Streptococcus pneumoniae, resistance and disease in adults in the era of conjugate vaccines

Jordi Càmara Mas

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PhD Thesis

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Programa de Doctorado en Medicina e Investigación Traslacional
Facultad de Medicina-Campus de Bellvitge
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Streptococcus pneumoniae, resistance and disease in adults in the era of conjugate vaccines

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La **Dra. Carmen Ardanuy Tisaire**, facultativa especialista del Servicio de Microbiología y Parasitología del Hospital Universitario de Bellvitge, hace constatar que la tesis titulada:

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que presenta el licenciado **Jordi Càmara Mas** ha sido realizada bajo su dirección en el Servicio de Microbiología y Parasitología del Hospital Universitario de Bellvitge, en el marco del Programa de Doctorado en Medicina e Investigación Traslacional de la Universidad de Barcelona. La considera finalizada y autoriza su presentación para que sea defendida ante el tribunal que corresponda.

L'Hospitalet de Llobregat, a 19 de mayo de 2021.

Dra. Carmen Ardanuy Tisaire

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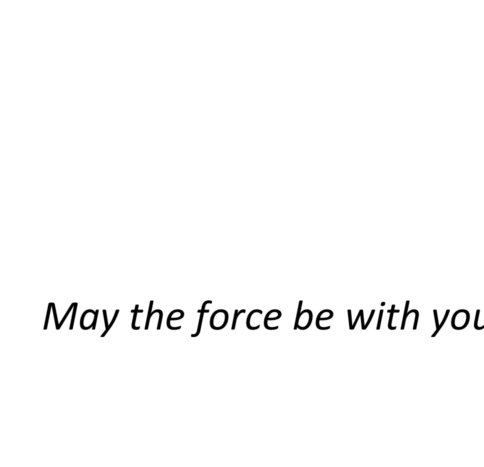
  - **PI18/00339:** Impacto del genoma accesorio de *Streptococcus pneumoniae* en la resistencia y la patogenia de la enfermedad.



May the force be with you

LIST OF ABBREVIATIONS **3**

PUBLICATIONS **7**

ABSTRACT **11**

RESUMEN **17**

1. INTRODUCTION **23**

1.1. *Streptococcus pneumoniae*, historical background 25

1.2. Taxonomy and genetic evolution 28

1.3. Microbiological characteristics and laboratory identification 31

1.4. Pneumococcal capsule 34

1.5. Antimicrobial resistance 37

1.6. Pneumococcal typing 45

1.7. Burden of pneumococcal diseases 48

1.8. Pneumococcal vaccines 51

2. HYPOTHESES **55**

3. OBJECTIVES **59**

4. METHODOLOGY AND RESULTS **63**

4.1. Decrease of invasive pneumococcal disease (IPD) in adults after introduction of pneumococcal 13-valent conjugate vaccine in Spain 65

4.2. A historical perspective of MDR invasive pneumococcal disease in Spanish adults 87

4.3. Evolution of the β-lactam-resistant *Streptococcus pneumoniae* PMEN3 clone over a 30 year period in Barcelona, Spain 103

5. DISCUSSION **137**

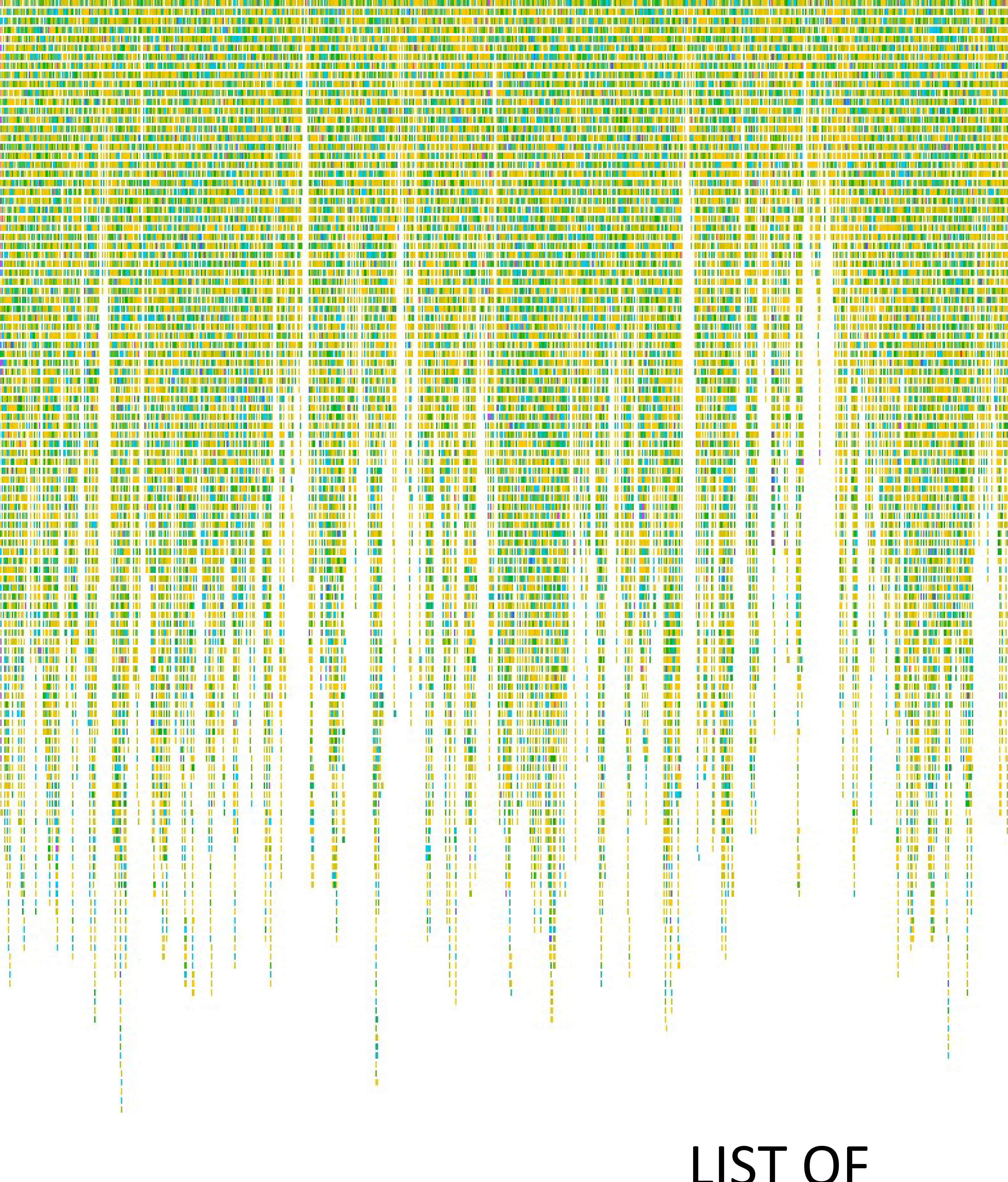
6. CONCLUSIONS **153**

7. REFERENCES **157**

8. ANNEXES **179**

8.1. Pneumococcal disease and conjugate vaccines (Editorial comment) 181

8.2. Enfermedad neumocócica en el adulto.
Serotipos, clones y resistencia antibiótica (Spanish, book chapter) 183



LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CAP	Community-acquired pneumonia
cART	Combination antiretroviral therapy
COPD	Chronic Obstructive Pulmonary Disease
CLSI	Clinical and Laboratory Standards Institute
DALYs	Disability-Adjusted Life-Years
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GPS	Global Pneumococcal Sequencing Project
IPD	Invasive pneumococcal disease
IRR	Incidence risk ratio
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MDR	Multidrug-resistance
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
NAATs	Nucleic acid amplification tests
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PCVs	Pneumococcal conjugate vaccines
PCV7	7-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PFGE	Pulsed-field gel electrophoresis
PMEN	Pneumococcal molecular epidemiology network
PPV23	23-valent pneumococcal polysaccharide vaccine
RR	Risk ratio
SNP	Single nucleotide polymorphism
ST	Sequence type
WGS	Whole genome sequencing
WHO	World Health Organization

PUBLICATIONS

Research publications in international peer-reviewed journals:

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Collaborations related to this thesis:

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González-Díaz A, **Càmara J**, Ercibengoa M, Cercenado E, Larrosa N, Quesada MD, Fontanals D, Cubero M, Marimón JM, Yuste J, Ardanuy C. **Emerging non-13-valent pneumococcal conjugate vaccine (PCV13) serotypes causing adult invasive pneumococcal disease in the late-PCV13 period in Spain.** *Clin Microbiol Infect* 2020 Jun;26(6):753-759. Impact Factor (2017) is: 7.72 D1.

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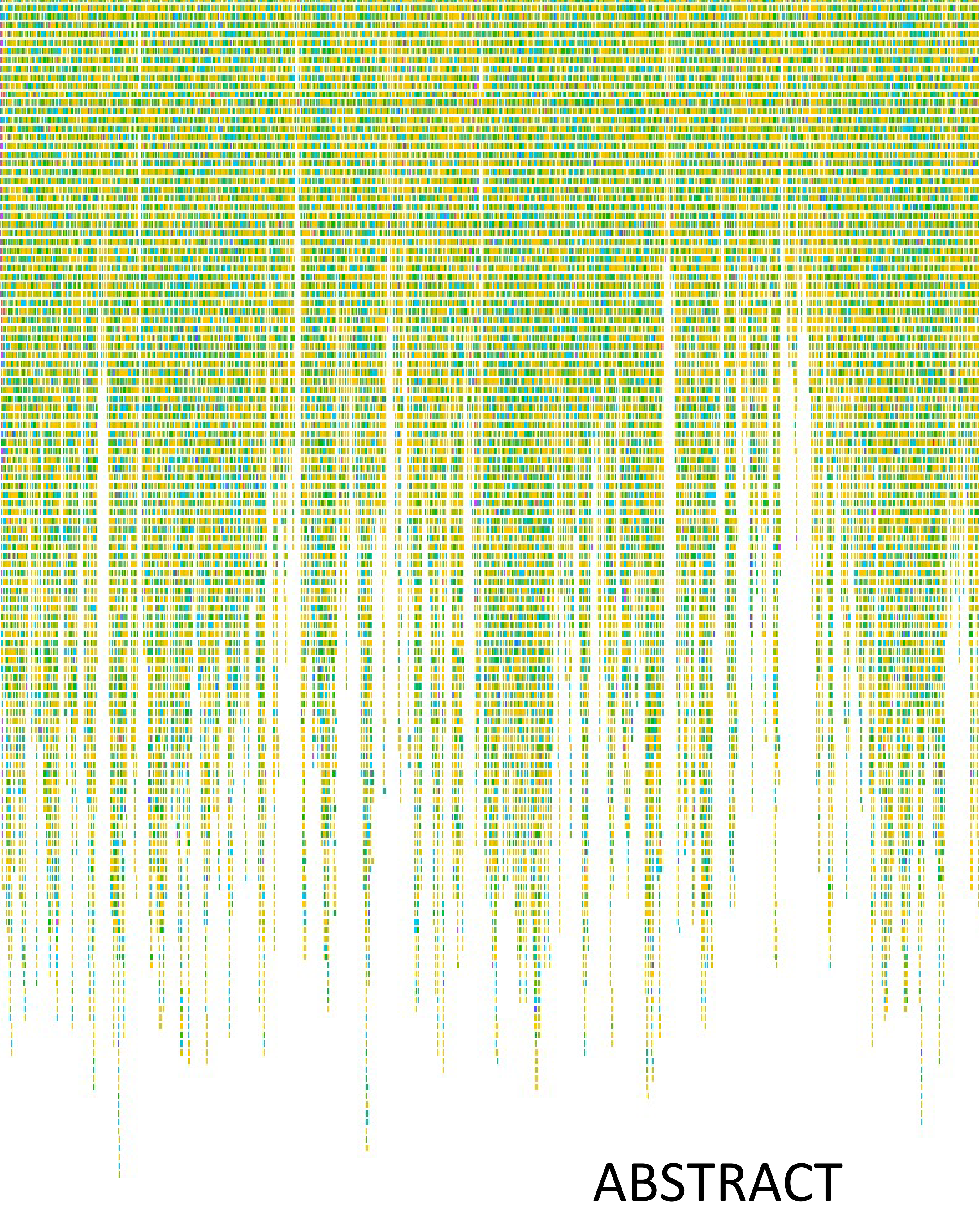


ABSTRACT

Streptococcus pneumoniae is an important human pathogen, being one of the main etiological agents of serious infectious diseases such as pneumonia or meningitis and other less serious like otitis media. *S. pneumoniae* adheres to the respiratory epithelium being part of the nasopharyngeal microbiome. This nasopharyngeal colonization is more frequent in young children (colonization percentages of around 27-65%) than in adults with a frequency of colonization around 10%. In recent years, the introduction of the pneumococcal conjugate vaccines (PCVs) for children has changed the epidemiology of pneumococcal diseases. Since vaccination by PCVs prevents the carrier status of those strains expressing serotypes included in the vaccine, their transmission is reduced and a beneficial group protection ("herd protection") occurs in the non-vaccinated population. Three PCVs have been marketed in Spain: PCV7 (introduced in 2001), PCV10 (in 2009) and PCV13 (which replaced PCV7 in 2010). All of them were administered voluntarily until 2015, when PCV13 became part of the routine vaccination schedule for children and were subsidized by the government.

The main objective of this thesis is to study the impact of the introduction of PCVs in children on invasive pneumococcal disease in adults (IPD). The works included in this thesis focus on describing the effects of the introduction of PCVs in Spain on the epidemiology of IPD in adults. Specifically, this thesis has studied the changes in the incidence of IPD, in the rates of antibiotic resistance, in the clones responsible for IPD and in the clinical characteristics of patients in the era of PCVs.

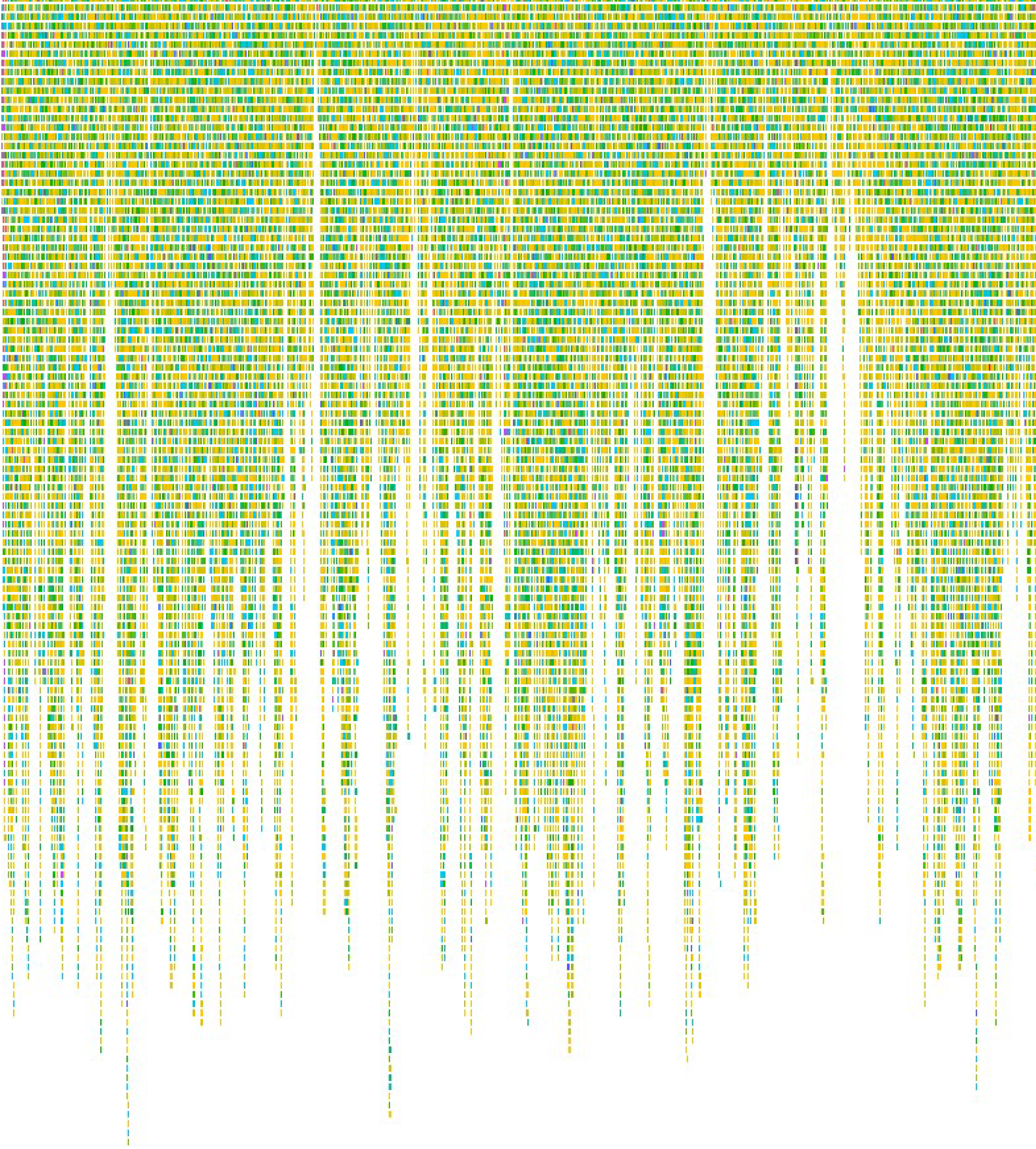
The first part of this thesis shows how the introduction of PCV13 in Spain caused an early decrease in the incidence of adult IPD. The global incidence of IPD decreased by 33.9% from 2008-2009 to 2012-2013. These results were related to a decrease in the incidence of those serotypes included in PCV7 (decrease of 52.7%) and of those additional PCV13 (decrease of 55.0%), while disease caused by non-vaccine serotypes remained stable. The reduction in IPD rates was greater in the Community of Madrid, a community in which PCVs (PCV7 and later PCV13) were included in the routine childhood vaccination schedule in 2009. Regarding the additional PCV13 serotypes all decreased except serotype 3 which remained stable, possibly indicating a lower effectiveness of PCV13 in preventing IPD due to this serotype. On the other

hand, a change was observed in two non-vaccine serotypes: first, a decrease in the incidence of serotype 8, probably due to the disappearance of an outbreak in young adults in the Madrid region in previous years. And, second, an increase in serotype 6C that may indicate a minor effect of the described cross-reactivity with serotype 6A (included in PCV13). Regarding antibiotic susceptibility, a non-statistically significant increase in the frequency of isolates non-susceptible to penicillin (MIC ≥ 0.12 mg/L, 22.7% vs 26.8%, p = 0.065), cefotaxime (MIC > 0.5 mg/L, 10.1% vs 12.5%, p = 0.14) and MDR isolates (13.1% vs 16.2%, p = 0.08) was observed. In spite of that, the incidence of IPD caused by these resistant isolates remained stable over time.

Because we detected an increase in the rates of non-susceptibility to penicillin and cefotaxime after PCV13, in the second work we aimed to study the local impact of PCVs on the incidence of IPD due to MDR and penicillin non-susceptible strains. The period analysed (1994-2018) includes two key moments for the study of the evolution of IPD in Spain: the introduction of PCV7 and PCV13 vaccines in 2001 and 2010, respectively. In this study, we analysed changes in the incidence of IPD, antimicrobial susceptibility, evolution of clones responsible for multidrug-resistance, and clinical characteristics of patients. Our results demonstrated that PCVs have been beneficial in reducing the incidence of resistant strains (risk ratio [RR] comparing the period 1994-2000 with the period 2016-2018: 0.70 [95% CI 0.53–0.93]) while the effect on the incidence of IPD due to susceptible strains was limited (RR 0.96 [95% CI 0.80–1.16]). Therefore, while the overall incidence of IPD in the adult population has shown a slight non-statistically significant decrease (RR 0.87 [95% CI 0.74–1.02], the rates of resistance to most antimicrobials have been reduced. With respect to genotypes associated with antimicrobial resistance, about 50% of IPD due to resistant strains in the 2016-2018 period was caused by two clones: Spain^{9V}-ST156 (serotype 11A) and Denmark^{14}-ST230 (serotype 24F). We also evaluated the putative coverage of two vaccines that are under development (PCV15 and PCV20), concluding that almost half of the current resistant strains express serotypes not included in these vaccines, so their efficacy with respect to antibiotic resistance may be limited. Finally, we also analysed the clinical characteristics of the patients with resistant IPD, showing that, despite not being an independent factor associated with mortality, resistant strains

appear in patients with worse prognosis (advanced age, more comorbidities, nosocomial acquisition and previous antibiotic therapy) which leads to higher mortality.

The last part of this thesis focuses on the study by WGS of the genetic evolution of the clone Spain^{9V}-ST156 (PMEN3) over a period of 30 years (1987–2016). In Spain, this clone has been the most frequent among β-lactam resistant strains causing IPD and has classically been associated with serotypes 9V and 14. In our study, this clone was responsible for more than 12% of all cases of IPD in adults during the 90s. The introduction of PCV7 in 2001 (which included serotypes 9V and 14) caused a progressive decrease in the incidence of the PMEN3 clone. Despite this, the appearance of strains expressing serotype 11A (not included in PCV13) in recent years has allowed this clone to persist as a cause of IPD. The WGS study allowed us to identify 6 lineages within this clone with different prevalence over time. All lineages were derived from the original ST156^{9V} except the last one (ST6521^{11A}) which originated from the ST838^{9V} lineage through a capsular switching event and the acquisition of capsular type 11A. Using WGS, it was also possible to verify the existence of two different serotype 14 lineages (ST156) that independently emerged during the 1990s and that could not be easily detectable with the usual typing techniques (PFGE or MLST). Furthermore, the study showed evidence of recombination events in three chromosomal regions: the capsular operon (capsular switching) and the adjacent regions containing *pbp2x* and *pbp1a* genes, the *murM* region, and the region containing *pbp2b–ddl* genes. Then, all the chromosomal regions that had undergone changes were associated with capsular switching or β-lactam resistance, which evidenced the enormous impact of the introduction of PCVs and broad-spectrum β-lactams on the evolution of this clone. Finally, despite the genetic changes detected, no differences in the clinical characteristics of patients were found, which highlighted the importance of the PMEN3 genetic background. In this sense, the majority of strains harboured prophages with genes coding for PblB proteins, which have recently been associated to platelet aggregation and increased mortality in IPD.



RESUMEN

Streptococcus pneumoniae es uno de los patógenos humanos más importantes, siendo uno de los principales agentes etiológicos de enfermedades graves como neumonía o meningitis y otras menos graves como puede ser la otitis media. *S. pneumoniae* se adhiere al epitelio respiratorio y forma parte de la microbiota nasofaríngea. Esta colonización nasofaríngea es más frecuente en los niños pequeños (porcentajes de colonización de alrededor 27-65%) mientras que en adultos la frecuencia de colonización suele ser inferior, alrededor del 10%. En los últimos años la introducción de las vacunas conjugadas neumocócicas (VCNs) en niños ha cambiado la epidemiología de las enfermedades neumocócicas. Debido a que la vacunación mediante VCNs previene el estado de portador de aquellas cepas con serotipos incluidos en la vacuna, la transmisión de estos serotipos se ve reducida y se produce un efecto beneficioso de protección de grupo ("herd protection") en la población no vacunada. En España se han comercializado tres PCVs: PCV7 (introducida en 2001), PCV10 (en 2009) y PCV13 (que sustituyó a la PCV7 en 2010). Todas ellas se administraron de forma voluntaria sin estar incluidas en el calendario de vacunación hasta el año 2015, en el que la PCV13 pasó a formar parte de las vacunas incluidas en el calendario de vacunación sistemática gratuita en niños.

Esta tesis tiene como objetivo principal estudiar el efecto de la introducción de las PCVs en niños en la enfermedad neumocócica invasiva en adultos (ENI). Los trabajos incluidos en esta tesis se centran en describir los efectos de la introducción de las PCVs en España sobre la epidemiología de la ENI en adultos. En concreto, en esta tesis se han estudiado los cambios en la incidencia de ENI, en las tasas de resistencia antibiótica, en los clones responsables de ENI y en las características clínicas de los pacientes en la era de las PCVs.

La primera parte de esta tesis muestra como la introducción de la PCV13 en España en el año 2010 causó un rápido descenso en las tasas de incidencia de ENI en adultos. La incidencia global de ENI se redujo un 33.9% desde el periodo 2008-2009 hasta el 2012-2013. Estos resultados se relacionaron con un descenso en la incidencia de aquellos serotipos incluidos en la PCV7 (descenso del 52.7%) y de los adicionales PCV13 (descenso del 55.0%), mientras que los serotipos no vacunales se mantuvieron estables. La reducción en las tasas de ENI fue mayor en la Comunidad de Madrid,

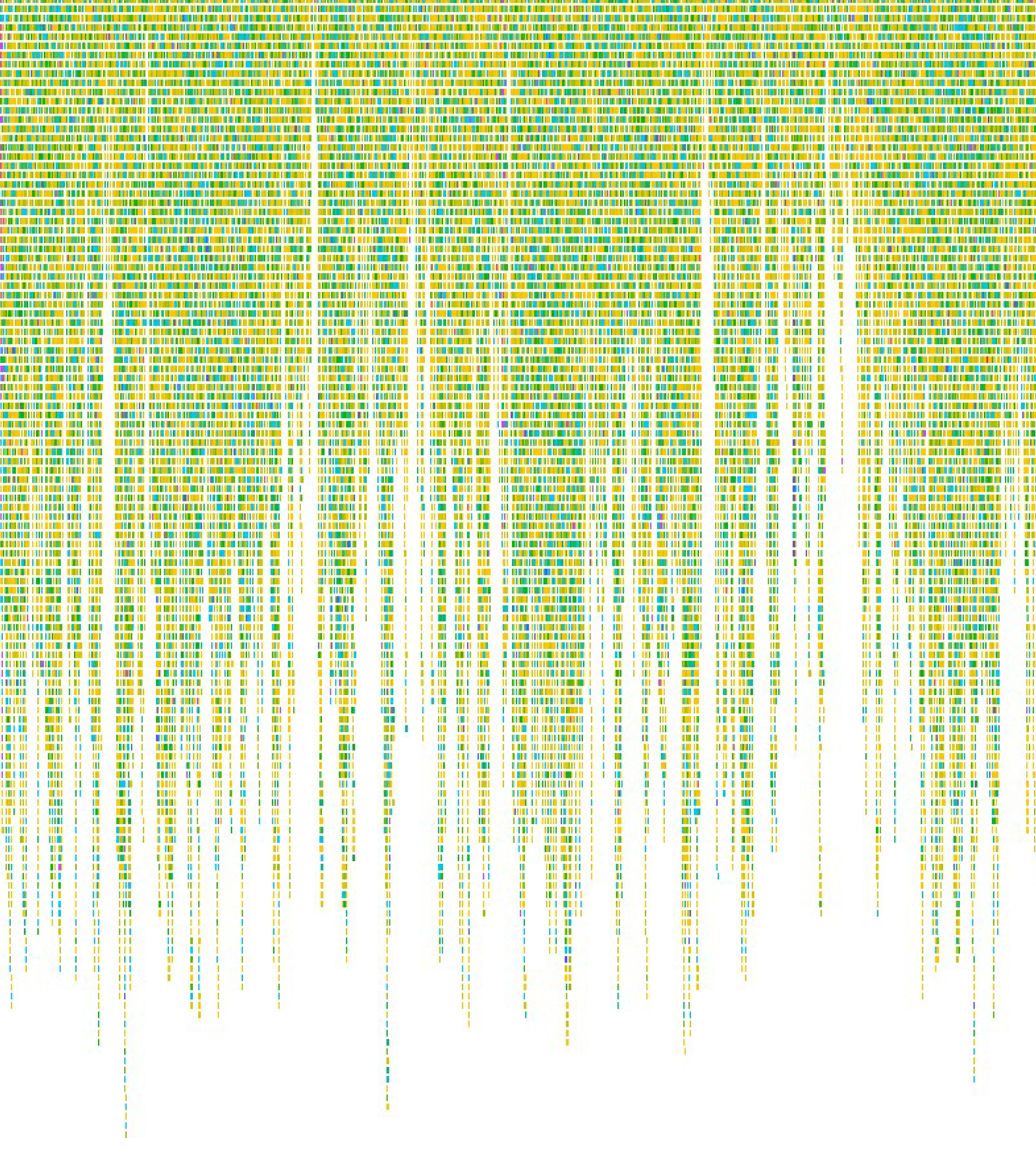
comunidad en las que las PCVs (PCV7 y posteriormente PCV13) se incluyeron en la vacunación sistemática infantil en 2009. Respecto a los serotipos adicionales PCV13, todos descendieron excepto el serotipo 3 que se mantuvo estable lo que puede indicar una menor efectividad de la PCV13 en prevenir la ENI debida a este serotipo. Por otro lado, se observó un cambio en dos serotipos no vacunales: un descenso en el serotipo 8, probablemente debido a la desaparición de un brote en adultos jóvenes en la región de Madrid en los años previos, y un incremento en el serotipo 6C que puede indicar una ausencia de la descrita reactividad cruzada con el serotipo 6A (incluido en la PCV13). Respecto a los datos de sensibilidad antibiótica, se observó un incremento no estadísticamente significativo en la frecuencia de aislados no sensibles a penicilina (MIC ≥ 0.12 mg/L, 22.7% vs 26.8%, $p = 0.065$), cefotaxima (MIC > 0.5 mg/L, 10.1% vs 12.5%, $p = 0.14$) y en la de aislados MDR (13.1% vs 16.2%, $p = 0.08$). A pesar de ello, la incidencia debido a estos aislados resistentes se mantuvo estable.

Como detectamos un aumento de las tasas de no susceptibilidad a la penicilina y a la cefotaxima tras la PCV13, en el segundo trabajo nos propusimos estudiar el impacto de las PCV sobre la incidencia de la ENI debido a las cepas no sensibles a penicilina y MDR. El período analizado (1994-2018) incluye dos momentos clave para el estudio de la evolución de la ENI como son la introducción de las vacunas PCV7 y PCV13 en los años 2001 y 2010, respectivamente. En el estudio analizamos los cambios en la incidencia de la enfermedad neumocócica invasiva, la evolución de los clones responsables de la multirresistencia y las características clínicas de los pacientes durante los últimos 25 años. Los resultados mostraron que las PCVs han tenido un efecto beneficioso sobretodo en la disminución de la incidencia de cepas resistentes (riesgo relativo [RR] comparando el periodo 1994-2000 con el periodo 2016-2018: 0.70 [95%IC 0.53–0.93]) mientras que su impacto sobre la incidencia de ENI debida a cepas sensibles ha sido limitado (RR 0.96 [95%IC 0.80–1.16]). Estos datos muestran que mientras la incidencia global de ENI en la población adulta ha mostrado un leve descenso, no estadísticamente significativo (RR 0.87 [95%IC 0.74–1.02], las tasas de resistencia a la mayoría de antimicrobianos han descendido. Con respecto a los genotipos resistentes, alrededor del 50% de la ENI actual debida a cepas resistentes está causada por dos clones: Spain^{9V}-ST156 (serotipo 11A) y Denmark^{14}-ST230

(serotipo 24F). En el trabajo también evaluamos las tasas de cobertura de dos vacunas que actualmente están en desarrollo (PCV15 y PCV20) concluyendo que casi la mitad de las cepas multiresistentes actuales expresan serotipos no incluidos en estas vacunas por lo que su eficacia con respecto a la resistencia antibiótica puede ser limitada. Por último, también analizamos las características clínicas de los pacientes con ENI concluyendo que, a pesar de no ser un factor independiente asociado a mortalidad, las cepas resistentes aparecen sobretodo en pacientes con peor pronóstico (edad avanzada, más comorbilidades, adquisición nosocomial y antibioterapia previa) lo que conlleva una mayor mortalidad.

La última parte de esta tesis se centra en el estudio mediante WGS de la evolución genética del clon Spain^{9V}-ST156 (PMEN3) durante un periodo de 30 años (1987–2016). En España, este clon ha sido el más frecuente entre las cepas resistentes a β-lactámicos causantes de ENI y clásicamente se ha asociado a los serotipos 9V y 14. En nuestro estudio este clon ha sido responsable de más del 12% de todos los casos de ENI en adultos durante los años 90. La introducción de la PCV7 en el año 2001 (incluyendo serotipos 9V y 14) hizo que la incidencia del clon PMEN3 progresivamente disminuyera pero la aparición de cepas expresando el serotipo 11A (no incluido en la PCV13) durante los últimos años ha permitido a este clon mantenerse como una causa importante de ENI. El estudio mediante WGS permitió identificar 6 linajes dentro de este clon con prevalencias distintas a lo largo del tiempo. Todos los linajes derivaron del original ST156^{9V} excepto el último (ST6521^{11A}) que se originó a partir del linaje ST838^{9V} mediante un evento de intercambio capsular con la adquisición del tipo capsular 11A. Mediante WGS también se pudo constatar la existencia de dos linajes distintos del serotipo 14 con el mismo ST156 que emergieron de forma independiente durante los años noventa y que no podían ser fácilmente detectables con las técnicas de tipado habituales. Además, el estudio mostró evidencia de eventos de recombinación en tres regiones cromosómicas: el operón capsular (intercambio capsular) y las regiones adyacentes que contienen los genes *pbp2x* and *pbp1a*, el gen *murM* y la región con los genes *pbp2b–ddl*. De esta forma, todas las regiones cromosómicas que habían sufrido cambios a lo largo del tiempo estaban relacionadas con eventos de intercambio capsular o de resistencia a β-lactámicos, lo que

evidenciaba el impacto por un lado de las vacunas conjugadas y por el otro de la presión antibiótica en la evolución de este clon. Por último, a pesar de los cambios genéticos detectados, no se encontraron diferencias en las características clínicas de los pacientes a lo largo del tiempo lo que mostraba la importancia de su *background* genético. En este sentido, la mayoría de cepas presentaban profagos con la presencia de genes que codifican para proteínas PblB, recientemente relacionadas con agregación plaquetaria y aumento de mortalidad en ENI.



INTRODUCTION

1.1. *Streptococcus pneumoniae*, historical background

Streptococcus pneumoniae, also known as pneumococcus, has been one of the most studied bacteria throughout the history. One of the first reports aiming to investigate the bacterial origin of pneumonia comes from Klebs in 1875[1]. This author observed many different bacteria in the bronchial secretion of a patient with pneumonia ("*monadinen"*) and postulated that it could be the cause of the disease ("monas pulmonale"). However, it was not until 1880 when *S. pneumoniae* was first isolated from human sources by George M. Sternberg (1838-1915) and Louis J. Pasteur (1822-1895) in an independent way.[2,3] Both microbiologists injected human saliva into rabbits, which caused fatal septicaemia in the animals, and recovered streptococci from their blood. Besides being the first isolation of *S. pneumoniae*, it was also the demonstration of its pathogenic potential.

This microorganism has received several different names throughout history. For instance, Pasteur ("Microbe septicemique du salive") and Sternberg ("Micrococcus pasteuri") called it differently in their respective works. It seems that the first reference to "Pneumococcus" comes from Fraenkel in 1886.[4] Later, in an attempt to standardize the classification of bacteria into families and genera, "the committee of the society of American bacteriologists on characterization and classification of bacterial types" was set up by the Society of American Bacteriologists. The final report of this committee was released in 1920 and classified *S. pneumoniae* into the "Coccaceae" family (Family IV) and the "Diplococcus" Genus (Genus 2), a term adopted from Weichselbaum (1886).[5] In this beautiful report the pneumococcus is described as "Cells usually in pairs of somewhat elongated cells, often capsulated, sometimes in chains." Therefore, by 1920 the term *Diplococcus pneumoniae* was conventionally established and this nomenclature persisted throughout the twentieth century. Its current name, *Streptococcus pneumoniae*, was finally adopted in 1974.

Streptococcus pneumoniae has been involved in some of the most important events in the research and development of the clinical microbiology. For example, this pathogen had a key role during the development of the Gram Stain (Hans Christian Gram 1853-1938). The observation that the lung tissue of patients who died of

pneumonia contained cocci retaining the gentian violet stain ("Gram-positive") allowed their differentiation from other bacteria that did not retain it ("Gram negative"). Probably, what Dr. Gram was observing in lung preparations were two distinct bacterial aetiologies of pneumonia: *Streptococcus pneumoniae* on one hand and *Klebsiella pneumoniae (Friedländer's pneumonia)* on the other. The Gram Stain resulted in the most important and widely used bacterial classification of the clinical microbiology history.

Another cornerstone of the clinical microbiology that involved *S. pneumoniae* was the study and discovery of the bacterial transformation by Frederick Griffith (1879–1941).[6] This author reported in 1928 what is considered the first demonstration of the transfer of genetic material between bacteria. After preliminary work that showed the existence of two different pneumococcal morphologies (morphology smooth or S –virulent- and morphology rough or R –avirulent-), the author was able to convert an attenuated strain into a virulent one through its inoculation in mice together with a heated (and therefore killed) culture of a virulent strain. These observations allowed F. Griffith to hypothesise that "the R form of Type II, when inoculated together with a heated suspension of Type I, uses the antigen of the latter strain and an *S. pneumococcus* of Type I makes its appearance". The precise mechanism of bacterial transformation was elucidated twenty years later by Oswald Avery, Colin MacLeod, and Maclyn McCarty. These authors managed to purify the key components involved in the transformation process and were able to conclude that the transforming potential of a dead bacterium is transmitted in its DNA content instead of the protein content.[7]

S. pneumoniae has also played an important role in the study and development of vaccines. After some works demonstrating the existence of humoral immunity after *S. pneumoniae* infection in animals and the appearance of anti-pneumococcal sera (serum injected with heat-killed pneumococci) for human use, Almroth E. Wright conducted a randomized clinical trial among gold miners in South-Africa (1911-1912).[8] The trial included 5963 cases inoculated with a whole cell vaccine and 5671 controls that resulted in a significant lower incidence of pneumonia (2.6% vs 3.5%) and mortality (0.83% vs 1.53%) for the vaccinated group. Nevertheless, the effect was only

evident during the first-two months after inoculation. During the following years other experiences were carried out with anti-pneumococcal whole cell vaccines with unclear clinical efficacy. Other important steps in the development of pneumococcal vaccines were the discovery of the existence of soluble substances of pneumococcus by Dochez in 1917[9] and the following demonstration of immunogenicity of the pneumococcal capsular antigens by Avery in 1931.[10] The introduction of effective antibiotics to treat streptococcal infections such as sulfonamides or penicillin during the 1930s and 1940s led to the suspension of most vaccination programs. However, the prevention of pneumococcal disease through vaccination took a new impulse in the 1970s thanks to the work of R. Austrian who conducted several clinical trials trying to demonstrate the efficacy of pneumococcal vaccination.[11] All of these research programs were the foundation for the successful introduction and distribution of the pneumococcal polysaccharide vaccines (PPVs) during the 1970s and 1980s, especially the 23-valent polysaccharide vaccine (PPV23, Pneumovax®). Later, the conjugation of the capsular polysaccharide with a protein carrier improved the efficacy of pneumococcal vaccines.[12] Therefore, the introduction of the pneumococcal conjugate vaccines in the early 2000s (PCV7, Prevenar®) opened a new stage in the fight against pneumococcal infections.

1.2. Taxonomy and genetic evolution

S. pneumoniae is currently classified into the phylum *Firmicutes*, order *Lactobacillales*, family *Streptococcaceae* and genus *Streptococcus*. However, the taxonomy of the genus *Streptococcus* has changed over the years in parallel with the introduction of new available methodologies. An important and widely used standardization methodology has been the serological classification of β-haemolytic streptococci published by Lancefield in 1933.[13] This methodology consisted of the use of a precipitin reaction to classify β-haemolytic streptococci in five groups (Lancefield groups A, B, C, D and E). Some years later, Sherman released a new classification based on the Lancefield antigens but also adding other phenotypic and biochemical characteristics.[14] This classification separated the species of streptococci in four groups: Pyogenic, Viridans, Lactic and Enterococcus. This scheme was updated during the 1980s by the application of new sequencing and nucleic acid hybridization techniques that resulted in the separation of the genus *Streptococcus* into three new genera: *Enterococcus*, *Lactococcus* and *Streptococcus*.[15] Some years later, the introduction of the 16S rRNA sequencing[16] allowed to establish more accurately the phylogenetic relationship of streptococci. This technique allowed differentiating six clusters into the *Streptococcus* genus: anginosus, bovis, mitis, mutans, pyogenic and salivarius groups.[17] This classification has been widely used and has remained with few changes to date.[18]

The recent development of the whole genome sequencing technologies has made it possible to study in detail the genetic relationship of streptococci which also means *S. pneumoniae* and its close relatives. Within the genus *Streptococcus*, *S. pneumoniae* can be classified into de Mitis group (Figure 1). The Mitis group includes species that are usually members of the human commensal microbiota such as *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus pseudopneumoniae.* Interestingly, the recent WGS analysis has been able to identify that some species previously classified into the Mitis group, such as *S. gordonii* or *S. sanguinis*, must be considered part of an independent group.[19] Despite its low frequency among human infections, some of these Mitis group species can play a relevant role in severe infections like endocarditis.

Among the species that make up the Mitis group, *S. mitis* and *S. pseudopneumoniae* are the genetic closest genetic relatives of *S. pneumoniae.*[19] Recent studies on the evolution of the Mitis group members suggest that both *S. pneumoniae* and *S. mitis* have a common ancestor.[20] From the original ancestor these two species have evolved in two opposite ways: while *S. mitis* has evolved towards reducing its genome, emptying it of virulence genes, *S. pneumoniae* has evolved enhancing its genome plasticity which has resulted in high antigenic diversity of surface structures.[21] *S. pneumoniae* has been able to achieve this through the continuous import of genes, mostly from other pneumococci or other species of the Mitis group. In fact, it seems that this process of gene transfer is mostly unidirectional (from *S. mitis* to *S. pneumoniae*). As a result of this process of genes gain or loss, *S. mitis* has achieved a commensal status of the human flora while *S. pneumoniae* has acquired a high capacity to adapt to environmental changes and maintain its pathogenic potential. A recent study on the genetic adaptation of *S. pneumoniae* to the environment has shown that most part of the pneumococcus genome has experienced recombination events but some regions should be considered recombination hotspots.[22] These regions are related, as expected, to ICEs or transposable elements but also to genes codifying for major surface exposed and immunogenic structures such as the capsular locus (*cps*) or *pspA*, *pspC* and *psrP* genes. These findings highlight the importance of the selection driven by the human immune system but also the successful adaptation of *S. pneumoniae* to a changing environment.

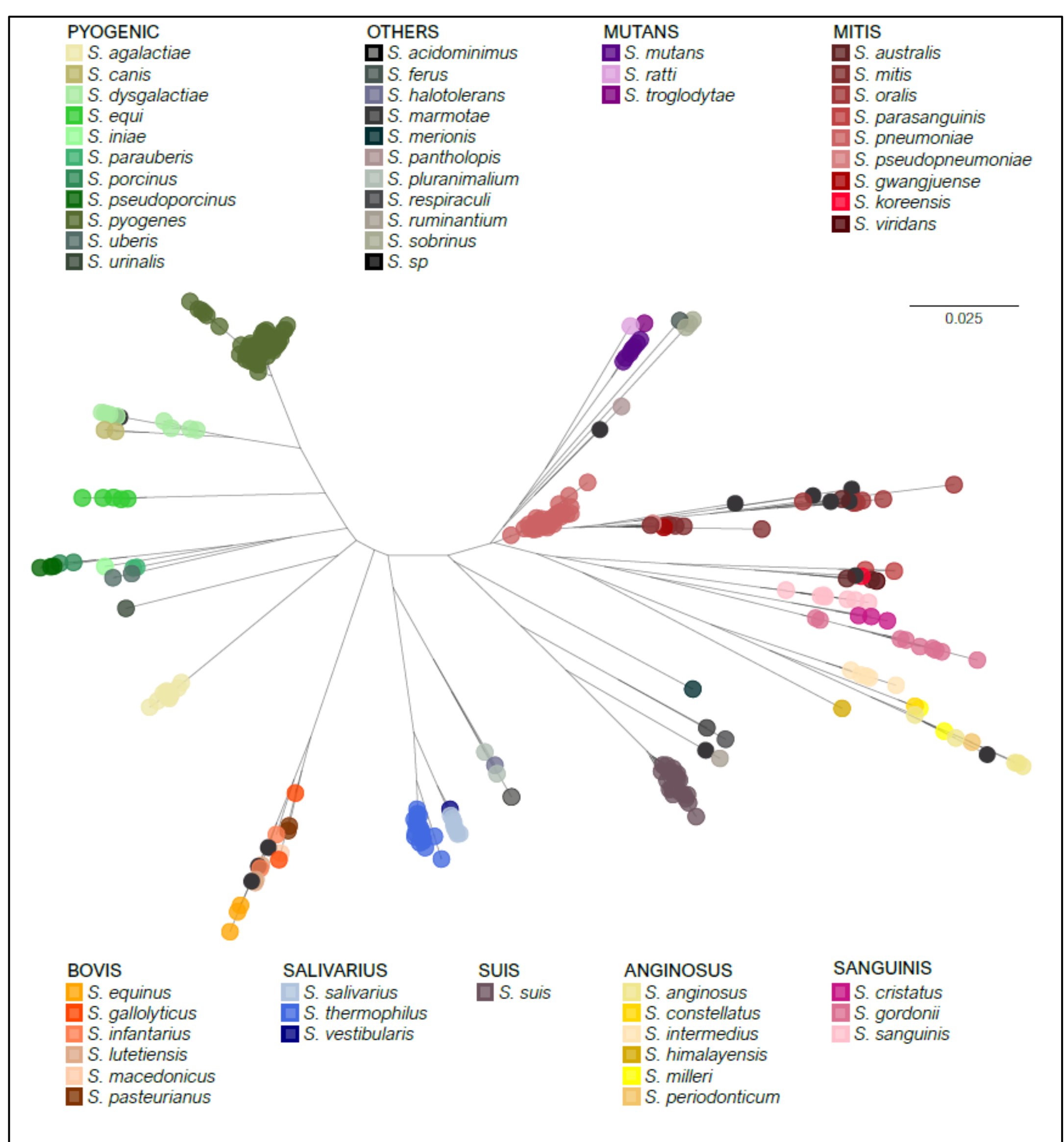


Figure 1. Phylogenetic SNP tree of 715 streptococcal genomes deposited in the National Center for Biotechnology Information webpage (NCBI, https://www.ncbi.nlm.nih.gov/). The search was performed using "*Streptococcus*" and "complete genome" terms in the Assembly database (genomes downloaded on 7h July 2020). The study of differences in SNPs was performed with CSI Phylogeny 1.4 (standard settings) using *Streptococcus pneumoniae* R6 as reference.

1.3. Microbiological characteristics and laboratory identification

Streptococcus pneumoniae is a bacterium found in the upper respiratory tract of humans, predominantly in the nasopharynx of children. Pneumococci are gram-positive rods of around 1-2 µm that can usually be visualized as diplococci or forming short-length chains on microscopy examination of clinical samples (Figure 2).

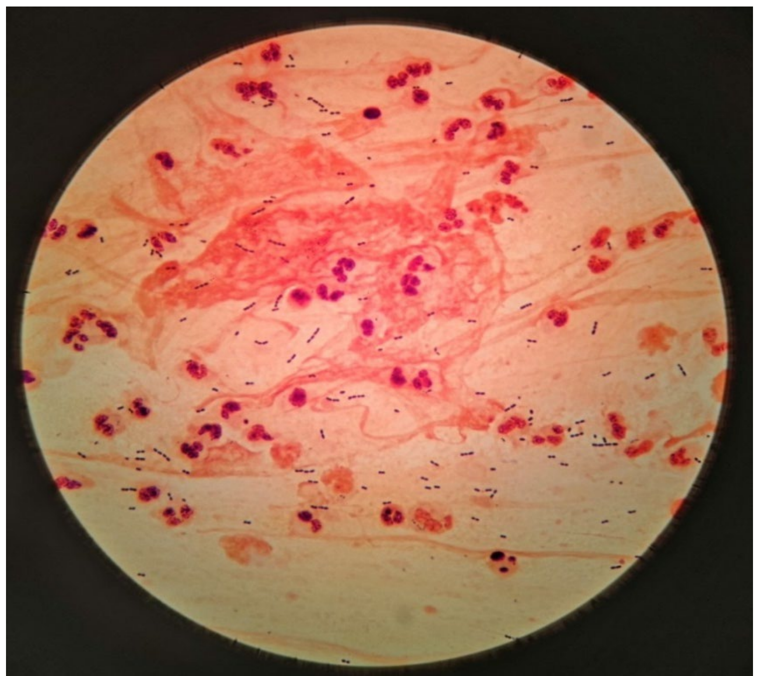


Figure 2. Gram stain of sputum (x1000) of a patient with community-acquired pneumonia. Note the high count of neutrophils and the absence of squamous epithelial cells. Pneumococci are primarily seen as short-length chains and a light area surrounding the bacteria indicates the presence of the capsule.

