



Streptococcus pneumoniae: a study of strains isolated from cerebrospinal fluid

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Abstract

Objective: To determine the frequency of capsular serotypes and the antimicrobial susceptibility of strains of *Streptococcus pneumoniae*, as well as to provide recommendations on the use of available vaccines and antimicrobial drugs.

Methods: In this retrospective study, standard procedures were followed to identify, serotype, and determine bacterial susceptibility to penicillin, cefotaxime, and vancomycin. Pneumococcal strains were isolated from the cerebrospinal fluid (CSF) of patients admitted to nine public and three private hospitals in Distrito Federal, Brazil, between January 1995 and December 2004. Identification and antimicrobial susceptibility tests were carried out at the Central Laboratory of Public Health (Laboratório Central de Saúde Pública). Serotyping was performed at Instituto Adolfo Lutz.

Results: A total of 232 pneumococcal strains were isolated, including 126 (54.31%) strains from male patients. Patients had an age range of 0 to 62 years and were distributed into four age groups: 0 to 5, 6 to 17, 18 to 50, and above 50. From the 36 distinct serotypes identified, eight were more prevalent: 14, 6B, 18C, 5, 19F, 23F, 9V, and 6A. The oxacillin test identified 67 penicillin-resistant strains, out of which 47 were confirmed by the E test as having intermediate level of resistance. None of the strains exhibited high-level resistance.

Conclusion: Pneumococcal resistance to penicillin has gradually increased over the last 10 years in Distrito Federal. Serotypes more frequently isolated in the 0 to 5 years age group were the same involved in penicillin-resistance, all of which are covered by the 7-valent vaccine.

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Introduction

Streptococcus pneumoniae is a bacterium that belongs to the family Streptococcaceae, commonly found in the

nasopharyngeal and oropharyngeal mucosa of healthy human beings. It is the bacterial pathogen most commonly associated with acute otitis media and pneumonia, and the second most important pathogen in cases of meningitis in children under 2 years of age. In the United States and Europe, 25% to 40% of the cases of meningitis are caused by pneumococcus.^{1,2}

Mortality from pneumococcal infection has decreased significantly over the first decades of the last century, following the introduction of antimicrobial drugs, such as sulphonamide and penicillin.³ Penicillin-resistant pneumococci were first reported in New Guinea in the 1960s. Ten years later, cases of resistance were described in Africa and Spain.^{4,5} The emergence of resistant strains is caused

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mostly by the frequent exposure to antimicrobial agents, especially in children,⁶ and also by community-acquired infections.⁷ In the last years, reports of infections caused by pneumococcus have become increasingly common, and penicillin-resistant *S. pneumoniae* infection is now observed throughout the world.^{3,4,6,8-10}

Penicillin has long been the drug of choice for the treatment of pneumococcal meningitis.^{8,11} Selection of the initial empirical treatment is based on resistance rates to penicillin and other antimicrobial agents, clinical suspicion, and rapid laboratory tests, such as Gram stain and latex agglutination test of the cerebrospinal fluid (CSF).

The worldwide high incidence of penicillin-resistant strains of *S. pneumoniae* entails the need to previously isolate, identify the etiological agent, and perform *in vitro* susceptibility tests in order to decide on whether or not to maintain the initial empirical therapy. Penicillin remains as the drug of choice when pneumococcus is sensitive to this antibiotic.⁵

Currently more than 90 pneumococcal serotypes have been identified based on antigenic differences in the capsular polysaccharides,² with distribution varying according to age group, clinical symptoms, and geographic area. Regional differences in the prevalence of specific serotypes highlight the importance of knowing the serotypes for each geographic area.^{9,12-15}

The vaccine, prepared from purified polysaccharides, has the advantage of including in its composition 23 different serotypes. It is recommended for children above 2 years of age, susceptible to pneumococcal invasive disorders, and for people over 65 years.¹⁶ Immunogenicity, however, increases when polysaccharide antigens are individually conjugated to a carrier protein, as in the 7-valent vaccine, justifying its indication in vulnerable populations (e.g., children under 2 years of age, the elderly, and immunocompromised persons).¹⁴

