Edusfarm 9 *(2017), 1-19*
ISSN: 1886-6271

Rebut: 23 de maig de 2017
Acceptat: 16 de juny de 2017

DYNAMICS OF INVASIVE DISEASE CAUSED BY *STREPTOCOCCUS PNEUMONIAE* CLONES RELATED TO THE PCV13 SEROTYPES NOT INCLUDED IN THE PCV7

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Abstract
Streptococcus pneumoniae is a significant cause of infectious diseases such as community-acquired pneumonia or meningitis. After diagnosis by MALDI-TOF identification and antibiotic susceptibility tests, monitoring of the disease is established by serotyping tests, genotyping tests, and characterization of resistance mechanisms, especially transferable by transposon Tn916 in ermB and mef genes associated with macrolide resistance, and the tetM gene associated with tetracycline resistance. This whole process is needed to confirm the existence of different multidrug resistant clones that emerged and spread throughout the world decades ago, generated by selective adaptation while naturally residing within the human upper respiratory pathways. Nowadays, the prevention of Pneumococcal disease is based on vaccination; the capsular polysaccharide is the major determinant of virulence of *Streptococcus pneumoniae* and it is considered the basis of current vaccine development.

Keywords: *Streptococcus pneumoniae*, resistance, serotyping, genotyping, vaccine.

Resumen
Streptococcus pneumoniae es una causa importante de enfermedades infecciosas como la neumonía adquirida en la comunidad o la meningitis. Tras el diagnóstico mediante identificación por MALDI-TOF y pruebas de susceptibilidad antibiótica, la monitorización de la enfermedad se establece mediante pruebas de serotipificación, genotipado y caracterización de mecanismos de resistencia, especialmente aquellos transferibles por el transposón Tn916 en genes ermB y mef, asociados a resistencia a macrólidos, y el gen tetM asociado a resistencia a la tetraciclina. Todo este proceso es necesario para confirmar la existencia de diferentes clones resistentes a múltiples fármacos surgidos y extendidos por todo el mundo desde hace décadas y generados por la adaptación selectiva mientras residen de forma natural en las vías respiratorias humanas. Hoy en día, la prevención de la enfermedad neumocócica se basa en la vacunación; el polisacárido capsular es el principal determinante de la virulencia de *Streptococcus pneumoniae* y se considera la base del desarrollo actual de las vacunas.

Palabras clave: *Streptococcus pneumoniae*, resistencia, serotipificación, genotipado, vacunas.

Resum
Streptococcus pneumoniae és una causa important de malalties infeccioses com la pneumònia adquirida en la comunitat o la meningitis. Després d'un diagnòstic mitjançant identificació per MALDI-TOF i proves de susceptibilitat antibiòtica, la monitorització de la malaltia s'estableix mitjançant proves de serotipificació, genotipat i caracterització de mecanismes de resistència, especialment aquells que són transferibles pel transposó Tn916 en gens ermB i mef, associats a resistència a macròlids, i el gen tetM associat a resistència a la tetraciclina. Tot aquest procés és necessari per confirmar l'existència de diferents clons resistents a múltiples fàrmacs sorgits i estesos arreu del món fa dècades i generats per l'adaptació selectiva mentre resideixen de forma natural en les vies respiratòries humanes. Avui dia, la prevenció de la malaltia pneumo-

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còccica es basa en la vacunació; el polisacàrid capsular és el determinant principal de la virulència de *Streptococcus pneumoniae* i es considera la base del desenvolupament actual de les vacunes.

Paraules clau: *Streptococcus pneumoniae*, resistència, serotipificació, genotipat, vacunes.

Introduction

Pneumococcus is an alpha-hemolytic, facultative anaerobic, motionless, catalase negative, non-endospore-forming bacteria that belongs to the *Streptococcaceae* family and *Streptococcus* genus, and is grouped by small Gram-positive *diplococcus* chains that can be recognized using an optical microscope (Figure 1).

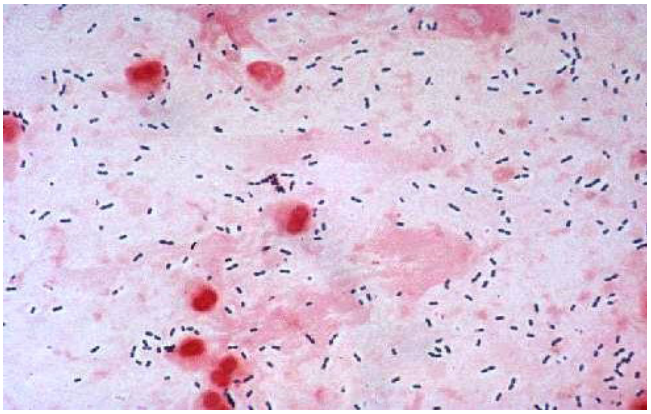


Figure 1. Gram stain of a respiratory sample from a patient with pneumonia. *Streptococcus pneumoniae* could be identified (blue) as Gram-positive *diplococcus*.

At the present time it is the cause of multiple invasive and serious diseases, such as community-acquired pneumonia, meningitis, blood stream infections in children and adults, and otitis (Austrian, 1999; García-Vidal *et al.*, 2010, Liñares *et al.*, 2010). Globally, pneumonia remains the most common cause of death in children under five years of age, with approximately 1.6 million deaths per year; the most at risk population groups are children under two years and adults over 65 years old, people with underlying chronic diseases, and those with immune-suppression due to congenital immunodeficiency, HIV, leukaemia, or routine corticosteroids, exceeding 80-100 cases per 100,000 population (Kim *et al.*, 2016). The incidence of invasive Pneumococcal disease (IPD) is also very variable and depends on many factors related to the patient, such as race, socioeconomic conditions and geographical area (Ardanuy *et al*., 2007; Ardanuy *et al*., 2010; Muñoz-Almagro *et al*., 2008; Picazo *et al*., 2010).

In addition, the capsule is the main virulence factor of *S. pneumoniae* and it has been used for Pneumococcal vaccine design. Although more than 93 capsular types are known, there are less than 25 which cause more than 90% of IPD cases worldwide (Liñares *et al.*, 2010; Ardanuy *et al*., 2007; Ardanuy *et al*., 2010; Dockrell, Whyte & Mitchell, 2012; Mitchell & Mitchell, 2010; Pilishvili *et al*., 2010). Nowadays there are three vaccines used for the prevention of Pneumococcal disease: 23-valent polysaccharide (PPSV23), 10-valent (PCV10), and 13-valent (PCV13). Until 2010 7-valent conjugate vaccine (PCV7) was available, replaced later by PCV13, which was developed by the same

pharmaceutical company. The effectiveness of PPSV23 in preventing Pneumococcal bacteraemia in adults is around 60%, but this vaccine is not immunogenic in children under two years old, so in this age group only PCV10 and PCV13 conjugate vaccines are used (Liñares *et al.*, 2010; Picazo *et al*., 2010; Pilishvili *et al*., 2010).

Besides this, the implementation of PCV7 in 2001 was associated with a dramatic decrease of IPD incidence caused by serotypes 4, 6B, 9V, 14, 18C, 19F and 23F in children under five years old, and also a decrease of IPD incidence in the non-vaccinated population (children over five years old and adults) due to the protecting population group (Liñares *et al.*, 2010; Ardanuy *et al*., 2007; Muñoz-Almagro *et al*., 2008; Pilishvili *et al*., 2010). However, a worldwide increase was described of IPD caused by non-PCV7 serotypes, especially 19A (Liñares *et al.*, 2010; Pilishvili *et al*., 2010; Ardanuy *et al*., 2009; Fenoll *et al*., 2009; Hicks *et al*., 2007). Although in Spain there was a significant decrease of IPD caused by PCV7 serotypes in children and adults, the overall rate has not decreased due to a significant increase caused by the 1, 5, 6C, 7F and 19A non-PCV7 serotypes (Liñares *et al.*, 2010). The declining PCV7 serotypes, most with antibiotic multi-resistances, were associated with a significant decrease of pneumococcus antibiotic resistance (Liñares *et al.*, 2010; Ardanuy *et al*., 2007; Ardanuy *et al*., 2010; Muñoz-Almagro *et al*., 2008; Picazo *et al*., 2010).