It is a facultative anaerobic microorganism, immobile and does not form spores. It shows α-haemolysis when incubated under aerobic conditions on blood agar plates due to the oxidation of haemoglobin to methaemoglobin by the production of hydrogen peroxide.[23] On the contrary, under anaerobic conditions it shows β-haemolysis because of the haemolytic activity of the pneumococcal pneumolysin.[24]

The diagnosis of pneumococcal infections is generally based on culture of the microorganism in the laboratory. Besides the observation of the colony morphology on

a blood agar plate, pneumococci are generally identified in clinical microbiology laboratories by the optochin susceptibility or bile solubility tests. Pneumococci are susceptible to optochin and are lysed in the presence of sodium deoxycholate (2% for the bile solubility test performed in tub). These two characteristics are useful to differentiate *S. pneumoniae* from other α-haemolytic streptococci, particularly other mitis group streptococci. In spite of that, both tests have their particular limitations: while the bile solubility test is difficult to interpret and mostly based on subjective interpretation, *S. pneumoniae* isolates resistant to optochin also exist.[25] The introduction of MALDI-TOF in clinical laboratories has improved the identification of many bacterial species. Bacterial identification is currently fast (less than 1 hour) and highly reliable in most cases. However, MALDI-TOF is currently unable to accurately differentiate between *S. pneumoniae* and other streptococci of the mitis group.[26] Despite having made efforts in recent years trying to fix this problem, current reliable identification of *S. pneumoniae* by MALDI-TOF require spectra analysis which is not automated and is therefore difficult to apply to the clinical routine.[27]

Currently, there are also culture-independent methodologies that are routinely used in clinical laboratories. For example, the urinary antigen detection is a tool that helps to elucidate the pneumococcal aetiology of pneumonia. It is a rapid, simple, and useful test but has several limitations. For instance, it is not useful in children or adults with COPD due to the high rates of colonization and the potential high rate of false-positives. Furthermore, its sensitivity depends on the population of circulating pneumococcal serotypes and a reduction in sensitivity has been detected after the introduction of PCVs.[28] Other culture-independent frequently used methodologies are NAATs which are mostly based on PCR. These techniques are very sensitive and add extra value to the culture, especially when patients are on antimicrobial therapy. In a recent work comparing culture and PCR for CAP diagnosis, the percentage of patients in which a bacterial pathogen was detected was 39,3% and 81,1% for culture and PCR, respectively.[29] Moreover, when analysing only those patients with prior antimicrobial therapy the performance of the culture dropped to 32,1% while that of the PCR remained acceptable at 77,6%. In spite of that, the high sensitivity of the NAATs has its own drawbacks. It doesn't imply viability of the detected pathogen, nor does it allow

antimicrobial susceptibility testing and it is difficult to discriminate between infection and colonization in non-sterile samples. To try to solve this specificity problem, the use of a quantitative PCR with a cut-off value has been proposed. However, it is difficult to obtain a cut-off value with good sensitivity and specificity and it varies with the target used.[30] Therefore, the use of NAATs for the diagnosis of pneumococcal infections on non-sterile samples is not yet standardized. For all these reasons, the identification of *S. pneumoniae* on a routinely basis in clinical laboratories is still a challenge that will not be solved until the implementation of an accurate and easy to perform methodology.

1.4. Pneumococcal capsule

The polysaccharide capsule of *S. pneumoniae* is its most important virulence factor. Although there are non-encapsulated strains that have been associated with superficial infections (for instance, non-encapsulated strains have been classically associated with conjunctivitis),[31] these isolates are exceptionally infrequent as a cause of invasive infections. The pneumococcal capsule prevents entrapment in the nasal mucus and also has a protective effect against phagocytosis.[32] The capsule forms a shield that reduces the accessibility of surface antigens to antibodies, and also reduces the total amount of complement deposited in the bacterial surface.[33]

The capsular polysaccharide is a polymer constituted by repeating units of two or more monosaccharides. It is a highly diverse structure and 100 different capsular types have been described so far.[34] The majority of pneumococcal capsular types are synthesised by the Wzy-dependent pathway with the exception of types 3 and 37, synthesised by the synthase pathway.[35–37] Genes that regulate the capsular synthesis are located in the *cps* locus, which is a structure of around 10-30 kb between *dexB* and *aliA* genes. In the Wzy-dependent pathway, a polysaccharide is synthesized by the sequential addition of sugars to a membrane-associated lipid carrier on the inner side of the cell membrane. The repeat unit is flipped across the cytoplasmic membrane by Wzx and then the Wzy polymerase links the repeat units to form the capsular structure. Finally, the Wzd/Wze complex translocates the capsular polysaccharides to the cell surface and are covalently attached to peptidoglycan.[35] The structure of the polysaccharides synthesized through the synthase-dependent pathway is simpler than those of the Wzy-dependent pathway, with only one or two repeating sugars. In this pathway, initiation, polymerization and transport of the formed chain are carried out by a single enzyme (encoded by *cps3S* and *tts* genes in capsular types 3 and 37, respectively).[38] Interestingly, type 3 capsule produced by the synthase-dependent pathway is not covalently linked to the cell wall.[39]

The presence of the capsule plays a fundamental role in pneumococcal virulence, but its composition also determines the invasive potential of pneumococcal isolates. For example, some serotypes such as 1, 5, 7F, or 8 show a high propensity to

cause invasive disease regardless of their genetic background.[40] Serotypes have been reported to have different propensities to cause certain pneumococcal diseases and different fatality rates.[41,42] In addition, the distinctive genetic structure of the capsular operon, containing non-homologous genes (specific for each serotype) flanked by highly homologous regions (mostly the regulatory region which includes genes wzg, wzh, wzd, and wze)[35] favours its genetic recombination (Figure 3). This phenomenon, called “capsular switching” allows the diversification of the pneumococcal population.[43] In an environment of selective pressure on some serotypes, as is the case with the introduction of pneumococcal conjugate vaccines, the capsular switching allows the survival of those clones expressing serotypes targeted by the PCVs. Furthermore, as the capsular operon is flanked by *pbp1a* and *pbp2x* genes, which encode PBPs whose amino acid changes are associated with β-lactam resistance, the capsular switching can also be accompanied by changes in susceptibility to β-lactams.[44]

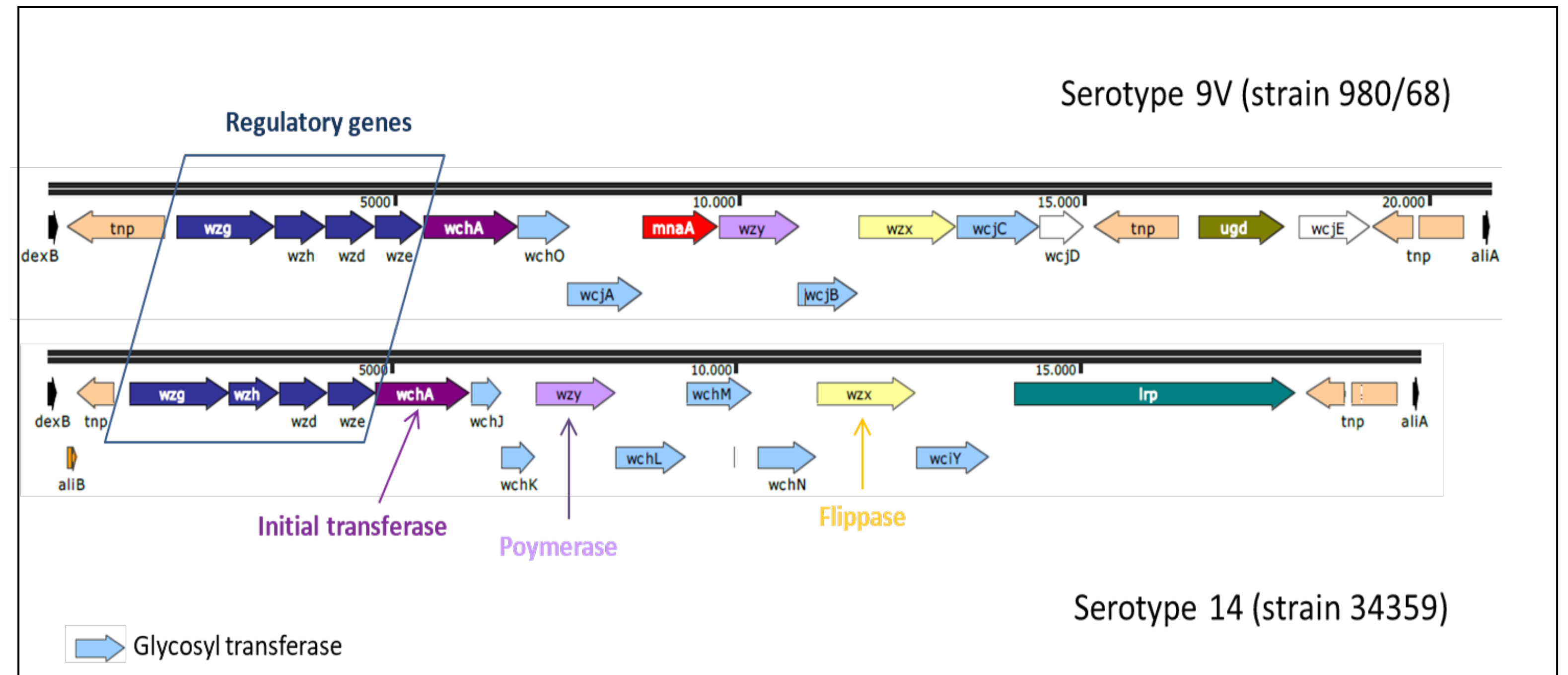


Figure 3. Genetic structure of the capsular operon of pneumococcal capsular types 9V and 14. Note the similar organization of the two serotypes, particularly among the regulatory region. Adapted from Bentley SD et al. Genetic analysis of the caps ular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet. 2006.

1.5. Antimicrobial resistance

Antibiotics are among the most commonly prescribed drugs in human and animal medicine. Antibiotic resistance occurs when bacteria acquire some characteristic that reduces the effectiveness of antibiotics. Although expansion of antibiotic resistance is closely associated to antibiotic use, antimicrobial resistance genes have existed since ancient times.[45] In fact, the use of antibiotics acts as a selection pressure that favours the spread of resistant bacteria. Antibiotic treatment can either promote the spread of pre-existing resistant bacteria or select resistance acquired during the course of treatment (Figure 4). The latter phenomenon typically occurs by acquiring specific amino acid changes in proteins that are targeted by antimicrobials. It is of particular importance to be aware of previous antimicrobials treatments when choosing empirical therapy for treatment of pneumococcal infections. For instance, quinolones treatment failures are well documented in patients who have previously received these antibiotics.[46]

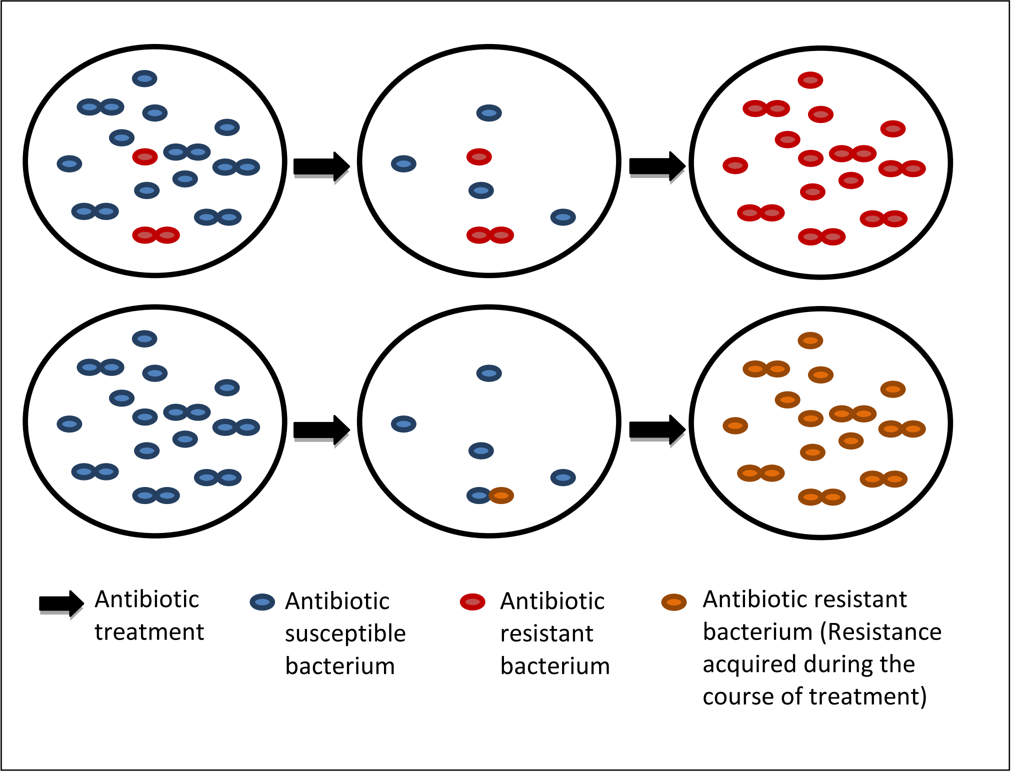


Figure 4. Selective pressure of antibiotic use on a population of bacteria. The figure shows the effect of antimicrobial use over a population of bacteria with a pre-existent resistant strain (top, red colour) and the selection of a bacterium that acquired a mutation (bottom, orange colour) during the course of treatment. In both cases the selective pressure of antimicrobials lead to the depletion of the susceptible strains and to the expansion of the resistant ones.

Antimicrobial resistance is a serious health threat. It has been estimated that infections due to antibiotic-resistant bacteria were responsible for 33.110 (28.480–38.430) attributable deaths and 874.541 (768.837–989.068) DALYs in the European Union in 2015.[47] For penicillin-resistant *S. pneumoniae*, estimates are 2.817 (2.552-3.104) infections and 171 (159-184) attributable deaths in the same year. It has to be noted that current infections caused by penicillin-resistant *S. pneumoniae* isolates have several effective treatments available. In spite of that, penicillin-resistant *S. pneumoniae* has been included in the WHO priority list of bacteria for which developing new antimicrobials is a priority because of its high mortality and high community burden (among other factors).[48]

S. pneumoniae is a pathogen that has been classically susceptible to a wide spectrum of antibiotics. For instance, amoxicillin alone -or combined in patients with comorbidities- is currently the recommended therapy for outpatients with CAP.[49] In spite of that, the description of penicillin and multidrug-resistant pneumococci in South-Africa in 1977[50,51] supposed a threat to the treatment of severe pneumococcal diseases, especially meningitis. The definition of resistance for a particular antibiotic depends on microbiological characteristics, pharmacokinetic data and clinical outcome of the infection. Regarding pneumococcal infections, it is necessary to take into account that for the same administered dose of penicillin, the levels reached in the lung will be 100 times greater than in the brain. Therefore, resistance breakpoints will depend on the site of infection. These considerations were incorporated into the CLSI breakpoints after 2008 which established different penicillin breakpoints depending on the route of administration and the site of infection.[52] Currently, both CLSI and EUCAST guidelines have different breakpoints for β-lactam antibiotics depending on the route of administration and the site of infection (Table 1). The most relevant difference between the two guides lies in the existence of the intermediate category in the CLSI guide that is absent in EUCAST which is important when considering non-meningeal infections.

Table 1. Clinical breakpoints of penicillin, amoxicillin and cefotaxime according to CLSI and EUCAST guidelines.

Antimicrobial	Guideline	Source of infection	Route of administration	S	I	R
Penicillin	CLSI	Meningitis	Intravenous	≤0.06	-	≥0.12
		Nonmeningitis	Intravenous	≤2	4	≥8
		Nonmeningitis	Oral	≤0.06	0.12-1	≥2
	EUCAST	Meningitis	-	≤0.06	-	>0.06
		Other than meningitis	-	≤0.06	-	>2
Amoxicillin	CLSI	Meningitis	-	-	-	-
		Nonmeningitis	-	≤2	4	≥8
	EUCAST	Meningitis	Intravenous	≤0.5		>0.5
		Other than meningitis	Intravenous	-	-	-
		Other than meningitis	Oral	≤0.5		>1
Cefotaxime	CLSI	Meningitis	Intravenous	≤0.5	1	≥2
		Nonmeningitis	Intravenous	≤1	2	≥4
	EUCAST	Meningitis	Intravenous	≤0.5	-	>0.5
		Other than meningitis	Intravenous	≤0.5	-	>2

Data was obtained from the documents "CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020." and "The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0, 2021 (http://www.eucast.org). Antimicrobial MIC values are expressed as mg/L. Categories: S: Susceptible, I: Intermediate, R: Resistant.

Among the groups of antibiotics that are currently available to treat pneumococcal infections, β-lactams, macrolides, and quinolones account for the majority of treatments administered either empirically or targeted. Therefore, resistance to these three antimicrobial groups is of great interest for the treatment of pneumococcal infections.

Resistance to β-lactams

In Spain, the prevalence of *S. pneumoniae* non-susceptible to penicillin has been higher than in most European countries, reaching peaks of 40% in the 1980s and 1990s.[53] After the introduction of PCV7 for children in 2001, there was a decrease in the rates of non-susceptibility to penicillin, although they are still around 30% in our environment (Figure 5). Resistance to β–lactam antibiotics is linked to antimicrobial consumption and, due to the fact that children are the main pneumococcal reservoir and they are also more frequently exposed to antimicrobials, selection of β–lactam resistance in *S. pneumoniae* occurs mostly in this population.[54]

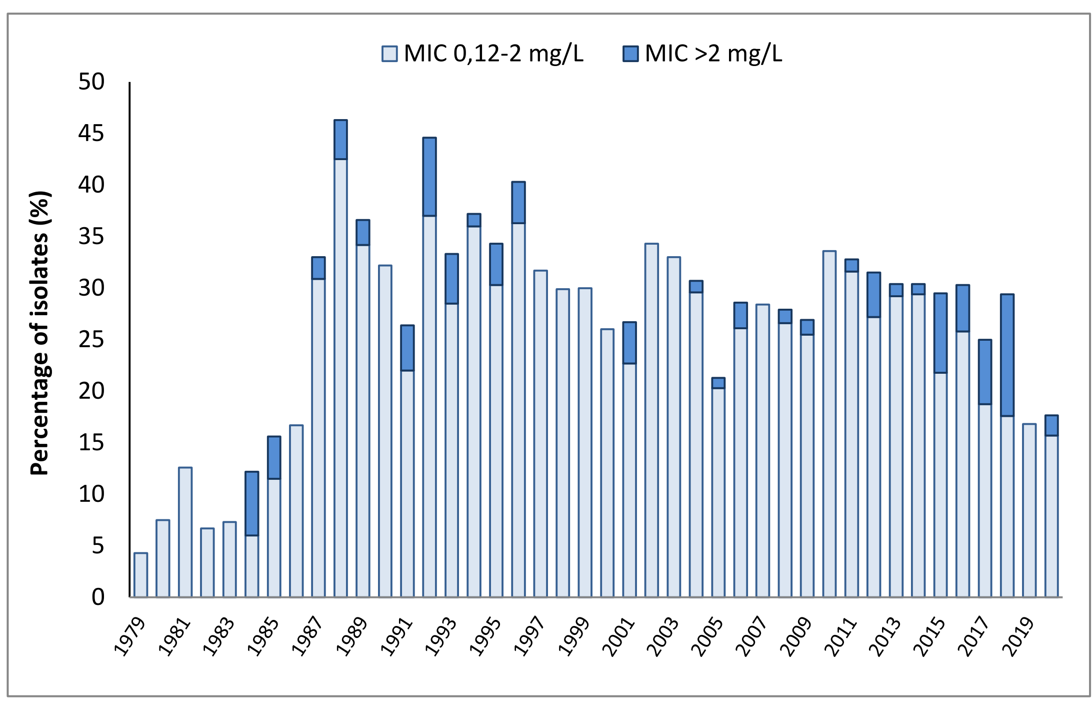


Figure 5. Evolution of penicillin non-susceptibility among *S. pneumoniae* invasive isolates collected in Hospital Universitari de Bellvitge (1979-2020). Percentage refers to the total population of invasive pneumococci.

β–lactam antibiotics inhibit the final steps of peptidoglycan synthesis by binding to penicillin-binding proteins (PBPs). Amino acid changes in PBPs resulting in reduced affinity for penicillin and other β–lactams are the main mechanisms of resistance. Three of the *S. pneumoniae* PBPs (PBP1a, PBP2b and PBP2x) have been mostly associated to β–lactam resistance (Figure 6). Mutations in genes that codify

PBPs are suggested to be acquired through horizontal gene transfer from commensal Mitis group streptococci, which could act as resistance reservoirs.[55] This fact is responsible for the characteristic mosaic structure of PBPs, with some divergent blocks acquired from commensal streptococci. In this sense, amino acid changes in PBP2b or PBP2x lead to low levels of penicillin resistance, and are the first step for high level β-lactam resistance, which can be achieved by additional substitutions in PBP1a.[56] Also, it has been described that changes in PBP2b have a fitness cost and this is compensated by the acquisition of PBP2x and PBP1a changes which could explain the frequent occurrence of resistant pneumococci with all three PBPs altered.[57] In general, amino acid changes in PBPs tend to have a greater impact on penicillin MICs than, for instance, cefotaxime or carbapenem MICs, which are compounds that can remain active.[58] In this regard, resistance to third-generation cephalosporines is associated to amino acid changes in PBP1a or PBP2x proteins.[59,60]

<u>Resistance to fluoroquinolones</u>

Fluoroquinolones are antibiotics that inhibit DNA synthesis by noncovalent binding to gyrase and topoisomerase IV enzymes, resulting in the formation of DNA-drug-enzyme complexes. These two enzymes are essential for DNA replication and bacterial survival. The formed DNA-drug-enzyme complexes act as permanent chromosomal breaks that lead to cell death if bacterial DNA repair systems are overwhelmed.[61] Therefore, fluoroquinolones convert gyrase and topoisomerase IV into cellular toxins so these drugs are also termed “topoisomerase poisons”.[62] Fluoroquinolones also act by inhibiting the DNA ligation activity of gyrase and topoisomerase IV enzymes so they are also catalytic inhibitors.

Resistance to fluoroquinolones is mediated by the accumulation of point DNA mutations in the quinolone resistance-determining region (QRDR), mostly in *gyrA* (gyrase) and *parC or parE* (topoisomerase IV) genes. The effect of these mutations is accumulative so mutations in both genes are required to observe high-level resistance to fluoroquinolones. In pneumococci, amino acid substitutions leading to fluoroquinolones resistance are typically located in positions S79 or D83 for ParC, D435 for ParE and S81 or E85 for GyrA.[63] Isolates harbouring amino acid changes related to

resistance in both enzymes usually show levofloxacin MICs ≥ 8 mg/L whereas those isolates with only ParC changes show low-level resistance, usually with levofloxacin MICs between 1-4 mg/L.[64]

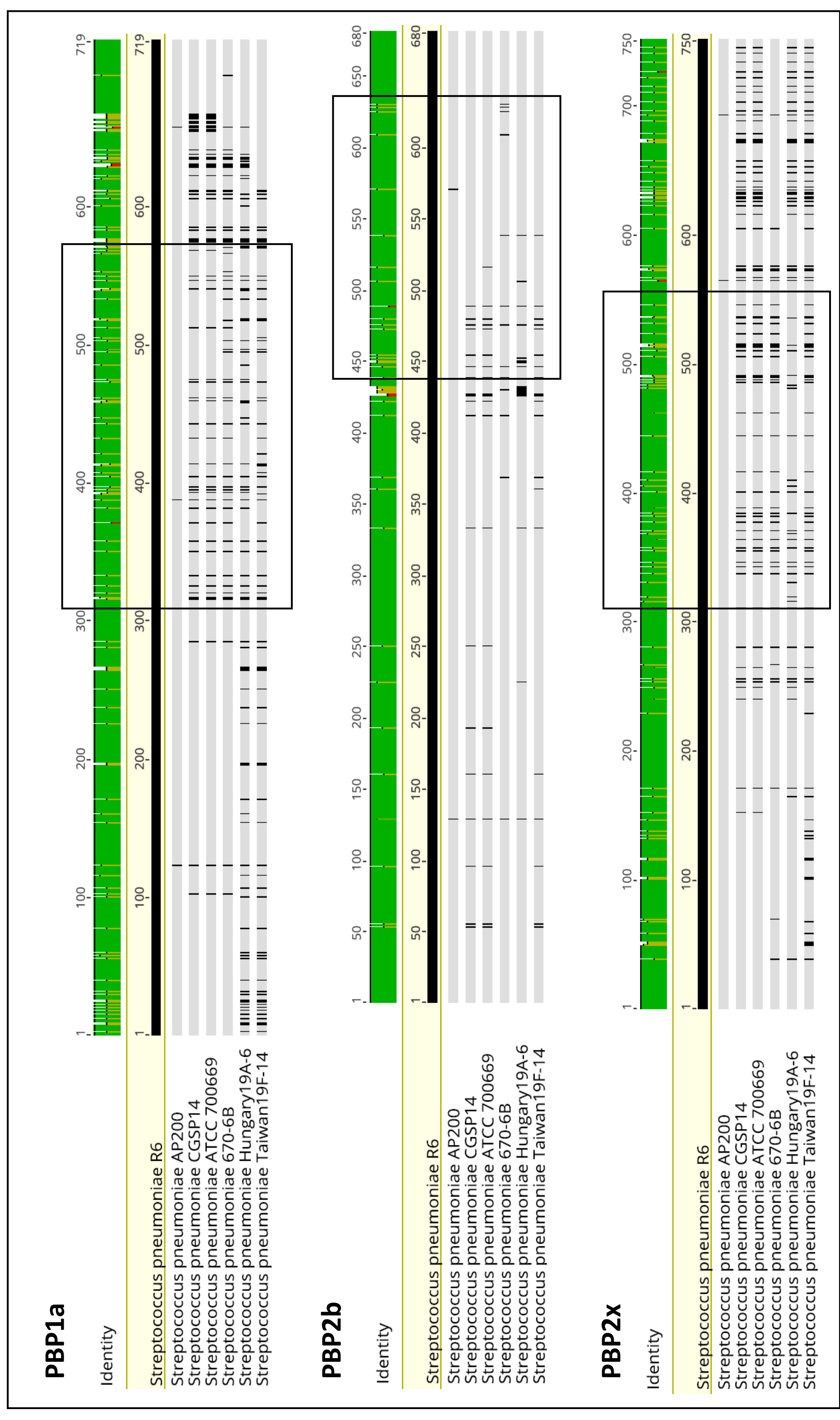


Figure 6. Amino acid sequence variation among PBP1a, PBP2b and PBP2x proteins of six representative reference strains. Black lines show amino acid changes with respect to *Streptococcus pneumoniae* R6. Transpeptidase domains of PBPs are highlighted. *Streptococcus pneumoniae* AP200 is included in the analysis as a representative of susceptible isolates. Penicillin and cefotaxime MICs (mg/L) of the studied isolates are as follows: AP200 (<0.12/<0.5), CGSP14 (2/1), ATCC700669-PMEN1 (1/1.5), 670-6B-PMEN2 (0.5/0.75), Hungary^{19A}-PMEN6 (1/0.75), Taiwan^{19F}-PMEN14 (2/0.75).

In Spain, rates of fluoroquinolones resistance in pneumococci collected from adults remain low, between 2-3%.[65] The fact that fluoroquinolones are not indicated in children could explain the low incidence of fluoroquinolone-resistant pneumococci in adults.[66] Despite this, some populations with high exposure to fluoroquinolones (such as COPD patients) show a higher proportion of infections caused by resistant strains.[67] Also, clonal dissemination of fluoroquinolone-resistant pneumococci has been described in Spain and abroad.[68–70]

Resistance to macrolides

Macrolides are a common treatment option for community-acquired pneumonia when β–lactam antibiotics cannot be used or even as empirical therapy.[49] Macrolides and lincosamides (also streptogramin B) are chemically distinct compounds that share a similar mechanism of action. Therefore they are usually referred to as MLS_B antibiotics. These compounds inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit.[71] The binding to the bacterial ribosome causes an obstruction of the nascent peptide exit tunnel and stops bacterial translation. Despite this classic view, recent evidences show that macrolides are not global inhibitors of translation but they can selectively inhibit the production of a subset of proteins.[72]

Two major mechanisms of macrolide resistance have been described in *Streptococcus pneumoniae:* ribosomal methylation and macrolide efflux. Other mechanisms such as mutations in 23S rRNA or in ribosomal proteins L4 and L22 appear to be less frequent.[71] Ribosomal methylation by Erm(B) is usually the most common macrolide resistance mechanism in *Streptococcus pneumoniae,*[73] conferring resistance to macrolides, lincosamides and streptogramin B (MLSB phenotype).[74] Erm(B) catalyses the dimethylation of an adenine residue in the 23S rRNA (A2058, *Escherichia coli* numbering) and prevents the binding of macrolides to the ribosomal target.[75] This resistance mechanism can be expressed as constitutively or inducible depending on the sequence of the promoter region of the gene.[76] Resistance mediated by Erm(B) causes high-level resistance to macrolides (erythromycin MICs ≥ 64mg/L). A second frequent mechanism of macrolide resistance in pneumococci is due to the presence of macrolide efflux pumps encoded by the *mef(A)/msr(D)* operon which confers

resistance to 14- and 15-membered macrolides (M phenotype). Resistance mediated by macrolide efflux is expressed as erythromycin resistance (MICs 2-32 mg/L) and susceptibility to clindamycin and streptogramin B.[77] Characteristically, both mechanisms of resistance are associated with the acquisition of integrative conjugative elements (ICEs) which allows its spread among bacteria and that frequently carry resistance determinants to other antimicrobials such as tetracycline (*tetM*) or chloramphenicol (*cat*).

In Spain, the prevalence of resistance to erythromycin increased steadily during the 1980s and 1990s, reaching a peak of 23-24% of invasive adult isolates in the early 2000s.[53] Regarding the isolates collected in children, the figures reached rates above 40%. After the introduction of PCV7 in 2001 the percentage of macrolide resistant isolates decreased to 18% in adults and 24% in children.[78,79]

1.6. Pneumococcal typing

Bacterial typing, the characterization of bacterial strains attempting to determine whether they are derived from the same unique ancestor, has important applications in clinical microbiology laboratories. Surveillance of resistant clones, outbreak investigation or study of transmission dynamics are just some examples of its routine application. Bacterial typing methodologies have evolved over time but can be classified in two major groups: phenotypic or genotypic methods. Currently, bacterial genotypic methods are preferred due to their higher performance and higher discrimination power so phenotypic typing is only applied in particular situations. Focusing on pneumococcal typing, description of a particular isolate by naming its capsular type (serotype) and its genetic background (ST is the most used description), is a relatively understandable and widely accepted methodology.

The pneumococcal serotype is usually determined by the Quellung reaction.[80] This method uses specific antisera against the different pneumococcal capsules allowing their microscopic visualization. An easy to make and widely used alternative is the use of latex agglutination.[81] The current nomenclature of pneumococcal serotyping is based on the Danish system of the Statens Serum Institut in Copenhagen, published in 1940.[82] It has to be noted that serotyping can also be indirectly obtained by some genotypic methods such as PCR or WGS.[83] Another phenotypic approach that can give some information on the genetic background of a particular isolate is to study its antibiotic resistance profile. Some internationally distributed antibiotic-resistant *Streptococcus pneumoniae* clones can be easily recognized by their serotype and their antibiotic resistance profile. Other phenotypic typing methods such as phage typing or biotyping through differences in biochemical tests have become obsolete.

Genotyping methods have become crucial to understand the genetic evolution and transmission dynamics of bacterial populations. Early genotyping methods were based on either PCR amplification (such as Random Amplified Polymorphic DNA, RAPD) or DNA fragmentation through restriction enzymes and subsequent fragment separation (Pulsed-field gel electrophoresis, PFGE). PFGE is a technology used for the separation of large DNA molecules (entire genomic DNA) after digesting it with

restriction enzymes. PFGE for *Streptococcus pneumoniae* isolates was standardized in 1993.[84] It is a powerful technique with high discrimination power but has some important limitations. First, it is an indirect measure of DNA sequence, so it cannot provide detailed information on the genetic differences found between two isolates. Second, it is a laborious and time-consuming technique that presents difficulties for standardization. In spite of that, PFGE has been a useful technique for bacterial typing, particularly in short-time outbreak situations. Moreover, the incorporation of standardized criteria for the interpretation of bands, such as the Tenover's criteria,[85] represented a great advance for the correct interpretation of PFGE results.

Another widely used genotyping methodology is Multilocus Sequence Typing (MLST).[86] This methodology consists of partially sequencing a set of housekeeping genes assigning them a numeric allele based on their nucleotide sequence. The combination of the numeric alleles defines the sequence type (ST). The methodology and the defined alleles and sequence types are publicly available and it is continuously updated (https://pubmlst.org/organisms/streptococcus-pneumoniae). The ability to obtain standardized and easily comparable results around the world is the most notable advantage of MLST over PFGE. On the contrary, MLST is significantly more expensive than PFGE and has low discrimination power in outbreak situations, particularly when an endemic clone is involved. The nomenclature and definition of antibiotic-resistant *Streptococcus pneumoniae* clones (including PFGE and MLST) has been standardized by The Pneumococcal Molecular Epidemiology Network (PMEN, https://www.pneumogen.net/pmen/).

Currently, whole genome sequencing (WGS) technologies have become the gold standard for bacterial typing. Although WGS is rapidly evolving, it has already been established as the reference tool for outbreak studies and population dynamics. Available technologies can be separated in two main groups: short reads (Illumina®) and long reads (PAcBio®, Oxford Nanopore®). Long reads offer advantages over short reads during the assembly process, but to date it is at the expense of a higher error rate. Both technologies can be combined to obtain good assemblies and reliable data.[87] WGS is the most discriminative tool for bacterial typing; beyond just getting MLST data, WGS is capable of discriminating single nucleotide polymorphisms along

the entire genome. Furthermore, as virtually the whole bacterial genome is sequenced, data on antimicrobial resistance, virulence factors and the presence of prophages, among others, can also be studied. Currently WGS is still an expensive tool and requires some bioinformatics knowledge, which makes it unsuitable for all laboratories. Despite this, as the technology has evolved, new online tools have appeared to allow WGS analysis without bioinformatics background. For instance, the study of antimicrobial resistance, virulence profile or just to getting epidemiological data such as MLST (among others) can be performed with online tools from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/), an application from the Technical University of Denmark. Another online tool provided by a consortium of several scientific institutions in the USA (https://usegalaxy.org/) allows to assemble raw WGS data and even to perform hybrid assemblies of long- and short-reads. These two examples serve to show applications available online that can help researchers to perform analysis with minimum bioinformatics skills and computational resources. In regard of WGS applied to the pneumococcal epidemiology, it is important to highlight the GPS project (https://www.pneumogen.net/gps/project_outline.html), which aims to study the pneumococcal evolution during the PCVs introduction through a WGS approach. Data from the GPS project has been used to define genetic clusters (GPSC) that may serve as reference for further WGS-based studies on pneumococcal epidemiology.[88]

1.7. Burden of pneumococcal diseases

Streptococcus pneumoniae is responsible for a wide spectrum of diseases. Despite this variability, nasopharyngeal colonization is the key and necessary step in pneumococcal pathogenesis.[89] Because colonization is also necessary for pneumococcal transmission, strategies that eradicate pneumococci from the nasopharynx are important from an epidemiological point of view. Carriage rates are higher in young children, which can be up to 60%,[90] and decreases to 10-20% in adults.[91] Nasopharyngeal colonization is the gateway to cause disease, either by local spread, by aspiration or by invasion of the bloodstream. In this way, pneumococcus can cause infection of the middle ear and lungs by direct spread from the nasopharynx and metastatic infections (endocarditis, joint infections…) by haematogenous spread. Central nervous system or pleural infections can be caused by either direct extension or haematogenous spread.

It is interesting to note that pneumococcal infections are seasonally related (Figure 7), occurring more often in winter, and that could be related to the presence of viral respiratory infections.[92]

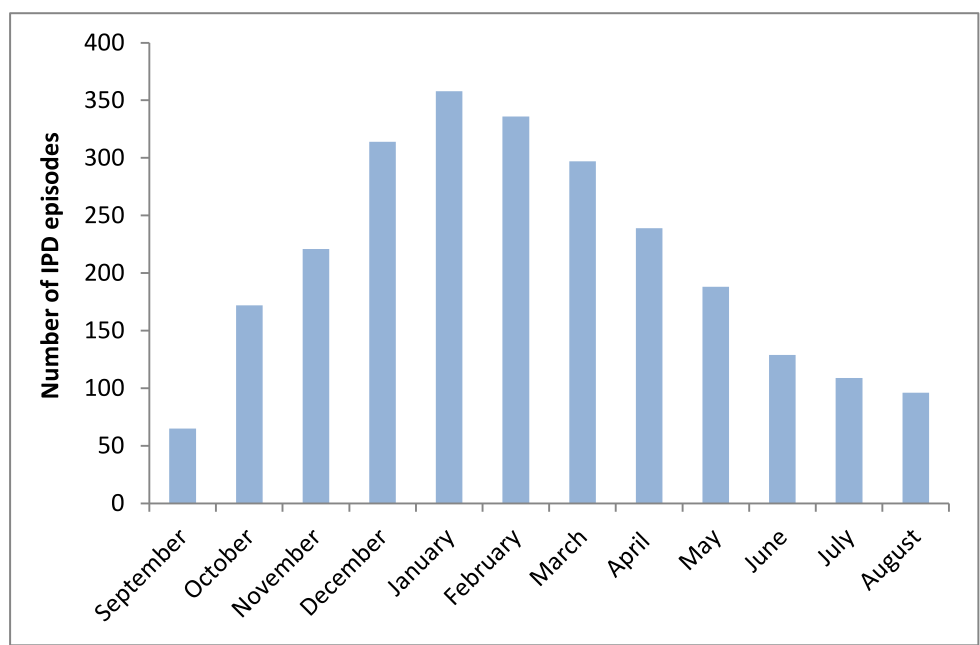


Figure 7. Monthly number of IPD episodes of patients attended at Hospital Universitari de Bellvitge (1981-2016). Only one IPD episode per patient and year was included.

In fact, inflammatory conditions of the respiratory tract such as viral infections favour the colonization by *S. pneumoniae*. It has been described that Influenza virus promotes *S. pneumoniae* colonization in mice[93] and it also produces some changes in lung physiology, such as epithelial damage or surfactant dysfunction, that favours *S. pneumoniae* superinfection.[94] Moreover, Influenza virus and *S. pneumoniae* coinfection has been associated to severe disease, more complications and poorer outcomes.[95,96]

S. pneumoniae is among the first causes of otitis media and purulent sinusitis together with non-typeable *Haemophilus influenza* and *Moraxella catarrhalis*.[97] It is also the first cause of CAP and meningitis in developed countries.[98] Among the pneumococcal infections, severe diseases such as pneumonia or meningitis represent one of the first causes of death worldwide. The population group with the highest risk of suffering severe pneumococcal diseases are children (mostly children under 5 years old), the elderly and immunocompromised people. A recent study estimated that the incidence of severe disease caused by *S. pneumoniae* was of 559 episodes (uncertainty range of 411–658) per 100,000 children in 2015.[99] These episodes were responsible for 294,000 deaths worldwide. Since these calculations excluded 23,300 deaths occurred in HIV-infected, the final figure may be higher. Significantly, this study showed enormous differences in the rate of mortality between countries. While developed countries from Europe or North America showed rates of mortality below 10 deaths per 100,000 children, rates from developing countries were up to 92 (uncertainty range of 60-114) and 50 (uncertainty range of 32-62) deaths per 100,000 children in Africa and Southeast Asia, respectively. These estimates show that most of the burden of severe pneumococcal disease falls on developing countries.

In adults, the burden of pneumococcal diseases is mostly determined by CAP. In our setting, *S. pneumoniae* is the first cause of CAP in adults accounting for around one third of all pneumonia episodes.[100,101] It has also been estimated that direct hospitalization costs associated to pneumococcal pneumonia are higher than those caused by other microorganisms (€2,864.7 vs. €2,259.8).[102] A recent report with data of 195 countries from 2016 , shows that the incidence of lower respiratory infections attributable to *S. pneumoniae* is 27 (95% uncertainty interval: 15-39) episodes per

1,000 population (all ages) and rises to 73 (95% uncertainty interval: 28-141) episodes per 1,000 in adults older than 70 years.[103] It is important to highlight that in this group of elderly adults *S. pneumoniae* is responsible for 122 (95% uncertainty interval: 52-222) deaths per 100,000 population. In this study, *S. pneumoniae* was the leading cause of lower respiratory infections and caused more deaths than all other aetiologies combined. In our setting, mortality associated with IPD remains above 20% in people ≥65 years despite the fact that it has decreased after the introduction of PCVs.[104] Therefore, pneumococcal infections are among the most common preventable diseases and one of the leading causes of death worldwide.

The risk of suffering pneumococcal diseases in adults is increased with some common underlying diseases: COPD, asthma, chronic heart diseases or diabetes mellitus have been associated to increased risk for both CAP and IPD.[105] In the same way, toxic habits such as alcohol abuse or smoking are among the most frequent preventable factors associated to invasive pneumonia.[106] There are also some underlying conditions that lead to notably high rates of IPD. For example, when compared to the annual incidence of IPD in the adult population of Canada (11,0 cases/100,000 population), patients with some malignancies such as lung cancer (143,6 cases/100,000 population) or multiple myeloma (673,9 cases/100,000 population) showed extremely high incidence rates.[107] Similarly, another study in the Netherlands showed incidence rates of IPD and CAP in HIV-infected individuals of 111 and 1529 per 100,000 patient-years, respectively.[108] In this population, the incidence of IPD rose up to 490 and 246 per 100,000 in patients without cART or with a CD4 count <500 cells/µL, respectively. Therefore, achieving high rates of pneumococcal vaccination in these high-risk groups should be essential.

1.8. Pneumococcal vaccines

The polysaccharide capsule has been the basis for the design of pneumococcal vaccines due to its immunogenic properties. Vaccines based on capsular polysaccharides induce type-specific antibodies, activate complement and stimulate bacterial phagocytosis.[109] However, the diversity of the capsular polysaccharide, with around 100 different types identified to date, makes it difficult to obtain a single vaccine with high coverage. Furthermore, vaccines composed of purified capsular polysaccharides produce a T cell-independent response, are not immunogenic in young children and do not generate immunological memory.[110] Therefore, new vaccines have been developed in recent years trying to induce a stronger immune response against bacterial capsular polysaccharides. These vaccines (conjugate vaccines) attach the polysaccharide components to a protein carrier which is strongly antigenic. The conjugation with the protein carrier stimulates a T cell-dependent immunological response which has an increased immunogenicity, is effective in young children and also prevents the carriage state.[111,112] This last property has important benefits from the epidemiological point of view by reducing the total bacterial load of the population and inducing herd protection. In Spain, four pneumococcal vaccines have been licensed so far: the pneumococcal polysaccharide vaccine PPV23 and three pneumococcal conjugate vaccines (PCV7, PCV10 and PCV13).

Pneumococcal polysaccharide vaccine

PPV23 (Pneumovax™, Merk and Pnu-Immune™23, Wyeth Lederle; targeting 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) has been used since the 1980's, mostly in adults over 65 years old or people over 5 years old at risk of having IPD. This vaccine was included in the Spanish systematic vaccination calendar in 2004, and the target was all adults over 65 years old, with low uptake.[113] In Spain, periodic booster doses are not recommended except for certain risk groups. As this vaccine does not affect the carriage state and is ineffective in young children, who have the higher carriage rates, the impact on the epidemiology of the pneumococcal population is limited. PPV23 has been shown to be effective in preventing invasive disease in immunocompetent individuals[114] and in

patients at high risk of pneumococcal infections (vaccine's protective efficacy between 60-70%).[115,116] Moreover, PPV23 has demonstrated effectiveness in preventing invasive disease in patients with some comorbidities such as diabetes mellitus, coronary vascular disease, congestive heart failure, chronic pulmonary diseases or anatomic asplenia (efficacy between 65-77%).[117] On the other hand, PPV23 has not clearly shown efficacy in reducing pneumococcal pneumonia, though this topic is controversial.[118–122] Finally, PPV23 has been helpful in reducing mortality and ICU admissions due to pneumococcal diseases.[123,124]

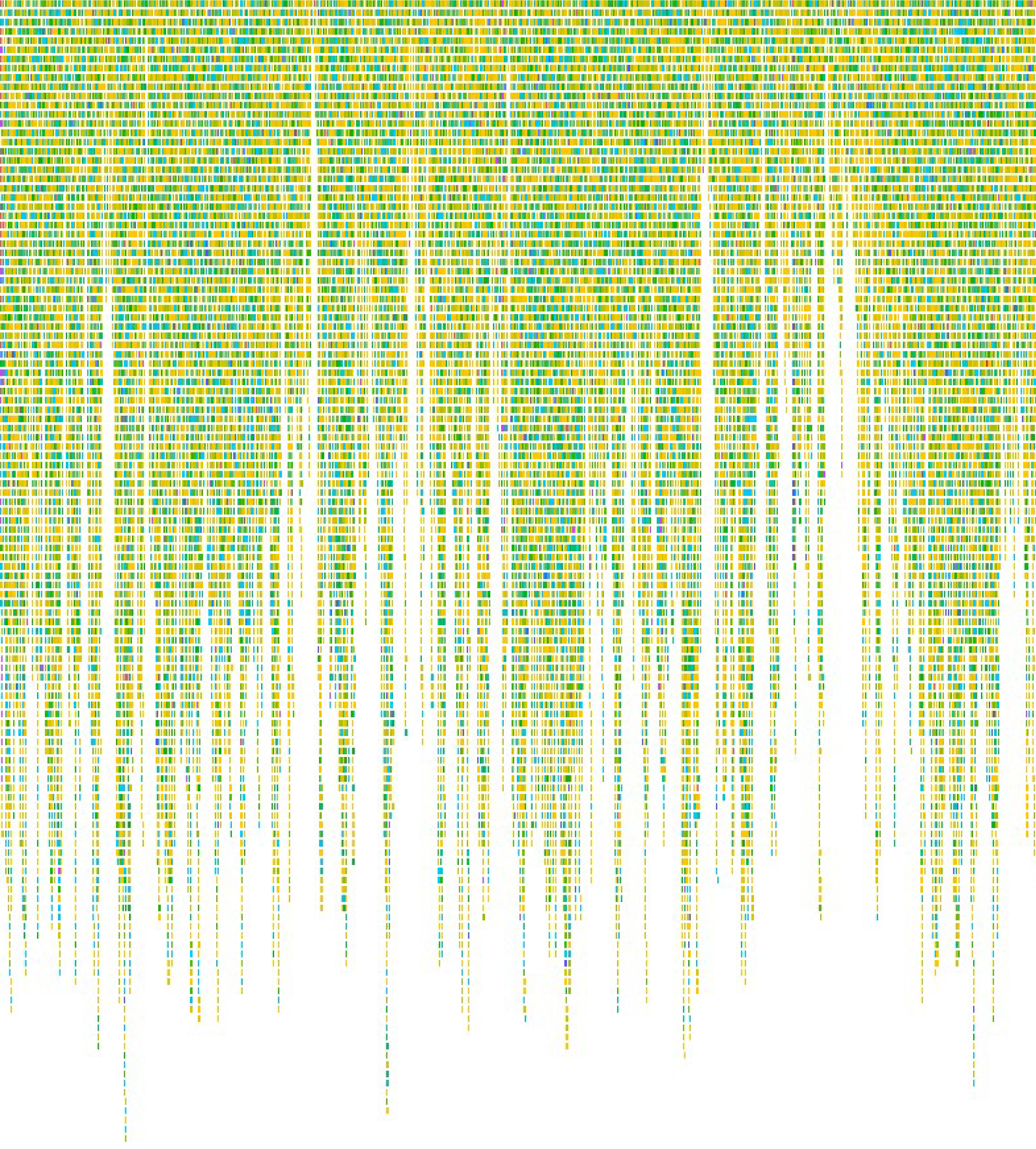
Pneumococcal conjugate vaccines

During the first decade of the 2000s, pneumococcal conjugate vaccines were introduced for the prevention of pneumococcal diseases. In Spain, the first conjugate vaccine, PCV7 (Prevenar®, Pfizer; targeting serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) was licensed in 2001 under voluntary basis. PCV7 included the seven most common serotypes isolated from blood or cerebrospinal fluid of children under 6 years old in the United States. It showed immunogenicity, safety and effectiveness in reducing the incidence of pneumococcal diseases in children.[12] Efficacy against invasive disease, pneumococcal pneumonia and acute otitis media caused by vaccine-types in children was 82-97%, 90% and 57%, respectively.[125] PCV7 prevented the carriage of vaccine-types, reducing the transmission from children to other children or adults, which resulted in a decline of pneumococcal diseases in unvaccinated populations through herd protection.[126,127] The introduction of PCV7 was also associated with a worldwide reduction of pneumococcal resistance rates.[128] In spite of that, a paradoxical increase in the incidence of IPD in children and adults was observed in Spain and other European countries some years after its introduction.[78,79] This was linked to the expansion of some epidemic serotypes not covered by PCV7 (mainly 1, 5 and 7F). Furthermore, a recombinant serotype 19A lineage (former 19F) with a multidrug-resistant pattern spread globally demonstrating the pneumococcal ability to evade the vaccine impact by means of capsular switching.[43]

Thereafter, new conjugate vaccines with expanded coverage were developed and licensed. In Spain, two other conjugate vaccines were introduced: PCV10 in 2009 (Synflorix®, GlaxoSmithKline; targeting PCV7 serotypes plus 1, 5 and 7F) and PCV13,

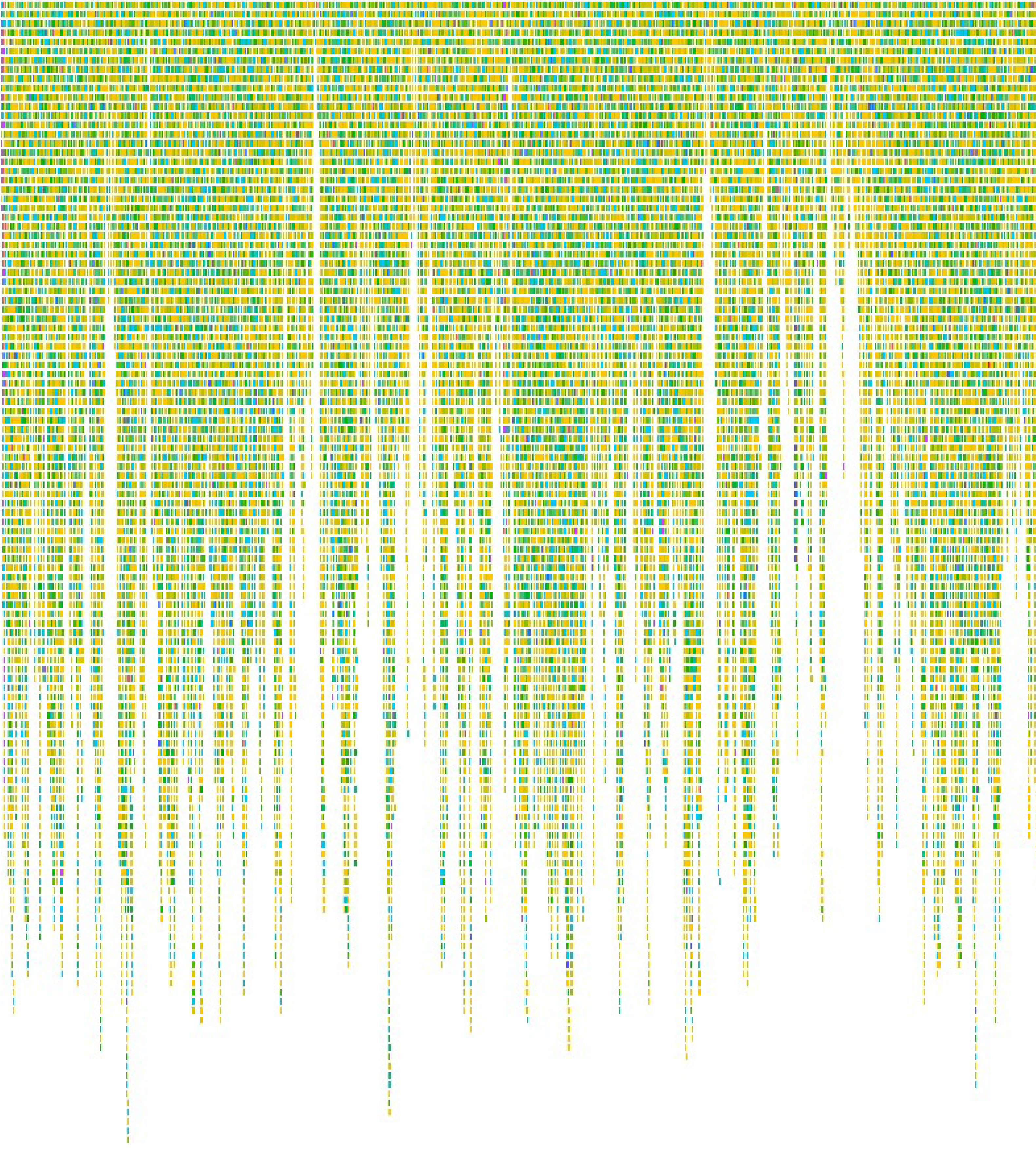
which replaced PCV7 -and was the most distributed- in 2010 (Prevenar 13®, Pfizer; targeting PCV10 serotypes plus 3, 6A and 19A). Both were licensed under a voluntary basis until 2015 when PCV13 was introduced into the paediatric vaccine calendar. After the introduction of PCV13, a sharp decrease of the incidence of IPD in children and adults was observed.[129–131] The introduction of PCV13 was also linked to a reduction in IPD-related mortality, even in the non-vaccinated population.[132] PCV13 has also demonstrated effectiveness in reducing pneumococcal pneumonia due to vaccine types in adults.[133]

New conjugate vaccines are currently in the final steps of development. A new 10-valent conjugate vaccine (Pneumosil®; 1, 5, 6A, 6B, 7F, 9V, 14, 19A, 19F and 23F) has been developed by the Serum Institute of India in collaboration with PATH and funding from the Bill & Melinda Gates Foundation. Recently this vaccine completed Phase 3 trial to evaluate the safety and immunogenicity in children from Gambia. As this vaccine is expected to be released at $2 per dose (https://www.path.org/articles/new-pneumococcal-vaccine/), it offers an opportunity to expand pneumococcal vaccination programs to low income countries. Two other conjugate vaccines (PCV15 -targeting serotypes PCV13 plus 22F and 33F- and PCV20 –targeting PCV15 serotypes 8, 10A, 11A, 12F and 15B-) have successfully completed clinical trials to evaluate their safety and immunogenicity in children and adults[134,135] so they are expected to be released in the following years.



