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Universidad de Barcelona, Facultad de Farmacia

"Dynamics of invasive disease caused by *Streptococcus pneumoniae* clones related to the PCV13 serotypes not included in the PCV7."

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INDEX

1. Abstract

Streptococcus pneumoniae is an important cause of some infectious diseases that affect the population such as community-acquired pneumonia or meningitis. The diagnosis of Pneumococcal disease is based on a variety of laboratory tests that confirm the presence of the bacteria as the causal agent, starting with a Gram staining for the previous microscopic identification and the bacterial growth in a culture media. Then, it is followed by the MALDI-TOF identification once the bacterial strain is isolated; in the case of urine samples, it proceeds with an lateral flow immunoassay test. Finally the antimicrobial susceptibility profile is required to establish an appropriate treatment as well as other useful complementary properties in clinical studies.

After completing this process, the monitoring of the disease is established by serotyping tests, genotyping tests, and characterization of resistance mechanisms, especially transferable by Tn916 transposon 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 emerged and spread throughout the world during the early use of antibiotics decades ago and generated by selective adaptation while naturally residing within the human upper respiratory pathways, also inhabited by other species of nosocomial streptococcus able to generate and transmit antibiotic resistances.

Nowadays, the prevention of Pneumococcal disease is based on vaccination; the capsular polysaccharide is the major determinant of virulence of *S. pneumoniae* and it is considered the basis of the current vaccine development. The introduction of the 7-valent Pneumococcal conjugate vaccine or PCV7 has changed the epidemiology of Pneumococcal disease worldwide (2001), and later the PCV13 in 2010. However, some serotypes of PCV13 not included in the PCV7 remained as a cause of invasive disease.

Resumen

Streptococcus pneumoniae es un causante importante de algunas de las enfermedades infecciosas que afectan a la población como la neumonía adquirida en la comunidad o la meningitis. El diagnóstico de la enfermedad neumocócica se basa en una variedad de pruebas de laboratorio confirmatorias de la presencia de la bacteria como agente causal, empezando con una tinción de Gram para la previa identificación microscópica junto con la siembra de la muestra en medios de cultivo enriquecido, y seguido posteriormente de la identificación por MALDI-TOF una vez aislada la cepa bacteriana; en el caso de las muestras urinarias, se procede con una prueba inmunocromatográfica en tira reactiva. Finalmente se requieren las pruebas de sensibilidad a antimicrobianos para establecer un perfil adecuado de tratamiento, así como otras propiedades complementarias útiles en estudios clínicos.

Una vez completado este procedimiento, se establece la vigilancia de la enfermedad mediante pruebas de identificación del serotipo, del genotipo, y la caracterización de los mecanismos de resistencia, especialmente los transferibles a través de transposones Tn916 relacionados con los genes *ermB* y *mef* asociados a resistencias a macrólidos, y al gen *tetM* asociado a resistencias a la tetraciclina. Todo este proceso es necesario para confirmar la existencia de diferentes clones multirresistentes que surgieron y se difundieron por todo el mundo durante los primeros usos de antibióticos décadas atrás y los cuales se generan a través de la adaptación selectiva mientras residen de forma natural dentro de las vías respiratorias superiores humanas, habitadas también por otras especies de estreptococos nosocomiales capaces de generar resistencias y transmitirlas.

Actualmente, la prevención de la enfermedad neumocócica se basa en la vacunación; el polisacárido capsular es el principal determinante de virulencia de *S. pneumoniae* y también es la base del desarrollo de la vacuna actual. La introducción de la vacuna neumocócica conjugada heptavalente o PCV7 ha cambiado la epidemiología de las enfermedades neumocócicas en todo el mundo (2001) y posteriormente la PCV13 en el año 2010. Sin embargo, algunos serotipos de la PCV13 no incluidos en la vacuna PCV7 se mantuvieron como causa de la enfermedad invasiva.

2. Justification of the study

This project is focused on invasive Pneumococcal disease (IPD) on adults. The incidence of IPD is higher at extreme ages of life (under 5 years and over 65 years old), and previous underlying diseases diagnostics. In these age groups the mortality may reach 30% in the severe bacteremic pneumonia. In recent decades, the structure of the population has increased the average age, the number of immune-suppressed people and the underlying diseases prevalence; these facts cause a major risk of people getting IPD and also make necessary a new molecular approach. Therefore, the epidemiological IPD analysis by sequencing the entire genome will be a valuable test to improve knowledge in the respective field. The study of persistent majority clones which have been adapted to the different antibiotic and vaccine pressure can contribute to understand in a better way the adaptive mechanisms of *Streptococcus pneumoniae* and also improve the current treatments or prophylaxis.

The work pretends to collaborate with the current CIBERES project, based on the dynamics of *Streptococcus pneumoniae* invasive disease clones related to the PCV13 serotypes not included in the PCV7 and then complete the data base. Supported by the learning of laboratory techniques and the appropriate bibliography, I will perform this project that is an interesting work to consolidate the knowledge acquired in the disciplines of microbiology, molecular biology, and public health.

3. Introduction

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

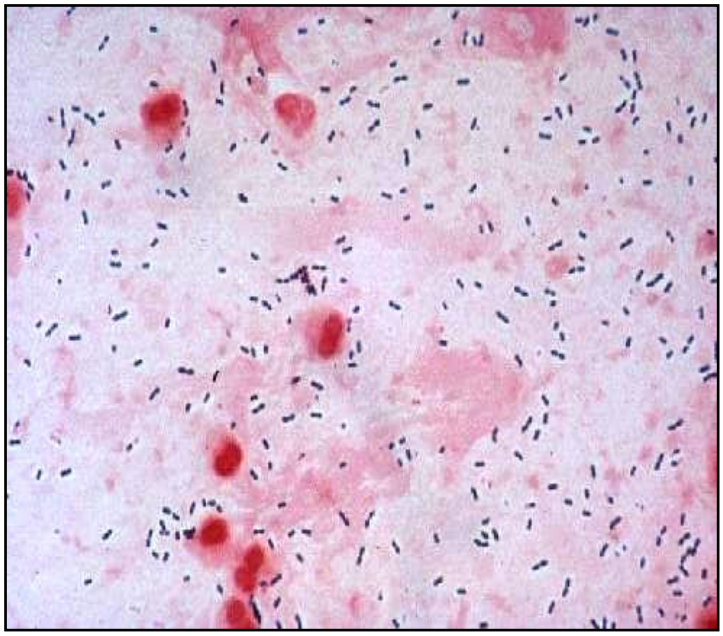


Figure I. Gram stain of a respiratory sample from a patient with pneumonia. *Streptococcus pneumoniae* could be identified (blue) as gram-positive *diplococcus*.