In 1993, the Regional System of Vaccines (Sistema Regional de Vacinas, SIREVA) was implemented in Brazil, sponsored by the Pan American Health Organization and the Brazilian Health Ministry. This project is responsible for laboratory vigilance of *S. pneumoniae* in Latin America, significantly contributing to the investigation of prevalent serotypes in each area and levels of resistance to antimicrobial agents commonly used.^{1,9,12}

The goal of the present study was to determine the frequency of capsular serotypes of *S. pneumoniae* and their susceptibility to penicillin and other antimicrobial drugs, identifying isolates with high- (PEN-R) and intermediate-level (PEN-IR) penicillin resistance in a sample of patients with suspected meningitis. This information can provide

evidence to support the indication of available vaccines and the use of antimicrobial agents, especially penicillin.

Methods

Patients

We performed a retrospective study of *S. pneumoniae* strains isolated from the CSF of patients with meningitis. Material was collected from nine public (Hospital Regional de Taguatinga – HRT; Hospital Regional da Asa Sul – HRAS; Hospital Regional do Gama – HRG; Hospital Regional da Ceilândia – HRC; Hospital Regional da Asa Norte – HRAN; Hospital Regional de Planaltina – HRP; Hospital Regional de Sobradinho – HRS; Hospital Regional de Brazlândia – HRBz; Hospital de Base do Distrito Federal – HBDF) and three private (Hospital Santa Luzia, Hospital Anchieta e Hospital da UNIMED) hospitals in Distrito Federal.

A total of 232 pneumococcal isolates were included in this study, 126 being from male patients and 83 from female subjects. Age ranged from 0 to 62 years, and patients were distributed into four age groups: 0 to 5, 6 to 17, 18 to 50, and above 50. Twenty three (9.91%) of the tests ordered did not indicate patients' sex, and 21 (9.05%) did not include patients' age. This study covers the period between January 1995 and December 2004. The study was approved by the Research Ethics Committee (report number 088/2006 in reference to research project number 084/06).

Microbiological identification

Bacterial growth in cultures was identified as *S. pneumoniae* based on colony morphology, alpha-hemolysis on Mueller-Hinton agar supplemented with 5% sheep blood, Gram stain, sensitivity to optochin (5 µg) and bile solubility.¹⁷

Storage

Strains were grown in brain heart infusion (BHI) broth, supplemented with 16% glycerol and frozen at -70 °C.

Serotyping

This procedure was performed at Instituto Adolfo Lutz, in São Paulo, Brazil, which is the national reference center for bacterial meningitis. Quellung reaction was performed using antiserum from the Statens Seruminstitut Copenhagen Denmark.¹⁸

Quality control

Susceptibility tests were performed in accordance with international standards,¹⁹ with *Staphylococcus aureus* ATCC 25923 and *S. pneumoniae* ATCC 49619 as reference strains.

Antimicrobial susceptibility tests

Disk diffusion

Oxacillin (1 µg) disk screening was used for the detection of penicillin resistance. Disk diffusion to determine vancomycin susceptibility used vancomycin 30 µg. Both tests were performed on Mueller-Hinton agar supplemented with 5% sheep blood, and test samples were incubated for 20 to 24 h at 35 °C±2 °C in a 5% CO₂ atmosphere.

