkP in *S. pneumoniae,* its role in susceptibility to β-lactams has been assessed by mutational analysis. Deletion replacement mutation in *stkP* conferred hypersensitivity to penicillin G and was epistatic on mutations in PBP2X, PBP2B and PBP1A from the nonsusceptible 9V clinical isolate URA1258. This result reveals that StkP is involved in the bacterial response to penicillin and suggests that StkP activity allows bacteria to bypass cell wall injury due to penicillin up to a critical concentration defined as the MIC for a given strain. Genetic analysis of 55 clinical isolates allowed identifying 11 StkP alleles with regard to the reference R6 allele. Nevertheless the MIC values of these strains appeared rather to be determined by their PBP alleles suggesting strong functional conservation of StkP among clinical isolates.

9.2 - Introduction

Streptococcus pneumoniae is one of the main aetiological agents of invasive infectious disease. Penicillin-resistant pneumococci were first observed in the 70s, and resistance to penicillin and multidrug-resistance have since then increased worldwide. Cell-wall biosynthetic enzymes named Penicillin Binding Proteins (PBP) are the targets for β-lactam antibiotics; mutations in these proteins constitute a major mechanism of resistance in clinical isolates. In laboratory strains, *murMN*, *ciaRH* and *cpoA* genes are also involved in penicillin susceptibility suggesting their involvement in cell wall metabolism (Guenzi *et al.*, 1994; Hakenbeck *et al.*, 1999; Filipe & Tomasz, 2000). One of the first steps of cell wall biosynthesis is catalysed by phosphoglucosamine mutase GlmM (Mengin-Lecreulx & van Heijenoort, 1996). GlmM of *S. pneumoniae* is substrate for the serine/threonine kinase StkP consistent with regulation of its activity by phosphorylation, as shown in other species (Jolly *et al.*, 1999; Novakova *et al.*, 2005). StkP is also required for virulence expression in the mouse model of infection and for induction of competence for genetic transformation in cultures (Echenique *et al.*, 2004). We report an analysis of the role of StkP in β-lactam resistance in various strains carrying wild-type and mutated PBPs and we assess the contribution of StkP to the resistance level of clinical isolates.

9.3 - Material and methods

Bacterial strains, plasmids and growth conditions. The plasmids and strains used in this study are described in Table 9.1. *Escherichia coli* was grown in LB (Difco, Sparks, Maryland) supplemented or not with ampicillin (100 mg/L) (Atral, Castanheira do Ribatejo, Portugal). *S. pneumoniae* clinical isolates were grown at 35 °C on Columbia agar plates supplemented with 5 % horse blood (Biomerieux, Carnaxide, Portugal), in an atmosphere enriched with 5 % CO_2. Serotyping was performed by the Quellung reaction with antisera produced by the Statens Seruminstitut, Copenhagen, Denmark (Sorensen, 1993). Laboratory unencapsulated strains derived from RX were routinely grown in casitone and tryptone (CAT) medium at 37 °C (Morrison *et al.*, 1983). CTM transformation medium was used to induce competence and for transformation, as described previously (Pestova & Morrison, 1998). The CSP concentration was 100 ng/mL and DNA concentration was 1 mg/L. The chromosomal source of DNA carrying mutated PBP alleles was the 9V derivative Spain^{23F}-1 clone (strain URA1258) which carries the following mutations near or within the conserved motifs on the PBPs: Gln443Glu, Thr451Ala, Glu481Gly, Ser485Ala and Thr494Ala in PBP2B, Thr338Ala, Met343Thr, Ala346Ser, Ala347Ser, Leu364Phe, Ile371Thr, Arg384Gly, Leu546Val and Asn605Thr in PBP2X, and Thr371Ala, Glu388Asp, Pro432Thr, Asn546Gly, Thr574Asn, Ser575Thr, Gln576Gly, Phe577Tyr, Leu606Ile, Asn609Asp, Leu611Phe and Thr612Leu in PBP1A. Transformants were selected on plates containing 0.1 mg/L and 0.5 mg/L penicillin, and appropriate integration of PBP mutations was confirmed by nucleotide sequencing. Plates containing 2 mg/L rifampicin and 10 mg/L chloramphenicol were used to select *rif-23* and ∆*stkp::cat* transformants. All constructions were verified by PCR with the primers described in Table 9.2. Spontaneous mutation to penicillin in DNA free medium was $<10^{-9}$. Penicillin G was from Atral, Castanheira do Ribatejo, Portugal, and rifampicin was from Aventis Pharma. To assess StkP and PBPs conservation 50 strains were randomly selected among those isolated between 1994 and 2005 in various areas in Portugal; they included forty invasive isolates from blood and CSF and ten colonizing isolates from the nasopharynx of asymptomatic carriers. Half of the isolates (n = 25) were nonsusceptible to penicillin (MIC >0.1 mg/L). These isolates were compared to the following reference strains: the highly resistant serotype 9V strain URA1258, two susceptible and three nonsusceptible strains provided by the ATCC and the unencapsulated strain R6 (Table 9.1).

Measurements of penicillin susceptibility. The MIC of penicillin G for the strains constructed were determined in duplicate by E-test (AB Biodisk, Solma, Sweden) according to the manufacturer's recommendations (incubation at 35 ºC in 5 % CO_2 for 18 to 24 H), and for clinical isolates by an agar dilution method with the testing conditions and susceptibility interpretation standards proposed by the CLSI (Clinical and Laboratory Standards Institute, 2007). Strains were considered penicillin susceptible for MIC values ≤0.06 mg/L,

intermediate MIC for values of 0.1 – 1 mg/L, and highly resistant for MIC values ≥1.6 mg/L. Strains were classified as nonsusceptible for MIC values ≥0.1 mg/L, according to CLSI criteria.

Table 9.1 - Strains and plasmids used in the study

Strain or plasmid	Genotype or description	Phenotype[a]	Source or reference
S. pneumoniae			
R6	Non-capsulated D39 derivative, susceptible reference strain; genome sequence available (R6)	AtbS	Laboratory stock
ATCC BAA-334	Virulent reference strain, genome sequence available (TIGR4)	AtbS	ATCC
ATCC 51916	Reference strain Tennessee 23F-4	PenR, EryR,	ATCC
ATCC 700670	Reference strain Spain 6B-2	PenR, CmR, TetR	ATCC
ATCC 700673	Reference strain Hungary 19A-6	PenR, EryR, CmR, TetR	ATCC
URA1258	Multiresistant strain closely related to Spain 23F-1 clone	PenR, CmR, TetR	Caniça *et al.*, 2003
Cp1015	Rx derivate, *str1*; *hexA*	SmR	Echenique *et al.*, 2004
Cp1016	Rx derivate, *str1*; *hexA*, *rif23*	RifR	Echenique *et al.*, 2004
Cp7000	Cp1015, *stkP*::*cat*	CmR	This study
Pen1	Cp1015, *penA,* and *pbpX* from URA1258	PenR	This study
Pen2	Cp1015, *penA*, *pbpX* and *pbp1A* from URA1258	PenR	This study
Pen1STK	Cp1015Pen1, *stkP*:: *cat*	PenR CmR	This study
Pen2STK	Cp1015Pen2, *stkP*:: *cat*	PenR CmR	This study
E. coli			
DH5α	F^-, φ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk^-, mk^+), *pho*A, *sup*E44, λ^-, *thi*$^-$1, *gyr*A96, *rel*A1	NalR	White *et al.*, 1988
plasmid			
pISTK	3.5 kb EcoRI/SacII fragment containing *stkP* and flanking regions with *cat* cassette inserted	ApR, CmR	Novakova *et al.*, 2005

[a] ApR, resistant to ampicillin; AtbS, susceptible to all tested antibiotics; CmR, resistant to chloramphenicol; EryR, resistant to erythromycin; NalR, resistant to nalixidic acid; PenR, nonsusceptible to penicillin G; RifR, resistant to rifamycin; SmR, resistant to streptomycin; TetR, resistant to tetracycline.

***stkP* genotyping by amplification and nucleotide sequencing.** The *stkP* gene of clinical strains was amplified by PCR using the primers listed in Table 9.2 and a Qiagen multiplex PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, this routinely involved 40 cycles with an annealing temperature of 56 ºC for 1 minute. The PCR products were purified on ExoSAP-IT (USB, Cleveland, Ohio) and the nucleotide sequence was established (BigDye Cycle sequencing kit v1.1 from Applied Biosystems, Foster City, California). BioNumerics software v3.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for contig assemblages of the DNA sequences.

