Antimicrobial resistance in *Streptococcus pneumoniae*: mechanisms and current epidemiology

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ABSTRACT

Infections caused by *Streptococcus pneumoniae* are a worrisome public health problem worldwide. Young children and the elderly are the main age groups affected and the highest burden of the disease is found in developing countries. Pneumococcal infections cause 11% of the total infant deaths, representing the leading cause of child death currently preventable by vaccination. Epidemiologic information about pneumococci in Brazil is somehow restricted, but available data reinforce the worrisome occurrence of pneumococcal diseases, which are commonly treated empirically. Limitations in the diagnostic methods, along with the severity of disease contribute to this behavior. Thus, surveillance studies are crucial to define the prevalence of resistant strains both globally and in a particular region, as these strains may compromise empirical therapeutic choices. However, although different clones of penicillin non-susceptible pneumococci are internationally distributed, and considering diseases other than meningitis, the prevalence of resistance to penicillin is quite low, making this old, safe, and inexpensive drug an attractive first choice to treat pneumococcal infections. The widespread use of conjugate vaccines among children, influencing the circulation of resistant clones and the distribution of serotypes reinforces the need of surveillance studies to define the prevalence of resistance.

Keywords: *Streptococcus pneumoniae; public health; antimicrobial resistance*

Infections caused by *Streptococcus pneumoniae* are a public health problem worldwide, especially considering young children and the elderly, and developing countries are clearly the most affected regions. Pneumococcal disease presents with a variable degree of severity, ranging from mild infections (acute otitis, sinusitis, and uncomplicated pneumonia) to invasive pneumococcal disease (IPD), such as bacteremic pneumonia and meningitis, which are associated with elevated morbidity and mortality, even when treated adequately. Indeed, case-fatality rates of pneumococcal meningitis can be as high as 37%[1] and around 20% of survivors experience long-term disabling sequelae[2].

Around 14.5 million episodes of severe pneumococcal disease occur annually in the world, causing 1,612,000 deaths[3], 825,000 of them among children under 5 years old, representing 11% of the total number of infant deaths[4]. Indeed, pneumococcal infections are the leading cause of child death currently preventable by vaccination[3].

Epidemiologic information about pneumococcal disease is lacking in many parts of Latin America. Information is mostly based on laboratory

surveillance of *S. pneumoniae* isolates from hospitalized patients with IPD, such as Pan-American Health Organization's SIREVA II database. It is estimated that around 20,200 to 33,000 children die annually in Latin America due to infections caused by pneumococci[3]. Brazilian data for pneumococcal disease are also scarce (considering the whole geographical area), but some studies from specific regions (Goiania, a Brazilian Midwest city) report that among children from 28 days to less than 3 years old, the incidence of IPD was 57.5/100,000 inhabitants from 2007 to 2009[5].

Pneumococci are the major cause of community-acquired pneumonia (CAP) and Brazil is among the 15 countries with the highest estimated numbers of new cases of pneumonia worldwide[6]. The mean rate of hospitalization due to CAP was 2,100/100,000 inhabitants in Brazil, from 2000 to 2008; 45% of them occurred in children aged less than 5 years and were caused by pneumococci[7].

According to the Brazilian Ministry of Health, it was notified of an average of 1,227 cases/year of pneumococcal meningitis, from 2000 to 2010, with a mortality rate around 30%[7]. Among children under 5 years of age, the incidence was 5.9/100,000 inhabitants, and this value increased to 9.5 cases per 100,000 inhabitants when younger children were taken into consideration (≤2 years old); mortality was high for both groups: 33 and 34%, respectively[5].

As an exclusively human pathogen, pneumococci colonize the nasopharynx, especially in children aged younger than 5, and transmission occurs by contact with respiratory secretions. From the primary colonization niche, they can migrate to other sites, such as middle ear, sinus, lung, blood, or cerebrospinal fluid and cause damage, leading to invasive disease. In this context, pneumococci have a robust arsenal of virulence factors, among which the polysaccharide capsule plays a central role[8]. Opsonophagocytosis is avoided by the presence of the capsule and differences in the polysaccharide composition distinguish over 94 distinct serotypes among pneumococci[9].

Commonly, antimicrobial therapy against pneumococci is defined empirically and the severity of the disease determines the medical approach[10]. Besides, specific features of microbiological diagnostic tests may also justify the empirical therapy. Indeed, some culture-based methods to identify *S. pneumoniae* have an intrinsic low sensitivity. For instance, blood cultures from patients with pneumococcal bacteremia detect the microorganism only in around 10 to 30% of cases[11]. On the other hand, some specimens, such as sputum, should be carefully analyzed, as low specificity may lead to false-positive results[11]. Another feature that substantiates the broad use of empirical therapy is the originally excellent activity of antimicrobials against pneumococci, especially the β-lactams. Pneumococci, in general, present very low Minimal Inhibitory Concentration (MIC) values[13] to these drugs.

However, this scenario has changed in recent years. The widespread and/or inadequate use of antimicrobials exerts a selective pressure on pneumococcal populations by picking out resistant isolates. In addition, selection of non-susceptible pneumococci may be a result of the dissemination of specific clones, which have an advantageous genetic background, including virulence and spread features, as well as resistance genes. The increased occurrence of these particular clones may be a natural event, where variations of frequency are expected during a certain period, or may be a result of another selective pressure force, such as vaccination[14].

