Characterization of *Streptococcus pneumoniae* associated with invasive disease in children in Portugal (2015-2017)

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Declaração

Declaro que o presente documento é um trabalho original da minha autoria e que cumpre todos os requisitos do Código de Conduta e Boas Práticas da Universidade de Lisboa.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Abstract

Streptococcus pneumoniae is an important human pathogen causing high mortality and morbidity worldwide. Pneumococcal conjugate vaccines (PCVs) have been available in Portugal since 2001 and in 2010 higher valency vaccines were introduced through the private market. In 2015, PCV13 was introduced in the National Immunization Plan. Despite more than a decade of vaccine use, vaccine serotypes still remain a major cause of paediatric invasive pneumococcal disease (IPD) in Portugal.

During the work presented in this thesis, *S. pneumoniae* associated with paediatric IPD in Portugal between 2015-2017 were characterized by phenotypic (serotyping and antimicrobial susceptibility testing) and molecular methods (DNA sequencing to obtain MLST profiles). The results were compared with those from the period of 2012-2015 to evaluate the effect of the vaccine in serotype distribution, antimicrobial resistance and clonal composition of the pneumococcal population.

Serotype 3, which was mainly detected by molecular methods, remained the major cause of paediatric IPD (29.1%). Non-vaccine types were responsible for 50.7% of IPD, and the main NVTs found were serotypes 8, 10A and 15B/C. Non-susceptibility to penicillin and resistance to erythromycin were present in 13.2% and 16.5% of isolates, respectively. The MLST analysis of the isolates revealed a diverse population, with the most frequent CCs (CC156, CC63, CC180, CC460, CC393, CC433 and CC1262) accounting for 70.9% of IPD.

Routine surveillance should continue to be performed in the future to evaluate the effects of PCV13 vaccination in serotype distribution, antimicrobial resistance and in the genetic population of *S. pneumoniae.*

Key words: *Streptococcus pneumoniae*; invasive pneumococcal disease; PCV13; serotype distribution; antimicrobial resistance; genetic lineages.

Resumo

Streptococcus pneumoniae é um microrganismo patogénico importante, responsável por elevadas taxas de mortalidade e morbilidade. Vacinas pneumocócicas conjugadas (PCVs) estão disponíveis em Portugal desde 2001 e em 2010 foram introduzidas vacinas de maior valência no mercado privado. Em 2015, a PCV13 foi introduzida no Plano Nacional de Vacinação. Apesar de mais de uma década de uso de vacinas, serótipos vacinais permanecem uma das principais causas de doença invasiva pneumocócica (DIP) pediátrica em Portugal.

Durante este trabalho, *S. pneumoniae* responsáveis por DIP pediátrica em Portugal (2015-2017) foram caracterizados por métodos fenotípicos (serotipagem e testes de suscetibilidade a antimicrobianos) e moleculares (sequenciação de DNA para obtenção de perfis de MLST). Os resultados foram comparados com os de 2012-2015 para avaliar o efeito da vacina na distribuição de serótipos, resistência a antimicrobianos e composição clonal da população pneumocócica.

O serótipo 3 foi a principal causa de DIP pediátrica (29.1%). Os serótipos não-vacinais (NVTs) foram responsáveis por 50.7% de DIP, sendo que os principais NVTs encontrados foram os serótipos 8, 10A e 15B/C. Não suscetibilidade à penicilina e resistência à eritromicina foram detetadas em 13.2% e 16.5% das estirpes, respetivamente. A análise por MLST revelou uma população diversa, sendo que os complexos clonais (CCs) mais frequentes (CC156, CC63, CC180, CC460, CC393, CC433 e CC1262) foram responsáveis por 70.9% de DIP pediátrica.

É importante continuar o estudo da epidemiologia de doença invasiva pneumocócica no futuro, para avaliar os efeitos da PCV13 na distribuição de serótipos, resistência a antimicrobianos e na população genética de *S. pneumoniae.*

Palavras-chave: *Streptococcus pneumoniae*; doença invasiva pneumocócica; PCV13; distribuição de serótipos; resistência a antimicrobianos; linhagens genéticas.

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Abbreviations

AW	Adjusted Wallace
BHI	Brain Heart Infusion
CAP	Community Acquired Pneumonia
CBP	Choline binding protein
CC	Clonal Complex
CDC	Centers for Disease Control and Prevention
CI95%	95% confidence intervals
CLSI	Clinical and Laboratory Standards Institute
$cMLS_B$	Constitutive MLS_B phenotype
CSF	Cerebrospinal fluid
DLV	Double-locus variant
DNA	Deoxyribonucleic acid
ECDC	European Centers for Disease Control and Prevention
EPNSP	Erythromycin and penicillin non-susceptible pneumococci
ERP	Erythromycin resistant pneumococci
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDR	False Discovery Rate
$iMLS_B$	Inducible MLS_B phenotype
IPD	Invasive pneumococcal disease
MDR	Multidrug resistance
MIC	Minimal inhibitory concentration
MLST	Multilocus Sequence Typing
NIP	National Immunization Plan
NVT	Non-vaccine type
OR	Odds Ratio
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction

PCV7	7-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PMEN	Pneumococcal Molecular Epidemiology Network
PNSP	Penicillin non-susceptible pneumococci
PPV23	23-valent polysaccharide pneumococcal vaccine
RNA	Ribonucleic acid
rPCR	Real time PCR
SID	Simpson's Index of Diversity
SLV	Single locus variant
ST	Sequence Type
SXT	Trimethoprim-sulfamethoxazole
TLV	Triple-locus variant
TSA	Tryptone soya agar
TSB	Tryptone soya broth
WGS	Whole genome sequencing
WHO	World Health Organization

1. Introduction

1.1 The importance of *Streptococcus pneumoniae*

Streptococcus pneumoniae, also known as the pneumococcus, was first isolated by Pasteur and Steinberg more than a hundred years ago [1]. It is one of the most common bacterial respiratory pathogens and the most frequent cause of community acquired pneumonia (CAP) [2], with high morbidity and mortality risks worldwide [2,3], affecting mainly young children, immunocompromised individuals and the elderly [4]. It is a colonizer of the human nasopharynx and carriage rates are higher among young children [3].

The World Health Organization (WHO) estimates that 1.6 million people, including up to 1 million children <5 years old die annually due to invasive pneumococcal disease (IPD) [5].

The ability of the pneumococcus to colonize its host, adapt through genetic exchange and to express diverse virulence factors are properties that contribute to its success as a pathogen [6].

Prevention strategies against pneumococcal infection have been developed over the years, such as vaccination [7]. However, the differences in the epidemiology of *S. pneumoniae* according to geographic area, age group, time period and other factors pose a challenge in developing a universal strategy to prevent pneumococcal disease [7].

1.2 General description of *S. pneumoniae*

S. pneumoniae is part of the viridans group of streptococci [1]. It is a gram-positive coccus bacteria that can appear in pairs (diplococcus) or in short chains [1,8]. Cells are non-motile, non-spore forming with a diameter between 0.5 μm and 1.2 μm [1,9]. Encapsulated strains appear as big, round and mucoid in blood agar, while non-encapsulated strains appear as small and flat [1].

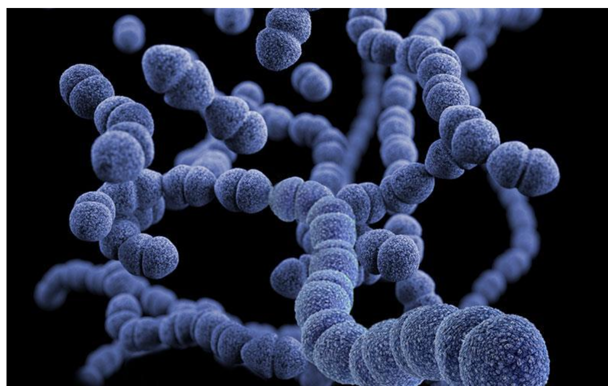

Figure 1 – Illustration of *S. pneumoniae* organized in short chains. Reproduced from https://www.cdc.gov/pneumococcal/about/photos.html.

Pneumococci are fastidious bacteria that require an enriched media supplemented with blood and incubation in an atmosphere enriched with 5-10% CO_2 and an optimal temperature of 35-37ºC. They ferment carbohydrates producing lactic acid, however in certain concentrations it becomes toxic to the cells [1]. Cells are catalase negative, therefore if a source of catalase is not provided, accumulation of

H_2O_2 will inhibit the growth of pneumococci [1]. On blood agar, *S. pneumoniae* colonies are α-hemolytic usually with a visible central depression in the colonies [1,10].

1.3 Identification of *S. pneumoniae*

The correct diagnosis of *S. pneumoniae* infections remains a challenge. The confirmation of an invasive pneumococcal disease case is given by isolation of *S. pneumoniae* from a normally sterile body site (such as blood, cerebrospinal, pleural, synovial or peritoneal fluid) [10]. However, it can be difficult to obtain a pneumococcal isolate from some biological products, so evidence of pneumococcal infection is only achieved in a minority of IPD cases, which leads to limitations in the capacity to obtain accurate data on disease burden, serotype prevalence and to evaluate the effectiveness of vaccination [10,11].

Laboratory identification relies on conventional methods such as microscopy, culture and phenotypic tests. Under microscopic observation, *S. pneumoniae* appear as gram-positive lancet-shaped diplococci or organized in short chains. Culture requires inoculation of samples on enriched media, such as blood agar, supplemented with blood [1,10].

Figure 2 – Schematic illustration of *S. pneumoniae* colonies on blood agar with alpha hemolysis (green halo). Reproduced from: https://www.microbiologyinpictures.com/bacteria%20photos/streptococcus%20pneumoniae%20photos/STPN2.html.

To differentiate *S. pneumoniae* from other viridans group streptococci, two important tests should be performed: the optochin susceptibility and bile solubility tests [1,10,11].

In the optochin susceptibility test, the isolate is inoculated in a blood agar plate and an optochin disk is used. If there is observation of a clear zone of inhibited growth around the disk after overnight incubation, the isolate is susceptible to optochin (halo ≥ 14 mm) which is characteristic of *S. pneumoniae* isolates [10]. However, optochin-resistant *S. pneumoniae* isolates were already described [12,13].

The bile solubility test is based on the lysis of bacteria in the presence of sodium deoxycholate. *S. pneumoniae* produces autolysins which degrade the cell wall peptidoglycan. When exposed to bile salts, those autolysins are activated and lead to the lysis of *S. pneumoniae* cells which dissolve in the bile, while other α-hemolytic streptococci remain unchanged (solution remains turve) [1,10]. Some strains of pneumococci give inconsistent results with the typical phenotypic tests [11].

Additionally, *Streptococcus pseudopneumoniae* can sometimes be mistakenly identified as *S. pneumoniae* even though *S. pseudopneumoniae* lacks a polysaccharide capsule, is insoluble in bile and presents resistance or indeterminate susceptibility to optochin if incubated in 5% CO_2, but is susceptible to optochin when incubated in ambient air [10].

Additionally, other tests are available for the identification of *S. pneumoniae*. The Quellung reaction can help detect *S. pneumoniae* in pure culture or sputum samples. Anticapsular antibodies are mixed with bacteria and if the reaction is positive the capsule becomes visible and the cells seem to be surrounded by a halo upon microscopic visualization [1,10].

The API 20 Strep System (bioMerieux Vitek, Inc., Hazelwood, Mo.) is a commercial, standardized, easy-to-use test system composed of strips that contain biochemical tests, allowing the identification of *Streptococcus* species [13,14].

MALDI-TOF (Matrix-assisted laser desorption ionization time of flight mass spectrometry) has been used as a routine tool for microbial identification of many pathogens, although sometimes differentiation between *S. pneumoniae* and other streptococci from the *Streptococcus mitis* group can be difficult [15].

More recently, an immunochromatographic test that targets the C polysaccharide cell wall antigen that is common to all strains of *S. pneumoniae* was developed, the *S. pneumoniae* urinary antigen test; Binax NOW. When applied to urine samples, it presents high sensibility. However, it also presents the disadvantage of high cost and the limitation of its use in children which can lead to false positives due to nasopharyngeal colonization [10].

In the last decades, molecular methods have been under development. PCR (Polymerase Chain Reaction) can be useful in the detection of pneumococcal DNA in CSF (cerebrospinal fluid) samples to diagnose pneumococcal meningitis. It has also been successfully used in samples like pleural fluid [10,16]. Nowadays, the WHO recommends that for molecular detection of pneumococci, the method that should be used is *lytA* (encodes an autolysin) real-time PCR [11]. However, some streptococcal species possess a *lytA* homologue, so an additional real-time PCR of *piaB* (component of the iron uptake ABC (ATP binding cassete) transporter [17,18]) is recommended [11]. PCR schemes using other genes such as *ply* (encodes a pneumolysin) and *psaA* (codes for pneumococcal surface antigen A) have also been developed [11]. In our laboratory, we have been using a PCR scheme that targets both *lytA* and *wzg* (encodes a membrane regulatory protein [19]), for the molecular detection of *S. pneumoniae* in CSF and pleural fluid samples. In 2010-2015, molecular methods were able to identify *S. pneumoniae* in 68% of culture-negative samples of pleural fluid from paediatric patients [16].

Despite the development of other techniques for the detection of *S. pneumoniae*, culture should still be performed, since it is the only technique that allows antimicrobial susceptibility testing [4].