At present time it is the cause of multiple invasive and serious diseases, such as community-acquired pneumonia, meningitis, blood stream infections on children and adults, and otitis [1,2,3]. Globally, pneumonia remains the most common cause of death in children under 5 years of age, approximately 1.6 million deaths per year; the most risked population groups are children of 2 years and adults of 65 years old, people with underlying chronic diseases and those with immune-suppression due to congenital immunodeficiency, HIV, leukemia, or corticosteroid routine, exceeding 80-100 cases per 100,000 population [4]. 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 [5,6,7,8].

In addition, the capsule is the main virulence factor of *S. pneumoniae* and it has been used for the Pneumococcal vaccines design. Although more than 93 capsular types are known, there are less than 25 which cause more than 90% of worldwide IPD cases [3,5,6,9,10,11]. 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 was available 7-valent conjugate vaccine (PCV7) which was replaced later by the PCV13, developed by the same pharmaceutical company. The PPSV23 effectiveness in preventing Pneumococcal bacteremia on adults is around 60% but this vaccine is not immunogenic on children under two years old, so in this age group only PCV10 and PCV13 conjugate vaccines are used [3,8,11].

Besides this, the implementation of PCV7 in 2001 was associated with a dramatic decrease of the IPD incidence caused by serotypes 4, 6B, 9V, 14, 18C, 19F and 23F on children under 5 years old, and also a decrease of the IPD incidence on non-vaccinated population (children over 5 years old and adults) due to the protecting population group [3,5,7,11]. However, it was described a worldwide increased of IPD caused by non-PCV7 serotypes, especially 19A [3,11,12,13,14]. Although in Spain there was a significant decrease of IPD caused by PCV7 serotypes on 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 [15]. The declining PCV7 serotypes, most with antibiotic multi-resistances, were associated with a significant decrease of pneumococcus antibiotic resistance [3,5,6,7,8].

Furthermore, the application of molecular typing techniques such as pulsed field gel electrophoresis (PFGE) and the multi-locus sequence typing (MLST), have demonstrated that only a few clones reach a successful worldwide spread and are capable of causing IPD in population [5,16,17,18]. Genetic diversity of *Streptococcus pneumoniae* is very high due to their ability to acquire homologous DNA by genetic recombination [16,17,19,20,21]. 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 is usually susceptible to antibiotics [19,22].

Regarding antimicrobial resistances, the first is dated on 1912 describing optoquine resistance in experimental mice. In 1939, it was reported the first resistance to sulfonamides in a case of Pneumococcal meningitis, 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.

For testing the antibiotics it is required to grow the bacterial strain being able to recognize the colony by their greenish appearance, sometimes mucosa, and distinctly susceptible to optoquine (Figure II). In addition, to perform the antibiotic susceptibility tests there are different resources like disc-diffusion antibiogram which has tabulated susceptibility or resistance criteria according to the bacterial diameter inhibition halo (Figure III). Other methods are based on the MIC (minimum inhibitory concentration) by agar diffusion as the epsilometry test also called *e-test* and concentration gradients in liquid medium as the *Sensititre*^{TM} *test* (Figure IV).

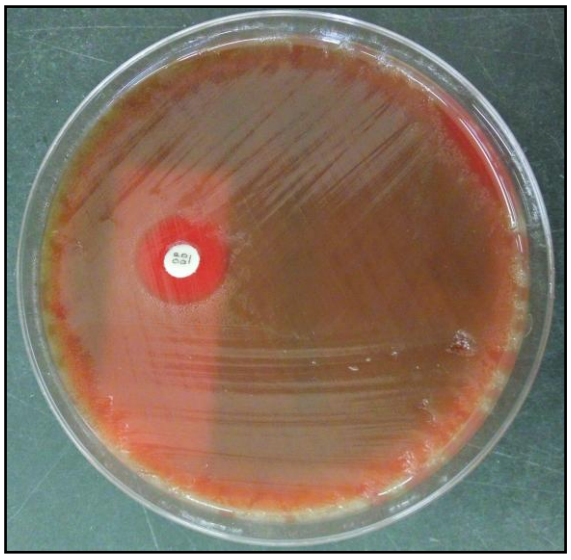


Figure II. Optoquine susceptibility test used for S*treptococcus pneumoniae* identification in Mueller-Hinton Fastidiosus medium..

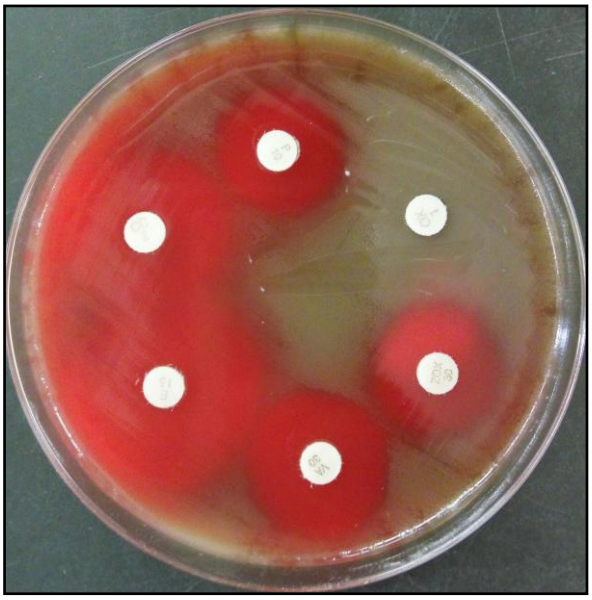


Figure III. *Streptococcus pneumoniae* Antibiotic susceptibility test by disc-diffusion method in Mueller-Hinton Fastidiosus medium.