Therefore, treatment of pneumococcal infections may be severely hampered by the isolation of non-susceptible strains. Indeed, it is well established that the delay in the implementation of the correct therapy in cases of CAP significantly increases in-hospital mortality, as well as 30-day mortality. Thus, it is reasonable to conclude pneumococcal resistance may directly affect patient's outcome[10].

RESISTANCE MECHANISMS

According to different guidelines, therapy against pneumococcal infections is primarily based on the use of β-lactams and macrolides[15-17]. Glycopeptides may also be an important therapeutic choice and their resistance among pneumococci has not been described so far. Some other drugs, such as fluoroquinolones, tetracycline, sulfamethoxazole-trimethoprim, lincosamines, and chloramphenicol also have good activity against pneumococci, but resistance against these drugs may occur in variable frequencies around the world as demonstrated bellow.

Defining resistance to some β-lactams (penicillins and cephalosporins) is a complex issue. The Clinical and Laboratory Standards Institute[18] determines breakpoints for the interpretation of antimicrobial susceptibility testing based on the

site of the infection (meningitis and non-meningitis) and the route of administration (oral and parenteral). The decision of the Institute to change breakpoints was based on the pharmacokinetic and pharmacodynamic properties of penicillin (low penetration across the blood-brain barrier). The term "penicillin non-susceptible *S. pneumoniae*" (PNSP) refers to isolates classified as resistant or intermediately resistant, according to current interpretative breakpoints[19]. Thus, according to CLSI meningitis criteria, pneumococci with MIC > 0.06 µg/mL are considered resistant to penicillin. On the other hand, by non-meningitis breakpoints, MICs of 4 µg/mL and ≥ 8 µg/mL define the isolate as intermediate and fully resistant to penicillin, respectively[18].

β-lactams act by binding to the penicillin-binding proteins (PBP), compromising cell wall formation, which leads to osmotic induced pneumococcal lysis[20]. This mechanism is highly effective and penicillin MICs for pneumococci are, in general, very low (as low as 0.01 µg/mL for benzylpenicillin). The first reports of higher penicillin MICs are from the 1960s and 1970s, but were neglected because of β-lactams excellent activity against pneumococci. Indeed, little regard was given to antimicrobial resistance in pneumococci until 1977 when the attention of the medical community was drawn to reports of an IPD epidemic in South Africa caused by highly resistant *S. pneumoniae*[21]. Following this report, multidrug-resistant (resistance to three or more antimicrobial classes) pneumococci were reported with greater frequency worldwide[20,22,23].

There are six physiological PBPs in the pneumococcal cell: PBP1a, PBP1b, PBP2a, PBP2b, PBP2x, and PBP3. Resistance to β-lactams is basically related to mutations in three of those enzymes: mutations in PBP2x and PBP2b being strongly related to resistance; mutated PBP1a, in tandem with PBP2b and PBP2x increases penicillin MICs further; altered PBP2a also seems to be related to resistance, but to a lesser degree[13,24,25].

While PBP genes are highly conserved among pneumococci susceptible to penicillin, in most clinical isolates of PNSP, PBPs are codified by mosaic genes, which are continuous nucleotide sequences that differ from the non-mosaic allele by up to 20%,[13] strongly suggesting a non-pneumococcal origin of these genes. Indeed, interspecies gene transfer, followed by recombination events involving closely related commensal species, such as *Streptococcus mitis* and *Streptococcus oralis* seems to be the origin of the mosaic PBP resistant genes[13,20].

Some of those mosaic genes have become stable in specific clones. For instance, some PNSP clones (Spain^{23F}-1, Spain^{6B}-2 and Spain^{9V}-3) are internationally disseminated (see section "international clones" below) and present a highly conserved PBP2x among them; this suggests that the mutated gene may generate some evolutionary advantage. Indeed, apart from the wondrously transformation potential of pneumococci, dissemination of resistant clones are also important for the increase of penicillin non-susceptibility among pneumococci[13].

PNSP will only disseminate if a delicate balance occurs between antimicrobial selective pressure and the cost that resistance imposes on bacterial fitness. In this context, mutations in *pbp2b* carry an energy expenditure that affects fitness. However, *pbp2b* mutants that also carry *pbp1a* and *pbp2x* mutated genes not only compensate energetic costs, but also increase MICs, leading to evolutionary better adapted bacterial isolates[13].

Along with PBP changes, mutations in non-PBP genes also occur in PNSP and, depending on the selective antibiotic, distinct genes are affected[19]. A mutation in the GlcNAc deacetylase (*pdgA*) has been detected by genome sequencing in high-level resistant transformants obtained in four selection steps using chromosomal DNA of a high-level resistant *S. pneumoniae* strain. Moreover, deletion of the peptidoglycan O-acetyl transferase has also been shown to cause an extensive reduction of resistance in several PNSP strains[26].

The worldwide increase in penicillin resistance coincided with an increase in macrolide resistance. In many parts of the world, macrolide resistance now exceeds penicillin[13]. Macrolides act by inhibiting protein synthesis as a result of their binding to the 23S portion of the ribosomal RNA[27]. Resistance is due to two major mechanisms: (i) alteration of the target site of the antimicrobial by producing a bacterial methylase codified by *erm* genes; (ii) expression of *mef* genes that codify an active efflux pump. Another mechanism may be associated with macrolide resistance but its clinical relevance is very low: through mutations in the 23S rRNA gene or in L4 and L22 ribosomal proteins[28,29].