Table 9.2 - Primers used for PCR amplification

Primer Name	Primer sequence	Gene targeted	Reference
STKP-F	5'-AGGATGCCATATGATCCAAATCGGCAA-3'	*stkP*	Novakova *et al.*, 2005
STKP-R	5'-TTGATTATGAATTCGCTTTTAAGGAGTAGC-3'	*stkP*	Novakova *et al.*, 2005
STKP-F2	5'-GTAGGACAGAATTCAAGACAAGTCTACATACA-3'	*stkP*	Novakova *et al.*, 2005
pbp1aF	5'-CCAGCAACAGGTGAGAGTC-3'	*pbp1A*	Sanbongi *et al.*, 2004
pbp1aR	5'-GTAAACACAAGCCAAGACAC-3'	*pbp1A*	Sanbongi *et al.*, 2004
pbp1aF2	5'-GAACTTCAAGACAAGGCAGT-3'	*pbp1A*	Sanbongi *et al.*, 2004
pbp2bF	5'-CCGTCTTAATCCCGATACC-3'	*penA*	Sanbongi *et al.*, 2004
pbp2bR	5'-ATTTTTGGGTGACTTGTTGAG-3'	*penA*	Sanbongi *et al.*, 2004
pbp2xF	5'-GGAATTGGTGTCCCGTAAGC-3'	*pbpX*	Sanbongi *et al.*, 2004
pbp2xR	5'-CATCTGCTGGCCTGTAATTTG-3'	*pbpX*	Sanbongi *et al.*, 2004

Genetic diversity of the StkP kinase in 56 pneumococcal strains. The amino acid sequences deduced from the 56 *stkP* genes were aligned using the CLUSTALW program built in the MEGA version 4 software package (Tamura *et al.*, 2007). There were a total of 637 positions in the final dataset, of which 8 were parsimony informative. The evolutionary history was inferred using the maximum parsimony method (Eck & Dayhoff, 1966). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the *stkP* gene (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The maximum parsimony tree was obtained using the Close-Neighbor-Interchange algorithm (Nei & Kumar, 2000) with search level 3 (Felsenstein, 1985; Nei & Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale with branch lengths calculated by the average pathway method (Nei & Kumar, 2000) and with the number of changes over the whole sequence as units.

Estimates of Average Evolutionary Divergence over Sequence Pairs of *stkP* within penicillin susceptibility groups. The number of amino acid and of nucleotide substitutions per site was averaged over all sequence pairs within each group by the Poisson correction method and the Maximum Composite Likelihood method, respectively, using MEGA version 4 software (Tamura *et al.*, 2007). Standard error estimates were obtained by the bootstrap procedure (1000 replicates).

StkP modelling. A 3D-model of the kinase domain of the StkP protein (271 residues long) of strain R6 was obtained using the sequence (accession number NP_359169). BLASTP analysis indicated that the serine-threonine kinase from strain R6 has 63 % sequence identity with serine-threonine kinase of *Mycobacterium tuberculosis* (PDB ID: 1o6yA). The following structure PDB ID: 1o6yA; 1mruA.pdb, 1mruB.pdb, 1y8gB.pdb and 1zmwB.pdb were used as a template for building a homology model for the kinase domain of StkP with the SWISS-MODEL server (Guex & Peitsch, 1997; Schwede *et al.*, 2003). Ramachandran plot analysis for phi and psi torsion angles indicated that 95.9 % of residues were in the allowed region of the plot, which is more than the average cut-off of 90 % used in

most reliable models (Ramachandran *et al.*, 1963). The final alignment adjustments and visualisation were undertaken with Deep View/Swiss-PdbViewer version 3.7.

Genotyping of *pbp* genes. Genetic polymorphism of *penA, pbpX* and *pbp1A* genes (encoding PBP2B, PBP2X and PBP1A, respectively) of all clinical strains was investigated first by RFLP analysis. A number was given to each restriction pattern for each of the three *pbp* genes analysed, so the PBP profile has three numbers (for example: 4-9-7). The full genes were amplified by PCR using the primers described in Table 2 and 0.8 U of iProof Polymerase (Bio-Rad, Hercules, California) according to the manufacturer's instructions, with 35 cycles at an annealing temperature of 56 °C for 30 seconds. The amplification products of *penA* and *pbpX* were digested for 1 H with 5 U of both *Hae*III and *Rsa*I restriction endonucleases. The amplification product of *pbp1A* was similarly digested with *Hae*III and *Dde*I (all restriction enzymes supplied by New England Biolabs, Beverly, Mas.). The digested products were separated on agarose gel. Dice coefficient of similarity was used for cluster analysis with the unweighted pair group method with arithmetic averages using BioNumerics software v3.5 (Applied Maths, Sint-Martens-Latem, Belgium). The position tolerance was set to 1.0 % and the optimization 1.5 %. For each *pbp* gene restriction pattern identified, one isolate was randomly chosen and re-amplified by PCR for nucleotide sequencing. Contig assemblages of the DNA sequencing were performed as described above.

Nucleotide sequence accession numbers. Sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank database under accession numbers AM889231 to AM889284 for *stkP*, AM779386 to AM779409 for *penA*, AM779338 to AM779361 for *pbpX*, and AM779362 to AM779385 for *pbp1A*.

9.4 - Results

Influence of *stkP* mutation on penicillin susceptibility. To assess the role of StkP in penicillin resistance, the laboratory transformable strain Cp1015 (Table 9.1) was transformed with DNA from the serotype 9V nonsusceptible strain URA1258 related to the international multiresistant clone Spain^{23F}-1 (Caniça *et al.*, 2003). Penicillin-nonsusceptible transformants were selected on plates containing 0.1 mg/L of penicillin. One transformant was isolated: strain Pen1, isogenic to Cp1015 but with mutations in PBP2X and 2B and resistant up to 0.125 mg/L of penicillin. Strain Pen1 was then transformed with DNA from URA1258 and transformants were selected on plates containing 0.5 mg/L penicillin; this gave strain Pen2 isogenic to Pen1 but for mutations in *pbp1A* and resistant to 0.5 mg/L penicillin. Transformation of strains Cp1015, Pen1 and Pen2 with plasmid pISTK (Table 9.1) and selection on chloramphenicol plates gave the corresponding isogenic strains differing by their PBP and StkP alleles. The MICs of these strains were determined: the StkP$^-$ allele significantly and reproducibly increased penicillin susceptibility (Table 9.3). The StkP$^-$ mutations not only increased the penicillin susceptibility of strain Cp1015 carrying wild-type penicillin binding proteins, but was also epistatic on mutations PBP2B, 2X and 1A; therefore StkP acts upstream from the PBPs.

In control transformation assays with DNA from the Rif^R strain Cp1016 as donor the penicillin resistance mutants Pen1 and Pen2 were 10^{-1} and 10^{-3} times less transformable, respectively, than the wild-type strain Cp1015. The effect of the StkP$^-$ mutation on penicillin resistance led us to investigate the contribution of StkP mutations to the MIC levels of clinical isolates. We studied the genetic diversity of StkP in clinical isolates in relation with their PBP alleles.

Table 9.3 - Resistance phenotype and transformability of RX derivatives with different combinations of PBP and StkP alleles

Strain	Genotype	MIC Pen (mg/L)[a]
URA1258	Multiresistant strain closely related to Spain 23F-1 clone	0.5 -1
Cp1015	Rx derivate, *str1*; *hexA*	0.016
Cp7000	Cp1015, *stkP*::*cat*	0.008
Pen1	Cp1015, *penA* and *pbpX* from URA1258, allelic exchange mutant	0.064 - 0.125
Pen2	Cp1015, *penA*, *pbpX* and *pbp1A* from URA1258, allelic exchange mutant	0.38 - 0.5
Pen1STK	Pen1, *stkP*::*cat*	0.016 - 0.032
Pen2STK	Pen2, *stkP*::*cat*	0.032 - 0.125

[a] MIC Pen, Minimum inhibitory concentration for penicillin.