HYPOTHESES

The aim of this work is to evaluate the impact of the introduction of PCVs for children on the epidemiology of the adult IPD in Spain. As observed for PCV7, the PCV13 introduction in children should have a high impact on the adult IPD. It is expected to detect a decrease in isolates harbouring serotypes included in PCV13 but the niche left by them could be filled with other non-vaccine serotypes. Also, as the study includes Spanish regions with different vaccination schemes, it will be interesting to compare its different impact on adult IPD. Because both PCV7 and PCV13 include several serotypes associated to β-lactam and multidrug-resistance, its introduction should lead to a decrease in the overall rates of antimicrobial resistance and a decrease in the incidence of MDR clones. Despite this, the impact of multidrug-resistance on mortality in patients with IPD has not yet been elucidated. In regard of the persistence of PMEN3 clone over time despite being targeted by PCVs, it is expected to find recombination events that helped this clone to avoid its extinction.



OBJECTIVES

This work focuses on three main objectives:

Objective 1. To study the early impact of PCV13 introduction for children on the incidence of invasive pneumococcal disease, antimicrobial resistance and serotype and genotype distributions in Spanish adults.

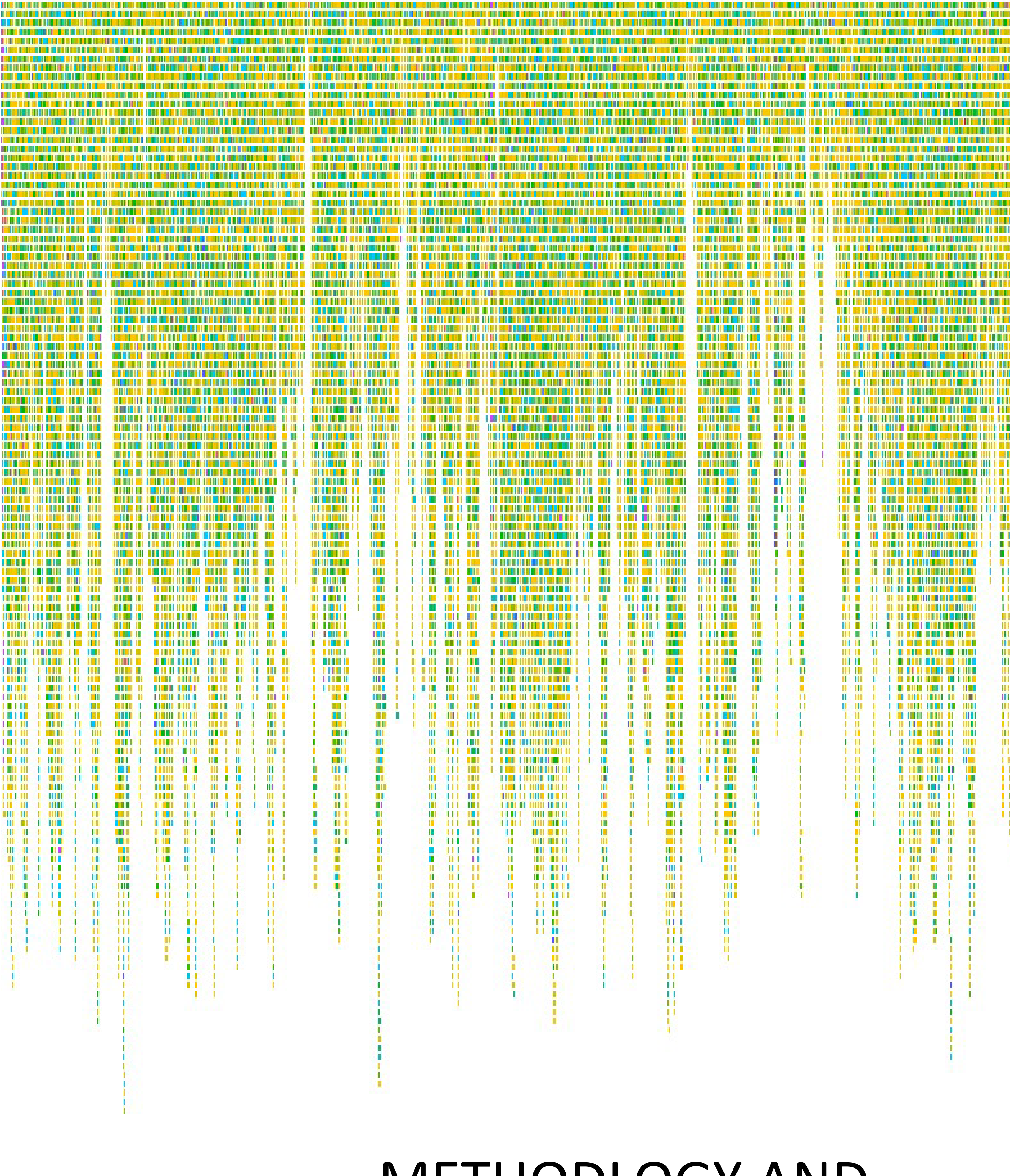
This study aims to explore differences in the incidence of IPD, in antimicrobial resistance and in the distribution of serotypes and genotypes among adult IPD episodes occurred before and after the introduction of PCV13 in Spain.

Objective 2. To explore the impact of the introduction of pneumococcal conjugate vaccines on multidrug-resistant *Streptococcus pneumoniae* isolates over a 25-year period and its impact on the incidence and mortality of IPD.

This study focuses on evaluating differences in the incidence of IPD, clinical features, antimicrobial susceptibility and genetic structure of *S. pneumoniae*-MDR isolates in the PCVs era.

Objective 3. To analyse the major recombination events occurred in the β-lactam-resistant PMEN3 (CC156) *Streptococcus pneumoniae* clone that allowed it to persist over the time as a cause of invasive disease despite the introduction of PCVs.

This study aims to elucidate the genetic changes occurred in the PMEN3 clone over the past years that allowed it to persist as an important cause of IPD in Spain.



METHODLOGY AND RESULTS

Objective 1. To study the early impact of PCV13 introduction for children on the incidence of invasive pneumococcal disease, antimicrobial resistance and serotype and genotype distributions in Spanish adults.

Càmara J, Marimón JM, Cercenado E, Larrosa N, Quesada MD, Fontanals D, Cubero M, Pérez-Trallero E, Fenoll A, Liñares J, Ardanuy C. **Decrease of invasive pneumococcal disease (IPD) in adults after introduction of pneumococcal 13-valent conjugate vaccine in Spain.** PLoS One. 2017 Apr 6;12(4):e0175224.

RESEARCH ARTICLE

Decrease of invasive pneumococcal disease (IPD) in adults after introduction of pneumococcal 13-valent conjugate vaccine in Spain

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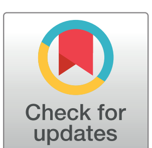

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Abstract

A prospective laboratory-based multicenter study that collected all adult invasive pneumococcal disease (IPD) episodes from 6 Spanish hospitals before (2008–2009) and after (2012–2013). The 13-valent pneumococcal conjugate vaccine (PCV13) licensure was conducted in order to analyze the impact of PCV13 introduction for children on adult IPD. A total of 1558 IPD episodes were detected. The incidence of IPD decreased significantly in the second period by -33.9% (95% CI, -40.3% to -26.8%). IPD due to PCV7 serotypes (-52.7%; 95% CI, -64.2% to -37.5%) and to PCV13 additional serotypes (-55.0% 95% CI, -62.0% to -46.7%) significantly decreased whereas IPD due to non-PCV13 serotypes remained stable (1.0% 95% CI, -12.9% to 17.2%). IPD due to all PCV13 additional serotypes significantly declined with the exception of serotype 3 (-11.3%; 95%CI -35.0% to 21.1%). IPD due to two non-PCV13 serotypes varied: serotype 6C that rose (301.6%; 95% CI, 92.7% to 733.3%, p<0.001), related to the expansion of $ST386^{6C}$, and serotype 8 that decreased (-34.9%, 95%CI, -57.1 to -1.2, p = 0.049), related to a decline of the $ST63^{8}$. The recombinant clone $ST6521^{11A}$ (variant of $ST156^{9V}$) increased in frequency. The decrease of serotype 19A IPD was linked to a fall in those antibiotic susceptible clones. In the last period, rates of penicillin- and cefotaxime-resistance remained under 10% and 4%, respectively. Adult IPD decreased after the PCV13 introduction in Spain due to herd protection. The spread of multidrug resistant clones ($ST386^{6C}$, $ST6521^{11A}$) related to non-PCV13 serotypes needs further surveillance.

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Introduction

Streptococcus pneumoniae is a leading cause of severe disease worldwide, mainly affecting children and the elderly populations. Pneumococcus infection can cause a broad spectrum of invasive disease including bacteremic pneumonia, sepsis and meningitis. In 2015, it was estimated that pneumoccal pneumonia was responsible for more than 1.5 million deaths worldwide [1].

Based on the seven most frequent pediatric serotypes in the 1990's (4, 6B, 9V, 14, 18C, 19F, and 23F), the heptavalent conjugate vaccine (PCV7) was developed and introduced in 2000–2001. Thereafter, the incidence of invasive pneumococcal disease (IPD) dramatically decreased among children due to PCV7-serotypes, but also among adults due to herd protection [2,3]. This change was accompanied by a general decline in antibiotic resistance [3,4]. However, new emerging serotypes were detected in the late PCV7 period [5–7] and the vaccine was improved by including six additional serotypes (PCV13: 1, 3, 5, 6A, 7F, and 19A), whose frequency increased after the PCV7 introduction. This PCV13 was licensed in Spain in 2010 for children and in 2012 for adults. Children's vaccination occurs mainly in the private market, with the exception of the Autonomous Community of Madrid, which included universal PCV7 vaccination for children in 2009 (3+1 schedule) that was replaced by PCV13 in June 2010 with a coverage higher than 94% [8].

The purpose of our study was to evaluate the impact of the PCV13 introduction on the incidence of adult IPD in Spain and its relationship with the dynamics of genotypes and serotypes. To assess it, we designed a prospective multicenter study involving six Spanish teaching hospitals from three Spanish regions that serve a population of approximately 4.000.000 adult inhabitants. The study was conducted throughout two periods: prePCV13 (2008–2009) and PCV13 (2012–2013).

Material and methods

A 4 year laboratory-based multicenter study involving 6 Spanish hospitals was conducted. The included hospitals serve a global population of around 4.000.000 adult inhabitants from three Spanish regions: Catalonia (Hospital Universitari de Bellvitge, Hospital Universitari Vall d'Hebron, Hospital Universitari Germans Trias i Pujol and Corporació Sanitària Parc Taulí), Madrid (Hospital General Universitario Gregorio Marañón) and the Basque Country (Hospital Universitario Donostia). An IPD episode was defined as the isolation of pneumococci from a normally sterile body site in a patient with clinical symptoms of infection (http://wwwn.cdc.gov/nndss/conditions/invasive-pneumococcal-disease/case-definition/2010/). All episodes of IPD among adults (≥18 years old) were prospectively collected. Only one isolate per episode was included. Data collection included age, sex, source of isolate and focus of infection. For comparison purposes, two periods were defined: pre-PCV13 (2008–2009) and PCV13 (2012–2013). The PCV7 (targeting serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) was licensed in 2001 and the PCV13 in 2010 (targeting PCV7 serotypes plus 1, 3, 5, 6A, 7F and 19A). The incidence of IPD was calculated using the total number of people as the denominator which was obtained from data published by the regional governments (http://www.madrid.org/iestadis/, http://www.idescat.cat/es/ and www.eustat.eus/). The distribution of IPD episodes (using previous description) with missing serotype was assumed to be identical to those episodes with serotype information (45 episodes in the pre-PCV13 period and 10 in the PCV13 period).

This study was approved by the Clinical Research Ethics Committee of Hospital Universitari de Bellvitge. Written informed consent was considered not necessary. Patients' data were anonymized for the purposes of this analysis.

Bacterial strains, serotyping and antimicrobial susceptibility

All available isolates [904 of 949 (95.3%) from the pre-PCV13 period and 599 of 609 (98.4%) from the PCV13 period] were serotyped by Quellung reaction at the Spanish Reference Laboratory. Susceptibility to 8 antimicrobials (penicillin, cefotaxime, erythromycin, clindamycin, chloramphenicol, tetracycline, co-trimoxazole and levofloxacin) was tested by microdilution method following The Clinical and Laboratory Standards Institute (CLSI) recommendations and criteria [9]. For the epidemiological analysis of penicillin resistance, the oral therapy breakpoints were applied. A multidrug resistant (MDR) isolate was defined as non-susceptible to penicillin (MIC$\geq$0.12) plus resistant to $\geq$2 classes of non-βlactam antimicrobials [10].

Molecular typing

A selection of isolates was genotyped through PFGE and/or MLST. We analysed 378 isolates obtained in the pre-PCV13 period (39.8%) including most penicillin- or macrolide- resistant isolates (n = 162) and a selection of susceptible isolates (n = 206). We also analysed 474 isolates collected in the PCV13 period (77.8%). The stains were selected according to major serotypes and were representative of all the regions. All 852 isolates were typed by PFGE (*SmaI*). Band patterns were visually compared following the criteria described by Tenover [11,12]. Major PFGE patterns were described as those accounting for at least 5 isolates. In addition, 344 isolates (22.1%) were typed using MLST (Multi Locus Sequence Type) [13] including at least one representative isolate of each serotype-PFGE pattern combination.

Statistical analysis

Statistical analysis was performed using the SPSS software package (SPSS, version 14.0; SPSS, Chicago, Illinois, USA). Statistical differences were assessed using the x2 or Fisher's exact test when appropriate. Statistical significance was established at $\alpha = 0.05$. All reported p values are two tailed. Incidence rates of IPD were defined as the number of episodes per 100.000 population and 95% confidence intervals (CIs) were calculated.

Results

Study population

A total of 1558 IPD episodes were detected. Of these, 949 (60.9%) were collected in the pre-PCV13 period and 609 (39.0%) in the PCV13 period. The overall mean age of patients was 61.6 years (range 18–107) and 925 (59.4%) episodes were detected in men. 839 episodes (53.9%) occurred in young adults (18–64 years), of these 458 (54.6%) were aged below 50. On the other hand, 719 episodes (46.1%) occurred in older adults ($\geq$65 years), of these 434 (60.4%) were aged 75 or over. Demographic characteristics of the study population are shown in Table 1. No differences were found between the two periods in male percentage or source of isolates. The average age of the patients increased significantly during the study period (59.5 vs 64.7 years, $p<0.001$). The incidence of bacteremic pneumonia statistically decreased (9.03 vs 6.00 per 100 000 population, $p<0.001$) whereas the incidence of meningitis remained stable (1.03 vs 0.87 per 100 000 population, p = 0.33).

Incidence of invasive pneumococcal disease (IPD)

The overall incidence of IPD decreased by -33.9% (95% CI, -40.3% to -26.8%) from 12.3 to 8.1 episodes per 100,000 population (Table 2). This decrease was statistically significant for all age groups. IPD due to PCV7 serotypes decreased 2.0 to 1.0 episodes per 100,000 (-52.7%; 95% CI, -64.2% to -37.5%) and IPD due to PCV13 additional serotypes declined from 5.7 to 2.6

Table 1. Demographic characteristics, source of isolates and focus of infection of the study population.

		pre-PCV13 (2008–2009)		PCV13 (2012–2013)		p*
Demographics	**Age mean (years)**	59.5		64.7		<0.001
	Male (%)	60.0		58.5		0.56
Source of isolates		**Episodes(n)**	**%**	**Episodes(n)**	**%**	
	Blood culture	822	86.6	520	85.4	0.43
	Cerebrospinal fluid	47	5.0	41	6.7	0.14
	Pleural fluid	54	5.7	28	4.6	0.35
	Ascitic fluid	15	1.6	13	2.1	0.42
	Other	11	1.2	7	1.1	0.99
Focus of infection		**Episodes(n)**	**Incidence****	**Episodes(n)**	**Incidence****	
	Pneumonia	700	9.03	451	6.00	<0.001
	Meningitis	80	1.03	66	0.87	0.33
	Peritonitis	33	0.43	23	0.31	0.22
	Unknown focus	102	1.31	48	0.64	<0.001
	Other	34	0.44	21	0.28	0.10

* p-value comparing pre-PCV13 and PCV13 periods

** Number of episodes per 100.000 population.

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Table 2. The incidence of IPD among adult patients before and after the introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) in Spain.

Age group (years)	Serotypes	Number of episodes		Incidence*		IPD change from pre-PCV13 to PCV13 (95% CI)	P**
		pre-PCV13	PCV13	pre-PCV13	PCV13		
18–50	PCV7	52	19	1.1	0.4	-60.9 (-77.0 to -33.8)	<0.001
	Additional PCV13	155	39	3.3	0.9	-73.0 (-81.0 to -61.7)	<0.001
	non-PCV13	120	73	2.6	1.7	-34.8 (-51.3 to -12.8)	0.004
	All	327	131	7.1	3.0	-57.1 (-65.0 to -47.4)	<0.001
51–64	PCV7	38	19	2.5	1.2	-50.2 (-71.3 to -13.7)	<0.001
	Additional PCV13	109	56	7.2	3.6	-48.8 (-62.9 to -29.4)	<0.001
	non-PCV13	76	84	5.0	5.5	10.1 (-19.3 to 50.1)	0.58
	All	223	158	14.7	10.3	-29.5 (-42.5 to -13.5)	<0.001
65–74	PCV7	25	10	3.2	1.3	-60.6 (-81.1 to -17.9)	<0.001
	Additional PCV13	74	36	9.3	4.5	-52.0 (-67.8 to -28.6)	<0.001
	non-PCV13	61	79	7.7	9.8	27.7 (-8.6 to 78.4)	0.15
	All	160	125	20.2	15.6	-23.0 (-39.0 to -2.6)	0.03
≥75	PCV7	42	24	5.2	2.8	-46.8 (-67.8 to -12.2)	<0.001
	Additional PCV13	102	61	12.6	7.0	-44.4 (-59.5 to -23.6)	<0.001
	non-PCV13	95	110	11.7	12.6	7.74 (-18.1 to 41.8)	0.63
	All	239	195	29.5	22.4	-24.1 (-37.2 to -8.3)	0.005
All groups	PCV7	157	72	2.0	1.0	-52.7 (-64.2 to -37.5)	<0.001
	Additional PCV13	440	192	5.7	2.6	-55.0 (-62.0 to -46.7)	<0.001
	non-PCV13	352	345	4.5	4.6	1.0 (-12.9 to 17.2)	0.91
	All	949	609	12.3	8.1	-33.9 (-40.3 to -26.8)	<0.001

*Estimated episodes per 100.000 population (95% CI)

** p-value comparing pre-PCV13 (2008–2009) and PCV13 (2012–2013) periods.

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episodes per 100,000 (-55.0%; 95% CI, -46.7% to -62.0%). The incidence of IPD due to non-PCV13 serotypes remained stable: 1.0% (95%CI, -12.9% to 17.2%), from 4.5 to 4.6 episodes per 100 000 population. Among patients aged 18–50 years, the incidence of IPD due to both PCV13 and non-PCV13 serotypes showed a statistically significant decrease. In those groups of patients aged >50 years, the IPD incidence due to PCV13 additional serotypes decreased ($p<0.001$) whereas the IPD incidence due to non-PCV13 serotypes increased, although this difference was not statistically significant. Since the hospitals are located in three different Spanish regions, we also analyzed IPD changes by regions. The decrease of IPD due to PCV7 and PCV13 additional serotypes was more noticeable in Madrid than in the Basque Country or Catalonia, although these differences were not statistically significant (Fig 1).

Serotypes

The overall IPD due to each of PCV13 additional serotypes (1, 3, 5, 6A, 7F and 19A) significantly declined with the exception of IPD due to serotype 3 which remained stable (Fig 2 and S1 Table). In addition, IPD due to serotypes 4, 14 and 23F, included in PCV7, also showed a statistically significant decrease. No significant changes in the IPD due to non-PCV13 serotypes was observed with the exception of IPD due to serotype 6C, which rose (301.6%; 95%CI, 92.7% to 733.3%), and IPD due to serotype 8, which decreased (-34.9%, 95%CI, -57.1% to -1.2%). This decrease of serotype 8 was only linked to a reduction in the region of Madrid

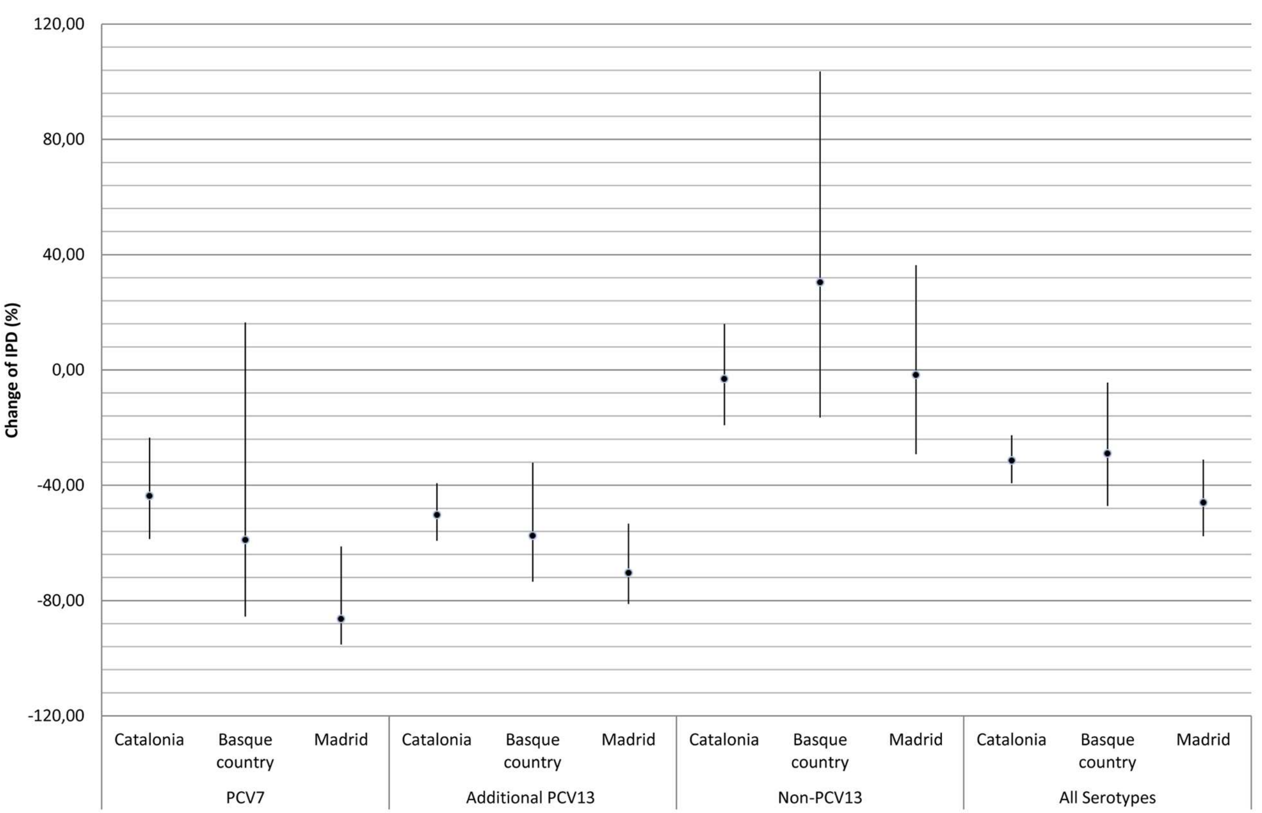

Fig 1. Regional changes in IPD by serotype group. Dots expressed the percentage of change and lines the limits of 95% CI. The decrease of IPD due to PCV7 and PCV13 additional serotypes was more noticeable in Madrid (the only region that included universal children vaccination with PCV13) than in the Basque Country or Catalonia.

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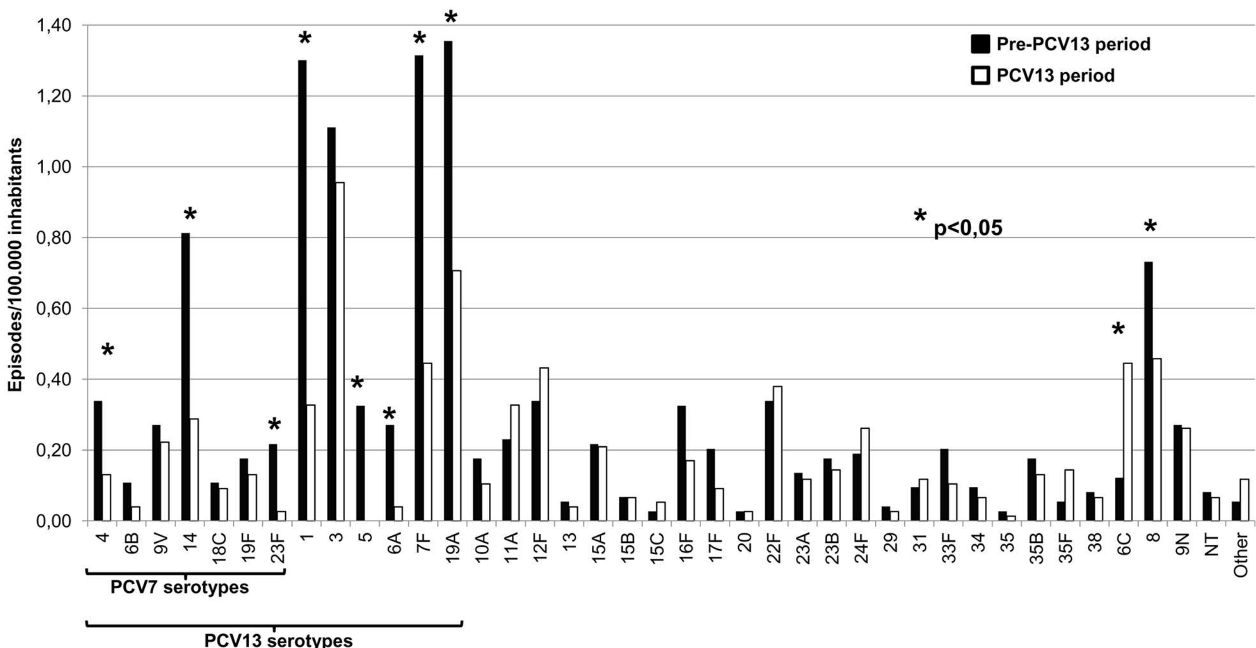

Fig 2. Incidence of invasive pneumococcal disease by serotype and by period. Asterisks indicate serotypes with statistically significant changes (p≤0.05). All p values were ≤ 0.001 with the exception of serotypes 4 (0.012) and 8 (0.049).

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(-53.4%, 95% CI, -74.7% to -14.2%), remaining stable in the other regions (-9.8%, 95% CI, -49.8% to 62.1%).

Antibiotic susceptibility

Table 3 shows the results of the antibiotic susceptibility to the 8 antimicrobials tested. Percentages of non-susceptibility to penicillin (MIC≥0.12 mg/L, 22.7% vs 26.8%, p = 0.065), cefotaxime (MIC>0.5 mg/L, 10.1% vs 12.5%, p = 0.14), as well as the proportion of MDR isolates (13.1% vs 16.2%, p = 0.08), showed a non-significant increase. Moreover, the proportion of

Table 3. Changes in antimicrobial susceptibility to eight antimicrobials before and after the PCV13 introduction.

Antimicrobial	pre-PCV13 (2008–2009)			PCV13 (2012–2013)			p*
	S[#]	I	R	S	I	R	
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	
Penicillin	734 (77.3)	155 (16.3)	61 (6.4)	446 (73.2)	107 (17.6)	56 (9.2)	0.043
Cefotaxime	852 (89.8)	83 (8.7)	14 (1.5)	533 (87.5)	54 (8.9)	22 (3.6)	0.006
Erythromycin	748 (78.8)	0 (0)	201 (21.2)	463 (76.1)	0 (0)	146 (23.9)	0.219
Clindamycin	770 (81.1)	0 (0)	179 (18.9)	483 (79.3)	0 (0)	126 (20.7)	0.399
Tetracycline	741 (78.1)	74 (7.8)	134 (14.1)	463 (76.1)	38 (6.2)	108 (17.7)	0.350
Chloramphenicol	880 (92.7)	. . .	69 (7.3)	571 (93.7)	. . .	38 (6.3)	0.415
Co-trimoxazole	675 (71.1)	54 (5.7)	220 (23.2)	441 (72.4)	21 (3.5)	147 (24.1)	0.607
Levofloxacin	926 (97.6)	. . .	23 (2.4)	601 (98.7)	. . .	8 (1.3)	0.147

* p-value comparing resistant isolates between periods. CLSI breakpoints were used.

[#] S: susceptible; I: intermediate; R: Resistant. Classical CLSI breakpoints for penicillin (oral: susceptible≤0.06mg/L; intermediate 0.12-1mg/L; and resistant ≥2mg/L) and cefotaxime (meningeal: susceptible≤0.5mg/L; intermediate 1mg/L; and resistant ≥2mg/L) were used.

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isolates fully resistant to penicillin or cefotaxime (MIC $\geq$ 2 mg/L) rose throughout the study period (6.4% vs 9.2%, p = 0.043 and 1.5% vs 3.6%, p = 0.006, respectively). Nevertheless, the incidence of IPD due to penicillin-resistant isolates was similar in both periods: 0.79 and 0.75 episodes per 100.000 population, respectively. Similarly, the incidence of IPD caused by those cefotaxime-resistant isolates showed a non-significant increase (62.0%, 95% CI, -17.1% to 216.6%, p = 0.154) from 0.18 to 0.29 episodes per 100.000 population. IPD caused by MDR isolates also remained stable: 1.60 and 1.31 episodes per 100.000 population, respectively (-17.7%, 95% CI, -36.8% to 7.1%, p = 0.14). There were no statistically significant differences for the remaining antimicrobials.

Two PCV13 serotypes 19A (26.9% and 26.5%, respectively) and 14 (25.5% and 26.5%, respectively) were the major serotypes associated with penicillin non-susceptibility in both periods. Regarding erythromycin-resistance, a shift in the most frequent serotypes was found: serotypes 8 (29.4%), 33F (12.9%), 19A and 23A (10.6% both) were the most frequent in the first period and serotypes 6C (25.6%), 11A (16.3%) and 33F (11.6%) in the second period. In this way, serotype 19A was the most frequent among dual-resistant strains (non-susceptibility to penicillin and resistance to erythromycin) in both periods (36.1% vs 37.6%, respectively). Two nonPCV-13 serotypes associated with dual resistance emerged in the second period: 24F and 6C (15.8% both).

Molecular typing

After the analysis of 852 isolates by PFGE/MLST we were able to identify the major clones related to specific serotypes previously described in Spain and other European countries [3, 14–17]. This analysis allowed us to classify genotypes according to their antimicrobial resistance profile (See S2 Table). When comparing the two periods, major differences were found in the genetic background of five serotypes (3, 6C, 9V, 11A and 19A). The increase in the IPD caused by serotype 6C was related to an expansion of the clone ST386^{6C} in all three regions, which accounted for 14.3% (1 out of 7 studied) of the first period serotype 6C isolates and for 82.6% (19 out of 23 studied) of the second period isolates. For serotype 3, two major clones were identified: ST180 and ST260. A shift in their proportions was observed, ST260 being predominant in the pre-PCV13 period (66.7%, 16 out of 24 studied), and ST180 in the PCV13 (61.4%, 35 out of 57 studied). In the second period, serotype 9V was only detected in Catalonia and was associated with the emergence of a new penicillin-susceptible clone (ST280^{9V}). This clone accounted for 64.7% (11 out of 17 studied) of serotype 9V isolates of Catalonia, while in the first period 42.1% of serotype 9V isolates (8 out 19 studied) belonged to the β-lactam-resistant clone and were detected in all three regions. Among serotype 11A, we detected the emergence of ST6521-MDR isolates (double locus variant of ST156) which accounted for 34.8% (8 out of 23) of the studied strains, mainly in Madrid (5 episodes). Finally, among serotype 19A isolates, the major decline of its incidence was due a decrease of the penicillin- and macrolide-susceptible clones. In fact, we could identify an increase of isolates belonging to the MDR ST320 clone (13.7%, 6 out of 44 and 57.1%, 24 out of 42 studied, respectively) in all three regions.

Discussion

The present study demonstrated a herd protection of the adult population in Spain after the PCV13 introduction for children, even when vaccination is mainly due under a private voluntary basis. Those results highlight the importance of the herd protection in the epidemiology of the IPD when the target of vaccination is the pediatric population.

In Spain, the estimated vaccine coverage for children remains around 60% for most regions, with the exception of the Autonomous Community of Madrid where the vaccine is included in the official vaccination schedule reaching a coverage above 95% [8, 18–19]. A recent pooled analysis showed that the level of herd protection could be associated with the initial vaccine coverage and the accumulated size of the vaccinated group [20]. In agreement with this, the highest adult IPD decrease due to herd protection was observed in the Madrid region which has the highest vaccine coverage. Although other factors could have influenced this higher decrease in the Madrid region, we were not able to identify them.

Our results are similar to what was reported previously from other European countries, from the US and from Israel [10, 21–25] and show a sharp decrease of the IPD only three years after the PCV13 introduction. This IPD reduction was observed in all age groups and was mainly linked to a reduction of the incidence of bacteremic pneumonia whereas the incidence of meningitis remained stable [26]. However, it is remarkable that the mentioned studies included pediatric populations with higher vaccine coverage than our study.

Overall, the incidence of non-PCV13 serotypes remained stable in the last period, with the exception of a decrease in people aged <50 years. Although we have no explanation for this decrease, it could be partially due to the existence of an outbreak of IPD due to Serotype 8-ST63 among young adults (mainly HIV-infected patients) in the region of Madrid over the 2004–2009 period (pre-PCV13); in fact among non-PCV13 serotypes, only serotype 8 IPD decreased [27,28]. As expected, a reduction of incidence was observed for all PCV13 additional serotypes (1, 3, 5, 6A, 7F and 19A) with the exception of serotype 3, which remained stable (-11.3%, 95%CI -35.0% to 21.1%, p = 0.477). The impact of the PCV13 on the incidence of serotype 3 is a controversial issue as there are different findings. For instance, a reduction of IPD due to serotype 3 has been recently reported in England and Wales by Waight [25], whereas no significant changes have been reported in the US and other European Countries [10, 17, 22, 24]. This serotype is rarely found colonizing children [29,30] whereas it is one of the first causes of IPD in adults as well as the fact that it is also associated with severe disease and high mortality rates [31, 32]. In this way, we detected that serotype 3 was the leading cause of IPD in the PCV13 period (12%) whilst it was only the fourth (9.1%) of the pre-PCV13 period (after serotypes 7F, 19A and 1). Probably, higher vaccine coverage and a longer vaccination period are needed to observe herd protection on the adult population [20]. However, the expansion of the PCV13 vaccination to the adult population at risk could be more successful to reduce the serotype 3 disease in adults. On the other hand, a change in their genetic background was observed when the local serotype 3 clone (ST260) has been partially replaced by the widely disseminated serotype 3 clone (ST180). This shift could not be explained by antimicrobial pressure (both STs are fully antimicrobial susceptible) and probably reflects clonal fluctuations of serotypes as previously described.

Regarding serotype 19A, despite the observed reduction of the IPD due to this serotype, the number of isolates belonging to $ST320^{19A}$ increased over the study period, which is a matter of concern since isolates of this clonal complex show a MDR pattern. These results are in agreement with those published in the USA from the late PCV7 period and the early PCV13 [5,7, 33–35] but, as data of molecular epidemiology after PCV13 introduction in Europe is scarce, we could not compare the occurrence of this phenomenon in other countries. Moreover, a reduction of isolates belonging to $ST320^{19A}$ has also been detected in a pediatric population from the USA [35] after the PCV13 introduction, even though this reduction was lower than that observed for the susceptible clones. In fact, it seems that the persistence of the $ST320^{19A}$ over the remaining 19A clones may be due to a combination of factors including an improved colonizing ability [36], a change in their metabolic profile [37] and an enhanced resistance to the antimicrobial stress due its MDR pattern.

Regarding the non-vaccine serotypes, their incidence remained stable for most of them with the exception of serotype 6C and the previously described serotype 8. In fact, serotype 6C was the only non-vaccine serotype that significantly increased over the study period, related to an expansion of the $CC386^{6C}$. This multidrug resistant clone was identified as an emerging lineage in Spain in 2009 [38] and a rise in its frequency has also been detected in the UK [39] and France [16] after the PCV7 introduction and in the early PCV13 period. These data could suggest a clonal replacement of those vaccine serotypes included in the serogroup 6 (6A and 6B). Nevertheless, it is remarkable that cross-reactivity between serotypes 6A and 6C has been reported [40] and, therefore, the PCV13 introduction should theoretically protect against serotype 6C colonization and reduce their impact on the adult IPD, as observed by some authors [10,41]. Our results could suggest a minor effect of the cross-reactivity between these serotypes but also the need for a higher coverage to observe this impact. Moreover, several works that were unable to detect a reduction of 6C isolates after the PCV13 introduction have been published [24, 25, 42]. In any case, the expansion of this clone showing non-susceptibility to penicillin, and macrolides and tetracycline resistance should be monitored.

Data regarding antimicrobial resistance after the PCV13 introduction is still scarce [10, 17, 23] but a decrease in the prevalence of penicillin- and cefotaxime-resistance has generally been reported. In our study, although we have observed an increase in the proportion of non-susceptibility to penicillin, cefotaxime, and MDR isolates, the incidence of IPD due to these isolates remained stable. These results were related to a drastic reduction of the IPD caused by those prevalent susceptible clones (mainly $ST306^{1}$, $ST289^{5}$, $ST392^{17F}$ and $ST1201^{19A}$), to a stabilization of the IPD caused by some multidrug resistant clones ($ST230^{24F}$, $ST320^{19A}$) and to an emergence of new resistant clones ($ST6521^{11A}$ and $ST386^{6C}$). Of note, $ST6521^{11A}$ is a recombinant clone previously associated to serotypes 14 and 9V (included in the PCV13) and it was mainly detected in Madrid, the region with higher coverage. This clone has been shown to have the ability to evade the immune system [43]. On the other hand, the emergence of $ST386^{6C}$ clone was detected in all three regions, even in Madrid. Then, probably, the cross-reactivity of 6A with 6C needs a longer period as occurred for 6B and 6A [38].

The main strength of our study is the valuable information that it gives through a well characterized population. Since the impact of the conjugate vaccines depends on, obviously, the pre-existing proportion of serotypes of the target population, but also depends on the circulating clones, it is mandatory to study the pneumococcal population from a global perspective of serotype-genotype. However, other factors could have contributed to the observed changes in IPD such as variations in the overall population at risk of suffering IPD, which is the main limitation of our study. In the same line, the selection of resistant isolates for molecular typing in the pre-PCV13 period could have hidden the emergence or spread of antibiotic susceptible clones. However, the clonal composition of Spanish invasive pneumococci in the prePCV13 period has been previously analyzed. Other limitations of this study are the lack of information about the 23-valent pneumococcal polysaccharide vaccination (with coverage nearly 40% in adults over 65), the immune status of the patients and the existence of comorbidities, whose changes could have affected the incidence of IPD [44]. Finally, since up to 70% of the strains were from Catalonia, this could be a bias of our study.

Conclusions

The decrease in the incidence of IPD observed in the period 2012–2013 was due to a decrease in the incidence of PCV13 serotypes which demonstrates the importance of the herd immunity. In spite of that, after the PCV13 introduction new clones have appeared ($ST386^{6C}$, $ST6521^{11A}$) and others have increased despite being clones related to serotypes included in the

vaccine (ST180[3] and ST320[19A]). In addition, in our geographical area, the rates of resistance to penicillin and cefotaxime have increased in association to an expansion of MDR clones. On account of all this, the surveillance of the pneumococcal invasive disease continues being necessary as well as the development of broader conjugate vaccines.

Supporting information

S1 Table. Change of adult IPD incidence by serotype from pre-PCV13 to PCV13.
(PDF)

S2 Table. Results of molecular typing.
(XLSX)

S3 Table. Demographic characteristics of the study population by hospital.
(XLSX)

S1 Fig. Hospital's location, demographics and number of episodes by period.
(TIF)

Author Contributions

Conceptualization: CA JL JC.

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Formal analysis: JC CA JL.

Funding acquisition: CA JL.

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Project administration: CA.

Resources: NL DF MDQ JMM EC EPT MC JC JL AF CA.

Supervision: CA.

Validation: MC JC CA AF JMM EPT EC.

Visualization: JC CA.

Writing – original draft: JC CA.

Writing – review & editing: NL DF MDQ JMM EC EPT MC JC JL AF CA.

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Supplementary material

S1 Table. Change of adult IPD incidence by serotype from pre-PCV13 to PCV13.

