Spatial exploration of *Streptococcus pneumoniae* clonal clustering in São Paulo, Brazil

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ABSTRACT

Objectives: To examine the spatial distribution of *Streptococcus pneumoniae* and its clonal patterns collected between 2002 and 2006 in São Paulo, Brazil. **Methods:** As part of an observational study in São Paulo city, Brazil, *S. pneumoniae* isolates routinely cultured from blood, respiratory specimens, or cerebrospinal and other profound fluids were selected. Additionally, only isolates with either penicillin (PEN) intermediate (I) or resistant (R) status on routine antibiogram were included, in order to obtain a higher probability of clonal isolates. A single I/R *S. pneumoniae* isolate per patient was included and submitted to genotypic determination by pulsed field gel electrophoresis (PFGE). Minimum inhibitory concentrations (MICs) were determined for the isolates by Etest® to PEN and other antimicrobials. Each isolate was geocoded in a digital map. The Kernel function and ratio methods between total isolates vs. clones were used in order to explore possible cluster formations. **Results:** Seventy-eight (78) *S. pneumoniae* community isolates from two major outpatient centers in São Paulo, Brazil, were selected from the databank according to their penicillin susceptibility profile, i.e. R or I to penicillin assessed by oxacillin disc diffusion. Of these, 69 were submitted to PFGE, 65 to MIC determination, and 48 to spatial analytical procedures. Preliminary spatial analysis method showed two possible cluster formation located in southwest and southeast regions of the city. **Conclusion:** Further analyses are required for precisely determining the existence of *S. pneumoniae* clusters and their related risk factors. Apparently there is a specific transmission pattern of *S. pneumoniae* clones within certain regions and populations. GIS and spatial methods can be applied to better understand epidemiological patterns and to identify target areas for public health interventions.

Keywords: *Streptococcus pneumoniae*; penicillin resistance; drug resistance, bacterial; molecular epidemiology.

INTRODUCTION

Streptococcus pneumoniae is the main bacterial agent of many respiratory tract infections (RTI). The importance of this pathogen is not only related to its prevalence, especially in the pediatric population, but also to the risks associated to resistance development and its therapeutic consequences.

Antimicrobial resistance in pathogens causing RTIs is a global problem and surveillance studies are of fundamental importance for identifying locations and patterns of these infections. If, on one hand, routine *in vitro* susceptibility tests are usually determined by a simple S, I, or R classification, on the other hand, this antibiogram method provides little information on the underlying level of susceptibility or resistance [i.e., the minimum inhibitory concentration (MIC)].[1] As an alternative, the determination of a pathogen's MIC allows better interpretation in terms of low or non-fully expressed resistance levels. Additionally, pulsed field gel electrophoresis (PFGE) technique holds a notorious discriminatory ability, high reproducibility, and well-determined interpretative criteria, although it is labor and cost-intensive. PFGE has been vastly applied and considered as one of the main tools for epidemiological and surveillance studies.[2,3] Furhermore, due to its ability in differentiating isolates of same species and correlating them with endemic clones, it has also been one of the most frequently used methods for *S. pneumoniae* molecular typing.[4-7]

Spatial analysis based on geographical information systems (GIS) is useful for understanding disease epidemiology. However, it has not been frequently used for understanding the patterns of specific bacterial infections and their related risk factors, despite the fact that spatial aspects are probably linked to many factors influencing antimicrobial resistance patterns. It is well-known that antimicrobial resistance prevalence in community-acquired infections varies greatly depending on location and its related patterns (antimicrobial usage density, socio-economic level, health care system).

The present study aimed at using all three techniques (MICs, PFGE and GIS) in order to explore possible spatial patterns among *S. pneumoniae* clones with similar characteristics isolated from a higher penicillin resistance prevalence population submitted to routine cultures [respiratory or other invasive sample – blood or cerebrospinal fluid (CSF)], between 2002 and 2006 in São Paulo, Brazil.

MATERIALS AND METHODS

The present study was part of the EUREQA project[8] (FAPESP). As such, it has been submitted and approved by the Ethics Committee of the Universidade Federal de São Paulo (CEP process 545/08) and it was based on data observation without patient identification.

Population data

The observational EUREQA study stored on its database all *S. pneumoniae* events by individual address. *S. pneumoniae* events had the following case definition: routine culture (respiratory, blood, CSF or other profound fluids) results positive for *S. pneumoniae*, collected on two large healthcare outpatient facilities in São Paulo between 2002 and 2006, encompassing a public and a private sector unit. All cases had to reside in São Paulo (with address in clinical request form).