Polymorphism of *stkP* in clinical isolates and relationship to penicillin resistance. StkP protein from *S. pneumoniae* contains a eukaryotic kinase domain (Hanks kinase domain) and a PASTA (penicillin-binding protein and serine threonine kinase) domain signature only found in prokaryotes and putatively involved in cell wall sensing (Jones & Dyson, 2006). The sequence of the *stkP* gene from 50 clinical isolates and 6 reference strains was determined. The *stkP* gene in each strain was amplified by PCR using oligonucleotides complementary to sequences at -10 and +1997 of the gene. In each case, a 2007bp DNA fragment was obtained and the nucleotide sequences confirmed that they corresponded to *stkP.* There were 61 segregating sites with a rate of segregating sites per site of 0.033, resulting in 27 allelic variants with an average of 10.26 nucleotides substitutions per sequence. Analysis of the encoded amino-acid sequences revealed 11 segregating sites and a rate of segregating sites per site of 0.020, resulting in 12 allelic variants (including strain R6) with an average of 1.37 amino acid substitution per sequence (Table 9.4 and Figure 9.1). This, the full-size StkP protein is well conserved in invasive and colonising clinical isolates and independent of their penicillin-resistance character.

We considered PASTA domains and kinase domains individually: nucleotide divergence was higher in the 5' terminal part of the gene encoding the kinase module (d = 0.0072; S.E.: 0.0013) than in the 3' part of the gene encoding the PASTA modules (d = 0.0048; S.E.: 0.0011). By contrast, amino acid divergence was higher in the PASTA domains (d = 0.0037; S.E.: 0.0011) than in kinase domain (d = 0.0012; S.E.: 0.0007). The distribution of the amino acid allelic variants of StkP into penicillin-resistance classes was assessed (Figure 9.1): alleles 2, 3, 5, 6, 7, 8, 10 and 11 were found in penicillin-susceptible strains and alleles 1, 4, 9 and 12 were found both in penicillin-nonsusceptible and -sensitive strains (Table 9.4). The StkP amino acid sequence divergence was similar among penicillin-susceptible strains (d = 0.0027; S.E.: 0.0009), penicillin-intermediate strains (d = 0.0015; S.E.: 0.0009) and highly resistant strains (d = 0.0017; S.E.: 0.0011).

To evaluate the effects of the StkP mutations on its kinase, a model of the enzymatic domain, amino acid 4 to 274, based on the sequence of the strain R6 was developed (Accession number: NP_359169) (Figure 9.2). The mutations carried by the various alleles were located outside of the catalytic site and appeared unlikely to affect the ATP binding site. Thus, these clinical isolates are unlikely to carry loss of kinase function mutants.

To evaluate the consequences of mutations in the PASTA domains on the penicillin susceptibility of clinical isolates we analysed the genetic polymorphism of PBP2B, PBP2X and PBP1A, in relation the PASTA alleles in the different isolates (Table 9.4). RFLP patterns 4, 5, 7, 9, 18 of PBP2B, patterns 5 to 9 of PBP2X and patterns 4 to 10, 13, 16 and 17 of PBP1A (Tables 9.5 – 9.7) are not associated with mutations involved in penicillin resistance, according to previous descriptions (Smith & Klugman, 1995; Grebe & Hakenbeck, 1996;

Gordon *et al.*, 2000; Dessen *et al.*, 2001; Smith & Klugman, 2003; Chesnel *et al.*, 2005; Smith & Klugman, 2005; Carapito *et al.*, 2006; Contreras-Martel *et al.*, 2006). Four PASTA alleles (StkP alleles: 3, 7, 10 and 11) were only found in sensitive strains (URA3826, URA5133, URA3537, URA3388, URA3444, URA6035, URA4549). These strains showed PBP profiles characteristic of sensitive strains, suggesting that their MICs were determined by their PBPs rather than mutation in their PASTA sequence. The other PASTA alleles were found in all the three classes of strains (high and intermediate resistance, and susceptibility) suggesting that this allele did not affect the MIC. We checked, for each strain, that the resistance character corresponded to the PBP profile (Table 9.4). Findings for strain URA5132 were, however, more ambiguous: it was susceptible with a MIC of 0.006 mg/L despite carrying the PBP2X mutations Arg384Gly and Gln552Glu related to resistance (Carapito *et al.*, 2006); it also carries the Val623Ala PASTA allele suggesting that it may have a putative suppressor function leading to the susceptible phenotype. However, we did not test whether the PASTA Val623Ala allele is directly involved as a suppressor of the PBP mutations, partly because mutation Val623Ala is the replacement of one non polar amino acid with another. Note that this mutation was also found in resistant (URA5805 and URA4203) and intermediate (URA4566, URA4731 and URA5779) strains and therefore it is unlikely that it determines the penicillin susceptibility of strain URA5132.

Table 9.4 - Deduced amino acid substitutions in StkP and related PBP profiles of 50 clinical strains and 6 reference strains[a]

Strains	Serotype	MIC[b]	Kinase domain					TMS	Pasta domains							Total[f]	RFLP profile[g]			StkP allele[h]
			45	113	227	237	Kinase[c]	310[d]	385	449	453	492	500	623	PASTA[e]		PBP 2B	PBP 2X	PBP 1A	
R6		0.0125	Arg	Ala	Asn	Ser		Val	Ala	Ser	Ala	Asp	Val	Val			4	9	7	5
URA3826	9V	0.006	.	Val	Lys	Pro	3	.	Glu	.	.	.	.	Ala	2	6	4	7	10	11
URA5132	6B	0.006	.	.	Lys	Pro	2	.	.	.	.	.	.	Ala	1	4	9	7	4	9
URA5995	1	0.006	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	5	5	4	1
URA4376	6B	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	9	5	13	1
URA3537	3	0.0125	.	.	Lys	Pro	2	.	.	.	Ser	.	.	.	1	4	5	5	9	3
URA4929	9V	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	4	9	10	12
URA4933	14	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	7	7	7	1
URA3388	4	0.0125	.	.	Lys	Pro	2	.	.	.	Ser	.	.	.	1	4	5	7	4	3
URA3444	33F	0.0125	.	.	Lys	.	1	.	.	.	.	Ala	.	.	1	3	5	8	4	7
URA3595	3	0.0125	Lys	.	Lys	Pro	3	.	.	.	.	.	.	Ala	1	5	4	5	4	8
URA3864	23A	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	4	8	4	1
URA4135	15B	0.0125	.	.	Lys	Pro	2	Ala	.	.	.	.	.	.	0	4	4	8	4	4
URA4549	11A	0.0125	.	.	Lys	Pro	2	.	.	Phe	.	.	.	Ala	2	5	5	8	4	10
URA4893	22F	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	7	5	4	1
URA5133	7F	0.0125	.	Val	Lys	Pro	3	.	Glu	.	.	.	.	Ala	2	6	4	7	4	11
URA5775	23A	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	4	8	4	1
URA5939	3	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	4	9	6	1
URA6035	33F	0.0125	.	.	Lys	.	1	.	.	.	.	Ala	.	.	1	3	5	8	4	7
ATCC BAA334	4	0.0125	.	.	Lys	Pro	2	.	.	.	.	.	.	Ala	1	4	7	8	4	2
URA2543	23B	0.025	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	5	9	17	1
URA4087	19F	0.025	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	4	6	7	1
URA3417	14	0.025	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	7	7	7	1
URA5468	35F	0.025	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	4	9	4	12
URA2542	22F	0.05	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	18	7	4	1
URA2520	6	0.05	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	9	8	4	1
URA2932	19F	0.05	.	.	Lys	.	1	.	.	.	.	.	.	.	0	2	4	6	7	6
URA4566	6B	0.1	.	.	Lys	Pro	2	.	.	.	.	.	.	Ala	1	4	6	3	8	9
URA4731	14	0.1	.	.	Lys	Pro	2	.	.	.	.	.	.	Ala	1	4	3	18	4	9
URA5391	23F	0.1	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	1	1	5	1
ATCC 51916	23F	0.1	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	8	12	12	1

(*continued*)