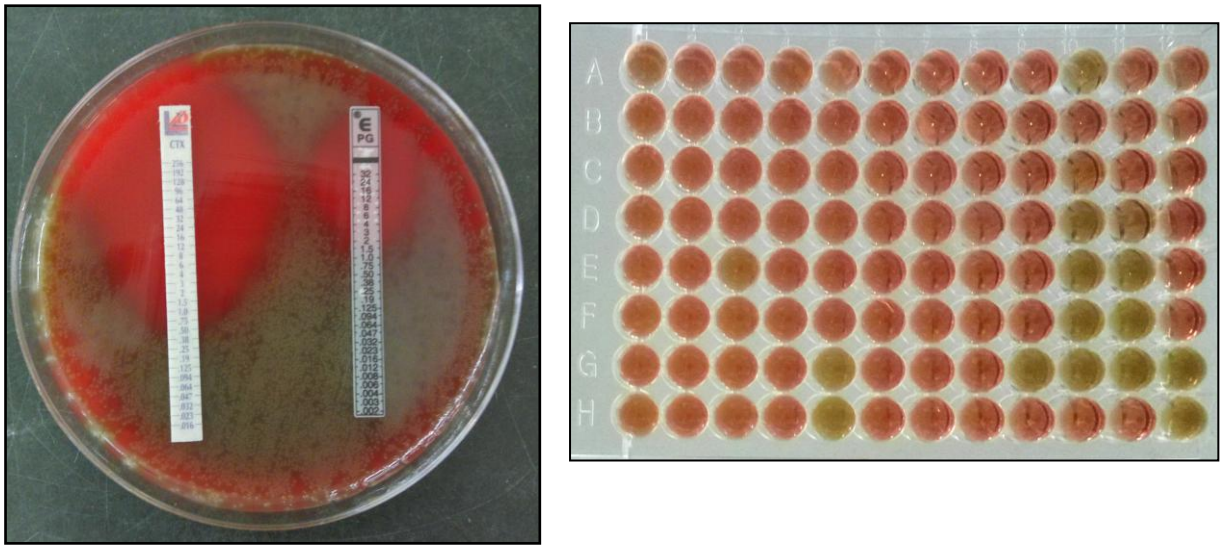


Figure IV. *Streptococcus pneumoniae* Antibiotic Susceptibility tests.
Quantitative diffusion method *e-test* on Mueller-Hinton Fastidiosus medium (left).
Commercially available microdilution method *Sensititre*^{TM} (right).

The polysaccharide capsule, as already mentioned before, is the major virulence factor of the pneumococcus and has been attributed full role in the invasive capacity of different serotypes [1,9,10,22,23]. The pneumococcus has been classified as invasive or non-invasive depending on their higher prevalence on IPD or nasopharyngeal colonization. Some serotypes as 1, 5 and 7F are considered primary IPD pathogens but rarely isolated in nasopharyngeal colonization. While other serotypes as 15A, 19F and 23F, considered opportunistic pathogens found in nasopharynx more frequently than IPD [22,23]. However, a recent study based on genotype analysis clarifies there are homogeneously invasive serotypes with its capsule, regardless of its genotype, while other serotypes are mucosal or noninvasive independently of the genotype. There is also a third group of serotypes with heterogeneous invasive capacity which varies according to genotype [23]. 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 on 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 on patients with underlying diseases, which can lead to a worse outcome [24,25,26]. 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 80s, most of the strains were serotype 9V, and a decade later, in the 90s, appeared the variant serotype 14; recently (2003) also emerged the 11th variant (not included in the PCVs) having high amoxicillin resistance, so its spread in Spain leads to advise 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 of differences in the capsule genes location, and flanked by homologous genes present in most serotypes [19,21,27]. Homologous genes facilitate genetic recombination where non-homologous genes are also switched and results in to the serotype changing [27].

In addition, CIBERES group of Bellvitge has characterized the appearance in Madrid and later to other communities of a new multi-resistant clone spread of serotype 8 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 new multidrug resistance of the recipient strain and the invasive capacity of the donor strain, provided by the serotype 8 capsules [28,29,30].

4. Objectives

I. Analysis of the impact of the PCV13 on the dynamics of *Streptococcus pneumoniae* serotypes that cause IPD on adults.

- Dynamic of the PCV7 serotypes.
- Dynamic of the PCV13 serotypes not included in PCV7.
- Dynamic of the non-PCV13 serotypes.

II. Molecular typing of invasive pneumococcus belonging to PCV13 serotypes and not included in PCV7.

- Molecular typing by pulsed field gel electrophoresis (PFGE). Identification of the majority clones in the medium.
- Molecular typing by *Multi Locus Sequence Typing* (MLST). Identification of international clones among major clones.

III. Detection of resistance genes associated with transposons of the Tn916 family.

- PCR detection of *ermB* and *mefA/E* genes associated with macrolide resistance in macrolide resistant strains.
- PCR detection of *tetM* gene associated with tetracycline resistance in tetracycline resistant strains.

5. Material

5.1. Instrumental

- Inoculation loop of 1 and 10 µL.
- Micropipettes with adjustable volume.
- Sterile pipette tips.
- Glass slides.
- Plastic spacers.
- Sterile *eppendorf* tubes of 1.5 ml and 50 µL (PCR tubes).
- Sterile disposable plastic tubes 4-6 ml.
- Cooler rack.

5.2. Equipment

- CHEF-DRII equipment: Pulse controller, electrophoresis cell, refrigeration system and buffer recirculation pump (Bio-Rad).
- Electrophoresis equipment (Bio-Rad).
- *Vortex.*
- Water bath.
- Camera and ultraviolet light transilluminator (Bio-Rad).
- Centrifuge (Heraeus).
- Precision balance.
- Thermoblock.
- PCR thermal cycler (Applied Biosystems).

5.3. Reagents

- Solution for the DNA agarose disc lysis:

- ST (basic lysis solution): 6 mM Tris-HCl (pH 8); 1 M NaCl; 0.1 M EDTA (pH 8); 0.2 % sodium desoxicolate; 0.5 % Sarcosyl.
- Brij-58: 10 % solution.
- Rnase-A: 10 mg/ml.
- Lisozime: 50 mg/ml.

- Gel agarose and low melting point agarose.
- PIV: 0.01 mM Tris-HCl (pH 8); 1 M NaCl.
- ES solution: 0.5 M EDTA (pH 9), 1 % Sarcosyl.
- TE buffer: 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8).
- TBE solution (10x): 890 mM Tris, 890 mM Boric acid, 20 mM EDTA pH 8.
- *Syber®Safe.*
- Proteinase-K.
- Restriction enzymes: *SmaI* and *ApaI*.
- Load buffer 1/3 and log-ladder marker.