1.4 Virulence factors

S. pneumoniae produces a variety of virulence factors that contribute to its success as a pathogen. The study of virulence factors is important for the understanding of infection and for the development of treatment and vaccination strategies [20].

Below, a schematic illustration of a pneumococci and its virulence factors is presented.

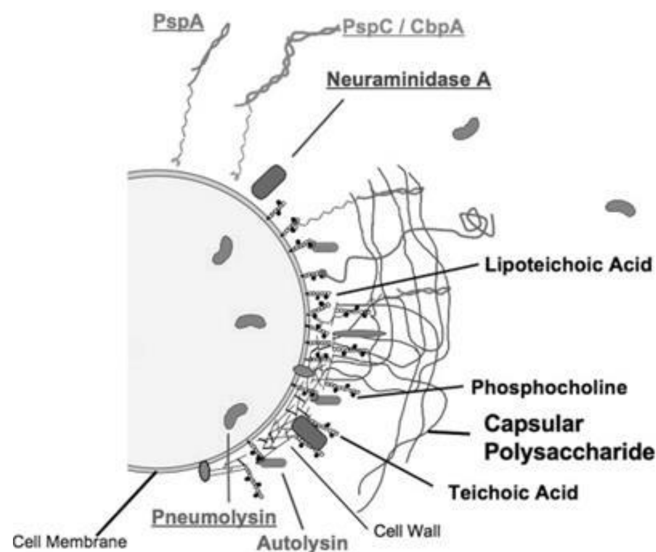

Figure 3 – Schematic illustration of a pneumococcus and some of its virulence factors. Adapted from [21].

1.4.1 Polysaccharide Capsule

The polysaccharide capsule of *S. pneumoniae* is a very important virulence factor. The capsule is antiphagocytic, it prevents the action of the complement system by inhibiting C3b opsonization of bacterial cells [17,20]. Furthermore, it also prevents the mechanical action of mucus and it can reduce the exposure to antibiotics [20].

Non-encapsulated strains tend to be less virulent and can cause superficial infections, however they may cause invasive disease in immuno-compromised patients [22]. Isolates from other sterile sites are encapsulated, though the non-encapsulated variants of those strains are mainly avirulent [23].

There are at least 97 distinct types of capsular polysaccharide, that differ both serologically and structurally [17,24]. The capsular serotypes differ chemically, and virulence is also influenced by the serotype, with some having more ability to cause invasive disease than others [17,25]. Additionally, some serotypes are more associated with susceptibility or resistance to antimicrobials and the ability to colonize the nasopharynx [26]. It has also been shown that pneumococcal serotypes causing disease differ between geographic areas and distribution is dependent on the time period in study [27].

It has been reported that in infants and young children, serotypes/serogroups 3, 6A, 6B, 9V, 14, 15, 19A, 19F, 21, 23F and 35 colonize the nasopharynx [28]. Serotypes 6A, 6B, 9V, 14, 19A, 19F and 23F are often associated with antimicrobial resistance [28,29]. Furthermore, serotype 3 was associated with increased risk of empyema [4].

The capsule is the target of currently available vaccines, because capsular polysaccharides are immunogenic, inducing antibodies that protect against pneumococcal infection [26].

1.4.2 Cell wall components – lipoteichoic acid (LTA) and teichoic acid (TA)

The cell wall of the pneumococcus is composed of a peptidoglycan layer, which is typical of gram-positive cocci [1]. Other components of the cell wall are teichoic acids (TA) and lipoteichoic acids (LTA). Teichoic acid, also known as C-polysaccharide, is exposed on the cell surface and lipoteichoic acid, also known as F antigen, is a teichoic acid bound to lipids in the cytoplasmic membrane [1].

Both LTA and TA are composed of phosphocholine residues, which are required for the action of autolysins and for binding a family of surface proteins which is discussed below [30].

LTA and TA lead to activation of the alternative complement pathway and induce the production of cytokines such as interleukin-1 (IL-1), playing an important role in the inflammation process [1,31].

1.4.3 Choline binding proteins (CBPs)

Choline binding proteins (CBPs) include several proteins produced by *S. pneumoniae* that are anchored to the cell surface via interaction with phosphocholine which resides in lipoteichoic acids and cell wall teichoic acids under the protection of the capsule [20]. The variety of CBPs produced by *S. pneumoniae* is dependent on the strain of pneumococcus [20]. Examples of CBPs include LytA, an autolysin which is responsible for the pneumococcal cell lysis, resulting in the release of other virulence factors such as pneumolysin and pro-inflammatory molecules [20,32].

Pneumococcal surface protein A, PspA is a choline binding protein involved in the evasion from the host complement system, blocking the binding of complement component C3, therefore inhibiting phagocytosis and opsonization [32,33].

Another important CBP is pneumococcal surface protein C, PspC which acts as an adhesin and recognizes sialic acid on the epithelial cells of the host [34]. Therefore, it binds secretory IgA and the polymeric immunoglobulin receptor (pIgR) promoting adherence, uptake of bacteria into host cells and invasion of the respiratory epithelium and blood-brain barrier. Additionally, it also binds complement C3, and the complement regulatory factor H, providing resistance to the complement system [18,35].

1.4.4 Pneumolysin (Ply)

Pneumolysin is a cytoplasmic toxin which is part of a group of proteins known as cholesterol-dependent cytolysins [32]. Ply is released upon cell lysis and it binds to the cholesterol on the host cells cytoplasmic membrane and forms transmembrane pores, leading to lysis of the host cells [20,32]. The toxin affects the immune system, causing induction of the proinflammatory response and production of reactive oxygen intermediates and it activates the classical complement pathway [17,20].

1.4.5 LPxTG proteins

Pneumococci express a group of LPxTG proteins that are anchored to the peptidoglycan of the cell wall by sortases, important in the pathogenesis of pneumococcal infection [20]. Examples of those proteins are hyaluronidase, neuraminidase, IgA1 protease and pilus proteins among others [17,20].

Hyaluronidase is an enzyme that breaks down the extracellular matrix components and facilitates tissue invasion, increasing tissue permeability to pneumococci [20].

Neuraminidase cleaves the terminal sialic acid from glycoconjugates (glycolipids, glycoproteins) and oligosaccharides in the mucus and on cell surfaces [32,36], potentially exposing receptors for adherence [17].

IgA1 protease is another protein belonging to this group. It is a zinc metalloprotease that targets human immunoglobulin IgA1 (the most abundant class of immunoglobulin on mucosal surfaces) [37]. Previous studies revealed that instead of inhibiting adherence, IgA1 enhanced bacterial attachment to host lung epithelial cells, but only when cleaved by IgA1 protease [38].

Pili are filamentous surface structures that are composed of proteins with LPxTG motifs that are recognized by sortases which attach pili to the peptidoglycan cell wall [17,39]. There are two pathogenicity islets that encode pili (PI-1 and PI-2) [17]. Not all pneumococci strains have pili, however some strains can express both types [20]. Those structures are involved in virulence and mediate binding of pneumococci to host cells, promoting colonization. Furthermore, they also stimulate proinflammatory cytokine production [20,39].

1.4.6 Lipoproteins

Lipoproteins are tethered to the bacterial surface with a variety of functions, from transport to protein maturation [20].

One example is PsaA, an extracellular lipoprotein located on the cell membrane [40]. It is part of an ABC transporter involved in magnesium transport into the cells [20,40]. PiuA and PiaA are lipoprotein components of two iron ABC transport systems designated Pia (pneumococcal iron acquisition) and Piu (pneumococcal iron uptake) [41].

1.5 Host colonization

Carriage by *S. pneumoniae* is very common. In children, the nasopharyngeal colonization is established in the first months of life [27] and they are the main carriers of *S. pneumoniae* [3]. A study from 2010 regarding day-care centers in Lisbon reported a pneumococcal carriage rate of 63.8% in children from 0 to 6 years [42]. *S. pneumoniae* is transmitted through respiratory droplets [3,27].

Pneumococcal disease is anteceded by asymptomatic colonization [27]. When colonizing organisms from the upper respiratory tract gain access to body sites such as the middle ear, lung, and bloodstream, disease occurs. Hence, colonization appears to be the first step in the pathogenesis of pneumococcal disease [6] and carriage is a pre-requisite for infection [3,17].

The reported rates of acquisition and carriage of bacteria depend on many factors, such as age, genetic background, socioeconomic conditions and geographical area [27]. Pneumococcal carriage is an important source of horizontal spread of the pathogen in the community. Overcrowded spaces such as hospitals, day-care centres, prisons, military barracks, orphanages and slums represent situations where colonization rates are higher, and the horizontal spread of pneumococcal strains increases [3,27]. Previous antibiotic treatment promotes carriage of antibiotic resistant strains [3].

1.6 Pneumococcal Infections

S. pneumoniae is responsible for a variety of invasive and non-invasive infections. It can cause acute otitis media, sinusitis and non-bacteremic pneumonia, but also meningitis, bacteremic pneumonia, bacteraemia and sepsis [3,27].

The burden of disease is higher in children and in the elderly populations [27]. Important risk factors for IPD are immunodeficiency states, like asplenia and cirrhosis of the liver, congenital deficiencies in immunoglobulin or complement system, human immunodeficiency virus, sickle cell disease (red blood cell disorder) and anatomical disorders such as cerebrospinal fluid leaks [8,26,27].

In the case of children, some risk factors such as attendance of day care centre are important for infections [8].

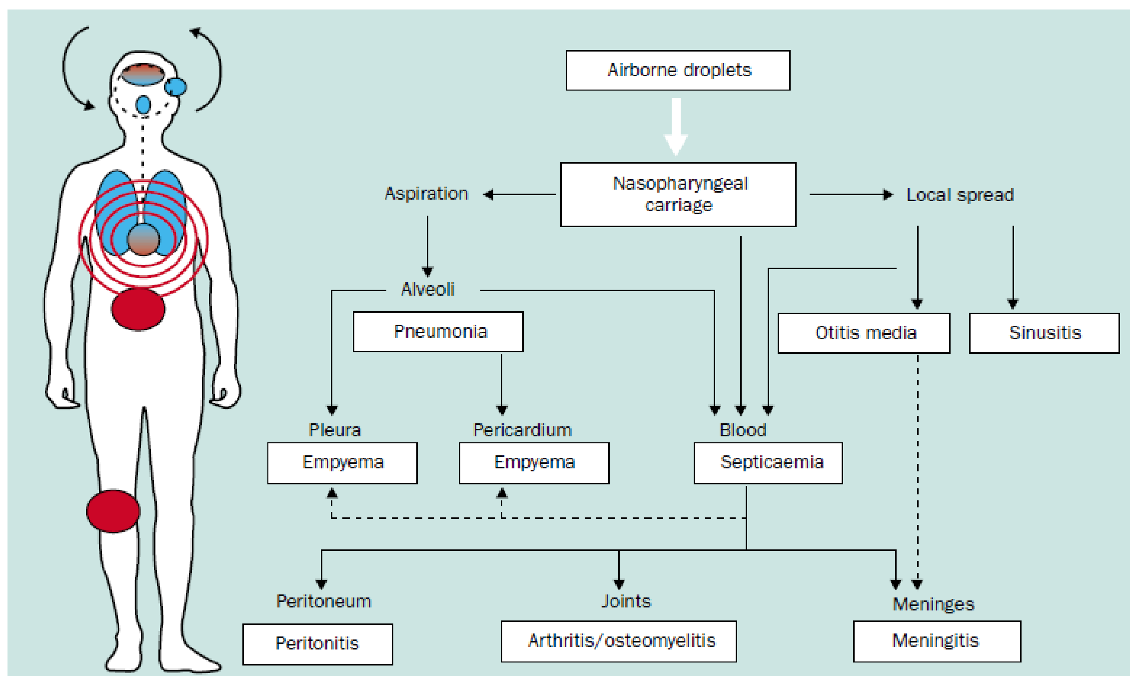

Figure 4 – Illustration of pathogenic routes for *S. pneumoniae* infection. Transmission is through airborne droplets. After colonization, pneumococci can disseminate to other organs and cause infection. Reproduced from [27].

1.6.1 Pneumonia

Streptococcus pneumoniae is the most common cause of CAP [2]. It is the leading cause of death in developing countries, responsible for approximately 5 million deaths annually. In adults, the most common clinical presentation of pneumococcal disease is also pneumonia [8]. In children, in some cases patients develop pleural effusions and a small proportion progress to empyema, the accumulation of pus in the pleural space [1].

When bacteria are able to spread to other normally sterile sites such as the blood (bacteraemia), they can be carried to the meninges, joint spaces, bones and other places causing diseases such as meningitis, peritonitis and osteomyelitis [27].

1.6.2 Meningitis

Meningitis is the inflammation of meninges, the brain's protective membranes [43]. The incidence of pneumococcal meningitis varies with age, being less frequent in neonates. However, in children aged 1-23 months, *S. pneumoniae* is one of the major causative pathogens [8].

When pneumococci come in contact with the blood-brain barrier endothelial cells, they cross the tight junctions of the epithelium and can entry into the cerebrospinal fluid and access the brain parenchyma where they cause damage [1,17].

Pneumococci penetrate into the blood as part of other conditions such as pneumonia, acute otitis media or sinus infections, or they can penetrate directly from the nasopharynx [1,17]. The diagnosis can be difficult, and in case the patient has already started antibiotic treatment, results may be negative. In those cases, diagnosis can be achieved using molecular methods such as PCR [44,45].