Serotypes			Incidence*		Number of episodes		IPD change from pre-PCV13 to PCV13 (95% CI)	*p*-value**
			pre-PCV13	PCV13	pre-PCV13	PCV13		
PCV7	PCV13	4	0.34	0.14	26	10	-60.36 (-80.88 to -17.83)	0.012
		6B	0.11	0.04	8	3	-61.35 (-89.75 to 45.77)	0.227
		9V	0.27	0.23	21	17	-16.60 (-55.99 to 58.23)	0.628
		14	0.81	0.30	63	22	-64.00 (-77.85 to -41.52)	<0.001
		18C	0.11	0.09	8	7	-9.83 (-67.30 to 148.76)	1
		19F	0.18	0.14	14	10	-26.36 (-67.30 to 65.84)	0.542
		23F	0.22	0.03	17	2	-87.87 (-97.20 to -47.51)	0.001
		1	1.30	0.34	101	25	-74.49 (-83.53 to -60.47)	<0.001
		3	1.11	0.99	86	74	-11.35 (-35.02 to 21.07)	0.477
		5	0.33	0.00	25	0	-95.88 (-99.44 to -69.58)	<0.001
		6A	0.27	0.04	21	3	-85.28 (-95.61 to -50.64)	<0.001
		7F	1.31	0.46	102	35	-64.64 (-75.91 to -48.08)	<0.001
		19A	1.36	0.73	105	55	-46.00 (-61.04 to -25.21)	<0.001
non-vaccine serotypes		6C	0.12	0.46	9	35	301.61 (92.68 to 733.33)	<0.001
		8	0.73	0.47	57	36	-34.90 (-57.12 to -1.19)	0.049
		10A	0.18	0.11	14	8	-41.11 (-75.29 to 40.45)	0.287
		11A	0.23	0.34	18	25	43.06 (-21.88 to 162.47)	0.286
		12F	0.34	0.45	26	34	34.77 (-19.09 to 124.72)	0.302
		13	0.05	0.04	4	3	-22.7 (-82.7 to 244.8)	1
		15A	0.22	0.22	17	16	-3.01 (-50.98 to 91.94)	1
		15B	0.07	0.07	5	5	3.1 (-70.2 to 255.9)	1
		15C	0.03	0.05	2	4	106.2 (-62.2 to 1023.6)	0.446
		16F	0.33	0.18	25	13	-46.41 (-72.58 to 4.71)	0.075
		17F	0.20	0.09	16	7	-54.91 (-81.45 to 9.65)	0.094
		20	0.03	0.03	2	2	3.1 (-85.5 to 629.9)	1
		22F	0.34	0.39	26	29	18.91 (-29.68 to 101.21)	0.593
		23A	0.14	0.12	10	9	-15.68 (-65.06 to 103.67)	0.824
		23B	0.18	0.15	14	11	-19.03 (-63.24 to 78.25)	0.691
		24F	0.19	0.27	15	20	37.36 (-29.63 to 168.10)	0.400
		29	0.04	0.03	3	2	-31.3 (-88.5 to 311.5)	1
		31	0.09	0.12	7	9	32.45 (-50.64 to 255.87)	0.624
		33F	0.20	0.11	16	8	-48.48 (-77.94 to 20.48)	0.153
		34	0.09	0.07	7	5	-26.36 (-76.64 to 132.02)	0.775
		35	0.03	0.01	2	1	-48.5 (-95.3 to 468.2)	1
		35B	0.18	0.14	14	10	-26.36 (-67.30 to 65.84)	0.542
		35F	0.05	0.15	4	11	183.29 (-9.75 to 792.86)	0.073
		38	0.08	0.07	6	5	-14.09 (-73.79 to 181.69)	1
		9N	0.27	0.27	21	20	-1.86 (-46.78 to 81.16)	1
		NT	0.08	0.07	6	5	-14.1 (-73.8 to 181.7)	1
		Other	0.08	0.12	6	9	54.6 (-45.0 to 334.4)	0.565

* Episodes per 100,000 population

**p-value comparing pre-PCV13 (2008-2009) and PCV13 (2012 2013) periods

S2 Table. Results of molecular typing. Isolates are shown according their antimicrobial susceptibility: **S=** Isolates susceptible to Penicillin and Erythromycin, **PE=** Penicillin- and Erythromycin-resistant, **P=**Penicillin-resistant and **E=**Erythromycin-resistant isolates. Only serotypes accounting for more than 10 isolates are shown

Vaccine type	Serotype	Resistance profile	pre-PCV13			PCV13		
			Number of isolates	Isolates typed	Clonal complex	Number of isolates	Isolates typed	Clonal complex
PCV7	4	S	23	7	CC247(n=6) CC800(n=1)	10	8	CC1866 (n=2) CC247(n=2) CC4127 (n=1) CC8064 (n=1) CC770 (n=1) Unrelated (n=1)
	6B	PE	5	4	CC315(n=2) CC90(n=2)	2	1	CC315(n=1)
		P	0	0		1	1	Unrelated(n=1)
		E	1	1	CC138(n=1)	0	0	
		S	2	0		0	0	
	9V	PE	1	0		0	0	
		P	16	8	CC156(n=8)	5	5	CC156(n=5)
		S	3	3	CC62(n=1) CC406(n=1) Unrelated (n=1)	12	12	CC280(n=11) CC156(n=1)
	14	PE	13	12	CC156(n=11) CC9(n=1)	4	3	CC156(n=3)
		P	40	26	CC156(n=26)	18	15	CC156(n=14) Unrelated (n=1)
		E	3	3	CC9(n=3)	0	0	
		S	4	2	CC4818(n=1) Unrelated (n=1)	0	0	
	18C	PE	0	0		1	1	CC4819(n=1)
		E	1	0		1	1	CC113(n=1)
		S	7	3	CC241(n=1) CC4819(n=1) CC113(n=1)	5	3	CC113(n=2) Unrelated (n=1)
	19F	PE	9	6	CC63(n=3) CC88(n=3)	5	4	CC88(n=1) CC87(n=1) Unrelated(n=2)
		P	2	2	CC88(n=2)	1	1	CC202(n=1)
		S	2	1	CC177(n=1)	4	4	CC177(n=1) CC271(n=1) Unrelated(n=2)
	23F	PE	6	4	CC81(n=4)	1	0	
		P	4	3	CC156(n=1) CC338(n=1) CC42 (n=1)	0	0	
		E	1	1	CC42 (n=1)	1	1	CC242(n=1)
		S	4	1	Unrelated (n=1)	0	0	

PCV13	1	E	4	4	CC306(n=3) Unrelated (n=1)	0	0	
		S	90	25	CC306(n=23) CC304(n=2)	25	19	CC306(n=17) CC304(n=2)
	3	E	0	0		2	2	CC180(n=2)
		S	80	24	CC260(n=16) CC180(n=8)	70	57	CC180(n=35) CC260(n=19) Unrelated (n=3)
	5	S	24	7	CC289(n=6) Unrelated (n=1)	0	0	
	6A	PE	5	4	CC473(n=2) CC315(n=1) CC1518(n=1) Unrelated (n=1)	0	0	
		E	4	2	CC2591(n=1) Unrelated (n=1)	0	0	
		S	10	2	CC1143(n=1) CC2611(n=1)	3	1	CC1143(n=1)
	7F	S	96	31	CC191(n=31)	32	27	CC191(n=27)
	19A	PE	39	16	CC230 (n=8) CC320 (n=6) CC81 (n=1) CC156 (n=1)	38	34	CC230 (n=9) CC320 (n=24) CC81 (n=1)
		P	17	4	CC230 (n=4)	5	4	CC230 (n=3) CC63 (n=1)
		E	9	7	CC193 (n=5) CC62 (n=1) CC202 (n=1)	1	1	CC1201 (n=1)
		S	35	17	CC1201 (n=10) CC199 (n=3) CC994 (n=1) CC193 (n=1) CC645 (n=1) CC416 (n=1)	10	6	Unrelated (n=3) CC199 (n=1) CC994 (n=1) CC3259 (n=1)
Non-vaccine	10A	E	2	1	CC97(n=1)	0	0	
		S	11	6	CC97(n=6)	8	7	CC97(n=5) Unrelated(n=1)
	11A	PE	0	0		2	2	CC156(n=1) Unrelatd (n=1)
		P	1	0		9	8	CC156(n=7) Unrelated (n=1)
		E	3	3	CC62(n=3)	7	7	CC62(n=7)
		S	13	4	CC62(n=4)	7	5	CC62(n=5)
	12F	S	25	8	CC989 (n=8)	32	28	CC989 (n=26) Unrelated (n=2)
	15A	PE	14	10	CC63(n=10)	14	12	CC63(n=12)
		E	0	0		1	1	CC63(n=1)
		S	2	1	Unrelated (n=1)	1	1	Unrelated (n=1)
	16F	P	0	0		1	1	Unrelated (n=1)
		E	8	7	CC30(n=7)	1	1	CC30(n=1)
		S	16	5	CC30(n=5)	11	10	CC30(n=7) CC7438(n=1) Unrelated (n=2)

	17F	S	14	6	CC392(n=5) CC4006(n=1)	7	6	CC392(n=4) Unrelated (n=2)
	22F	S	24	9	CC433(n=7) CC1372(n=1) Unrelated (n=1)	29	25	CC433(n=23) CC1372(n=1) Unrelated (n=1)
	23A	E	9	6	CC42 (n=6)	4	4	CC42(n=4)
		S	0	0		4	3	CC42(n=2) Unrelated(n=1)
	23B	P	7	4	CC338(n=4)	9	7	CC338(n=7)
		S	5	4	CC439(n=4)	2	2	CC338(n=1) Unrelated(n=1)
	24F	PE	9	8	CC230 (n=8)	16	15	CC230 (n=14) Unrelated (n=1)
		P	2	0		1	1	CC230 (n=1)
		S	3	1	CC72 (n=1)	3	2	CC156(n=1) CC177(n=1)
	31	S	7	4	CC1684(n=4)	8	5	CC1684(n=5)
	33F	E	11	9	CC717(n=9)	5	4	CC717(n=2) Unrelated(n=2)
		S	3	1	CC717(n=1)	3	2	CC717(n=1) CC1012(n=1)
	34	S	7	5	CC1046(n=4) CC1884(n=1)	5	2	CC1046(n=1) Unrelated(n=1)
	35B	P	6	1	CC558(n=1)	4	2	Unrelated(n=2)
		S	6	4	CC198(n=3) Unrelated(n=1)	5	2	CC198(n=1) Unrelated(n=1)
	35F	S	4	2	CC558(n=1) Unrelated(n=1)	10	7	CC2217(n=2) CC2203(n=2) CC1635(n=1) Unrelated(n=2)
	38	S	6	3	CC393(n=3)	5	4	CC393(n=4)
	6C	PE	1	1	CC386 (n=1)	16	14	CC386 (n=13) CC224 (n=1)
		P	3	3	CC224 (n=2) Unrelated (n=1)	2	2	CC224 (n=2)
		E	0	0		11	8	CC386 (n=7) Unrelated (n=1)
		S	5	5	CC224 (n=1) CC4011 (n=1) CC1143 (n=1) CC61 (n=1) CC4534(n=1)	5	0	
	8	E	25	1	Unrelated (n=1)	3	3	CC63 (n=3)
		S	29	7	CC53 (n=4) CC404 (n=3)	31	20	CC53 (n=13) CC404 (n=5) Unrelatd (n=2)
	9N	P	0	0		2	1	CC67(n=1)
		S	20	6	CC67(n=5) CC4811(n=1)	18	15	CC67(n=12) Unrelated(n=3)

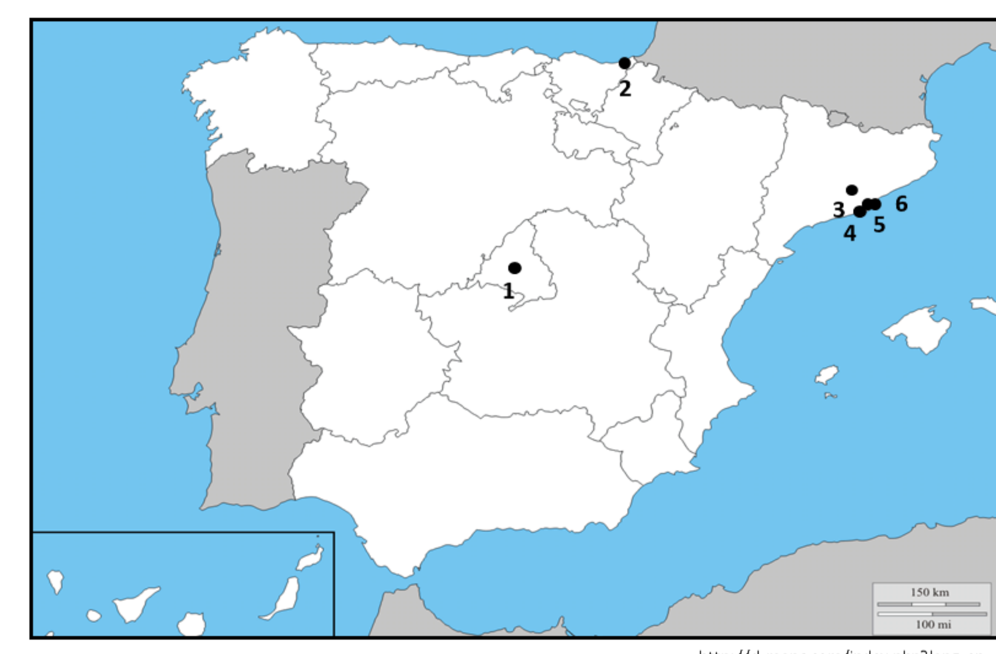
S3 Table. Demographic characteristics of the study population by hospital.

	Madrid					Catalonia				
	pre-PCV13 (2008-2009)		PCV13 (2012-2013)		*p*-value*	pre-PCV13 (2008-2009)		PCV13 (2012-2013)		*p*-value*
Demographics										
Age mean (years)	53,5		65,6		<0,001	60,9		64,5		<0,001
Male (%)	63,4		63,2		1	59,9		57,1		0,38
Source of isolates	**Episodes(n)**	**%**	**Episodes(n)**	**%**		**Episodes(n)**	**%**	**Episodes(n)**	**%**	
Blood culture	162	87,1	86	87,8	1	567	86	375	86	1
Cerebrospinal fluid	10	5,4	6	6,1	0,7916	32	4,9	27	6,2	0,3418
Pleural fluid	8	4,3	2	2	0,5023	42	6,4	21	4,8	0,2925
Ascitic fluid	2	1,1	3	3,1	0,3441	12	1,8	8	1,8	1
Other	4	2,2	1	1	0,6625	6	0,9	5	1,1	0,7617
Focus of infection	**Episodes(n)**	**Incidence****	**Episodes(n)**	**Incidence****		**Episodes(n)**	**Incidence****	**Episodes(n)**	**Incidence****	
Pneumonia	136	24,01	66	11,94	<0,001	486	7,48	333	5,31	<0,001
Meningitis	14	2,47	15	2,71	0,8065	59	0,91	40	0,64	0,1026
Peritonitis	4	0,71	6	1,09	0,7226	28	0,43	15	0,24	0,0866
Unknown focus	26	4,59	7	1,27	0,0022	68	1,05	35	0,56	0,0029
Other	6	1,06	4	0,72	0,7814	18	0,28	13	0,21	0,5356

	Basque country					All regions				
	pre-PCV13 (2008-2009)		PCV13 (2012-2013)		*p*-value*	pre-PCV13 (2008-2009)		PCV13 (2012-2013)		*p*-value*
Demographics										
Age mean (years)	61,7		64,8		0,2139	59.5		64.7		<0.001
Male (%)	53,8		60		0,4474	60.0		58.5		0.56
Source of isolates	**Episodes(n)**	**%**	**Episodes(n)**	**%**		**Episodes(n)**	**%**	**Episodes(n)**	**%**	
Blood culture	93	89,4	59	78,7	0,0579	822	86.6	520	85.4	0.43
Cerebrospinal fluid	5	4,8	8	10,7	0,1542	47	5.0	41	6.7	0.14
Pleural fluid	4	3,8	5	6,7	0,4945	54	5.7	28	4.6	0.35
Ascitic fluid	1	1	2	2,7	0,5726	15	1.6	13	2.1	0.42
Other	1	1	1	1,3	1	11	1.2	7	1.1	0.99
Focus of infection	**Episodes(n)**	**Incidence****	**Episodes(n)**	**Incidence****		**Episodes(n)**	**Incidence****	**Episodes(n)**	**Incidence****	
Pneumonia	78	11,38	52	7,47	0,0228	700	9.03	451	6.00	<0.001
Meningitis	7	1,02	11	1,58	0,4993	80	1.03	66	0.87	0.33
Peritonitis	1	0,15	2	0,29	0,5724	33	0.43	23	0.31	0.22
Unknown focus	8	1,17	6	0,86	0,768	102	1.31	48	0.64	<0.001
Other	10	1,46	4	0,57	0,1725	34	0.44	21	0.28	0.10

* *p*-value comparing pre-PCV13 and PCV13 periods ** Number of episodes per 100.000 population.

S3. Figure. Hospital's location, demographics and number of episodes by period



http://d-maps.com/index.php?lang=en

Hospital	Region	pre-PCV13 (2008-2009)			PCV13 (2012-2013)		
		Number of episodes	Age mean (years)	Male (%)	Number of episodes	Age mean (years)	Male (%)
1.- Hospital General Universitario Gregorio Marañón	Madrid	186	53,5	63,4	98	65,6	63,2
2.- Hospital Universitario Donostia	Basque Country	104	61,7	53,8	75	64,8	60
3.- Corporació Sanitària Parc Taulí	Catalonia	101	62,1	59,4	79	62,3	46,8
4.- Hospital Universitari de Bellvitge	Catalonia	286	62,4	62,6	161	64,8	59,6
5.- Hospital Universitari Vall d'Hebron	Catalonia	168	56,8	54,8	107	64,4	52,3
6.- Hospital Universitari Germans Trias i Pujol	Catalonia	104	62,3	61,5	89	66,1	67,2

Objective 2. To explore the impact of the introduction of pneumococcal conjugate vaccines on multidrug-resistant *Streptococcus pneumoniae* isolates over a 25-year period and its impact on the incidence and mortality of IPD.

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A historical perspective of MDR invasive pneumococcal disease in Spanish adults

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Objectives: To analyse the clonal dynamics and clinical characteristics of adult invasive pneumococcal disease (IPD) caused by MDR and penicillin-non-susceptible (PNS) pneumococci in Spain.

Methods: All adult IPD episodes were prospectively collected (1994–2018). *Streptococcus pneumoniae* isolates were serotyped, genotyped and tested for antimicrobial susceptibility. Changes in the incidence of IPD were analysed and risk factors contributing to MDR were assessed by logistic regression.

Results: Of 2095 IPD episodes, 635 (30.3%) were caused by MDR/PNS isolates. Over the study period, the incidence of MDR/PNS-IPD decreased (IRR 0.70; 95% CI 0.53–0.93) whereas that of susceptible isolates remained stable (IRR 0.96; 95% CI 0.80–1.16). A reduction of resistance rates to penicillin (−19.5%; 95% CI −37% to 2%) and cefotaxime (−44.5%; 95% CI −64% to −15%) was observed. Two clones, Spain^{9V}-ST156 and Denmark^{14}-ST230, accounted for 50% of current resistant disease. Among current MDR/PNS isolates, 45.8% expressed serotypes not covered by the upcoming PCV15/PCV20 vaccines. MDR/PNS episodes were associated with older patients with comorbidities, nosocomial acquisition and higher 30 day mortality. MDR/PNS pneumococci were not independently associated with 30 day mortality in multivariate analysis [OR 0.826 (0.648–1.054)].

Conclusions: Our study shows an overall reduction of MDR/PNS isolates in adults after the introduction of pneumococcal conjugate vaccines. However, a significant proportion of current resistant isolates are not covered by any of the upcoming PCV15/PCV20 vaccines. The burden of resistant disease is related to older patients with underlying conditions and caused by two major clones. Our data show that MDR is not a statistically significant factor related to increased mortality.

Introduction

Penicillin-non-susceptible (PNS) and MDR pneumococci emerged as a serious health threat in the late 1970s.[1] In Spain, the rate of penicillin-resistant pneumococci increased rapidly reaching percentages of 40% among invasive disease throughout the 1990s.[2,3] Likewise, macrolide resistance rose in parallel with the introduction of new drugs such as clarithromycin or azithromycin.[4] Both phenomena were associated with the spread of a few clones, the nomenclature for which was standardized by the Pneumococcal Molecular Epidemiology Network (PMEN).[5] In Spain, antibiotic resistance was linked to four PMEN clones (Spain^{23F}-ST81, Spain^{6B}-ST90, Spain^{9V}-ST156 and Spain^{14}-ST18) and the ST88 clone (serotype 19F).[4,6] Three of them (Spain^{23F}-ST81, Spain^{6B}-ST90 and Spain^{9V}-ST156) were globally detected and contributed to the worldwide increase of antibiotic resistance.[6–11]

In pneumococci, β-lactam resistance is due to amino acid changes within the transpeptidase domain of PBPs (mainly PBP1A, 2B and 2X).[12] These modifications are usually acquired by homologous recombination resulting in mosaic genes. Resistance to other antimicrobials such as erythromycin, tetracycline or chloramphenicol is associated with the acquisition of integrative conjugative elements (ICEs), which frequently harbour multiple resistance determinants.[13]

The introduction of pneumococcal conjugate vaccines (7-valent –PCV7–, 10-valent –PCV10– and 13-valent –PCV13–) resulted in a major change in the epidemiology of pneumococcal disease. After PCV7 introduction, major MDR clones expressing

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vaccine types decreased reducing the rate of penicillin resistance.[14,15] In contrast, the effect on macrolide resistance varied in different countries and in patients in different age groups. In adults from our area the rate of macrolide resistance remained stable, associated with both dissemination of ICE-borne resistance genes and the spread of macrolide-resistant clones,[13] while the rate of macrolide resistance in isolates from children decreased.[4] A similar reduction in macrolide resistance among isolates from children was also observed after the introduction of PCV7 in Germany.[16] In Spain, PCV7 was licensed in June 2001, but it was only recommended for children at high risk of infection. Its introduction occurred gradually (on a voluntary basis) and the estimated vaccine uptake for children under 2 years of age ranged from 22%–29% in 2002–04 to 45%–50% in 2005–06.[17,18] PCV7 was replaced by PCV13 in 2010 and the estimated coverage for children remained around 55%[19] until it was finally included into the paediatric vaccine calendar in 2016 (2+1 schedule). Although the 23-valent pneumococcal polysaccharide vaccine (PPV23) is recommended for vaccination of people over 65 years of age, the reported vaccination rate in our area is low (38.8%).[20] In adults, PCV13 is only recommended for patients with underlying diseases.[21]

In the present study, we analysed the dynamics of PNS and MDR clones causing invasive pneumococcal disease (IPD) in adults over a 25 year period. Our results are an overview of past and present pneumococcal resistant clones in Spain in the era of conjugate vaccines.

Materials and methods

Study setting and invasive disease surveillance

This study was conducted at Hospital de Bellvitge, a teaching hospital that serves an adult population of nearly 600 000 inhabitants. Since 1987, all laboratory-detected IPD episodes in adults (≥18 years old) are collected and recorded in a database. Since genotyping (PFGE) is routinely performed from 1994 onwards, this study includes isolates from a 25 year period (1994–2018). IPD was defined as the growth of *Streptococcus pneumoniae* in normally sterile fluids in a patient with signs and symptoms of infection (https://wwwn.cdc.gov/nndss/conditions/invasive-pneumococcal-disease/case-definition/2017/). Trends in the incidence of IPD were studied using the total number of adult inhabitants as denominator (available at https://www.idescat.cat/). The serotype incidence was estimated assuming that the distribution of isolates with missing serotype was the same as from the typed strains. Based on PCVs introduction, the study was divided into five periods: pre-PCV (1994–2001), early-PCV7 (2002–05), late-PCV7 (2006–10), early-PCV13 (2011–15) and late-PCV13 (2016–18). In order to analyse changes in blood culture practices or demographic changes, we added data on the incidence of community acquired bacteraemia due to *Escherichia coli* and *Staphylococcus aureus*, which are also prospectively collected.

Bacterial strains, susceptibility test, serotyping and molecular typing

Pneumococcal isolates were identified and serotyped at the Spanish Pneumococcal Reference Laboratory. Susceptibility to seven antimicrobials (penicillin, cefotaxime, erythromycin, clindamycin, tetracycline, co-trimoxazole and levofloxacin) was tested by microdilution following the CLSI criteria.[22] Because of the key importance of β-lactams in clinical practice, MDR was defined as non-susceptible to penicillin (MIC ≥0.12 mg/L) and to ≥2 other antimicrobials among the following: erythromycin or clindamycin, chloramphenicol, tetracycline, co-trimoxazole and levofloxacin.[23] Penicillin-non-susceptible isolates resistant to less than two other groups were categorized as PNS. XDR isolates were defined as PNS additionally resistant to ≥4 antimicrobials.

PFGE (SmaI) was performed on all available isolates and PFGE patterns were compared with the reference PMEN clones.[5] A selection of isolates belonging to major clusters (>10 isolates) were further studied by MLST.[24]

Serotypes were classified into: PCV7 (4, 6B, 9V, 14, 18C, 19F and 23F), additional PCV13 (1, 3, 5, 6A, 7F and 19A), and non-PCV13 serotypes. There are two upcoming PCVs: PCV15 (PCV13 serotypes plus 33F and 22F) and PCV20 (PCV13 plus serotypes 8, 10A, 11A, 12F, 15B, 22F and 33F).

Clinical data

Clinical data were prospectively collected, including age, sex, origin of infection (pneumonia, meningitis and others), acquisition (extrahospitalary or nosocomial), comorbidities, severity of underlying diseases (McCabe & Jackson score) and 30 day mortality. Differences were assessed through SPSS software package (SPSS, version 23) using the χ^2 test or Fisher's exact test, when appropriate. Adjusted analyses for mortality (prognostic variables adjusted included age, sex, acquisition, severity of underlying diseases and source of infection) were carried out by means of logistic regression models and fitted in the final model to determine the adjusted ORs and 95% CI. The statistical significance level was set at $P < 0.05$ (two-sided).

Ethics

This work was approved by The Clinical Research Ethics Committee of Hospital Universitari de Bellvitge (PR395/19). Since the study involved no intervention and the results would not be accurate without the collection of all episodes, written informed consent was waived. Patient data were always protected according to the local committee and national standards.

Results

Overall incidence of IPD

Over the study period, 2095 episodes were collected. As previously described, the incidence of IPD increased after PCV7 introduction.[15] The incidence rose from 13.9 to 19.4 episodes/100 000 population from the pre-PCV to the late-PCV7 period (IRR 1.39; 95% CI 1.24–1.56) and decreased to 12.7/100 000 in the early-PCV13 (IRR 0.65; 95% CI 0.57–0.75; late-PCV7 versus early-PCV13) (Figure 1). Thereafter, the incidence of IPD stabilized, being 12.1/100 000 in 2018. Since 2012, non-PCV13 serotypes have been predominant (73.7% of IPD during 2016–18). Overall, there was a non-significant reduction in the incidence of IPD of −13.1% (IRR 0.87; 95% CI 0.74–1.02; pre-PCV versus late-PCV13). It should be noted that the incidence of *E. coli* and *S. aureus* bacteraemia progressively increased over time (IRR 1.49; 95% CI 1.39–1.61 for *E. coli* and IRR 1.33; 95% CI 1.14–1.55 for *S. aureus*).

IPD caused by MDR and PNS isolates

There were 396 MDR (18.9%) and 239 PNS (11.4%) isolates (Table 1). Their proportions over the study fluctuated from 16.5% to 21.0% (MDR isolates) and from 9.3% to 13.8% (PNS isolates). The incidence of resistant disease (MDR plus PNS) remained stable after PCV7 introduction (IRR 1.09; 95% CI, 0.89–1.34; pre-PCV versus late-PCV7) and decreased after PCV13 introduction (IRR 0.64; 95% CI, 0.48–0.87; late-PCV7 versus late-PCV13), which led to an overall decrease throughout the period studied (IRR 0.70; 95% CI, 0.53–0.93). Interestingly, among MDR isolates, those classified as

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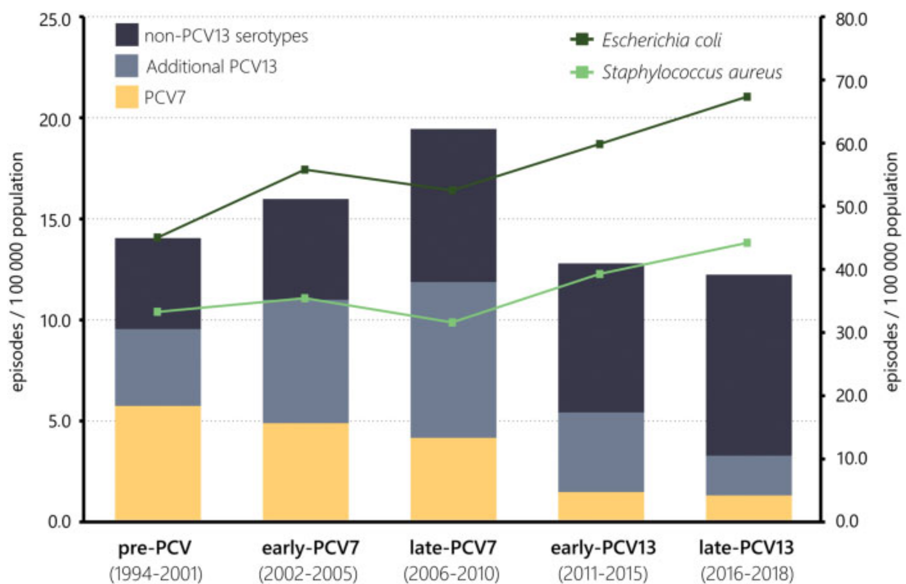

Figure 1. Evolution of the incidence of the adult IPD. Bars show the incidence of IPD caused by PCV7 (yellow), additional PCV13 (grey) and non-PCV13 serotypes (dark blue) by period. Lines show the incidence of community acquired bacteraemia due to *E. coli* (dark green, referred to secondary axis) and *S. aureus* (light green). PCV7 serotypes: 4, 6B, 9V, 14, 18C, 19F and 23F. Additional PCV13 serotypes: 1, 3, 5, 7F, 6A, 19A. Non-PCV13 serotypes: serotypes not included in PCV13. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

XDR (PNS plus resistance to ≥4 antimicrobials) drastically fell. This was related to the disappearance of the historical Spain^{23F}-ST81 (PMEN1) and Spain^{6B}-ST90 (PMEN2) clones, which were mainly expressing serotypes included in PCV7. In the last period, 62.7% of MDR/PNS isolates expressed non-PCV13 serotypes and 47.5% expressed serotypes not covered by the upcoming PCV15 and PCV20 vaccines [Figure S1 (Figure S1 is available as Supplementary data at *JAC* Online)].

Antimicrobial susceptibility

Antimicrobial resistance rates throughout the study are shown in Figure 2(a). Penicillin-non-susceptibility decreased from 34.8% to 28.0% ($P=0.07$) and resistance to cefotaxime fell from 18.5% to 10.3% ($P=0.006$). Resistance rates of chloramphenicol and co-trimoxazole also showed a statistically significant decrease (from 15.5% to 5.1% and from 42.8% to 25.7%, respectively). Resistance rates of erythromycin increased (from 18.7% to 24.8%, $P=0.06$). Resistance rates remained stable for the remaining antimicrobials. In regard to MIC_{50} and MIC_{90} values (Figure 2b), declines over time were observed for penicillin MIC_{90} (from 2 to 1 mg/L) and chloramphenicol MIC_{90} (from >8 mg/L to 4 mg/L).

Evolution of major PNS and MDR clones throughout the study period

A total of 607 (95.6%) MDR or PNS isolates were available for molecular typing. Figure 3 shows the proportion of major genotypes by period. Four clones (Spain^{23F}-ST81, Sweden^{15A}-ST63, ST88^{19F} and Spain^{6B}-ST90) were responsible for 70% of the MDR-IPD before PCVs. Three of them, namely Spain^{23F}-ST81, Spain^{6B}-ST90 and ST88^{19F}, have disappeared. In regard to the emerging clones, two capsular switching variants of Denmark^{14}-ST230 clone expressing serotypes 24F and 19A, and a serotype 6C variant of the Poland^{6B}-ST315 (ST386) clone accounted for half of MDR isolates in the late-PCV13 period. The emergence and expansion of a serotype 19A variant of the Taiwan^{19F}-ST236 clone (ST320) in the late-PCV7 period has been contained after the introduction of PCV13 in 2010.

With respect to PNS isolates, a single clone (Spain^{9V}-ST156) dominated all periods. Even though it has shown a decreasing trend over years, this genotype (mainly expressing serotype 11A) accounted for more than 50% of IPD episodes caused by PNS after PCV13. Among the remaining PNS clones, only the Colombia^{23F}-ST338 is a current significant cause of IPD, related to the non-vaccine serotype 23B.

Clinical characteristics

Clinical characteristics of 2083 patients were analysed. Because patients with MDR or PNS pneumococci showed no clinical differences, they were grouped for the analysis (Table S1). Our data showed that patients with IPD due to MDR/PNS isolates were significantly older ($P<0.001$) and had more comorbidities ($P=0.032$) in the last period (Table S2). Furthermore, the percentage of clinical presentations other than pneumonia or meningitis increased significantly ($P=0.035$). 30 day mortality remained stable over time ranging from 26% to 22% ($P=0.693$).

When comparing IPD episodes caused by MDR/PNS isolates with those caused by penicillin-susceptible pneumococci, MDR/PNS episodes were associated with older patients ($P=0.004$), higher McCabe score ($P<0.001$), nosocomial acquisition ($P<0.001$), existence of comorbidities ($P<0.001$) and prior antibiotic therapy ($P<0.001$) (Table 2). Also, 30 day mortality was significantly higher for MDR/PNS disease (24.1% versus 16.6%, $P<0.001$). In spite of that, the estimation through logistic regression models after adjusting for other prognostic variables (age,

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Table 1. Incidence of MDR, PNS and penicillin-susceptible pneumococci by period

Group	Pre-PCV (1994–2001)		Early-PCV7 (2002–05)		Late-PCV7 (2006–10)		Early-PCV13 (2011–15)		Late-PCV13 (2016–18)		Risk ratio[b] (95% CI)		
	n (%)	incidence[a]	*n* (%)	incidence[a]	*n* (%)	incidence[a]	*n* (%)	incidence[a]	*n* (%)	incidence[a]	pre-PCV versus late-PCV7	late-PCV7 versus late-PCV13	pre-PCV versus late-PCV13
MDR+PNS	207 (35)	4.9	110 (29)	4.6	162 (27)	5.3	96 (31)	3.9	60 (28)	3.4	1.09 (0.89–1.34)	**0.64 (0.48–0.87)**[c]	**0.70 (0.53–0.93)**[c]
MDR	125 (21)	2.9	68 (18)	2.8	98 (16)	3.2	65 (21)	2.6	40 (19)	2.3	1.09 (0.84–1.42)	0.71 (0.49–1.02)	0.77 (0.54–1.10)
PNS	82 (14)	1.9	42 (11)	1.8	64 (11)	2.1	31 (10)	1.3	20 (9)	1.1	1.08 (0.78–1.50)	**0.54 (0.33–0.90)**[c]	**0.59 (0.36–0.96)**[c]
XDR[d]	48 (8)	1.1	22 (6)	0.9	10 (2)	0.3	4 (1)	0.2	1 (0)	0.1	**0.29 (0.15–0.57)**[c]	0.17 (0.02–1.36)	**0.05 (0.01–0.36)**[c]
Susceptible	387 (65)	9.1	270 (71)	11.3	432 (73)	14.1	217 (69)	8.8	154 (72)	8.7	**1.55 (1.35–1.78)**[c]	**0.62 (0.52–0.74)**[c]	0.96 (0.80–1.16)
All IPD	594	13.9	380	15.9	594	19.4	313	12.7	214	12.1	**1.39 (1.24–1.56)**[c]	**0.63 (0.54–0.73)**[c]	0.87 (0.74–1.02)
E. coli	1919	45.0	1335	55.8	1611	52.5	1477	59.8	1189	67.3	**1.17 (1.09–1.25)**[c]	**1.28 (1.19–1.38)**[c]	**1.49 (1.39–1.61)**[c]
S. aureus	443	10.4	265	11.1	303	9.9	303	12.3	244	13.8	0.95 (0.82–1.10)	**1.40 (1.18–1.66)**[c]	**1.33 (1.14–1.55)**[c]

[a]Incidence is shown as episodes per 100 000 population.
[b]Risk ratios between periods representing the impact of the introduction of PCV7, PCV13 and the impact over the whole study period.
[c]Statistically significant results are highlighted in bold.
[d]XDR isolates are also included into the MDR group.

sex, acquisition, severity of underlying diseases and source of infection) showed that mortality was related to factors other than MDR/PNS disease [OR 1.234 (0.968–1.573), Table S3].

Discussion

Data on temporal analysis of the evolution of clones related to penicillin-non-susceptibility or MDR in pneumococci are scarce. In this work, we used a historical series to analyse MDR/PNS disease over the last 25 years adding important data to the knowledge of pneumococcal infections.

First, we showed that the incidence of IPD in adults showed a modest reduction over the past 25 years. In Spain, the expansion of some non-PCV7 serotypes (1, 7F and 19A) resulted into an overall increase in the incidence of IPD after PCV7.[15,25] This situation was reversed after PCV13 introduction[26,27] and the current incidence of IPD is lower than it was before PCV7, although the differences were not statistically significant. However, over this long period of time other factors could have influenced the burden of IPD such as increased life expectancy or changes in blood culture protocols,[28] among others. For this reason we also analysed community acquired bacteraemia due to *E. coli* and *S. aureus*, which significantly increased. In parallel, we have observed a progressive increase in the mean age of patients with IPD. All things considered, our data seem to indicate that the benefits of PCVs could be underestimated.

A second important issue is the impact of successive introduction of PCVs on the incidence of IPD caused by MDR/PNS pneumococci. In the 2000s, with low vaccine coverage, the introduction of PCV7 weakly affected the burden of resistant disease in Spain. In fact, the decrease of antibiotic-resistant PCV7 serotypes 19F, 23F, 14 and 9V was balanced by the emergence of MDR non-PCV7 serotypes 19A and 24F.[13,29] After PCV13, with increasing vaccine coverage in children, a sharp decrease of both susceptible and resistant disease was observed, the latter mainly due to a decrease in serotype 19A isolates, which in our area were associated with clones Denmark[14]-ST230 and Taiwan[19F]-ST236. Currently, the niche left by serotype 19A has not been filled, which means a significantly lower incidence of resistant disease. Taken together, these findings highlight the importance of PCVs targeting serotype 19A in terms of reducing the incidence of MDR, as described.[30,31]

The decrease of antimicrobial resistance as a collateral benefit of the PCVs was described in the USA after PCV7.[14] Our data show a reduction of resistance rates for most antimicrobials, with the exception of erythromycin, clindamycin and tetracycline. However, antimicrobial consumption should also be taken into account.[32] Published data on the evolution of antimicrobial consumption in Spain showed an increase of penicillin consumption (from 7.9 to 13.8 DDDs per 1000 inhabitants per day) and a reduction of macrolides (from 3.2 to 2.8 DDDs per 1000 inhabitants per day) over 1998–2018 (https://www.ecdc.europa.eu/en/antimicrobial-consumption/database/trend-country). These data support the beneficial impact of PCVs on reducing β-lactam resistance, but also show a variable effect on resistance to macrolides depending on age group and countries. All things considered, it suggests greater success in controlling highly clonal chromosomal resistance than that mediated by ICEs, which spreads horizontally. However, the shift from short- to long-acting macrolides (azithromycin), which

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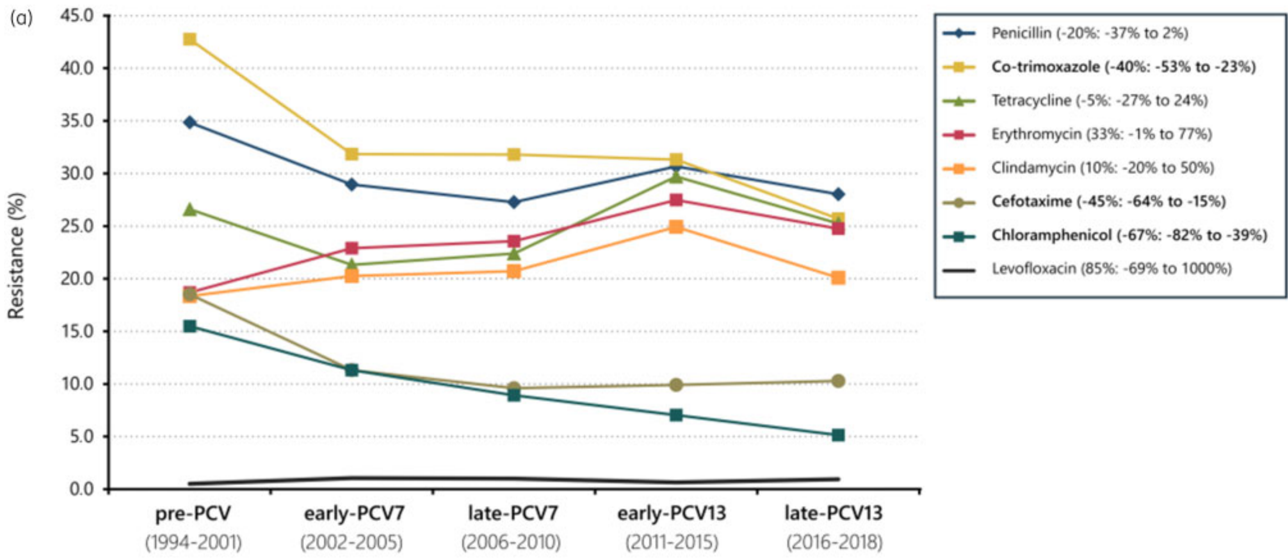

(b)

ANTIMICROBIAL MIC (mg/L)		pre-PCV (1994-2001)	early-PCV7 (2002-2005)	late-PCV7 (2006-2010)	early-PCV13 (2011-2015)	late-PCV13 (2016-2018)
Penicillin	MIC_{50}	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03
	MIC_{90}	2	2	1	1	1
Cefotaxime	MIC_{50}	<0.06	<0.06	<0.06	<0.06	<0.06
	MIC_{90}	1	1	0.5	0.5	1
Erythromycin	MIC_{50}	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
	MIC_{90}	>16	>16	>16	>16	>16
Clindamycin	MIC_{50}	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
	MIC_{90}	>0.5	>0.5	>0.5	>0.5	>0.5
Tetracycline	MIC_{50}	≤1	≤1	≤1	≤1	≤1
	MIC_{90}	>4	>4	>4	>4	>4
Chloramphenicol	MIC_{50}	4	4	4	4	4
	MIC_{90}	>8	>8	4	4	4
Co-trimoxazole	MIC_{50}	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
	MIC_{90}	>2	>2	>2	>2	>2
Levofloxacin	MIC_{50}	≤1	≤1	≤1	≤1	≤1
	MIC_{90}	≤1	≤1	≤1	≤1	≤1

Figure 2. Evolution of antimicrobial susceptibility (a) and MIC_{50}/MIC_{90} values (b) over the study period. The percentage of change and the 95% CI are shown in parentheses (differences between the pre-PCV and the late-PCV13 periods). Statistically significant differences ($P<0.01$) are highlighted in bold. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

has been associated with increased antimicrobial resistance,[33] could also explain these findings.

A third conclusion of our work is the high proportion of penicillin-non-susceptibility (always above 25%) over the whole period. Penicillin-non-susceptibility was initially associated with a few worldwide distributed clones[7] that for a large part disappeared after the PCVs introduction. The exception was Spain^{9V}-ST156 clone (PMEN3) that has been the major contributor to PNS over the whole period, currently expressing serotype 11A (formerly PCV7 serotypes 9V and 14).[34,35] Isolates related to the Spain^{9V}-ST156 clone have been described among MDR pneumococci in Canada[23] and as emerging lineages related to serotypes 35B and 11A in the USA.[36,37] Interestingly, it has recently been described that capsular switching between serotypes 9V and 11A occurs more frequently than expected, probably because they share some polysaccharide components.[38] Hopefully, the introduction of a next generation of vaccines targeting serotype 11A could reduce its incidence. Nevertheless, as up to half of PNS isolates still express vaccine

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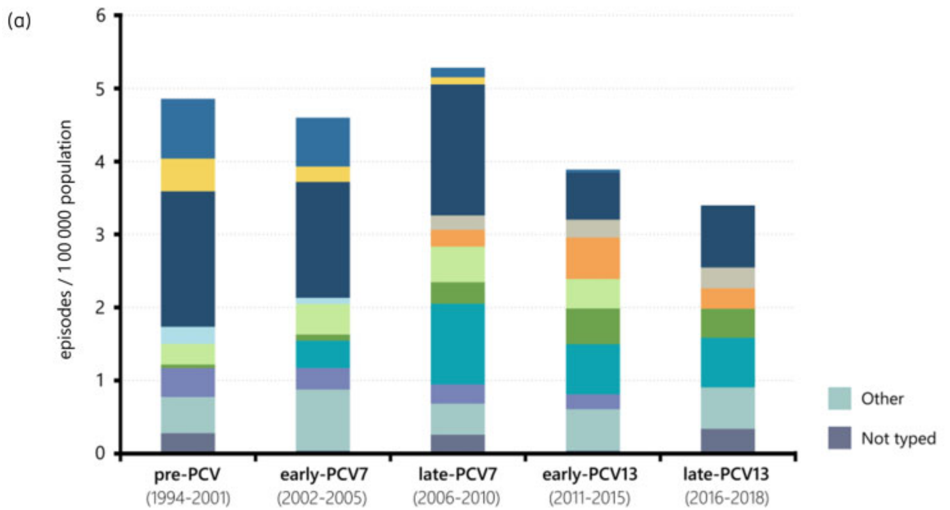
(a)



(b)

	PMEN	CLONE	ISOLATES	SEROTYPES (n)	RESISTANCE PHENOTYPE
	PMEN1	Spain^{23F} (ST81)	56	23F (40), 19A (10), 19F (6)	PEN, CTX, ERY, CLI, CHL, TET, SXT
	PMEN2	Spain^{6B} (ST90)	27	6B (27)	PEN, ERY, CLI, CHL, TET, SXT
	PMEN3	Spain^{9V} (ST156)	203	14 (103), 9V (82), 11A (15), Other (3)	PEN, CTX, SXT
	PMEN5	Spain^{14} (ST18)	12	14 (12)	PEN, CTX, ERY, CLI, CHL, TET, SXT
	PMEN26	Colombia^{23F} (ST338)	17	23B (12), 23F (3), Other (2)	PEN, SXT
	PMEN14	Taiwan^{19F} (ST236)	26	19A (26)	PEN, CTX, ERY, CLI, TET, SXT
	PMEN32	Denmark^{14} (ST230)	72	24F (47), 19A (24), Other (1)	PEN, ERY, CLI, TET, SXT
	PMEN20	Poland^{6B} (ST315)	32	6C (24), 6B (8)	PEN, ERY, CLI, TET
	PMEN25	Sweden^{15A} (ST63)	47	15A (29), 19F (11), 15F (4), Other (3)	PEN, ERY, CLI, TET
	-	ST88	37	19F (35), Other (2)	PEN, ERY, CLI, CHL, TET, SXT

Figure 3. Contribution of major genotypes to the incidence of MDR/PNS-IPD over the study period (a), related serotypes and resistance profiles (b). Genotypes representing less than 10 isolates over the study were grouped into 'other'. 'Not typed' refers to isolates not available for molecular typing. Resistance phenotype refers to the most frequent phenotype of each clone. PEN, penicillin; CTX, cefotaxime; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; TET, tetracycline; SXT, co-trimoxazole. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

serotypes, herd protection seems not to be enough to decrease resistant disease in adults. It has to be noted that vaccination has been voluntary in Catalonia with a low estimated coverage among children under 5 years of age (55% in 2013).[39] Probably, the recent introduction of PCV13 into the official paediatric schedule in 2016 will help to reduce MDR/PNS disease as we found in 2016–18.

Although most classic MDR clones, namely Spain^{23F}-ST81,[11] Spain^{6B}-ST90 and Spain^{14}-ST18, disappeared, others replaced them. For example, a serotype 24F variant of Denmark^{14}-ST230 emerged and is the primary cause of current MDR IPD. This serotype has been recently linked to the increase of paediatric meningitis in France.[40] Our data also proved the success of the ST386^{6C} clone (a 6C variant of Poland^{6B}-ST315 clone), which emerged after PCV7 in our geographical area[41] and abroad.[42–44] PCV13 has been associated with *in vitro* cross-reactivity between serotypes 6A and 6C.[45] Moreover, cross-protection after childhood vaccination with PCV13 has been suggested in Sweden when comparing the stabilization of IPD caused by serotype 6C in counties using PCV13 with those using PCV10 where 6C-IPD increased.[46] In this way, our study showed a maintained rate of IPD due to serotype 6C mainly associated with the pre-existent ST386-6C MDR clone.[41] In any case, as both clones (Denmark^{14}-ST230 and ST386^{6C}) share non-susceptibility to penicillin and macrolides/lincosamides/tetracycline resistance, this could limit the oral therapeutic options for pneumonia. Furthermore, both serotypes are not included in the next conjugate vaccines PCV15 and PCV20 so its incidence is not expected to be reduced in the short term.

The fourth important issue of this work is the analysis of the impact of antimicrobial resistance on the outcome of patients with pneumococcal bacteraemia, which remains the subject of debate.[47–49] On the contrary, host factors such as older age or the existence of comorbidities have been associated with worse prognosis.[50,51] Our series shows that MDR/PNS isolates are associated to factors related to poor prognosis: older age, high McCabe score, nosocomial acquisition, existence of comorbidities and prior antibiotic therapy. All this makes mortality at 30 days significantly

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Table 2. Differences in clinical characteristics between susceptible and MDR/PNS isolates causing IPD episodes

Clinical characteristics	Susceptible isolates (n = 1452), number of episodes (%)	MDR/PNS isolates (n = 631), number of episodes (%)	*P* value
Age (years), mean (±SD)	60.69 (±17.43)	63.10 (±17.58)	**0.004**[b]
Male	929 (64)	417 (66)	0.191
Bacteraemia	1349 (93)	574 (91)	0.077
Nosocomial acquisition[a]	93 (6)	79 (13)	<**0.001**[b]
Clinical presentation			
pneumonia	1104 (76)	463 (73)	0.216
meningitis	133 (9)	60 (10)	0.862
others	215 (15)	108 (17)	0.203
Underlying conditions			
current smoking	509 (35)	193 (31)	**0.026**[b]
alcohol abuse	204 (14)	77 (12)	0.143
Prior antibiotic therapy	278 (19)	299 (47)	<**0.001**[b]
Comorbidities			
one or more	1104 (76)	566 (90)	<**0.001**[b]
chronic pulmonary disease	286 (20)	124 (20)	0.516
chronic heart disease	236 (16)	121 (19)	0.060
diabetes mellitus	300 (21)	134 (21)	0.404
malignancies	355 (24)	206 (33)	<**0.001**[b]
liver cirrhosis	186 (13)	88 (14)	0.262
HIV infection	132 (9)	81 (13)	**0.007**[b]
chronic renal failure (E IV-V)	43 (3)	24 (4)	0.192
cerebrovascular disease/dementia	80 (6)	42 (7)	0.178
immunosuppressive therapy	244 (17)	167 (26)	<**0.001**[b]
Shock at presentation	257 (18)	111 (18)	0.503
McCabe & Jackson II-III	571 (39)	351 (56)	<**0.001**[b]
30 day mortality	241 (17)	152 (24)	<**0.001**[b]

Because patients with MDR or PNS pneumococci showed no clinical differences, they were grouped for the analysis (see Table S1).
[a]Acquisition has two categories: nosocomial or extrahospitalary.
[b]Statistically significant results are highlighted in bold.

higher in these patients, though not significant after adjusting. These findings highlight the importance of pneumococcal vaccination of the population at risk of IPD, which could also contribute to decrease the burden of resistant disease in adults.[52] However, a significant proportion of current MDR/PNS isolates is not covered by the current or upcoming vaccines, so due to the risk of having a resistant isolate, IPD in older patients with comorbidities continues to deserve special attention.

The main strength of our study is the systematical and prospective collection of IPD episodes performed by the same team minimizing potential biases. Also, the inclusion of extensive microbiological characterization gives comprehensive information of the evolution of MDR-IPD over time. Our study has also limitations: it focuses on a population of a specific geographical area and our findings could not be translated to other settings. Also, we have not estimated changes in the severity of seasonal influenza that could have influenced the incidence of IPD. However, since the potential impact of these factors should equally affect the population of susceptible isolates and MDRs, the overall resistance rates and the proportion of MDR isolates should not depend on them.

In summary, our study shows that the introduction of PCVs in children has had a modest impact on adult IPD caused by penicillin-susceptible isolates in our setting. However, a beneficial effect was observed on the incidence of MDR/PNS isolates and therefore on the overall rates of resistance. The emergence of some MDR lineages expressing non-vaccine serotypes such as 11A or 24F needs close surveillance. Although MDR isolates appear in older patients with comorbidities, our data show that this factor is not related to significantly increased mortality.

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Transparency declarations

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Supplementary data

Tables S1 to S3 and Figure S1 are available as Supplementary data at *JAC* Online

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Supplementary data

Figure S1. Proportion of susceptible (top) and MDR/PNS (bottom) isolates covered by current and upcoming conjugate vaccines by period. The figure shows the percentage of episodes by period. PCV7 serotypes (blue): 4, 6B, 9V, 14, 18C, 19F and 23F; Additional PCV13 serotypes (red): 1, 3, 5, 6A 7F and 19A; Additional PCV15 serotypes (green): 33F and 22F; Additional PCV13 serotypes (grey): 8, 12F, 11A, 10A, and 15B. Non-vaccine serotypes refers to serotypes not included in PCV7, PCV13, PCV15 or PCV20.