Table 9.4 - *Continued*

Strains	Serotype	MIC[b]	Kinase domain					TMS	Pasta domains							Total[f]	RFLP profile[g]			StkP allele[h]
			45	113	227	237	Kinase[c]	310[d]	385	449	453	492	500	623	PASTA[e]		PBP 2B	PBP 2X	PBP 1A	
URA5779	15A	0.1	.	.	Lys	Pro	2	.	.	.	.	.	.	Ala	1	4	13	11	6	9
URA3635	19A	0.1	.	.	Lys	Pro	2	Ala	.	.	.	.	.	.	0	4	11	14	5	4
URA3557	19C	0.1	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	1	1	4	1
URA3891	23F	0.1	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	1	1	7	1
URA3558	23F	0.2	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	5	16	16	1
URA3699	19F	0.5	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	2	2	18	1
URA5450	24F	0.5	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	15	8	11	1
URA5128	14	0.5	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	10	13	2	12
URA4806	19A	0.5	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	15	13	11	1
URA4835	19A	0.5	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	15	13	11	1
URA4926	9V	0.5	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	10	13	1	12
URA3432	9V	0.8	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	10	13	1	12
URA3706	9V	0.8	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	10	13	1	12
URA5464	19A	0.8	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	15	13	11	1
URA3420	19A	1	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	15	13	11	1
URA5805	19A	1.6	.	.	Lys	Pro	2	.	.	.	.	.	.	Ala	1	4	17	19	14	9
URA4203	14	1.6	.	.	Lys	Pro	2	.	.	.	.	.	.	Ala	1	4	10	13	3	9
URA1258	9V	1.6	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	10	13	1	1
URA2884	14	1.6	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	10	13	1	12
URA5316	19C	1.6	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	10	13	1	1
ATCC 700673	19A	2	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	14	4	15	1
URA3043	14	2	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	10	10	1	12
URA3543	14	2	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	16	15	1	12
ATCC 700670	6B	2	.	.	Lys	Pro	2	.	.	.	.	.	.	.	0	3	19	17	1	1
URA6056	14	3.2	.	.	Lys	Pro	2	.	.	.	.	.	Ile	Ala	2	5	12	13	1	12

[a] The amino acid positions in strain R6 were used as reference. All amino acid substitutions found are shown.
[b] MIC, Minimum Inhibitory Concentration of penicillin (mg/L).
[c] Kinase, total number of amino acid substitutions in kinase domain of StkP (12-273).
[d] TMS, transmembrane segment
[e] PASTA, total number of amino acid substitutions in PASTA domain of StkP (366-651).
[f] Total number of amino acid substitutions in StkP.
[g] RFLP profile, PBP restriction fragment length polymorphism pattern for PBP2B - PBP2X - PBP1A.
[h] Numbers correspond to each genetic lineage obtained from the Maximum Parsimony analysis (according to Figure 9.1).

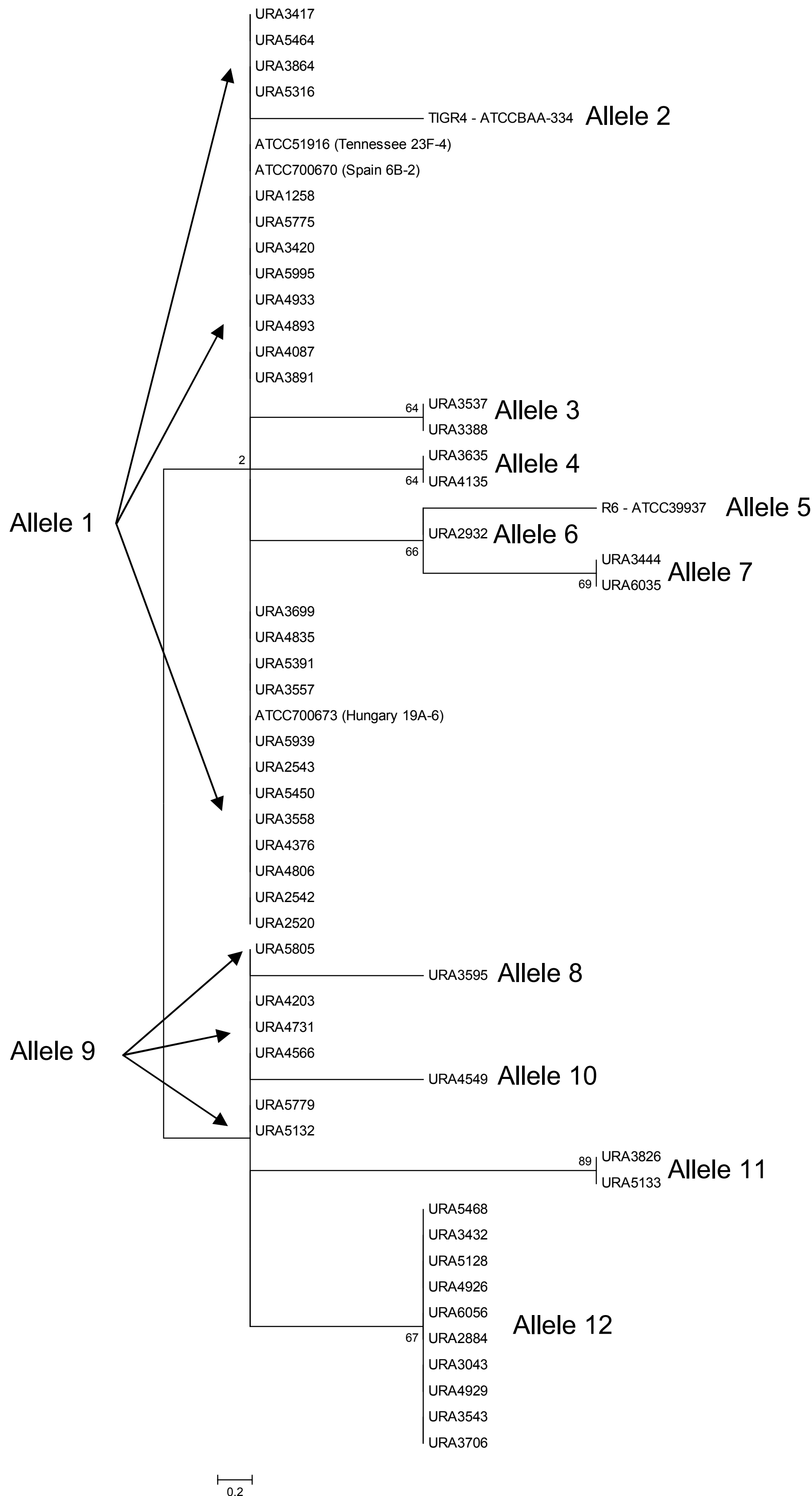

Figure 9.1 - Inference of phylogenetic history of StkP from 56 strains using the Maximum Parsimony method. A number was given to each branch corresponding to the StkP alleles. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