1.6.3 Bacteraemia and sepsis

Bacteraemia is the presence of bacteria in the blood. It is estimated that 25-30% of patients with pneumococcal pneumonia and more than 80% of patients with meningitis develop bacteraemia [1,8].

In case of sepsis, there are signs of systemic illness caused by activation of multiple cascades that lead to systemic inflammation. If left untreated, sepsis can lead to multisystem organ failure and death [8].

1.6.4 Acute otitis media and sinusitis

Pneumococci can also pass from the nasopharynx to the middle ear, causing acute otitis media (inflammation in the middle ear) [1]. *S. pneumoniae* causes 30-50% of all cases of acute otitis media [46]. Children aged 6 to 12 months have the highest incidence of acute otitis media [47,48]. Viral infections such as influenza increase the susceptibility to otitis media causing disfunctions that enhance the ability of colonizing bacteria to ascend and become trapped in the middle ear [8].

Besides otitis media, pneumococci can also cause sinusitis, which is usually preceded by viral infection of the upper respiratory tract and after leucocytes infiltrate and obstruct the sinuses [1].

1.7 Vaccines

Studies on pneumococcal disease prevention started in 1911 when a crude whole-cell pneumococcal vaccine was developed by Wright *et al.* [8]. In 1977, a 14-valent polysaccharide vaccine was licensed in the USA, but it was replaced by a 23-valent vaccine in 1983 [8].

The 23-valent PPV (polysaccharide pneumococcal vaccine) contains purified capsular polysaccharide antigens of *S. pneumoniae* 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 which are responsible for at least 85–95% of the IPD between adults and children in the United States [8].

However, PPV23 is a T-cell independent vaccine, inducing antibody production through interaction between polysaccharide antigens and B cells. Children have a poor B cell response, so vaccines that do not induce T-cell response cannot provide adequate protection. To face this problem,

pneumococcal conjugate vaccines were developed [2,8]. Conjugate vaccines produce a T-cell response, leading to higher immunogenicity [2].

The first pneumococcal conjugate vaccine was the PCV7 (heptavalent pneumococcal conjugate vaccine) which includes serotypes 4, 6B, 9V, 14, 18C, 19F and 23F [2,49], the most common serotypes in paediatric IPD before the PCV7 era in the USA, which accounted for 80% of infections [50]. PCV7 was registered for use in children in 2000 in the USA and in 2001 in Europe [51]. In 2001, it was introduced in Portugal, but only available through the private market [52].

The ten-valent vaccine (PCV10), which includes the PCV7 serotypes plus the additional serotypes 1, 5 and 7F became available for childhood vaccination in Portugal, in the private market since mid-2009 [53]. The more recent 13-valent vaccine (PCV13) which includes PCV10 serotypes with the addition of the serotypes 3, 6A, and 19A has been available in Portugal since early 2010. However, it was only included in the National Immunization Plan (NIP) in July 2015, for children born from January 2015 onwards [54].

In Portugal, DGS ("Direção Geral de Saúde") recommends the use of PCV13 in children aged <5 years with the administration of 3 doses at 2, 4 and 12 months of life. It also recommends the use of PCV13 and PPV23 in high-risk individuals [54,55].

Overall, after the introduction of PCVs there was a decrease in incidence of IPD and a reduction in the incidence of vaccine serotypes along with a decline in antimicrobial resistance by targeting the most resistant serotypes [53,56,57]. Moreover, a reduction in IPD was also observed in adults due to a herd effect [26,58,59].

The introduction of conjugate vaccines could lead to serotype replacement with serotypes not included in the vaccines [60]. Serotype replacement can be due to "unmasking" of non-vaccine types (NVT) that were present but in lower frequency [61] or it can happen by "capsular switching" since pneumococci are able to acquire foreign DNA by natural transformation and they can recombine with other strains [62].

After the introduction of PCV7, 19A emerged as an important serotype in America, Western Pacific regions and Europe [56,63]. However, after PCV13 introduction, the proportion of IPD cases associated with serotype 19A decreased in many countries, including Portugal [53]. It is also important to recognize the importance of population dynamics, since fluctuations in the prevalence of serotypes may occur naturally, even in the absence of vaccine pressure [64,65] and the properties of each serotype, because in some cases, vaccinal serotypes still persist, even in the presence of vaccination, such as serotype 3 that is associated with vaccine failure and was the most common serotype detected in cases of paediatric complicated pneumonia in Portugal from 2010-2015 [16].

In Europe, the non-PCV13 serotypes causing disease were mainly 12F, 10A, 24F, 22F and 15C accounting for 30% of IPD. Additional studies have also demonstrated that the non-PCV13 serotypes 15B, 15C, 15A, 23B, 24F and 35B are important causes of IPD, but until now none of these serotypes emerged significantly as important causes of IPD [63].

Regarding the serotype distribution of paediatric IPD in Portugal, before PCV13 introduction, the most common serotypes found in IPD were 1 (24%), 19A (20%), 7F (12%), 14 (7%) and 3 (6%). Serotype 1 was associated with empyema and 19A was more frequent in younger children. After the

introduction of PCV13, the incidence of IPD due to serotypes 1 and 19A decreased, with an increase in serotype 10A [53].

The current licensed pneumococcal polysaccharide conjugate vaccines have some limitations, namely high cost, which is worrying mainly for developing countries where the burden of disease is heaviest, and the fact that vaccines only provide protection against serotypes included in the formulation [26,62,66]. Serotypes to be included in conjugate vaccines represent another issue since data suggest different formulation based on geography, age group and type of disease [67]. Thus, there is an urgent demand for the development of a universal vaccine that is based on invariable and conserved antigens, common to all pneumococcal strains. Examples of current candidates are pneumolysin, PsaA, PspC and pilus proteins among others [62].

1.8 Typing Methods

There are several methods available for the typing of *S. pneumoniae*. Serotyping is based on the differences in capsular polysaccharide structure [31]. However, molecular typing is important for the comparison of populations in terms of genetic features and the study of strains involved in outbreaks [68].

1.8.1 Serotyping

The gold standard method for pneumococcal capsule serotyping is the Quellung reaction, which was first described by Neufeld in 1902. After an isolate is identified as a pneumococcus, it is sequentially tested with commercially available antisera pools until a positive reaction is observed [69].

There are two different nomenclature systems for the pneumococcal serotypes, the Danish system and the American system. The American system numbers serotypes sequentially, in the order they were identified. The Danish system groups serotypes by antigenic similarities, based on cross-reactions between different types. Types that serologically cross-react are assigned to a common serogroup. This system is more widely accepted [8,70,71]. It involves antisera developed in the Statens Serum Institute, Copenhagen [72] and it can identify 48 main serogroups. In some cases, those serogroups are further divided into serotypes, e.g. 19A, 19B, 19C or 19F.

The Quellung reaction is a technique that is laborious, requires specific expertise and uses antisera with high cost [69,73]. A latex agglutination test (Pneumotest-Latex) was developed for serotyping and its major advantage compared to the Quellung reaction is the simple procedure [73].

Several methods for “genetic serotyping” have also been developed. The most adopted are based in PCR amplification of specific serogroup or serotype genes [74]. However, those methods also have disadvantages such as high cost, they require specific expertise and only detect a certain number of serotypes [16].

Serotyping is important for the study of epidemiology and tracking of global or local spread of pneumococci, but it can also give information about vaccine efficacy and impact studies [75].

1.8.2 Molecular typing

Phenotypic methods, such as serotyping have a limited discriminatory power, hence molecular typing methods are important to obtain detailed epidemiological data. An example of a molecular typing method is multilocus sequence typing (MLST).

MLST is used for characterization of bacterial isolates based on the sequence of seven housekeeping genes. The MLST scheme generates sequences of approximately 450 bps nucleotides for internal fragments of the seven housekeeping genes for each isolate. Different sequences at each locus are assigned different allele numbers, and each strain is defined by an allelic profile. Each unique allelic profile is assigned a sequence type (ST) [76,77]. In the case of *S. pneumoniae*, the housekeeping genes used are [78]: *aroE* (shikimate dehydrogenase); *ddl* (D-alanine-D-alanine ligase); *gdh* (glucose-6-phosphate dehydrogenase); *gki* (glucose kinase); *recP* (transketolase); *spi* (signal peptidase I) and *xpt* (xanthine phosphoribosyltransferase).

MLST is a powerful tool that allows the study of genetic relatedness between clinical isolates, investigation of the origins of newly identified serotypes and tracking of the emergence and spread of multidrug-resistant isolates [76]. It is a highly discriminatory technique as it detects all the nucleotide polymorphisms within that gene fragment. Furthermore, any isolate characterized by MLST can be rapidly compared with the previous characterized isolates, through a single database available at http://pubmlst.org/ [79].

In the last decades, there has been a development of sequencing methods. Nowadays, WGS (whole genome sequencing) has become so inexpensive that it is a fast and economical way of obtaining information at multiple loci for determining MLST or other sequence types. WGS can provide more useful information, such as identification of serotype, antibiotic resistance profile, virulence determinants and can also help identify possible vaccine candidates [11,80]. In fact, in 2014 the USA Centers for Disease Control and Prevention (CDC) began using WGS as the serotype surveillance tool [24]. Furthermore, WGS has already been used to study the evolution of successful antimicrobial resistant clones and for detection and characterization of capsular switching events [74].

1.8.2.1 eBURST and PHYLOViZ

eBURST is an algorithm that is used for subdividing large MLST data sets into groups of related STs or clonal complexes (CCs) [77]. This algorithm uses the differences in the allelic profile of isolates assigning them to clonal complexes to construct an unrooted tree representation of the relationship of the isolates analysed [74].

The model for emergence of clonal complexes is that a founding genotype increases in number in the population, due to a random genetic drift or fitness advantage, becoming a predominant clone in the population. The clone gradually diversifies either by recombination or by mutation, originating a cluster of strains that are phylogenetically related. Clones that differ from the founder in only one of the seven MLST loci are called single locus variants (SLVs). Over time, SLVs continue to diversify giving rise to double-locus variants (DLVs) and triple-locus variants (TLVs) and so on [74,77,81].

Later, an improved version of this tool was developed, an algorithm called goeBURST which is

a globally optimized implementation of eBURST, that is able to find alternative patterns of descent [81].

PHILOViZ is a user-friendly software that allows the analysis of sequence-based typing methods, integrating information from several sources such as phenotypic and epidemiological data and interacting with many public databases. It has a goeBURST and Minimum Spanning Tree expansions for visualization of the possible relationships between isolates, freely available at http://www.phyloviz.net [74,82].

1.9 Antimicrobial resistance

β-lactams and macrolides are among the most frequently used antimicrobials to treat pneumococcal infections [83]. Unfortunately, the emergence and spread of antimicrobial resistance in *S. pneumoniae* is a worldwide problem [8]. Moreover, vaccine introduction can exert selective pressure on the prevalence of resistance, due to the possible emergence of non-vaccine serotypes associated with antimicrobial non-susceptibility, but it can also decrease the resistance, if vaccine serotypes associated with resistance decrease [17].

Prevalence of resistant pneumococci varies according to geographic region [29]. Therefore, surveillance studies are important to determine local data concerning antimicrobial resistance and to access the impact of conjugate vaccines in the prevalence of resistant isolates [17,63].

In the last decades, new classes of antimicrobials were developed. The overuse of antibiotics contributes to antimicrobial resistance in many ways such as selection of antibiotic-resistant pathogens that have an opportunity to spread due to elimination of susceptible organisms, competition between opportunistic and drug-resistant microorganisms after commensal microbiota is eliminated by the antibiotic and horizontal gene transfer (HGT) of antibiotic resistance genes to other bacteria [84]. Antimicrobial resistance is also associated with age, presence of comorbidities and attendance of day care centres [84].

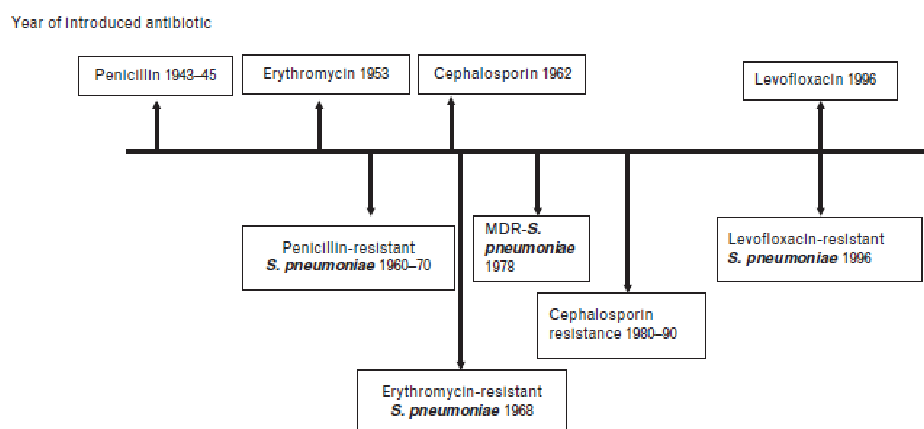

Figure 5 – Schematic illustration of a timeline of antimicrobial resistance in *S. pneumoniae*. Adapted from [84].

