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ORIGINAL ARTICLE

Prevalence of serotypes and antimicrobial resistance of invasive strains of Streptococcus pneumoniae

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Abstract

Objective: To determine the prevalence of serotypes and antimicrobial susceptibility of invasive strains of *Streptococcus pneumoniae* and to discuss the implications of these findings for vaccine formulation.

Method: Strains of *Streptococcus pneumoniae* obtained from normally sterile fluids from patients admitted with invasive diseases were isolated and identified at the Hospital de Clínicas, Universidade Federal de Uberlândia, state of Minas Gerais, and forwarded to Instituto Adolfo Lutz, state of São Paulo, for further identification, serotyping and determination of antimicrobial susceptibility.

Results: From April 1999 to March 2003, 148 invasive strains of *Streptococcus pneumoniae* were obtained. The age of patients ranged from 1 day to 88.83 years (mean: 21.33 ± 25.82 years; median: 4.42 years). Eighty-four (56.7%) patients were male. The most common diagnoses were pneumonia (91 cases; 61.4%), meningitis (32 cases; 21.6%) and occult bacteremia (15 cases; 10.1%). Strains were isolated mostly from blood (76 occasions; 51.3%), pleural fluid (39 occasions; 26.3%) and cerebrospinal fluid (30 occasions; 20.2%). There were 23 different serotypes, and the most common were 14, 3, 1, 5, 6A, 6B and 18C. Among 30 (20.2%) oxacillin-resistant strains, 23 (15.5%) were confirmed as resistant to penicillin (12.8% intermediate resistance and 2.7% full resistance). Oxacillin-resistant strains were restricted to serotypes 14, 23F, 19A and 6B. Resistance to penicillin varied with age, being more common in children under two years of age ($p = 0.0008$). We observed decreased sensibility to sulfamethoxazole-trimethoprim (92 isolates; 63.4%), to erythromycin (12 isolates; 8.3%), to clindamycin (12 isolates; 8.7%), to ofloxacin (one strain; 0.8%) and to cefotaxime (three strains; 2%; also resistant to penicillin). All isolates were susceptible to chloramphenicol, rifampin and vancomycin.

Conclusion: The decreased susceptibility to penicillin, detected in 15.5% of the strains was predominant in children under two years of age. There were 23 different *Streptococcus pneumoniae* serotypes. The 23-valent polysaccharide vaccine covers 82.6% of the serotypes and 90.2% of the invasive strains isolated in this population. In addition, 46.7% of the serotypes and 63.6% of the strains isolated from children until five years of age are covered in the currently available 7-valent conjugated vaccine (PN CRM7).

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Introduction

Streptococcus pneumoniae continues to be a major cause of morbidity and mortality among people of all ages, all over the world. Children less than two years old, people over 65 and sufferers from certain chronic debilitating and/or immunosupressing diseases are particularly susceptible.[1,2] Treatment with antibiotics is not capable of eliminating pneumococcal pneumonia lethality, probably because drugs reduce lethality very little during the first three to five days of the disease.[3,4] Furthermore, during the last decade, pneumococcus resistance to penicillin and other antimicrobials has increased in many countries.[5,6] This being the case, prevention is the strategy most indicated to reduce still further the incidence rates of this disease.[7]

Prevention of invasive diseases is basically founded on active immunization.[2,7,8] A vaccine containing purified pneumococcus capsular polysaccharide antigen is effective for young, immunologically competent adults, but for particularly susceptible individuals its efficacy is reduced or even null.[2,7] Under these conditions - which are the target for recommending vaccination - polysaccharide antigens have little immunogenic effect, produce a weak humeral response, are of short duration and have no memory.[2,7] The vaccine includes 88% of the serotypes that cause bacteremia and meningitis in adults, around 100% of those responsible for bacteremia and meningitis in children and 85% of the serotypes recovered from children with acute otitis media in the USA.[2,8] In Brazil, according to a survey performed in greater São Paulo between 1977 and 1992, 89.7% of the serotypes that predominate in children with bacteremia and pneumonia (14, 1, 5, 6B, 9V, 4 and 6A) and 76.1% of those isolated from cerebrospinal fluid (1, 6B, 14, 6A, 18C, 3, 5 and 23F, among others), are present in the vaccine.[9,10]