Indeed, the production of methylase is the major mechanism of macrolide resistance and it commonly confers the MLS_B phenotype (resistance to Macrolides, Lincosamines and B Streptogramins). Two major acquired genes are responsible for this

resistance: *erm*(B) and *erm*(TR). The *erm*(TR) gene is a subclass of *erm*(A) and has a very narrow distribution compared to *erm*(B). The bacterium *Streptococcus pyogenes* seems to be the origin of *erm*(TR) and nasopharynx co-colonization with pneumococci. which may have allowed interspecies dissemination. Among pneumococci, after its first isolation, only a few isolates carrying this gene have been reported[13,30].

The presence of *erm*(B) usually leads to elevated MICs (> 64µg/mL). This gene is carried on members of Tn*916* family of transposons (in pneumococci: Tn*3872*, Tn*6002*, Tn*6003* and Tn*1545*), which also carry *tet*(M), an important determinant of tetracycline resistance. Although *tet*(M) is not always expressed, tetracycline resistance is very common among macrolide resistant pneumococci[27,31].

Clonal dissemination seems to play a more relevant role for macrolide resistance than gene acquisition by single strains as a result of the selective pressure of antimicrobial usage. Indeed, 77.1% of *erm*(B) are located into Tn*916* transposons family, suggesting that the increased occurrence of macrolide resistant pneumococci is a result of the clonal dissemination of these transposons. Indeed, an expressive number of pneumococci presenting macrolide resistance with the MLS_B phenotype (more than 50%) belong to a few international pneumococcal clones: Sweden^{15A}-25, Spain^{23F}-1, Spain^{6B}-2, clone^{19F}-ST276, and clone^{19A}-ST276 (see section "international clones" below)[32].

Efflux pump, codified by *mef* genes, confer lower macrolide MICs than *erm*(B): 1 to 32µg/mL. In this case, M phenotype (only macrolide resistance) occurs and lincosamines and B Streptogramins may have activity. Among pneumococci, there are three related genes: the abundant *mef*(E) and *mef*(A), and a third gene, *mef*(I), with a very narrow distribution so far[13]. *Mef*(E) and *mef*(A) show 90% of genetic identity and present a distinct geographical distribution: the former is widely distributed in USA, Asia, and South Africa, while the latter is more commonly recovered from European countries, as well as South America[33-35].

The increase in β-lactam resistance spurred the development of fluoroquinolones active against Gram-positive pathogens. Fluoroquinolone resistance is increasing amongst pneumococci. These drugs act by binding to the DNA gyrase (formed by GyrA and GyrB subunits) and topoisomerase IV (formed by ParC and ParE subunits) thus disrupting DNA synthesis. The primary target varies according to microorganisms (Gram-positive or Gram-negative) and the fluoroquinolone drug: among pneumococci, ciprofloxacin and levofloxacin act primarily in ParC topoisomerase subunit, while moxifloxacin firstly binds to GyrA DNA gyrase subunit[36].

Resistance to fluoroquinolone occurs because of gradual accumulation of point mutations in the Quinolone Resistance Determinant Region (QRDR) of the GyrA and/or ParC. Mutations in *parC* QRDR guarantee resistance to ciprofloxacin, but not to the newer fluorquinolones. Indeed, QRDR *parC* mutations are the primary step in fluorquinolone resistance. They do not substantially increase MICs but enhance the risk of new mutations. On the other hand, isolates presenting QRDR regions of *gyrA* and *parC* mutated have elevated MICs (> 16µg/mL). Mutations in *gyrB* and *parE* are infrequent and seem to be unexpressive[37-40].

Although some studies demonstrate an heterogeneous genetic background, fluoroquinolone resistance appears to be strongly associated with a single mutation in ParC and GyrA: substitution of a phenylalanine in positions 79 and 81, respectivelly. Indeed, a multicentric study demonstrated that 51% of pneumococci resistant to fluorquinolone showed only those point mutations[41].

Besides alterations in *parC* and *gyrA* nucleotide composition, the overexpression of efflux pumps, such as PmrA or the ABC pumps PatA and PatB, may have a role in fluorquinolone resistance. Although MICs in those isolates are not as high as the *gyrA* and *parC* mutated ones, overexpression of efflux pumps seems to increase chances of occurrence of point mutations[13].

Recombination does not play a central role in the dissemination of fluorquinolone resistance. Indeed, pneumococcal QRDR has been shown to have low homology with viridans QRDR, a species more frequently related to this phenotype than pneumococci[40]. In fact, some studies demonstrated the occurrence of mosaic genes shared by viridans and pneumococci but this was not common among the pneumococcal population. One reason for this may be bacterial fitness, even though supportive data are lacking. Unlike macrolide resistance, clonal dissemination of fluorquinolone resistance does not have a major participation in the increase of this resistance and, again, bacterial fitness may justify this observation[40].