Minimum inhibitory concentration (MIC)

Penicillin and cefotaxime E-test strips were applied to the surface of Mueller-Hinton sheep blood agar plates and incubated at 35 °C±2 °C in a 5% CO₂ atmosphere for 20 to 24 h. Values were read from the scale at the point of intersection between the inhibition ellipse edge and the E-test strip by use of reflected light. Care was taken to determine the margin of pneumococcal growth, not the area of alpha-hemolysis of the medium.²⁰

Results

Distribution according to location, sex, and age group

Hospitals with the highest number of *S. pneumoniae* isolates were HRT, with 64 isolates (27.59%); HRAS, with 49 isolates (21.12%); HRG, with 45 isolates (19.40%); and HRC, with 17 isolates (7.33%). Prevalence of *S. pneumoniae* was higher in males than females, with 126 isolates (54.31%) in the male population. In 23 cases (9.91%), patients’ sex was not specified in the tests ordered.

The highest number of *S. pneumoniae* strains was found in the age group 0 to 5 years, with 140 isolates (60.35%). The other age groups presented the following rates: 6 to 17 years – 33 strains (14.22%), 18 to 50 years – 34 strains (14.65%) and above 50 years – 04 strains (1.72%). In 21 cases (9.05%) patients’ age was not specified.

Serotyping

Among the 232 isolates characterized as *S. pneumoniae*, 36 distinct serotypes were identified, distributed by age group (Table 1).

Susceptibility to penicillin and other antimicrobial agents

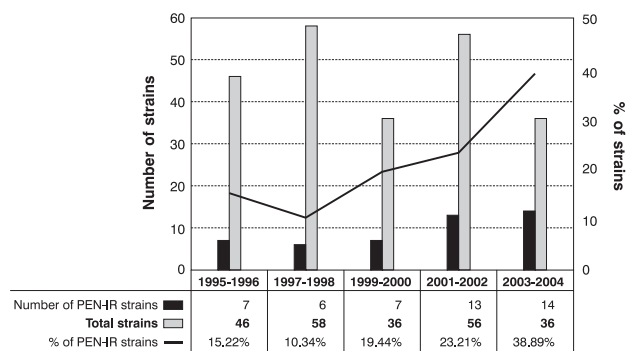
From all the isolates analyzed, 67 showed a zone of inhibition of <20 mm around the oxacillin disk, thus identifying 67 penicillin-resistant strains. After the E-test, 47 strains (70.15%) were confirmed as having intermediate-level resistance to penicillin, while 20 (29.85%) proved to be sensitive to the drug. None of the strains exhibited high-level resistance, based on MIC.

values for penicillin were as follows: ≤ 0.06 µg/mL, sensitive; 0.12 to 1.0 µg/mL, intermediate-level resistance; and ≥ 2.0 µg/mL, high-level resistance.

MIC values for cefotaxime were ≤ 0.5 µg/mL, sensitive; 1.0 µg/mL, intermediate; and ≥ 2.0 µg/mL, resistance. It is important to emphasize that all investigated strains presented values equal to or below 0.5 µg/mL, thus characterizing sensitivity to cefotaxime. In addition, all isolates showed a zone size equal to or greater than the cut-off diameter (17 mm), therefore showing sensitivity to vancomycin.

In the age group 0 to 5 years, which concentrated the highest number of isolates, 34 PEN-IR strains were found, corresponding to 72.34% of the 47 strains isolated with this level of penicillin susceptibility (Table 2).

A gradual increase in the number of PEN-IR isolates was observed for the period between 1995 and 2004, as shown in Figure 1.



Source: Distrito Federal, Secretaria de Estado de Saúde, Subsecretaria de Vigilância à Saúde, Laboratório Central de Saúde Pública do Distrito Federal, Gerência de Biologia Médica, Núcleo de Bacteriologia.

Figure 1 - Number and percentage of PEN-IR strains of *Streptococcus pneumoniae* isolated from cerebrospinal fluid, according to age group, in Distrito Federal, from 1995 to 2004

Relationship between serotype and penicillin-susceptibility

Analysis of PEN-IR isolates revealed that 19 strains were serotype 14; 10 were serotype 6B; and five were 23F. These serotypes also predominated in the 0 to 5 years age group (Table 3).