Recently a heptavalent vaccine containing capsular polysaccharides from the 4, 6B, 9V, 14, 18C, 19F and 23F serotypes, conjugated to a protein carrier, was licensed in the USA.[11,12] These serotypes are responsible for around 85% of pneumococcal diseases in that country.[13,14] The immunogenicity of the polysaccharide is amplified by the covalent bond with the protein molecule, in particular in children less than 2 years old,[11,12] in addition to the vaccine offering protection against oropharynx colonization by the serotypes it contains.[11,12,15]

Epidemiological features of pneumococcal disease vary from country to country and over time, which results in the need for periodic local assessments in order that control strategies may be established.[13,16] Depending on the capsular polysaccharide antigen, to date 90 serotypes of pneumococcus are recognized and decisions on the formulation and application of vaccines depend on regional and temporal data on which of these serotypes are causing diseases.[13,16] Additionally, antimicrobial resistance monitoring is of great importance to clinical practitioners, supplying a rationale for the choice of initial empirical treatment of pneumococcal diseases.[5,6]

The SIREVA (Sistema Regional de Vacinas) regional vaccine project, sponsored by the Pan American Health Organization and the Health Ministry (National Health Foundation), provide laboratory vigilance of Streptococcus pneumoniae in Latin America. In Brazil, the project was started in 1993 and, since then, a large number of different publications have revealed a certain amount of diversity in serotype distribution and antimicrobial resistance, in different parts of the country and over time.[9,17-20] It is important that each community is aware of the profile of prevalent serotypes in their area and the levels of in vitro resistance to the antimicrobial agents commonly employed as the basis of measure to combat and prevent disease caused by pneumococcus.

Therefore, the objective of this study is to document the profile of serotypes and sensitivity to antimicrobial agents of the strains of streptococcus pneumoniae found in the clinical specimens of individuals treated for invasive diseases at the Hospital de Clínicas of the Universidade Federal de Uberlândia, Minas Gerais and to evaluate the implications for the formulation of antipneumococcal vaccines.

Patients and methods

Strains of pneumococcus isolated at the Bacteriological Unit of the Clinical Analysis Laboratory of the Hospital de Clínicas of the Universidade Federal de Uberlândia (HCUFU), from clinical specimens (cerebrospinal fluid, blood, pleural fluid, peritoneal fluid, joint fluid and secretions from closed abscesses) collected from patients with invasive pneumococcal disease at the HCUFU were sent to the Instituto Adolfo Lutz (IAL) for confirmation of identification, serotyping and antimicrobial sensitivity testing. The specimens, obtained aseptically were duly processed and seeded in sodium thioglycollate broth (blood samples) or on chocolate agar or blood agar (all other samples) plates as soon as possible after collection and immediately on arrival at the laboratory. After incubation at 37 °C and in the event of clouding of the content of the flasks or of growth on the plates, colonies were submitted for identification.[10,21] Positive culture results were only taken into account once for each patient, irrespective of the number of positive samples. The project was approved by the Committee for Ethics in Research of the UFU.

Pneumococcus strains were isolated and identified according to usual methods.[10,21] In brief, after an incubation period of 12 to 18 hours, a suspicion of pneumococcus was established if colonies were identified that were small, round, transparent or opaque, mucoid and with a discrete greenish halo on the surface of blood agar or chocolate agar discs, revealing lancet-shaped

Gram positive diplococcus when stained. Discrimination from other alpha hemolytic streptococcus was performed using optochin discs (inhibition zone > 14 mm). Samples were then immediately forwarded to the Bacteriology Section at the IAL, São Paulo, SP, for confirmation of species, serotyping and in vitro antimicrobial susceptibility testing. On arrival species was confirmed by standard procedures[10,21] and the pneumococcus samples were lyophilized in skimmed milk at 20% and duly catalogued. Serotyping was performed by the quellung reaction according to a technique that has been described before,[22] using polyclonal antisera.[23]