Less clinically significant phenotypes among pneumococci include resistance to tetracycline, sulfamethoxazole plus trimethoprim, and

chloramphenicol. Tetracycline acts through binding to the 30S ribosomal unit, preventing protein synthesis. Resistance to this antimicrobial may be due to the presence of Tet(M) and, occasionally, Tet(O) proteins, which prevent tetracycline binding by a methylation reaction onto the target site; or, less frequently, the occurrence of efflux pumps, Tet(K) and Tet(L), respectivelly[41,42]. The *tet*(M) gene is located in genetic mobile elements widely found and transmitted among many Gram-positive bacteria justifying the frequent occurrence of this phenotype in pneumococcal population. Efficacy and low cost are the main reasons for sulfamethoxazole-trimethoprim therapy against pneumococci, especially in developing countries, where reports of resistance are increasing. Prophylactic usage of this antimicrobial to prevent secondary infections in HIV positive patients may also explain the high rates of resistance observed. Sulfamethoxazole-trimethoprim acts on folic acid synthesis and mutations in genes (*folA* and *folP*) that codify the binding target of these drugs are responsible for resistance[43]. Finally, chloramphenicol resistance occurs through production of chloramphenicol acetyltransferases, codified by *cat* genes. The enzyme converts the antimicrobial into a non-functional molecule, preventing chloramphenicol binding to 50S ribosomal subunit[44].

Table 1 summarizes the main resistance mechanisms to antimicrobials in *S. pneumoniae*.

PREVALENCE OF RESISTANCE

Global

Although the incidence of IPD caused by PNSP, pneumococci resistant to erythromycin or multiresistant pneumococci had decreased significantly after the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), including serotypes in 2000, the increased isolation of resistant non-vaccine serotypes promoted a rise in the frequency of resistant pneumococci in some parts of the world[45]. Indeed, from 1998 to 2003, the proportion of PNSP decreased from 32% to 19.4%, followed by a post-vaccine increase to 30.1% in 2005. Some serotypes are consistently related to the decrease of the susceptibility, especially the so-called pediatric serotypes (6A, 6B, 9V, 14, 19A, 19F, and 23F), as well as some other non-vaccine serotypes: 19A and 35B[46,47].

A recent multicentric study encompassing 2,173 IPD-recovered pneumococci from patients of all ages and from all continents (2004-2009) showed that 33.3% of all isolates were non-susceptible to penicillin (MIC < 0.06 µg/mL). Resistance to erythromycin was quite lower (22.9%) and 16.2% of all *S. pneumoniae* were resistant to both penicillin and erythromycin. Isolates resistant to levofloxacin represented only 0.5% of the total. Some serotypes were significantly associated with PNSP: 19A, 6A, 19F, 14, 6B, 9V, 35B, 23A, and 15A. Similarly, serotypes 19A, 6A, 15A, 19F, 9V, 6B, and 14 had a statistically significant relationship with erythromycin resistance[23].

If the populations with the highest risk for pneumococcal infections are taken into consideration[21], resistance to penicillin considerably increased in all continents compared to the general (all ages) population[23]. Brandon and Dowzicky included in their study pneumococci recovered from clinically relevant sites of pediatric populations (0 to 18 years old), from 2004 to 2011. Globally, PNSP was 46.1% and levofloxacin remained very low 0.3%[22].

Geographic occurrence of resistance is not homogeneous and both selective pressure by antimicrobial use and circulation of some specific clones/serotypes are responsible for the differences in the prevalence of resistant pneumococci worldwide[14].

Hackel et al. demonstrated that, for patients of all ages, erythromycin resistance ranges from 15.3% to 28.8% among all continents, with the lowest frequency in Latin America and the highest among Asian countries. On the other hand, Africa presents the highest frequency of isolation of PNSP (64.3%), while only 18.6% of pneumococci

Table 1: Mechanisms of resistance against the most clinically relevant antimicrobials.

Antimicrobial	Resistance mechanism	Reference
Penicillin	Mutations in *pbp* genes = mosaic genes	12
Erythromycin	Expression of *erm* (methylation) and/or *mef* (efflux pumps) genes	27
Tetracycline	Expression of *tet* genes: methylation [*tet*(M) and *tet*(O)] and/or efflux pump [*tet*(K) and *tet*(L)]	41
Fluoroquinolones	Mutations in QRDR of *par*C and *gyr*A.	35
Chloramphenicol	Expression of *cat* gene (acetyltransferases)	43
Sulfamethoxazole-trimethoprim	Mutations in *folA* and *folP*	42

recovered from European countries show this phenotype[23].

Geographical variation of frequency among the pediatric population presents a similar pattern. The lowest frequency of isolation of PNSP (34.4%) occurred in Europe, with a frequency almost twofold higher than that observed among all ages (18.6%). Africa had, once again, the highest rates of PNSP, 85.7%. All continents but Africa (no resistance detected) had levofloxacin-resistant pneumococci in a low frequency of isolation: from 0.2 in Europe to 1.1% in the Middle East[22].

Although data from Europe commonly demonstrate low rates of PNSP, a large recent European study including more than 21,000 pneumococcal isolates showed that some countries may have frequencies of PNSP and resistance to macrolides as high as 42.2% and 38.1%, respectively. Once again, serotypes 14, 19A, and 19F were the most commonly involved[48].