Furthermore, the application of molecular typing techniques, such as pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST), has demonstrated that only a few clones reach a successful worldwide spread and are capable of causing IPD in the population (Ardanuy *et al*., 2007; Croucher *et al*., 2011; Domenech *et al*., 2014; McGee *et al*., 2001). Genetic diversity of *Streptococcus pneumoniae* is very high due to their ability to acquire homologous DNA by genetic recombination (Croucher *et al*., 2011; Domenech *et al*., 2014; Coffey *et al*., 1998; Croucher *et al*., 2013; Donati *et al*., 2010). However, this variability is not equal for all serotypes; strains of the same serotype, such as 14, 19A, 19F and 23F, may be genotypically different from each other and present different antibiotic resistance patterns, whereas the strains of other serotypes, such as 1, 5 and 7F, have low genetic diversity and are usually susceptible to antibiotics (Coffey *et al*., 1998; Brueggemann *et al.*, 2004).

Regarding antimicrobial resistance, the first is dated 1912 and describes optoquine resistance in experimental mice. In 1939, the first resistance to sulfonamides in a case of Pneumococcal meningitis was reported, and in 1965 a strain was isolated with reduced susceptibility to penicillin. During the 1970s and 1980s, resistance to penicillin, erythromycin, and cotrimoxazole quickly spread throughout the world, including Australia, Papua New Guinea, Israel, Spain, Poland, South Africa and the United States. Resistance to chloramphenicol and tetracycline were also identified, with variations depending on region and population. Fluoroquinolone resistance has been described in relatively low levels compared with the other antibiotics.

Testing the antibiotics requires growing the bacterial strain, being able to recognize the colony by its greenish appearance, sometimes mucosa, and distinct susceptibility to optoquine (Figure 2). In addition, to perform antibiotic susceptibility tests there are different resources like the disc-diffusion antibiogram, which has tabulated susceptibility or resistance criteria according to the bacterial diameter inhibition halo (Figure 3). Other methods are based on the MIC (minimum inhibitory concentration) by agar diffusion, the epsilometry test or *e-test*, and concentration gradients in liquid media such as the *SensititreTM test* (Figure 4).

The polysaccharide capsule, as mentioned before, is the major virulence factor of pneumococcus and has been attributed a full role in the invasive capacity of different

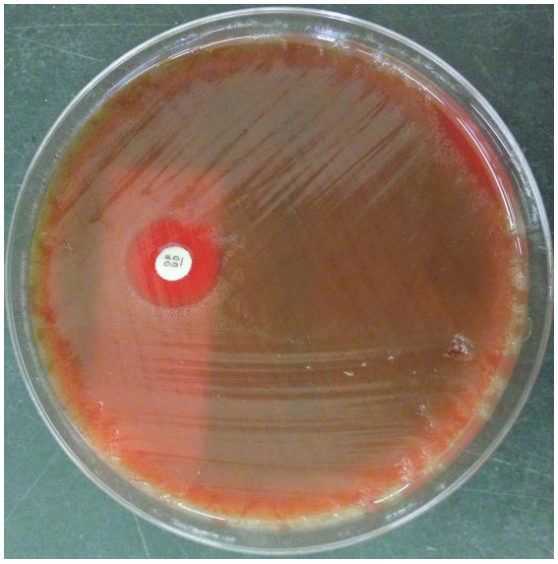


Figure 2. Optoquine susceptibility test used for *Streptococcus pneumoniae* identification in Mueller-Hinton Fastidiosus medium.

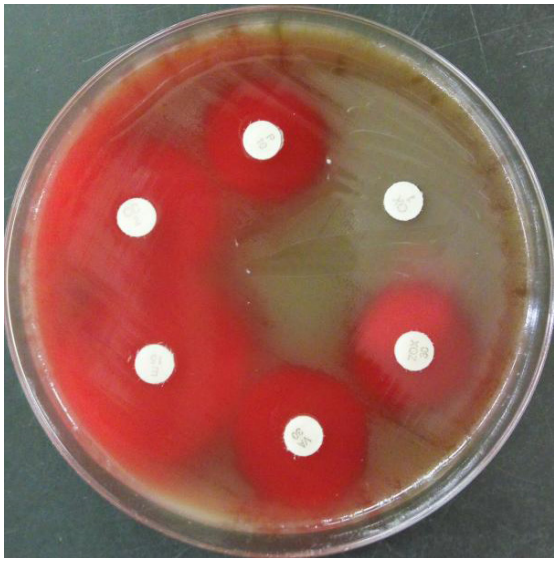


Figure 3. *Streptococcus pneumoniae* Antibiotic susceptibility test by disc-diffusion method in Mueller-Hinton Fastidiosus medium.

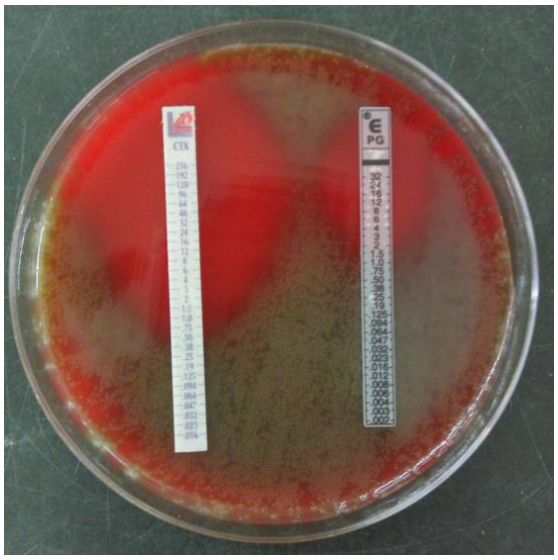


Figure 4. *Streptococcus pneumoniae* antibiotic susceptibility tests: quantitative diffusion method *e-test* on Mueller-Hinton Fastidiosus medium (right); commercially available microdilution method *SensititreTM* (left).

serotypes (Austrian, 1999; Dockrell, Whyte & Mitchell, 2012; Mitchell & Mitchell, 2010; Brueggemann *et al.*, 2004; Sá-Leão *et al*., 2011). Pneumococcus has been classified as invasive or non-invasive depending on its higher prevalence in IPD or nasopharyngeal colonization. Some serotypes, such as 1, 5 and 7F, are considered primary IPD pathogens but rarely isolated in nasopharyngeal colonization. While other serotypes, including 15A, 19F and 23F, are considered opportunistic pathogens found in nasopharynx more frequently than IPD (Brueggemann *et al.*, 2004; Sá-Leão *et al*., 2011). However, a recent study based on genotype analysis clarifies that there are homogeneously invasive serotypes with its capsule, regardless of its genotype, while other serotypes are mucosal or non-invasive independently of the genotype. There is also a third group of serotypes

with a heterogeneous invasive capacity that varies according to genotype (Sá-Leão *et al*., 2011). It is important to note that the invasiveness is not always synonymous of virulence or increased mortality. Thus, a recent meta-analysis has compared the mortality association with different serotypes in adults with IPD, using serotype 14 as a reference. In the study, serotypes 3, 6A, 6B, 9N and 19F were associated with increased mortality while serotypes 1, 7F and 8 were associated with lower mortality.