The strains were subjected to penicillin susceptibility screening by the oxacillin diffusion method on a Mueller-Hinton agar plate supplemented with 5% lamb blood according to a standardized technique.[24] All strains presumed to be resistant to penicillin (inhibition zone < 19mm) were subjected to a minimum inhibitory concentration (MIC) test for penicillin and cefotaxime, by the broth microdilution technique.[25] Values for MIC result interpretation were ≤ 0.06 µg/ml was taken to be susceptible; 0.12 to 1.0 µg/ml as intermediate resistance and ≥ 2.0 g/ml as total resistance to pennicillin.[26] For cefotaxime, the values of ≤ 0.5 g/ml, 0.5 to 1.0 g/ml and ≥ 2.0 µg/ml as susceptible, intermediately resistant and completely resistant, respectively, for samples from cerebrospinal fluid and ≤ 1.0 µg/ml as susceptible, 2.0 µg/ml as intermediately resistant and ≥ 4.0 µg/ml as completely resistant for samples from patients without meningitis.[27]

Additionally, four antimicrobial agents were tested by broth microdilution (chloramphenicol, erythromycin, trimethoprim-sulfamethoxazole and vancomycin) and three others (tetracycline, ofloxacin and clindamycin), by the disc diffusion method.[24]

The chi-square ($\chi 2$) test was run on SPSS (Statistical Package for Social Analysis) 8.0 for Windows to compare proportions of different susceptibility patterns by age group. Tests of normality and homogeneity were performed on the samples whenever necessary. The level at which the null hypothesis was to be rejected was fixed at 5% ($p < 0.05$).

Results

In the four-year period from April 1999 to March 2003, 150 samples were sent to the IAL laboratory. Of these, the species was not confirmed in two cases. Of the remaining 148, 84 (56.7%) were from male patients and age varied from one day to 88.83 years, with a mean of 21.33 ± 25.82 years and a median of 4.42 years, with 25 - 75 interquartiles of 1.16 and 31.91 years. The number of valid samples obtained in each year between 1999 and 2003 was 22, 53, 29, 39 and 5, respectively.

The number of positive isolations per age group was 4 among newborns, 50 among infants from 29 days to 24 months old, 23 among children from 25 months to 60 months, eight among individuals 61 to 119 months old, 51 among those from 120 to 779 months of age and 12, in adults over 65. The most common clinical diagnosis was pneumonia [91 cases (61.4%)], followed by meningitis [32 cases (21.6%)] and then bacteremia with no obvious focus [15 cases (10.1%)] and the most common sources were blood [76 samples (51.3%)], pleural fluid [39 (26.3%)] and cerebrospinal fluid [30 (20.2%)]. A total of 23 different serotypes (ten of them members of five different serogroups) were identified from 143 samples tested (Table 1).

Table 1 - Distribution of *Streptococcus pneumoniae* serotypes isolated from patients admitted to the hospital with invasive disease from April 1999 to March 2003

Serotype	Amount	(%)
14	35	23.65
1	10	6.75
3	10	6.75
5	10	6.75
18C	8	5.41
6A	8	5.41
6B	8	5.41
10A	6	4.05
19F	6	4.05
9V	6	4.05
9N	5	3.38
4	4	2.70
7F	4	2.70
19A	4	2.70
23F	4	2.70
8	3	2.03
18A	3	2.03
15B	2	1.35
34	2	1.35
7	1	0.68
7C	1	0.68
11A	1	0.68
12F	1	0.68
22F	1	0.68
Nontypable	5	3.38
Total	148	100.00

Of the 148 strains tested, 30 (20.2%) were oxacillin resistant and, of these, 23 had penicillin resistance confirmed, of which 19 (12.8%) were at the intermediate level and four (2.7%) had total resistance. The maximum minimum inhibitory concentration found was 4 µg/ml, detected in a sample of serotype 14. Of the 23 samples with confirmed penicillin resistance, 16 were serotype 14 (12 with intermediate resistance and four with total), three were serotype 23F (with intermediate resistance), two serotype 19A (intermediate resistance) and, finally, two were serotype 6B (with intermediate resistance). Of the 23 strains with in vitro resistance to penicillin, 16

(69.5%) were obtained from patients aged less than two years. When this result was compared with the resistance among individuals above this age, the results were statistically significant (Table 2).