- PCR reagents:

- Taq polymerase.
- $MgCl_2$ (Mg^{2+}).
- dNTPs.
- Buffer (10x) or Red buffer (which includes dNTP and $MgCl_2$).
- Primers (forward and reverse).
- Distilled water.

- Differentiation between *mefA* and *mefE* by restriction enzymes:

- Buffer (10x).
- *BamHI* restriction enzyme.
- Distilled water.

6. Methods

Patients and strains: This is a prospective, laboratory-based study collecting all episodes of invasive Pneumococcal disease occurred in adults patients (≥18 years old) from 2011 until 2016 at Hospital Universitari de Bellvitge (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 denominator the total number of people that can be obtained by the regional government publication (*web de l'Estadística de Catalunya*). This study was approved by the Clinical Research Ethics Committee of Hospital Universitari de Bellvitge. 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 *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 its use for further studies. The method used is based on the following techniques:

1. - DNA extraction
2. - Molecular Typing by PFGE
3. - Molecular typing by MLST
4. – Serotyping by PCR
5. - PCR detection of resistance genes

6.1. 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 other streptococci (resistant) than *Streptococcus pneumoniae* (susceptible).

Several colonies of this culture were re-suspended in 150 µL of PIV into 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 into 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 for 3-5 times). Finally, the plugs were preserved in 1 ml of TE buffer at 4°C.

Table I. Lysis solution composition for one plug.

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

6.2. 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: i) bacterial chromosomal DNA extraction, ii) DNA restriction by low frequency restriction enzymes, iii) fragments 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 reference method for molecular typing, although it has limitations such as the high cost of the equipment and the laboriousness of the procedure. To analyze the results, there was proposed a standardized system for the interpretation of the results according to the band patterns: 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 agarose plug (or disc) was placed in a tub containing 40 µl of the restriction solution and incubated according to the manufacturer recommendations (Table II).

Table II. Restriction solution composition to perform the PFGE typing.

	Enzyme	**H_2O_2**	**Buffer**	**BSA**	**Tº Restr.**	**Pulses PFGE**	**Tº PFGE**	**Hours PFGE**
Serotype 3	0.5 µl *ApaI*	26.5 µl	3 µl	0.8 µl (200 µl/ml)	25ºC	1-30 s	11 ºC	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 III), boiled for 5-10 minutes and cooled for 30 minutes. Plugs were carefully placed into the agarose gel before solidification that occurs after 30 minutes.

Table III. Composition for the gel electrophoresis used in 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 during 30 minutes. Finally, the gel was visualized using the UV transilluminator (Figure V).

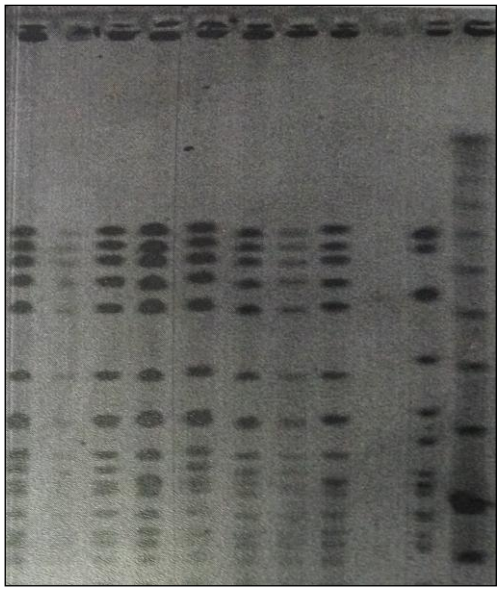


Figure V. Band patterns of bacterial DNA separated by pulsed field gel electrophoresis (PFGE) after restriction with *SmaI* enzyme.

Lanes 1-10: samples; Lane 11: Log-ladder marker.

6.3. Molecular typing by multilocus sequence typing (MLST)

The MLST is a molecular typing method based in 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. 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 I.
- *xpt*: xanthine phosphoribosyltransferase.
- *ddl*: D-alanine-D-alanine ligase.

To obtain a DNA solution the agarose plugs were melted re-suspended in 380 µl of TE solution and incubated for 15 minutes at 70°C. The PCR mix was prepared according to Table IV, 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 ºC x 10’
2. *Denaturation* 94 ºC x 30”
Annealing 55 ºC x 30”
Elongation 72 ºC x 60”
x 35 cycles
3. 72 ºC x 10’

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 during 25 minutes, and then the gel was visualized under UV light (Figure VI). The sequence of primers used for PCR amplification is described in Table V.

Table IV. MLST mix solution composition 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

Table V. Primers required to perform the 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	

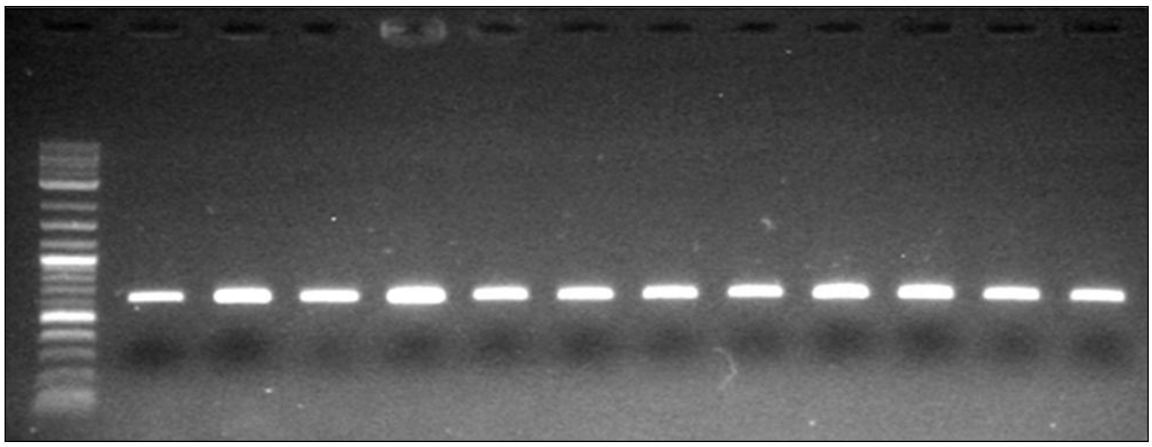


Figure II. Electrophoresis gel of g*dH* gene amplification by PCR sequencing.