Serotypes associated with higher mortality were the colonizers; they can behave as opportunistic pathogens causing IPD in patients with underlying diseases, which can lead to worse outcomes (Weinberger *et al*., 2010; Rolo *et al*., 2013; Rolo *et al*., 2011). An important epidemiological pneumococcus aspect is the appearance of new emerging clones or IPD outbreaks in the community caused by the expansion of existing clones. In Spain, in the 1980s, most of the strains were serotype 9V, and a decade later, in the 1990s, the variant serotype 14 appeared; recently (2003) the eleventh variant also emerged (not included in the PCVs) having high amoxicillin resistance, so its spread in Spain leads to recommendations against the use of oral penicillin in the empirical treatment of community-acquired pneumonia. This phenomenon of changing serotype, known as capsular switching, appears due to the characteristics of the capsular Pneumococcal operon. This operon, regardless of serotype, has a fixed location between *dexB* and *aliA* genes and is organized in a cassette form, which includes non-homologous genes, responsible for differences in the capsule genes' locations, and flanked by homologous genes present in most serotypes (Coffey *et al*., 1998; Donati *et al*., 2010; López & García, 2004). Homologous genes facilitate genetic recombination, where non-homologous genes are also switched and which results in the serotype changing (López & García, 2004).

In addition, the CIBERES group at Bellvitge has characterized the appearance in Madrid and later in other communities of a new multi-resistant clone spread of serotype 8, as a result of homologous recombination between a strain of serotype 8 sensitive to antibiotics (ST53) and another serotype 15A with penicillin, macrolides, tetracycline and ciprofloxacin resistances (ST63). The resulting clone presents the new multidrug resistance of the recipient strain and the invasive capacity of the donor strain, provided by the serotype 8 capsules (Ardanuy *et al*., 2014; Grau *et al*., 2016; Harboe *et al*., 2014).

Methods

Patients and strains: This is a prospective, laboratory-based study collecting all episodes of invasive Pneumococcal disease occurring in adult patients (≥18 years old) from 2011 until 2016 at Bellvitge University Hospital (Barcelona, Southern Metropolitan Area). Demographics (age, sex), source of isolation and focus of infection were recorded. The PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) was licensed in 2001, and PCV13 in 2010 (PCV7 + serotypes 1, 3, 5, 6A, 7F and 19A). The incidence of invasive Pneumococcal disease (IPD) was calculated using as a denominator the total number of people that could be obtained through the regional government publication (*web de l'Estadística de Catalunya*). This study was approved by the Clinical Research Ethics Committee of Bellvitge University Hospital. Invasive Pneumococcal isolates were serotyped. All available isolates belonging to PCV13 serotypes not included in the PCV7 (serotypes 1, 3, 6A, 7F and 19A) were selected for molecular typing by PFGE or MLST. The presence of *ermB* and *mefA/E* genes and the *tetM* gene was screened by PCR in all erythromycin- and tetracycline-resistant strains, respectively.

Invasive Pneumococcal disease was defined as the isolation of *Streptococcus pneumoniae* from a normally sterile fluid, such as blood, cerebrospinal fluid, joint fluid, pleural fluid, peritoneal fluid, etc. Strains were preserved frozen in glycerol until required for further studies. The method used is based on the following techniques:

DNA extraction and immobilization in agarose plugs

The objective of this procedure is to obtain DNA immobilized in agarose plugs in order to avoid their fragmentation during the manipulation process. Firstly, strains were streaked on TSA with 5% sheep blood medium and incubated overnight at 37°C in a 5% CO_2 atmosphere. An optoquine disc (plug) was placed in order to check the absence of streptococci (resistant) other than *Streptococcus pneumoniae* (susceptible).

Several colonies of this culture were re-suspended in 150 µL of PIV in a 1.5 ml *eppendorf* tube. This mixture was mixed with 150 µL of low-melting-point agarose and several 20 µL drops were placed onto a glass slide. After their solidification the agarose plugs were treated with a lysis solution and incubated at 37°C for 5-6 hours. Then, the lysis solution was removed and 1 ml of ES solution containing 1mg/ml of proteinase-K was added. This solution was incubated at 50°C for 18 hours. Following this, the solution was discharged and 1 ml of TE buffer was added and the tubes were shaken for 30-45 minutes (the process was repeated 3-5 times). Finally, the plugs were preserved in 1 ml of TE buffer at 4°C.

Table 1. Lysis solution composition for one plug.

ST lysis	RNAse	Lysozyme	Bridj
1 ml	5 µl	2 µl	2 µl

Molecular typing by pulsed field gel electrophoresis (PFGE)

The term clone or clonal group in epidemiology refers to group of isolates with a common ancestor. Clonally related isolates maintain a higher genetic identity than isolates arbitrarily selected without epidemiological relation. The molecular typing of microorganisms by pulsed field gel electrophoresis aims to recognize the relationship between epidemiologically linked isolates and, therefore, recent derivatives of a common ancestral microorganism. At the same time, it must differentiate unrelated isolates, regardless of whether they belong to the same microbiological species or taxon. This process had several steps: a) bacterial chromosomal DNA extraction; b) DNA restriction by low-frequency restriction enzymes, and c) fragment separation by pulsed field gel electrophoresis. Through this technique the bacterial chromosome is resolved into simple patterns (10-20 bands) that facilitate the comparison between isolates, allowing the establishment of genetic similarities between the studied bacteria. Currently, it is considered a benchmark method for molecular typing, although it has limitations, such as the high cost of the equipment and the laboriousness of the procedure. For analysis, a standardized system for interpretation of the results according to band patterns was proposed: bacterial isolates presenting differences from one to three bands would reflect a simple mutation and are considered related, while those differing in four to six bands represent two independent

mutations and are considered possibly related. Finally, isolates with differences in more than six bands represent three mutations and are considered unrelated. The DNA embedded in an agarose plug (or disc) was placed in a tub containing 40 µl of the restriction solution and incubated according to the manufacturer's recommendations (Table 2).

Table 2. Restriction solution composition for PFGE typing.

	Enzyme	H_2O_2	Buffer	BSA	T° Restr.	Pulses PFGE	T° PFGE	Hours PFGE
Serotype 3	0.5 µl *ApaI*	26.5 µl	3 µl	0.8 µl (200 µl/ml)	25°C	1-30 s	11 °C	18 h
Other serotypes	0.5 µl *SmaI*	25.5 µl	4 µl					

For the electrophoresis, a 1% agarose gel in TBE 0.5x solution was prepared. The components were mixed (Table 3), boiled for 5-10 minutes, and cooled for 30 minutes. Plugs were carefully placed into the agarose gel before solidification, which occurs after 30 minutes.

Table 3. Composition of electrophoresis gel used in the PFGE technique.

	Agarose	TBE 10x	Distilled water
100 ml (16 wells)	1 g	5 ml	95 ml
150 ml (30 wells)	1.5 g	7.5 ml	142.5 ml

The electrophoresis was done in a CHE-DRII apparatus. The electrophoresis buffer (TBE 0.5x) was prepared by adding 100 ml TBE 10x in 1900 ml of distilled water. After the electrophoresis, the gel was removed and stained with an ethidium bromide solution for 30 minutes. Finally, the gel was visualized using the UV transilluminator (Figure 5).