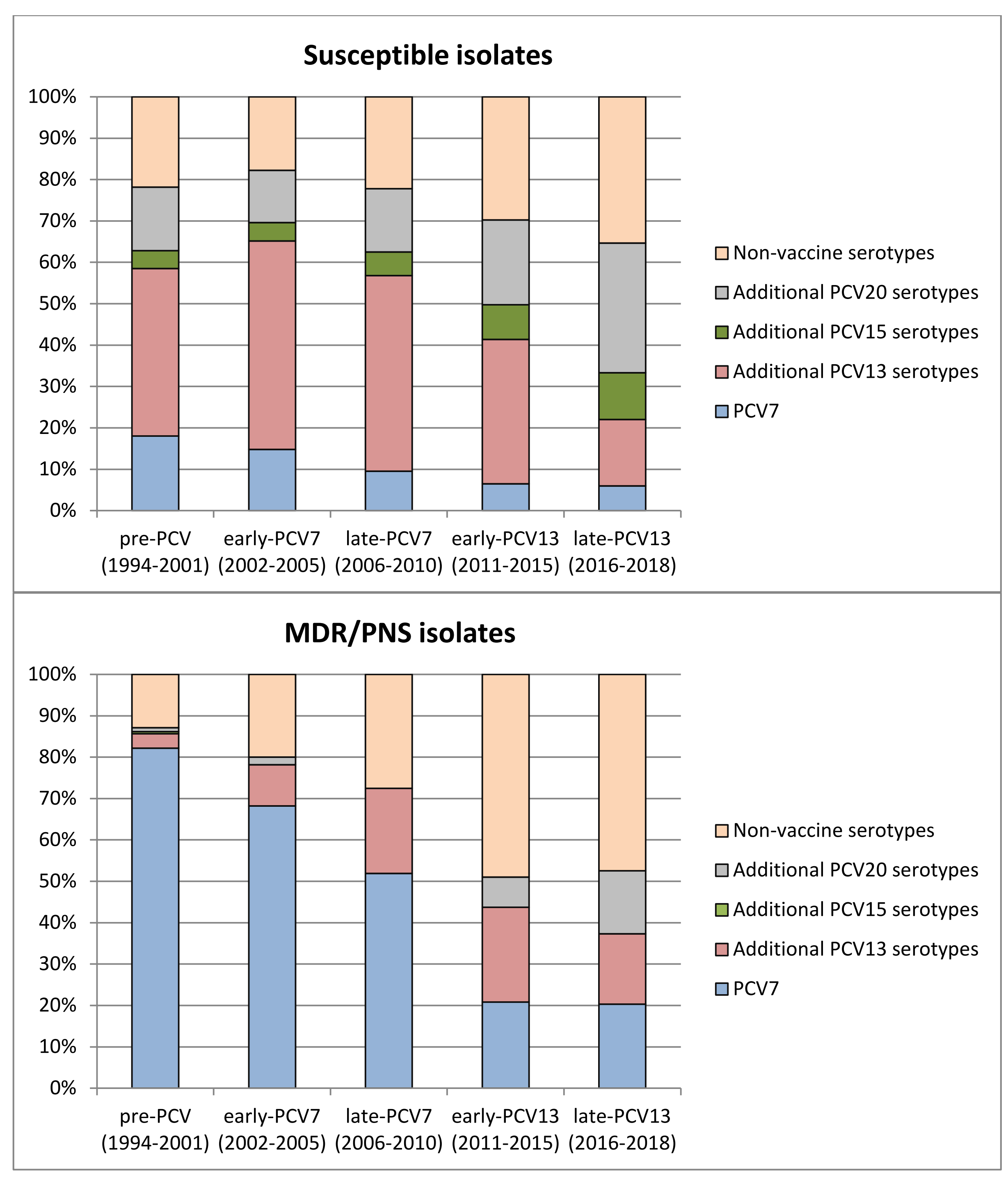

Table S1. Differences in clinical characteristics between susceptible, MDR and PNS isolates. For categorical variables we used two-by-three contingency tables. Variables have two categories (ex. male vs female) and the "isolates" have three categories (susceptible, PNS and MDR isolates). A significant *p*-value means that patients from one category group (Susceptible, PNS and MDR isolates) have significant differences from the others.

	Susceptible isolates n = 1452	PNS isolates n = 237	MDR isolates n = 394	*p*-value
Age mean (SD)	60.69 (+/- SD 17.4)	62.71 (+/-SD 17.4)	63.31 (+/-SD 17.7)	**0.009**[b,c]
Male Sex, n (%)	929 (64)	158 (67)	259 (66)	0.635
Bacteraemia	1349 (93)	222 (94)	352 (89)	**0.044**[b]
Nosocomial Acquisition[a]	93 (6)	30 (13)	49(12)	**<0.001**[b]
Clinical presentation				
Pneumonia	1104 (76)	187 (79)	276 (70)	**0.019**[b]
Meningitis	133 (9)	22 (9)	38 (10)	0.958
Others	215 (15)	28 (12)	80 (20)	**0.007**[b]
Underlying conditions				
Current smoking	509 (35)	79 (33)	114 (29)	0.074
Alcohol abuse	204 (14)	34 (14)	43 (11)	0.249
Prior antibiotic therapy	278 (19)	103 (43)	196 (50)	**<0.001**[b]
Comorbidities				
One or more	1104 (76)	212 (89)	354 (90)	**<0.001**[b]
Chronic pulmonary disease	286 (20)	40 (17)	84 (21)	0.684
Chronic heart disease	236 (16)	50 (21)	71 (18)	0.163
Diabetes mellitus	300 (21)	45 (19)	89 (23)	0.794
Malignancies	355 (24)	75 (32)	131 (33)	**0.003**[b]
Liver cirrhosis	186 (13)	28 (12)	60 (15)	0.656
HIV infection	132 (9)	30 (13)	51 (13)	**0.034**[b]
Chronic renal failure (E IV-V)	43 (3)	11 (5)	13 (3)	0.683
Cerebrovascular disease/Dementia	80 (6)	14 (6)	28 (7)	0.488
Immunosuppressive therapy	244 (17)	60 (25)	107 (27)	**<0.001**[b]
Shock at presentation	257 (18)	43 (18)	68 (17)	0.960
McCabe&Jackson II-III	571 (39)	118 (50)	233 (59)	**<0.001**[b]
30-day mortality	241 (17)	57 (24)	95 (24)	**<0.001**[b]

[a]Acquisition have two categories: nosocomial vs. extrahospitalary
[b]Statistically significant results are highlighted in bold.
[c]Mean age was significantly different between patients with susceptible isolates and those with MDR isolates (*p*=0.009; Student t-test)

Table S2. Differences in clinical characteristics among MDR/PNS isolates between prePCV (1994-2001), PCV7 (2002-2010) and PCV13 (2011-2018) periods. For categorical variables we used two-by-three contingency tables. Variables have two categories (ex. male vs. female) and the "PCV-periods" have three categories (PrePCV7-period, PCV7 period and PCV13-period). A significant *p*-value means that patients from one category group (PrePCV7-period, PCV7 period and PCV13-period) have significant differences from the others.

	PrePCV7-period 1994-2001 n = 203	**PCV7-period 2002-2010 n = 272**	**PCV13-period 2011-2018 n = 156**	***p*-value**
Age mean (SD)	60.35 (+/- SD 18.42)	61.66 (+/-SD 17.53)	69.11 (+/-SD 15.2)	**<0.001**[b,c]
Male Sex, n (%)	140 (69)	179 (66)	98 (63)	0.472
Bacteraemia	181 (89)	252 (93)	141 (90)	0.406
Nosocomial Acquisition[a]	39 (19)	26 (10)	14(9)	**0.002**[b]
Clinical presentation				
Pneumonia	160 (79)	202 (74)	101 (65)	**0.010**[b]
Meningitis	15 (7)	27 (10)	18 (11.5)	0.394
Others	28 (14)	43 (16)	37 (24)	**0.035**
Underlying conditions				
Current smoking	75 (37)	90 (33)	28 (18)	**<0.001**[b]
Alcohol abuse	46 (23)	23 (8.5)	8 (5)	**<0.001**[b]
Prior antibiotic therapy	103 (51)	124 (46)	72 (46)	0.506
Co-morbidities				
One or more	184 (91)	235 (86)	147 (94)	**0.032**[b]
Chronic pulmonary disease	36 (18)	46 (17)	42 (27)	**0.030**[b]
Chronic heart disease	27 (13)	49 (18)	45 (29)	**<0.001**[b]
Diabetes mellitus	30 (15)	50 (18)	54 (35)	**<0.001**[b]
Malignancies	51 (25)	90 (33)	65 (42)	**0.003**[b]
Liver cirrhosis	29 (14)	37 (14)	22 (14)	0.976
HIV infection	37 (18)	39 (14)	5 (3)	**<0.001**[b]
Chronic renal failure (E IV-V)	10 (5)	6 (2)	8 (5)	0.188
Cerebrovascular disease/Dementia	13 (6)	17 (6)	12 (8)	0.834
Immunosuppressive therapy	40 (20)	75 (28)	52 (33)	**0.031**[b]
Shock at presentation	31 (15)	62 (23)	18 (11.5)	**0.008**[b]
McCabe&Jackson II-III	120 (59)	144 (53)	87 (56)	0.405
30-day mortality	53 (26)	64 (23.5)	35 (22)	0.693

[a]Acquisition have two categories: nosocomial vs. extrahospitalary
[b]Statistically significant results are highlighted in bold.
[c]Mean age was significantly different between patients in the PrePCV7-period and those in the PCV13-period ($p<0.001$; Student t-test)

Table S3. Logistic regression analysis of factors associated to MDR/PNS-IPD.

Risk factor	Adjusted OR (95% CI)	*p*-value
Age[a]	1.007 (1.002-1.013)	**0.012**[b]
Sex, male	1.038 (0.845-1.275)	0.725
Nosocomial acquisition	1.859 (1.338-2.583)	**<0.001**[b]
McCabe&Jackson II and III	1.825 (1.482-2.248)	**<0.001**[b]
30-day mortality	0.826 (0.648-1.054)	0.125
Source of infection		
Pneumonia	reference	
Meningitis	0.951 (0.726-1.245)	0.714
Other	1.368 (0.978-1.912)	0.067

[a]Age was included in the model as a continuous variable
[b]Significant results are highlighted in bold

Objective 3. To analyse the major recombination events occurred in the β-lactam-resistant PMEN3 (CC156) *Streptococcus pneumoniae* clone that allowed it to persist over the time as a cause of invasive disease despite the introduction of PCVs.

Càmara J, Cubero M, Martín-Galiano AJ, García E, Grau I, Nielsen JB, Worning P, Tubau F, Pallarés R, Domínguez MÁ, Kilian M, Liñares J, Westh H, Ardanuy C. **Evolution of the β-lactam-resistant *Streptococcus pneumoniae* PMEN3 clone over a 30 year period in Barcelona, Spain.** J Antimicrob Chemother. 2018 Nov 1;73(11):2941-2951.

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Evolution of the β-lactam-resistant *Streptococcus pneumoniae* PMEN3 clone over a 30 year period in Barcelona, Spain

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Objectives: To analyse the epidemiology and genetic evolution of PMEN3 (Spain^{9V}-156), a penicillin-non-susceptible clone of *Streptococcus pneumoniae*, causing invasive pneumococcal disease (IPD) in Barcelona during 1987–2016.

Methods: WGS was performed on 46 representative isolates and the data were used to design additional molecular typing methods including partial MLST, PCR-RFLP and detection of surface-exposed proteins and prophages, to assign the remaining isolates to lineages. The isolates were also subjected to antimicrobial susceptibility testing.

Results: Two hundred and twenty-seven adult cases of IPD caused by PMEN3 were identified. PMEN3 caused mainly pneumonia (84%) and the 30 day mortality rate was 23.1%. Evidence of recombination events was found, mostly in three regions, namely the capsular operon (associated with capsular switching) and adjacent regions containing *pbp2x* and *pbp1a*, the *murM* gene and the *pbp2b–ddl* region. Some of these genetic changes generated successful new variant serotype lineages, including one of serotype 11A that is not included in the current PCV13 vaccine. Other genetic changes led to increased MICs of β-lactams. Notably, most isolates also harboured prophages coding for PblB-like proteins. Despite these adaptations, the ability of this clone to cause IPD remained unchanged over time, highlighting the importance of its core genetic background.

Conclusions: Our study demonstrated successful adaptation of PMEN3 to persist over time despite the introduction of broader antibiotics and conjugate vaccines. In addition to enhancing understanding of the molecular evolution of PMEN3, these findings highlight the need for the development of non-serotype-based vaccines to fight pneumococcal infection.

Introduction

Invasive pneumococcal disease (IPD) is an important cause of mortality worldwide, mainly affecting children under 5 years old, the elderly and immunocompromised patients.[1,2] The pneumococcus is a highly recombinant microorganism and its genomic plasticity has given it the ability to respond efficiently to environmental challenges.[3,4] This has also contributed to the emergence of new genotypes that have ensured its persistence over time, driven by pressure from antimicrobials and the human immune system. The introduction of antibiotics has driven the emergence of β-lactam-resistant and MDR pneumococci since the late 1970s.[5] Even when isolated in different countries, some of these strains have shown close relationships; these were the major successful clones, nomenclature for which was established by the Pneumococcal Molecular Epidemiology Network (PMEN).[6] On the other hand, the pneumococcal capsular polysaccharide is a major virulence factor and has been the basis of vaccine formulation.

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In Spain, four pneumococcal vaccines have been licensed: the 23-valent polysaccharide vaccine (PPSV23), from the mid-1980s, and three pneumococcal conjugate vaccines (PCVs; PCV7, PCV10 and PCV13, licensed in 2001, 2009 and 2010, respectively). The introduction of PCVs has led to a worldwide reduction in the incidence of IPD and has changed the epidemiology of pneumococcal serotypes.[2,7]

During the 1990s, four major pneumococcal clones were associated with penicillin resistance in Spain: PMEN1 (Spain^{23F}-1), PMEN2 (Spain^{6B}-2), PMEN3 (Spain^{9V}-3) and PMEN5 (Spain^{14}-5).[8] Of these, the extensively studied PMEN1 has been proposed as the donor of antibiotic-resistant determinants.[9] Besides penicillin resistance, the Spanish clones share a high propensity to occur in older people with comorbidities. While PMEN1, PMEN2 and PMEN5 have almost disappeared, PMEN3 has remained prevalent, accounting for up to 10% of all IPD isolates.[10] Originally associated with serotype 9V and ST156, the PMEN3 clone is known to express several capsular types.[8] For example, a serotype 14 variant emerged in the late 1990s and progressively replaced the former MDR serotype 14 clone (PMEN5).[2,8,11] Thereafter, the introduction of PCV7 (targeting both the 9V and 14 serotypes) resulted in a sustained reduction in PMEN3 until now, when an increase in isolates has been detected following the emergence of a serotype 11A variant.[12]

In this study, we analysed the clinical and molecular epidemiology of the PMEN3 clone over a 30 year period in Barcelona. WGS was used to reveal the successive adaptations of this clone that allowed it to persist over time.

Methods

Bacterial strains, serotyping and antibiotic susceptibility testing

Since 1979, all pneumococci recovered from adult patients admitted to Hospital Universitari de Bellvitge have been prospectively collected as a part of an ongoing research database. However, the first available isolate dates from 1987, so we included IPD isolates collected over a 30 year period from 1987 to 2016. Isolates were identified by conventional methods and serotyped at the Spanish Pneumococcal Reference Laboratory. Antimicrobial susceptibility was tested by microdilution following CLSI methods and criteria.[13]

Molecular typing

Molecular typing was performed by PFGE and MLST. PFGE (SmaI or ApaI) was introduced in our laboratory in 1995 and all available isolates were tested retrospectively. Band patterns were compared with known PMEN clones.[6] A selection of isolates were additionally typed by MLST.[14]

WGS analysis

Forty-six isolates, representing all PMEN3 serotype/genotype combinations and covering the whole period, were studied by WGS (Table S1, available as Supplementary data at *JAC* Online). Genomic DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN) and adjusted to 0.2 ng/μL using Qubit® (Thermo Fisher Scientific, USA). Libraries were prepared with Nextera XT® and multiplexed in two MiSeq runs (Illumina, USA) with a 2×150 bp (paired) read length protocol.

The quality of raw data was assessed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads were further processed using Geneious 9.1.7 (Biomatters). Ends were trimmed, duplicate reads were removed, errors were corrected and reads were assembled *de novo* using the Geneious assembler (medium sensitivity).

Initial analyses included the study of differences in SNPs using CSI Phylogeny 1.4.[15] The generated data were subsequently managed with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). To localize regions with low identity within the chromosome, we constructed a basic local alignment search tool (BLAST) atlas in the CGview Comparison Tool,[16] using the oldest isolate (156_9V_1987) as a reference. Assembled contigs from strain 156_9V_1987 were mapped and annotated according to ATCC 700699, which produced a unique annotated chromosome. The remaining 45 isolates were also mapped to ATCC 700699 and were compared with the inferred chromosome, constructing a BLAST atlas with regions coloured by identity.

In silico analysis included the study of genes encoding resistance to β-lactams (i.e. *pbp1a*, *pbp2x*, *pbp2b* and *murM*), quinolones (*gyrA* and *parC*) and co-trimoxazole (*folA* and *folP*). Additionally, a search was performed to identify acquired resistance genes (ResFinder 2.1).[17] Lineages were named based on MLST, serotype and isolation year of the oldest isolate for which we had WGS data (e.g. MLST/serotype/year). The differentiation of lineages within serotype 14 and 9V isolates not included in the WGS analysis was performed by PCR-RFLP (Table S2).

Raw data were deposited in the European Nucleotide Archive with accession numbers from ERS2201200 to ERS2201245.

Surface protein analysis

Surfotyping was performed based on the presence/absence of full/truncated versions of 17 genes.[18] The genes were detected by Prodigal[19] and annotated using BLAST and the Pfam database.[20] Homologues were aligned by Clustal Omega and Muscle. Phylogenetic trees were created using Mega 7.0[21] and branch quality was estimated by the bootstrap method with 1000 replicates.

Prophage analysis

The genomic sequences were searched for the presence of prophages (or remnants) using PHASTER[22] and *lytA*-like genes using BLAST, because most (if not all) pneumococcal prophages code for LytA-like lytic endolysins.[23] After finding a phage-like endolysin-coding gene, other potential prophage-coding genes were identified by sequence comparisons in Pfam.[20]

Analysis of clinical characteristics

Clinical data were collected, including age, sex, source of infection, acquisition, comorbidities and 30 day mortality. Differences were analysed by IBM SPSS, Version 23 (IBM Corp., Armonk, NY, USA), using the χ^2 test or Fisher's exact test, as appropriate. A two-sided P value of <0.05 was considered statistically significant.

Ethics

The clinical research ethics committee of Hospital Universitari de Bellvitge approved this research (PR001/18). Written informed consent was not considered necessary and any confidential information was protected according to national standards.

Results

Burden of the PMEN3 clone among adults with IPD in Barcelona

Over the 30 year period, we collected data for 2808 IPD-causing pneumococci. Serotype and molecular typing data were available

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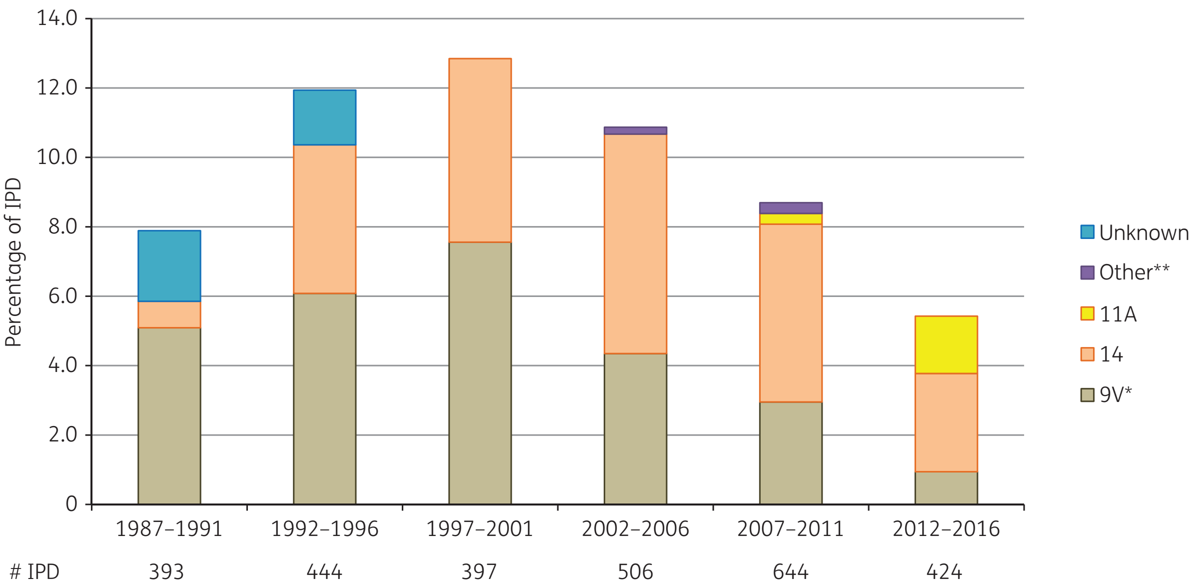

Figure 1. Distribution of major PMEN3 serotypes during the study period. Each bar reflects the frequency of PMEN3 among IPD isolates. Each colour represents a different capsular type. The first isolates expressing capsular types 9V, 14 and 11A were isolated in 1987, 1990 and 2011, respectively. *The serotype 9V group includes all serogroup 9 isolates in the first period. **Includes four isolates: two expressing serotype 19A, one expressing 23F and one expressing 9N/L. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

for 2441 isolates (86.9%) and 2136 isolates (76.1%), respectively. Given that the frequency of serotyped/genotyped strains was lower before 1996, the rate of PMEN3 isolates was estimated for that period (Table S3). Overall, this clone was a major cause of IPD, peaking at 12.8% in the 1997–2001 period (Figure 1) and decreasing progressively to 5.4% after PCV7 was introduced in 2001. Among the 2136 genotyped isolates, 227 belonged to PMEN3, mainly serotypes 9V ($n = 101$) and 14 ($n = 113$), which predominated in 1987–2001 and 2002–16, respectively. Other serotypes (19A, 23F and 9N/L) were sporadically detected until the emergence and further spread of serotype 11A in 2011.

Evolution of PMEN3 lineages over the study period

WGS data were used to construct a phylogenetic tree (Figure 2a). Six major clusters (lineages) could be identified, all originating from the ancestral $ST156^{9V}$ clone: lineage 1 (ST156/9V/1987), lineage 2 (ST838/9V/1996), lineage 3 (ST44/14/1991), lineage 4 (ST156/14/1993), lineage 5 (ST156/14/2001) and lineage 6 (ST6521/11A/2011). Of note, the emergence of lineage 6 (ST6521/11A/2011) occurred in a second step from lineage 2 (ST838/9V/1996). Lineage emergence and proportional changes are shown in Figure 2(b). Lineage 1 (ST156/9V/1987) was the most frequent until 1996, but was progressively replaced by lineage 2 (ST838/9V/1996). After 2001, lineages expressing serotype 14 predominated, with the serotype 11A lineage accounting for 29% of PMEN3 isolates in the 2012–16 period.

Major genetic differences between lineages

Most genetic regions had a high degree of identity (>99%, blue colours) but there were three regions with significantly lower identity (≤97%, red colours): the capsular operon and flanking regions (*pbp1a* and *pbp2x* genes), the *murM* region and the *pbp2b*–*ddl* region (Table 1 and Figure 3). Therefore, we hypothesized that these regions were generated by recombination events and decided to study them in detail. Sequences were aligned, alleles were assigned and regions were compared, using the BLAST algorithm to search for putative donors.

Capsular operon

The capsular operon was identical for all serotype 9V isolates and, in the same way, for all those harbouring capsule 11A. However, some differences were detected among serotype 14 strains, with the nucleotide sequences of lineages 3 (ST44/14/1991) and 5 (ST156/14/2001) being identical to each other, but different to those of lineage 4 (ST156/14/1993). These differences consisted of 13 SNPs in the *wzg* gene (seven amino acid substitutions) and 1 SNP in both the *wzh* gene and the *wzd* gene (one amino acid change each). These three genes of the capsular operon of lineages 3 and 5 were identical to PMEN5 (accession number FWTC00000000), whereas those of the lineage 4 isolates differed from PMEN5 and were identical to CGSP14 (accession number CP001033).

pbp1a

Four lineages (1, 2, 3 and 6) harboured a *pbp1a* gene identical to that of PMEN1 (ATCC 700669, allele A). Lineage 5 harboured a *pbp1a* that was identical to PMEN5 (ATCC 700902, allele C) and lineage 4 harboured a *pbp1a* that was identical to URAspn5128, a serotype 14 strain from Portugal (allele B; accession number AM779378). The corresponding PBP1a alleles showed 10 (allele B) and 28 (allele C) amino acid substitutions compared with allele A.

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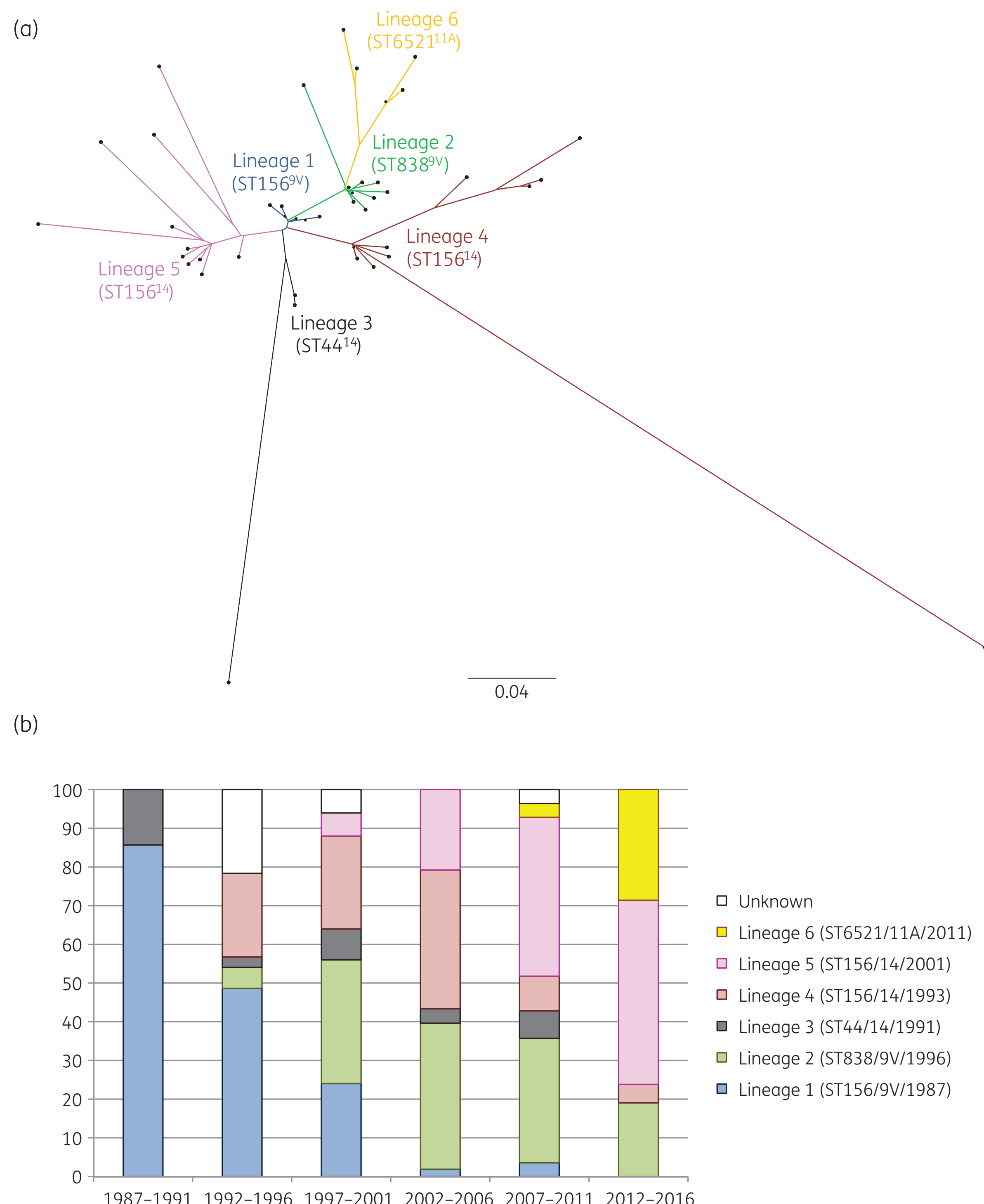

Figure 2. Phylogenetic tree of 46 PMEN3 pneumococcal isolates (a) and major genotypes (b) during the study period. (a) Branches are coloured according to the lineage to which they belong: lineage 1 (ST156/9V/1987), blue; lineage 2 (ST838/9V/1996), green; lineage 3 (ST44/14/1991), black; lineage 4 (ST156/14/1993), red; lineage 5 (ST156/14/2001), pink; and lineage 6 (ST6521/11A/2011), yellow. (b) Bars show the relative frequency of each lineage among the cases of IPD caused by PMEN3. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

pbp2x

Allele A, which was identical to the *pbp2x* gene of PMEN1, was found in the oldest strains, lineage 1 (ST156/9V/1987), lineage 3 (ST44/14/1991) and five isolates of lineage 4 (ST156/14/1993). Allele B (five amino acid differences to PMEN1) was found in lineages 2 (ST838/9V/1996) and 6 (ST6521/11A/2011).

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Table 1. Characteristics of the major recombination areas by lineages and MICs

		Region 1			Region 2		Region 3	Penicillin MIC (mg/L)		Amoxicillin MIC (mg/L)		Cefotaxime MIC (mg/L)	
Lineage	Description	*pbp2x*[a]	Cps operon	*pbp1a*[a]	*pbp2b*[a]	*ddl*[a]	*murM*[a]	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
1	ST156/9V/1987	A	9V	A	A	1	A	1	2	1	2	1	1
2	ST838/9V/1996	**B**	9V	A	**B**	**90**	**B**	2	4	4	8	1	2
3	ST44/14/1991	A	**14**	A	A	1	A	1	2	1	8	1	2
4	ST156/14/1993	A	**14**[b]	**B**	A	1	A	1	2	2	2	1	2
5	ST156/14/2001	**C**	**14**	**C**	A	1	**C**	2	2	2	8	1	2
6	ST6521/11A/2011	**B**	**11A**	A	**B**	**90**	**B**	2	4	4	8	1	1

Cps, capsular.
Major genetic differences are shown in bold.
[a]Assigned alleles.
[b]When compared with the remaining serotype 14 isolates, the capsular operon of the strains of this lineage had 13 SNPs in the *wzg* gene (seven amino acid substitutions) and 1 SNP in both the *wzh* gene and the *wzd* gene (one amino acid change each).

The remaining isolates showed SNP variability within this region (Table S4).

pbp2b–ddl region

Lineages 1, 3, 4 and 5 shared *pbp2b* (allele A) and *ddl* (MLST allele 1) genes with PMEN1. A second group included lineages 2 (ST838/9V/1996) and 6 (ST6521/11A/2011) that shared the *pbp2b* (allele B) and *ddl* (allele 90) genes. Allele B of *pbp2b* shared a 98.5% nucleotide identity with *Streptococcus mitis* B6 (accession number NC_013853) while it had 95% identity with allele A (23 amino acid differences).

murM region

Allele A (closest to PMEN1, with two SNPs) was found in lineages 1, 3 and 4. Allele B was found in lineages 2 and 6 (93% nucleotide identity and 27 amino acid substitutions compared with PMEN1) and was related to the previously described *murM* allele, *murMB5* (nine amino acid differences, 98% nucleotide identity).[24] Allele C was found in lineage 5 (21 amino acid differences and 95% nucleotide identity with the PMEN1 allele).

***In silico* study of antimicrobial resistance**

Detailed information of the results of the *in silico* study of antimicrobial resistance is shown in Table S4.

β-Lactams

The original lineage 1 harboured PBPs (1a, 2b and 2x) that were identical to PMEN1 (penicillin non-susceptible), so penicillin non-susceptibility was deemed characteristic of this clone. It was associated with changes in the main domains of PBP1a (T371A in the STMK373 motif, P432T close to the SRN430 motif and the alteration of four consecutive residues from 574TSQF to 574NTGY), PBP2b (T446A in the 443SSNT motif) and PBP2x (T338A in the 337STMK motif, I371T, and R384G and L546V close to the 547KSG motif). Additionally, an increase in the amoxicillin MIC (from 1–2 to 4–8 mg/L) was observed in lineages 2 and 6 (ST838/9V/1996 and ST6521/11A/2011), which was associated with changes in PBP2b between residues 590 and 641, as previously described.[24]

Quinolones

All except one of the studied isolates were susceptible to levofloxacin and, consequently, only the resistant isolate showed an amino acid substitution related to quinolone resistance (ParC, S79F).

Co-trimoxazole

Co-trimoxazole resistance is associated with mutations in genes coding for dihydropteroate synthetase (*folP*) and dihydrofolate reductase (*folA*). Characteristic of PMEN3, all but one isolate were resistant to co-trimoxazole and harboured amino acid substitutions/insertions associated with resistance in both proteins. Forty-three isolates harboured an Arg insertion (P59GSS→P59G**R**SS) and two harboured a Ser-Tyr insertion (P59GSS→P59GS**SY**S) in dihydropteroate synthetase. Additionally, all isolates but two harboured the I100L change in dihydrofolate reductase.

Acquired resistance carried by integrative conjugative elements (ICEs)

Acquired ICEs were detected in five serotype 14 isolates: two harboured the macrolide efflux genetic assembly (mega) element [*mef*(A) and *msr*(D)], two had both *tet*(M) and *erm*(B) genes associated with the Tn*3872* transposon [one of them also had the Ω*cat* (pC194) element] and one harboured a Tn*6003* element carrying *tet*(M), *erm*(B) and *aph(3′)*-II genes (Table S1).

Changes in pneumococcal surface proteins

As intra-clonal phenotypic variability is usually linked to differences in surface proteins,[18] this subset of the proteome was analysed and compared with the lineage structure described above. A total of 28 strains showed an identical profile of accessory genes coding for surface proteins (i.e. *cbpJ*, *cbpL*, *diiA*-long, *nanC*, *rrgB*, *sp1796*, *srtD* and *zmpD* genes) as a hallmark of PMEN3 (Figure S1). A notable outlier was a lineage 4 strain (156SLV/14/2007), which lacked genes coding for the StrD and RrgB pilus proteins, but that had acquired PsrP, a virulence factor involved in pulmonary disease via binding to keratin 10. The allelic variability of the seven most immunogenic surface proteins encoded in the core genome was also evaluated (data not shown).[25] Among them, the CbpD variant of lineage 5 isolates, a murein hydrolase involved in competence

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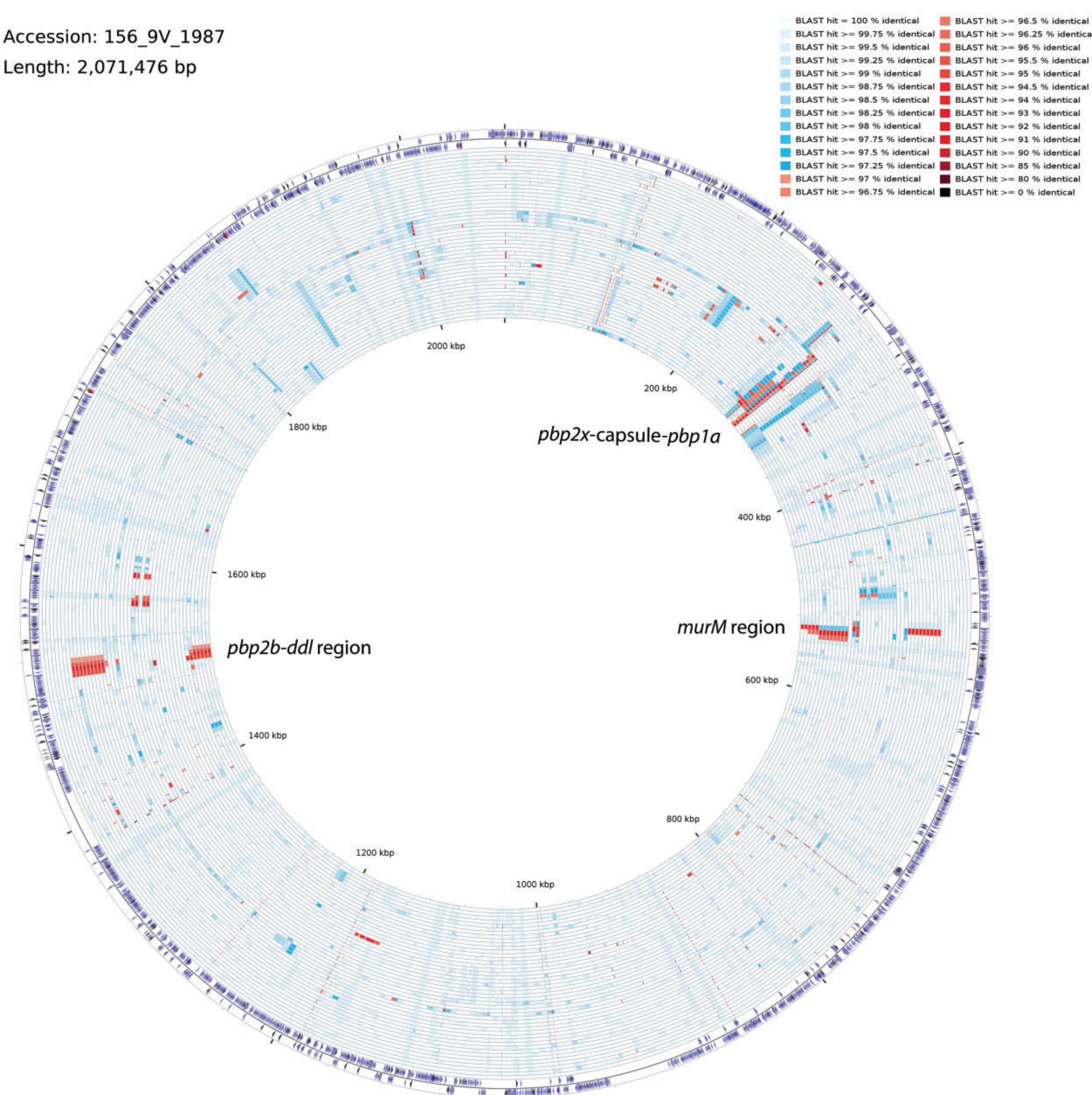

Figure 3. Chromosomal BLAST atlas of the PMEN3 isolates. Isolates are centripetally ordered by their lineage (lineages 1 to 6 from the outer to inner circles) and regions are coloured by their percentage of nucleotide identity with respect to the oldest isolate (ST156/9V/1987). Red regions show nucleotide sequence identities of ≤97%. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and fratricide,[26] showed five residue changes (three affecting the peptidoglycan-binding SH3 domain) with respect to the fully conserved variants of the other lineages. Ten isolates also carried an extra homologue of CbpA that showed differences in the amino-terminal region, resulting in a different affinity to factor H, the recruitment of which by CbpA minimizes the activity of the alternative complement pathway and increases the isolate's capacity to evade opsonophagocytosis.[27]

Detection of phage-related proteins

All isolates contained a 13 kb prophage remnant (PG1) and most ($n = 41$) were polylysogenic. Prophages were classified into five

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Table 2. Characteristics of the prophages found in this study; pneumococcal genomic sequences were aligned with the nucleotide sequences of strain D39

Prophage group	No. of lysogenic strains	*att*	nt positions[a]	Location[a]	Genome size (bp)	Integrase accession number (*n*)	Integrase number of amino acid residues	Endolysin accession number (*n*)
1	46	5′-TCCCTTTTTGTGTTA-3′	2946–2960	SPD_0003–SPD_0004	13662	WP_000704684 (44)	388	none
2A	8	5′-CTTTTTCATAATAATCTCCCT-3′	24016–24036[b]	SPD_0024–SPD_0025	34278	WP_000876735 (3)	382	WP_000350506 (1)
						WP_000876722 (1)		WP_050197950 (1)
						WP_000876726 (1)		new alleles (3)
						WP_024475415 (1)		unknown (3)
						WP_050216447 (1)		
						WP_050225784 (1)		
2B[c]	1	5′-CTTTTTCATAATAATCTCCCTTAAC-3′	231378–231402	SPD_0240–SPD_0241	>30000	WP_024475415 (1)	382	new allele (1)
3	40	5′-TTATAATTCATCCGC-3′	1415147–1415161	SPD_1394	41257	WP_000266847 (38)	375	WP_000350525 (32)
								new allele (1)
								unknown (5)
4	3	5′-TTCCTCCTACTTATCTATTCGTA-3′	1712414–1712436[d]	SPD_1711–SPD_1712	>22000	WP_000859011 (1)	475	new alleles (3)
						WP_000859013 (1)		
						WP_023396450 (1)		
5	7	5′-ACCWACATYTCMAYCAA-3′	1841057–1841073[e]	SPD_1861	>40000	WP_000633505 (4)	481	WP_088778480 (1)
						WP_024478469 (1)		WP_000350534 (1)
						WP_061597704 (1)		new alleles (3)
								unknown (1)

[a]Refers to nt positions and genes of the *S. pneumoniae* D39 genome (accession number CP000410).
[b]Two additional *att* sequences are present in the D39 genome, i.e. between genes SPD_0240 and SPD_0241 (231378–231398) and between genes SPD_0242 and SPD_0243 (233750–233770).
[c]More than 99% identical to the group 21A prophages described in this study.
[d]One additional *att* sequence is present in the D39 genome between genes SPD_1740 and SPD_1741 (1735090–1735112).
[e]The *att* sequence in D39 is 5′-ACCAACATCTCCACCAA-3′.

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Table 3. Clinical characteristics and outcomes of 212 IPD episodes caused by PMEN3 isolates

Characteristics	Number of episodes (%), unless otherwise stated
Age (years), average ± SD	63.1 ± 17.7
Male	133 (62.7)
Bacteraemia	192 (90.6)
Acquisition	
community acquired/healthcare related	182 (85.8)
nosocomially acquired	30 (14.2)
Current smoking	66 (31.1)
Alcohol abuse	32 (15.1)
Comorbidities	
one or more	180 (84.9)
chronic pulmonary disease	32 (15.1)
chronic heart disease	38 (17.9)
diabetes mellitus	35 (16.5)
malignancies	59 (27.8)
liver cirrhosis	21 (9.9)
HIV infection	22 (10.4)
immunosuppresive therapy	47 (22.2)
Source of infection	
pneumonia	178 (84.0)
meningitis	23 (10.8)
primary	5 (2.4)
abdominal/biliary tract	3 (1.4)
others	3 (1.4)
Shock	40 (18.9)
30 day mortality	49 (23.1)

groups (PG1 to PG5) by their integrase-coding gene (*int*) and the location of the attachment core sequence (*att*) in the genome (Table 2 and Figure S2a). Interestingly, as has previously been observed,[28] two closely related prophages (PG2A and PG2B) were inserted into two different locations in diverse isolates. Although prophages tend to integrate outside of coding sequences,[29] PG3 and PG5 were inserted into the coding regions of SPD_1394 and SPD_1861, respectively. The latter gene encodes for ComGC, the competence pilus major pilin, which is required for transformation.[30,31] Targeted disruption of competence genes is a mechanism by which mobile elements inhibit transformation events and prevent their elimination.[32]

Sequence comparisons with other pneumococcal genomes revealed the existence of similar prophages in otherwise unrelated strains (Figure S2b). Sequence alignments showed that only genes like *pblB* (coding for the phage antireceptor) and those located downstream of *pblB* and belonging to the lysis module[33] were conserved in all prophages (Figure S2c). Although the *pblB* gene is difficult to assemble with short-read sequence data, as noted previously, two apparently complete *pblB* genes were found (from PG2 and PG3). When compared with other homologues, the whole sequence diversity of *pblB* was significant (Figure S2d), which is perhaps not surprising since the PblB protein is responsible for interacting with the surface-located phage receptor(s). It should be underlined that whereas >90% amino acid identity existed among the 16 endolysin alleles (not shown), significant differences were found among the 15 different integrases (Figure S2e).

Clinical characteristics of patients

Clinical data were available for 212 episodes (Table 3 and Table S2). Most episodes were community acquired (85.8%), involved pneumonia (84.0%) and occurred in patients with at least one comorbidity (84.9%). The overall 30 day mortality was 23.1%. In addition, two findings were remarkable, though without reaching significance. First, the incidence of meningitis and the 30 day mortality were considerably higher for episodes involving serotype 11A. Second, there were fewer patients with comorbidities among IPD episodes due to lineage 4 (67.4%) than other lineages.

Discussion

Streptococcus pneumoniae is a pathogen that has adapted to the changing conditions of its environment. In this paper, we have described the genetic evolution of the PMEN3 clone[6] in Barcelona over a 30 year period, using WGS to explore successive adaptations to the introduction of new antimicrobials and PCVs.

Several major episodes of horizontal DNA transfer were detected in our study (Figure 4). One of the first was the emergence of a serotype 9V lineage (lineage 2, ST838), which showed increased β-lactam resistance in the 1990s when community use of this antibiotic drug class was extensive. The relationship between oral cephalosporin use and the increase in high-level penicillin resistance has been studied in Spain.[34] However, as the most remarkable difference between the former and the replacing lineage is a significant increase in the amoxicillin MIC (from 1–2 to 4–8 mg/L), the increased aminopenicillin consumption (e.g. amoxicillin/clavulanic acid) could have influenced this replacement. Anyhow, the rise of this lineage was associated with the acquisition of a region including the transpeptidase domain of PBP2b, which was related to *S. mitis*, highlighting the importance of commensal streptococci as resistance reservoirs.[35]

A second major event was the emergence of serotype 14 isolates (lineage 4 ST156/14/1993 and lineage 3 ST44/14/1991) representing one of the first recombination events described for *S. pneumoniae*.[11] The expansion of these isolates has been related to the rise of penicillin resistance in paediatric cases of IPD in Uruguay (29% in 1994 to 40% in 1997).[36] Notably, these lineages could spread after capsular switching, a phenomenon that did not occur with the sporadic serotypes 19A, 23F or 9N/L. Probably, there are synergisms between serotype and genotype that confer higher invasiveness and likelihood of spread.[37] Focusing on PMEN3, the supremacy of capsular type 14 over 9V, especially in combination with ST156, is supported by several reports linking serotype 14 with invasiveness.[38,39] However, we were unable to find differences in the clinical characteristics of patients with IPD due to PMEN3 in terms of the lineages or serotypes. The PMEN3 genetic background appeared to have equal clinical invasiveness, irrespective of whether it expressed capsular type 9V, 14 or 11A.

WGS was extremely helpful in revealing horizontal DNA transfer events that are not easily recognized by analysing classical markers (e.g. same serotype, MLST and antibiotic profile). For example, ST156[14] isolates were noted to differ in *pbp1a* and capsular genes in the USA in the late 1990s;[40] using WGS, we were able to

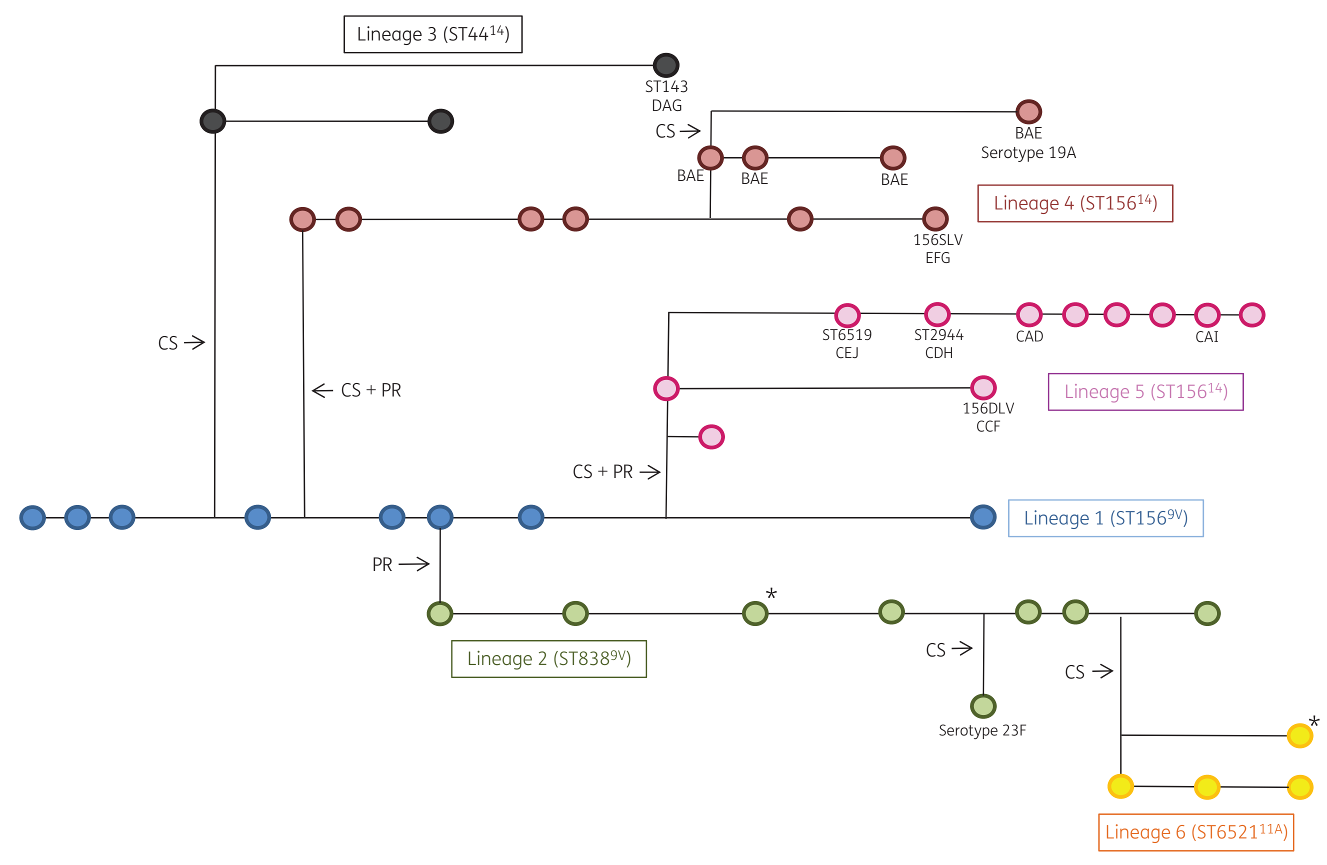

Figure 4. Proposed genetic evolution of PMEN3 over the study period. The timeline is represented by the bottom horizontal line. Each circle represents a single isolate studied by WGS. Isolates of the same lineage have the same colour. Arrows indicate the major recombination events in each lineage. Strains with variation in MLST and/or PBP profile are indicated below the circle. A three-letter code was used for the PBP profile (PBP1a, 2b and 2x). CS, capsular switching; DLV, double-locus variant; PR, *pbp* recombination; SLV, single-locus variant. *Two strains isolated in the same year. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

show that lineages 4 and 5 (serotype 14) emerged independently from the original 156^{9V} lineage through two different genetic events that led to major differences in *pbp1a*, *pbp2x* and *murM*. Although we were unable to find the putative donors of all regions, the PMEN5[41] clone could be a good candidate to be a donor for lineage 5, at least regarding the capsule and the *pbp1a* gene that they share. However, the diversity of *pbp2x* observed among some of the isolates of this lineage suggests that additional recombination occurred. Given that this occurred with serotype 9V and the newest lineage showed higher β-lactam MICs, this again highlighted the role of these antibiotics in pneumococcal evolution.