Lane 1: Log-ladder marker; Lane 2-13: samples.

6.4. 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 VI). Also it is used a cpsA gene primer (expressed in bacterial capsule) as a control.

A mixture of the PCR reagents (Table VII) 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 program conditions (same for all serotypes):

1. 95 ºC x 1’

2. *Denaturation* 95 ºC x 30”
Annealing 54 ºC x 1’
Elongation 72 ºC x 2’

x 35 cycles

3. 72 ºC x 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 during 45 minutes, and then the gel was visualized under UV light (Figure VII).

Table VI. Primers required to perform the 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

Table VII. PCR mix solution composition to perform the 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

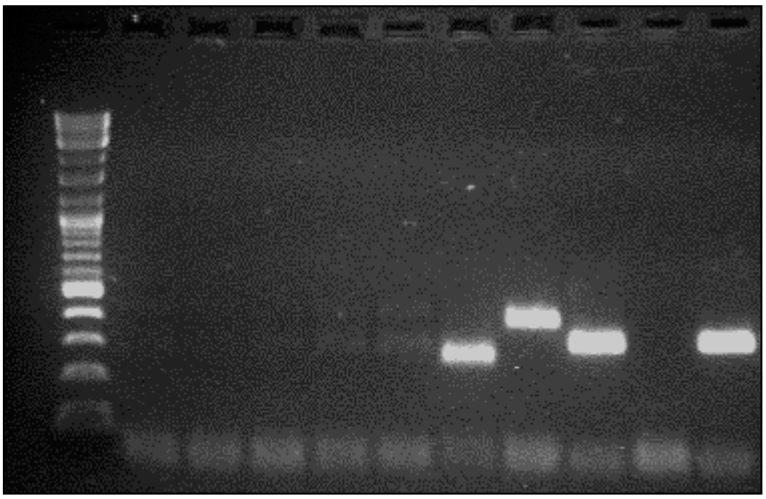


Figure VII. Electrophoresis gel of serotype amplification by PCR sequencing.

Lane 1: Log-ladder marker; Lane 2-10: samples; Lane 11: Positive control.

6.5. Resistance gene genotyping by PCR

The macrolide resistance may be occurs due to the acquisition of encoding genes which usually belong to the 916 transposons 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 by the selection of the resistant strains using the clinical breakpoints criteria indicated in EUCAST website (Table VIII) and proceed with the same way as MLST technique explained before. Subsequently, a PCR mixture is made according to the number of samples (Table IX). In this case, a different buffer (Red Buffer) has been used which includes the $MgCl_2$ and the dNTPs already. 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 ºC x 1’

2.

	Denaturation	94 ºC x 30”
	Annealing	58 ºC x 60”
	Elongation	72 ºC x 90”

x 35 cycles

3. 72 ºC x 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 120 V during 20 minutes, and then the gel was visualized under UV light (Figure VIII). 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 at the same conditions.

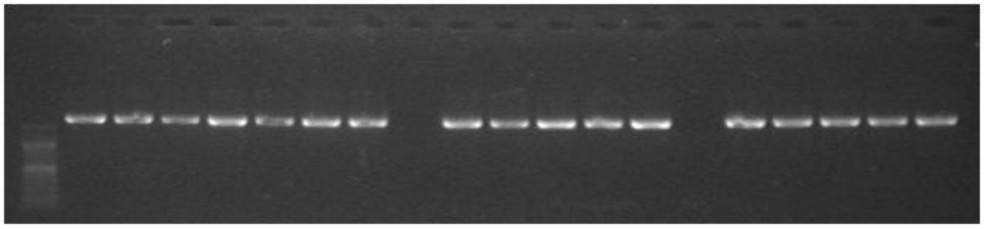


Figure VIII. Electrophoresis gel of *tetM* gene amplification by PCR sequencing.
Lane 1: Log-ladder marker; Lane 2-19: samples; Lane 20: Positive control

Table VIII. 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 IX. PCR mix solution composition to perform the 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 X. 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

6.6. Sequence results edited and analyzed

- Sequence edition: *SeqScape v2.7* program.

The chromatogram (Figure IX) 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 labeled 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, Madrid.

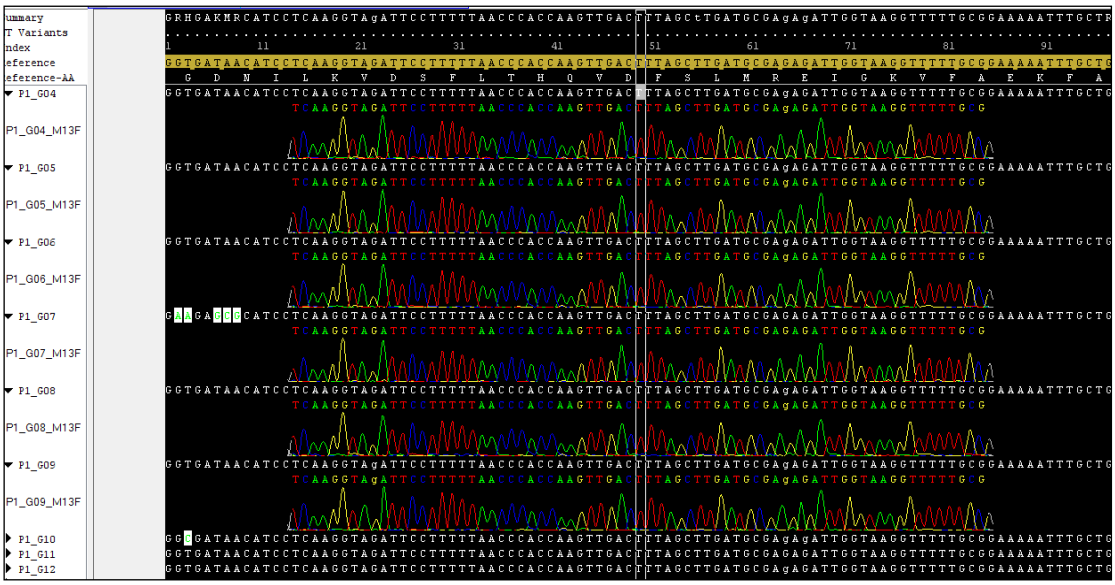


Figure IX. Image of *SeqScape v2.7* in which the nucleotide sequences can be edited.