1.9.1 Definition of resistance

Antimicrobial resistance can be defined based on minimum inhibitory concentrations (MIC) using Etest strips or zone diameter measures of disk diffusion susceptibility testing, based on breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). According to those breakpoints, isolates can be classified as being susceptible, intermediately resistant or resistant [85].

The breakpoints for penicillin were first established in the late 1970s to prevent treatment failures in patients with pneumococcal meningitis due to penicillin non-susceptible strains [86]. However, in 2008 the CLSI modified the penicillin breakpoints to take into account the site of infection and route of administration (oral or parenteral) [86].

Table 1 – Former and current penicillin breakpoints established by CLSI for treatment of *S. pneumoniae* infections [86].

Period, syndrome and route of administration	Minimum inhibitory concentration (MIC) µg/mL		
	Susceptible	Intermediate	Resistant
Before January 2008	≤0.06	0.12-1	≥2
After January 2008 until nowadays			
Meningitis (parenteral)	≤0.06	None	≥0.12
Nonmeningitis (parenteral route)	≤2	4	≥8
Nonmeningitis (oral route)	≤0.06	0.12-1	≥2

1.9.2 Mechanisms of antibiotic resistance

1.9.2.1 β-lactams

β-lactam antibiotics such as penicillins, cephalosporins and carbapenems act by binding to penicillin-binding proteins (PBPs) which are responsible for cell wall biosynthesis [84]. Six PBPs have been described in *S. pneumoniae*: high molecular weight PBPs 1a, 1b, 2x, 2a and 2b and low molecular weight PBP 3 [28,87].

Resistance involves the alteration of target PBPs, decreasing their affinity to the antibiotic [29,88]. Altered PBPs are encoded by mosaic genes that have emerged due to intraspecies and interspecies gene transfer, specially from *Streptococcus mitis* and *Streptococcus oralis* [28,87]. Mutations in PBP2b and PBP2x result in β-lactam resistance and high-level resistance requires mutations in PBP1a [89,90].

1.9.2.2 Macrolides and lincosamides

Macrolides such as erythromycin are agents that bind to the 50S ribosomal subunit leading to the inhibition of bacterial protein synthesis [91]. Lincosamides and streptogramin have overlapping binding sites with macrolides, even though they have distinct chemical structures [91].

There are two main mechanisms of macrolide resistance in *S. pneumoniae*: target site modification or efflux of the antimicrobial agent [89]. The first mechanism is by acquisition of the *erm*(B) gene, which encodes a methylase that modifies the 23S rRNA at the post transcriptional level [89,91]. Ribosomal methylation confers resistance to macrolides, lincosamides and streptogramin B, which is named the MLS_B phenotype. The *erm*(B) expression can be inducible ($iMLS_B$ phenotype) when the presence of a macrolide induces resistance to lincosamides and streptogramin B or constitutive ($cMLS_B$ phenotype) when the gene is always expressed, therefore strains are always resistant to macrolides, lincosamides and streptogramin B [91].

The second mechanism involves the acquisition of *mef* genes which encode a proton dependant efflux pump. This mechanism confers resistance to 14- and 15-member macrolides (M phenotype) [84,89]. In Europe *mef*(A) is the most predominant, while in the USA, Asia and South Africa, *mef*(E) dominates [89].

1.9.2.3 Fluoroquinolones

Fluoroquinolones target DNA gyrase (encoded by *gyr*A and *gyr*B) and topoisomerase IV (encoded by *par*C and *par*E) which are involved in DNA supercoiling and chromosome segregation respectively, therefore inhibiting DNA synthesis [89,92].

In *S. pneumoniae*, fluoroquinolone resistance is mainly due to accumulation of mutations in the quinolone resistance determinant region (QRDR) of *gyrA* and/or *par*C [89,92]. Mutations involving subunits *par*E or *gyr*B are uncommon [92]. For most fluoroquinolones, such as levofloxacin and ciprofloxacin the primary target is topoisomerase IV leading to low level resistance, while mutations in a secondary target (*gyr*A) lead to high level resistance [84]. In addition to mutations, the over expression of drug efflux pumps such as PmrA has been implicated in the development of fluoroquinolone resistance [89].

1.9.2.4 Tetracyclines

Tetracycline binds to the 30S subunit of the bacterial ribosome preventing the binding of tRNA, inhibiting protein synthesis [87].

In *S. pneumoniae*, tetracycline resistance is achieved usually by ribosomal protection by the presence of the *tet*(M) and *tet*(O) genes [84,87]. The *tet*(M) gene is often carried by transposons of the Tn916-family which usually contain *erm* and/or *mef* genes, leading to macrolide and tetracycline resistant pneumococci [84].

1.9.2.5 Other antibiotics

Chloramphenicol inhibits protein synthesis by targeting peptidyl transferase, an enzyme involved in the peptide bond formation. In pneumococci, resistance is achieved by production of chloramphenicol acetyltransferase (encoded by gene *cat*) which transforms chloramphenicol in its derivatives that are not able to bind the 50S ribosomal subunit [87,93]. The *cat* gene is frequently carried in the family of transposons Tn916 [84].

Trimethoprim-sulfamethoxazole (SXT) interferes with folic acid production [87]. Pneumococci resistance to SXT is due to mutations in the gene that encodes dihydrofolate reductase, the target of the antibiotic [84,94].

Glycopeptides such as vancomycin prevent the transglycosylation and transpeptidation reactions that mediate the formation of the cell wall [87]. Vancomycin is used as a last-resort treatment in penicillin-resistant *S. pneumoniae* [87,93] and to date no vancomycin resistant pneumococci have been described.

Linezolid belongs to a new class of synthetic antibiotics. It binds to the 50S ribosomal subunit via interaction with the 23S rRNA, inhibiting protein synthesis. Resistance to linezolid is usually caused by point mutations in the 23S rRNA [95].

Rifampicin binds to DNA-dependant RNA polymerase inhibiting RNA synthesis [96]. Resistance to this antibiotic is due to a mutation in the gene encoding the beta subunit of RNA polymerase [93]. Usually, rifampicin is combined with one or more antibiotics, since resistance can develop rapidly [93].

1.9.3 Antimicrobial epidemiology

As mentioned above, antimicrobial resistance can vary according to geographic location. The first penicillin non-susceptible *S. pneumoniae* strain was found in Australia in 1967. By the late 1980s and 1990s, antibiotic-resistant pneumococci had already emerged all over the globe [29].

According to the report on antimicrobial resistance surveillance in Europe 2016 by the ECDC (European Centre for Disease Prevention and Control) for *S. pneumoniae*, penicillin non-susceptibility in Europe varied from 0.4% to 41.1%, being lower in Belgium, The Netherlands and Austria and higher in Romania, Cyprus, France and Spain [97]. Regarding macrolide resistance, between 2013-2016 it varied between 0% (Island) and 60% (Cyprus) [97].

The antimicrobial epidemiology is directly associated with antibiotic consumption, since countries with the highest resistance rates such as Cyprus, France and Romania also present the highest antibiotic consumption rates according to ECDC [98].

1.9.4 PMEN clones

Since the emergence of antimicrobial resistant pneumococci worldwide became a concern, there was a need for the identification of resistant clones and monitoring their spread.

Thus, in 1997 the Pneumococcal Molecular Epidemiology Network (PMEN) was established with the objective of standardizing the nomenclature and classification attributed to pneumococcal resistant clones worldwide [99]. To include clones in the PMEN (https://www.pneumogen.net/pmen, accessed April 2018), they should meet certain criteria such as: the clone must be widely distributed throughout a country or have spread internationally, it must be resistant to one or more antibiotics that are in wide clinical use or a global susceptible clone which is important in disease and data from the clone should be published or in press before approval by the network (among others).

Presently, there are 43 clones included in the network, with the first one to have been described being Spain^{23F}-1 (first described in Spain, with serotype 23F) [100].

Multidrug resistance (MDR) is defined as being resistant to at least three distinct classes of antibiotics. In the 1990s, MDR pneumococci emerged worldwide, which was associated with spread of successful clones such as Spain^{23F}-ST81, Spain^{6B}-ST90, and Taiwan^{19F}-ST236 [84]. Nowadays, a high proportion of MDR strains are associated to serotype 19A [84].

1.10 Genome of *S. pneumoniae*

As previously mentioned, the WGS approach has been applied to a variety of pathogenic bacteria with the aim of obtaining new strategies to deal with disease [101]. In the case of *S. pneumoniae,* the first complete genome sequence was presented in 2001 [101].

Pneumococci have a circular genome with a size of 2.1 megabases (Mb). There is a level of genomic variability between isolates, which emphasizes the genomic plasticity of this organism [102].

The genes that are not conserved in all members of the species are denominated “accessory” genome [102]. Previous data from genome sequencing suggested that 20-30% of pneumococcal DNA is part of the accessory genome [25].

S. pneumoniae is a naturally competent bacterium, therefore it is able to uptake exogenous DNA and integrate it in the genome through recombination [102,103]. This phenomenon introduces genomic variation which is important to adapt to the host environment and avoid the immune response [102].

Through recombination, strains can acquire genes which confer resistance to antibiotics and genes encoding a different capsular locus (capsular switching) [89,104]. Furthermore, HGT of mobile elements also contributes to the emergence of resistant strains [105]. Recombination and HGT occur not only between pneumococci, but also between other species present in the nasopharynx such as *S. mitis* and *S. oralis* [105].

1.11 Aims of the thesis

The main objectives of the work presented in this thesis were:

- The phenotypic characterization of *S. pneumoniae* responsible for invasive pneumococcal disease in children, isolated from 2015 to 2017 in Portugal, by serotyping and antimicrobial susceptibility testing.
- The molecular characterization of the isolates, by whole genome sequencing to extract information relative to the MLST profiles of the isolates.
- To compare data obtained with data from previous routine surveillance to evaluate the effect of the PCV13 vaccine in serotype distribution, levels of antimicrobial resistance and to evaluate the circulating population of *S. pneumoniae* after the introduction of the 13-valent pneumococcal conjugate vaccine in the NIP.

2. Materials and Methods

2.1 Bacterial isolates

The isolates included in this study were provided by the Portuguese Group for the Study of Streptococcal Infections and the Portuguese Study Group of Invasive Pneumococcal Disease of the Paediatric Infectious Disease Society, involving microbiology laboratories and paediatric departments of 61 hospitals throughout Portugal.

A case of IPD was defined as the isolation of *S. pneumoniae* from a normally sterile body site (only one isolate was considered per patient). However, in cases where culture was negative, we performed pneumococcal DNA detection by real time PCR targeting *lytA* and *wzg* in CSF and pleural fluid samples.

The strains were identified as *S. pneumoniae* through colony morphology, haemolysis in blood agar, optochin susceptibility and bile solubility tests. In cases where the identification was made using molecular methods, total DNA was extracted from patient samples using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions [16]. A multiplex PCR amplification of 2 human genes (human β-actin and RNaseP) was used as control for the quality of the purified DNA. For the identification of *S. pneumoniae*, singleplex rPCR of *lytA* and *wzg* genes was performed on the Rotor-Gene 6000 (Corbett Research, Cambridge, United Kingdom), using the Platinum quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Massachusetts, USA) [16].

The isolates characterized in the present study were recovered from patients under 18 years, in two epidemiological years, 2015-2016 and 2016-2017 in Portugal. Epidemiological years were defined as starting in week 26 of one year and ending in week 25 of the following year. Four different age groups were considered: infants aged less than 12 months, children aged 12–23 months, children aged from 2 to 4 years and children and adolescents from 5 to less than 18 years.

A subset of the samples had already been previously analysed. Furthermore, antimicrobial susceptibility testing and MLST were performed in cases where an isolate was available.

2.2 Serotyping

Bacteria were grown in Tryptone soya agar (TSA) (Oxoid, Hampshire, United Kingdom) supplemented with 5% sheep blood (Probiológica, Belas, Portugal) and incubated overnight at 35ºC with an atmosphere enriched in 5% CO_2. Serotyping was performed by the capsular reaction using the chessboard system [72] and specific sera (Statens Serum Institut, Copenhagen, Denmark).

Serotypes 15B and 15C; 25A and 38; 29 and 35B were included in three single groups due to the difficulty in distinguishing isolates positive for those serotypes.

Serotypes were divided in PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), PCV13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) and non-vaccine types (NVT).

In the cases where identification was made by molecular methods, serotyping was performed by rPCR, using 7 multiplex reactions that target the following serogroups/serotypes: 3, 7F/7A, and 19A; 1, 15B/15C, and 23F; 14, 18C, and 19F; 4, 6, and 9V/9A; 5, 11A/11D, and 16F; 8, 12F/12A/12B, and 22F/22A; and 15A, 23A, and 33F/33A/37 [16].

All bacteria were stored at -80ºC in Tryptic Soy Broth (TSB) (bioMérieux, Marcy-l'Étoile, France) supplemented with 15% glycerol.

2.3 Antimicrobial susceptibility testing

To study the susceptibility to antimicrobials, Mueller-Hinton agar (Oxoid, Hampshire, United Kingdom) supplemented with 5% sheep blood (Probiológica, Belas, Portugal) plates were inoculated using a 0.5 McFarland colony suspension, and bacteria were incubated overnight at 35ºC with the antibiotic with an atmosphere enriched in 5% CO_2.