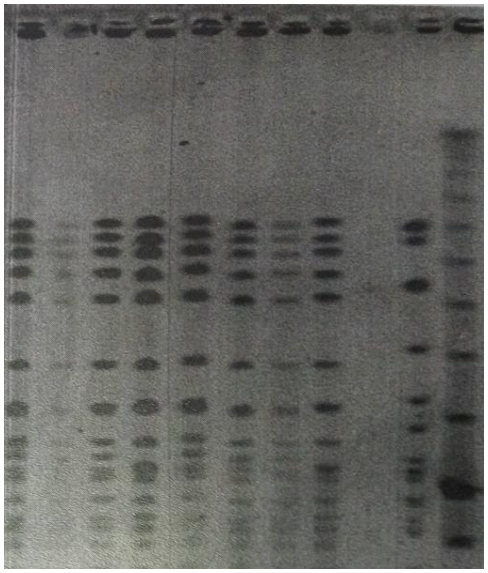


Figure 5. Band patterns of bacterial DNA separated by pulsed field gel electrophoresis (PFGE) after restriction with *SmaI enzyme*. Lanes 1-10: samples; lane 11: log-ladder marker.

Molecular typing by multilocus sequence typing (MLST)

MLST is a molecular typing method based on DNA sequencing that is precise, reproducible, and with a high discrimination power. This technique uses allele combination of several housekeeping genes. The sequence type is an arbitrary number given to a single allele combination. In the *Streptococcus pneumoniae* MLST scheme, seven metabolic genes are amplified by PCR and sequenced:

- *aroE*: shikimate dehydrogenase;
- *gdh*: glucosa-6-phosphate dehydrogenase;
- *gki*: glucosa kinase;
- *recP*: transketolase;
- *spi:* signal peptidase 1;
- *xpt*: xanthine phosphoribosyltransferase;
- *ddl*: D-alanine-D-alanine ligase.

To obtain a DNA solution the agarose plugs were melted and re-suspended in 380 µl of TE solution and incubated for 15 minutes at 70°C. The PCR mix was prepared according to Table 4, dispensed into PCR tubs and 2 µl of DNA sample was added. The tubs were placed in the PCR thermal cycler using the following cycling conditions for all reactions:

1. 94°C × 10'
2. Denaturation 94°C × 30"
   Annealing 55°C × 30"
   Elongation 72°C × 60"
   × 35 cycles
3. 72°C × 10'

Table 4. Composition of MLST mix solution for one reaction.

Buffer (10x)	5 µL
$MgCl_2$ (25 mM)	4 µL
dNTPs	0,5 µL
Primers (forward and reverse)	0.5 µL
Taq polymerase	0.5 µL
Distilled water	37.2 µL

The PCR products were visualized after electrophoresis. After this, 100 ml of 1 % agarose in TBE gel was prepared (1 g of agarose in 100 ml of TE solution 0.5x), which contained 5 µl of *Syber®Safe*. Samples were loaded after mixing 5 µl of sample and 5 µl of loading buffer. A ladder DNA marker was included in each electrophoresis. The electrophoresis was performed at 130 V for 25 minutes, and then the gel was visualized under UV light (Figure 6). The sequence of primers used for PCR amplification is described in Table 5.

Table 5. Primers required for MLST genotyping.

TARGET	PRIMERS	SEQUENCE (5' → 3')	SIZE
aroE	aroE-F	GCCTTTGAGGCGACAGC	405 pb
	aroE-R	TGCAGTTCA(G/A)AAACAT(A/T)TTCTAA	
gdh	gdh-F	ATGGACAAACCAGC(G/A/T/C)AG(C/T)TT	459 pb
	gdh-R	GCTTGAGGTCCCAT(G/A)CT(G/A/T/C)CC	
gki	gki-F	GGCATTGGAATGGGATCACC	483 pb
	gki-R	TTCTCCCGCAGCTGACAC	
recP	recP-F	GCCAACTCAGGTCATCCAGG	448 pb
	recP-R	TGCAACCGTAGCATTGTAAC	
spi	spi-F	TTATTCCTCCTGATTCTGTC	472 pb
	spi-R	GTGATTGGCCAGAAGCGGAA	
xpt	xpt-F	TTATTAGAAGAGCGCATCCT	486 pb
	xpt-R	AGATCTGCCTCCTTAAATAC	
ddl	ddl-F	TGC(C/T)CAAGTTCCTTATGTGG	441 pb
	ddl-R	CACTGGGT(G/A)AAACC(A/T)GGCAT	

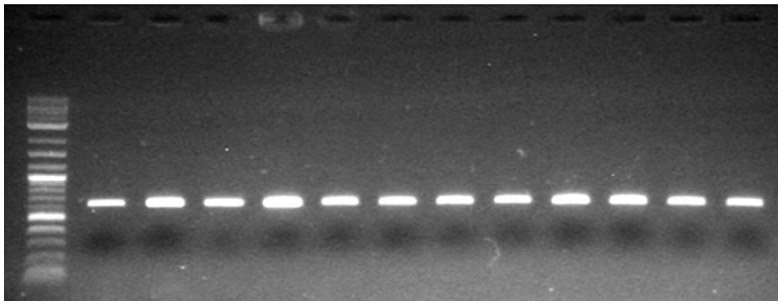


Figure 6. Electrophoresis gel of g*dH* gene amplification by PCR sequencing. Lane 1: log-ladder marker; lane 2-13: samples.

Serotyping by PCR

The PCR test is a molecular typing technique in which primers are used to hybridize the sequences distributed throughout the bacterial genome. Thus, the regions of interest are amplified to obtain different patterns using the corresponding primers according to the serotypes (Table 6). A cpsA gene primer is also used (expressed in bacterial capsule) as a control.

Table 6. Primers required to perform PCR serotyping.

TARGET	PRIMERS	SEQUENCE (5' → 3')	SIZE
1	1-F 1-R	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	280 pb
3	3-F 3-R	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	371 pb
5	5-F 5-R	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG	362 pb
6A	6A-F 6A-R	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	250 pb
7F	7F-F 7F-R	CCT ACG GGA GGA TAT AAA ATT ATT TTT GAG CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	826 pb
8	8-F 8-R	GAT GCC ATG AAT CAA GCA GTG GCT ATA AAT C ATC CTC GTG TAT AAT TTC AGG TAT GCC ACC	201 pb
19A	19A-F 19A-R	GTTAGTCCTGTTTTAGATTTATTTGGTGATGT GAGCAGTCAATAAGATGAGACGATAGTTAG	478 pb
cpsA	cpsA-F cpsA-R	GCAGTACAGCAGTTTGTTGGACTGACC GAATATTTTCATTATCAGTCCCAGTC	160 pb

A mixture of the PCR reagents (Table 7) was made in the quantity required according to the number of samples. After mixing all components, 46 µl of the mixture and 4 µl of DNA sample were dispensed into a PCR *eppendorf* tube and placed in the thermal cycler using the following programme conditions (same for all serotypes):

1. 95°C × 1'
2. Denaturation 95°C × 30"
   Annealing 54°C × 1'

Table 7. Composition of PCR mix solution for PCR serotyping.

Red Buffer (10x)	10 µL
cpsA primers	0.2 µL
Distilled water	34.8 µL
Primers (forward and reverse)	0.3 µL
Taq polymerase	0.7 µL

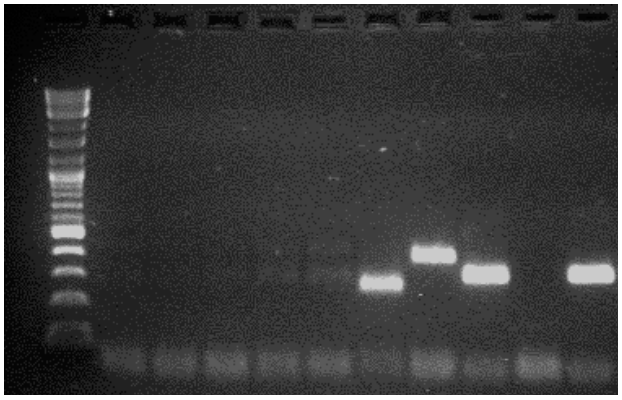


Figure 7. Gel electrophoresis of serotype amplification by PCR sequencing. Lane 1: log-ladder marker; lanes 2-10: samples; lane 11: positive control.