Discussion

Although data on strains of *S. pneumoniae* in Distrito Federal have been previously reported in other national^{1,21} and international^{9,12} studies, our study presents results specifically for this region, showing with greater clarity, and for the first time, the susceptibility pattern of these strains to penicillin, cefotaxime, and vancomycin, as well as indicating the most common capsular serotypes.

Table 1 - Distribution of capsular serotypes of *Streptococcus pneumoniae* isolated from cerebrospinal fluid, according to age group, in Distrito Federal, from 1995 to 2004

Serotype	Total		Age group				
	n	%	0-5 years	6-17 years	18-50 years	> 50 years	Age unknown
14	41	17.67	33	2	0	0	6
6B	22	9.48	17	3	0	0	2
18C	16	6.90	15	0	1	0	0
5	11	4.74	8	2	1	0	0
19F	11	4.74	7	1	0	0	3
23F	8	3.45	4	2	1	0	1
9V	8	3.45	5	0	0	0	3
6A	7	3.02	5	0	1	0	1
19A	6	2.60	2	2	1	0	1
3	6	2.60	0	1	3	1	1
4	6	2.60	2	1	2	1	0
10A	5	2.16	2	1	1	0	1
9N	4	1.72	2	0	2	0	0
1	3	1.29	2	1	0	0	0
15B	3	1.29	0	2	0	1	0
18B	3	1.29	3	0	0	0	0
18F	3	1.29	2	1	0	0	0
12F	3	1.29	0	2	1	0	0
8	2	0.86	1	0	1	0	0
17F	2	0.86	1	1	0	0	0
12	2	0.86	2	0	0	0	0
13	2	0.86	0	0	2	0	0
15C	2	0.86	0	0	2	0	0
34	2	0.86	1	1	0	0	0
18A	2	0.86	2	0	0	0	0
7F	2	0.86	0	0	1	0	1
11A	2	0.86	0	1	1	0	0
21	2	0.86	1	1	0	0	0
33F	1	0.43	0	0	1	0	0
23B	1	0.43	1	0	0	0	0
24F	1	0.43	0	0	1	0	0
28A	1	0.43	1	0	0	0	0
9	1	0.43	1	0	0	0	0
7	1	0.43	0	0	1	0	0
31	1	0.43	0	0	0	1	0
22F	1	0.43	1	0	0	0	0
No serology*	38	16.39	19	8	10	0	1
Total	232	100.00	140	33	34	4	21

Source: Distrito Federal, Secretaria de Estado de Saúde, Subsecretaria de Vigilância à Saúde, Laboratório Central de Saúde Pública do Distrito Federal, Gerência de Biologia Médica, Núcleo de Bacteriologia.

* Strains were not serotyped because isolates became non-viable after transportation to Instituto Adolfo Lutz.

The elevated number of pneumococcal isolates in the age group 0 to 5 years (60.43%) is an important finding, also reported by other authors, with male children being

more predisposed to invasive pneumococcal infections in this age group.^{3,9,12} The small number of patients above 50 years of age (4 strains) prevents a proper evaluation of

Table 2 - Penicillin susceptibility profile of *Streptococcus pneumoniae* strains isolated from cerebrospinal fluid, according to age group, in Distrito Federal, from 1995 to 2004

Age group	Total	Susceptibility to penicillin		
	n (%)	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
0-5 years	140 (60.35)	106 (75.71)	34 (24.29)	0 (0.00)
6-17 years	33 (14.22)	28 (84.85)	05 (15.15)	0 (0.00)
18-50 years	34 (14.66)	32 (94.12)	02 (5.88)	0 (0.00)
> 50 years	04 (1.72)	04 (100.00)	00 (0.00)	0 (0.00)
Age unknown	21 (9.05)	15 (71.43)	06 (28.57)	0 (0.00)
Total	232 (100.00)	185 (79.74)	47 (20.26)	0 (0.00)

Source: Distrito Federal, Secretaria de Estado de Saúde, Subsecretaria de Vigilância à Saúde, Laboratório Central de Saúde Pública do Distrito Federal, Gerência de Biologia Médica, Núcleo de Bacteriologia.