Some other limited studies have shown quite different frequencies of resistance, especially if differentiated populations are taken into consideration. For instance, pneumococci recovered from nasopharynx of healthy children in China revealed 39.4% of PNSP during 2012 to 2013[49]. In the same study, all 175 pneumococcal isolates were resistant to erythromycin. Over again, serotype 19F (precisely Taiwan1^{9F}-14 clone) was significantly associated with β-lactam resistance[49].

Resistance to erythromycin was also extremely high among Japanese pneumococcal isolates, recovered from noninvasive or colonization sites during 2011: 96.8%, and serotypes 23F and 6B were the most commonly related to this phenotype. On the other hand, resistance to penicillin was very low[50].

Other studies focusing on pneumococcal from carriage demonstrate a similar scenario[51-55], although some specific regions may show higher frequencies. Indeed, frequency of PNSP was 78.6% among isolates from healthy Korean children[56].

Of note, despite the worrisome occurrence of PNSP considering CLSI meningitis breakpoints, isolates presenting non-susceptibility to penicillin following non-meningitis breakpoints (MIC ≥4μg/mL) are very low worldwide[48].

BRAZIL

According to SIREVA II, the prevalence of PNSP (MIC > 0.06 μg/mL) in Brazil was 25.7%, while 11.5% showed erythromycin resistance[57]. Some regional studies present quite similar data. Of note, all studies used CLSI meningitis breakpoints to define PNSP.

Andrade et al. evaluated pneumococci recovered from children with IPD previous to implementation of vaccination in Brazil (207-2009) and identified 13.3% as PNSP, all of them belonging to serotypes included in PCV7. No levofloxacin resistant isolates were found and 13.3% presented macrolide resistance[5].

On the other hand, Mott et al. firstly evaluated 159 invasive pneumococcal isolates recovered in post-vaccination period (2010 to 2012) in the country. An increase of PNSP was observed (21.4%) compared to the above-cited study and serotypes 14, 9V, 19F, 23F, and 19A were the most commonly related to this phenotype. Only one isolate, belonging to serotype 19A, had a MIC=4 mg/mL to penicillin (intermediate resistance, according to CLSI criteria for non-meningitis), and isolates showing MICs≥8 mg/mL were not found. Resistance to erythromycin was 12% and only one isolate was resistant to fluoroquinolone[35].

Among pneumococci recovered from patients with meningitis during 2000-2007, the frequency of PNSP was very similar (22.2%) but erythromycin resistance was considerably lower: 0.8%[58]. Similar results were found when pneumococcal from carriage were taken into consideration[59].

Resistance rates to tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim were found to be more heterogeneous in different Brazilian studies[5,35,57,58,60,61]. As an example, non-susceptibility to sulfamethoxazole-trimethoprim varied from 28.5%[56] to 80%[5], while the percentage of pneumococci non-susceptible to tetracycline seems to be more homogeneous (around 20-30%)[35,58].

Continuous surveillance of pneumococci focusing on antimicrobial susceptibility, as well as serotype distribution is of great concern in developing countries such as Brazil and should be performed systematically to better understand the impact of vaccination on resistance rates.

PNEUMOCOCCAL INTERNATIONAL CLONES AND THE INFLUENCE OF VACCINATION IN THE DISSEMINATION OF RESISTANCE

Although *S. pneumoniae* is a genetically diverse species capable of expressing over 94 different capsular types[9], only a limited number of these serotypes associated with a few pandemic

clones have been responsible for the increase of pneumococcal drug resistance worldwide[14]. The origin of these drug resistant clones is believed to be the nasopharynx of young children, from where they are transferred from person-to-person. These circumstances, combined with frequent antibiotic use, constitute ideal conditions for the selection, amplification, and transmission of drug-resistant clones[62].

Created in 1997, the Pneumococcal Molecular Epidemiology Network (PMEN - http://web1.sph.emory.edu/PMEN/) intended to develop a global surveillance of antibiotic-resistant *Streptococcus pneumonia*e clones. In order to standardize the nomenclature and classification of these clones, their names are composed by the location of the first isolation, the serotype (superscript), plus a number indicating the chronological order of nomination by PMEN. For example, the first PMEN clone was isolated in Spain and the strains were serotyped as 23F: Spain^{23F}-1. Forty-three important disease-causing clones have been identified[63]. Although resistant strains are the primary focus of surveillance, some susceptible clones with relevant importance in invasive disease worldwide are also considered by PMEN. Table 2 presents characteristics of PMEN clones resistant to penicillin considering meningitis breakpoints (MIC> 0.06 µg/mL).

To be included into the network, clonality must be determined based on Pulsed-Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), and Penicillin-Binding Protein (PBP) fingerprinting results. Despite the high discriminatory power of PFGE, this technique has low reproducibility and, consequently, data may not be homogeneous among geographically distinct laboratories. On the other hand, MLST generates unambiguous data, making it easy to compare strains from different regions. Indeed, it increases the understanding of the pneumococcal population dynamics and their patterns of dissemination worldwide.

Apart from the β-lactams, resistance to erythromycin and tetracycline are the most prevalent phenotypes among PMEN clones (19/43; 44.2%).

Table 2: Pneumococcal Molecular Epidemiology Network clones presenting resistance to penicillin (meningitis breakpoints: MIC > 0.06 µg/mL).

