Characterisation of *Streptococcus pneumoniae* isolates from invasive disease in adults following the introduction of PCV10 in Brazil

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Abstract

**Purpose.** Invasive pneumococcal disease (IPD) in the elderly is an important public health issue due to the increased proportion of this population in many countries including Brazil. We aimed to characterise pneumococci isolates in adults >50 years with IPD, following the introduction of the 10-valent pneumococcal conjugate vaccine (PCV10) as part of the National Childhood Immunisation Program for children ≤2 years in March 2010.

**Methodology.** Between 2013 and 2015, pneumococcal isolates were collected and serotypes were determined using multiplex PCR and/or Quellung reaction. Antimicrobial susceptibility was defined by E-test (bioMérieux); genetic diversity was determined using Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) and, in selected isolates, Multi Locus Sequence Typing (MLST) was performed.

**Results/Key findings.** Among 102 pneumococcal isolates, the most frequent serotypes were 19A, 13 of 102 (12.7 %) and 22F, 10 of 102 (9.8 %). Ninety-eight isolates were tested for antimicrobial susceptibility. Intermediate resistance to penicillin was present in 2/98 (2.0 %), ceftriaxone in 7/98 (7.1 %) and meropenem in 7/95 (7.4 %) of the isolates (non-meningitis breakpoint: 4 µg ml⁻¹/2 µg ml⁻¹/0.5 µg ml⁻¹, respectively). Resistance to penicillin (meningitis breakpoint ≥0.12 µg ml⁻¹) was observed in 31/98 (31.6 %) of the isolates. Genetic analysis presented two relevant clonal groups, belonging to non-PCV10 serotypes: 19A (ST320, linked to non-susceptibility) and 22F (ST6403).

**Conclusion.** Our data suggest a predominance of non-PCV10 serotypes among IPD in the elderly population in circulating strains ca. 3 to 5 years after the introduction of PCV10 in Brazil.

INTRODUCTION

Pneumococcal infections are a major cause of morbidity and mortality in children and the elderly; it is considered a significant public health problem worldwide [1–3]. The progressive increase in the proportion of elderly people globally, including Brazil, makes this group of particular interest in studying pneumococcal disease [4–8].

The introduction of the 7-valent conjugate vaccine (PCV7) in children against pneumococcal infections significantly reduced the occurrence of Invasive Pneumococcal Disease (IPD), even in the adult group, by an indirect effect, termed herd immunity [9]. Although, a relative increase in the rates of IPD caused by non-vaccine serotypes was also observed [10]. Thus, the emergence of serotype 19A (a non-PCV10 serotype) has been a cause of concern in several regions of the world, including Brazil [11–14].

In 2010, the PCV10 was introduced in Brazil for children <24 months old as part of the National Childhood Immunisation Program [15, 16]. Also, we can emphasise that Brazil represents the fifth largest population in the world and that studies bringing data on serotyping, genotyping and epidemiology after the implementation of PCV10 have not yet been performed in this country. The PCV10 vaccine includes three additional serotypes (1, 5, 7F) to those of the PCV7.
contained in the PCV7 vaccine (4, 6B, 9V, 14, 18C, 19F and 23F). It is formulated to cover ≥80.0% of the serotypes that cause IPD in most regions of the world [17, 18]. In Brazil, in a study involving 316 children with IPD and 1219 controls, the effectiveness of the vaccine was 83.8% for vaccine serotypes and 77.9% for serogroups (serotypes belonging to the same group of a vaccine serotype) [19]. Compared to a broad distribution of PCV10 for children, the 23-valent polysaccharide vaccine (PPSV23) is available for the elderly with a restricted access. Studies performed in other countries with PCV13, recently considered the use in combination with PPSV23 [20–22] for the prevention of invasive infections in adults.

Since the introduction of PCV10 in Brazil, there have been few studies to determine the effects of this intervention on IPD during the post-vaccination period amongst the elderly population. Surveillance of pneumococcal disease helps to inform vaccine policy and, the phenotypic and genotypic characterisation of pneumococci provides important data regarding changes in serotype, clonal shifts and the emergence of antimicrobial resistance which may be linked to an increase in IPD [12, 23].

In this context, the present study aims to characterise isolates of S. pneumoniae obtained ca. 3 to 5 years after the introduction of PCV10 from invasive pneumococcal diseases in individuals over fifty years old in Porto Alegre, South Brazil.