Elongation 72°C × 2'
× 35 cycles
3. 72°C × 10'

After this, 100 ml of 1.5 % agarose in TBE gel was prepared (1.5 g of agarose in 100 ml of TE solution 0.5x), which contained 5 µl of *Syber®Safe*. Samples were loaded after mixing 5 µl of sample and 5 µl of loading buffer. A ladder DNA marker was included in each electrophoresis. The electrophoresis was performed at 110 V for 45 minutes, and then the gel was visualized under UV light (Figure 7).

Resistance gene genotyping by PCR

Macrolide resistance may occur due to the acquisition of encoding genes, which usually belong to the 916 transposon family and often lead determinants for tetracycline resistance. In particular, this technique was used to sequence the macrolide resistance genes (*ermB* and *mefA/E*) and tetracycline (*tetM*). The protocol begins with the selection of resistant strains using the clinical breakpoints criteria indicated on the EUCAST website (Table 8), and proceeds in the same way as the MLST technique explained above. Subsequently, a PCR mixture is made according to the number of samples (Table 9). In this case, a different buffer (Red Buffer) has been used, which already includes MgCl2 and the dNTPs. The controls used in the electrophoresis gel are ATCC England 14-9 as a positive control. The configuration of the thermal cycler for all genes is:

1. 94°C × 1'
2. Denaturation 94°C × 30"
   Annealing 58°C × 60"
   Elongation 72°C × 90"
   x 35 cycles
3. 72°C × 10'

After this, 100 ml of 1.5 % agarose in TBE gel was prepared (1.5 g of agarose in 100 ml of TE solution 0.5x), which contained 5 µl of *Syber®Safe*. Samples were loaded after mixing 5 µl of sample and 5 µl of loading buffer. A ladder DNA marker was included in each elec-

trophoresis. The electrophoresis was performed at 120 V for 20 minutes, and then the gel was visualized under UV light (Figure 8). Finally, it was necessary to differentiate between *mefA* and *mefE* genes, performing a digestion with *bamHI* for 1 hour at 37°C (Table X) and another electrophoresis under the same conditions.

Table 8. Pneumococcal susceptibility (CMI in mg/l) to macrolides and tetracycline according to EUCAST clinical breakpoints criteria.

	Susceptible	Intermediate	Resistant
Tetracycline	≤1	1-2	>2
Erythromycin	≤0.25	0.25-0.5	>0.5
Clindamycin	≤0.25	0.25-0.5	>0.5

Table 9. Composition of PCR mix solution for PCR resistance gene genotyping.

Red Buffer	10 µL
Primer-forward	0.4 µL
Primer-reverse	0.4 µL
Taq *polymerase*	0.3 µL
Distilled water	36.9 µL

Table 10. Reagents required for the digestion of *mefA* and *mefE* genes.

Buffer (10x)	4 µL
bamHI	0.5 µL
Distilled water	16 µL
PCR product sample	20 µL

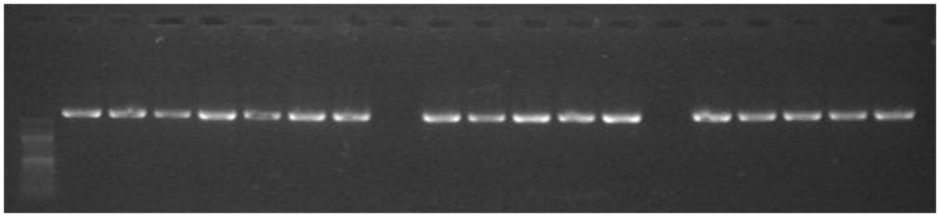


Figure 8. Gel electrophoresis of *tetM* gene amplification by PCR sequencing. Lane 1: log-ladder marker; lane 2-19: samples; lane 20: positive control.

Sequence results edited and analysed

The chromatogram (Figure 9) was the result of loading the *Macrogen, Inc* files where the MLST genotyping products were sent to be sequenced. This sequencing is made by the *Sanger method*, which is based on the presence of nucleotides labelled with fluorophores and a capillary electrophoresis. To obtain the serotypes and the antibiotic susceptibility of each strain, the samples were also sent to the Health Institute Carlos III in Madrid.

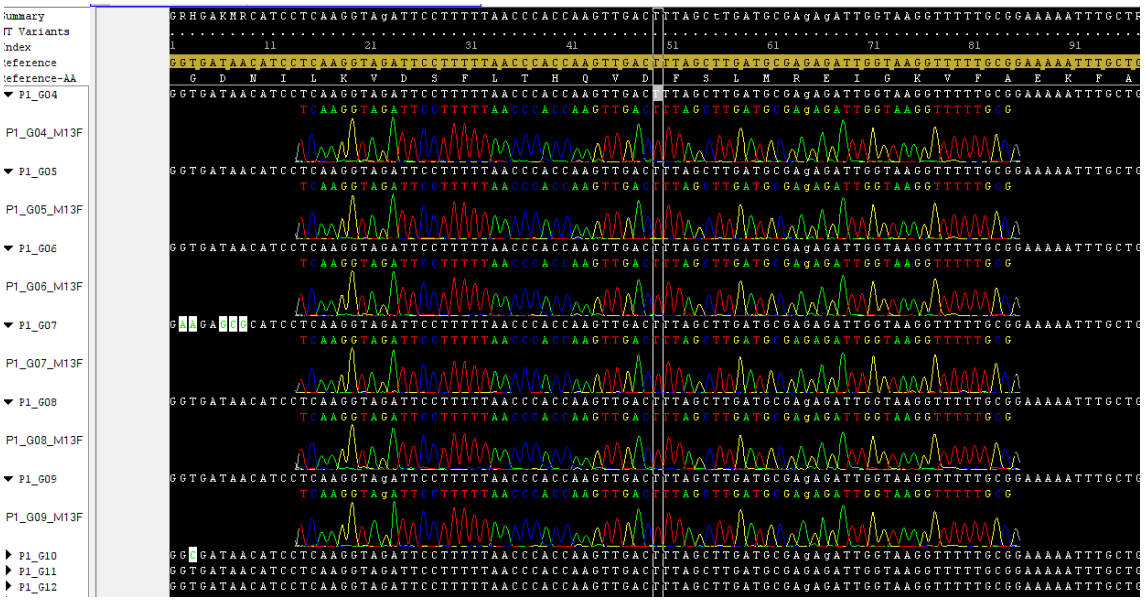


Figure 9. Image of *SeqScape v2.7* in which the nucleotide sequences can be edited.

After processing all data files, we obtain allelic numbers of each metabolic gene that we can use to acquire the serotype and clonal complex number, using the programme that *PubMLST website* has on its database (Figure 10).