Identification and susceptibility testing procedures

Bacterial isolates were manually identified, with the GPI VITEK system card (bioMérieux,, Inc., Hazelwood, Missouri, USA) and conventional biochemical tests applied when indicated. Susceptibility testing was determined by disc diffusion with oxacillin (1 mg disc, Oxoid) and by agar diffusion with Etest® (AB BIODISK, Solna, Sweden) according to the manufacturer's procedures. Interpretative criteria used were those described in CLSI document M100-S20.[9]

Genotyping

Evaluation of chromosomal polymorphisms was performed by PFGE as described by Denton et al.[10] with minor modifications. Each plug was digested with 10 U of SmaI restriction endonuclease (Invitrogen, Carlsbad, CA) at 37°C for 12 hr. Electrophoresis was performed by 1% PFGE agarose gel run on CHEF-DR III system (Bio-Rad Laboratories, Richmond, CA) over 22 hr at 14°C with 5 to 35 s of linear ramping at 6 V/cm. Electrophoretic patterns were analyzed with GelCompar II v. 2.5 (Applied Maths, Kortrijik, Belgium) using the interpretative criteria by Dice similarity coefficient.

Geo-codification spatial analysis procedures

All spatial analytical procedures were performed with TerraView 4.01 software (Instituto Nacional de Pesquisas Espaciais, São José dos Campos, Brasil, 2003). Digital maps containing different layers with streets and districts information were used as the basis for including each individual case in the map by their addresses (point events, i.e. *S. pneumoniae* cases). In order to explore possible cluster formation, point events were submitted to Kernel function method,[11] which is an initial exploratory technique for interpolating and smoothing point events and is mainly used for identifying possible cluster formations. Point events were submitted to an adaptive radius with a quartic density Kernel. The analytical procedure was: (I) total point events distribution in a digital map; (II) Kernel function application on total point events; (III) Kernel function application on major clonal (A to D) point events; (IV) Kernel ratio application between total point events vs. total clonal events, in order to compare possible cluster formations and exclude bias from total sample distribution.

RESULTS

Seventy-eight (78) *S. pneumoniae* community isolates from two major outpatient centers in São Paulo, Brazil, were selected from the databank (with susceptibility R or I to penicillin). Of these, 69 were submitted to PFGE, 65 to MIC determination, and 48 to spatial analytical procedures (differences due to either isolate viability in the period studied or address loss during geocoding techniques). The median MIC of all isolates was 1.0 µg/mL, with an MIC range of 0.016-8.0 µg/mL (full MIC results are not shown in the present report). Based on CLSI criteria for invasive (CSF) isolates, 100% were R to penicillin. Based on non-invasive isolates, 18% were R or I to penicillin. From the 65 isolates, 43.1% (n = 28) were collected from respiratory tract (sputum, middle ear fluid, nasopharyngeal swab or bronchoalveolar lavage), 41.5% (n = 27) from blood, and 15.3% (n = 10) from CSF or other profound fluids.

Genotyping

All 69 isolates submitted to PFGE were compared by Dice similarity coefficient with 80% cutoff and 2% tolerance (BioNumerics v 5.1, Applied Maths, Kortrijk, Belgium).
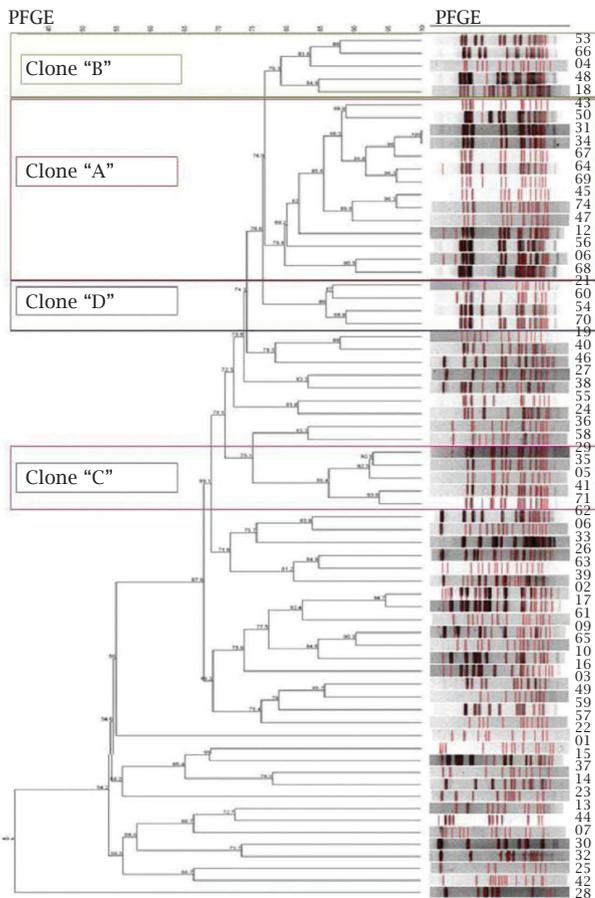
Figure 1: Genetic similarity dendogram of the 69 *Streptococcus pneumoniae* isolates by PFGE based on Dice coefficient.