The latest event detected in the evolution of PMEN3 in Spain has been the emergence of serotype 11A isolates. Although a similar episode was reported in 2000 among pneumococci identified in Israeli children,[42] the isolates reported here seem to have a distinct origin. In fact, WGS analysis shows that they emerged from lineage 2 rather than the original 156^{9V} clone, which appeared to have been the origin of the isolates from the Israeli children. In Spain, serotype 11A isolates related to PMEN3 emerged in 2004 and spread after the introduction of PCV13.[12] Of note, the use of oral amoxicillin to treat pneumonia could be compromised by the high amoxicillin MICs. Serotype 11A has classically been associated with carriage and low invasiveness,[38,39] mainly related to CC53 (ST62). Conversely, our data provide evidence for the potential virulence of 11A, indicating high rates in meningitis and in its 30 day mortality. These findings emphasize the importance of the genetic background of PMEN3 to invasiveness. The reduction in mortality during the PCV era has been linked to the decline of PCV7 serotypes[43,44] and it is likely that the genetic background of the bacteria played a major role. The emergence of an invasive vaccine escape clone linked to high mortality, such as PMEN3 with serotype 11A, is a worrying complication that warrants further surveillance. For instance, an MDR serotype 35B (non-PCV13) variant of PMEN3 is emerging in the USA and could fill the gap left by the PCV13 serotypes of this clone.[45]

The presence of temperate phages is a background characteristic that supports the invasiveness of PMEN3. Recently, their role in virulence and mortality has been linked to PblB, a prophage-encoded

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protein with an important role in different infectious disease processes.[46] With the exception of defective PG1, all detected prophages encode PblB proteins. There is also evidence of LytA involvement in pneumococcal virulence[47] and, taking its similarity to the prophage endolysins into account, the latter may also demonstrate immunogenic relevance. It should be realized that fluoroquinolones can induce pneumococcal prophages[46] with a concomitant increase in PblB expression and, presumably, the endolytic enzyme, which may lead to increased virulence. Besides phages, surface proteins also have a role in the pathogenic potential of pneumococci and we found variability in emerging lineages that indicated a wide capacity to interact with host molecules to either increase adhesion or evade the immune system.[18] Nevertheless, the higher prevalence of the preferred combination of surface proteins indicates that such combinations are important to the virulence of PMEN3 through its genetic background.

Our study gives valuable insight into the evolution of PMEN3 over a long period, but does have some important limitations. The selection of only invasive isolates could have meant that we missed events that occurred exclusively in non-invasive isolates, though we consider these putative missed recombinants to be irrelevant because they lose their invasiveness. Limitations also arise from the methodology used; because the identity analysis was based on whether a reference isolate was present, the acquisition of novel genetic material not present in the reference could have remained undetected. Nevertheless, most genetic events that drive the evolution of pneumococci exist in its core genome (e.g. *pbp* genes, *murM* and the capsular operon) or are related to transferable elements (phages and resistance associated with ICEs). Given that these have been studied independently, the impact of this limitation should be marginal.

In summary, we have shown how the PMEN3 clone has adapted over the past 30 years in response to the use of broad-spectrum β-lactams and the introduction of conjugate vaccines. The recent emergence of a serotype 11A lineage is a perfect illustration of the ability of this clone to persist over time, regardless of human interventions. In the future, vaccines not based on serotypes may be needed to fight these successful and highly transformable clones.

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Transparency declarations

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Supplementary data

Tables S1 to S4 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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Table S1. Complete list of isolates studied by Whole genome sequencing (WGS).

Isolate	Identification	Year	Serotype	MLST	Acquired Resistance	Lineage	Surfotype profile	Pbp1a Profile	Pbp2b Profile	Pbp2x profile	MurM profile
1	156_9V_1987	1987	9V	156	No	Lineage 1 (ST156/9V/1987)	A	A	A	A	A
2	156_9V_1988	1988	9V	156	No	Lineage 1 (ST156/9V/1987)	A	A	A	A	A
3	156_9V_1989	1989	9V	156	No	Lineage 1 (ST156/9V/1987)	A	A	A	A	A
4	44_14_1991	1991	14	44	No	Lineage3 (ST44/14/1991)	H	A	A	A	A
5	156_9V_1992	1992	9V	156	No	Lineage 1 (ST156/9V/1987)	E	A	A	A	A
6	156_14_1993	1993	14	156	No	Lineage 4 (ST156/14/1993)	A	B	A	A	A
7	156_14_1994	1994	14	156	No	Lineage 4 (ST156/14/1993)	A	B	A	A	A
8	156_9V_1995	1995	9V	156	No	Lineage 1 (ST156/9V/1987)	A	A	A	A	A
9	156_9V_1996	1996	9V	156	No	Lineage 1 (ST156/9V/1987)	A	A	A	A	A
10	44_14_1996	1996	14	44	No	Lineage3 (ST44/14/1991)	F	A	A	A	A
11	838_9V_1996	1996	9V	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
12	156_14_1998	1998	14	156	No	Lineage 4 (ST156/14/1993)	A	B	A	A	A
13	156_9V_1998	1998	9V	156	No	Lineage 1 (ST156/9V/1987)	A	A	A	A	A
14	156_14_1999	1999	14	156	tet(M), cat(pC194), erm(B)	Lineage 4 (ST156/14/1993)	A	B	A	A	A
15	838_9V_1999	1999	9V	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
16	156_14_2001	2001	14	156	No	Lineage 5 (ST156/14/2001)	E	C	A	C	D
17	143_14_2001	2001	14	143	tet(M), erm(B), aph(3')-III	Lineage3 (ST44/14/1991)	G	D	A	G	F
18	156_14_2002	2002	14	156	No	Lineage 4 (ST156/14/1993)	J	B	A	E	A
19	156_14_2002_2	2002	14	156	No	Lineage 5 (ST156/14/2001)	A	C	A	C	A
20	838_9V_2003	2003	9V	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
21	156_14_2003	2003	14	156	mef(A),msr(D)	Lineage 4 (ST156/14/1993)	A	B	A	E	A
22	838_9V_2003_2	2003	9V	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
23	156_14_2004	2004	14	156	No	Lineage 4 (ST156/14/1993)	A	B	A	A	A
24	6519_14_2005	2005	14	6519	No	Lineage 5 (ST156/14/2001)	F	C	E	J	C
25	156_14_2006	2006	14	156	mef(A),msr(D)	Lineage 4 (ST156/14/1993)	A	B	A	E	A
26	838_9V_2006	2006	9V	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
27	156SLV_14_2007	2007	14	156SLV	No	Lineage 4 (ST156/14/1993)	I	E	F	G	A
28	2944_14_2007	2007	14	2944	No	Lineage 5 (ST156/14/2001)	F	C	D	H	C
29	156DLV_14_2008	2008	14	156DLV	tet(M), erm(B)	Lineage 5 (ST156/14/2001)	A	C	C	F	E
30	838_23F_2008	2008	23F	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
31	156_9V_2008	2008	9V	156	No	Lineage 1 (ST156/9V/1987)	A	A	A	A	A
32	838_9V_2009	2009	9V	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
33	156_14_2009	2009	14	156	No	Lineage 5 (ST156/14/2001)	A	C	A	D	C
34	156_19A_2009	2009	19A	156	No	Lineage 4 (ST156/14/1993)	A	B	A	E	A
35	838_9V_2010	2010	9V	838	No	Lineage2 (ST838/9V/1996)	A	A	B	B	B
36	156_14_2010	2010	14	156	No	Lineage 5 (ST156/14/2001)	B	C	A	C	C
37	156_14_2011	2011	14	156	No	Lineage 5 (ST156/14/2001)	A	C	A	C	C
38	6521_11A_2011	2011	11A	6521	No	Lineage 6 (ST6521/11A/2011)	D	A	B	B	B
39	156_14_2012	2012	14	156	No	Lineage 5 (ST156/14/2001)	A	C	A	C	C
40	156_14_2013	2013	14	156	No	Lineage 5 (ST156/14/2001)	C	C	A	I	C
41	838_9V_2013	2013	9V	838SLV	No	Lineage2 (ST838/9V/1996)	E	A	B	B	B
42	6521_11A_2013	2013	11A	6521	No	Lineage 6 (ST6521/11A/2011)	D	A	B	B	B
43	156_14_2014	2014	14	156	No	Lineage 5 (ST156/14/2001)	C	C	A	C	C
44	6521_11A_2015	2015	11A	6521	No	Lineage 6 (ST6521/11A/2011)	D	A	B	B	B
45	838_11A_2015	2015	11A	838	No	Lineage 6 (ST6521/11A/2011)	D	A	B	B	B
46	838_11A_2015_2	2015	11A	838	No	Lineage 6 (ST6521/11A/2011)	D	A	B	B	B

Isolate	Focus of infection	Source	Minimal inhibitory concentration (mg/L)									
			penicillin	amoxicillin	cefotaxime	erythromycin	clindamycin	Phenotype	tetracycline	chloramphenicol	co-trimoxazole	levofloxacin
1	Meningitis	CSF	1	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
2	Meningitis	CSF	2	2	2	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
3	Meningitis	CSF	2	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
4	Pneumonia	Bronchoalveolar lavage	2	1	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
5	Pneumonia	Bronchoalveolar lavage	1	1	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
6	Meningitis	CSF	2	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
7	Pneumonia	Blood culture	2	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
8	Pneumonia	Blood culture	2	1	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
9	Pneumonia	Blood culture	2	1	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
10	Pneumonia	Blood culture	2	1	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
11	Pneumonia	Blood culture	2	4	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
12	Pneumonia	Blood culture	1	1	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
13	Pneumonia	Transthoracic neddle aspiration	1	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
14	Pneumonia	Blood culture	2	1	1	>32	>2	MLSBc	>4	16	>2	≤1
15	Pneumonia	Blood culture	2	8	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	2
16	Pneumonia	Blood culture	2	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
17	Pneumonia	Blood culture	1	1	0,25	>32	>2	MLSBc	>4	≤4	≤0,5	≤1
18	Pneumonia	Blood culture	1	0,5	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
19	Pneumonia	Blood culture	1	1	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
20	Pneumonia	Blood culture	2	8	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
21	Pneumonia	Blood culture	1	2	0,5	8	<0,25	M	≤1	≤4	>2	≤1
22	Pneumonia	Blood culture	2	8	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
23	Pneumonia	Blood culture	1	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
24	Pneumonia	Blood culture	2	8	2	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
25	Pneumonia	Blood culture	2	2	1	8	<0,25	M	≤1	≤4	>2	≤1
26	Pneumonia	Blood culture	2	4	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
27	Pneumonia	Transthoracic neddle aspiration	0,25	0,25	0,25	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
28	Pneumonia	Blood culture	2	8	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
29	Pneumonia	Bronchoalveolar lavage	2	8	2	>32	>2	MLSBc	>4	≤4	>2	≤1
30	Pneumonia	Blood culture	2	8	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
31	Pneumonia	Blood culture	1	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
32	Pneumonia	Blood culture	2	8	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
33	Pneumonia	Blood culture	1	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
34	Pneumonia	Blood culture	2	2	1	16	<0,25	M	≤1	≤4	>2	≤1
35	Pneumonia	Blood culture	2	8	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
36	Peritonitis	Blood culture	1	1	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
37	Pneumonia	Blood culture	1	1	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
38	Pneumonia	Blood culture	4	4	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
39	Pneumonia	Blood culture	2	2	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
40	Pneumonia	Blood culture	1	2	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
41	Pneumonia	Blood culture	2	4	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
42	Pneumonia	Blood culture	2	4	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
43	Primary	Blood culture	2	2	2	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
44	Meningitis	CSF	2	4	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
45	Pneumonia	Blood culture	4	4	0,5	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1
46	Pneumonia	Blood culture	4	4	1	<0,25	<0,25	Susceptible	≤1	≤4	>2	≤1

Table S2. Clinical characteristics and outcomes of IPD episodes caused by PMEN3 lineages. Major lineages were identified based on WGS data, particularly through the study of SNPs differences. To differentiate lineages within serotype 14 and 9V isolates not included in the WGS analysis, two PCR-RFLP protocols were designed. For serotype 14 isolates, the *murM* region was amplified (murN-F: 5'-TGGCACTAACAACACTCACGA-3' Nucleotide position ref R6: 541545 – 541565; murN-R: 5'-GAATGCCAAAGGTTTCAGCCT-3' Nucleotide position ref R6: 542079 – 542099) and PCR products were cut with *Hinf*. This allowed us to differentiate lineage 5 isolates from isolates of lineages 3 and 4. To differentiate lineages 3 (ST44) and 4 (ST156), a partial MLST (*aroE* and gdh) was performed. For serotype 9V, we used a PCR targeting the region *estA-murM* (estA-F 5'-CTGAAAGTCAAAATCTGGGAAG-3' Nucleotide position ref R6: 539888 – 539909; estA-R 5'-CCCATAATCCAATATAGGTCCTCT-3' Nucleotide position ref R6: 540535 – 540558) that yielded a positive PCR result for ST156 isolates and negative for ST838. Lineages were named according the combination of MLST, serotype and year of isolation of the oldest isolate for which we had WGS data (MLST/serotype/year). For instance, lineage 1 (ST156/9V/1987) identifies a collection of serotype 9V-ST156 isolates whose oldest representative was collected in 1987.

	Lineage 1 ST156^{9V} n = 35	**Lineage 2 ST838^{9V} n = 57**	**Lineage3 ST44^{14} n = 11**	**Lineage 4 ST156^{14} (1993) n = 43**	**Lineage 5 ST156^{14} (2001) n = 44**	**Lineage 6 ST6521^{11A} n = 8**
Age, mean (+/-SD)	56.6 (+/-16.7)	66.7 (+/-17.1)	69.1 (+/-14.1)	61.6 (+/-18.1)	68.6 (+/- 12.8)	56.2 (+/-17.3)
Male Sex, n (%)	25 (71)	34 (60)	7 (64)	24 (56)	27 (61)	6 (75)
Bacteraemia, n (%)	31 (89)	51 (89.5)	11 (100)	39 (91)	43 (98)	6 (75)
Acquisition, n (%)						
CA/HCR	27 (77)	50 (88)	10 (91)	38 (88)	40 (91)	5 (62.5)
NOS	8 (23)	7 (12)	1 (9)	5 (12)	4 (9)	3 (37.5)
Current smoking	13 (37)	14 (25)	5 (45.5)	16 (37)	9 (20.5)	2 (25)
Alcohol abuse	7 (20)	5 (9)	3 (27)	7 (16)	7 (16)	0
Comorbidities						
One or more	28 (80)	53 (93)	10 (91)	29 (67)	39 (89)	8 (100)
Chronic pulmonary disease	6 (17)	8 (14)	2 (18)	5 (12)	8 (18)	3 (37.5)
Chronic heart disease	6 (17)	13 (23)	1 (9)	6 (14)	11 (25)	-
Diabetes mellitus	4 (11)	6 (10.5)	1 (9)	11 (26)	9 (20.5)	-
Malignancies	5 (14)	18 (32)	5 (45.5)	10 (23)	13 (29.5)	4 (50)
Liver cirrhosis	6 (17)	4 (7)	-	3 (7)	6 (14)	-
HIV infection	4 (11)	6 (10.5)	2 (18)	4 (9)	3 (7)	-
Immunosuppresive therapy	2 (6)	17 (30)	3 (27)	7 (16)	13 (29.5)	2 (25)
Source of infection						
Pneumonia	24 (68.5)	51 (89)	11 (100)	38 (88)	35 (79.5)	6 (75)
Meningitis	9 (26)	6 (11)	-	3 (7)	3 (7)	2 (25)
Primary	-	-	-	1(2)	3 (7)	-
Abdominal/biliary tract	1 (3)	-	-	1 (2)	1 (2)	-
Others	1 (3)	-	-	-	2 (4.5)	-
Shock	4 (11)	13 (23)	5 (45.5)	8 (19)	8 (18)	1 (12.5)
30-day mortality	4 (11)	15 (26)	3 (27)	10 (23)	8 (18)	3 (37.5)

Table S3. Burden of S.pneumoniae CC156 among IPD by 5-year periods. The table shows the total number of isolates (serotyped and genotyped -PFGE and/or MLST-) and those belonging to CC156. Among CC156 isolates, they are divided in genotyped and estimated (according to their serotype and the characteristic antimicrobial resistance pattern of penicillin- and co-trimoxazole-resistance).

Period	All isolates			CC156 isolates		
	Number	Serotyped (%)	Genotyped (%)	All	Genotyped	Estimated
1987–1991	393	178 (45.3%)	36 (9.2%)	31 (7.9%)	7	24
1992–1996	444	333 (75.0%)	208 (46.8)	53 (11.9%)	37	16
1997–2001	397	389 (98.0%)	362 (91.2%)	51 (12.8%)	50	1
2002–2006	506	505 (99.8%)	500 (98.8%)	55 (10.9%)	55	
2007–2011	644	618 (96.0%)	615 (95.5%)	56 (8.7%)	56	
2012–2016	424	418 (98.6%)	415 (97.9%)	23 (5.4%)	22	1
All	2808	2441 (86.9%)	2136 (76.1%)	269 (9.6%)	227	42

Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: PBP1A protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

ID	Lineage	103	124	285	316	318	321	326	333	351	358	370	371	372	373	382	388	393	395	397	405	414	428	429	430	431	432	443	459	462	473	475	495	497	503	512	517	533	540	546	550	553	566	568	570	574	575	576	577	583
S.pneumoniae R6		T	A	E	T	E	A	E	I	S	I	S	T	M	K	L	E	I	H	E	N	G	S	R	N	V	P	N	I	S	D	K	T	Y	H	E	N	D	S	N	A	P	E	I	N	T	S	Q	F	L
44 14 1991	Lineage3	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
44 14 1996	Lineage3	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 1987	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 1988	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 1989	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 1992	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 1995	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 1996	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 1998	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 9V 2008	Lineage1	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 1996	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 1999	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 2003 2	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 2003	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 2006	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 2009	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 2010	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 9V 2013	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 11A 2015 2	Lineage6	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 11A 2015	Lineage6	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
838 23F 2008	Lineage2	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
6521 11A 2011	Lineage6	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
6521 11A 2013	Lineage6	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
6521 11A 2015	Lineage6	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 1993	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 1994	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 1998	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 1999	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 2002	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 2003	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 2004	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 2006	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 19A 2009	Lineage4	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
143 14 2001	Lineage3	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	.	.	.	K	.	.	T	G	P	.	.	V	.	N	T	G	Y	M
156 14 2001	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156 14 2002 2	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156 14 2009	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156 14 2010	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156 14 2011	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156 14 2012	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156 14 2013	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156 14 2014	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156DLV 14 2008	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
2944 14 2007	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
6519 14 2005	Lineage5	S	T	Q	S	Q	S	D	V	A	T	.	A	.	.	I	D	M	N	I	S	A	.	.	.	.	T	D	M	A	N	Q	I	H	N	K	D	E	A	G	P	A	D	.	K	N	T	G	Y	M
156SLV 14 2007	Lineage4	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

585	606	609	611	612	622	628	629	630	635	636	638	641	654	655	657	658	660	661	664	665	666	667	682
A	L	N	L	T	M	G	S	N	N	I	E	Y	S	T	S	S	A	P	P	P	S	T	S
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	P	I	T	E	S	T	S	S	T	A	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	I	D	T	H	T	M	D	F	.	.	N	.	.	.	.	.	.	.	.
V	I	D	F	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
V	I	D	F	L	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: PBP2B protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

Isolates	Lineage	54	56	96	130	161	193	199	225	251	322	333	361	412	422	426	427	438	443	444	445	446	455	473	476	480	489	497	508	512	516	538	542	545	552	561	565	566	567	568	569	571	578	582
S.pneumoniae R6		I	S	T	I	Q	A	D	V	A	A	E	I	S	N	T	Q	Q	S	S	N	T	L	S	E	S	T	D	S	Y	A	N	V	R	G	D	Q	L	Q	P	T	M	D	S
44 14 1991	Lineage3	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
44 14 1996	Lineage3	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
143 14 2001	Lineage3	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1987	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1988	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1989	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1992	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1995	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1996	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1998	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 2008	Lineage1	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1993	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1994	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1998	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1999	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2001	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2002 2	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2002	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2003	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2004	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2006	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2009	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2010	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2011	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2012	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2013	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2014	Lineage5	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156 19A 2009	Lineage4	V	T	I	T	P	V	.	.	S	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
156SLV 14 2007	Lineage4	.	.	.	T	.	.	.	.	.	.	.	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	.												
156DLV 14 2008	Lineage5	V	T	I	T	P	.	.	I	.	.	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 1996	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 2003	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 2003	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 2006	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 2009	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 2010	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 2013	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 11A 2015	Lineage6	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 11A 2015 2	Lineage6	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 23F 2008	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
6521 11A 2011	Lineage6	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
6521 11A 2013	Lineage6	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
6521 11A 2015	Lineage6	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
838 9V 1999	Lineage2	V	T	I	T	P	.	E	I	.	S	G	L	P	Y	K	L	E	.	.	.	A	I	T	G	A	V	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
6519 14 2005	Lineage5	V	T	I	T	P	.	E	I	.	.	G	.	P	Y	K	L	E	.	.	.	A	I	T	G	A	A	.	.	.	S	D	.	.	.	.	.	.	.	.	.	.	.	.
2944 14 2007	Lineage5	V	T	I	T	P	.	.	.	.	.	G	L	P	Y	K	L	E	.	.	.	A	I	.	G	.	S	Y	N	F	.	.	L	H	D	E	A	I	D	T	K	I	E	A

590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	659	660	664	674	675	676
Q	V	A	H	G	T	S	G	L	T	T	G	R	A	F	S	N	G	A	L	V	S	I	S	G	K	T	G	T	A	E	S	Y	V	A	D	G	Q	Q	A	T	N	T	N	A	V	A	Y	A	P	S	D	N	G	S	Q	K	Y
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Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: PBP2X protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

Isolates	Lineage	6	39	60	71	102	103	116	117	133	135	138	153	165	172	241	250	254	256	268	281	311	310	320	337	338	339	340	343	346	347	355	358	364	371	378	382	384	389	400	401	417	444	446	462	465	486	488
Streptococcus pneumoniae R6		R	I	K	T	E	N	K	T	E	S	R	A	S	A	L	Q	R	M	I	P	Q	D	E	S	T	M	K	M	A	A	G	V	L	I	E	G	R	S	M	T	N	N	A	I	F	P	D
44 14 1991	Lineage3	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
44 14 1996	Lineage3	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 1988	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 1989	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 1992	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 1995	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 1996	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 1998	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 2008	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 9V 1987	Lineage1	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 1993	Lineage4	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 1994	Lineage4	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 1998	Lineage4	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 1999	Lineage4	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2004	Lineage4	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 1996	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 2003 2	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 2003	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 2006	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 2009	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 2010	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 11A 2015 2	Lineage6	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 11A 2015	Lineage6	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 23F 2008	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
6521 11A 2011	Lineage6	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
6521 11A 2013	Lineage6	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
6521 11A 2015	Lineage6	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 1999	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
838 9V 2013	Lineage2	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	V	L	.	T	N
156 14 2001	Lineage5	.	V	.	.	.	.	.	.	.	.	.	.	A	T	.	.	Q	V	.	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2002 2	Lineage5	.	V	.	.	.	.	.	.	.	.	.	.	A	T	.	.	Q	V	.	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2010	Lineage5	.	V	.	.	.	.	.	.	.	.	.	.	A	T	.	.	Q	V	.	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2011	Lineage5	.	V	.	.	.	.	.	.	.	.	.	.	A	T	.	.	Q	V	.	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2012	Lineage5	.	V	.	.	.	.	.	.	.	.	.	.	A	T	.	.	Q	V	.	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2014	Lineage5	.	V	.	.	.	.	.	.	.	.	.	.	A	T	.	.	Q	V	.	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	D	T	G	L	.	S	K	S	.	L	.	T	N
156SLV 14 2007	Lineage4	.	.	.	.	.	.	.	.	.	.	.	S	.	T	V	L	Q	V	L	.	L	N	.	.	A	F	.	.	S	S	S	Y	F	T	A	T	G	L	T	S	K	S	.	L	.	T	N
143 14 2001	Lineage3	.	.	.	.	.	.	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	A	.	.	T	.	.	S	Y	F	T	A	I	G	L	.	S	K	S	.	L	.	T	N
156 14 2002	Lineage4	K	V	R	.	K	K	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	G	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2003	Lineage4	K	V	R	.	K	K	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	G	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2006	Lineage4	K	V	R	.	K	K	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	G	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
156 19A 2009	Lineage4	K	V	R	.	K	K	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	G	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
2944 14 2007	Lineage5	K	V	.	.	K	K	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	G	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
156DLV 14 2008	Lineage5	.	V	.	.	.	.	.	.	.	.	.	S	.	T	.	.	Q	V	.	T	L	N	K	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2009	Lineage5	.	V	.	I	.	.	.	.	.	.	.	.	.	T	.	.	Q	V	.	.	L	N	.	.	A	.	.	T	S	S	S	Y	F	T	.	T	G	L	.	S	K	S	.	L	.	T	N
156 14 2013	Lineage5	K	V	.	.	K	K	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	L	T	N
6519 14 2005	Lineage5	K	V	.	.	K	K	D	S	D	A	K	S	.	T	.	.	Q	V	.	T	L	N	K	.	A	.	.	T	.	.	S	Y	F	T	A	T	G	L	.	S	K	S	.	L	.	T	N

490	491	501	504	505	506	507	510	513	514	516	523	531	536	537	546	547	548	549	550	565	567	572	574	576	605	616	622	623	626	628	629	631	632	633	635	637	641	648	652	657	671	672	674	678	688	693	696	703	710	715	721	726	734	740	745
T	A	N	S	K	D	A	L	T	N	V	V	S	T	V	L	K	S	G	T	L	D	A	S	S	N	D	T	T	A	E	Q	S	Q	Q	P	P	V	D	E	L	N	S	A	K	I	A	V	T	L	N	Q	T	A	D	T
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	P	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	G	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	K	.	E	.	T	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	.	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	E	A	I	E	T	V	T	L	V	I	K	F	D	E	V	T	N	K
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	V	T	N	T	E	S	P	N	D	K	T	T	E	S	A	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S	V	.	.	.	E	.	T	N	H	I	L	Y	I	I	V	.	.	.	.	S	N	G	T	N	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.

Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: MurM protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

Isolates	Lineage	6	10	11	15	19	21	22	23	25	33	37	39	42	43	47	48	49	50	54	60	61	71	76	80	82	86	88	89	101	102	115	117	120	124	127	129	131	132	133	135	137	140	144	146	149	175	186	190	193	197	201	208	231	237	291	292	293	305	310	315	340
Streptococcus pneumoniae R6		I	T	L	Q	E	E	L	A	V	E	N	Q	K	F	R	E	E	K	T	R	T	I	I	G	K	N	A	I	V	V	S	I	E	F	N	A	I	D	S	Q	M	R	K	E	G	K	L	Y	L	D	E	K	A	T	I	D	V	S	T	I	A
44 14 1991	Lineage3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
44 14 1996	Lineage3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1987	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1988	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1989	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1992	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1995	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1996	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 1998	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 9V 2008	Lineage1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1993	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1994	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1998	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 1999	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2002 2	Lineage5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2002	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2003	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2004	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 14 2006	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156 19A 2009	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
156SLV 14 2007	Lineage4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
143 14 2001	Lineage3	.	I	.	.	.	.	.	.	.	.	D	.	R	.	.	.	.	.	.	.	.	V	.	.	T	S	V	.	A	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	V	F	T	E	F	.	.	.	M	.	A	.	.	L	.
156 14 2009	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
156 14 2010	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
156 14 2011	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
156 14 2012	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
156 14 2013	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
156 14 2014	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
2944 14 2007	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
6519 14 2005	Lineage5	.	.	.	.	.	.	.	.	.	.	D	E	.	.	.	.	.	.	.	.	S	.	L	R	T	S	V	.	A	I	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	S	.	.	.	A	T	Q	L	T
156 14 2001	Lineage5	.	.	.	.	D	.	.	G	.	K	D	N	.	.	E	.	G	.	V	K	S	.	.	R	I	K	V	L	A	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	.	.	.	.	A	A	T	Q	L	.
156DLV 14 2008	Lineage5	L	L	S	G	.	P	M	V	L	K	D	N	R	L	E	G	.	N	V	K	S	.	.	R	T	K	V	L	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	F	T	E	F	N	.	A	.	.	A	T	Q	L	.
838 9V 1996	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 9V 1999	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 9V 2003 2	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 9V 2003	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 9V 2006	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 9V 2009	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 9V 2010	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 9V 2013	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 11A 2015	Lineage6	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 11A 2015 2	Lineage6	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
838 23F 2008	Lineage2	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
6521 11A 2011	Lineage6	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
6521 11A 2013	Lineage6	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.
6521 11A 2015	Lineage6	.	.	.	.	.	.	.	.	.	.	D	N	R	L	.	.	.	.	.	.	.	.	.	.	T	S	V	.	A	.	H	V	D	Y	K	S	V	E	I	G	L	K	R	T	D	.	V	F	T	E	F	.	.	.	.	.	.	.	.	.	.

Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: GyrA protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

Isolates	Lineage	413	489
Streptococcus pneumoniae R6		L	V
44 14 1991	Lineage3	.	I
44 14 1996	Lineage3	.	I
143 14 2001	Lineage3	.	I
156 9V 1987	Lineage1	.	I
156 9V 1988	Lineage1	.	I
156 9V 1989	Lineage1	.	I
156 9V 1992	Lineage1	.	I
156 9V 1995	Lineage1	.	I
156 9V 1996	Lineage1	.	I
156 9V 1998	Lineage1	.	I
156 9V 2008	Lineage1	.	I
156 14 1993	Lineage4	.	I
156 14 1994	Lineage4	.	I
156 14 1998	Lineage4	.	I
156 14 2001	Lineage5	.	I
156 14 2002 2	Lineage5	.	I
156 14 2002	Lineage4	.	I
156 14 2003	Lineage4	.	I
156 14 2004	Lineage4	.	I
156 14 2006	Lineage4	.	I
156 14 2009	Lineage5	.	I
156 14 2010	Lineage5	.	I
156 14 2011	Lineage5	.	I
156 14 2012	Lineage5	.	I
156 14 2013	Lineage5	.	I
156 14 2014	Lineage5	.	I
156 19A 2009	Lineage4	.	I
156DLV 14 2008	Lineage5	.	I
838 9V 1996	Lineage2	.	I
838 9V 1999	Lineage2	.	I
838 9V 2003 2	Lineage2	.	I
838 9V 2003	Lineage2	.	I
838 9V 2006	Lineage2	.	I
838 9V 2009	Lineage2	.	I
838 9V 2010	Lineage2	.	I
838 9V 2013	Lineage2	.	I
838 11A 2015 2	Lineage6	.	I
838 11A 2015	Lineage6	.	I
838 23F 2008	Lineage2	.	I
2944 14 2007	Lineage5	.	I
6519 14 2005	Lineage5	.	I
6521 11A 2011	Lineage6	.	I
6521 11A 2013	Lineage6	.	I
6521 11A 2015	Lineage6	.	I
156 14 1999	Lineage4	F	I
156SLV 14 2007	Lineage4	F	I

Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: ParC protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

Isolates	Lineage	79	137	373	473	589
S. pneumoniae R6		S	K	R	N	A
44 14 1991	Lineage3	.	N	H	K	E
44 14 1996	Lineage3	.	N	H	K	E
143 14 2001	Lineage3	.	N	H	K	E
156 9V 1987	Lineage1	.	N	H	K	E
156 9V 1988	Lineage1	.	N	H	K	E
156 9V 1989	Lineage1	.	N	H	K	E
156 9V 1992	Lineage1	.	N	H	K	E
156 9V 1996	Lineage1	.	N	H	K	E
156 9V 1998	Lineage1	.	N	H	K	E
156 9V 2008	Lineage1	.	N	H	K	E
156 14 1993	Lineage4	.	N	H	K	E
156 14 1994	Lineage4	.	N	H	K	E
156 14 1998	Lineage4	.	N	H	K	E
156 14 1999	Lineage4	.	N	H	K	E
156 14 2001	Lineage5	.	N	H	K	E
156 14 2002 2	Lineage5	.	N	H	K	E
156 14 2002	Lineage4	.	N	H	K	E
156 14 2003	Lineage4	.	N	H	K	E
156 14 2004	Lineage4	.	N	H	K	E
156 14 2006	Lineage4	.	N	H	K	E
156 14 2009	Lineage5	.	N	H	K	E
156 14 2010	Lineage5	.	N	H	K	E
156 14 2011	Lineage5	.	N	H	K	E
156 14 2013	Lineage5	.	N	H	K	E
156 14 2014	Lineage5	.	N	H	K	E
156 19A 2009	Lineage4	.	N	H	K	E
156DLV 14 2008	Lineage5	.	N	H	K	E
156SLV 14 2007	Lineage4	.	N	H	K	E
838 9V 1996	Lineage2	.	N	H	K	E
838 9V 2003 2	Lineage2	.	N	H	K	E
838 9V 2003	Lineage2	.	N	H	K	E
838 9V 2006	Lineage2	.	N	H	K	E
838 9V 2009	Lineage2	.	N	H	K	E
838 9V 2010	Lineage2	.	N	H	K	E
838 9V 2013	Lineage2	.	N	H	K	E
838 11A 2015 2	Lineage6	.	N	H	K	E
838 11A 2015	Lineage6	.	N	H	K	E
838 23F 2008	Lineage2	.	N	H	K	E
2944 14 2007	Lineage5	.	N	H	K	E
6519 14 2005	Lineage5	.	N	H	K	E
6521 11A 2011	Lineage6	.	N	H	K	E
6521 11A 2013	Lineage6	.	N	H	K	E
6521 11A 2015	Lineage6	.	N	H	K	E
156 9V 1995	Lineage1	.	N	H	K	E
156 14 2012	Lineage5	.	N	H	K	E
838 9V 1999	Lineage2	F	N	H	K	E

Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: DHPS protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

Isolates	Lineage	11	61	62	63	64	87	109	124	129	131	133	135	142	156	165	172	177	181	184	188	191	192	197	204	224	249	267	276	289	309
S. pneumoniae R6		V	S	-	-	S	D	D	P	E	R	Q	V	M	F	E	T	E	A	E	A	A	E	P	P	K	N	A	A	A	N
143 14 2001	Lineage3	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
44 14 1991	Lineage3	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
44 14 1996	Lineage3	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 9V 1988	Lineage1	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 9V 1992	Lineage1	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 9V 1995	Lineage1	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 9V 1996	Lineage1	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 9V 1998	Lineage1	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 9V 2008	Lineage1	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 9V 1987	Lineage1	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 1993	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 1994	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 1998	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2001	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2002 2	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2003	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2004	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2006	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2009	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2010	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2011	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2012	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2013	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2014	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 19A 2009	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156DLV 14 2008	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 1996	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 1999	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 2003 2	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 2003	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 2006	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 2009	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 2013	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 11A 2015 2	Lineage6	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 11A 2015	Lineage6	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 23F 2008	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
2944 14 2007	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
6519 14 2005	Lineage5	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
6521 11A 2011	Lineage6	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
6521 11A 2013	Lineage6	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
6521 11A 2015	Lineage6	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
838 9V 2010	Lineage2	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	E	.	Q	.	.	.	.	V	.	.
156 14 2002	Lineage4	A	R	S	-	.	N	N	A	K	G	K	.	I	.	K	.	D	T	D	.	D	.	Q	.	.	.	.	V	.	.
156 14 1999	Lineage4	A	.	S	Y	.	.	N	A	K	G	K	I	.	.	.	K	.	T	.	.	E	.	Q	S	.	.	.	V	.	.
156SLV 14 2007	Lineage4	A	.	S	-	.	.	N	A	N	G	K	.	.	.	.	K	.	T	.	.	E	.	Q	.	.	.	.	.	V	D
156 9V 1989	Lineage1	A	.	S	Y	V	.	N	A	K	G	K	.	.	I	.	.	D	.	D	S	N	Q	Q	.	R	S	V	.	.	.

Table S4. Amino acid sequence variation among proteins involved in antimicrobial resistance: DHFR protein. First line: *S. pneumoniae* R6 (reference). Polymorphic sites are highlighted. Isolates are named according to: MLST_Serotype_Year of isolation.

Isolates	Lineage	16	20	26	53	60	70	78	92	100	111	120	135	147	149
S. pneumoniae R6		L	E	H	M	K	P	A	D	I	P	H	L	F	A
6521 11A 2015	Lineage6	V	.	.	.	.	.	.	A	L	.	.	.	.	.
6521 11A 2013	Lineage6	V	.	.	.	.	.	.	A	L	.	.	.	.	.
6521 11A 2011	Lineage6	V	.	.	.	.	.	.	A	L	.	.	.	.	.
156SLV 14 2007	Lineage4	V	.	.	.	.	.	.	A	.	.	.	.	.	.
143 14 2001	Lineage3	V	D	.	.	.	.	.	A	.	A	.	.	.	.
6519 14 2005	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
2944 14 2007	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 23F 2008	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 11A 2015 2	Lineage6	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 11A 2015	Lineage6	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 2013	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 2010	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 2009	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 2006	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 2003	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 2003 2	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 1999	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
838 9V 1996	Lineage2	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 19A 2009	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2014	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2013	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2012	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2011	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2010	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2009	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2006	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2004	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2003	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2002	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2002 2	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 2001	Lineage5	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 1999	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 1998	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 1994	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 14 1993	Lineage4	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 1998	Lineage1	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 1996	Lineage1	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 1995	Lineage1	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 1992	Lineage1	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 1989	Lineage1	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 1988	Lineage1	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 1987	Lineage1	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
44 14 1996	Lineage3	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
44 14 1991	Lineage3	V	D	Y	I	Q	S	T	A	L	.	Q	F	S	T
156 9V 2008	Lineage1	V	D	Y	I	Q	S	T	T	L	.	Q	F	S	T
156DLV 14 2008	Lineage5	V	D	Y	I	Q	S	T	A	L	S	.	F	S	T

Figure S1. Changes in pneumococcal surface proteins. Differences in the accessory genome were evaluated by detecting the presence of the seventeen proteins with the highest discriminatory power.[18]

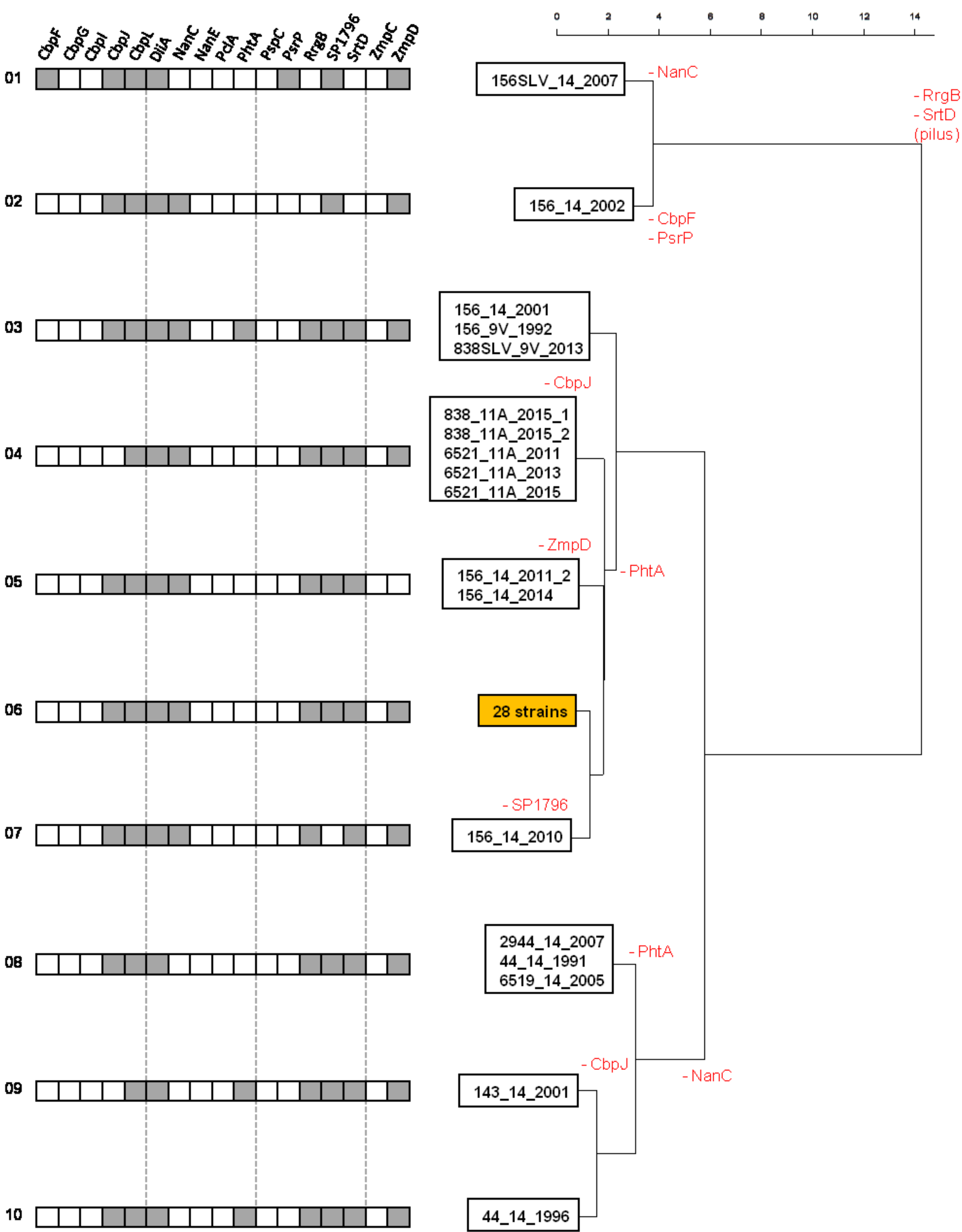


Figure S2(a). Gene organization of the five groups of prophages studied here. Genes are represented as arrows indicating the direction of transcription. The putative products of selected ORFs are indicated below each scheme. Yellow, dark blue, green, light blue, and red arrows correspond to genes involved in lysogeny regulation, DNA replication, packaging and head-assembly, tail morphogenesis, and lysis of the host cell, respectively.

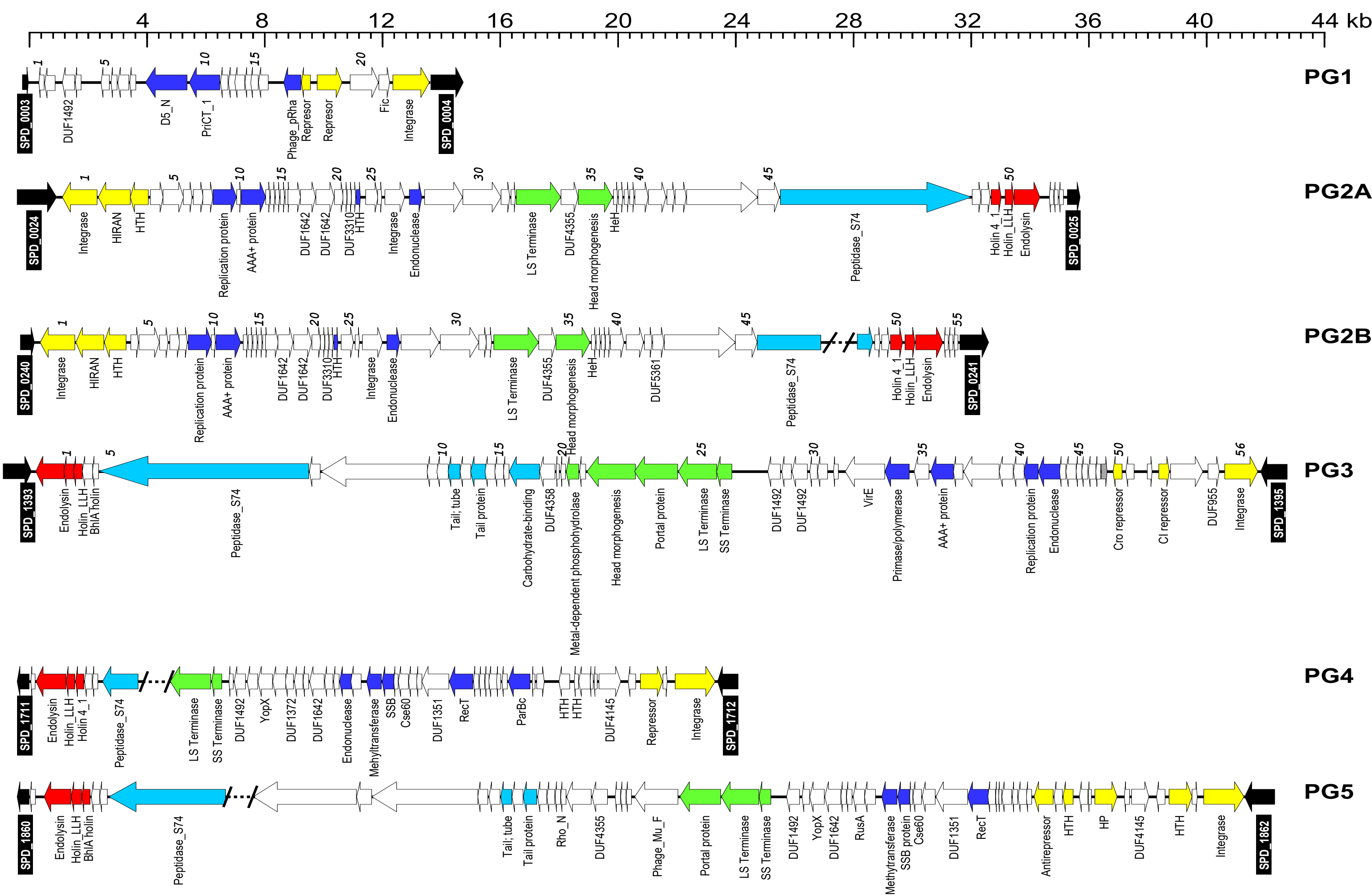


Figure S2(b). Diagram showing the similarities between the different prophages found in this study and the most similar prophages harbored by completely sequenced pneumococcal strains. Genes are shown with arrows pointing in the direction of transcription. Regions longer than 300 bp were aligned and those sharing ≥ 90% identical nucleotides are represented by identical color and shading. Chromosomal genes flanking the prophage are indicated in a black background. Dotted lines indicate prophage regions that could not be identified in our dataset. The prophages most similar to groups 1–4/5 prophages were found, respectively, in the following *S. pneumoniae* strains: ST556 (MYY; Acc. No. CP003357); 670-6B (SP670; Acc. No. CP002176); NT_110_58 (SpnNT; Acc. No. CP007593), and JJA (SPJ; Acc. No. CP000919). MYY_0003 and MYY_0033 correspond to SPD_0003 and SPD_0004 respectively; MYY_0257 and MYY_0272 correspond to SPD_0175 and SPD_0176 respectively. SP670_0044 and SP670_0099 correspond to SPD_0024 and SPD_0025 respectively; SpnNT_01571 and SpnNT_01640 correspond to SDP_1303 and SPD_1394 respectively; SPJ_1904 corresponds to SPD_1712 whereas SPJ_1843 appears to be absent in D39.

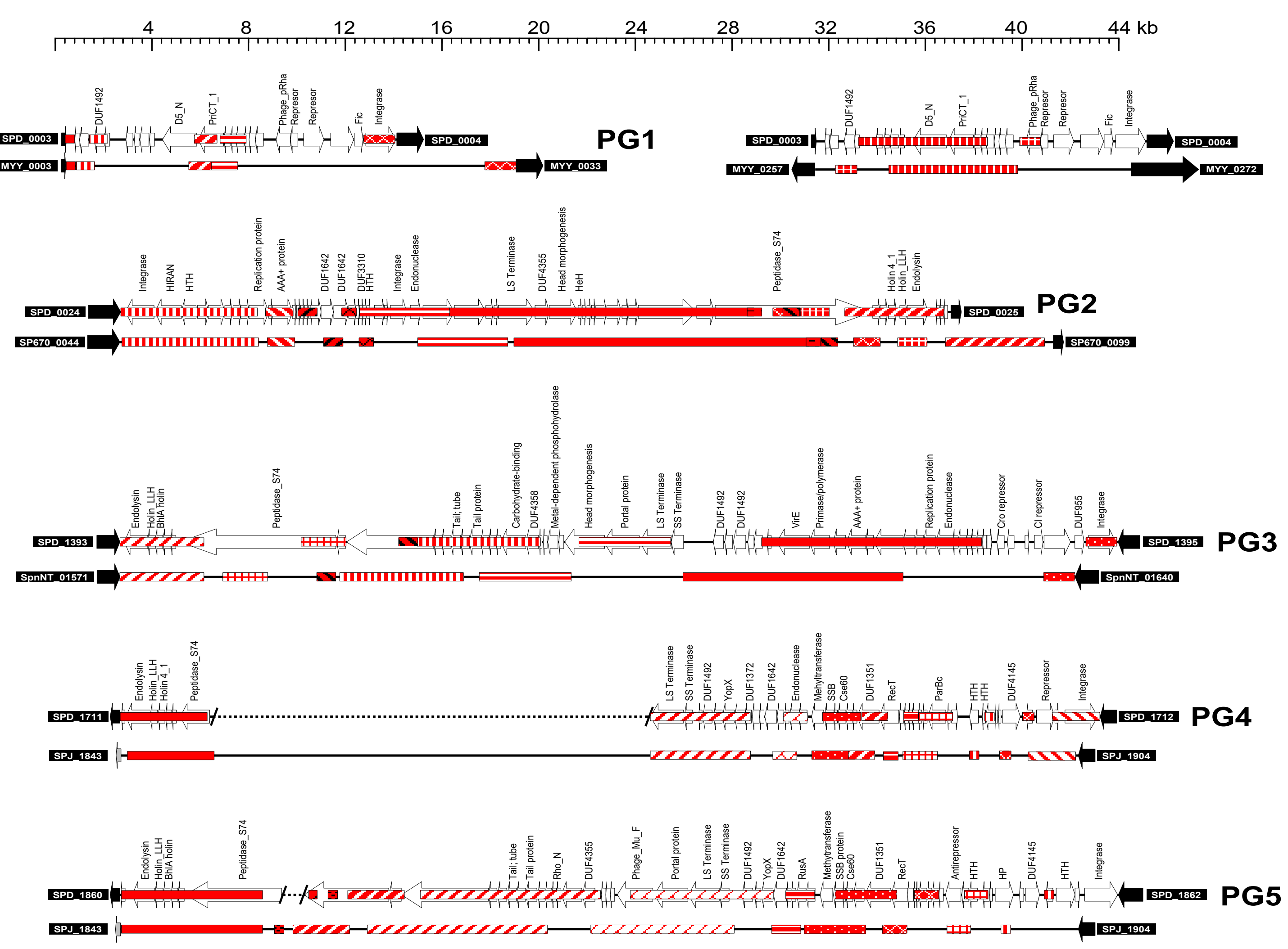


Figure S2(c). Similarities between prophages of the CC156 isolates. Pairwise sequence alignments were performed using BLAST. Regions longer than 300 nucleotides and sharing ≥ 90% sequence identity are represented by identical color and shading.