- Sample analysis: *PubMLST* website.

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 program that *PubMLST website* has on its database (Figure X).

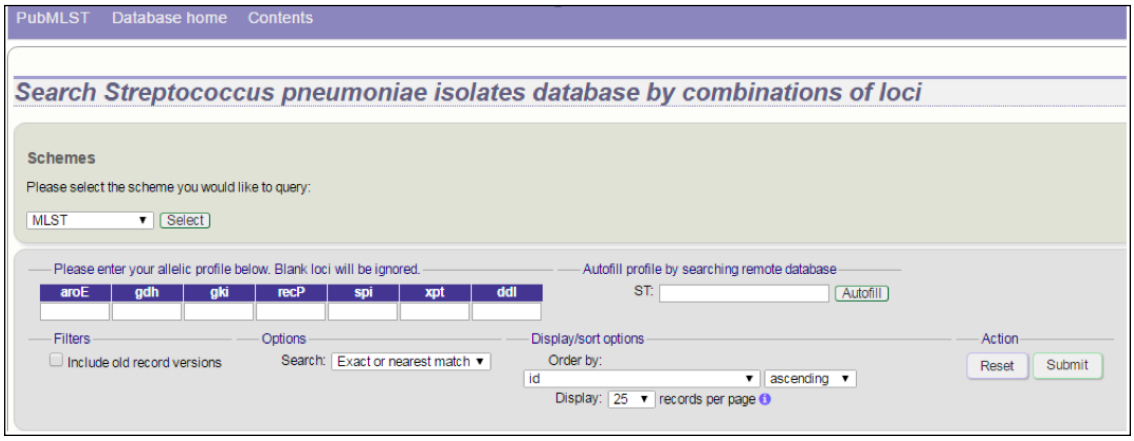
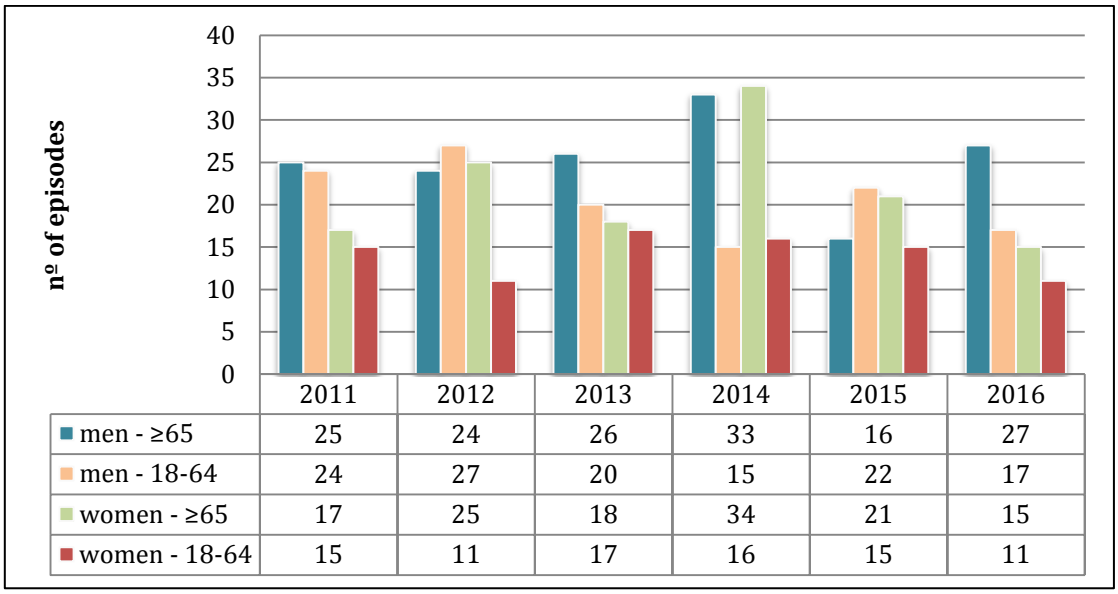


Figure X. Image of *pubMLST.org* website database in which the results, obtained through the previous sequence edition, can be inserted and processed.

7. Results and discussion

7.1. Invasive Pneumococcal Disease (IPD) in 2011-2016 period

The results obtained have been analyzed by age groups (those over 65 years old and those between 18 and 64 years) and sex (Figure XI). A total of 492 IPD strains were isolated among 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 groups differences 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 XI. Distribution of IPD episodes among age and sex group per year (2011-2016).

Figure XII shows the results of total number of IPD episodes analyzed 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 the IPD due to non-PCV13 serotypes was observed in 2014 and onwards.

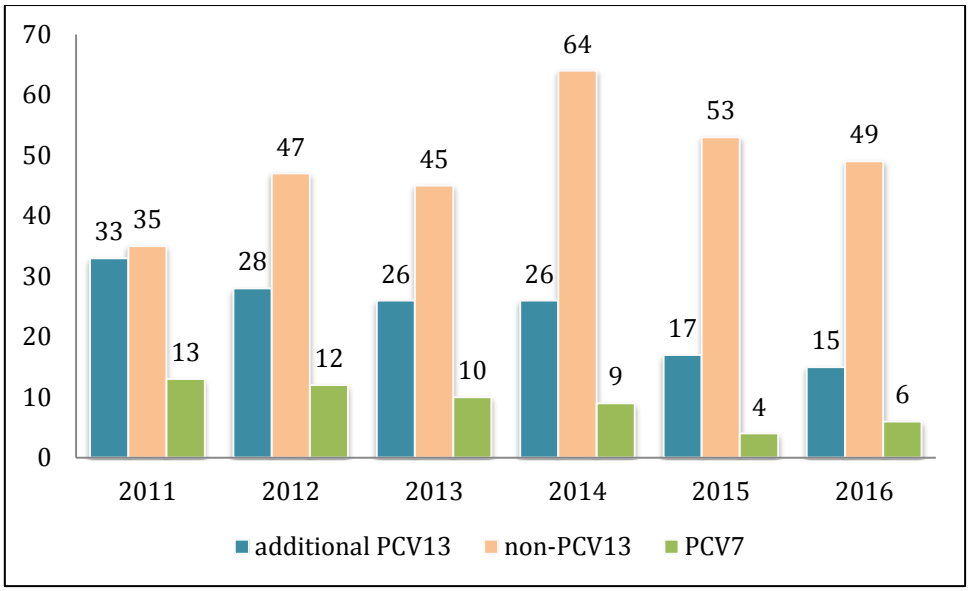


Figure XII. Total number of IPD episodes by serotype group (PCV7, non-PCV13 and additional PCV13) per year (2011-2016).