PMEN clone	ST	Serotype	Vaccine	Susceptibility profile [c] (MIC in µg/mL)						
				PEN	CTX	ERY	CLI	CHL	TET	SXT
Spain^{23F}-1	81	23F	all [b]	1	1.5	0.25	0.19	16	64	2
Spain^{6B}-2	90	6B	all	0.5	0.75	0.19	0.19	16	48	2
Spain^{9V}-3	156	9V	all	1.5	0.75	0.125	0.19	3	0.5	1.5
Tennessee^{23F}-4	37	23F	all	0.125	32	32	0.125	3	0.25	2
Spain^{14}-5	18	14	all	1.5	1	0.19	0.125	32	24	0.25
Hungary^{19A}-6	268	19A	PCV13	1	0.75	>256	>256	24	48	3
S.Africa^{19A}-7	75	19A	PCV13	0.19	0.094	0.25	0.19	4	1	4
S.Africa^{6B}-8	185	6B	all	0.19	0.125	0.19	0.19	2	0.5	1.5
CSR^{14}-10	20	14	all	8	1	>256	>256	32	48	0.25
CSR^{19A}-11	175	19A	PCV13	6	0.5	>256	>256	24	64	2
Finland^{6B}-12	238	6B	all	4	0.75	>256	>256	8	64	6
S.Africa^{19A}-13	41	19A	PCV13	1.5	0.5	>256	>256	24	48	4
Taiwan^{19F}-14	236	19F	all	2	0.75	16	0.25	16	48	2
Taiwan^{23F}-15	242	23F	all	0.75	0.75	>256	>256	4	48	0.25
Poland^{23F}-16	173	23F	all	8	4	>256	0.25	16	64	1.5
Maryland^{6B}-17	384	6B	all	1.5	1	24	0.19	3	0.75	2
Tennessee^{14}-18	67	14	all	4	12	>256	>256	3	32	2
N.Carolina^{6A}-23	376	6A	PCV13	1	0.75	4	0.19	3	0.5	1
Utah^{35B}-24	377	35B	NONE	1	0.75	0.125	0.125	3	0.38	0.38
Denmark^{14}-32	230	14	PCV7	1	0.75	0.125	0.125	3	64	1.5
Norway^{NT}-42	344	NT	NONE	0.094	0.125	4	0.064	2	32	0.38
USA^{NT}-43	448	NT	NONE	0.094	0.094	4	0.094	3	32	0.25

[a] ST: sequence type, according to MLST results
[b] All = PCV7, PCV10 and PCV13
[c] * PEN = penicillin; CTX = ceftriaxone; ERY = erythromycin; CLI = clindamycin; CHL = chloramphenicol; TET = tetracycline; SXT = sulfamethoxazole-trimethoprim

Considering CLSI meningitis criteria for penicillin and ceftriaxone, 20 (46.5%) and 16 (37.2%) clones exhibit nonsusceptibility: MIC ≥ 0.12µg/mL and ≥ 2µg/mL, respectively. If non-meningitis breakpoints are taken into consideration, clones CSR^{14}-10, CSR^{19A}-11, Finland^{6B}-12, Poland^{23F}-16, and Tennessee^{14}-18 are non-susceptible to penicillin (MICs ≥ 4µg/mL). Poland^{23F}-16 and Tennessee^{14}-18 are also fully resistant to ceftriaxone (MICs ≥ 4µg/mL). Besides, despite its penicillin susceptibility, Tennessee^{23F}-4 is characterized by a very high ceftriaxone MIC (32 µg/ml). In general, these β-lactams nonsusceptible clones are multiresistant and their occurrence worldwide may strongly compromise empirical therapy against IPD.

PCV7 was particularly designed against the most prevalent and/or resistant serotypes, i.e. 4, 6B, 14, 18C, 19F, and 23F, which are widely distributed around the world. Indeed, most PMEN clones (51.2%) encompass pneumococci from PCV7, especially serotypes 14, 6B and 23F (17/22; 72.3%), which is not surprising, since vaccine formulations were developed precisely against the serotypes most commonly related to IPD worldwide and/or those with worrisome resistance characteristics. PCV10 (PCV7 serotypes plus 1, 5 and 7F) and PCV13 (PCV10 serotypes plus 3, 6A and 19A) comprise 65.1% (28/43) and 81.4% (35/43) of the international pneumococcal clones, respectively. Of note, 18.6% of PMEN clones are composed of serotypes not included in any vaccine formulation currently available (including two non-typable clones: Norway^{NT}-42 and USA^{NT}-43) and they will be further discussed below.

Among all 43 PMEN clones, the most widely distributed seem to be Spain^{23F}-1, Spain^{6B}-2, and Spain^{9V}-3. Spain^{23F}-1 predominantly circulates as a vaccine serotype 23F, multilocus sequence type 81 (ST81). However, ST81 has also been associated with several other serotypes, including both vaccine and non-vaccine types[64,65]. After the first isolation, Spain^{23F}-1 disseminated worldwide[64,66-69]. Indeed, by the late 1990s, it was estimated that approximately 40% of the penicillin non-susceptible pneumococci circulating in the USA were members of this clone[70], corroborating the spread of penicillin resistance determinants among other pneumococcal clones. Besides penicillin, Spain^{23F}-1 is also commonly associated with fluoroquinolone resistance and some authors have suggested that genetic determinants for this resistance have been donated from Spain^{23F}-1 to numerous unrelated pneumococcal clones[71].