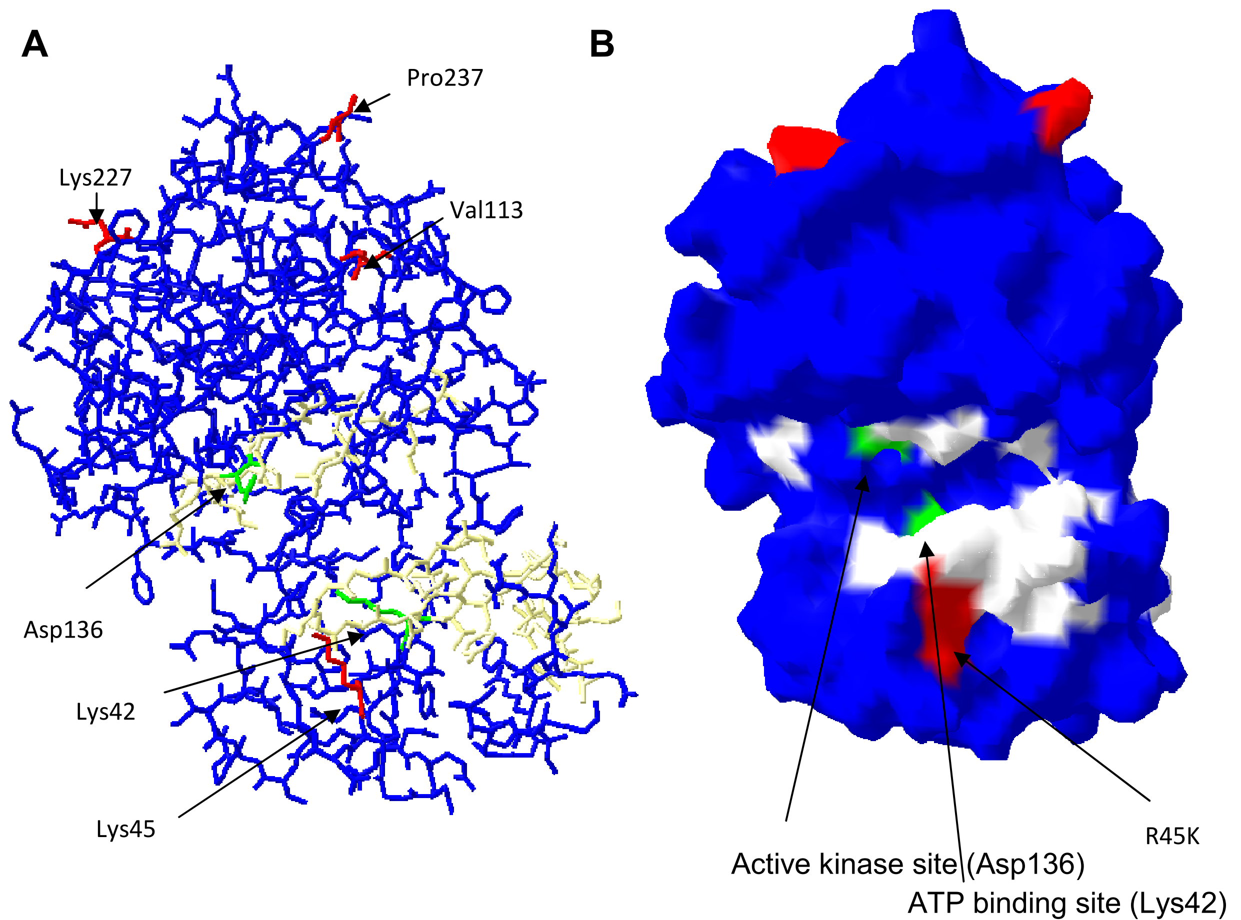

Figure 9.2 - Predicted structure of the kinase catalytic domain of StkP. (A) Image of backbone with oxygens of the StkP kinase domain (4-274). In white, predicted catalytic zones: ATP-binding site (18-42) and kinase motif (132-144). In green: Asp136 - phosphorylation site and Lys42 - ATP binding site. The amino acid substitutions, relative to the R6 sequence, are in red: Arg45Lys, Ala113Val, Asn227Lys and Ser237Pro. (B) Image of computed molecular surface of StkP kinase domain (4-274). The colours are otherwise as in Figure 9.2A.

Table 9.5 - Deduced amino acid substitutions in PBP2B in 25 pneumococcal strains

PBP 2B pattern[b]	Number of strains	MIC[c]	PBP2B[a]														
			443 Gln	451 Thr	481 Glu	485 Ser	488 Gly	494 Thr	614 Leu	624 Ala	629 Ala	630 Asp	633 Gln	635 Thr	DM[d]	TP[e]	ORF[f]
4, 5, 7, 9	8	0.0125 - 0.2	.	.	.	.	.	.	.	.	.	.	.	.	0 -3	0 -1	0 -4
6	1	0.1	.	Ala	Gly	.	.	Ser	.	.	.	Gly	Glu	Asn	0	13	18
18	1	0.05	.	Ala	Gly	.	.	Ser	Ser	.	Glu	Gly	Glu	Asn	1	12	17
11	1	0.1	.	Ala	Gly	.	.	Ser	.	.	.	.	.	.	6	6	13
3	1	0.1	Glu	Ala	Gly	.	Ala	Ala	Ala	.	Glu	Gly	Glu	Asn	1	32	37
1	1	0.1	Glu	Ala	Gly	.	Ala	Ala	Ser	.	Glu	Gly	Glu	Asn	1	34	40
13	1	0.1	.	Ala	Gly	.	.	Ser	Ala	.	Glu	Gly	Glu	Asn	1	10	16
19	1	2	Glu	Ala	Gly	.	.	Ser	Ala	.	.	Gly	Glu	Asn	2	11	14
14	1	2	.	Ala	Gly	.	.	Ser	.	.	.	.	.	.	2	15	18
17	1	1.6	Glu	Ala	Gly	Ala	.	Ala	Thr	Gly	.	Gly	Glu	Asn	9	31	50
8	1	0.1	.	.	.	.	.	.	.	Gly	.	Gly	Glu	Asn	2	3	14
16	1	2	Glu	Ala	Gly	Ala	.	Ala	Thr	Gly	.	Gly	Glu	Asn	5	21	37
12	1	3.2	Glu	Ala	Gly	.	.	Ser	Thr	Gly	.	Gly	Glu	Asn	6	33	50
10	3	0.5-2	Glu	Ala	Gly	Ala	.	Ala	.	.	.	.	.	.	7	12	20
2	1	0.5	Glu	.	Gly	.	Ala	Ala	Ala	.	.	Gly	Lys	Asn	37	33	83
15	1	0.5	.	Ala	Gly	.	.	Ser	.	.	.	.	.	.	4	12	17

[a] The amino acid positions of strain R6 were used as reference. The positions within or close to conserved motifs in the PBP2B are shown.
[b] PBP2B pattern, restriction fragment length polymorphism pattern of *penA* gene encoding PBP2B.
[c] MIC, Minimum Inhibitory Concentration of penicillin (mg/L).
[d] DM, number of amino acid substitutions in the dimer domain.
[e] TP, number of amino acid substitutions in the transpeptidase domain.
[f] ORF, number of amino acid substitutions in the open reading frame.

Table 9.6 - Deduced amino acid substitutions in PBP2X in 25 pneumococcal strains

PBP 2X pattern[b]	Number of strains	MIC[c]	PBP2X[a]																DM[d]	TP[e]	PASTA[f]	ORF[g]
			338 Thr	339 Met	343 Met	346 Ala	347 Ala	364 Leu	369 Ala	371 Ile	384 Arg	394 His	400 Met	546 Leu	550 Thr	552 Gln	595 Tyr	605 Asn				
5, 6, 7, 8, 9	5	0.0125-0.025	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1-2	0	1-2
7	1	0.0125	.	.	Thr	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	2
16	1	0.2	.	.	Thr	.	.	.	Val	.	.	Leu	.	.	.	.	.	.	16	12	3	37
6	3	0.025-0.5	.	.	.	.	.	.	.	.	Gly	.	.	.	.	E	.	.	0-2	13-24	3-5	25-44
3	1	0.1	Ala	.	Thr	.	.	.	Val	.	Gly	.	.	.	.	.	.	.	3	8	1	17
14	1	0.1	Ala	.	Thr	.	Ser	.	Val	.	Gly	.	.	.	.	.	.	.	1	9	4	19
7	1	0.05	.	.	Thr	.	.	.	.	.	Gly	.	.	.	.	E	.	.	0	10	0	10
1	1	0.1	.	.	Thr	.	.	.	.	.	Gly	.	.	.	.	E	.	.	4	23	15	54
11	1	0.1	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	1	21	2	27
4	1	2	Ala	.	Thr	Ser	Ser	Ser	Val	Thr	Gly	.	.	.	.	.	.	.	3	28	20	65
2	1	0.5	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	.	.	.	.	.	18	32	20	104
17	6	0.5 -2	Ala	.	Thr	Ser	Ser	Phe	.	Thr	Gly	.	.	Val	.	.	.	Thr	2-8	34-37	1-21	45-79
12	1	0.1	Ala	Phe	.	Ser	Ser	Phe	.	Thr	Gly	.	Thr	Val	Ala	.	.	Thr	2	37	21	74
13	1	3.2	Ala	Phe	.	Ser	Ser	Phe	Val	Thr	Gly	.	Thr	Val	.	.	Phe	Thr	3	42	23	82

[a] The amino acid positions of strain R6 were used as reference. The positions within or close to conserved motifs in the PBP2X are shown.
[b] PBP2X pattern, restriction fragment length polymorphism pattern fo *pbpX* gene encoding PBP2X.
[c] MIC, Minimum Inhibitory Concentration of penicillin (mg/L).
[d] DM, number of amino acid substitutions in the dimer domain.
[e] TP, number of amino acid substitutions in the transpeptidase domain.
[f] PASTA, number of amino acid substitutions in PASTA - Penicillin-binding protein and serine/threonine kinase associated domain.
[g] ORF, number of amino acid substitutions in the open reading frame.