METHODS

Bacterial isolates

Pneumococcal isolates were obtained between January 2013 and June 2015 from patients over 50 years of age with IPD in Porto Alegre. These isolates were recovered from normally sterile fluids, collected in three different hospitals in Porto Alegre, South Brazil: Grupo Hospital Conceição (GHC), Hospital Mãe de Deus (HMD), and Hospital de Clínicas de Porto Alegre (HCPA).

The isolates were preserved as bacterial suspensions, in a solution containing 10.0% Skim Milk (Becton, Dickinson and Company-BD) and 10.0% glycerol (Neon), maintained at −80°C. The isolates were subcultured on tryptic soy agar (TSA) containing 5.0% of defibrinated sheep blood and incubated at 35–37°C for 18–24 h in 5.0% CO2. Identification of S. pneumoniae was based on the following phenotypic characteristics: colonial morphology, haemolysis, optochin susceptibility and bile (sodium deoxycholate) solubility [24].

Serotyping

Capsular serotype was determined by sequential multiplex PCR as previously described [25] with modifications to target the most frequent pneumococcal serotypes in Latin America. Quellung reaction was used to characterise serotypes that could not be determined using the PCR [25, 26].

DNA extraction

DNA extraction was performed following recommendations from the Center for Disease Control and Prevention (CDC): one loop of a fresh growth of S. pneumoniae was suspended in 200 µl of 5.0% Chelex resin solution (Bio-Rad) containing 200 µg ml⁻¹ of Proteinase K (Invitrogen, Life Technologies). The suspension was incubated at 56°C for 1 h and at 100°C for 10 min. The process was concluded by centrifugation at 16,000 g for 3 min, and the supernatant was stored at −20°C [27].

Antimicrobial susceptibility test

Antimicrobial susceptibility was determined by the Minimum Inhibitory Concentration (MIC), which was evaluated using the E-Test (bioMérieux) for the following antimicrobials: penicillin (non-meningitis breakpoints: ≤2 µg ml⁻¹/4 µg ml⁻¹≥8 µg ml⁻¹; meningitis breakpoints: ≤0.06 µg ml⁻¹≥0.12 µg ml⁻¹), meropenem (≤0.25 µg ml⁻¹/0.5 µg ml⁻¹≥1 µg ml⁻¹), ceftriaxone (non-meningitis breakpoints: ≤1 µg ml⁻¹/2 µg ml⁻¹≥4 µg ml⁻¹; meningitis breakpoints: ≤0.5 µg ml⁻¹/1 µg ml⁻¹≥2 µg ml⁻¹), trimethoprim-sulfamethoxazole (≤0.5/9.5 µg ml⁻¹/1/19 µg ml⁻¹/2/38 µg ml⁻¹≥4/76 µg ml⁻¹), levofloxacin (≤2 µg ml⁻¹/4 µg ml⁻¹≥8 µg ml⁻¹), erythromycin (≤0.25 µg ml⁻¹/0.5 µg ml⁻¹≥1 µg ml⁻¹), tetracycline (≤1 µg ml⁻¹/2 µg ml⁻¹≥4 µg ml⁻¹) and vancomycin (≤1 µg ml⁻¹). The technique was performed following the manufacturer’s recommendations; the tests were interpreted according to the Clinical Laboratory Standards Institute [28]. The strain S. pneumoniae ATCC 49619 was used as internal quality control [28].

Genotyping

Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) was performed on the 102 isolates obtained, based on the protocol designed and adapted by Koeck and co-workers [29]. The strain S. pneumoniae ATCCBAA-2555 (R6) was used for the purpose of quality control [30]. The results generated by MLVA Types (MT) were considered as a Clonal Group (CG) if, for each isolate, at least 13 of the 18 loci were identical, which is equivalent to >70% similarity [31]. The website www.mlva.eu hosted by the Groupe d’Etudes en Biologie Prospective, Bordeaux, France provides an online database to allow comparison of the allelic profiles obtained [32]. Isolates belonging to the most frequent CGs with minor genotype diversity generated by MLVA analysis and representing the most relevant non-PCV10 serotype, were selected to be evaluated by Multi Locus Sequence Typing (MLST) using an adapted method described by Enright and Spratt [33]. Allele profiles and Sequence Type (ST) were obtained from the MLST database (http://pubmlst.org/spneumoniae/) [34]. All 13 isolates belonging to serotype 19A were submitted to MLST analysis; however, only six among ten 22F isolates were available for such analysis.