Antimicrobial susceptibility testing was performed using Etest strips (bioMérieux, Marcy-l'Étoile, France) to determine the MIC to penicillin, cefotaxime, ceftriaxone, meropenem and levofloxacin (the last one was only performed when the isolate was resistant to norfloxacin using the Kirby-Bauer disk diffusion technique). The recommended breakpoints for interpretation of MIC values for penicillin were changed in 2008, however in this case, the CLSI guidelines from 2007 were used to interpret the MIC values to allow comparison with the previous studies.

Susceptibility to levofloxacin, norfloxacin, erythromycin, clindamycin, telithromycin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, vancomycin, linezolid and rifampicin was determined by the Kirby-Bauer disk diffusion technique using commercial disks (Oxoid, Hampshire, United Kindgom), according to the CLSI recommendations [106]. Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin, where both discs were placed in proximity. The MLS_B phenotype is defined by resistance to both erythromycin and clindamycin (resistance to macrolides, lincosamides and streptogramin B) while the M phenotype is defined by resistance to erythromycin. The reference strain *S. pneumoniae* ATCC 49619 was used as control.

2.4 DNA extraction and sequencing

Bacteria were grown in BHI (Brain Heart Infusion) broth (Becton Dickinson, New Jersey, USA) overnight at a 37ºC bath until they reached an optical density between 0.7-0.9. The optical density was measured using the WPA CO 8000 Biowave Cell Density Meter (Biochrom Ltd., Cambridge, United Kingdom).

Pneumococcal DNA was extracted using the PureLink Genomic DNA Mini Kit (ThermoFisher Scientific, Massachusetts, USA), according to the manufacturer's instructions. The final elution volume was 50 µl. The purity of the DNA was evaluated using Nanodrop 2000 (ThermoFisher Scientific, Massachusetts, USA).

To check the quality of the pneumococcal DNA, 5 µl of sample with 2 µl of loading buffer were run in a 1% agarose gel along with a 1 Kb plus DNA ladder during approximately 45 minutes at 100 V. Gels were stained with ethidium bromide and were photographed using the AlphaImager system.

Qubit (Invitrogen by ThermoFisher Scientific, Massachusetts, USA) was used to check the DNA concentration. Finally, dilutions were performed in Tris-HCl 10mM pH 8 to obtain a final concentration of 10 ng/ µl and samples were sent for sequencing.

Whole genome sequencing was performed at Instituto Gulbenkian de Ciência, Gene Express Unit (Oeiras, Portugal). Whole genome sequencing libraries were prepared using paired-end Nextera

XT DNA Library Prep Kit, Index Kit v2 (Illumina, San Diego, CA, USA) and sequenced on Illumina NextSeq 500 system (Illumina) using NextSeq 500/550 Mid-Output v2 Kit (300 cycles).

The quality of the 151 bps paired-end reads obtained was assessed with INNUca pipeline (https://github.com/B-UMMI/INNUca, accessed June 2018), which also performs de novo assembly and curates the bacterial genomes. INNUca v3.1 was run using Docker image "ummidock/innuca:3.1" (https://hub.docker.com/r/ummidock/innuca/, accessed June 2018) providing Nextera XT adapter sequences for adapters removal using a predicted genome size of 2.1 Mb.

Briefly, the quality of the reads was checked with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed June 2018) and they were cleaned using Trimmomatic [107]. De novo assembly was performed using SPAdes [108] and subsequently, contigs were polished using Pilon [109]. The MLST type was determined for the final draft assembly through MLST software (https://github.com/tseemann/mlst, accessed June 2018), more specifically, alleles and sequence types were assigned using the pneumococcal database available at http://pubmlst.org/spneumoniae/.

When new alleles were found, they were submitted to the pneumococcal database curator for number attribution. Lineages (relationships between sequence types) were assigned using the goeBURST algorithm and the online pneumococcal MLST database (http://pubmlst.org/spneumoniae/). Results were visualized at PHYLOViZ [82]. Clonal complexes were defined at the single-locus-variant (SLV) level.

2.5 Statistical Analysis

Simpson's Index of Diversity (SID) was used to evaluate the population diversity and the respective 95% confidence intervals (CI95%) were calculated. This index measures the discriminatory ability of typing systems, representing the probability of two strains sampled randomly from a population belonging to different typing groups [110].

Adjusted Wallace (AW) coefficients were calculated in order to compare two sets of partitions, using a tool available at http://www.comparingpartitions.info. This coefficient compares the congruence between typing methods, allowing an estimation of how much new information is obtained from using another typing method [110,111].

The Odds ratio (OR) was calculated using the Fisher method implemented in the Epitools package for the R language to evaluate possible associations between variables and the obtained p-values were corrected using the false discovery rate (FDR) correction for multiple testing [112].

The Cochran-Armitage test was used for trends. A p-value < 0.05 was considered significant for all tests.

3. Results

3.1 Isolate collection

A total of 134 cases of paediatric invasive pneumococcal disease were reported between July 2015 and June 2017, with 91 isolates (67.9%) and 43 samples (32.1%). Regarding the source of the isolates, 70 were recovered from blood (76.9%), 13 from CSF (14.3%), 6 from pleural fluid (6.6%), 1 from peritoneal fluid (1.1%) and 1 from synovial fluid (1.1%). Among the cases detected by molecular methods, the majority were isolated from pleural fluid (n=40) and 3 were from CSF. The number of cases per epidemiological year remained constant with 64 from 2015-2016 and 70 from 2016-2017.

Among this collection, 27.6% (n=37) of the cases were from infants below 12 months of age, 15.7% (n=21) from children between 12 and 23 months, 30.6% (n=41) from children aged 2-4 years and 26.1% (n=35) from individuals aged 5-17 years.

Furthermore, 55.2% (n=74) of the cases were recovered from male patients and 44.0% (n=59) from female patients. No information regarding gender was available for one of the isolates.

3.2 Serotyping

Among this collection, 25 different capsular types were detected (SID=0.950; CI95%: 0.936-0.965). The most frequent serotype was serotype 3 (n=39), representing 29.1% of all cases.

When a serotype could not be determined or when the isolate was non-typable, the case was considered caused by a non-vaccine type (NVT).

Non-vaccine types represented a significant proportion of cases (50.7%, n=68), of which 8 and 10A (n=10, 7.5% each), 15B/C (n=6, 4.5%), 22F (n=5, 3.7% each), 11A, 29/35B and 33F (n=4, 3.0% each) were the most frequent (Figure 6). Furthermore, 6 of the cases were non-typable.

Serotypes included in the PCV7 and PCV13 constituted respectively 12.7% and 49.3% of the isolates and samples responsible for invasive pneumococcal disease in patients aged <18 years, in Portugal during the study period.

Some serotypes included in the vaccines were absent from this collection (6A, 7F and 18C) when compared with previous time period (2012-2015). Furthermore, serotype 14 which is included in PCV7, was present in 5 cases in 2015-2016 but absent from 2016-2017.

Table 2 – Serotypes responsible for invasive pneumococcal disease in Portugal from July 2015 to June 2017 in patients <18 years.

Serotype	Number of isolates and samples	
	2015-2016	2016-2017
1	1	3
3	16	23
6B	2	2
14	5	0
19A	1	5
19F	2	3
23F	2	1
NVT[(a)]	35	33
Total	64	70

[(a)] NVT: non-vaccine type

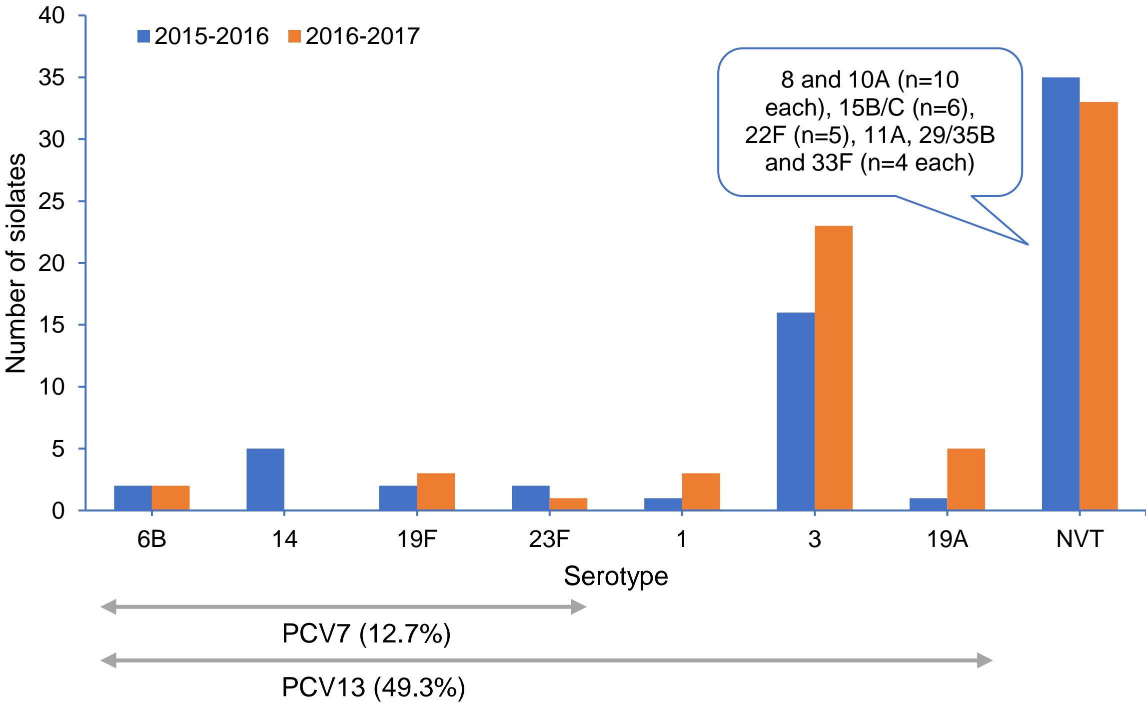

Figure 6 – Graphic representation of serotype distribution of *S. pneumoniae* isolates and patient samples causing children IPD in Portugal from July 2015 to June 2017.

Serotypes included in PCV13 are indicated by an arrow and percentage of the number of isolates and samples expressing the serotypes included in the PCV13 vaccine is indicated. Among serotypes included in PCV13, serotypes 4, 5, 6A, 7F, 9V and 18C were not detected in this collection.

To evaluate serotype diversity in each age group, the Simpson's Index of diversity was calculated. In infants aged <1 year and children between 1 and 2 years old, serotype diversity was high (SID=0.951; CI95%: 0.925-0.976 and SID=0.952; CI95%: 0.907-0.998 respectively). In the older age groups, which include children aged 2 to 4 years and from 5 to 17 years old, the SID values were 0.798 (CI95%: 0.677-0.918) and 0.805 (CI95%: 0.681-0.930), respectively. When comparing the SID values from infants <1 year and children aged 2-4 years, it can be concluded that serotype diversity was higher in the first age group.

The same analysis was done to evaluate serotype diversity per epidemiological year of study. It was concluded that serotype diversity was similar in the two epidemiological years of study, since the SID 95% confidence intervals overlapped, demonstrating no major changes between 2015 and 2017.

Table 3 – Simpson's index of diversity and respective 95% confidence intervals for serotype distribution per epidemiological year of study.

Epidemiological Year	SID (CI95%)
2015-2016	0.912 [0.868-0.957]
2016-2017	0.874 [0.810-0.938]

It should be taken into account that not all serotypes have the same relevance when considering age groups. Serotype 3 represented only 5.4% and 19.0% of the isolates and patient samples responsible for infection in infants <1 year of age and children from 1 to under 2 years old, respectively. Meanwhile, in the older age groups, serotype 3 alone included 43.9% (children aged 2 to 4 years old) and 42.9% (individuals from 5 to 17 years) of all cases.

However, when grouping the isolates and samples per age group, serotype 3 presented a statistically significant association with children from 2 to 4 years only before FDR correction ($p=0.022$).

Regarding isolation source, there was observation of a significant association between serotype 3 and pleural fluid, with 33 out of 39 isolates and samples (84.6%) from serotype 3 being collected from pleural fluid ($p < 0.001$ after FDR correction).

To evaluate possible trends in serotype distribution over time, the Cochran-Armitage test was used. When considering serotype variation from 2010 (year in which PCV13 was introduced in the private market in Portugal) to 2017 [53, unpublished data], only serotypes 1, 3, 7F and 19A showed a trend after FDR correction. More specifically, serotype 1 ($p < 0.001$), 7F ($p=0.008$) and 19A ($p=0.015$) decreased in incidence, while serotype 3 ($p < 0.001$) increased.

3.3 Antimicrobial susceptibility testing

Susceptibility to antimicrobials was tested among the 91 available isolates and it is resumed in Table 4 and Figure 7. No resistance to cefotaxime, ceftriaxone, meropenem, levofloxacin, telithromycin, vancomycin, linezolid and rifampicin was detected.

Table 4 – Antimicrobial resistance of *S. pneumoniae* isolates responsible for invasive pneumococcal disease in patients <18 years old, in Portugal from July 2015 to June 2017.