Table 9.7 - Deduced amino acid substitutions in PBP1A in 25 pneumococcal strains

PBP 1A pattern[b]	Number of strains	MIC[c]	PBP1A[a]														
			371 Thr	388 Glu	432 Pro	546 Asn	574 Thr	575 Ser	576 Gln	577 Phe	606 Leu	609 Asn	611 Leu	612 Thr	TG[d]	TP[e]	ORF[f]
4, 5, 6, 7, 8, 9, 10, 13, 16, 17	13	0.0125 - 0.2	.	Asp	.	.	.	.	.	.	.	.	.	.	1-4	1-28	7-56
4	1	0.1	.	Asp	.	.	Asn	Thr	Gly	Tyr	.	Asp	.	.	1	11	19
15	1	2	.	Asp	.	Gly	Asn	Thr	Gly	Tyr	Val	Asp	.	.	13	44	90
1, 2, 3	6	0.5 - 3.2	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	2	36-44	43-60
11, 18	2	0.5	Ala	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Tyr	1	42	70
12, 14	2	0.1-1.6	Ser	Asp	Thr	Gly	Asn	Thr	Gly	Tyr	Ile	Asp	Phe	Leu	1-12	43-47	60-80

[a] The amino acid positions of strain R6 were used as reference. The positions within or close to conserved motifs in the PBP1A are shown.
[b] PBP1A pattern, restriction fragment length polymorphism pattern of *pbpA* gene encoding PBP1A.
[c] MIC, Minimum Inhibitory Concentration of penicillin (mg/L).
[d] TG, number of amino acid substitutions in the transglycosylase domain.
[e] TP, number of amino acid substitutions in the transpeptidase domain.
[f] ORF, number of amino acid substitutions in the open reading frame.

9.5 - Discussion

We present genetic and physiological evidence of the involvement of the serine threonine kinase StkP in cell wall metabolism upstream from the steps catalysed by penicillin binding proteins PBP2B, 2X and 1A in *S. pneumoniae*.

The work by Giefing *et al.*, described the conservation of StkP among clinical strains and also observed the impact of *stkP* mutation on penicillin susceptibility on a susceptible genetic background (Giefing *et al.*, 2008). However the association between PBPs and StkP mutation were not assessed. Here, we showed that the role of StkP on penicillin susceptibility is not related to the major genetic determinants for penicillin susceptibility in pneumococci among a set of clinical and reference strains as well as in the set of penicillin resistant mutants.

This function, notably attributed to its PASTA domains, has already been proposed elsewhere (Yeats *et al.*, 2002), but there was previously no supporting experimental evidence. This role for StkP is consistent with previous observations showing that the phosphoglucomutase GlmM is involved in the first steps of peptidoglycan biosynthesis is a target for StkP (Novakova *et al.*, 2005). Consistent with this notion, GlmM in *E. coli* is activated by phosphorylation (Mengin-Lecreulx & van Heijenoort, 1996) and in *S. aureus* functional GlmM is needed for full expression of methicillin resistance (Wu *et al.*, 1996).

Although StkP is not essential and loss of function mutations can be obtained in laboratory conditions (Echenique *et al.*, 2004; Novakova *et al.*, 2005) and this work), it is strongly conserved in clinical isolates, reminiscent of housekeeping genes (Feil *et al.*, 2000a); presumably, it has an important role in natural niches. Extensive sequence analysis of StkP in susceptible and resistant pneumococcal isolates did not reveal any mutation significantly associated with susceptibility to penicillin. This suggests that *stkP* is of great importance for the cellular homeostatic mechanisms of *S. pneumoniae* and is not subject to the selective pressures caused by the β-lactams, unlike *pbp* genes presenting mosaic structures.

PASTA domains in prokaryotic serine-threonine kinases and PBP2X are involved in cell wall motif recognition (Jones & Dyson, 2006). Consistent with our study, Jones and Dyson reported that the PASTA domain of STK from several species showed high amino acid sequence divergence and Ka/Ks values, suggesting that PASTA domain interact with a wider range of stem-peptide ligands (Jones & Dyson, 2006). We report similar observations for invasive and colonizing strains. It is thus unlikely that mutation in the kinase or the PASTA domains contributes to the characteristics of the virulent strains in our collection.

Although the mechanism(s) regulating StkP activity remain(s) to be determined, it is likely that GlmM under the control of StkP is a control point for cell wall homeostasis and allows penicillin insult to the cell wall to be bypassed. Interestingly, the highly conserved serine threonine-kinase of *S. pneumoniae* is thus involved in the processes underlying three key features of bacterial physiology and evolution: virulence in animals, development of competence for genetic transformation culminating in gene transfers (Echenique *et al.*, 2004), and susceptibility to penicillin (this work). This makes StkP a potentially promising target in *S. pneumoniae*.

CHAPTER X

General Discussion

The aim of the studies presented in this thesis was to gain insight into some aspects related to the antimicrobial resistance in pneumococci. At the end of each of Chapters II to IX, which correspond to published or submitted publications, a discussion was presented about the results enclosed in that particular Chapter. In this Chapter, redundancy will be avoided.

We begin by analysing the antimicrobial resistance rates of pneumococci isolated from patients with invasive disease in the Portuguese population during an eleven year period (Chapter II). Until 1994, little data was available concerning antimicrobial resistance rates of pneumococci isolated from invasive source. We reported an increasing trend of pneumococci isolates that were nonsusceptible to penicillin until 2000, mainly intermediate-resistant pneumococci. Interestingly, in the following years, alterations to this trend were observed, with a decrease of penicillin nonsusceptibility between 2001 and 2003, mainly in pneumococci isolates which were highly resistant to β-lactams. This decline was thought to be due to the introduction of the heptavalent conjugate vaccine in Portugal in 2001.

However, apparent contradictory results were observed: the penicillin nonsusceptibility increased significantly in 2004. Furthermore, the rates of strains nonsusceptible only to macrolides, as well as the rate of isolates coresistant to penicillin and erythromycin showed the same increasing trend between 2002 and 2004. Also, the rate of macrolide-nonsusceptible strains was shown to be related to multiresistance. Indeed, multiresistance increased from 7.9% in 2002 to 15.6% in 2004.

To shed some light on the impact of the introduction of the PCV-7, we evaluated, by means of a longitudinal study, the association between IPD, serotypes and antimicrobial resistance in the paediatric population between 1999 and 2004 (Chapter III). As observed in Chapter II, which considers both the adult and paedriatric populations, the number of *S. pneumoniae* isolates nonsusceptible to penicillin declined initially in the latter, and then doubled in 2004. The multiresistance and the nonsusceptibility to both macrolide and tetracycline did not decrease following the introduction of the vaccine in 2001. The results presented in Chapter III suggest that the direct and herd effect of routine PCV-7 vaccination led to significant changes in the predominant *S. pneumoniae* serotypes found in IPD, as well as among antimicrobial resistant pneumococci. Our work also shows that the number of cases of IPD was not significantly reduced after the introduction of the vaccine.

The incidence of IPD described in this work was similar to the rates reported in other European countries (Reinert, 2004; Motlova *et al.*, 2008). However, it was quite different from those described in the United States (Whitney *et al.*, 2003). Thus, studies of vaccine effectiveness based on the United States population may lead to an overestimation of the benefits of its introduction in Europe (Jefferson *et al.*, 2006).

The incidence of pneumococcal disease may vary somewhat from year to year, and the distribution of pneumococcal serotypes may change as different strains pass through a particular geographic area. Extensive reports also show the lack of ability of PCV-7 to provide protection to the so called related vaccine serotypes, mainly 19A. Consequently, the new PCV-13 vaccine includes both serotypes 6A and 19A in its formulation (World Health Organization, 2007b). The increasing nonvaccine serotypes observed in Chapter III (mainly serotypes 19A, 7F and 33F), suggest a relation with the replacement effect, which has also been reported worldwide (Bettinger *et al.*, 2007; Clarke *et al.*, 2006; Kyaw *et al.*, 2006; Lett *et al.*, 2007; Moore *et al.*, 2008; Munoz-Almagro *et al.*, 2008; Pelton *et al.*, 2007; Zemlickova *et al.*, 2006).