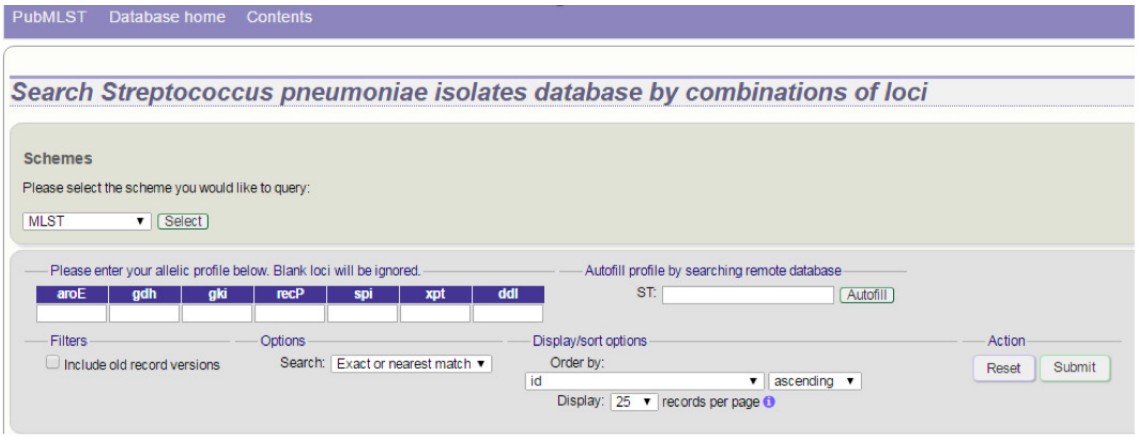
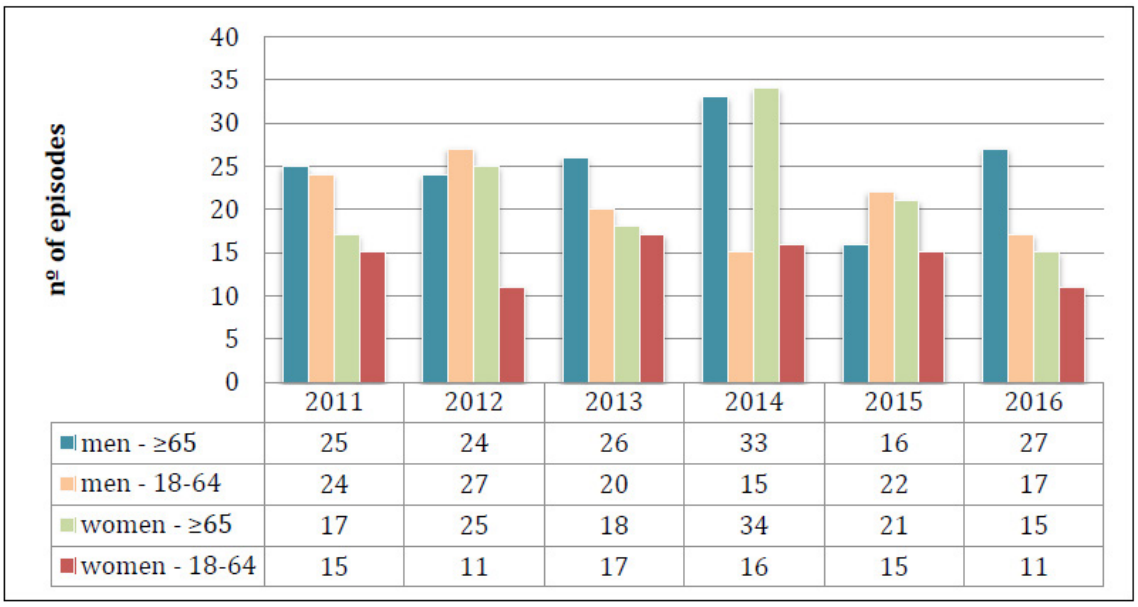


Figure 10. Image of *pubMLST.org* website database in which the results, obtained through the previous sequence edition, can be inserted and processed.

Results

Invasive Pneumococcal Disease (IPD) for the period 2011–2016

The results obtained have been analysed by age groups (those over 65 years old, and those between 18 and 64 years) and sex (Figure 11). A total of 492 IPD strains were isolated for the study period (2011–2016). The highest incidence was observed in men older than 65 and this difference was statistically significant ($p<0.01$); the remaining differences between groups were not statistically significant ($p>0.05$).



	2011	2012	2013	2014	2015	2016
men - ≥65	25	24	26	33	16	27
men - 18-64	24	27	20	15	22	17
women - ≥65	17	25	18	34	21	15
women - 18-64	15	11	17	16	15	11

Figure 11. Distribution of IPD episodes by age and sex group per year (2011–2016).

Figure 12 shows the total number of IPD episodes analysed by serotype group. While IPD due to PCV7 serotypes and those due to the additional PCV13 serotypes decrease, the IPD due to non-PCV13 serotypes did not change significantly. Nevertheless, an increase in IPD due to non-PCV13 serotypes was observed in 2014 and onwards.

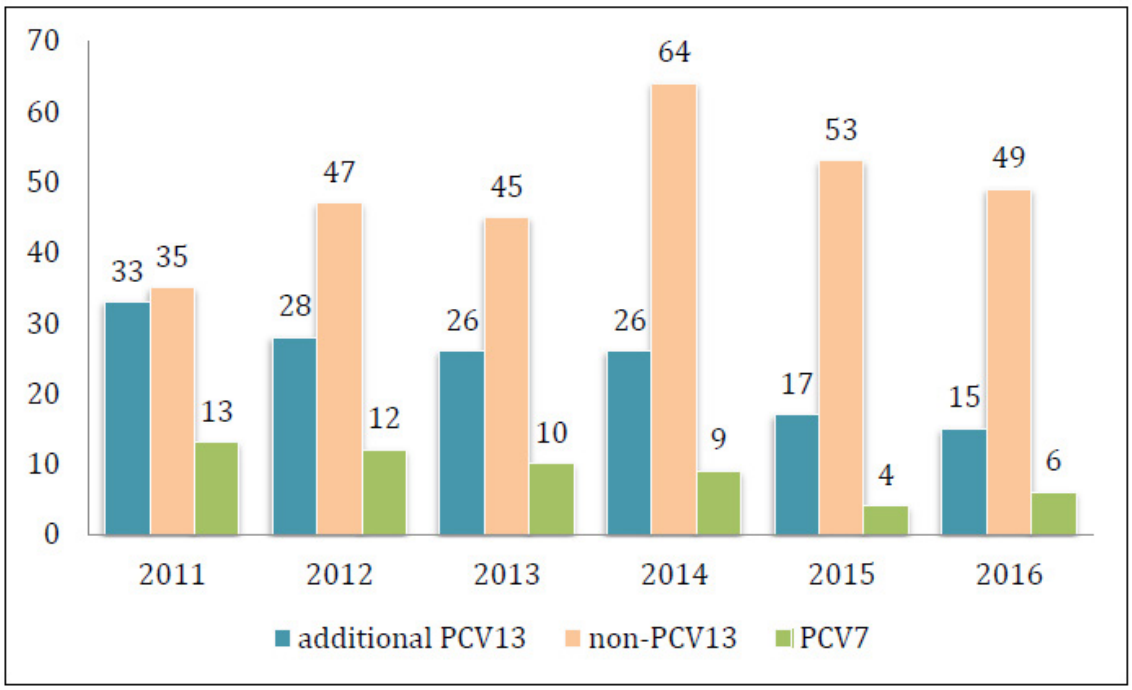


Figure 12. Total number of IPD episodes by serotype group (PCV7, non-PCV13, and additional PCV13) per year (2011–2016).

In regard to vaccine group distribution, we observed a decrease in IPD among young adults (18–64), while in adults over 65 it remained stable due to an increase of the disease caused by non-PCV13 serotypes (Figure 13). Despite this, if we observe the results with absolute numbers we will notice how the total number of isolates of invasive Pneumococcal strains has suffered a greater reduction per year; this trend suggests that the impact of vaccines on the population is relevant and its effectiveness can be evidenced by the total number of isolated invasive strains which have decreased more than half in the last decade.