Antibiotic	Number of resistant isolates (%)			
	0-11 months (n=35)	12-23 months (n=18)	2-4 years (n=20)	5-17 years (n=18)
PEN[b]	5 (14.3)	3 (16.7)	3 (15.0)	1 (5.6)
MIC_{50}	0.006	0.016	0.006	0.006
MIC_{90}	0.094	0.25	0.016	0.012
CTX	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MIC_{50}	0.012	0.016	0.008	0.008
MIC_{90}	0.047	0.125	0.032	0.012
CRO	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MIC_{50}	0.012	0.023	0.008	0.008
MIC_{90}	0.047	0.19	0.023	0.016
MP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MIC_{50}	0.006	0.006	0.003	0.003
MIC_{90}	0.016	0.125	0.006	0.006
LEVO	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
NOR	1 (2.9)	0 (0.0)	1 (5.0)	1 (5.6)
ERY	5 (14.3)	6 (33.3)	3 (15.0)	1 (5.6)
CLI	4 (11.4)	5 (27.8)	3 (15.0)	1 (5.6)
TEL	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TET	2 (5.7)	4 (22.2)	1 (5.0)	0 (0.0)
CHL	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)
SXT	1 (2.9)	4 (22.2)	3 (15.0)	0 (0.0)
VAN	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
LNZ	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
RIF	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

PEN-penicillin; CTX-cefotaxime; CRO-ceftriaxone; MP-meropenem; LEV-levofloxacin; MIC-Minimal inhibitory concentration; NOR-norfloxacin; ERY-erythromycin; CLI-clindamycin; TEL-telithromycin; TET-tetracycline; CHL-chloramphenicol; SXT- trimethoprim-sulfamethoxazole; VA-vancomycin; LNZ-linezolid; RIF- rifampicin
MIC_{50} and MIC_{90}: minimal inhibitory concentration necessary to inhibit 50% and 90% of the strains, respectively.

[b]Number and percentage of penicillin non-susceptible isolates

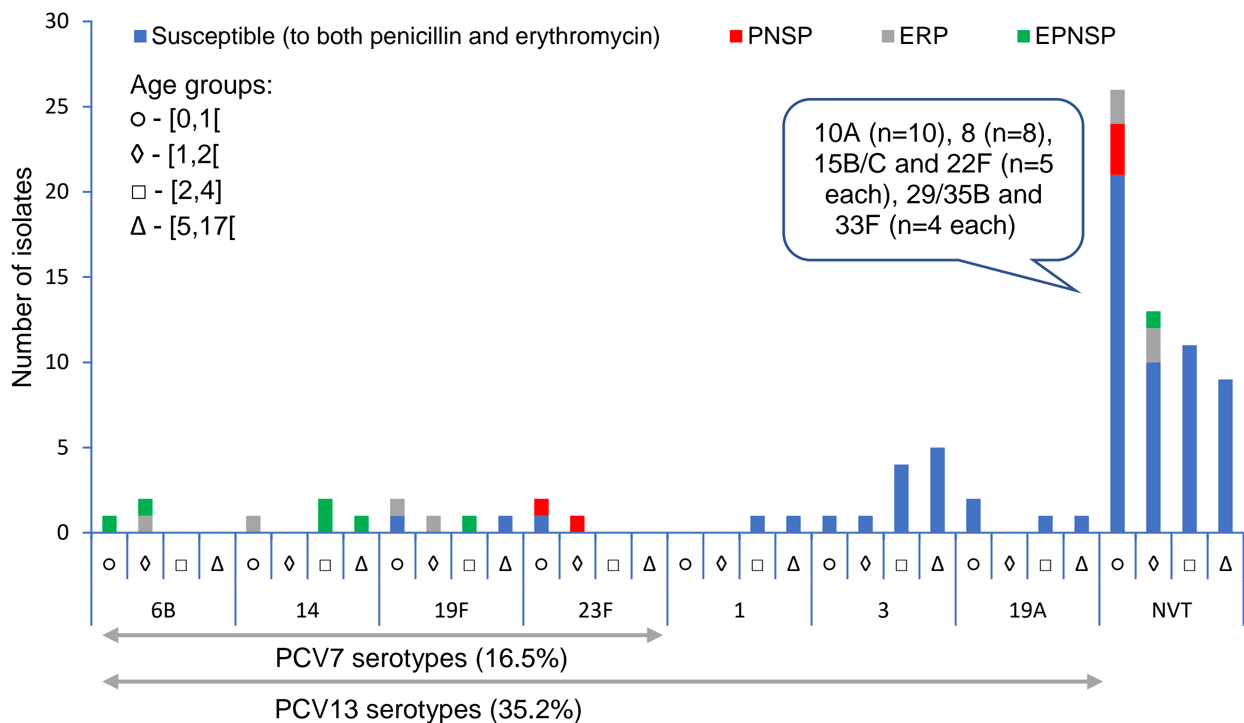

Figure 7 – Antimicrobial resistance of *S. pneumoniae* serotypes responsible for invasive pneumococcal disease in patients aged < 18 years, in Portugal from July 2015 to June 2017.

PNSP: penicillin non-susceptible isolates; ERP: erythromycin resistant isolates; EPNSP: isolates presenting both erythromycin resistance and penicillin non-susceptibility; NVT- non-vaccine types.

Overall, there were 12/91 (13.2%) isolates non-susceptible to penicillin, of which 7/12 (58.3%) were also resistant to erythromycin (EPNSP). If the current CLSI breakpoints for parenteral penicillin were considered [85], only 2/13 isolates from CSF would have been considered resistant to penicillin.

Resistance to erythromycin was found in 15/91 isolates (16.5%), of which 13 isolates (86.7%) expressed the $cMLS_B$ phenotype and 2/15 of the isolates (13.3%) the M phenotype. Resistance to norfloxacin was found in 3 isolates (3.3%) and resistance to tetracycline in 7 isolates (7.7%). All the tetracycline resistant isolates were resistant to both erythromycin and clindamycin ($cMLS_B$ phenotype).

Together, serotypes 6B and 14 contribute significantly to EPNSP (5/7; 58.3%) and serotypes 6B,14 and 19F to resistance to erythromycin (10/15; 66.7%). PCV13 serotypes represent 8/12 (66.7%), 10/15 (66.7%) and 6/7 (85.7%) of the penicillin non-susceptible, erythromycin resistant and EPNSP isolates respectively.

When evaluating the association between serotype and penicillin non-susceptibility, no significant associations were observed. However, serotypes 6B, 14 and 23F presented a significant p-value before FDR correction (p=0.045; p=0.007; p=0.045 respectively). Regarding erythromycin resistance, there was a significant association with serotypes 6B (p=0.048 after FDR correction) and 14 (p=0.013 after FDR correction).

Furthermore, no significant associations were observed between penicillin non-susceptibility or erythromycin resistance and any of the age groups.

Correlation between serotype and antimicrobial resistance was evaluated calculating the

Adjusted Wallace coefficients. The AW coefficient between serotype and penicillin non-susceptibility was 0.653 (CI95%: 0.496-0.809) and the AW between serotype and erythromycin resistance was 0.531 (CI95%: 0.261-0.800).

3.4 Molecular characterization

The molecular characterization by MLST of the isolates revealed 47 different STs (SID=0.971; CI95%: 0.957-0.985) that grouped into 23 CCs (SID=0.908; CI95%: 0.875-0.940), after goeBURST analysis using all STs deposited in the database (http://pubmlst.org/spneumoniae/). The most frequent STs were ST180 (n=9, 10.5%), ST53 (n=8, 9.3%), ST97 (n=6, 7.0%), ST393 and ST433 (n=4, 4.7% each), ST198 and ST994 (n=3, 3.5% each), ST72, ST143, ST306, ST338, ST558, ST717, ST1262, ST4083 and ST8126 (n=2, 2.3% each) which together accounted for more than half of all isolates analysed (64.0%).

Four new STs were detected and submitted to the *S. pneumoniae* MLST database (http://pubmlst.org/spneumoniae/). In two cases, there were new allele sequences, of which one was in *rec*P (427) and one in *xpt* (841) originating ST13864 and ST13863 respectively, while the remaining two cases were new allelic combinations, ST13669 and ST13811.

However, it should be mentioned that 3 isolates could not be analysed because they didn't grow in liquid media and 1 isolate with a new ST was sent to the curator of the MLST database for number attribution, but the ST number still hasn't been attributed yet.

The most prevalent CCs were CC156 (n=19, 22.1%), CC63 (n=12, 13.9%), CC180 and CC460 (n=9, 10.5 % each), CC393, CC433 and CC1262 (n=4, 4.7% each), which together represent 70.9% (n=61) of all isolates studied. Table 5 presents the serotypes of the STs in the CCs and figure 8 illustrates the STs and the main CCs that were identified.

Table 5 – Serotypes of the STs found among the 23 CCs identified by goeBURST.

CC (n)	ST	Total	Dominant serotype (n)	Other serotypes
CC156 (19)	72	2	24F (2)	
	143	2	14 (2)	-
	338	2	23A (1), 23F (1)	-
	8126	2	23F (2)	-
	66	1	9N (1)	-
	138	1	6B (1)	-
	162	1	24F (1)	-
	177	1	19F (1)	-
	271	1	19F (1)	-
	469	1	19F (1)	-
	1877	1	21 (1)	-
	2372	1	23B (1)	-
	4948	1	11A (1)	-
	13863	1	19F (1)	-
	13864	1	19F (1)	-
CC63 (12)	53	8	8 (8)	-
	62	1	11A (1)	-
	445	1	22F (1)	-
	673	1	33F (1)	-
	1012	1	33F (1)	-
CC180 (9)	180	9	3 (7)	10A (1) ,11A (1)
CC460 (9)	97	6	10A (4)	14 (1), 19A (1)
	461	1	10A (1)	-
	1551	1	10A (1)	-
	1635	1	35F (1)	-
CC393 (4)	393	4	25A/38 (3)	NT (1)
CC433 (4)	433	4	22F (4)	-
CC1262 (4)	1262	2	15B/C (2)	-
	8711	1	15B/C (1)	-
	9975	1	15B/C (1)	-
CC198 (3)	198	3	29/35B (3)	-
CC994 (3)	994	3	19A (3)	-
CC30 (2)	30	1	3 (1)	-
	12069	1	10A (1)	-
CC306 (2)	306	2	1 (2)	-
CC558 (2)	558	2	29/35B (1), NT (1)	-
CC717 (2)	717	2	33F (2)	-
CC1368 (2)	4083	2	10A (1), 34 (1)	-
CC15 (1)	9	1	14 (1)	-
CC199 (1)	199	1	15B/C (1)	-
CC315 (1)	13669	1	6B (1)	-
CC378 (1)	232	1	3 (1)	-
CC439 (1)	9579	1	23B (1)	-
CC896 (1)	896	1	15A (1)	-
CC1046 (1)	1046	1	34 (1)	-
CC1475 (1)	1475	1	27 (1)	-
CC13811 (1)	13811	1	16F (1)	-

Figure 8 – Illustration of all the sequence types identified and indication of the main CCs (CC156 (n=19), CC63 (n=12), CC180 and CC460 (n=9 each), CC393, CC433 and CC1262 (n=4 each)), which together account for 70.9% of all isolates. STs united by lines are SLVs and ST896 marked in blue is a singleton. The representation was made using PHYLOViZ [82].

The Adjusted Wallace coefficient between CC and PCV13 serotypes was 0.524 (CI95%: 0.239-0.809). Furthermore, the major CC (CC156) included PCV13 serotypes (n=11, 57.9%), such as 6B, 14, 19A, 19F and 23F. The second most prevalent CC (CC63) was composed of non-PCV13 serotypes, more specifically 8, 11A, 22F and 33F.

To evaluate the genetic diversity in each serotype, Simpson's index of diversity was calculated (in the case of serotypes with a number of isolates ≥ 3). It was observed that serotypes 14, 19F, 10A, 11A, 15B/C and 33F presented high genetic diversity (SID > 0.8), while serotypes 8 and 25A/38 had low diversity (SID < 0.3). In the case of serotypes 19F and 15B/C, the diversity in ST was not reflected in CC, since the STs mainly belong to the same CC.

Fig. 9 shows the STs expressing serotypes included in PCV13 and Fig. 10 the STs expressing each of the most frequent serotypes not included in PCV13.

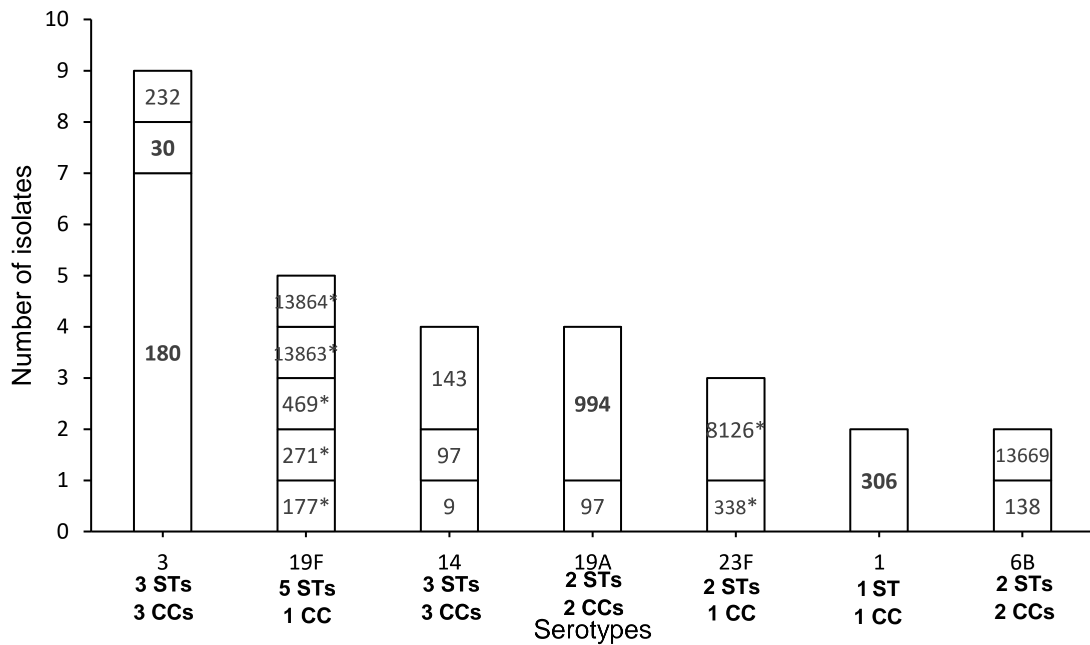

Figure 9 – Distribution of STs according to serotype of the isolates expressing serotypes included in PCV13 found among our collection. Vaccine serotypes that are not indicated were not detected in the analysed collection. The STs that were considered by goeBURST as founders of a CC are in bold. Marked with "*" are STs belonging to the same CC in each serotype.