Table 3 - Capsular serotypes associated with PEN-IR strains, according to age group, in Distrito Federal, from 1995 to 2004

Serotype	Total	Age group				
	n (%)	0-5 years	6-17 years	18-49 years	> 50 years	Unknown
14	19 (40.42)	15	1	0	0	3
6B	10 (21.27)	7	2	0	0	1
23F	5 (10.64)	2	1	1	0	1
19F	2 (4.25)	1	1	0	0	0
23B	1 (2.13)	1	0	0	0	0
5	1 (2.13)	0	1	0	0	0
6 ^a	1 (2.13)	1	0	0	0	0
19 ^a	1 (2.13)	0	1	0	0	0
9	1 (2.13)	1	0	0	0	0
9V	1 (2.13)	1	0	0	0	0
No serotype	5 (10.64)	3	1	1	0	0
Total	47 (100.00)	32	8	2	0	5

Source: Distrito Federal, Secretaria de Estado de Saúde, Subsecretaria de Vigilância à Saúde, Laboratório Central de Saúde Pública do Distrito Federal, Gerência de Biologia Médica, Núcleo de Bacteriologia.

serotype distribution in this age group, especially when considering that pneumococcal pneumonia is a serious disease that affects mostly this group of patients and that ideal isolates for such analysis should come from blood cultures.

From the 36 serotypes isolated in Distrito Federal, the following eight were more commonly found, in decreasing order of prevalence: 14, 6B, 18C, 19F, 5, 9V, 23F, and 6A, which is in accordance with other studies carried out in Brazil.⁵ In other countries, although these serotypes also figure among the most prevalent, data concerns mostly invasive strains isolated from CSF and blood.^{2,9,22}

The great diversity of serotypes identified in this study, and also described by other authors, is associated with the fact that the samples used in this study came from patients with meningitis, and not pneumonia.^{1,22} Serotype 14, the most commonly isolated serotype in cases of meningitis,¹ was the most frequent serotype in the whole sample, as well as in the 0 to 5 years age group. These findings are also in accordance with previous reports in the literature (United States, Canada, Europe, Oceania, and Latin America).^{1,2,9,22} In Brazil, serotype 14 predominates in the states of São Paulo and Pernambuco.²²

Serotypes 9V and 18C are included among the eight more prevalent serotypes, as described elsewhere for Belo Horizonte and Recife, Brazil.²² Serotype 5, uncommon in Europe, Canada, and the United States,^{1,9} appeared in our study as the fourth most prevalent serotype in Distrito Federal, which is in accordance with data from SIREVA.¹ On the other hand, this serotype shows reduced prevalence in Brazil when compared to other South American countries.¹

Serotype 1 showed a low prevalence in the 0 to 5 years age group, which has also been demonstrated in the city of Salvador, Brazil, where it was shown to be associated with pneumonia.³ Reports from other South American countries have highlighted the significance of serotype 1.^{9,12} This serotype predominates in Asian countries.²

Considering the cross-reaction between serotypes 6A and 6B,¹⁵ coverage of the 7, 9, 11 and 23-valent conjugate vaccines was calculated including serotype 6A. From the 36 serotypes identified in this study, 21 are included in the 23-valent pneumococcal polysaccharide vaccine, providing vaccine coverage for 73.28% of the isolated strains. According to bibliographical data, this vaccine is not effective for children between 0 and 2 years of age, being used most often in the adult and elderly populations.^{1,21}

Coverage of the 7-valent conjugate vaccine (14, 6B, 18C, 19F, 4, 9V, and 23F) was 51.30% of the total strains, and 61.42% of the strains found for the 0 to 5 years age group. Our data is equivalent to those found in studies in

Mexico⁹ and other Brazilian states,^{1,9} while vaccine coverage is higher than that reported for Argentina, Chile, and Uruguay.⁹ The 9-valent conjugate vaccine, which includes serotypes 1 and 5 in addition to those contained in the 7-valent vaccine, increased vaccine coverage to 57.33% of total strains and to 68.57% in the 0 to 5 years age group.