Table 2 - Distribution of *Streptococcus pneumoniae* strains with penicillin resistance, isolated in invasive disease, according to the patients' age

Age group (months)	Penicillin resistance		Total
	Susceptible *	Resistant†	
Up to 24	38	16	54
< 24	87	7	94
Total	125	23	148

Pearson's χ^2, with Yates correction, for 1 degree of freedom = 11.224 (p = 0.0008).
* Susceptibility detactable by the oxacillin disc screening test (1 µg): zone diameter > 19 mm.
† Reistance confirmed by the broth microdilution test: minimal inhibitory concentration > 0.06 µg/ml.

Total and intermediate resistance to cotrimoxazole was detected in 56.5% (82 strains) and 6.9% (10 strains) respectively of the 145 strains tested. With the exception of two strains, those that were resistant to penicillin were simultaneously resistant to cotrimoxazole (21 out of 23). Resistance to erythromycin was observed in the same 12 strains that were resistant to clindamycin, resulting in levels of 8.3% (12 of 144 samples tested) and 8.7% (12 of 138 samples tested), respectively. The level of resistance to ofloxacin was 0.8% (one out of 123 strains) and no resistance to chloramphenicol, rifampicin or vancomycin was observed. Resistance to cefotaxime was detected in three of the 30 strains that were tested (2% of 148), all of them with a confirmed resistance to penicillin.

Discussion

The most frequently isolated serotypes, in descending order of frequency, were 14, 3, 1, 5, 6A, 6B and 18C. Despite the limited dimensions of the population in question, it is possible to recognize similarities with results from surveys of a national [9,17-21] and international[14,16] scale. Of 1920 invasive strains obtained from 1993 to 1998, the seven most common serotypes found by Brandileone et al.[21] were 14, 1, 6B, 18C, 5, 3 and 6A and the results of the study when amplified to cover 23 years, from 1977 to 2000[20] show that, of 4,858 samples, the most often isolated invasive serotypes were 14, 1, 6B, 5, 18C, 6A and 3. In general, the profile of the most common serotypes found in developing countries was confirmed: 14, 6, 5, 1, 19, 9 and 23,[13,16] with prominence given to the elevated prevalence of serotypes 1 and 5, commonly found in Latin America.[19,21] The 23-valent polysaccharide vaccine offers protection from 23 antigens[2,7] and the conjugated heptavalent product, for serotypes 4, 6B, 9V, 14, 18C, 19F and 23F.[11,12] Therefore, of the 23 different serotypes isolated from this population, only serotypes 6A, 7C, 18A and 34 would not be covered by the polysaccharide vaccine, offering 82.6% cover for the serotypes and 90.2% cover against the strains that were isolated. The conjugated heptavalent vaccine, for children up to five years old, would cover 46.7% of the serotypes and 63.6% of the strains and would not cover, among others, serotypes 1, 3 and 5, which are often isolated, although they would cover serotypes 6B, 14, 19F and 23F, among which the penicillin resistant strains are included. In a recently published study, Brandileone et al.[20] estimated the potential impact of the heptavalent conjugated on Brazilian children aged 5 or less to be 58.2%, a value below the 70% - 88% achieved in the USA,[13,14] Canada and countries in Australasia, Africa and Europe.[13] The inclusion of serotypes 1 and 5 in a 9-valent product would raise cover for Brazilian children to 73.2%[20] and for the population in this study to 60% (of the serotypes) and 80.5% (of the strains).