It has been well demonstrated by Wyres et al. that Spain^{23F}-1 and related clonal variants (all belonging to the same clonal complex - CC81), exhibit extraordinary genetic diversity, which largely results from hundreds of recombination events[72]. These features indicate rapid genomic evolution and presumably allow rapid response to selective pressures such as those imposed by vaccine and antibiotic usage[65].

Indeed, although antibiotics are among the most influential global public health successes, selective pressures imposed by them drive bacterial genomic evolution. Spain^{23F}-1 is an excellent example of a bacterium that has become resistant to multiple antibiotics and that has evolved to become very successful in colonization, transmission, and causing disease. Moreover, Spain^{23F}-1 has subsequently shared its successful DNA with other unrelated pneumococci[72].

On the other hand, Spain^{9V}-3 belongs to ST156 (CC156), which, according to the MLST database, has been associated with a considerable diversity of capsular types (14, 9V, 19F, 11A, 9A, 15C, 13, 19A, and 15B), suggesting a high tendency of this clonal cluster to undergo capsular switching events. CC156, one of the largest CC presently found in the MLST database with frequent occurrence around the world[63], including Latin America and Brazil[61,73,74], is globally and consistently associated with important resistance profiles, including non-susceptibility to penicillin[75].

As PCV7 has been widely implemented worldwide, it is expected that these traditional resistant clones will lose ground because of selective pressure, given advantages to other clones/serotypes. A classic example of this natural biological event was the emergence of serotype 19A in both carriage and invasive disease soon after PCV7 implementation in the USA[76]. Of note, some clones of serotype 19A are consistently non-susceptible to penicillin, as well as resistant to other antimicrobials[77]. As a consequence, this capsular switching event dramatically increased the occurrence of penicillin non-susceptible isolates in many different regions of the world.

However, serotype 19A also increased in regions without vaccine selective pressure, suggesting the participation of other factors, such as temporal variations, dissemination of some specific clones, and antimicrobial pressure[78]. Four PMEN international clones are related to serotype 19A: Hungary^{19A}-6 (ST268), S.Africa^{19A}-7 (ST75), CSR^{19A}-1 (ST175), and S.Africa^{19A}-13 (ST41).

All but one (S.Africa[19A]-7) are multiresistant, including non-susceptible to penicillin (meningitis breakpoints).

Besides those above-mentioned clones, genotypic characterization of serotype 19A isolates by MLST showed that there are five major CC associated with them: CC81, CC193, CC199, CC276, and CC320[78]. ST320 (CC320), derived from Taiwan[19F]-14 (ST236) by capsular switching events, has become prevalent in many countries, and is strongly related to penicillin resistance[76,80-83]. Recently, it was observed that the genetic background of ST320 provides advantages associated with improved colonization in the nasopharynx when compared to ST199[77], another well-established serotype 19A clone, prevalent in the USA previously to PCV7. Indeed, this advantage may be responsible for the rapid shift of ST199 to ST320 in the USA soon after the introduction of PCV7[76].

As mentioned above, along with those antibiotic-resistant clones, PMEN also focus on some important disease-causing susceptible clones, such as the following related to serotypes included in one of the conjugate pneumococcal vaccines: Sweden[1]-27 (ST217), Sweden[1]-40 (ST304), Netherlands[3]-31 (ST180), Sweden[4]-38 (ST205), Portugal[6A]-41 (ST327), S.Africa[6B]-8(ST185), Netherlands[7F]-39 (ST191), and Colombia[23F]-26 (ST338).

Serotype 1 ranks among the most prevalent invasive serotypes in many countries[84-87] causing severe episodes of pneumonia and empyema in children[88]. In Brazil, since 1977, serotype 1 has been identified as one of the most frequent pneumococci causing severe infections in both adult and pediatric patients[89].

Some specific features are responsible for the epidemiological relevance of serotype 1, despite its susceptibility to most antimicrobials. First, a low colonization frequency, even in populations in which serotype 1 is a frequent cause of pneumococcal infections[90,91]. In addition, serotype 1 has the ability to cause outbreaks in communities and in crowded and closed institutions[92]. Besides, serotype 1 markedly presents low genetic diversity among the isolates, which has been associated with the short duration of carriage and/or a low density of this serotype in the nasopharynx, resulting in a reduced opportunity to exchange genes between strains[93].

Another serotype with high invasiveness power is serotype 3, which has been related with increased mortality in different regions[94-96]. Considering its genetic background, strains of serotype 3 belonging to ST180 have been associated with significant mortality[97]. Therefore, the high frequency of isolation of this serotype/ST and its relation with mortality needs continued surveillance to monitor for increases in this serotype post-PCV10 as this data may be important to consider the use of PCV13 in some regions.

Although somewhat controversial, serotype 7F also appears to be associated with high case-fatality[95]. Some authors have observed serotype 7F as one of the main serotypes associated with replacement following PCV7 introduction, through clonal expansion[98,99]. Pichon et al. demonstrated ST191 (serotype 7F) as the most prevalent clone causing meningitis 3 years after the introduction of PCV7 in England and Wales[99]. From reported studies, serotype 7F seems to be very rare in the nasopharynx of Brazilian children[59,73,90]. Besides, as it is part of the currently available pneumococcal vaccine (PCV10), it may not represent a worrisome occurrence in Brazil.