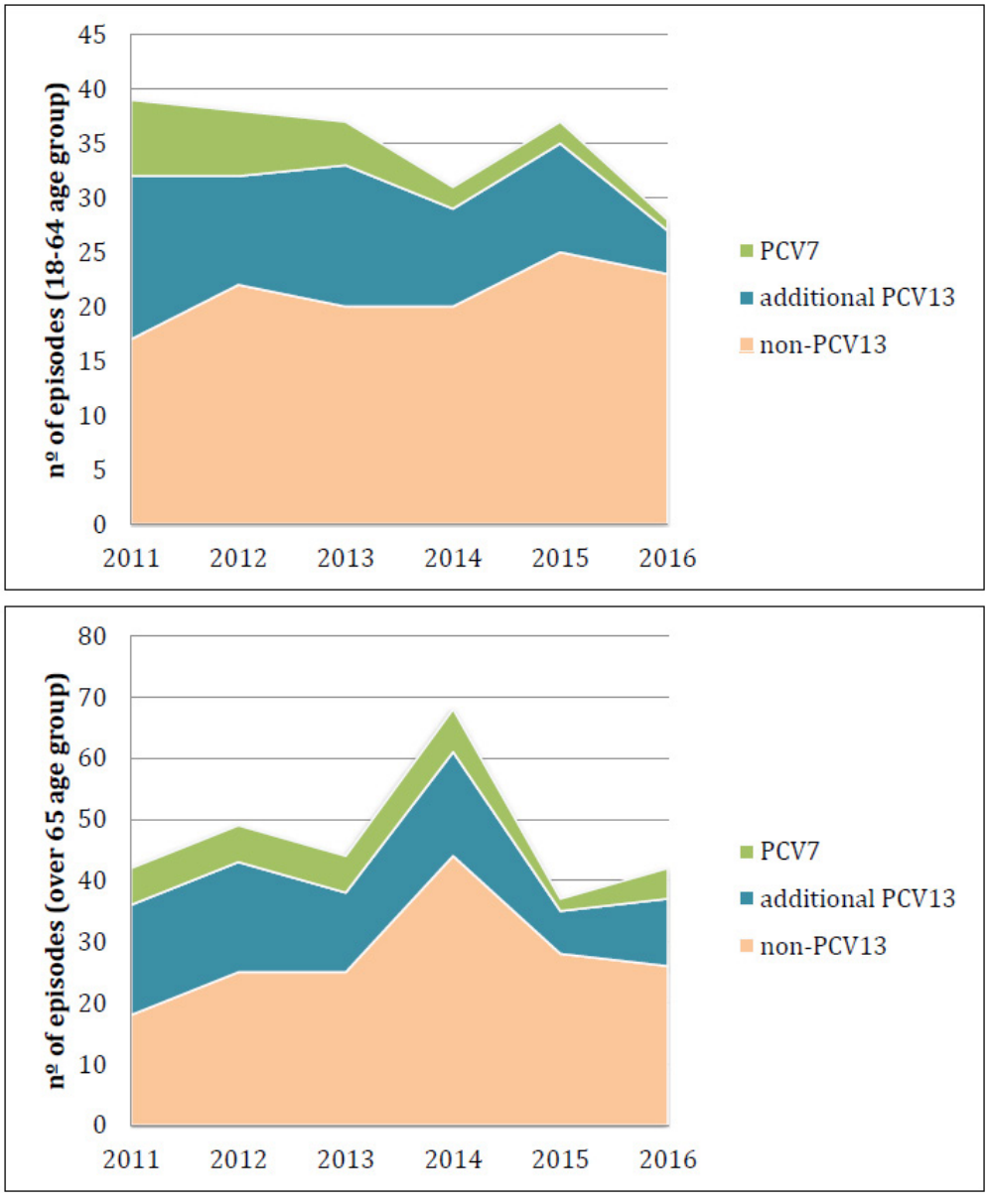


Figure 13. Annual distribution of IPD (episodes/100,000 people) related to vaccine serotypes and age groups in a southern metropolitan area of Barcelona. Top: Incidence of IPD in people aged 18–64. Bottom: Incidence of IP in people aged over 65. Green: PCV7 serotypes (4, 6B, 9V, 14, 19F, 18C and 23F). Blue: additional PCV13 serotypes (1, 3, 5, 6A, 7F and 19A). Orange: non-PCV13 serotypes (others).

Genetic relatedness of Pneumococcal isolates belonging to PCV13 serotypes not included in PCV7 (additional PCV13)

The IPD caused by additional PCV13 serotypes (1, 3, 5, 6A, 7F and 19A) shows differences over the study period. In general, IPD caused by the additional serotypes decreases with the exception of serotypes 3 and 19A. In fact, IPD caused by serotype 3 remains stable over the study period. On the other hand, IPD caused by serotype 19A remains stable between 2011 and 2014, decreasing in the last two years (Figure 14). These results show the impact of child vaccination among adult IPD due to herd protection, with the exception of serotype 3, which is rarely found in children.

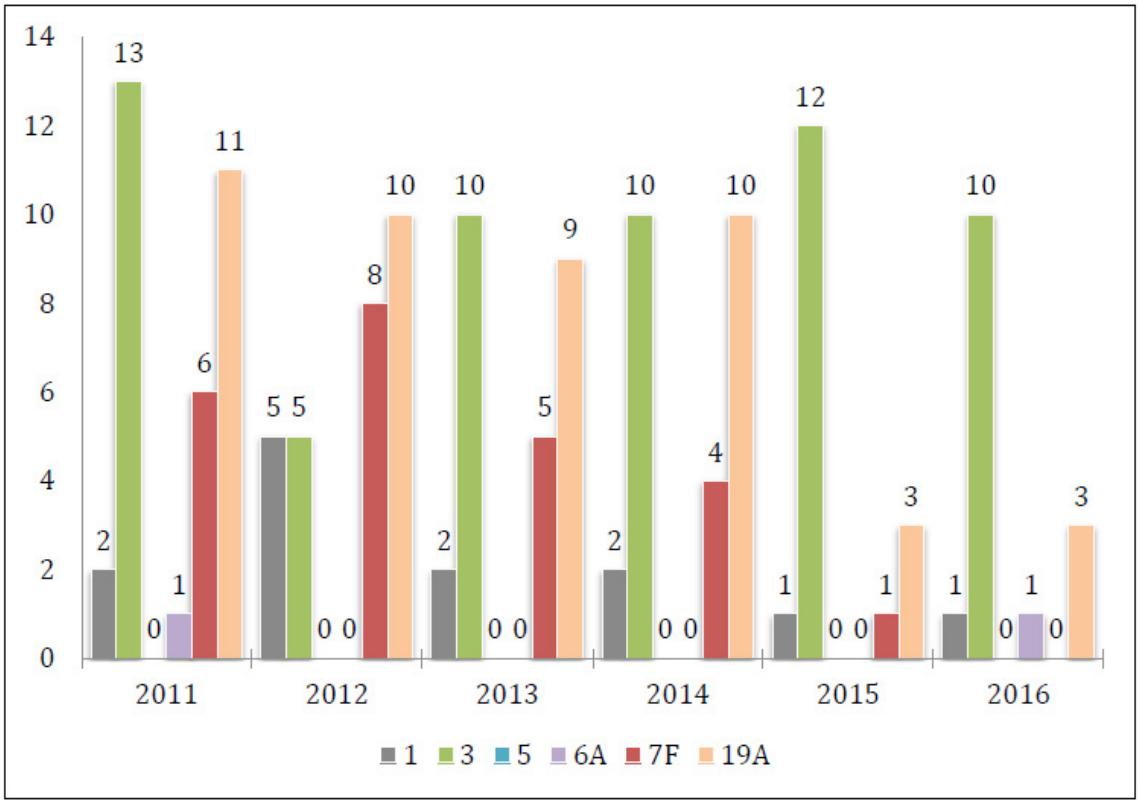


Figure 14. Incidence of PCV13 non-PCV7 serotypes (1, 3, 5, 6A, 7F and 19A) episodes per year (2011–2016).