In regard the vaccine group distribution, we observed a decrease in the IPD among young adults (18-64) while in adults over 65 remains stable due to an increase of the disease caused by non-PCV13 serotypes (Figure XIII). 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.

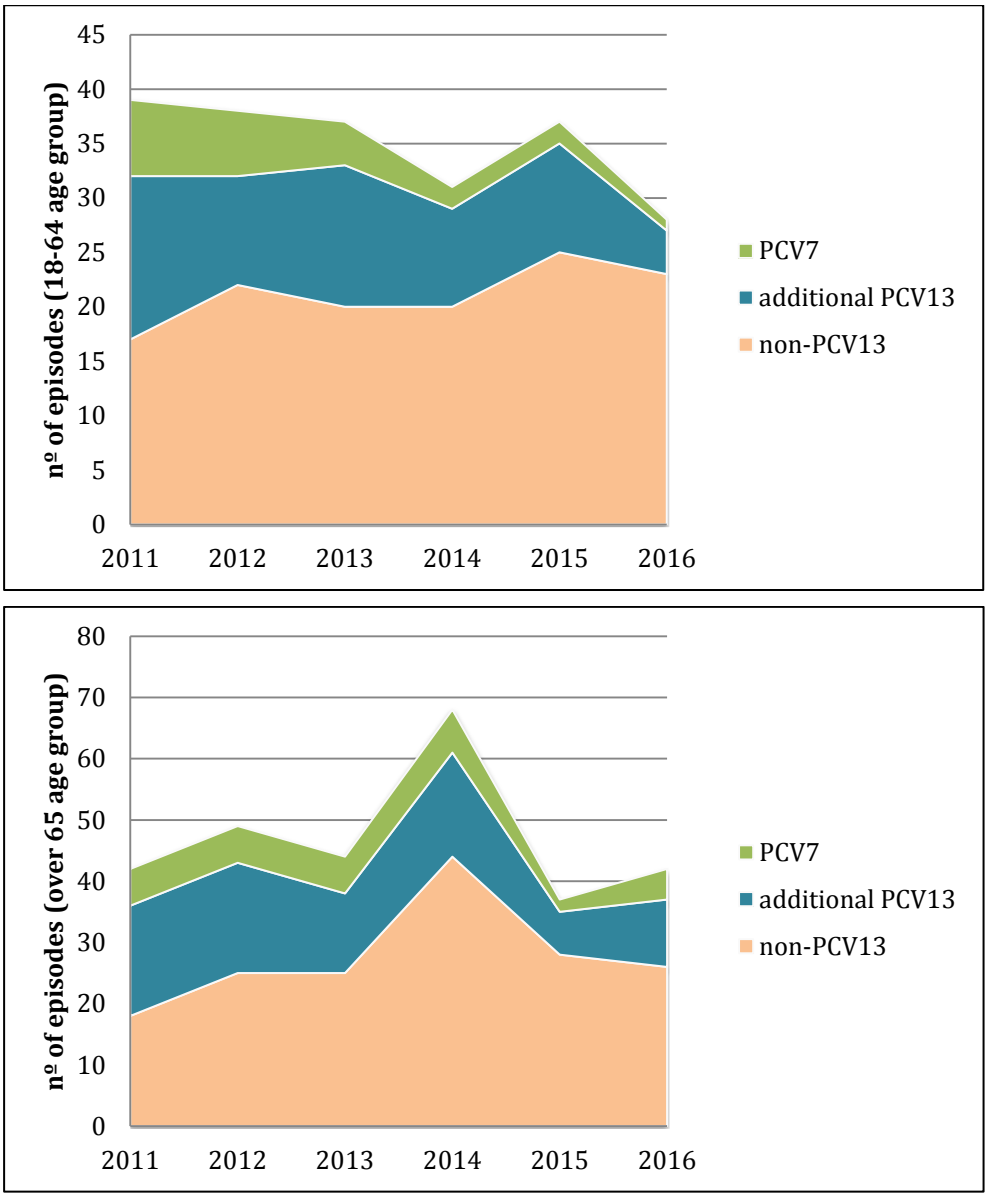


Figure XIII. Annual distribution of IPD (episodes/100.000 people) related to vaccine serotypes and age groups in a Southern Barcelona Area. Top: Incidence of IPD in people aged 18-64. Bottom: Incidence of IP in people aged over 65. Green: PCV7 serotypes (4, 6B, 9V, 14, 19F, 18C and 23F). Blue: additional PCV13 serotypes (1, 3, 5, 6A, 7F and 19A). Orange: non-PCV13 serotypes (others).

7.2. 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, the IPD caused by the additional serotypes decreases with the exception of serotypes 3 and 19A. In fact, the 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 XIV). These results show the impact of children vaccination among adult IPD due to the herd protection, with exception of serotype 3 which is rarely found in children.