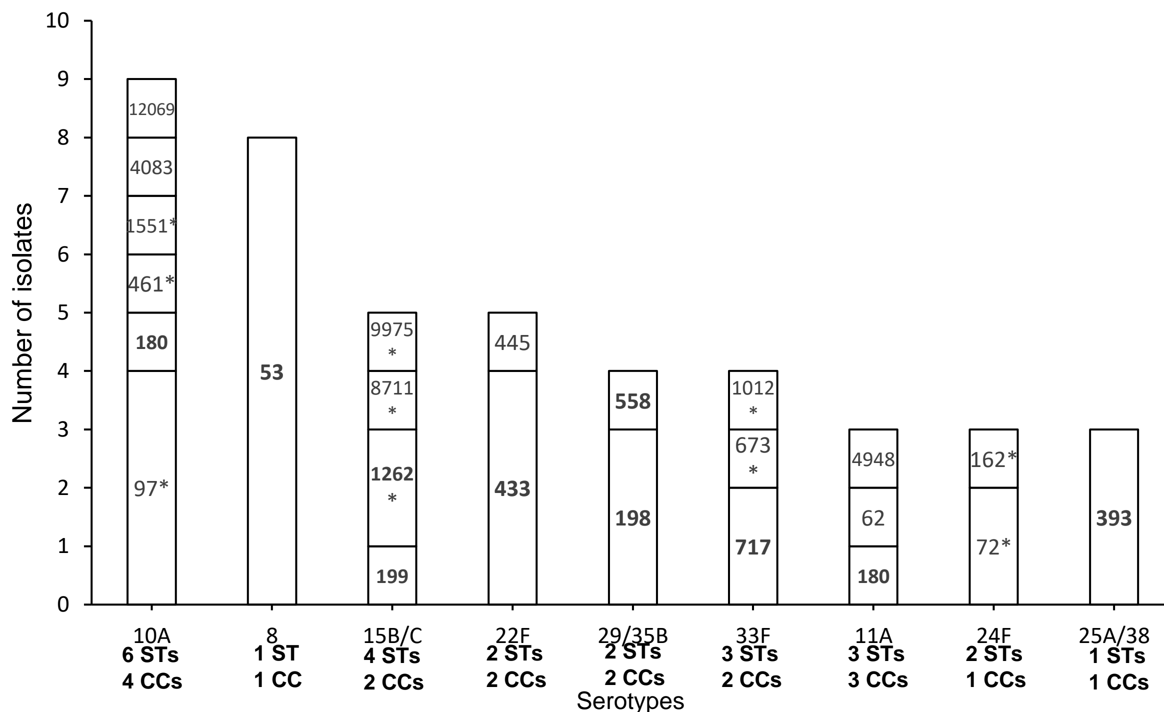

Figure 10 – Distribution of STs according to serotype of the isolates expressing the most frequent serotypes not included in PCV13 found among our collection. The STs that were considered by goeBURST as founders of a CC are in bold. Marked with "*" are STs belonging to the same CC in each serotype.

Regarding the PMEN clones, isolates related to 12 of the 43 recognized clones were found (England^{14}-ST9, Netherlands^{8}-ST53, Spain^{9V}-ST156, Portugal^{19F}-ST177, Netherlands^{3}-ST180, Netherlands^{15B}-ST199, Taiwan^{19F}-ST236, Hungary^{19A}-ST268, Sweden^{1}-ST306, Poland^{6B}-ST315, Columbia^{23F}-ST338 and Utah^{35B}-ST377). Among them, 19 isolates were representatives with the same serotype and ST as the PMEN clones but with a different antimicrobial profile. Furthermore, 5 SLVs and 4 DLVs of PMEN clones were identified.

The Adjusted Wallace coefficient between ST and serotype was moderately high (AW=0.703, CI95%: 0.549-0.857), indicating that there is a good correspondence between ST and serotype. However, some STs presented more than a single serotype as shown in table 5, which is consistent with the low AW between serotype and ST (AW=0.428, CI95%: 0.314-0.542).

Furthermore, there were STs to which some serotypes hadn't been previously associated. Table 6 illustrates the main differences found in serotypes between the analysed isolates and the *S. pneumoniae* MLST database (https://pubmlst.org/spneumoniae/).

Table 6 – Differences between ST and serotype association found in the study and MLST public database.

Sequence Type (ST)	Serotype in analysed collection	Serotype in MLST database
ST30	3	16F, 19A, 22F, 23F
ST97	14	19A
ST180	10A, 11A	6A, 6B, 15A, 19F, 37, NT
ST445	22F	33A, 33F
ST469	19F	6B
ST896	15A	6B
ST4083	10A	NT
ST4948	11A	14
ST12069	10A	16F

No significant statistical associations were detected between ST and penicillin non-susceptibility or erythromycin resistance.

The Cochran-Armitage test was used to evaluate ST variation from 2010 to 2017 (unpublished data). It was observed that ST53 (p=0.042), ST180 (p=0.033) and ST306 (p=0.044) which are associated to serotypes 8, 3 and 1, respectively, showed a trend after FDR correction. More specifically, ST53 and ST180 increased in prevalence, while ST306 decreased.

Additionally, some STs presented a significant p-value before FDR correction: ST97 (p=0.006), ST191 (p=0.013), ST276 (p=0.015) and ST338 (p=0.033) that are associated to serotypes 10A, 7F, 19A and 23F respectively. More specifically, ST97 increased in prevalence and ST191, ST276 and ST338 decreased overtime.

4. Discussion

During this work, *S. pneumoniae* responsible for pneumococcal invasive disease in children, recovered in Portugal from 2015 to 2017 were characterized. Firstly, they were characterized by phenotypic methods, namely by serotyping and antimicrobial susceptibility testing. Afterwards, DNA sequencing was used to extract information regarding the MLST profile of the isolates, determining their sequence types and the clonal lineages were then determined by the use of PHYLOViZ. Finally, the results from the 2015-2017 period were compared with data available from previous years of routine surveillance to understand and evaluate the effect of the vaccine in serotype distribution, antimicrobial resistance and the dynamics of the genetic lineages.

It is widely known that after the introduction of conjugate vaccines, the overall incidence of IPD decreased and the incidence of disease caused by serotypes included in the conjugate vaccines also decreased. However, some vaccinal serotypes still persist, more specifically, those associated with antibiotic resistance and serotype 3, which was already associated with infections in age-appropriately vaccinated children, representing vaccines failures [16,53,63,113]. In some countries, NVTs started to emerge, however in Portugal, none has still emerged as major cause of IPD.

PCV7 was introduced in Portugal in 2001, thought the private market [52]. Surveillance studies revealed that after seven years of PCV7 use, the overall proportion of IPD due to vaccine serotypes declined and serotypes 1, 7F and 19A were the major causes of children IPD [56]. A herd effect was also observed in the adult population [114]. In 2010, PCV13 was introduced in Portugal, also only through the private market. A study of *S. pneumoniae* IPD revealed that between 2008-2012, the incidence of disease continued to decrease, due to a reduction in the number of cases due to PCV13 serotypes, mainly due to serotypes 1 and 19A [53]. Nonetheless, PCV13 serotypes still remained an important source of IPD, being responsible for 63.2% of cases in 2011-2012 [53]. In Portugal, PCV13 was introduced in the NIP in 2015 [54]. Thus, a higher vaccinal coverage may lead to significant changes in the population of *S. pneumoniae.*

4.1 Population in study

When considering the population in study for this thesis, the majority of cases were within the age groups of children aged 2 to 4 years old (30.6%) and infants below 12 months of age (27.6%). Most of the patients were male (55.2%). A previous study in Madrid also reported similar results, with the majority of IPD cases happening between 0 and 5 years old [115].

4.2 Serotyping

During the study period, the most frequent serotypes causing disease were serotypes 3 (n=39, 29.1%), 8 and 10A (n=10, 7.5% each) and 19A and 15B/C (n=6, 4.5% each), together accounting for 52.9% of IPD. In the 3-year period prior to the introduction of PCV13 in the NIP (2012-2015), the most frequent serotypes were 3, 14, 1, 7F, 19A, 6B, 15B/C, 24F, 10A and 12B. The similarities between the two time periods are the persistence of serotype 3 as the major cause of IPD and the presence of vaccine serotype 19A and NVTs 10A and 15B/C among the most frequent serotypes. As for differences,

PCV13 serotypes 14, 1, and 6B were also present in 2015-2017, however in lower prevalence, not being among the most frequent serotypes.

Certain serotypes that were expressed in Portugal between 2012-2015 were absent in 2015-2017, such as vaccine serotypes 6A, 7F and 18C and NVT 12B. The cases of serotype 7F and 12B are the most surprising, since both serotypes were among the most frequent causes of IPD in 2012-2015, but were absent in 2015-2017. The case of serotype 7F could be related to the vaccine effect, since this serotype is included in PCV13. Therefore, a decrease and even absence of cases due to this serotype are within the possible effects of vaccination. As for NVT 12B, it may be a serotype that had no success in dissemination. It should also be mentioned, that vaccine serotypes 1 and 19A, which were also among the most frequent serotypes in 2012-2015, decreased in incidence when compared to 2015-2017, from 9.9% to 3.0% and from 5.6% to 4.5%, respectively. The decrease in vaccine serotypes should be associated with the vaccination effect, however it should always be taken into account that the natural fluctuations of serotypes can also play a part in the prevalence of serotypes. Additionally, serotype 14 which is included in PCV7, was present in 5 cases in 2015-2016 but absent from 2016-2017. Routine surveillance should be performed in the future to ascertain if this trend is permanent or if this was just a temporary decrease.

The number of IPD cases detected solely by molecular methods in 2015-2017 (n=43/134, 32.1%) was higher when compared to 2012-2015 (n=47/259, 18.1%) (unpublished data). Serotyping by molecular methods lead to an improvement in the identification of serotypes responsible for complicated pneumonias in which *S. pneumoniae* was identified using molecular methods in pleural fluid samples. Serotype 3, the most frequent serotype detected between 2015-2017 (figure 6 and table 2) was mainly identified in pleural fluid samples by molecular methods (n=33/39, 84.6%). This serotype was previously associated with cases of vaccine failure [16,116], which emphasizes the importance of using molecular methods which in this case contributed to the identification of a significant proportion of paediatric IPD cases.

PCV7 and PCV13 serotypes represented 12.7% and 49.3% of IPD cases in 2015-2017, respectively. When comparing with the values from 2012-2015 (21.6% for PCV7 and 57.8% for PCV13), it can be observed that the number of cases due to vaccine serotypes decreased from 2012-2015 to 2015-2017. However, even though the number of cases attributed to vaccine types has decreased, they still remain important causes of paediatric IPD.

Cases of IPD related with PCV7 types were mostly due to serotypes 6B, 14, 19F and 23F that are associated with antibiotic resistance [28,29] which could explain why they were still responsible for a fraction of IPD. Other vaccinal serotypes such as 6A, 7F and 18C which were expressed by a proportion of isolates in 2012-2015 were absent in 2015-2017. A study from England and Wales reported a decrease in serotypes 6A and 7F, however they were associated with a small number of IPD cases [117]. Furthermore, in a study from Japan, serotypes 6A, 7F and 18C were present, but in a very small number of cases (3 cases at most in each serotype) [118].

In the case of PCV13 types, the most problematic was serotype 3, being the major cause of IPD in Portugal both in 2012-2015 and 2015-2017 (after PCV13 was introduced in the NIP). One of the factors that may have contributed to the persistence of this serotype might be vaccine uptake. Vaccine

uptake in Portugal around 2008 reached 75% [53], but declined to between 58% and 65% in 2009-2014 (IMS and INE data). Additionally, there could be IPD cases in children that were not vaccinated, because when they were at the age when the vaccine is administered, PCV13 wasn't still included in the NIP. Furthermore, some studies suggested that since the synthesis of serotype 3 capsule is different from other serotypes and the polysaccharide is not covalently linked to the peptidoglycan, it can be released and potentially reduce opsonophagocytosis and interfere with antibody-mediated clearance, which may result in a higher anti-capsular antibody concentration being required for protection against serotype 3 [16,116].