Resistance to penicillin was detected in 15% of the strains tested, coinciding with the rate of 15.4% (14.5% with intermediate resistance and 0.9% total) described by Brandileone[21] on evaluating 2.050 invasive pneumococcus strains, collected between 1993 and 1998, originating from 14 different Brazilian states. The SIREVA Project vigilance system is voluntary and has attracted the collaboration of many different centers in different Brazilian states and Latin American countries over the last ten years[9,17,21] and the distribution of results by geographic region has revealed a certain amount of heterogeneous figures. For example, in the State of Minas Gerais, values of 12.8% for intermediate resistance and 2.1%, for total resistance were found from 94 strains collected between 1993 and 1998.[21] Widening the study to include samples collected up to 1999, but restricted to children up to six years old, found a global rate of resistance of 20.7%,[19] which value is similar to the 20% found in Salvador, Bahia, among 70 invasive pneumococcus strains collected from individuals aged between one month and 19.5 years.[28]

Resistance to penicillin was restricted to serotypes 14, 6B, 19A and 23F (respectively 70%, 15%, 10% and 5% of strains were resistant), which are classically associated with drug resistance.[5,6,19] They are known as "childhood serotypes" and are responsible for a large number of episodes of infection among children.[29] It is probable that the intrinsic characteristics of immunogenicity, depending upon age and the extent of exposure to antimicrobials contribute to, the predominance among young children and the development of resistance to penicillin, respectively.[2,5,6] In this study it was possible to confirm a tendency towards a

predominance of penicillin resistant strains, among children aged 2 years or less.

A redefinition of susceptibility categories has been proposed as a result of acceptable responses to treatment with beta lactams (penicillin or ampicillin) from patients with invasive pneumococcal disease (with the exception of meningitis), even when caused by strains that are resistant to penicillin (with a MIC up to 2.0 µg/ml).[30] For invasive strains, with the exception of those obtained from patients with meningitis, the proposed values are a MIC $\leq$ 1.0 µg/ml for susceptibility, 2.0 µg/ml for intermediate resistance and > 4.0 µg/ml for total resistance. By these new classifications, only three strains of pneumococcus, obtained from pneumonia patients, would be classed as intermediately resistant (MIC = 2.0 µg/ml) and none as totally so.

Certain observations can be made in respect of the susceptibility of the strains to other antimicrobial agents. The elevated level of resistance to cotrimoxazole (63.4%) is in agreement with reports in national studies (10,21,28,31) and could prejudice its indication for treatment of pneumococcal infections. Figures remain relatively low for erythromycin (8.3%), clindamycin (8.7%), ofloxacin (0.8%) and cefotaxime (2% of the strains that were resistant to penicillin), coinciding with reports by Brandileone (21) of a survey on a national scale (78.5% for cotrimoxazole, 2.4% for erythromycin) and by Nascimento-Carvalho et al.[28] in a recently published study, performed in Salvador, Bahia (65.7% for cotrimoxazole, 5.7% for erythromycin, 2.9% for clindamycin, 6.3% for ofloxacin and 5.9% for cefotaxime). Pneumococcus resistance to fluoroquinolones is considered uncommon or rare, but appears to be increasing in some parts of the world.[33-35] In contrast with what occurs with penicillin, resistance to these drugs is predominantly found in strains recovered from adults, particularly among those over 65 years of age, probably because of the elevated density of usage of these substances.[33] In a wide-ranging survey performed in the USA, it was possible to demonstrate a significant increase in resistance ton ofloxacin from 2.6% to 3.8%, in the period between 1995 and 1997 (significant differences when comparing groups above and below 18 years of age).

The fact that all of the strains that were resistant to erythromycin were also resitant to clindamycin, suggests a manifestation by the phenotype MLSB, which is characterized by inducible resistance to macrolides, lincosamides and streptogramin B.[32] There was no in vitro resistance tochloramphenicol, rifampicin or vancomycin.

Initial treatment for the majority of pneumococcal infections remains empirical in terms of etiology and drug sensitivity.[36] Community surveys are fundamental to determining the frequency and intensity of residence and are best interpreted when presented in strata, according to source of sample (nasopharyngeal swab, middle ear fluid, blood, cerebrospinal fluid, pleural fluid and others), nosology (otitis media, pneumonia, meningitis, bacteremia with no obvious focus, etc.) and age of patient.[36] Even so, there is no consensus on how to extract individual therapeutic recommendations from populational statistics and it is not possible to define the cut-off level at which resistance should impact on initial empirical treatment of a given pneumococcal disease.[36] The survey presented here may contribute to an understanding of the epidemiological behavior of pneumococcus within this population, particularly if monitored over time.

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