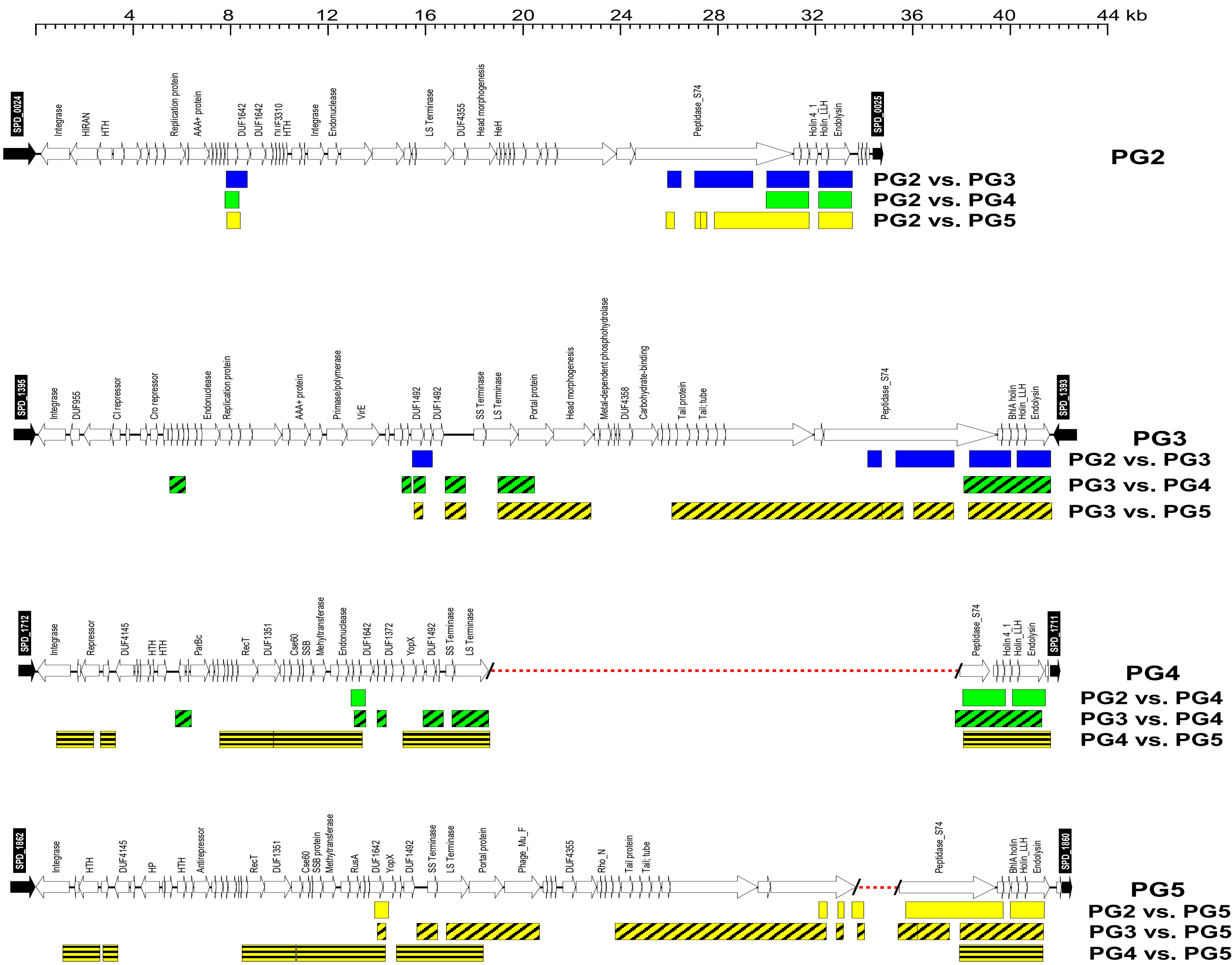


Figure S2(d). Comparison of the organization of several PblB proteins. The PblB protein of the *Streptococcus mitis* prophage SM1 (Acc. No. NP_862890) was used as the query for sequence alignments. Regions sharing sequence similarity are represented by identical color and shading. The percentage of identical/similar amino acid residues are indicated at the bottom. Stippled bars represent different repeated motifs. PG2 and PG3 correspond to PblB alleles from prophages of groups 2 and 3 respectively. The recently published sequence of PblB (Acc. No. AB679266) from the pneumococcal strain NTUH-P15 is also shown (P15).

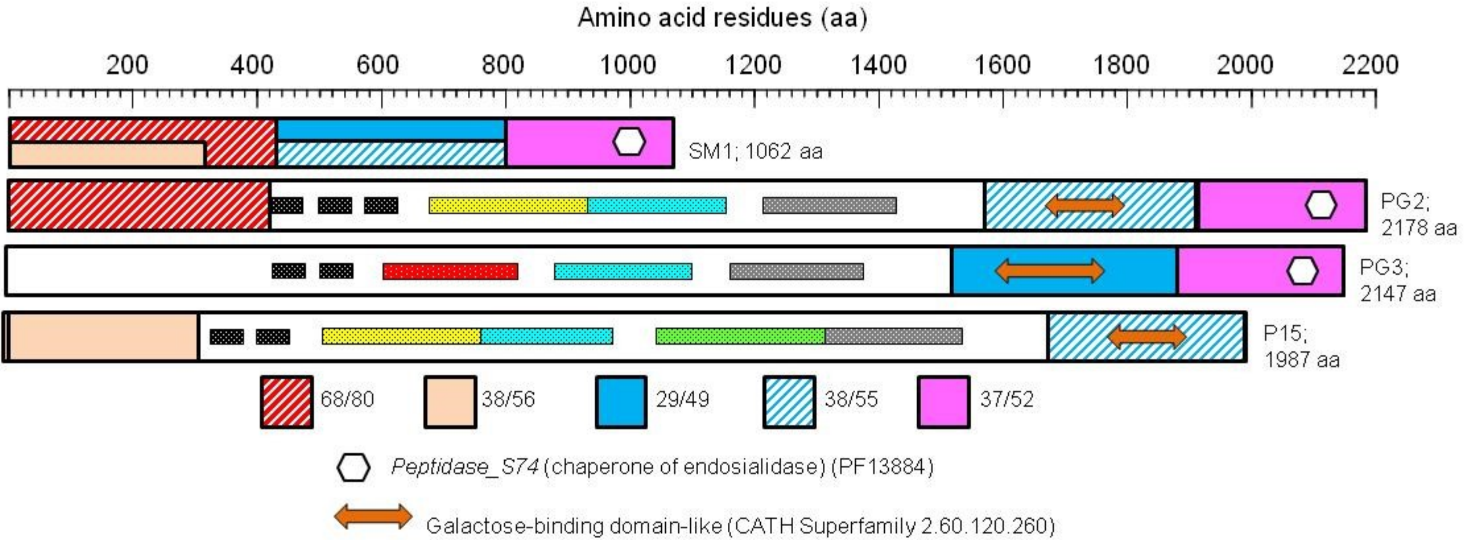
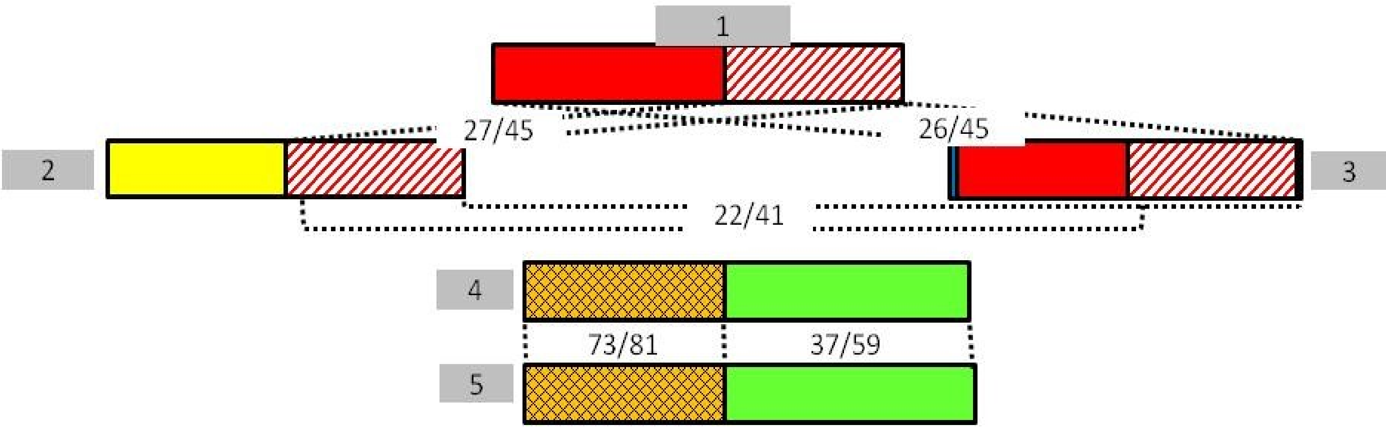
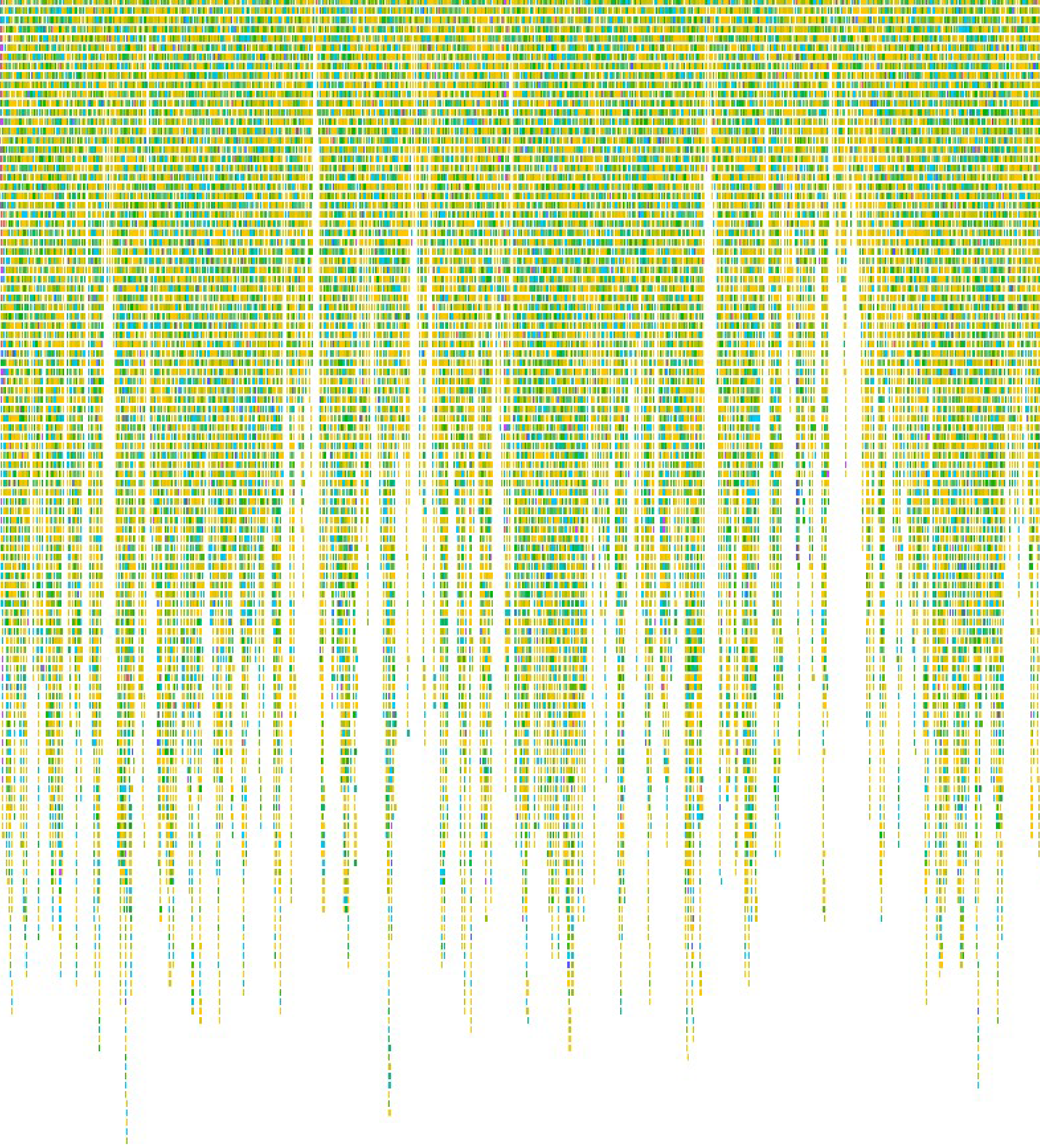


Figure S2(e). Schematic representation of the integrases of the five prophage groups. The phage groups are indicated on a grey background. Regions sharing sequence similarity are represented by identical color and shading. Identities/similarities of amino acid residues are shown as percentages.





DISCUSSION

The introduction of the PCVs at the beginning of the 21th century has changed the epidemiology of the pneumococcal diseases. In 2001, with the introduction of the first pneumococcal conjugate vaccine PCV7 a sharp decrease of the incidence of IPD was observed in children around the world.[136–138] Moreover, the incidence of IPD also decreased in the adult population due to herd immunity.[126] This two facts highlight the enormous impact of vaccination programs when the target of the vaccination is the main reservoir of disease. In spite of that, *Streptococcus pneumoniae* is a pathogen that is able to adapt to the changing environment[22] so new lineages can emerge and take the place left by others. As an example, a few years after the introduction of PCV7 in Spain, an increase in the incidence of IPD was observed among children and adults.[78,79] This was related to the clonal expansion of some epidemic serotypes such as 1, 5 and 7F. Therefore, surveillance of IPD and molecular epidemiology of the pneumococcal population are essential in order to design effective vaccination programs and identify new targets for developing vaccines.

This thesis focuses on the impact of the introduction of PCVs on the incidence and characteristics of IPD in the adult population in Spain. The first work aimed to analyse the early impact of PCV13 introduction in children on the incidence of IPD in the adult population. Then, in the second work we explored the impact of PCVs on the incidence of IPD caused by MDR isolates. Finally, we studied through WGS the genetic changes that occurred in isolates belonging to PMEN3 clone, which has been characteristically the most prevalent clone related to β-lactam-resistance in our geographical area. This thesis adds valuable knowledge to the study of pneumococcal diseases. As it combines laboratory, epidemiological and clinical data, the results give a comprehensive view of the evolution of IPD in Spain in recent years. In addition, the experience gained in pneumococcal epidemiology in recent years and the observations that the introduction of PCVs has yielded different results around the world makes it necessary to closely monitor the evolution of pneumococcal diseases.

In this chapter the main findings of this thesis will be discussed. The past and current situation will be discussed together to give a global vision of the evolution of adult IPD in Spain in the era of conjugate vaccines.

Early impact of PCV13 introduction for children on the incidence of invasive pneumococcal disease in Spain

In Spain, PCV13 was licensed in 2010 under a voluntary basis which means that vaccination coverage remained low (around 60%).[139] However, this situation has not been equal for all Spanish regions and also varied throughout time. For instance, in the Madrid autonomous community, PCVs were subsidized during the period 2006-2012 (initially PCV7 that was replaced by PCV13 in 2010) achieving a vaccination coverage over 90%.[140] PCV13 was finally included in the national vaccination paediatric schedule in 2015 and it was also recommended for some adults at risk of having IPD (VIH, immunosuppressed, transplantation…).[141]

Our results showed a prompt decrease in the incidence of adult IPD two years after the introduction of PCV13 for children (PCV7 period, years 2008-2009 compared to PCV13 period, years 2012-2013). Overall data show a decline of 33.4% (95% CI -40.3 to -26.8%) in the incidence of adult IPD (from 12.3 to 8.1 cases per 100,000 population) which was in concordance with published data from other countries. In Norway, the global incidence of IPD (all ages) showed a reduction from 15 to 13 episodes per 100,000 inhabitants when comparing 2010 (PCV7 year) to 2012 (PCV13 year).[142] In adults ≥65 years old the IRR between these two years was 0.79 (95%CI 0.70-0.92). In Denmark, the global incidence of IPD decreased from 18.0 to 15.6 episodes per 100.000 after PCV13 (2008-2010 vs 2011-2013, IRR 0.86 [95%CI 0.82-0.91]) and from 60.0 to 49.4 episodes per 100,000 in adults ≥65 years old (IRR 0.82 [95%CI 0.76-0.88]).[132] In the USA, it was estimated that the introduction of PCV13 resulted in a decrease in the incidence of adult IPD of 12-32% depending on age group (decrease of expected incidence in the absence of PCV13 during years 2012-2013).[130] Similarly, data from England and Wales showed that, when comparing 2008-2010 to 2013-2014 periods, IRR was 0.68 (95%CI 0.64-0.72) for the whole population and 0.75 (95%CI 0.69-0.81) for adults ≥65 years old.[131] All these data indicate that, besides the effect on children, replacement of PCV7 by PCV13 has had an early beneficial impact on the incidence of IPD in adults. This highlights the importance of the herd effect on pneumococcal diseases and also the importance of the vaccination programs targeting children as they are the main reservoirs of pneumococci. Our data also showed that

the greatest impact on the adult IPD was observed in the Madrid region, which had the highest vaccine coverage, though differences did not reach statistical significance. This remarks the importance of the accumulated size of the vaccinated group to achieve high levels of herd protection.[143] Thus, the introduction of PCV13 in the national vaccination paediatric schedule in 2015 should help to reduce the remaining disease caused by vaccine types. In spite of that, surveillance of emerging serotypes and clones continues to be crucial. For instance, we have recently shown that the initial reduction of IPD rates among Spanish adults after PCV13 introduction has been balanced by emerging non-PCV13 serotypes which could limit the benefits of current vaccination programs.[144] Therefore, new, broader conjugate vaccines or non-serotype-based vaccines are needed to continue fighting pneumococcal diseases.

Early impact of PCV13 introduction on vaccine and non-vaccine serotypes

As expected, the introduction of PCV13 caused a reduction in the incidence of the majority of PCV13 serotypes. The incidence of serotypes included in PCV13 and that were not in PCV7 (additional PCV13 serotypes) dropped from 5.7 to 2.6 episodes per 100,000 population (-55.0% [95%CI -62.0% to -46.7%]). Among these additional PCV13 serotypes, the incidence of IPD due to serotype 3 was the only one that did not decrease (-11.4% [95%CI -35.0% to 21.0%]).

The impact of PCV13 on the incidence of serotype 3 remains under debate. On one hand, a recent meta-analysis supports the efficacy of PCV13 in preventing IPD caused by serotype 3 in children.[145] In adults, a pooled analysis of published literature also suggested that PCV13 provides protection against CAP caused by serotype 3, although the analysis was limited to three studies.[146] A third work that compared the incidence of IPD in countries that have introduced PCV10 with others using PCV13 detected significant lower incidences of IPD due to serotype 3 in PCV13 countries.[147] On the other hand, some works have noticed an increase in the incidence of IPD and CAP due to serotype 3 in adults[148,149] and an increase of parapneumonic empyema in children after PCV13 introduction.[150] All these data illustrates that it is not easy to assess the efficacy of herd protection in preventing diseases caused by this serotype. Some hypotheses could explain the potential limited impact of PCV13 against serotype

3. First, data reported on the immunogenicity of PCV13 has shown lower levels of antibodies and less opsonophagocytic activity against serotype 3 than against other PCV13 serotypes.[151–153] Second, because of the particular synthesis of capsular type 3 (mediated by the synthase-dependent pathway), the capsular polysaccharide is not covalently linked to peptidoglycan. It has been described that serotype 3 isolates can release significant amounts of capsular polysaccharide during growth that can interfere with antibody-mediated killing and confer protection against anti-capsular antibodies.[154] And third, this serotype is not frequently found colonizing children (so studies on serotype 3 carriage must be interpreted with caution), but it seems that PCV13 does not prevent carriage acquisition of serotype 3 isolates as effectively as with other serotypes.[155] In our study, serotype 3 was the leading cause of IPD after PCV13 introduction, which is in concordance with the high prevalence of this serotype in the adult IPD, associated to septic shock and high mortality rates.[156] We also detected a shift in its genetic background: after PCV13, the local clone (ST260) has been partially replaced by a worldwide distributed clone (ST180). This finding is interesting since it has been reported that a lineage belonging to ST180 (“clade II”) has recently spread, particularly in North America and Asia, and this new clade could have played a major role in the persistence of disease caused by serotype 3.[157] Moreover, isolates belonging to clade II have also expanded in England and Wales after 2015 which have also contributed to increased rates of IPD.[158] As exposed in the last paragraph, a low herd immunity effect has been observed for serotype 3 after PCV13 introduction. The recent recommendations for PCV13 administration in adults at risk of suffering IPD could have a direct effect on the incidence of this serotype. In the meantime, more studies are needed to clarify the impact of PCV13 on pneumococcal diseases caused by serotype 3 in adults.

With respect to non-PCV13 serotypes their overall incidence remained stable after the introduction of PCV13 (1.0% [95%CI -12.9% to 17.2%]). However, two non-PCV13 serotypes showed statistically significant differences in their incidence between periods: IPD due to serotype 8, which fell (-34.9% [95%CI -57.1% to -1.2%) and IPD due to serotype 6C, which rose (301.6% [95%CI 92.7% to 733.3%). Regarding the fall in the incidence of IPD due to serotype 8, it should be noted that it was only observed in the

Madrid region and in adults younger than 65 years old, while it remained stable in the remaining regions. These results could be associated to the existence of an outbreak of IPD due to a recombinant serotype 8 clone (related to the multidrug-resistant Sweden^{15A}-ST63 clone) in HIV-infected patients in Madrid prior to the PCV13 introduction (2004-2009).[68,69] Furthermore, these results should not mask the current high burden of serotype 8 isolates in Spain, as it is the leading cause of IPD in adults, accounting for 30% and 19% in adults aged 18-64 and ≥65 years, respectively.[159] Hopefully, the introduction of the next PCV20 vaccine should help to control the expansion of serotype 8 isolates.

Regarding serotype 6C, we detected an increase of isolates related to the ST386^{6C} clone (a double locus variant of the Poland^{6B}-ST315 clone),[160] which accounted for 82.6% of serotype 6C isolates after PCV13. This increase could suggest a minor effect of the described cross-protection with serotype 6A, which is included in PCV13.[161] Nevertheless, data from Sweden comparing similar populations where either PCV10 (that does not includes serotype 6A) or PCV13 were administered demonstrated statistically significant increases in the incidence of IPD caused by serotype 6C for regions that used PCV10 instead of PCV13.[162] Therefore, we cannot rule out that the introduction of PCV13 has caused a similar effect in our geographical area. In any case, since serotype 6C is not included in the upcoming PCV15 and PCV20 conjugate vaccines it is likely that its incidence will not be significantly reduced in the short term.

Impact of PCVs on the incidence of IPD caused by MDR and penicillin-non-susceptible (PNS) pneumococci

When analysing the early impact of PCV13 on the adult IPD, we observed an increase in rates of non-susceptibility to β-lactams in the PCV13 period. Penicillin and cefotaxime non-susceptibility rates increased from 22.7% to 26.8% and from 10.1% to 12.5%, respectively. This was linked to an expansion of isolates expressing non-PCV13 serotypes related to MDR clones like ST386^{6C} and ST6521^{11A}. Then, we aimed to

analyse over a large period (1994-2018) what impact PCVs have had on antibiotic resistance rates among the adult IPD in our geographic area.

Besides reducing the incidence of invasive disease, it has been described that the introduction of PCVs could generate additional benefits such as decreasing rates of antimicrobial resistance.[128] In the second work of this thesis we showed that the sequential introduction of PCV7 and PCV13 has had a different impact on the population of susceptible and resistant (MDR/PNS) pneumococci. After PCV7 we detected an increase in the incidence of IPD caused by susceptible isolates (pre-PCV versus late-PCV7 RR: 1.55 [95%CI 1.35–1.78]) and stability in the incidence of resistant isolates (pre-PCV versus late-PCV7 RR: 1.09 [95%CI 0.89–1.34]). This means significant lower rates of antimicrobial resistance rates after PCV7 introduction. Penicillin non-susceptibility and cefotaxime resistance rates decreased from 34.8% to 27.3% and from 18.5% to 9.6%, respectively. It is important to highlight that these reductions were not linked to a decrease in the incidence of disease caused by resistant isolates but to an expansion of susceptible isolates. Therefore, the impact of PCV7 on the burden of resistant disease in our geographical area was limited. In contrast, the introduction of PCV13 caused a decrease in the incidence of IPD caused by both susceptible (late-PCV7 vs late-PCV13 RR: 0.62 [95%CI 0.52–0.74]) and resistant isolates (late-PCV7 vs late-PCV13 RR: 0.64 [95%CI 0.48–0.87]). Paradoxically, this reduction in the incidence of resistant isolates did not translate into a reduction in resistant rates that remained stable for most antimicrobials after PCV13 introduction. This remarks the importance of properly interpreting the impact of vaccines on antimicrobial susceptibility.

In Spain, published data after PCV7 introduction showed reductions in the rates of resistance to penicillin and cefotaxime in children and adults.[78,79] After PCV13, rates of PNS remained stable (from 22.7% to 21.1%, pre-PCV13 to late-PCV13) and cefotaxime resistance decreased (from 10.2% to 7.0%, pre-PCV13 to late-PCV13) in adults.[144] The impact of PCVs on the burden of resistant isolates mainly depends on two factors: the impact on carriage of the population of susceptible and/or resistant isolates and the existence of replacement by resistant non-PCVs serotypes.[163] Of these, the pre-existing population of resistant strains expressing serotypes included or not in

PCVs is critical. For instance, a study on carriage isolates from Alaska detected an increase in the proportion of PNS isolates after PCV13.[164] In this study, the pre-existing population of PNS isolates was diverse and up to 60% of them expressed non-vaccine serotypes. Therefore, PCV13 introduction was not useful to reduce resistant rates; rather, it resulted in the expansion of those PNS non-vaccine serotypes. In France, the rate of PNS and ceftriaxone non-susceptibility decreased among carriage isolates in children attending day-care centres over 1999-2012.[165] In this study, despite the fact that the percentage of children with *S. pneumoniae* remained fairly stable over time (54% in 1999 and 46% in 2012), serotype replacement was observed: PCV13 serotypes accounted for 88.2% before PCVs and only 6.5% after PCV13. Because replacement was due to a population of serotypes with lower resistance rates, PCVs introduction resulted in a beneficial impact on antimicrobial resistance. In the USA, the proportion of PNS isolates slightly increased among invasive and non-invasive disease over 1999-2011 (MIC ≥ 0.12 mg/L, from 33% to 39%).[166] These results were mostly associated to an expansion of isolates expressing 19A and 35B, which is a perfect illustration of a replacement of PCVs serotypes by resistant non-PCVs serotypes resulting in increased rates of antimicrobial resistance. This also highlights the importance of targeting serotype 19A to prevent the spread of a frequently antimicrobial resistant serotype, as it occurred in Spain with the clonal expansion of 19A isolates belonging to ST320 after PCV7 introduction.[167] In this regard, the experience of Belgium, where PCV13 was replaced by PCV10 in 2015-2016, is of interest. In this country, the switch from PCV13 to PCV10 has led to an increased number of IPD isolates[168] and increased PNS rates (from 12% to 16% among invasive isolates over 2015-2018) mostly due to the spread of serotype 19A, which became the first cause of IPD in 2017-2018.[169] Data published from Brazil, where PCV10 was introduced in 2010, show a similar pattern of increased PNS rates and expansion of serotype 19A isolates.[170,171]

Data regarding the impact of PCVs on the incidence of MDR isolates is scarce. The SENTRY Antimicrobial Surveillance Program reported increases in the frequency of MDR isolates (defined as non-susceptibility to ≥3 antimicrobial classes) for most regions over 1997-2016.[172] The percentages increased from 23.7% to 39.2% in the Asia-Pacific Region, from 8.9% to 17.3% in North America, from 16.6% to 19.1% in

Europe and from 5.3% (period 2003-2004) to 20.9% in Latin America. In Japan, where PCV7 was introduced in 2010, the frequency of MDR isolates among invasive and non-invasive samples rose from 0.8% to 2.4% in adults over 2001-2015.[173] Rates of MDR among *Streptococcus pneumoniae* isolates also increased from 1.4% to 4.3% (2007 and 2013, respectively) in Canada, which was mostly associated to isolates expressing serotype 19A (PCV13 was introduced in 2010).[174] It has to be noted that all these data refers to rates of MDR over the total population of studied pneumococci. Therefore, as occurs with PNS isolates, greater reductions of susceptible isolates may cause a rise in the proportion of MDR isolates. In Norway, where data on the incidence of IPD caused by MDR isolates is reported, an increase from 0.11 to 0.38 episodes/100,000 population was observed over 2004-2016 (PCV7 and PCV13 introduction in 2006 and 2011, respectively).[175] In this country, the non-PCV13 serotype 15A was mainly responsible for the increase in the incidence of IPD caused by MDR isolates.

All these data evidence that the impact of PCVs introduction on antimicrobial resistance rates has shown regional differences conditioned by the local epidemiology, previous antimicrobial resistance rates and which vaccine was introduced. As the overall incidence of IPD has been generally reduced, the burden of resistant isolates appears to be lower than it was before the PCVs era. However, as we have shown in our work, the initial reduction of antimicrobial resistance rates seen after PCV7 seems to need additional measures to be sustained. In this sense, reducing the consumption of antimicrobials could be a necessary measure to maintain the benefits seen after the introduction of PCVs.[176]

Evolution of genotypes related to MDR and PNS in the era of conjugate vaccines

Infections caused by PNS pneumococci were not reported until 1967.[177] Afterwards, PNS and MDR pneumococci became a health threat in the 1970s decade.[50] In Spain, the rate of PNS pneumococci reached percentages over 40% among pneumococcal isolates from invasive disease during the 1990s.[53] This was associated to the expansion of a few resistant clones (Spain^{23F}-ST81, Spain^{6B}ST90, Spain^{9V}-ST156, Spain^{14}-ST18 and ST88^{19F})[178,179] that characteristically expressed serotypes included in

PCV7. Our results show that although most of these clones disappeared after the introduction of PCVs, new clones emerged and replaced them. Furthermore, one of them (the Spain^{9V}-ST156 clone) persisted over time mainly due to a recombinant strain expressing serotype 11A.

Indeed, MDR clones that expressed PCV7 serotypes 6B, 14, 19F and 23F are no longer a significant cause of invasive disease in our area. Most of these clones were distributed worldwide and were part of the initial collection of antimicrobial-resistant clones of the Pneumococcal Molecular Epidemiology Network (PMEN1 [Spain^{23F}-ST81], PMEN2 [Spain^{6B}ST90] and PMEN5 [Spain^{14}-ST18]).[178] Their disappearance was in parallel to the emergence of new clones that expressed serotypes not included in PCV7 such as 19A-ST320 (a variant of the Taiwan^{19F}-ST236 clone)[167], 6C-ST386 (a variant of the Poland^{6B}-ST315 clone)[160] and 24F-ST230/19A-ST276 (both variants of Denmark^{14}-ST230). Because all these clones show non-susceptibility to penicillin and resistance to macrolides/lincosamides and tetracyclines they could compromise the oral treatment of pneumococcal pneumonia. Among these clones, the emergence and spread of ST320^{19A} isolates after PCV7 has been contained by the introduction of PCV13, which includes serotype 19A in its formulation. Despite this, serotype 19A continues to be a frequent cause of IPD in adults in Spain, currently being the second more common serotype (after serotype 3) among those included in PCV13.[159] More worrisome is the expansion of MDR isolates that express serotypes 6C or 24F, which are not included in the current PCV13 or the upcoming PCV15/PCV20 vaccines. With respect of serotype 6C, its expansion suggests a minor effect of the described cross-reactivity with serotype 6A, as discussed before. In regard to serotype 24F, it is important to note that this serotype has recently been associated to an increase of paediatric meningitis in France[180] and it is currently the leading cause of children IPD in Spain and France.[159,181] In fact, serotype 24F has been recognized among the serotypes with the highest invasive potential in children.[182] In adults, ST230 has also been associated to increased risk of developing acute cardiac events in pneumococcal pneumonia.[183] All of these data highlight the growing importance of serotype 24F isolates in both children and adult IPD and the need for their close surveillance, particularly those isolates related to the Denmark^{14}-ST230 clone.

In Spain, IPD caused by PNS pneumococci has been classically dominated by a single clone (Spain^{9V}-ST156) which accounted for more than half of IPD episodes caused by PNS isolates (not MDR) after PCV13. Formerly expressing PCV7 serotypes 9V and 14, this clone is currently associated to the non-vaccine serotype 11A. As the evolution of this clone has been studied in depth in this thesis, it will be discussed independently in a later section.

Impact of antimicrobial resistance on the outcome of patients with pneumococcal bacteraemia

The impact of antimicrobial resistance on the outcome of patients with IPD is a discussed topic. For instance, some works have found no association between antimicrobial resistance and worse outcome in bacteremic and non-bacteremic pneumococcal pneumonia.[184–187] On the other hand, some authors have found increased risk of adverse outcomes in invasive pneumococcal pneumonia caused by penicillin- or cefotaxime- resistant strains.[188–191] It is not easy to assess the impact of antimicrobial resistance on the outcome of pneumococcal infections. Resistant strains frequently appear in patients with comorbidities who often have a history of antimicrobial consumption. The emergence of macrolide or fluoroquinolone resistant *S. pneumoniae* isolates in COPD patients who previously have received these antimicrobials is well known.[192,193] The genetic background of the resistant isolates causing IPD may also display geographic differences which in turn could lead to different virulence patterns. In our work we have shown that, when comparing with susceptible isolates, PNS and MDR strains display similar clinical features: they appear more often in older patients with comorbidities, in nosocomial-related episodes, in patients with prior antimicrobial consumption and in patients with higher McCabe&Jackson score. Consequently, it is not uncommon for this group of patients to present with significantly higher mortality rates. However, our data showed that, after adjusting for another prognostic values, 30-day mortality was a factor not related to MDR/PNS disease. In fact, MDR/PNS episodes were only statistically associated to age, nosocomial acquisition and higher McCabe&Jackson score, factors mostly related to the host status.

These findings are similar to those published in the literature, which emphasize the importance of host factors as main contributors to mortality in adult patients with IPD. In a study on bacteremic pneumococcal pneumonia from Sweden, age was the strongest predictor of mortality.[194] Other host factors associated with mortality were smoking, alcohol abuse, solid tumour, liver disease and renal disease. A study on invasive pneumococcal pneumonia and mortality from the USA found that older age and the existence of comorbidities were the most important factors leading to death.[190] A third study on bacteremic pneumococcal pneumonia from Canada also found age and the existence of some underlying conditions (such as cancer and alcoholism, among others) as predictors of mortality.[195] Interestingly, this study found that polysaccharide pneumococcal vaccine was associated with reduced mortality, which is also a controversial issue. Finally, a study that collected data from 17 European countries reported again that age ≥65 years was the factor with more increased risk of mortality.[196] All in all, these data indicates that those IPD episodes that occur in fragile patients with comorbidities deserve special attention. Furthermore, the inclusion of these patients at risk of having IPD in vaccination programs could provide additional benefits.

Burden and evolution of the β-lactam-resistant Spain9V-ST156 (PMEN3) clone among adult IPD in Barcelona

In the previous work we showed that the burden of IPD caused by PNS isolates (not MDR) in Spain is mostly determined by the existence of a single clone ($Spain^{9V}$-ST156 [PMEN3]) that still accounts for more than half of PNS-IPD episodes. For this reason we conducted the following study in order to in-depth analyse the genetic evolution of this clone. The $Spain^{9V}$-ST156 (PMEN3) is a worldwide distributed clone, usually expressing serotypes 9V or 14,[44] and characterized by its non-susceptibility to penicillin (MICs 1-4 mg/L), cefotaxime (MICs 1-2 mg/L) and its resistance to co-trimoxazole (MICs >2 mg/L). In Spain, PMEN3 has been a significant cause of IPD over time.[179] Our data show that this clone, in association with serotypes 9V and 14, accounted for around 12% of adult IPD episodes during the 90s. The introduction of PCV7, which included serotypes 9V and 14, caused a decrease in the proportion of

PMEN3 isolates after 2001. Nevertheless, the percentage of PMEN3 isolates among IPD always remained over 5%, mostly because of the emergence of isolates expressing serotype 11A. When considering only invasive PNS isolates, this clone has been predominant (always over 50%), even after the introduction of PCV13. These results highlight the importance of this clone among the adult IPD in Spain. After WGS analysis of isolates belonging to PMEN3, we identified six lineages (all originating from the ancestral ST156^{9V} clone) that showed a different prevalence over time. The original ST156^{9V} lineage, prevalent during the 80s, was progressively replaced by a new serotype 9V lineage (ST838^{9V}) and other two other serotype 14 lineages (both ST156) that coexisted during the 90s and early 2000s. More recently, a new serotype 11A variant (ST6521^{11A}), originated from the ST838^{9V} lineage, emerged and spread.

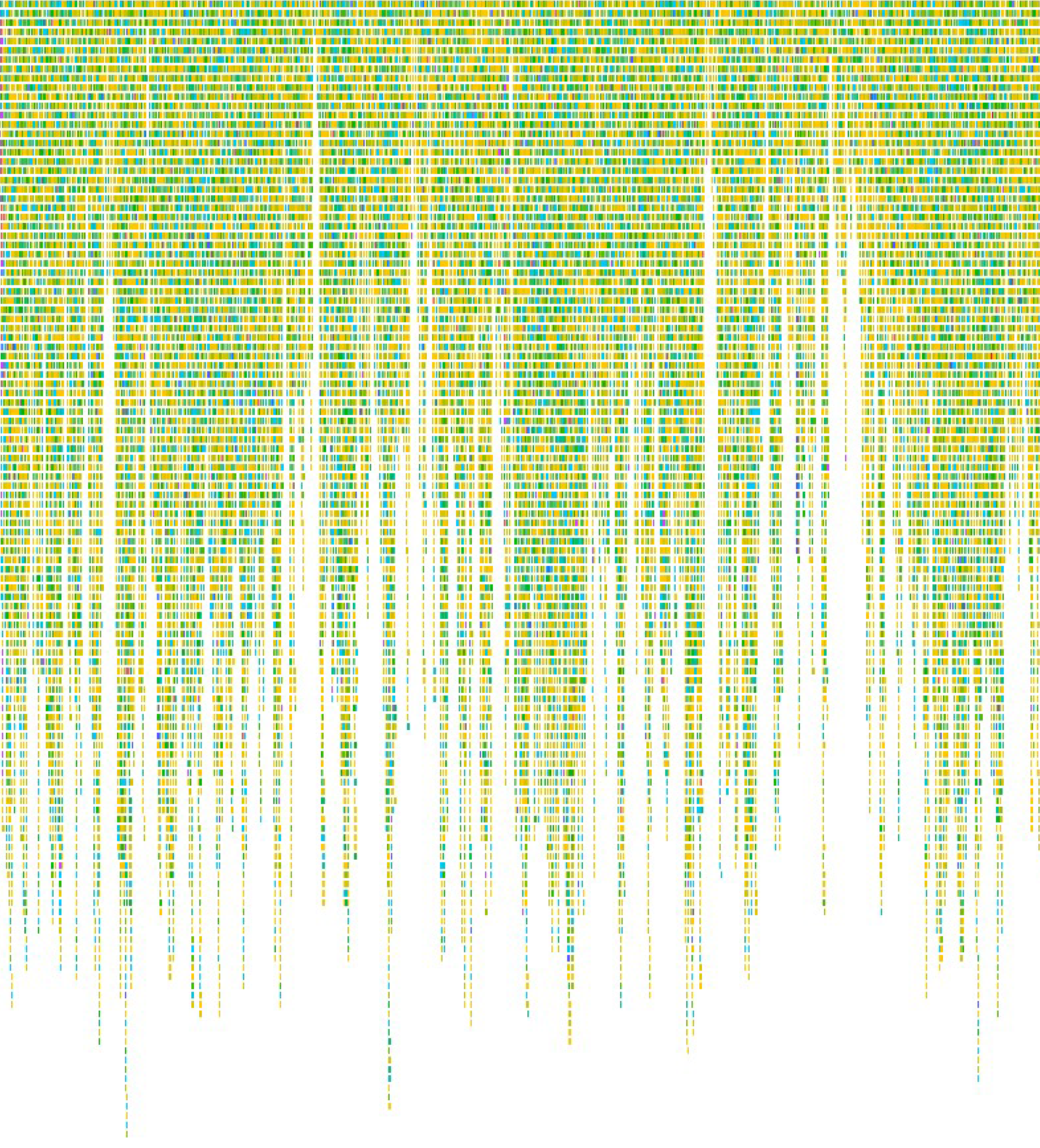
The ability to uptake DNA from other bacteria is a key process to understand the pneumococcal evolution. In our study we have found evidence of recombination events in three regions: the capsular operon (associated with capsular switching) and adjacent regions containing *pbp2x* and *pbp1a*, the *murM* gene and the *pbp2b–ddl* region. As all of these regions are related to either antimicrobial resistance or capsular switching, the PMEN3 clone evolution seems to have been driven by the use of broad-spectrum β-lactams and the introduction of conjugate vaccines. In fact, the emergence of lineage 2 (ST838^{9V}) during the 90s was associated to increased MICs to penicillin and amoxicillin, which could be related to the increased usage of β-lactam antibiotics in Spain during the 90s, as it has been described.[197] Our work also remarks the great importance of capsular switching events on the evolution of *Streptococcus pneumoniae*.[43] Although we detected the presence of other serotype variants (19A and 23F), successful lineages of the PMEN3 clone expressed serotypes 9V, 14 or 11A. It has been described that certain capsular switching are more likely to occur in the context of the same genetic background.[198] In fact, switching from serotype 9V to serotypes 14 or 11A is among those than occur more frequently than expected, probably because these serotypes share some polysaccharide components. The emergence of PMEN3 isolates expressing serotype 11A (a non-vaccine serotype) after the PCV7 introduction also illustrates the capacity of *Streptococcus pneumoniae* to avoid the effect of conjugate vaccines and persist over time. Besides, the fact that PMEN3 variants

express serotype 11A, a serotype usually related to CC62 and non-invasive disease, supports the hypotheses that recombination events are favoured in those environments that allow stable cell-to-cell contact such as colonization.[199] In the same way, as these environments also promote contact between pneumococci and other viridans group streptococci (reservoirs of β-lactam resistance) these streptococci can act as resistance donors.[55] Supporting this, we found that lineage 2 isolates harboured a transpeptidase domain of PBP2b closely related to *S. mitis*, which could have been the potential donor.

We have shown that the PMEN3 clone has successfully adapted to a changing environment of broader spectrum antibiotics and conjugate vaccines. Nevertheless, these genetic adaptations could have had a negative effect on the invasiveness or the virulence of PMEN3. On the contrary, we found that PMEN3 retained its potential virulence over time, regardless of lineage or serotypes. Then, we explored differences in surface proteins and the presence of prophages that could be related to changes in the virulence profile. The analysis through WGS detected the presence of temperate phages in all PMEN3 isolates. Interestingly, one of the detected prophages was inserted into the gene coding for ComGC, the competence pilus major pilin, which is an essential protein for transformation. Therefore, this could be a mechanism of targeted disruption of the competence machinery by which these prophages avoid their elimination.[200] Regarding its potential role in mortality, we found that most prophages encoded PblB proteins that have recently been associated to platelet activation and increased mortality in IPD.[201] Thus, these could be two key factors that have contributed to PMEN3 having retained its virulence potential. In regard of the pneumococcal surface proteins, although significant variability was detected, there was a predominant protein profile. The persistence of this surface protein profile over time suggests that this combination of proteins is important for the ability to cause disease and highlights the importance of the genetic background of PMEN3.

The β-lactam-resistant Spain^{9V}-ST156 clone has persisted over time despite expressing serotypes included in PCVs. The emergence of invasive PMEN3 isolates expressing non-vaccine serotypes such as serotype 11A in Europe[202] or serotype 35B in the USA[203] is an example of the ability of some *Streptococcus pneumoniae* clones to

quickly respond to environmental changes and allow its persistence.[22] A new generation of vaccines, whether or not based on the capsular polysaccharide, will be needed to continue the fight against pneumococcal diseases.



CONCLUSIONS

1. The introduction of the PCV13 for children in Spain caused an early decrease in the incidence of the adult IPD, mostly because of a reduction of PCV13 serotypes which demonstrates the importance of the herd immunity.

2. The stability of the incidence of the adult IPD caused by serotype 3 in the PCV13 period suggests a lack of effectiveness of this vaccine in preventing serotype 3 colonization and disease.

3. The expansion of some MDR clones expressing non-PCV13 serotypes such as ST386^{6C} and ST6521^{11A} was associated with an early increase of penicillin and cefotaxime resistance after the introduction of PCV13.

4. The increase in the incidence of community-acquired bacteraemia caused by other pathogens suggests an underestimation of the herd effect of the introduction of PCV13 on the adult IPD.

5. Over a 25-year period, sequential introduction of PCV7 and PCV13 has reduced the incidence of IPD caused by multidrug-resistant and penicillin non-susceptible isolates, mainly due to the decrease in resistant clones expressing vaccine serotypes.

6. The worldwide distributed clones Spain^{9V}-ST156 and Denmark^{14}-ST230 are responsible for nearly half of current multidrug-resistant and penicillin non-susceptible IPD and require further surveillance.

7. Due to the fact that a significant proportion of current resistant isolates are not covered by the upcoming PCV15/PCV20 vaccines, their efficacy with respect to antibiotic resistance could be limited.

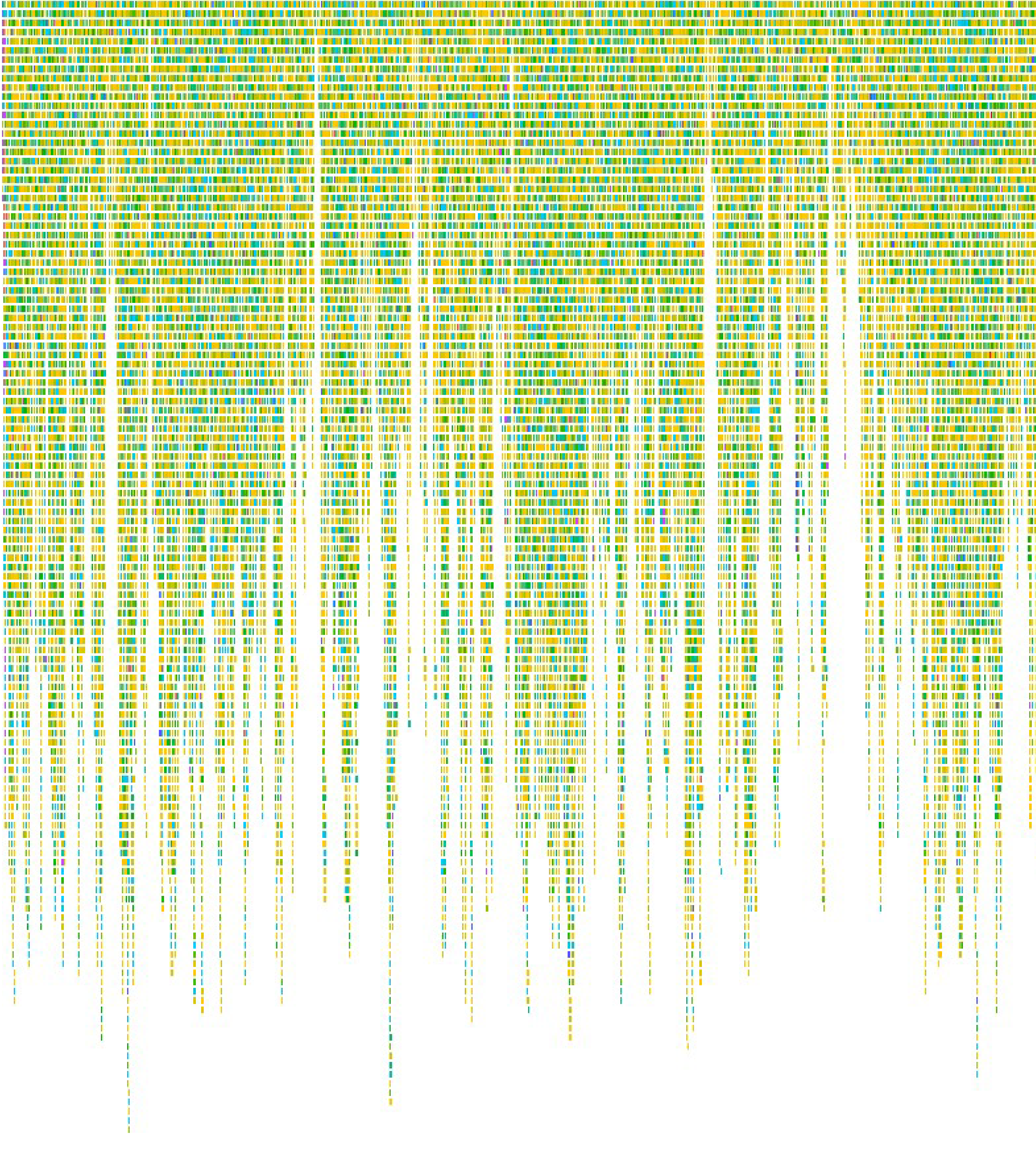
8. IPD caused by MDR/PNS isolates occurs in patients with factors related to poor prognosis (older age, high McCabe score) which leads to higher mortality. In spite of that, MDR or PNS are not factors related to statistically significant increased mortality.