Macrolide and tetracycline resistance genes

Among 492 IPD episodes from the 2011–2016 period, 137 were macrolide-resistant and 120 were tetracycline-resistant. Among macrolide resistant isolates, 104 had the *ermB* gene (76.47%), 10 had *mefE* (7.35%), and 22 (16.18%) had both genes *ermB-mefE*. The *tetM* gene was detected in all resistant strains tested. The association of macrolide and tetracycline resistance was found in 115 isolates. Of them, 93 (80.90%) had *ermB* and *tetM*; 1 (0.87%) had *mefE* and *tetM*, and finally 21 (18.26%) isolates had all three genes (*ermB, mefE* and *tetM*). Table 11 shows the serotypes associated with resistance and their resistance genotype. The frequent association of *ermB* and *tetM* genes is related to the presence of transposon of the Tn916-family.

Table 11. Serotypes associated with macrolide and tetracycline resistance genes.

	ermB	*mefE*	*tetM*	*ermB +mefE*	*ErmB +tetM*	*MefE +tetM*	*ermB+mefE +tetM*
2011	6B,6C,19F	6A,33F	-	6C,24F	19A, 15A, 23A, 24F, 33F	-	19A
2012	6C,7F,19F, 35B	11A,23F	19A	18C,19A	19A, 15A, 6F, 23A, 24F, 33F	-	19A
2013	6C,14,19F, 23A	11A	-	-	3,19A, 15A, 23A, 24F	-	19A
2014	6B, 19F, 23FF	22F	3,19A	-	19A, 15A,23A, 24F, 33F	9N	19A
2015	15A	-	12F,19A	-	3,6C,15A,23A	-	19A
2016	-	-	19F	-	24F,33F	-	6A,19A,23A,24F

Genotypes associated to additional PCV13 serotypes

As can be observed in Table 12, within the additional PCV13 group the ST306 associated to serotype 1 predominates, followed by ST180 and ST260 associated to serotype 3, and finally the ST191 associated to serotype 7F; the clonal complex related to serotypes 19A and 6A are varied.

Table 12. Clonal complex genotype related to additional PCV13 serotypes per year.

	2011	**2012**	**2013**	**2014**	**2015**	**2016**
1 (n=13)	ST306	ST306	ST306	ST306	ST306	ST306
3 (n=60)	ST180 ST260	ST180 ST260	ST180 ST260	ST180 ST260 ST458 ST53	ST180 ST260 ST1377	ST180 ST260
5 (n=0)	-	-	-	-	-	-
6A (n=2)	ST473	-	-	-	-	-
7F (n=24)	ST191	ST191	ST191	ST191	ST191 ST3544	-
19A (n=46)	ST1201 ST320 ST3261 ST230	ST320	ST320 ST81 ST199 ST3259 ST450 ST1201	ST1201 ST320 ST7737 ST1201 ST230	ST320 ST230	ST320

Figure 15 shows especially the clonal complexes related to serotype 19A in which multidrug-resistance caused by ST320 appears more frequently. Also, Figure 16 shows the clonal complex related to serotype 3, providing evidence of the greater presence of clonal complex ST180.

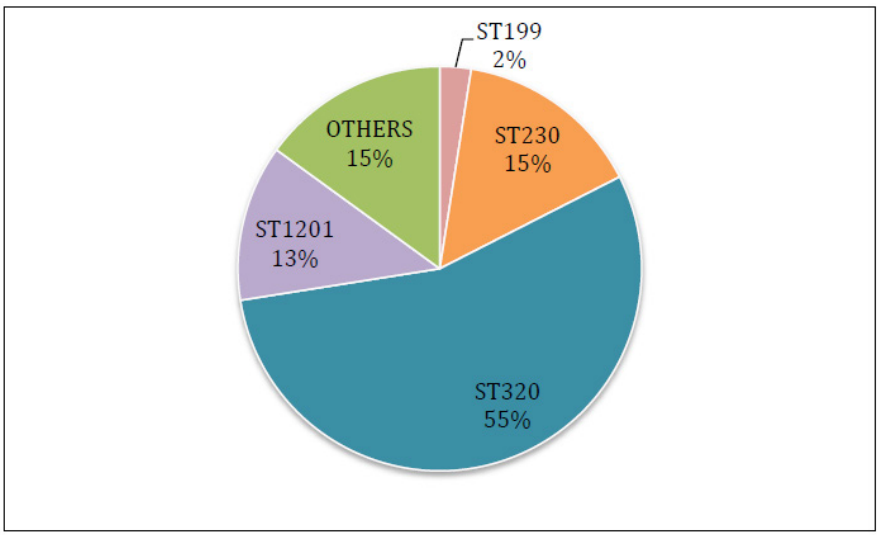


Figure 15. Clonal composition of serotype 19A (n=46).

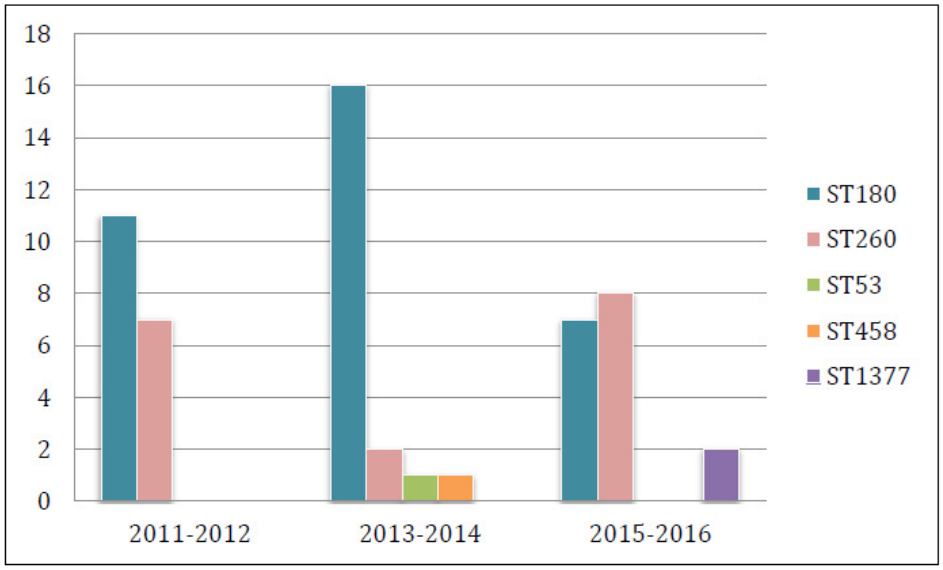


Figure 16. Clonal composition of serotype 3 (n=60) per years.

Conclusions

The incidence of IPD in adults decreases over the study period, demonstrating a significant impact of child vaccination in adults due to herd protection. The decrease in IPD was only remarkable in young adults (18-64), while in adults over 65 remained stable and, likewise, in adults over 65 the decrease of IPD caused by PCV13 serotypes was balanced by an increase of non-PCV13 serotypes.

Furthermore, there was no impact from child vaccination on IPD due to serotype 3, which is the major cause of invasive Pneumococcal disease in adults. The maintenance of serotype 3 was due to the predominant clonal complex ST180. Over the study period, a decrease of IPD due to macrolide resistant strains was observed, mainly linked to a felt in the IPD serotype 19A

Finally, the association of macrolide and tetracycline resistances was frequent, indicating the dissemination of Tn916-family transposons.

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