Data analysis

MLVA and MLST numerical profiles were analysed using BioNumerics version 7.1 (Applied Maths). Clustering of MLST and MLVA types were represented by a minimum
spanning trees (MST), where isolates were considered as CG if they presented at least 13 identical isolates of the 18 loci, which is equivalent to >70% similarity [31].

RESULTS

One hundred and two non-duplicated pneumococcal isolates were recovered from patients with IPD and included in the study. The mean age of the patients was 66 years (ranging from 50 to 90) and considering gender, 81.4% (n=83) of the patients were male. The majority of isolates were recovered from blood with 86.3% (n=88), followed by cerebrospinal fluid 7.8% (n=8), and pleural fluid 2.9% (n=3); one isolate was recovered from each of the remaining specimen types, ascites, pericardial and joint fluid (1.0%).

Among the 102 pneumococcal isolates, twenty-six different capsular serotypes were detected and the predominant serotypes identified were 19A, 13 of 102 (12.7%) and 22F, 10 of 102 (9.8%), representing the non-PCV10 serotypes group. A serotype could not be determined for five of the isolates, although PCR amplification of the cps gene was achieved. Three isolates whose serotype was determined as 6A/B by PCR could not subsequently be recovered by culture, thus their serotype could not be determined using the Quellung reaction. Serotypes included in PCV10 were represented by 31/102 (30.4%) of the isolates, and serotypes 4 and 23F were the most frequently found and represented by 8/102 (7.8%) of the isolates for both. It is important to highlight that isolates belonging to serotype 14 were not detected (Fig. 1).

Antimicrobial susceptibility testing was performed on 98/102 (96.1%) pneumococcal isolates. One isolate was not viable for erythromycin and three for meropenem testing. All isolates were susceptible to vancomycin (≤1 μg ml⁻¹) and levofloxacin (≤2 μg ml⁻¹). Considering non-meningitis breakpoints, resistance was not found among β-lactam agents tested (penicillin ≥8 μg ml⁻¹/ceftriaxone ≥1 μg ml⁻¹/meropenem ≥4 μg ml⁻¹); however, 2/98 (2.0%) of the isolates presented intermediate resistance to penicillin (4 μg ml⁻¹), 7/98 (7.1%) of the isolates to ceftriaxone (2 μg ml⁻¹), and 7/95 (7.4%) of the isolates to meropenem (0.5 μg ml⁻¹). According the breakpoints for meningitis, resistance to penicillin (≥0.12 μg ml⁻¹), and ceftriaxone (≥2 μg ml⁻¹) was found in 31/98 (31.6%) and in 7/98 (7.1%) of the isolates respectively; on the other hand intermediate resistance to ceftriaxone (1 μg ml⁻¹) was observed in 7/98 (7.1%) of the isolates. The prevalence of resistance to tetracycline (≥4 μg ml⁻¹) and trimethoprim-sulfamethoxazole (≥4/76 μg ml⁻¹) was observed in 6/98 (6.1%) of the isolates for both, and to erythromycin (≥1 μg ml⁻¹) it occurred in 16/97 (16.5%) of the isolates. Regardless of resistance rates, the most powerful drugs in vitro against pneumococci were meropenem (MIC₅₀=0.012 μg ml⁻¹ and MIC₉₀=0.38 μg ml⁻¹), followed by penicillin (MIC₅₀=0.023 μg ml⁻¹ and MIC₉₀=1.0 μg ml⁻¹) (Table 1).

Isolates belonging to serotype 19A and presenting MICs ≥1.0 μg ml⁻¹ for both penicillin and ceftriaxone were represented by 9/13 (69.2%) of the isolates. Furthermore, the preponderance of this particular serotype regarding the intermediate resistance to meropenem (0.5 μg ml⁻¹) was evident in 5/13 (38.5%) of the isolates. Successively, serotype 19A showed resistance to erythromycin (≥1 μg ml⁻¹) in 9/13 (69.2%), to tetracycline (≥4 μg ml⁻¹) and trimethoprim-sulfamethoxazole (≥4/76 μg ml⁻¹) in 4/13 (30.7%) of the isolates, respectively (Table 2).