The emergence of the replacement of serotypes can also be related to replacement of disease and/or replacement of antimicrobial susceptibility. In the state of Utah, in the United States, an increase of necrotizing pneumonia was described as being associated with the increase of penicillin-nonsusceptible strains after the introduction of the vaccine (Bender *et al.*, 2008). Also in the United States, a replacement of vaccine serotypes by nonvaccine serotypes was associated with an increase in incidence of empyema and severe IPD (Byington et al., 2005). In a 10-year prospective study, which included all children with culture-proven IPD admitted to a children's center in the southern area of Barcelona, the authors showed that in children aged <2 years there was an increase in the rate of IPD from 32.4 episodes per 100,000 population to 51.3 episodes per 100,000 population (Munoz-Almagro *et al.*, 2008). In the period after the introduction of the vaccine, the authors observed an increased rate of pneumonia and/or empyema among children aged <5 years associated with the emergence of serotype 1. The increased rates of IPD in the Spanish study were related to an increase of nonsusceptible strains with nonvaccine serotypes. This increase was mainly dominated by multiresistant strains from serotype 19A (Munoz-Almagro *et al.*, 2008). The work presented in Chapter III also describes this serotype as the major contributor for the increase of antimicrobial resistance and multiresistance in Portugal.

Nevertheless, some other European countries, such as Greece, presented results which differ from those reported in Chapter III. In Central Greece, the PCV-7 vaccine became available in October 2004 and it was incorporated into the national immunization schedule in January 2006. Between February 2005 and May 2007, a decrease of the nasopharyngeal proportion of PCV-7 serotypes was observed in vaccinated and unvaccinated carrier children aged 13-76 months and the carriage of serotype 19A did not increase in this study. Among vaccinated carriers, the rate of highly penicillin-resistant isolates also decreased, whereas the proportion of penicillin-intermediate pneumococci increased only slightly (Grivea *et al.*, 2008). This study showed a serotype pattern which is quite different from that observed in Portugal and Spain.

In Belgium, where the PCV-7 was not used, the overall prevalence of paediatric serotypes decreased significantly, and an increase of serotypes not included in the vaccine was observed (Flamaing *et al.*, 2008). The authors suggest that this effect may be linked to chronological trends in serotypes not included in the PCV-7 and/or herd effects at the international level. Furthermore, penicillin resistance also decreased and this may be due to a shift towards susceptible serotypes and/or a decrease in antibiotic use in that country (Flamaing *et al.*, 2008). Hausdorff *et al.* (2000a) had already reported that serotype distribution varies with geographic localization, having direct consequences on the efficiency of the vaccine in decreasing the incidence of IPD; the geographic differences of serotype distribution had been attributed to different blood-culture rates and practices and to the probable underdiagnosed and under-reported mild IPD in western Europe when compared to the United States (Hausdorff *et al.*, 2001). However, this argument does not explain the differences observed in serotype distribution among asymptomatic carriers nor the differences in diversity of serotypes recovered from blood and CSF. It may be probable that the geographic differences of serotype distribution are related to several factors such as: (i) differences between the strains recovered from blood or CSF (different mechanism of transversal of the blood-brain barrier); (ii) differences in the rate of colonization; (iii) different genetic background of the host population; (iv) immigration and population movements.

Concerning the first factor that we suggest contributes to serotype geographic distribution, most authors stated that the classic pathway of pathogenicity of a pneumococcal infection is based on the evolution from asymptomatic nasopharyngeal carriage to septicaemia and then to meningitis (Ring *et al.*, 1998; Koedel *et al.*, 2002; Bogaert *et al.*, 2004a). However, van Ginkel *et al.* (2003), using an experimental model, described meningitis infection in the absence of bacteremia. They showed that this process of infection is consistent with pneumococci reaching the brain directly from the nasal cavity by retrograde axonal transport through the olfactory nerves. Another previously described pathway of infection is based on the evolution of otitis media to meningitis (Marra & Brigham, 2001). Further studies must be undertaken to analyse the possible “serotype permeability” of theses pathways.

Nasopharyngeal carriage of pneumococci is a key factor in the development of IPD and in the spread of resistant strains, which have an impact on serotype distribution, within the community. Also, replacement effect had been reported internationally and happened fast among nasopharyngeal carriers in Portugal (Bogaert *et al.*, 2004b; Pelton *et al.*, 2004; Frazão *et al.*, 2005). This can be due to some of the factors described previously, as well as to the higher rate of colonization with pneumococcus in children attending DCC in Portugal when compared to the rate observed in DCC in the United States (Bogaert *et al.*, 2004a). The high rate of colonization may imply a higher frequency of transmission and,

consequently, a larger number of circulating clones and serotypes, under the selection pressure of the vaccine (Sá-Leão *et al.*, 2008). As a consequence, the serotype coverage of the vaccine in Portugal, as presented in Chapter III (63.8%), is much lower than that in the United States (83%) (Whitney *et al.*, 2003). The high rates of colonization observed in Portugal might also provoke a broader and more regular natural immunization in the population, providing more protection against IPD and, consequently, lower IPD rates than those observed in the United States.

A third factor related to differences of serotype geographic distribution may be the genetic background of the host, which may also have an impact on the rate of IPD and on antimicrobial resistance in a given population. In the studies undertaken in the United States, the incidence of IPD was observed to be highly associated with the high proportion of black infants in the population, twice the incidence observed in white infants. However, after the introduction of the PCV-7, there was a significant decrease of IPD in black children (17.1 vs 5.3 cases per 100.000 live births), but not in white infants (9.6 vs 6.8 cases per 100.000 live births) (Poehling et al., 2006). As we previously stated, black infants in Portugal were also observed to have an increased risk of nasopharyngeal carriage of *S. pneumoniae* (Neto *et al.*, 2003), suggesting that the serotype distribution is not directly related to geographic differences, but to genetic differences in the populations. Furthermore, in Israel, serotype differences between the Bedouin population of southern Israel and the neighbouring Jewish population were observed (Dagan *et al.*, 2008). In the first population there was a large increase in serotype 19A, despite the absence of PCV-7 vaccination, due to the multiresistant clone ST276. Conversely, in the Jewish population, this clone has not been observed. In Chapter VI, we also noticed that the erythromycin-resistant clones found in Portugal are different from those found in the United States. These observations suggest that the genetic background of the population may be of major importance in the colonization specificity and, consequently, in the dissemination of some of the clones involved in antimicrobial resistance. Studying the association of the HLA (human leukocyte antigen system) genotype with circulating clones at an international level could be of major importance to clarify this issue.

Immigration and population movements are factors associated with serotype distribution which are also not frequently studied. Immigration may imply importation of foreign clones to a determined population, and it can modify the genetic background of the host population. The Portuguese census of 2001 revealed 480.158 inhabitants of other nationalities living in Portugal, representing approximately 5% of the resident population (Instituto Nacional de Estatística, 2007). The main countries contributing to this number are Brazil (85,124), Ukraine (74,033), and Portuguese-speaking African countries, such as Cape Verde (67,404), Angola (34,122) and Guiné Bissau (24,741). However, these numbers do

not take into account the immigrant descendants with Portuguese nationality. Serotype 19A, which is the most prevalent nonvaccine serotype, is also the fourth most prevalent serotype among penicillin-nonsusceptible strains in Brazil, decreasing the level of coverage of PCV-7 to penicillin-nonsusceptible pneumococci recovered from carriage isolates (34.5%) and invasive isolates (28.2%) in that country (Laval *et al.*, 2006). The studies we undertook on the genetic structure of macrolide-nonsusceptible strains in Portugal, which are presented in Chapters V and VI, have shown the emergence of several clones, one of which, ST143, had only been described previously in Poland. In Chapter VI, it is suggested that the emergence of erythromycin resistance was also due to the importation of a specific number of clones.