Among NVTs, serotypes 10A and 15B/C remained in the most frequent serotypes both in 2012-2015 and 2015-2017. In fact, between 2008-2012, serotype 10A was already reported to have increased significantly in Portugal [53]. A portuguese study on carriage revealed that serotype 15B/C was among the most frequently carried NVTs in 2009-2010, therefore this could be related to the prevalence of this serotype. However, serotype 10A was not associated with carriage [42] but since there is no carriage data from the same time period (2015-2017), it is possible that 10A is actually an important NVT in carriage in Portugal. In the same study, serotypes 3 and 19A were also among the most frequently carried serotypes [42], which could also be related to their persistence. Furthermore, a study regarding invasiveness of pneumococcal serotypes reported that serotype 3 and NVT 8 have an enhanced propensity to cause invasive disease [25], which can also explain how they were among the more frequent serotypes in 2015-2017. A previous study reported that serotypes 3, 8 and 19A were also among the major serotypes responsible for invasive disease in adults in Portugal from 2012-2014, which makes sense since children are the main carriers of *S. pneumoniae* and transmission to adults can occur [59].

It is known that serotype distribution varies according to geographic location. Nevertheless, the most frequent serotypes found in this study were also found among other countries. An ECDC report stated that in 2015, serotypes 8, 3, 19A and 10A were among the most frequent serotypes responsible for IPD in Europe, along with serotypes 12F and 7F [119], which were not expressed by any isolate in Portugal between 2015-2017. A study in England and Wales reported that in children < 5 years, serotypes 12F, 8, 10A, 15B/C and 3 were among the most frequent [117], however serotype 12F was not observed in Portugal. Furthermore, in a study from Denmark, NVTs 24F, 12F and 23B were the most prevalent non-vaccine types [120]. This situation is different from what was observed in Portugal between 2015-2017, where none of those NVTs were among the most frequent.

Regarding serotype dynamics in the various age groups, there was not a clear trend. The fact that the number of isolates was small and the population very diverse limited the statistical analysis and, in some cases, significant associations between variables could not be found.

4.3 Antimicrobial resistance

Between 2015-2017, antimicrobial resistance in vaccine serotypes was associated with serotypes 6B, 14, 19F and 23F, which are all included in PCV7, and were already related to antimicrobial resistance in Portugal [53].

In the case of NVTs, antimicrobial resistance was observed in serotypes 10A, 23A, 23B, 29/35B

and 33F. Any significant increase in the incidence of a NVT associated with antimicrobial resistance can be worrying in the future, such as serotype 10A that was frequent in Portugal in 2015-2017 and in which antimicrobial resistance was observed. Therefore, routine surveillance of antimicrobial resistance in the future is important.

With the introduction of vaccines, it was expected that the rates of antimicrobial resistance would decrease, since vaccines include serotypes which are associated with resistance such as 6B, 14,19F and 23F [28,29,53]. However, PCV13 was still responsible for 66.7%, 66.7% and 85.7% of the penicillin non-susceptible, erythromycin resistant and EPNSP isolates in 2015-2017, respectively.

When considering antibiotic resistance, penicillin non-susceptibility decreased from 23.2% (43/185) in 2012-2015 to 13.2% (12/91) in 2015-2017. Erythromycin resistance decreased from 22.7% (42/185) in 2012-2015 to 16.5% (15/91) in 2015-2017. Isolates presenting both resistance to erythromycin and non-susceptibility to penicillin decreased from 13.5% (25/185) to 7.7% (7/91) in 2015-2017 (unpublished data). The proportion of erythromycin resistant isolates with MLS_B and M phenotypes in 2015-2017 remained similar when compared to 2012-2015 (86.7% vs 83.3% in MLS_B phenotype; 13.3% vs 16.7% in M phenotype).

The overall decrease in resistance must be due to the effect of vaccination in decreasing the incidence of vaccine serotypes associated with resistance, as seen with serotype 6B that decreased from 5.2% to 3.0% and serotype 14 from 9.9% to 3.7% when comparing 2012-2015 to 2015-2017. It should also be mentioned that in the time period in study, none of the 19A isolates presented antimicrobial resistance, even though this serotype has been associated with antimicrobial resistance in the past [28,53].

Antibiotic resistance also varies according to geographic location, and it can be influenced by variations in serotype distribution and antibiotic consumption in each region. A study in France revealed a different situation from what was observed in Portugal. The French study reported that in children < 2 years, vaccine serotypes 14, 19A and 19F were associated with resistance, together with NVTs 15A, 24F and 35B [121]. The similarities were that serotypes 14 and 19F are related to antimicrobial resistance in both countries, however in Portugal none of the 19A isolates present antimicrobial resistance. Other than serotype 35B, the other most common serotypes associated with antimicrobial resistance in France, were not found in Portugal. Another study in the UK reported serotypes 15A, 23B, 23A, 19F, 19A and 10A as being associated with resistance [122]. In Portugal, antimicrobial resistance was also detected in serotypes 23B, 23A, 19F and 10A. As observed in France, serotypes 15A and 19A were also associated with antimicrobial resistance in the UK [122], however the same as not observed in Portugal.

4.4 Molecular characterization

After molecular characterization of the isolates, 47 different STs and 23 CCs were detected, with a SID of 0.971 which reveals a diverse population. The seven most frequent STs were ST180 (n=9, 10.5%, ST53 (n=8, 9.3%), ST97 (n=6, 7.0%), ST393 and ST433 (n=4, 4.7% each).

The most frequent clonal complex was CC156 (n=19, 22.1%), which expressed mainly PCV7 serotypes (n=11, 57.9%). As mentioned above, after the implementation of pneumococcal conjugate

vaccines, there was a reduction of vaccine serotypes causing disease and a decrease of the total cases of IPD [53,63], therefore it could be expected that CC156 would lose its dominance. However, even after years of administration of conjugate vaccines, CC156, mainly expressing vaccine serotypes, was still the most frequent CC. The persistence of this clonal complex can be related to a few hypotheses such as antibiotic resistance, due to serotypes such as 6B, 14, 19F and 23F. In fact, 11/19 (57.9%) of the CC156 isolates were resistant to at least one antimicrobial. Furthermore, even though there was a decrease in vaccine serotypes responsible for IPD, PCV7 and PCV13 serotypes still remained important causes of paediatric IPD, with 78.6% (n=11/14) of PCV7 and 37.9% (n=11/29) of PCV13 isolates belonging to CC156. Additionally, CC156 has high genomic diversity which may facilitate adaptation to selective pressures [123].

After CC156, the most frequent CCs were CC63 (n=12, 13.9%), CC180 and CC460 (n=9; 10.5% each), CC393, CC433 and CC1262 (n=4, 4.7% each). The most frequent CCs were mainly composed of isolates expressing one of the major serotypes responsible for paediatric IPD between 2015-2017, excluding CC156 that presented a high genetic diversity and several STs and serotypes. In a previous study in Spain, CC156, CC180 and CC433 were also reported to be among the most frequent CCs found in IPD, along with CC191 (associated with serotype 7F), CC230 and CC320 (associated with serotype 19A) [124], which were absent in Portugal in 2015-2017.

The major CCs found in paediatric IPD in Portugal between 2015-2017 were also detected previously in adult IPD in Portugal [125], with the exception of CC994 which was associated with serotype 19A and CC1262 associated with serotype 15B/C in paediatric IPD, while serotype 19A and 15B/C were mostly associated with CC230 and CC199 in adult IPD, respectively. This indicates there was expansion of distinct clonal lineages in serotypes 15B/C and 19A in paediatric and adult IPD, emphasizing the differences in genotypes found in those 2 age groups.

Among the main CCs, CC63, CC460 and CC1262 are of special importance, since they were associated with NVTs 8, 10A and 15B/C, respectively, which were among the most frequent serotypes detected in IPD cases in 2015-2017. Therefore, it is important to see if those CCs will expand in the future, since they could represent the emergence of NVT genetic lineages.

When considering the clonal composition of the six most frequent serotypes (3, 10A, 8, 15B/C, 19F and 22F), both differences and similarities were found with other regions. Serotype 3 was mainly represented by ST180 (Netherlands^{3}-ST180), which was already reported to have a wide geographic dissemination, being present in countries such as Spain, Japan and Sweden [118,124,126]. However, in Portugal ST30 and ST232 were also detected among 2 serotype 3 isolates, but not in Spain where the second most frequent was ST260 [124], not found in Portugal. As for serotype 8, Netherlands^{8}-ST53 dominated as previously seen in Spain. However, in contrast to what was reported in Spain, where a higher genetic diversity was associated with serotype 8 strains, in Portugal all the serotype 8 isolates were included in the same genetic lineage, defined by ST53 (CC63) [124]. Furthermore, a study in Canada revealed that ST53 was not the most prevalent ST found among serotype 8 isolates in this region, but ST1480 was the most frequent [127]. Among serotype 10A isolates, a higher genetic diversity was noted. In spite of this, six of the nine isolates belonged to the same genetic lineage (CC460), which included ST97 (n=4) as well as ST461 and ST1551 (n=1 each). Similarly to what was reported here,

serotype 10A was also reported to be an important cause of IPD among NVT, also associated with ST97 in Spain [124]. Another study in Japan reported that ST97 was absent and ST5236 was the most prevalent [118]. Serotype 19F was the only PCV7 serotype detected among the 6 most frequent serotypes in this collection. Although grouped in the same clonal complex, CC156, these isolates presented high genetic diversity (which is frequent among isolates included in CC156). In a study in Japan, ST236 (Taiwan^{19F}-ST236) was the most prevalent [118]. Even though this ST was not observed in Portugal, ST13863 present in one isolate was identified as a DLV of ST236. In the case of serotype 15B/C, CC1262 is the most prevalent and there is no dominant ST. In Spain, ST1262 was the most frequent, however in Japan ST1262 was absent and ST199 (Netherlands^{15B}-ST199) was the most prevalent [118,124]. ST199 was also observed in Portugal, however only in one isolate. As for serotype 22F, ST433 was the most commonly found ST as observed in a previous study in Spain [124]. The same was also detected in Sweden and Canada in the post PCV13 period [126,127]. However, ST445 which was found in Portugal (n=1) was not detected in any of those countries.

The Simpson's index of diversity is a good measure to evaluate the diversity of populations and in the case of serotypes 15B/C and 10A, SID values were high indicating a high diversity of STs when compared with other serotypes. However it should be taken into account that the number of isolates was small which can compromise the statistical significance, therefore SID values are affected, since some STs might have only one isolate.

Overall, after the introduction of PCVs, there was an increase in incidence of non-vaccine types [63,113]. An increase in non-vaccine serotypes can be the result of capsular switching, when a successful clonal lineage expresses a different serotype not covered by conjugate vaccines and is able to persist in the population. In this collection, new associations between serotypes and STs were reported, mainly serotype 10A associated with ST180, ST4083 and ST12069 and serotype 11A associated with ST180 or ST4948 (according to the public MLST database (http://pubmlst.org/spneumoniae), accessed September 2018). However, in this collection, the capsular switching events seemed to have occurred at a low frequency, involving single isolates without clonal expansion and dissemination. Nevertheless, it is important to keep track of those cases since they may disseminate in the future as successful lineages.

When considering the ST variations since 2010 (unpublished data), the most significant differences were the decline of ST306 and the rise of ST180 and ST53. The decline of ST306 can be associated to the reduction of serotype 1 and the increase in ST53 and ST180 is related to the rise of serotypes 8 and 3, respectively.

Even though they were only significant before FDR correction, ST97, ST191, ST276 and ST338 variations should also be mentioned. The decrease in ST191, ST276 and ST338 are related to vaccine serotypes 7F, 19A and 23F, respectively. The case of ST97 is associated with the increase of serotype 10A in 2015-2017, an important NVT that should be monitored in the future.

It is important to continue routine surveillance to keep track of serotype changes in the context of universal vaccination, allowing the identification of vaccinal serotypes still persisting in the population and the possible emergence of NVT as important causes of IPD. Moreover, it is also important to monitor antimicrobial resistance rates, as they can be affected by the use of PCVs and to study the genetic

relatedness of *S. pneumoniae* isolates, in order to gain a better understanding of the dynamics of the population causing paediatric IPD.

5. Conclusion and Future Work

The main conclusions of this work were that after the introduction of PCV13 in the NIP, there were important changes in the serotype distribution of *S. pneumoniae*, however serotype 3 remained the major cause of paediatric IPD. The use of molecular methods contributed greatly for the identification of cases due to this serotype, therefore those methods should also continue to be used in the future. There were increases in non-vaccine serotypes, however none has still emerged as the major cause of IPD in Portugal. Antimicrobial resistance decreased when compared to the previous time period. The molecular analysis of the isolates revealed a diverse population and some of the most frequent clonal complexes identified in paediatric IPD in 2015-2017 were already observed in paediatric and adult IPD in Portugal before.

Routine surveillance studies should continue to be performed in the future to evaluate the effects of PCV13 vaccination, to see if serotype 3 and other vaccine serotypes will decrease in the future and if any NVTs will emerge as major causes of IPD. It is also crucial to study antimicrobial resistance to determine if changes in serotypes will lead to changes in antimicrobial resistance, either by the decrease in the proportion of resistant isolates presenting vaccine serotypes or by the increase in the proportion of NVTs associated with non-susceptibility. It is also important to keep track of changes in the bacterial population of *S. pneumoniae* to find out if the current genetic lineages remain in circulation or if any new clonal complex appears and disseminates successfully.

6. References

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