Figure 1 shows the genetic similarity dendogram of the 69 *S. pneumoniae* isolates by PFGE based on Dice coefficient. The percent similarity by the Dice coefficient among the isolates varied from 40.4 to 79.3%. Clones A, B, C, and D presented inter-clonal similarity above 75.1% and, in the present study, they were grouped together for spatial analytical purposes due to their relatedness pattern. Twenty-one isolates were considered individual isolates, presenting less than 80% intra-clonal similarity. Table 1 shows the main genotyping and susceptibility characteristics detected in the four main *S. pneumoniae* clones, named A, B, C, and D.

Geo-codification spatial analysis procedures

From a total 69 isolates collected in the 2002-2006 period, 48 were geocoded in the city of São Paulo. Of those, 18 belonged to clones A to D. Figure 2 shows both the spatial distribution of the total 48 *S. pneumoniae* isolates (A) and of the 18 *S. pneumoniae* clones A to D (B). Figure 3 shows the Kernel function of the total 48 *S. pneumoniae* isolates (A) and the Kernel ratio of clones A to D vs. total *S. pneumoniae* isolates (B) in the city of São Paulo.

DISCUSSION

Respiratory tract infections (RTIs) are amongst the most common causes of morbidity in the community worldwide. *S. pneumoniae* is the most common bacterial cause of upper and lower respiratory tract community infections, particularly pneumonia.[12] Additionally, it is one of the most frequent causative agents of meningitis and bacteremia, as well as the main cause of upper respiratory non-invasive infections, such as otitis media and sinusitis.[13-15] Infections caused by *S. pneumoniae* can occur in all age groups, but are more prevalent
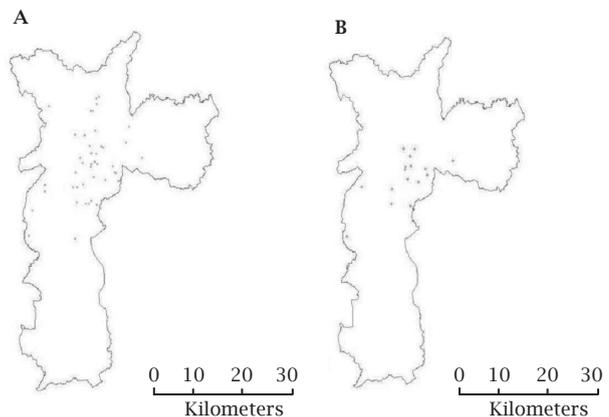

Figure 2: Spatial distribution of 48 *Streptococcus pneumoniae* isolates **(A)** and of the 18 *S. pneumoniae* clones A, B, C, and D isolates **(B)** in the city of São Paulo, collected in the 2002-2006 period.

Table 1. Streptococcus pneumoniae *clones A-D characteristics*

Clone	Dice Coefficient (%)		Penicillin %R	Azithromicin %R	% invasiveness*	% from children**
	Intra-clone	Inter-clone				
A	79,8	76,6	100	85	72	100
B	79,3	76,9	100	93	60	60
C	86,4	75,1	100	93	100	80
D	86,1	76,6	100	75	100	75

* *S. pneumoniae* isolated in CSF (fluids), blood cultures and respiratory tract.
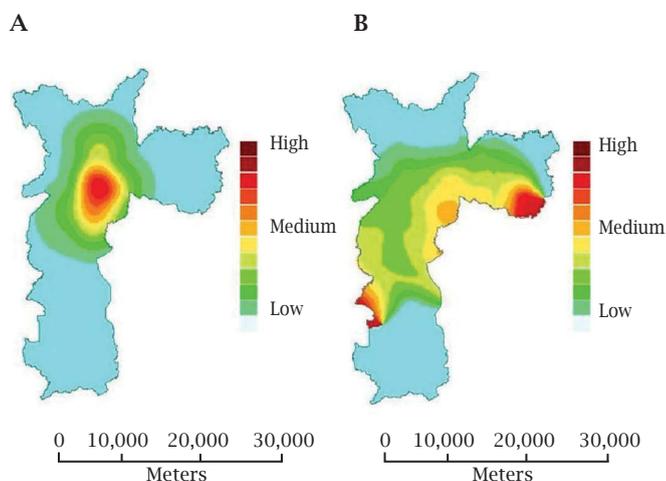** 7 years old.