9. Over a 25-year period, six major lineages were identified in the PMEN3 clone, originating primarily after recombination events in PBPs and capsular operon.

10. The use of WGS was extremely useful in revealing horizontal DNA transfer events that are not easily recognized by analysis of classical markers such as serotype, MLST, or antibiotic resistance profile.

11. The ability to gain exogenous DNA has allowed PMEN3 to persist over time despite the introduction of broad-spectrum antibiotics and conjugate vaccines.

12. Despite these adaptations, the maintenance of the virulence potential of PMEN3 highlights the importance of its genetic background as a key element for pathogenicity.



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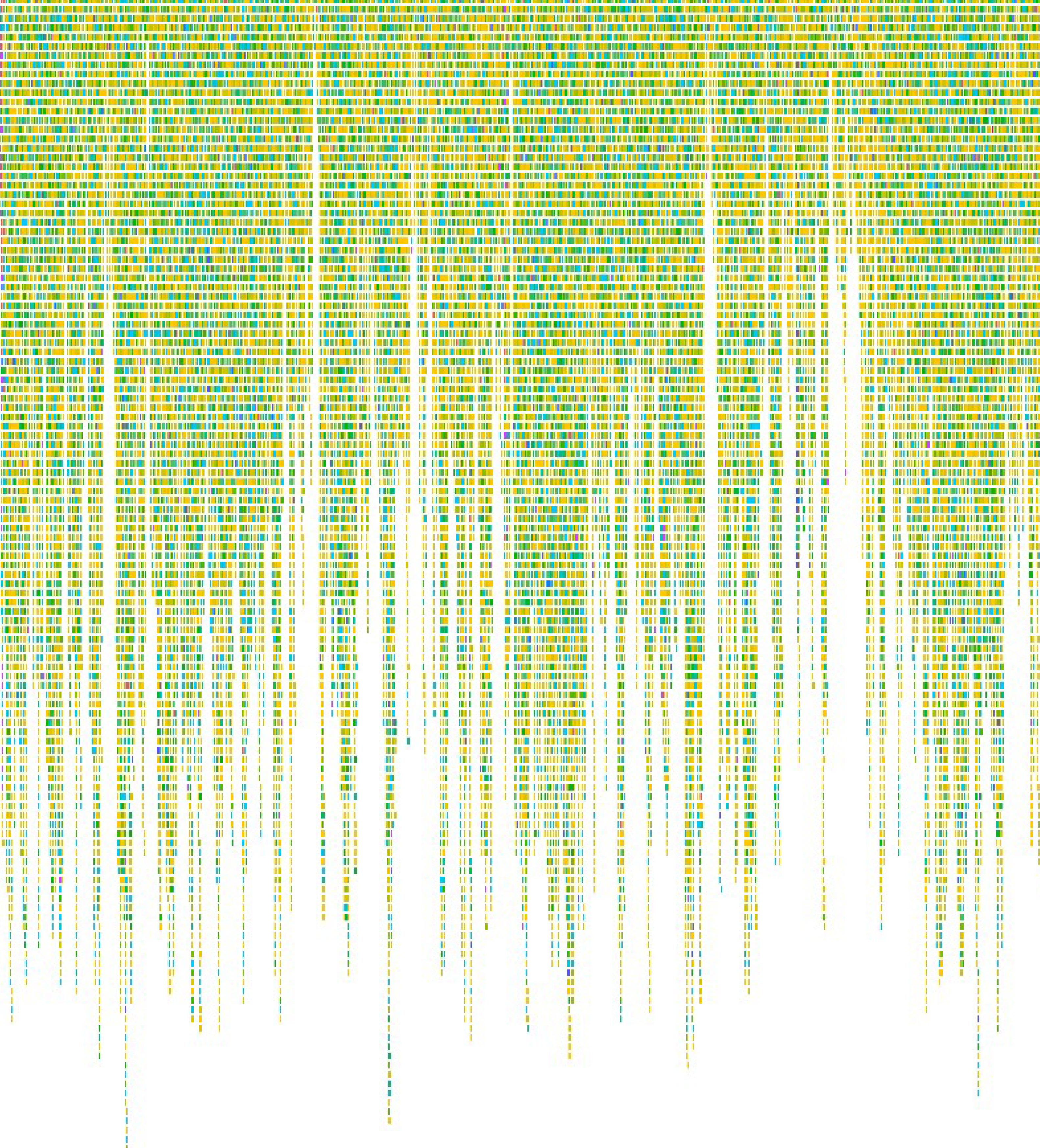

ANNEXES

Enferm Infecc Microbiol Clin. 2018;36(10):605–606

Enfermedades Infecciosas y Microbiología Clínica

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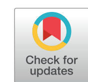
Editorial

Pneumococcal disease and conjugate vaccines



Enfermedades neumocócicas y vacunas conjugadas

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The prevention of pneumococcal diseases is a global challenge that has been partially achieved through vaccination. Over the 20th century, pneumococcal polysaccharide vaccines (PPV) were introduced. Among them, the 23-valent pneumococcal polysaccharide vaccine (PPV23) has been linked to a decrease in the mortality of pneumococcal pneumonia. However this PPV23 did not exert a noticeable impact on the incidence of invasive pneumococcal disease (IPD).[1,2] On the contrary, the introduction of the pneumococcal conjugate vaccines (PCVs) at the beginning of this century has shown high efficacy preventing IPD caused by vaccine-types.[3,4] This protection is achieved in two ways: a direct effect in the vaccinated population and an indirect effect in the non-vaccinated population (herd protection) due to a reduction of the global pneumococcal load. This reduction is at the expense of the vaccine-serotype pneumococci that are colonizing the nasopharynx of children, which are the main pneumococcal reservoir. However, other non-vaccine serotype pneumococci could fill the gap led by the PCV-ones and replace their role in invasive disease.[5]

In Spain, three conjugate vaccines have been licensed, all of them under a voluntary basis: PCV7 in 2001 (which included serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F), PCV10 in 2009 (adding serotypes 1, 5 and 7F) and PCV13 (which replaced PCV7 in 2010 adding serotypes 3, 6A, and 19A). Even so, the government of the Madrid autonomous community decided to subsidize the PCVs in 2006 and this policy was maintained until May 2012. Then, the vaccine coverage in Madrid (around 95% 2009–2012)[4] has always been higher than in most of the remaining Spanish regions (around 50–60%).[6–8] Other regions progressively introduced the PCVs such as Galicia for PCV13 in 2011. After the national approval in 2015, all the autonomous communities included PCV13 (2+1 scheme) in the pediatric schedule throughout the 2015–2016 period. Also, different approaches for adult vaccination with PCV13 have been introduced over the last years.

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In this issue two studies analyze the evolution of pneumococcal serotypes in the PCVs era in two regions with different vaccination strategies.[9,10] One of them performed in the island of Gran Canaria that had a low percentage of vaccinated children (48% in 2006 and 49% in 2014) until 2015. The second one from Madrid, where the percentage of vaccinated children was above 90% until 2012, when PCV13 was removed from the official vaccine schedule (though the uptake always remained above 65%).[11] It is not easy to analyze the impact of vaccines in the trends of pneumococcal disease. The influence of other factors such as natural fluctuations of serotypes and clones, geographic differences in the distribution of serotypes, or changes in flu activity could make the final analysis favorable or not. For instance, the marked fall of IPD after the PCV7 introduction in the US in the early 2000s was not observed in Europe.[3] In Spain, with a limited vaccine uptake, IPD increased after the PCV7 introduction due to an expansion of non-vaccine serotypes in both children and adults, especially those called epidemic serotypes (1, 7F, 5) which did not increase in the US .[12,13] The replacement of PCV7 by PCV13 in 2010 was followed by a sharp reduction in the overall incidence of IPD in Spain and other countries.[14–16]

In this way, the two studies showed a beneficial impact of the PCV13 introduction in the burden of IPD. The study from Gran Canaria[9] shows a significant decrease in the incidence of IPD after the PCV13 introduction (overall reduction of 66.4%) in the pediatric population (2001–2016 period). Although this decline was observed in young and older children, it was higher in children under 2 years old. Similarly, the results of the pneumococcal surveillance program of the Madrid autonomous community over 2008–2015[10] showed a significant decrease in the incidence of IPD after the PCV13 introduction (decrease of 32%). In fact, in the target population (children under 5) the remnant disease due to PCV13 serotypes was very low. Both studies linked the IPD reduction to a fall in the PCV7 and the additional PCV13 serotypes. However, a reduction of bacteraemia without focus was observed in Gran Canaria after the PCV13 introduction and this could reflect changes in the blood culture practice. This is a common limitation of the surveillance studies. Beside the overall reduction of the PCV13 serotypes 1, 5, 7F and 19A the impact on the incidence of serotype 3 has been controversial.[15] It is remarkable that both studies showed

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a marked fall in serotype 19A, a serotype usually associated to penicillin- and multidrug-resistance, which nearly disappeared in young children. Although less prominent, the same phenomenon was also observed in Madrid in the non-vaccinated population (older children and adults). On the other hand, the decrease of serotype 3 disease was only observed in the target population (children under 5) in Madrid and herd protection was not successfully achieved.[10] This is probably because of the low prevalence of this "adult" serotype colonizing children. Since Madrid recently started adult PCV13 vaccination, the analysis of the ongoing surveillance will be of high interest for the definition of future vaccine strategies.

The study from Gran Canaria did not detect any IPD related deaths in children throughout the PCV13 period. Before PCV13 introduction fatal outcomes were mostly linked to cases of meningitis or bacteremic pneumonia, whose number of episodes declined substantially. The reduction of pneumococcal meningitis in the pediatric population has been observed in other studies which is an important benefit for the severity of the disease, the mortality and sequelae.[11] Moreover, the decrease in mortality after the PCVs introduction agrees with recent reports that showed a decline in the mortality of children and also of adults as an indirect benefit of vaccination .[1,15] This fact was related to a reduction in the PCV7 serotypes that associated the higher fatality rates together with serotype 3.[1]

Another important finding of Santana et al. is an overall reduction in the rates of penicillin- and erythromycin-resistance after the PCV13 introduction linked to a reduction in serotypes 14 and 19A. This effect is related to a reduction of the multidrug resistance clones related to PCV7 serotypes [clonal complex (CC)156^{9V,14,} CC15^{14} CC88^{19F} and CC81^{23F}] and serotype 19A (CC320 and CC230), respectively.[12,16,17]

Finally, data from Madrid show a worrying increase of the non-PCV13 serotypes, suggesting serotype replacement.[5] This is in agreement with recently reported data from other countries such as England and Wales, where the PCVs have been included into the routine vaccination program since 2006.[18] In Madrid, these results are related to an increase in IPD mainly in two age groups: children under 5 years old, the target of the vaccination program, and adults over 59 years old, mostly a non-vaccinated population. Then, the connection between the reservoirs of pneumococci (children) and the people that are at a higher risk of having IPD (older adults) is emphasized. Among non-PCV13 serotypes, the rise in the incidence of serotype 8, which has become the first cause of IPD in the 2013–2015 period, is especially alarming. This serotype has demonstrated a high invasive disease potential among children after the PCV introduction.[19] Consequently, the emergence of non-vaccine serotypes showing invasiveness, such as serotypes 12F or 8, is worrying. Moreover, even when by Latasa et al. reported a decrease in the incidence of IPD after the PCV13 introduction, an increasing trend of disease has been observed since 2013, especially for children under 5 years old and adults over 59.

The introduction of the PCVs had a direct impact on the prevention of IPD and on the pneumococcal epidemiology. In England and Wales, it has been estimated that 40,000 episodes of IPD have been prevented since the introduction of PCV7.[18] Nevertheless, some doubts arise about the increase of the non-vaccine serotypes that could minimize the benefits of the vaccination programs. Thus, monitoring the incidence of IPD through surveillance programs continues being critical. In this issue, Santana and Latasa present their respective works regarding the pneumococcal epidemiology in two different Spanish regions after PCV13 introduction. Although differences in vaccine coverage between the two regions exist, a decrease in IPD after PCV13 introduction was clearly established by both works. However, the evidence for serotype replacement (especially serotype 8) detected in Madrid could compromise the effectiveness of the current vaccines in the prevention of the disease. These results highlight the need for developing new broader conjugate vaccines or preferably non-serotype based vaccines to preserve the efficacy of the immunization programs in the overall population.

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Enfermedad neumocócica en el adulto. Serotipos, clones y resistencia antibiótica

INTRODUCCIÓN

Streptococcus pneumoniae se adhiere al epitelio respiratorio formando parte de la microbiota respiratoria nasofaríngea. La colonización nasofaríngea, un punto clave en la patogenia del neumococo, es mayor en los niños pequeños, en los que puede estar presente en un 27-65%, mientras que solo se observa en el 10% de los adultos. Así, la colonización del epitelio respiratorio es la vía principal de diseminación, ya que los portadores pueden eliminar neumococos en sus secreciones nasales y alcanzar así a otros huéspedes[1]. Por otro lado, la colonización también es la puerta de entrada para causar enfermedad, ya sea por diseminación local, por aspiración o por invasión del torrente sanguíneo. El paso de colonización a infección se ve favorecido por una serie de factores, como pueden ser la inmunosupresión, el hábito tabáquico, el alcoholismo o la existencia de enfermedades crónicas. Los cambios inducidos en el epitelio respiratorio debido a procesos inflamatorios, especialmente infecciones virales, favorecen la colonización por el neumococo y que esta sea de alta densidad, lo que facilita la transmisión del neumococo y también su llegada al pulmón mediante microaspiraciones.

La enfermedad neumocócica invasiva (ENI), que se define como la invasión de la sangre o de otros fluidos estériles, es una enfermedad grave. Aunque la incidencia de la ENI es más alta en los niños menores de 5 años y en los adultos mayores de 65 años, hay otros condicionantes que incrementan el riesgo de padecerla. Así, las tasas de incidencia de la ENI en adul-

tos sin enfermedades de base se multiplican entre 5 y 10 veces en aquellos que tienen diabetes o enfermedades crónicas pulmonares o cardiacas. Este incremento es aún mayor en los pacientes con cáncer, infección por el virus de la inmunodeficiencia humana o una enfermedad oncohematológica[2]. También algunos hábitos de riesgo, como el abuso de alcohol o el fumar, se identifican con mayor frecuencia entre los pacientes que sufren ENI, y son importantes factores de riesgo en los adultos jóvenes[3].

La mortalidad de la ENI es especialmente alta en los mayores de 65 años y en los pacientes inmunodeprimidos, pudiendo superar el 15-20% a pesar de recibir un tratamiento antibiótico adecuado[4]. Por este motivo, la prevención de las enfermedades neumocócicas invasivas y no invasivas es un importante reto en nuestro medio, en especial en los grupos de edad de mayor incidencia y en otras poblaciones con riesgo incrementado de padecer ENI.

La mayoría de las ENI en los adultos son neumonías neumocócicas bacteriémicas. En un estudio multicéntrico español que recogió 639 episodios de ENI en pacientes adultos en el periodo 2015-2016, el 69,3% de los episodios fueron neumonías bacteriémicas y el 11,4% fueron meningitis[5]. En el grupo de mayores de 65 años (n = 351), las neumonías fueron la causa de un porcentaje similar (70,6%). Cuando se utilizan diferentes técnicas diagnósticas, el neumococo es el causante de, al menos, el 30% de las neumonías comunitarias del adulto que requieren ingreso hospitalario. Sin embargo, más del 80% de estas cursan sin bacteriemia, por lo que los estudios sobre la ENI son solo la punta del iceberg de la carga de enfermedad de la neumonía neumocócica[6].

LA CÁPSULA POLISACÁRIDA Y SU PAPEL EN LA INVASIVIDAD, LA MORTALIDAD Y LA VIRULENCIA

La cápsula es el principal factor de virulencia de *S. pneumoniae*. Es una estructura compuesta por polisacáridos situada externamente y que confiere al neumococo capacidad antifagocítica. La composición de la cápsula, que determina el reconocimiento por anticuerpos específicos, permite clasificar a los neumococos en serotipos, y es el método de tipificación neumocócica más usado. Aunque hasta el momento se conocen más de 100 serotipos, menos de 25 de ellos son causa del 90% de los casos de ENI[6,7]. De hecho, el serotipo tiene un papel clave en la invasividad y en la mortalidad de la ENI[8]. La capacidad invasiva de los diferentes serotipos se analiza comparando

la frecuencia de estos en la colonización nasofaríngea y en la enfermedad invasiva en poblaciones similares en un periodo de tiempo concreto. Estos estudios se realizan normalmente en población pediátrica, ya que la colonización por neumococo es baja en los adultos. En este tipo de análisis, algunos serotipos (1, 5, 7F, 14 y 19A) se clasifican como invasivos debido a que su frecuencia en la colonización nasofaríngea es muy inferior a la que tienen en la enfermedad invasiva. Por otro lado, otros serotipos se encuentran con mayor frecuencia colonizando que causando ENI (23F, 6B, 19F y 11A). Un aspecto que debe tenerse en cuenta es que los estudios de capacidad invasiva de los serotipos pueden variar a lo largo del tiempo y en función de la localización geográfica, probablemente debido a diferencias en los factores de virulencia de los clones circulantes asociados a estos serotipos. Un estudio realizado por Lindstrand et al.[9] que incluía población pediátrica y adulta (padres) encontró, además de los serotipos invasivos ya conocidos (1, 3, 4 y 7F), otros que también presentan gran capacidad invasiva (12F, 22F, 8 y 9N). En nuestro medio, los serotipos 1, 3 y 7F son los que se han asociado con mayor capacidad invasiva en los niños[10].

Los estudios de colonización en población adulta son escasos y por tanto es más difícil determinar la capacidad invasiva de los serotipos en los adultos. Una aproximación se basa en la comparación de la diferente composición de serotipos encontrada en pacientes con exacerbaciones de su enfermedad pulmonar obstructiva crónica (EPOC) y ENI. En un estudio publicado por Shoji et al.[11] que incluía los años 2013 a 2016 se observó que los serotipos 3, 7F, 8 y 12F presentaban mayor capacidad invasiva, ya que aparecían con mayor frecuencia en ENI que en exacerbaciones de la EPOC, mientras que el serotipo 11A, más frecuente en las exacerbaciones de la EPOC, se comportaba como no invasivo. En este estudio, la frecuencia de algunos serotipos invasivos (1 y 5) o no invasivos (6B y 23F) clásicos fue muy baja, por lo que no se les pudo atribuir su capacidad invasiva.

La mortalidad de la ENI, que puede llegar a superar el 25% en grupos de riesgo, se ha relacionado también con el serotipo, aunque esta relación no siempre coincide con la invasividad. Un metaanálisis[8] mostró que los serotipos invasivos 1, 5, 7F y 8 son los que se asocian con menor riesgo de muerte en comparación con el serotipo 14. Por otro lado, los pacientes con ENI causada por los serotipos 3, 6A, 6B, 9N o 19F presentaron mayor mortalidad. En nuestro medio, un estudio reciente encontró la mayor tasa de mortalidad (28%) en la ENI causada por el serotipo 3. Además, la ENI causada por los serotipos 4, 6B, 9V, 14, 19F y 23F tuvo una mortalidad

similar a la producida por el serotipo 3 después de ajustar por edad, comorbilidad y otros factores de riesgo[12].

Otro estudio realizado en la neumonía neumocócica bacteriémica en adultos encontró como factores de riesgo independientes de fallo respiratorio tanto la presencia de algunas características del huésped (edad mayor de 50 años, enfermedad crónica pulmonar o cardiaca) como el estar causada por determinados serotipos (3, 19A y 19F)[13].

Una de las principales causas de la mortalidad de la neumonía adquirida en la comunidad es la ocurrencia de eventos cardiovasculares adversos en el transcurso de la neumonía. De hecho, hasta el 30% de los pacientes ingresados con neumonía adquirida en la comunidad tiene dichos efectos cardiovasculares, lo que duplica la mortalidad. Un estudio ha investigado la presencia de eventos cardiovasculares adversos durante el trascurso de la ENI y su relación con los serotipos, y ha encontrado un riesgo más alto cuando la ENI estaba causada por los serotipos 3 y 9N[14]. La capacidad del neumococo de producir estos efectos cardiovasculares se ha estudiado en modelos experimentales, en los que se ha demostrado su capacidad para translocar a través del endotelio vascular e invadir el miocardio; allí, tras replicarse, produce daño cardiaco mediado por depósitos de colágeno[14].

En la patogenia de la infección neumocócica, la cápsula polisacárida tiene un importante papel al promover la adhesión del neumococo y la evasión de las defensas del huésped, e inhibir la fagocitosis. Sin embargo, el neumococo necesita otros factores de virulencia para causar enfermedad. La superficie de *S. pneumoniae* está recubierta por una serie de proteínas ancladas a la pared celular que exponen regiones al exterior. Estas proteínas actúan como importantes factores de virulencia debido a su interacción con receptores del huésped durante el proceso infeccioso. Este conjunto de factores de virulencia está más asociado al *background* genético (genotipo) de las cepas de neumococo que al serotipo, lo que condiciona diferencias geográficas en las características de la ENI a pesar de estar causada por un mismo serotipo. La ENI producida por el serotipo 1 es un ejemplo: mientras que en Europa se ha asociado con el ST306 causando neumonía complicada con empiema en adultos y niños, en África, donde predomina el ST217, el serotipo 1 es la causa principal de meningitis neumocócica[15]. Probablemente existen otros factores adicionales al tipo capsular que moderan el tropismo del neumococo hacia diferentes órganos. Así, el estudio de los genotipos asociados a los princi-

pales serotipos que causan ENI es una herramienta eficaz para entender las diferencias geográficas de la enfermedad neumocócica.

La cápsula del neumococo está codificada por un operón de 10-30 kb flanqueado por los genes *aliA* y *dexB*. Los genes incluidos en este operón son los que condicionan la estructura de la cápsula y, en consecuencia, el serotipo. Este tipo de estructura favorece el intercambio genético entre neumococos, resultando en un proceso llamado intercambio capsular *(capsular switching)*. Mediante este fenómeno, el operón capsular puede ser intercambiado entre neumococos, un proceso favorecido por la presión que ejerce el sistema inmunitario humano contra determinados serotipos (como ocurre con la vacunación), lo que produce una selección positiva de aquellas cápsulas no incluidas en las vacunas. Por este mecanismo, el neumococo es capaz de evadir el efecto de las vacunas. Además, debido a que el operón capsular se sitúa próximo a los genes *pbp2x* y *pbp1a*, que están directamente implicados en la resistencia a la penicilina, es frecuente que el fenómeno de intercambio capsular se acompañe de cambios en la sensibilidad a los betalactámicos[16].

CLONES Y RESISTENCIAS ANTIBIÓTICAS

Las técnicas de tipificación molecular, electroforesis en campo pulsátil tras restricción (ECP), *Multi Locus Sequence Typing* (MLST) y más recientemente la secuenciación del genoma completo (SGC) han permitido demostrar que solo unos pocos clones se diseminan con éxito y son capaces de causar ENI en todo el mundo[12,17]. Por otro lado, también han demostrado la diversidad genética de *S. pneumoniae* debido a su capacidad de adquisición de DNA homólogo mediante recombinación genética *(horizontal DNA transfer*, HDT), que permite la adquisición de determinantes de resistencia o de virulencia procedentes de otras cepas de *S. pneumoniae* o de otros estreptococos.

Los estudios moleculares han ayudado también a conocer mejor los cambios en la incidencia de algunos serotipos en particular. Un estudio demostró que la expansión clonal de dos linajes (ST1223 y ST289) del serotipo 5 en el área metropolitana de Barcelona se asociaba con brotes de ENI ocurridos en los años 1998, 2005 y 2009[7]. El intercambio capsular también desempeña un papel determinante en la dinámica poblacional de clones causantes de ENI, como es el caso del PMEN3 (ST156) en nuestro medio. Este clon fue uno de los más frecuentes causantes de ENI en las décadas de 1980, 1990 y 2000, asociado a los serotipos 9V y 14 (incluidos

en las vacunas conjugadas). En la actualidad persiste en nuestro medio gracias a la adquisición del tipo capsular 11A. Mediante estudios de SGC se ha determinado que este clon ha sufrido diversas recombinaciones importantes: una primera deriva genética (sin cambio de serotipo) apareció por transferencia horizontal de ADN a mediados de los años 1990 (9V-ST838), incrementando especialmente la resistencia a la amoxicilina (concentración mínima inhibitoria [CMI]: 8-16 mg/l); una segunda recombinación, a finales de la década de 2000, se originó con un cambio de cápsula (11A-ST838); y una tercera (11A-ST6528) le confirió una mayor capacidad de formación de biofilm, y este último linaje se diseminó por España, Francia y Portugal[18].

La exploración del genoma del neumococo mediante SGC está siendo de gran utilidad para identificar factores de virulencia importantes en la patogenia de la enfermedad neumocócica. Así, un estudio demostró no solo la diferente capacidad invasiva de los linajes genéticos definidos por MLST, sino también la variabilidad intraclonal del serotipo 6B-ST138. Esta variabilidad comporta diferencias en la capacidad invasiva en los niños y en la virulencia en modelos experimentales en ratones[19].

En los estudios epidemiológicos sobre el neumococo, la determinación de la resistencia a los antibióticos, frecuentemente asociada a determinados linajes, es clave. En España, la prevalencia de la resistencia de *S. pneumoniae* a la penicilina y a otros antimicrobianos ha sido y sigue siendo más alta que en la mayoría de los países europeos, alcanzando máximos del 40% en las décadas de 1990 y 2000[20]. Pese a que la ENI no meníngea puede ser tratada eficazmente con penicilina (la mayoría de las cepas presentan CMI <4 mg/l), la emergencia de linajes como el antes mencionado 11A-ST6521, con CMI de amoxicilina de 8-16 mg/l, es preocupante. El aumento de las CMI de los betalactámicos se asocia con alteraciones en las proteínas fijadoras de penicilina (PBP, *penicillin-binding proteins*), siendo PBP1A, PBP2B y PBP2X las que tienen un papel principal en la resistencia. Estas PBP presentan con frecuencia estructuras en mosaico como resultado de la recombinación con genes homólogos de otros estreptococos, principalmente los del grupo viridans[17].

Los macrólidos, solos o en combinación, son ampliamente utilizados en el tratamiento de las infecciones respiratorias[21]. En nuestro medio, la prevalencia de la resistencia a los macrólidos aumentó progresivamente desde un 5% en 1986 hasta un 28% en 2001. Aunque al principio se relacionó con la diseminación de clones multirresistentes asociados a los serotipos

vacunales, las tasas de resistencia se han mantenido elevadas debido a la emergencia de otros clones multirresistentes y a la diseminación horizontal de los genes que codifican resistencia a los macrólidos[22].

La resistencia a las quinolonas, en cambio, se mantiene por debajo del 5% en nuestro medio y se asocia principalmente al tratamiento previo con este grupo de antimicrobianos. En este caso, la resistencia es más alta en los pacientes con EPOC u otras enfermedades crónicas respiratorias que reciben cursos de tratamiento con quinolonas en las exacerbaciones de su enfermedad. Sin embargo, también se ha descrito en parte de la geografía española la diseminación de un clon recombinante del serotipo 8 asociado con resistencia a las quinolonas[23].

Los cambios en la resistencia a los antimicrobianos se asocian con frecuencia a las variaciones en la frecuencia de los serotipos y, especialmente, de sus clones multirresistentes asociados. De este modo, la introducción de la vacuna conjugada 7-valente (VCN7) conllevó la desaparición de los clones multirresistentes asociados a los serotipos vacunales 23F (PMEN1), 6B (PMEN2) y 14 (PMEN5), lo que causó un descenso de las tasas de resistencia a los betalactámicos en nuestro medio, tanto en la población adulta como en los niños.

Existen diferencias en las tasas de resistencia antibiótica en función del tipo de enfermedad neumocócica. La tabla 1 muestra los datos de sensibilidad antibiótica de las cepas de *S. pneumoniae* aisladas de pacientes mayores de 65 años con enfermedad invasiva según el foco, y también de aislamientos recogidos en episodios de exacerbación de EPOC. Las mayores tasas de resistencia se observan entre los aislamientos de exacerbaciones de EPOC. Así, la resistencia a la penicilina (incluyendo las cepas con sensibilidad intermedia) supera el 45% en los aislamientos de exacerbaciones de EPOC, mientras que en los procedentes de pacientes con neumonía bacteriémica esta tasa no llega al 25%. Algo similar ocurre con la resistencia a los macrólidos, que se sitúa en el 38,1% en las exacerbaciones de la EPOC y es del 18,1% en la neumonía bacteriémica. Estas diferencias también se observan en la resistencia al levofloxacino, que pasa del 9,5% en un grupo al 1,1% en el otro. Esto se puede explicar por el elevado consumo de antibióticos en los pacientes con EPOC, que favorece la selección de cepas resistentes[11]. Por otro lado, también es importante destacar la tasa de resistencia a los betalactámicos observada en cepas aisladas de pacientes con meningitis neumocócica, en especial la presencia de aislamientos con disminución de la sensibilidad a la cefotaxima (4,5%), ya que esta es el tratamiento de elección.

Tabla 1. Sensiblidad antibiótica de los aislamientos de neumococo de pacientes adultos según el tipo de infección.

Antibiótico	Punto de corte CMI (mg/l)	Exacerbación EPOC		Enfermedad neumocócica invasiva: Neumonía		Meningitis		Peritonitis		Bacteriemia sin foco		Otras	
		N	%	N	%	N	%	N	%	N	%	N	%
Penicilina	0,12-1	60	28,6	58	16,6	11	25,0	5	27,8	6	13,3	3	14,3
	2	26	12,4	22	6,3	1	2,3	3	16,7	2	4,4	1	4,8
	≥4	10	4,8	7	2,0	0	0,0	1	5,6	1	2,2	1	4,8
Cefotaxima	1	20	9,5	24	6,9	2	4,5	3	16,7	3	6,7	2	9,5
	≥2	5	2,4	10	2,9	0	0,0	0	0,0	0	0,0	2	9,5
Amoxicilina	4	21	10,0	13	3,7	1	2,3	1	5,6	2	4,4	1	4,8
	≥8	4	1,9	4	1,1	0	0,0	1	5,6	0	0,0	0	0,0
Eritromicina	≥0,5	80	38,1	63	18,1	9	20,5	7	38,9	7	15,6	5	23,8
Clindamicina	≥0,5	72	34,3	63	18,1	7	15,9	7	38,9	5	11,1	5	23,8
Tetraciclina	≥2	87	41,4	73	20,9	6	13,6	7	38,9	9	20,0	7	33,3
Cloramfenicol	≥8	9	4,3	22	6,3	1	2,3	1	5,6	19	42,2	0	0,0
Cotrimoxazol	≥1	69	32,9	80	22,9	10	22,7	6	33,3	8	17,8	3	14,3
Levofloxacino	>2	20	9,5	4	1,1	0	0,0	1	5,6	0	0,0	0	0,0
Aislamientos estudiados		210		349		44		18		45		21	
Periodo de estudio		2013-2016		2013, 2015-2016		2013, 2015-2016		2013, 2015-2016		2013, 2015-2016		2013, 2015-2016	
Edad, años		Media 73 (38-92)		>64		>64		>64		>64		>64	

CMI: concentración mínima inhibitoria; EPOC: enfermedad pulmonar obstructiva crónica.
Adaptada de refs. 5 y 11.

IMPACTO DE LA INTRODUCCIÓN DE LAS VACUNAS ANTINEUMOCÓCICAS DEL ADULTO (VPN23) Y DEL NIÑO (VCN)

La cápsula polisacárida se ha utilizado como base para el diseño de vacunas antineumocócicas debido a sus propiedades inmunógenas y a la estimulación de la producción de anticuerpos[16]. La diversidad del polisacárido capsular, con al menos 100 tipos diferentes identificados hasta la fecha, dificulta la cobertura amplia de las vacunas y ha hecho que estas sean de diferentes tipos[24].

En la actualidad se dispone de dos tipos de vacunas para la prevención de la enfermedad neumocócica. La primera está compuesta por polisacáridos capsulares purificados y se introdujo por primera vez en 1977 incluyendo 14 serotipos, que posteriormente se ampliaron a 23 (VPN23): 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14,15B, 17F, 18C, 19A, 19F, 20, 22F, 23F y 33F. La VPN23 está recomendada para adultos y niños mayores de 5 años para la prevención de la ENI. Esta vacuna no elimina el estado de portador asintomático, lo cual, unido a su no recomendación para niños pequeños, hace que el efecto de protección de grupo sea prácticamente nulo[25].

El segundo tipo de vacunas son las conjugadas neumocócicas (VCN). La conjugación del polisacárido con una proteína transportadora estimula la respuesta mediada por linfocitos T, y por ello las vacunas conjugadas presentan una mayor efectividad tanto en la prevención de la enfermedad causada por los serotipos vacunales como en la eliminación de estos serotipos de la nasofaringe. La acción sobre el estado de portador limita la transmisión de los serotipos incluidos en la vacuna, teniendo un efecto de protección de grupo frente a estos serotipos[24]. Se han comercializado cuatro vacunas conjugadas y actualmente otras dos se encuentran en fase de desarrollo. La vacuna conjugada 7-valente (VCN7) se introdujo en 2001 en nuestro país e incluía los serotipos 4, 6B, 9V, 14, 18C, 19F y 23F. La vacuna conjugada 10-valente (VCN10) se comercializó en 2009 e incorpora, además, los serotipos 1, 5 y 7F. En la vacuna conjugada 13-valente (VCN13) se añadieron los serotipos 3, 6A y 19A, y sustituyó a la VCN7[26]. En 2019, el Serum Institute of India desarrolló una nueva VCN10 incluyendo los serotipos 1, 5, 6A, 6B, 7F, 9V, 14, 19A, 19F y 23F, con la finalidad de prevenir la enfermedad neumocócica en los países en vías de desarrollo (www.gavi.org/sites/default/files/document/pcv-

profilespdf.pdf). Hay dos vacunas conjugadas en fase de desarrollo: la VCN15, que incorpora los serotipos 22F y 33F a los de la VCN13, y la VCN20, que añade a los anteriores los serotipos 8, 10A, 11A, 12F y 15B. Además se está trabajando en la búsqueda de otras vacunas no basadas en el polisacárido capsular, con la finalidad de tener una cobertura mayor y minimizar el fenómeno del reemplazo de serotipos no vacunales causado por el vacío dejado en el nicho ecológico por los serotipos vacunales.

En España, las vacunas conjugadas se han administrado en la población pediátrica bajo prescripción privada desde 2001, y la incorporación de la VCN13 en el calendario vacunal infantil se produjo durante el periodo 2015-2016 en todas las comunidades autónomas (https://www.mscbs.gob.es/profesionales/saludPublica/prevPromocion/vacunaciones/docs/Vacunacion_poblacion_adulta.pdf). Las tasas de primovacunación en España en el año 2017 se situaron en el 97,7% (https://www.mscbs.gob.es/profesionales/saludPublica/prevPromocion/vacunaciones/docs/CoberturasVacunacion/Tabla1.pdf). Por otro lado, desde 2004 se mantiene la vacunación de manera sistemática en la población mayor de 65 años con la VPN23, administrando en algunos grupos de riesgo una dosis de recuerdo a los 5 años de la primovacunación, mientras que la VCN13 solo está recomendada en población adulta perteneciente a grupos de riesgo (https://www.mscbs.gob.es/profesionales/saludPublica/prevPromocion/vacunaciones/docs/Vacunacion_poblacion_adulta.pdf).

La introducción de vacunas conjugadas ha cambiado la epidemiología global de *S. pneumoniae*. La implementación primero de la VCN7 y después de la VCN13 se ha asociado a un descenso drástico en la incidencia de la ENI causada por serotipos incluidos en estas vacunas. En nuestro medio, con unas tasas de vacunación infantil inferiores al 50%, la introducción de la VCN7 en 2001 tuvo un efecto importante en la ENI del adulto[20]. El efecto sobre la colonización nasofaríngea en los niños disminuyó la transmisión de serotipos incluidos en la VCN7 y a su vez la ENI causada por ellos. Sin embargo, el incremento de la ENI causada por serotipos no incluidos en la VCN7 con mayor potencial invasivo (1, 5, 7F y 19A) resultó en un incremento global de la ENI, especialmente en los adultos jóvenes y en los niños. Este efecto fue más variado, dependiendo principalmente del área geográfica, en los mayores de 65 años[27]. La VCN10 ha tenido un escaso impacto en nuestro medio debido a su poca utilización en España.

La introducción de la VCN13 con unas tasas superiores de vacunación infantil condujo a una importante protección de grupo, con un descenso

significativo de la ENI en la población adulta. Un estudio multicéntrico realizado en España mostró este impacto al comparar los periodos 2009-2010 y 2012-2013, debido a un descenso en los serotipos incluidos en la VCN13 (*incidence rate ratio* [IRR]: 0,46; intervalo de confianza del 95% [IC95%]: 0,4-0,53). Este efecto protector fue mayor en Madrid, donde la VCN13 ya estaba incluida en el calendario vacunal[28]. El análisis del periodo posterior, 2015-2016, mostró una estabilización de la ENI debido a que el incremento de los serotipos no incluidos en la VCN13 (IRR: 1,31; IC95%: 1,14-1,51) compensó el descenso significativo de la ENI causada por los serotipos incluidos en la VCN13 (IRR: 0,65; IC95%: 0,54-0,78).

La figura 1 muestra un análisis detallado de los cambios en la incidencia de la ENI en los mayores de 65 años incluyendo la contribución de los serotipos de la VCN7, los adicionales incluidos en la VCN13 (VCN13no7), los incluidos en la VPN23 y no en la VCN13 (VPN23noVCN13), y los no incluidos en ninguna vacuna (noVPN23). Tras la introducción de la VCN13 se produjo un descenso de la ENI total en un periodo temprano (2012-2013) tanto en el grupo de 65-75 años como en los mayores de 75 años. Este declive se asoció con una disminución de la ENI causada por los serotipos VCN13. En un periodo más tardío (2015-2016), aunque continuó el descenso de los serotipos VCN13, este se vio compensado por un aumento de los serotipos noVCN13. Es interesante el análisis de estos VPN23noVCN13, ya que este grupo de edad se incluye entre las recomendaciones de vacunación con VPN23, con unas tasas de vacunación en nuestro medio

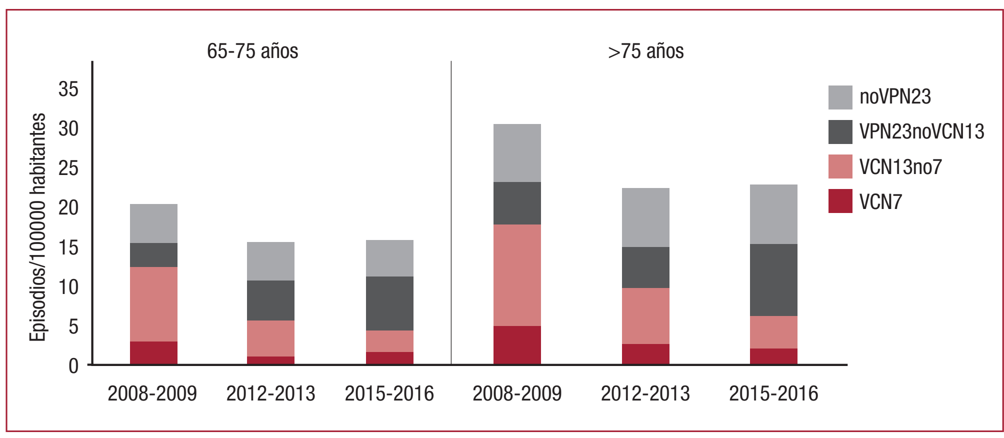

Figura 1. Incidencia de la enfermedad neumocócica invasiva causada por diferentes serotipos en mayores de 65 años.

de alrededor del 60%[29]. En este último periodo se ha observado un incremento de la ENI causada por serotipos VPN23no13 tanto en el grupo de edad de 65-75 años (IRR: 1,48; IC95%: 0,99-2,19) como en el de mayores de 75 años (IRR: 1,76; IC95%: 1,23-2,52), mientras que la ENI producida por serotipos noVCN se mantiene estable en ambos grupos de edad.

Un estudio del Centro Nacional de Microbiología ha analizado la incidencia de la ENI hasta el año 2019 en un contexto general de vacunación infantil alta, después de la introducción de la vacuna en todos los calendarios de forma oficial en 2016. En este estudio se observa una continuación del aumento de la ENI por serotipos noVCN13 en los mayores de 65 años, en especial de la causada por serotipos VPN23no13. La principal causa del aumento de los serotipos noVCN13 es el incremento de la ENI debida al serotipo 8[30].

COBERTURA DE SEROTIPOS DE LAS VACUNAS ACTUALES Y FUTURAS EN LA ENFERMEDAD NEUMOCÓCICA INVASIVA Y EN LA NEUMONÍA ADQUIRIDA EN LA COMUNIDAD

Los cambios ocurridos tras la introducción sucesiva de la VCN7 y la VCN13 han modificado la distribución de los serotipos causantes no solo de la ENI, sino también de la neumonía neumocócica (incluyendo la no bacteriémica) y de otras enfermedades neumocócicas del adulto, como las exacerbaciones de la EPOC. Los datos más recientes de la ENI en España muestran un continuo descenso en la incidencia de los serotipos incluidos en la VCN13. De hecho, en la serie de datos del año 2019 del Centro Nacional de Microbiología, estos serotipos solo representan el 25% de las ENI en la población a partir de los 65 años[30]. Respecto a las vacunas que están en desarrollo, este porcentaje aumenta hasta el 31% para la VCN15 y hasta el 62% para la VCN20. Hay que destacar que en este grupo de edad casi un tercio de los episodios están causados por solo dos serotipos (ambos incluidos en la VCN20): el serotipo 8 (18,7%) y el serotipo 3 (13,2%). Por otro lado, en este mismo estudio, la vacuna de polisacáridos VPN23 sigue mostrando las mayores tasas de cobertura, ya que incluye el 70% de los serotipos de neumococo actuales[30].

La figura 2 muestra la distribución de los serotipos en tres estudios independientes: ENI en adultos mayores de 65 años, neumonía neumocócica bacteriémica y no bacteriémica, y exacerbaciones de la EPOC, en un periodo de tiempo similar. En el periodo 2015-2016, los datos de un estudio

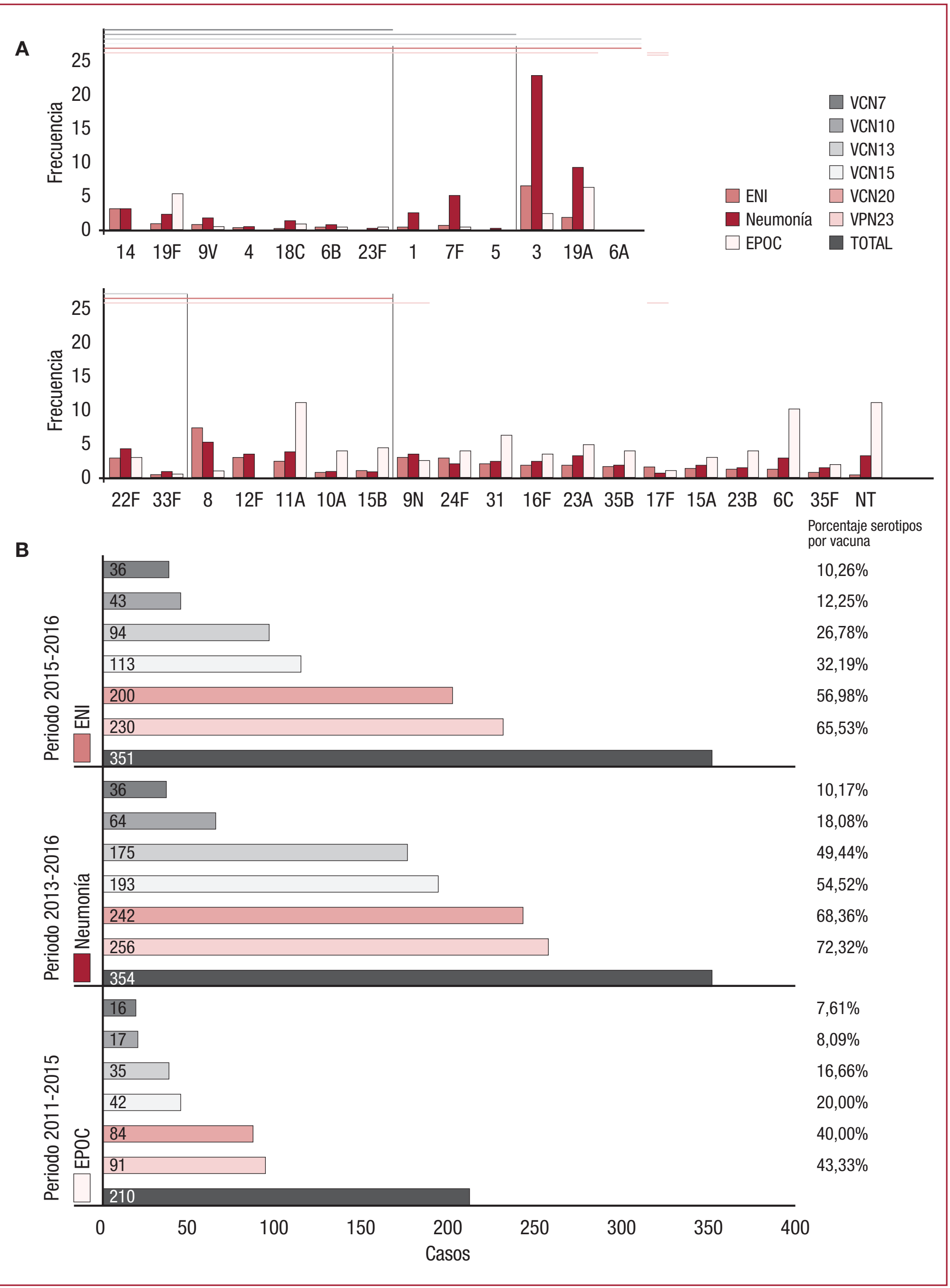

Figura 2. A: Distribución de serotipos que causan enfermedad neumocócica invasiva (ENI) en mayores de 65 años, neumonía y exacerbación de enfermedad pulmonar obstructiva crónica (EPOC). B: Cobertura de serotipos de las diferentes vacunas. NT: no tipificable.

multicéntrico, realizado en seis hospitales españoles de Cataluña, Madrid y Guipúzcoa, muestran que el porcentaje de serotipos causantes de ENI en adultos mayores de 64 años fue del 26,8% para la VCN13 y del 65,5% para la VPN23. En este mismo grupo, las vacunas conjugadas VCN15 y VCN20 que están en desarrollo cubrirían el 32,2% y el 57,0%, respectivamente.

Los datos referentes a la neumonía neumocócica en pacientes adultos, incluyendo la no bacteriémica, son considerablemente distintos a los de la enfermedad invasiva. En este subgrupo de pacientes se observan frecuencias más altas de serotipos VCN13 (el 49,4% según los datos del periodo 2011-2015) con incrementos de la cobertura modestos para VCN15 (54,5%) y VCN20 (68,3%). Esto se debe principalmente al predominio del serotipo 3 en este tipo de patología (22,3% en esta serie), un serotipo que se ha visto débilmente afectado por la introducción de las vacunas conjugadas[31]. Un estudio multicéntrico realizado en un periodo similar (2011-2014) utilizó una técnica de detección en orina de antígeno de neumococo que detecta los serotipos incluidos en la VCN13[6]. Este estudio incluye 368 neumonías neumocócicas y el 60% de ellas fueron causadas por serotipos incluidos en la VCN13. No es posible conocer los datos de cobertura de otras vacunas, ya que la detección urinaria de los serotipos era exclusiva para los incluidos en la VCN13.

Finalmente, las exacerbaciones agudas de la EPOC son otra carga importante de la enfermedad neumocócica en los adultos. En la figura 2 se muestran los datos de un estudio realizado en el periodo 2013-2016, con 210 episodios incluidos. En esta puede verse que el porcentaje de serotipos incluidos en la VCN13 es muy bajo (16,7%), en especial debido a la enfermedad residual por los serotipos 19A (6,2%) y 19F (5,2%). Entre los serotipos no vacunales, el 11A (11,0%), el 6C (10,0%) y el 31 (6,2%) son los más frecuentes. Estos datos probablemente indican tanto un reemplazo clonal debido a la introducción de la VCN13 como la dominancia de un grupo de serotipos más relacionados con colonización y sobreinfección en este tipo de patología, a diferencia de los serotipos más invasivos incluidos en la VCN13[11]. De hecho, en estas exacerbaciones de EPOC, la frecuencia de los serotipos 8 y 3 es muy baja, a diferencia de lo mencionado para la ENI y la neumonía.

La introducción de las vacunas, la utilización de los antibióticos y la expansión de linajes de neumococo con alta capacidad invasiva determinan los cambios constantes no solo en la carga de las enfermedades neumocócicas en el adulto, sino también en la distribución de los serotipos que las causan. La vigilancia constante de la resistencia antibiótica, de los serotipos

y de sus linajes genéticos es imprescindible para conocer los cambios en la enfermedad neumocócica que mejoren el abordaje de la prevención, así como el manejo clínico.

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