PCV7 was introduced in the United States in 2000, when almost half of all IPD was caused by pneumococci resistant to penicillin and/or macrolides[100]. As expected, following the introduction of pneumococcal vaccination, there was a substantial reduction in penicillin non-susceptible pneumococci occurrence[101]. However, subsequently to PCV7 usage, there has been an increase in pneumococcal disease due to non-PCV7 type pneumococci[102], many of which are now also penicillin non-susceptible, such as 19A and 6A, that are part of other vaccine formula, as well as other serotypes absent in any pneumococcal vaccine so far[103]. Therefore, despite the unquestionable beneficial effects of vaccination, the problem of resistance among pneumococci is far from solved.

In this context, eight PMEN clones include strains with serotypes not present in any of the currently available vaccine formula. They are related to the following STs: ST53 (Netherlands[8]-*33),* ST63 (Sweden[15A]-25), ST193 (Greece[21]-30), ST199 (Netherlands[15B]-*37),* ST218 (Denmark[12F]-34), ST377 (Utah[35B]-24), ST448 (USA[NT]-43), and ST344 (Norway[NT]-42). In general, they are multi-susceptible.

Some molecular characteristics of the Netherlands[8]-33 may explain its well-succeeded clonal spread: Jefferies et al. identified a pneumolysin allele 5 in ST53, a common worldwide-distributed ST related to serotype 8, that could facilitate the clonal expansion of those strains[104]. Besides, some authors include serotype 8 into the group of more invasive and/or the ones related to the worst outcomes. Therefore, as it

may become an important serotype in the post-vaccination era, and as antimicrobial usage may stimulate resistance occurrence, surveillance of these widely distributed serotype 8 clones is a subject of major concern.

Grabenstein et al. performed a systematic review to characterize differences in serious outcomes between pneumococcal serotypes. The authors show that serotype 8 was consistently related to an increase in severity of the disease, as well as serotype 15B[105]. The Netherlands^{15B}-37 is part of ST199, which also encompasses serotype 19A (among others), strongly related to penicillin resistance. The occurrence of the same genetic background (ST199) between serotype 19A and serogroup 15 is indicative of capsular switching.

Sweden^{15A}-25 belongs to ST63, which is worldwide distributed, including Latin America. This ST is essentially related to serotypes 15A, 14, 19F, and 19A. The capsular type 15A strain was found to only differ from the fully sequenced 19F clinical isolate G54 in the chemical composition of the capsular polysaccharide indicating that this lineage has the capacity to undergo *in vivo* capsular switch. A capsular switch may produce "vaccine escape" recombinants[94] that can avoid the vaccine-induced immune pressure, allowing pneumococcal survival as a species.

Of note, the serotype 19A (ST276) and 15A (ST63) clones have been identified as the *S. pneumoniae* clonal types most frequently recovered from pneumococcal infections in countries that introduced the PCV7 vaccine[80,106,107]. For unknown reasons, representatives of the third major colonizing clone with serotype 6A (ST2191) have not been recovered from pneumococcal disease. In contrast, colonization by each of the three major non-PCV7 clonal lineages has been widely reported[108].

Although Utah^{35B}-24 (ST377) presents a susceptible phenotype (albeit resistance to penicillin, considering meningitis CLSI criteria – MIC 1µg/mL), some post-vaccine works have demonstrated a relationship between this serotype and resistant profiles[109], as well as increased occurrence of this serotypes (and others such as 15A and 15B) in both carriage and invasive disease. Surveillance of serogroup 15, considering dissemination and resistance, may be of great relevance to the development of new vaccine formulations.

Similarly for serotype 8, serotype 12F has demonstrated increased occurrence after vaccination in some regions. Besides, it has been shown to cause outbreaks in human populations with identifiable risk factors[110]. This serotype has a high case/carriage ratio (CCR), i.e., it is a hyper invasive serotype, rarely found in nasopharynx[73,110]. One could expect that, after vaccine selective pressure, the pneumococcal population is supposed to suffer considerable changes, which may affect the behavior of such non-vaccine serotypes.

Based on the above, it is reasonable to conclude that the pneumococcal population is constantly changing, either because of biologically expected temporal changes or due to selective pressure exerted by vaccination and antibiotics. This situation may significantly alter the occurrence of antimicrobial resistance, and, in this context, epidemiological surveillance is consistently required to understand and monitor these changes, as they may directly affect patient care, as discussed below.

CONCLUSION

Pneumococcal infections are treated empirically. Limitations in the diagnostic methods, together with the severity of disease contribute to this procedure. Surveillance studies are crucial to define the prevalence of resistant strains both globally and in a particular region. Data obtained from such studies are generated by culture-dependent methods. Although different clones of PNSP are internationally distributed, and considering diseases other than meningitis, the prevalence to penicillin is quite low, making this old, safe, and inexpensive drug an attractive first choice to treat pneumococcal infections. The widespread use of conjugate vaccines among children, influencing the circulation of resistant clones, reinforces the need of surveillance studies to define the prevalence of resistance.

Finally, it is important to consider that almost all that is known about pneumococcal resistance comes from culture-insensitive methods. Culture independent methods are, in a certain sense, modifying some concepts about pneumococcal disease and once applied to the detection of resistant strains, they may also contribute to a better knowledge about resistance in pneumococci.

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