A   **B**

Figure 3: Kernel function of the total *Streptococcus pneumoniae* isolates **(A)** and the Kernel ratio of clones A to D vs. total *S. pneumoniae* isolates **(B)** in the city of São Paulo, collected in the 2002-2006 period.

in children and in the elderly.[12,16,17] In the present study, 78 pre-selected community *S. pneumoniae* isolates either R or I to penicillin (by disc diffusion) were selected. From these, 65 isolates were submitted to MIC determination and 43.1% (n = 28) were from respiratory tract (sputum, middle ear fluid, nasopharyngeal swab or broncho-alveolar lavage), 41.5% (n = 27) from blood, and 15.3% (n = 10) from CSF or other fluids. Isolates from respiratory samples could not be determined as causing infections, since they could represent respiratory colonization. It is well-established that certain host factors are associated with higher colonization frequency by *S. pneumoniae* (ethnic groups, co-inhabitants, smoking status, previous use of antimicrobials and socio-economic factors).[18,19] Additionally, previous regional studies with community respiratory isolates causing infection detected approximately 30% intermediate resistance and 10% full resistance to penicillin.[20,21] For the present study, resistance rate was 18% (using non-invasive CLSI interpretation) and 100% (if invasive CLSI interpretation is applied), due to previous selection of isolates with higher chance of being resistant and, thus, to belonging to a clone. The purpose of the present study was not affected by isolates causing colonization or infection. Since the study's main objective was to investigate possible clonal distribution pattern, PFGE was applied on a penicillin resistant *S. pneumoniae* population, irrespective of their clinical status (four main *S. pneumoniae* clones, named A, B, C, and D). All clones were represented mainly by invasive isolates with multiple resistance (Table 1).

Although there is an increasing number of studies applying different epidemiological techniques (including spatial and temporal) to quantitatively correlate resistance emergence with different risk factors, most are still surveillance or molecular epidemiology studies. The present study

applied an exploratory spatial analytical procedure and apparently detected a specific transmission pattern of clones A-D, relative to the general *S. pneumoniae* population. This possibly means a higher acquisition risk of certain clones (i.e. resistant and/or invasive) within certain city areas, which is supported by the finding of apparent independent clusters of clones A-D *S. pneumoniae* in southeast and southwest regions of the city (Kernel ratio technique).

Recently, it has been demonstrated[22] that a single clonal *Escherichia coli* group resistant to trimethoprim–sulfamethoxazole accounted for nearly half of community-acquired urinary tract infections in women in three geographically diverse communities. In a different study, a geographical information system and a regression model were applied to detect clusters of higher *Staphylococcus aureus* soft tissue abscesses acquisition risk.[23] Also, other strategies based on spatial scan statistics helped identifying bacterial clusters and detecting areas with significantly high or low sampling rates with a national antimicrobial resistance monitoring program.[24] It has been shown by time-series studies that antimicrobial usage in a restricted hospital environment was temporally linked to the emergence of bacterial resistance.[25,26] Additionally, it is worth mentioning that one very recent study from our group[8] has demonstrated a correlation of a population characteristic (i.e. antimicrobial usage) and emergence of bacterial resistance in a community based on a spatial correlation technique. However, space and time patterns and their correlations with specific resistances or clonal spreads are still not fully understood. Different environmental and individual determinants are probably present and responsible for many resistance acquisition risks.

CONCLUSIONS

Exploratory spatial approach of *S. pneumoniae* clones: there is an apparently non-random pattern of *S. pneumoniae* clones A to D distribution in the city of São Paulo, Brazil; further analyses are required for precisely determining the existence of these independent clusters and their related risk factors; Kernel methods are easy-to-use as an initial exploratory technique for interpolating and smoothing point events, being able to possibly identify clusters. However, biased samples may influence results; GIS and spatial methods can be applied to better understand epidemiological patterns and to identify target areas for public health interventions.

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