The 11-valent conjugate vaccine, which includes serotypes 3 and 7F in addition to those contained in the 9-valent, increased vaccine coverage to 60.78% of total strains. However, for the 0 to 5 years age group the coverage remains the same as in the 9-valent, since serotypes 3 and 7F were not identified in isolates from this age group.

Until today, PEN-R strains have not been a serious local problem. Up to the last year analyzed in this study, no PEN-R strains had been identified. The reality of countries such as Spain,^{6,11,13} Hungary, France, Japan, and Canada,⁶ where the prevalence of high-resistance strains is significant, differs considerably from the situation observed in Brazil, where data show that high-level resistance to penicillin is still rare.^{8,9,23} During the studied period, 47 PEN-IR strains were identified, corresponding to 20.26% of total isolates. This percentage is in accordance with previous data reported for Brazil,^{3,9} and differs from other Latin American countries, where PEN-IR strains surpass 30%.⁹ Nevertheless, 10-year analysis of penicillin resistance levels suggests that the PEN-IR strains have been gradually increasing, emphasizing the need to monitor resistance not only to penicillin, but also to other antimicrobial agents.

The decrease in the percentage of PEN-IR strains observed in the period from 1997 to 1998 may have been influenced by technical problems in the transportation of samples to Instituto Adolfo Lutz during this period, halting the serotyping of most isolated strains. Since there was not a reduction in the absolute number of isolates in this period, we can assume that these serotypes are not related to those more frequently associated with penicillin resistance. On the other hand, in the period between 2003 and 2004, when we observed an increase in the percentage of PEN-IR strains (38.89%), the most frequent serotypes were penicillin-resistant.

Resistance to cefotaxime was not observed in this study; however, other reports from Brazil have demonstrated low rates of resistance.^{3,8} This issue has started to cause some concern in face of the indiscriminate use of oral cephalosporin in children, exerting a selective pressure and inducing an increase in pneumococcal resistance.⁶

Considering that vancomycin, associated with other drugs, is a therapeutic option in the treatment of meningitis, and since, until now, there has been no report of pneumococcal resistance to this agent,^{6,8,10} prescription of vancomycin must be reserved for situations in which other agents can not be used.

Penicillin-resistant serotypes were, in decreasing order of prevalence, 14, 6B, 23F, and 19F. These data are similar to those reported previously.^{8,9,12,23} Serotype 19A, described as involved in penicillin resistance,^{8,9,12} occurred only once in this study. Among the 36 isolated strains, 10 show PEN-IR, out of which five (14, 6B, 23F, 19F e 9V) are targeted by the 7-valent conjugate vaccine.

Conclusion

Results obtained in this study allow us to conclude that penicillin, the drug of choice in the treatment of pneumococcal meningitis, shows a gradual increase in resistance over the last 10 years in Distrito Federal. Nevertheless, high-level resistance strains were not found.

Despite the limited number of *S. pneumoniae* strains analyzed in this study and the fact that some were not serotyped, the authors suggest the use of the 7-valent conjugate vaccine as an important strategy in the prevention of invasive diseases, since the vaccine targets the serotypes most commonly identified in children from 0 to 5 years, which are also the most frequently identified among PEN-IR strains.

Data collected here reinforce the importance of epidemiological vigilance of these infections, as well as the need to send to central reference laboratories not only CSF of patients with suspected meningitis, but all pneumococcal isolates from sterile sites.

Finally, it must be highlighted that, due to rapid changes in patterns of bacterial resistance, it is important to monitor and serotype these infectious agents with local standards of reference, since there is significant variability across different regions of the country and abroad.

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