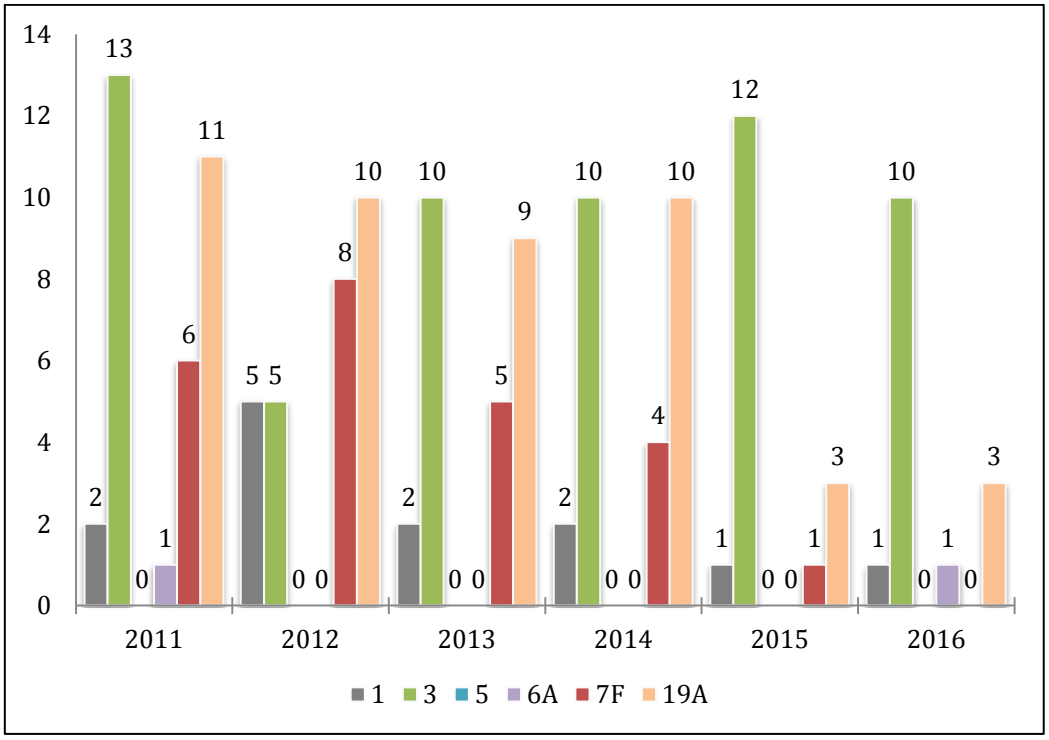


Figure XIV. Incidence of PCV13 non-PCV7 serotypes (1, 3, 5, 6A, 7F and 19A) episodes per year (2011-2016).

7.3. Macrolide and tetracycline resistance genes

Among 492 IPD episodes collected in 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 XI 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 Tn916-family.

Table XI. 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

7.4. Genotypes associated to additional PCV13 serotypes

As can be observed in Table XII, within the additional PCV13 group predominates the ST306 associated to serotype 1, 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 XII. 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

The Figure XV shows particularly the clonal complexes related to serotype 19A in which multidrug-resistant caused by ST320 appears more frequently. Also, the Figure XVI shows the clonal complex related to serotype 3, making evidence of the greater clonal complex ST180 presence.

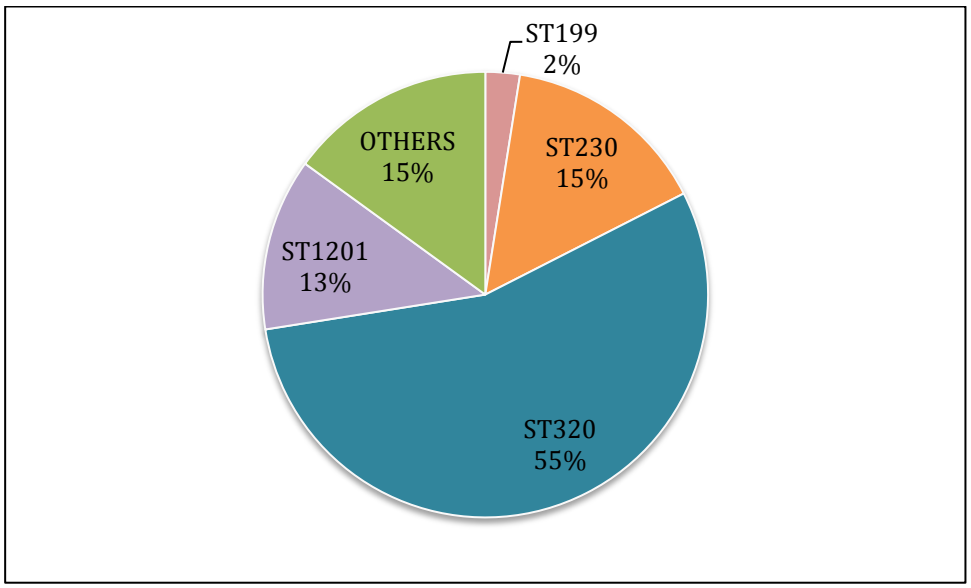


Figure XV. Clonal composition of serotype 19A (n=46).

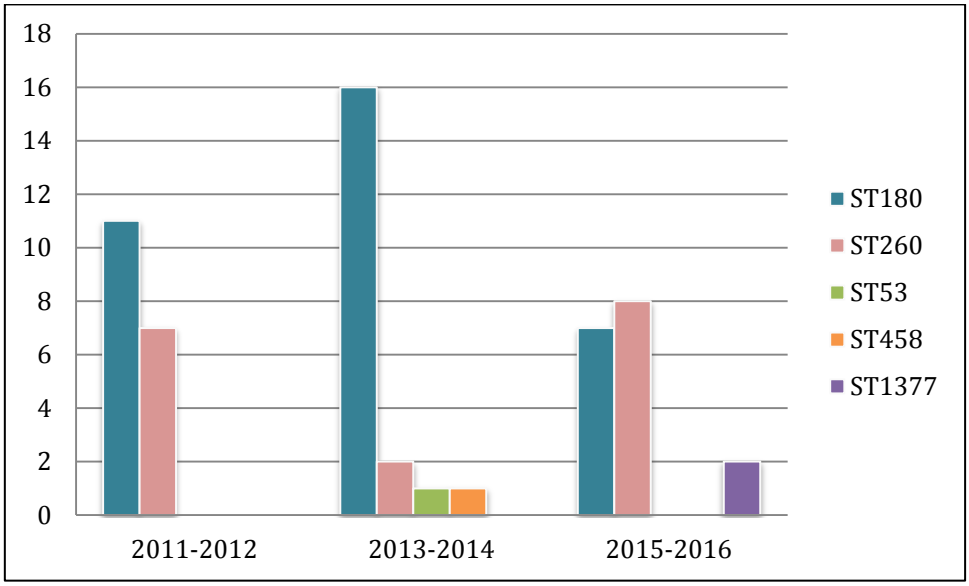


Figure XVI. Clonal composition of serotype 3 (n=60) per years

8. Conclusions

- The incidence of IPD in adults decreases over the study period demonstrating a significant impact of children vaccination in adults due to herd protection.
- The decrease on IPD was only remarkable in young adults (18-64) while in adults over 65 remained stable.
- In adults over 65 the decrease of IPD caused by PCV13 serotypes was balanced by an increase of non-PCV13 serotypes.
- There was not an impact of children vaccination on IPD due to serotype 3 that 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.
- The association of macrolide and tetracycline resistances was frequent, indicating the dissemination of Tn916-family transposon.

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