The efficiency of the vaccine in reducing the prevalence of strains which are nonsusceptible to antimicromial agents can also be limited by high exposure to antibiotics, as suggested previously by mathematical modelling (Temime *et al.*, 2005). To provide useful information about the main factors involved in the selection of penicillin- and erythromycin-nonsusceptible strains isolated from adults and children, we evaluated the role of antimicrobial agents and vaccine use in Chapter IV. The results show a slight decrease in the rates of penicillin-nonsusceptible pneumococci strains between the prevaccine and vaccine periods. The model we used indicates that the nonsusceptibility of isolates to penicillin, among children, correlated positively with the use of amoxicillin and azithromycin, and was negatively associated with the use of the pneumococcal conjugate vaccine and flucloxacillin. These antibiotics were the main β-lactams and macrolides used in Portugal, as found in Chapter IV and other studies (Goossens *et al.*, 2005), and a decreased use of the main β-lactam agents was evident in the vaccination period. This model also suggests that if the vaccine had not been introduced in Portugal, nonsusceptibility to penicillin would have been expected to achieve values greater than 55% in 2004; this means that the pneumococcal conjugate vaccine had potentially reduced the rate of penicillin nonsusceptibility by more than 15%. Surprisingly, it also suggests that the use of azithromycin is a limiting factor to the efficiency of the pneumococcal conjugate vaccine. Indeed, if the pneumococcal conjugate vaccine had been used but not azithromycin, the nonsusceptibility to penicillin was predicted to be reduced to 17% in 2004; a similar reduction was experienced by Whitney *et al.* (2003) after the introduction of the anti-pneumococcal conjugate vaccine in the United States. The model used for erythromycin resistance also allowed us to observe that the introduction of the conjugate vaccine in Portugal did not significantly decrease the incidence of erythromycin-nonsusceptible strains, due to an increased use of azithromycin, and it did not reduce penicillin-nonsusceptibility because of coresistance to macrolides.

The use of macrolides has been reported to be a more effective selector of strains nonsusceptible to macrolides and β-lactams than the use of aminopenicillins (Arason *et al.*, 1996; Baquero *et al.*, 2002). Furthermore, the use of azithromycin has been linked to other

resistances in pneumococci, as described in Chapters IV and V and in other studies (Barkai *et al.*, 2005). We also observed that the increasing use of azithromycin caused the expansion of macrolide resistance in part due to the spread of several clones in Portugal (Chapters V and VI). The analysis of the genetic structure of erythromycin-nonsusceptible isolates in Chapter VI shows that the emergence of this resistance, beginning in 1998, was mainly related to the clonal expansion and genetic diversification of pre-existing clones, and to importation of nonsusceptible international clones in association with the recent increase of coresistance to penicillin and macrolides.

Like previous studies undertaken worldwide, our results suggest that the vaccine covers the majority of penicillin- and erythromycin-nonsusceptible *S. pneumoniae* strains of our studied population. However, as pointed out before, resistance was also found in serotypes which are absent from the vaccine formulation. The use of a vaccine in a multi-strain environment, such as is the case of pneumococci, in addition to providing herd immunity, can act as a "serotype filter", which results in the emergence of nonvaccine clones; some of these clones lack any data regarding invasive potential or ability to acquire new genetic traits. Lipsitch *et al.* (2007) suggested that it is presently unlikely that strains covered by PCV-7 displaying drug resistance or high virulence ability can escape to nonvaccine serotypes. Nevertheless, Brueggemann *et al.* (2007) had already described the emergence of a novel "vaccine escape recombinant" pneumococcal strain. This strain expresses nonvaccine serotype 19A, but has a genotype associated with vaccine serotype 4. Furthermore, molecular analysis revealed a 39 kb recombinational fragment, which included the capsular locus, flanking regions, and two adjacent PBPs encoding genes, resulting in penicillin nonsusceptibility and a capsular switch with a single genetic event. Since 2003, 37 such vaccine escape strains had been detected in the United States, some of which had evolved further. Indeed, vaccine escape by genetic recombination at the capsular locus has the potential to reduce PCV-7 effectiveness in the long term (Brueggemann *et al.*, 2007). Interestingly, the potential of a strain to escape vaccine can also be related to the fact that the degree of competence of the various serotypes differs significantly, just as reported by Hsieh *et al.* (2006) for serotype 6B, which had the highest competence, among serotypes 14, 19F, 9V, 23F, 3 and 18C. In this case, isolates belonging to serotype 6B had greater genetic diversity than isolates belonging to serotype 3, which had higher genetic clustering (Hsieh *et al.*, 2006).

We also identified a greater diversity of serotypes among erythromycin-nonsusceptible pneumococci than among penicillin-nonsusceptible strains (Chapters II, III and IV), which cannot be explained by drug use alone, but may also be due to the fact that macrolide resistance is encoded by several genetic elements, which can be chromosomally inserted in different clonal lineages. In fact, in addition to the selective pressure caused by

vaccine and antibiotic consumption, the greater diversity of macrolide-nonsusceptible strains could enhance horizontal transfer of resistance-encoding genes between nonvaccine serotypes and/or capsular switching in nonsusceptible vaccine serotypes. On the other hand, it was also found that variations in susceptibility to penicillin in *S. pneumoniae* are mainly clonal, as described in Chapter VII.

We observed that the genetic divergence found in *pbp* genes from intermediate-resistant pneumococci strains was higher than that from highly resistant strains (Chapter VIII). The high clonality of these highly resistant strains, the low diversity of their *pbp* genes and the decrease of the frequency of these strains (Chapter II) suggest that high resistance may have a high fitness cost, making it more difficult for the dissemination of new alleles. Indeed, alteration of the 3 main PBPs is needed to provide a high level of resistance (Laible & Hakenbeck, 1987). These alleles can, however, be spread among a certain number of unrelated clones, as found in Chapter VII in highly resistant strains from serotypes 23F and 9V.

In Chapter IX we observed that the efficiency of transformation of penicillin-resistant mutants with mutations in all three PBP encoding genes was quite low when compared to the observed for mutants with only mutated *penA* and *pbpX* genes. The high clonallity observed among penicillin-resistant strains may be related to the loss of competence, resulting from the acquisition of altered PBPs. An association between genetic competence and antimicrobial susceptibility had already been suggested by Hsieh *et al.* (2006). However, they reported penicillin-susceptibille isolates belonging to serotypes 3 and 18C which were significantly less competent than isolates belonging to serotypes 6B, 14, 19F, 9V, and 23F, which were frequently resistant to penicillin (Hsieh *et al.*, 2006). This implies a need for further studies to better understand the responsibility of serotype and/or specific bacterial genetic backgrounds on the competence development and spread of antimicrobial resistance among the major circulating clones. In fact, the introduction of PCV-7 has emphasized the importance of studies regarding molecular surveillance of virulent clones with higher competence and their relation with penicillin susceptibility in the near future.

Resistance to penicillin in streptococci is much more than the alteration of the major *pbp* genes. In Chapter IX, we also provide some experimental evidence about the role of StkP in susceptibility to penicillin. Giefing *et al.* has suggested that the *stkP* mutation causes a reduction of MIC values of penicillin in susceptible mutants, but their work did not include nonsusceptible mutants. In our work we observed the same effect in both susceptible and nonsusceptible mutants, but more notorious among the latter. The reduction of penicillin MICs caused by the *stkP* mutation is not related to the *pbp* genes, suggesting that StkP acts upstream of the PBPs. Possibly, most of the genes involved in the functioning of enzymes that modify the stem-peptide ligands of cell wall precursors, such as *murMN*, *ciaHR*, *adr*, and

stkP, have an impact in penicillin susceptibility, just as we, and others, have suggested (Crisóstomo *et al.*, 2006; Filipe & Tomasz, 2000; Giefing *et al.*, 2008; Hakenbeck *et al.*, 1999). The study of these targets is quite important for the development of new therapeutic opportunities, as suggested recently regarding StkP (Giefing *et al.*, 2008).

Certainly, the control of antimicrobial resistance in pneumococci is of major relevance in terms of public health. This can only be achieved by helping the physicians to adequately prescribe antibiotics without taking risks that may lead to therapeutic failure, by controlling antimicrobial prescription, and by means of integrated networking for monitorization of antimicrobial resistance. It is theoretically possible that the observed trends of antibiotic resistance in Portugal may be reversed by applying these measures, contributing to the control of invasive pneumococcal disease (Levin *et al.*, 1999; Wang & Lipsitch, 2006). Mathematical modelling has shown that new antimicrobial agents and their criterious use can minimize resistance and treatment failure (Wang & Lipsitch, 2006). If antimicrobial use were to be kept below a level that limits the population-wide selection of highly resistant strains, then it might be possible to combine low failure rate of the most severe cases with continued suppression of resistance (Lipsitch, 2001).

We believe that the findings presented in this Thesis provide new insights into the understanding of the genetic diversity and epidemiology of antimicrobial resistance in pneumococci, one of the most extraordinarily versatile human pathogens. Many questions remain unanswered and many others will emerge as future work on this field is pursued. In sequence with the work provided in this Thesis, future prospects will be concerned with providing answers to the several questions raised herein.

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