Considering the MLVA technique, we identified 96 different genotypes distributed in 16 clonal groups (CGs) and 21 singletons between the 102 pneumococcal isolates analysed. Among the 16 CG found, we considered two predominant CG (Fig. 2); a major one with 11 isolates (nine belonging to serotype 19A, one to serotype 22F and one isolate to serotype 19F) and the second CG containing a total of eight isolates (seven belonging to serotype 22F and one isolate to serotype 19A). Serotype 19A isolates (n=13) were distributed in two different CGs (n=11), one singleton and one isolate belonging to the same CG containing most of the 22F isolates. Isolates of serotype 22F (n=10) were grouped into one main CG (n=7) and two singletons with two and one isolates, respectively.

Only 19 isolates were selected and submitted to MLST, which belong to serotype 19A (n=13) and 22F (n=6). Unfortunately, four 22F isolates were not available to be performed by MLST because of technical issues. The majority of 19A isolates were considered as ST320 for 10/13 (77.0%), and the six 22F isolates available and submitted to MLST were characterised as ST6403 (Table 2). Resistance to different antimicrobial agents was quite evident in isolates belonging to serotype 19A (9/13), especially associated to ST320. However, isolates belonging to serotype 22F (9/10), mostly represented by ST6403 were completely susceptible to the antimicrobial agents tested. A single serotype 22F presenting intermediate resistance to tetracycline was not available for MLST analysis.

DISCUSSION

Infections due to S. pneumoniae represent a global public health concern, particularly because of the major burden of pneumococcal diseases and the limited epidemiological data available in developing countries. Investigators are more interested in conducting studies on pneumococcal diseases among children under five years old, which may explain why studies focusing on elderly population are less frequent [2, 9–13, 18, 19]. As the proportion of elderly people is increasing over the world quickly, particularly in medium income countries like Brazil, special attention is required for this group of individuals [4–8].

The PCV10 was introduced in 2010 as part of National Immunisation Program in Brazil specifically targeting infants with a schedule comprising three primary doses at ages 2 months, 4 months and 6 months, and a booster dose at age 12 months. A single catch-up dose was offered for
children aged 12–23 months at the time of introduction. Pneumococcal conjugate vaccines are not available for adults ≥65 years in the Brazilian public healthcare system. However, the polysaccharide formulation (PPSV23) is available in the public healthcare system for adults belonging to risk groups [15, 16]. Despite the well characterised benefits that conjugate vaccines promote in the decrease of the incidence of pneumococcal diseases, a rise of non-vaccine serotypes can occur, including of those associated with antimicrobial resistance. Data evaluating the capsular type distribution and antimicrobial resistance in the post-vaccine period are still scarce in Brazil in the elderly group. Therefore, our study aimed to highlight the importance of serotyping and genotyping methods in order to characterise and establish the genetic diversity of the pneumococcal isolates circulating in Brazil.

**Table 1.** Antimicrobial susceptibility profile from 98 pneumococcal isolates collected among elderly people with invasive diseases from 2013 to 2015

<table>
<thead>
<tr>
<th>Susceptibility</th>
<th>MIC50 (µg ml⁻¹)</th>
<th>MIC90 (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Penicillin (non-meningitis)</strong></td>
<td>S (%)</td>
<td>I (%)</td>
</tr>
<tr>
<td>Penicillin (meningitis)</td>
<td>67 (68.4)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Ceftriaxone (non-meningitis)</strong></td>
<td>91 (92.9)</td>
<td>7 (7.1)</td>
</tr>
<tr>
<td>Ceftriaxone (meningitis)</td>
<td>84 (85.7)</td>
<td>7 (7.1)</td>
</tr>
<tr>
<td><strong>Meropenem</strong></td>
<td>88 (92.6)</td>
<td>7 (7.4)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>98 (100)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Erythromycin</strong></td>
<td>80 (82.5)</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td><strong>Tetracycline</strong></td>
<td>85 (86.8)</td>
<td>7 (7.1)</td>
</tr>
<tr>
<td><strong>Levofloxacin</strong></td>
<td>98 (100)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Trimethoprim-sulfamethoxazole</strong></td>
<td>66 (67.3)</td>
<td>26 (26.6)</td>
</tr>
</tbody>
</table>

*Three isolates were not available to test for meropenem and one for erythromycin.

![Fig. 1. Relative frequency of the distribution of PCV10 vaccine serotypes and non-PCV10 serotypes during the 2013–2015 period.](image-url)
Table 2. Characteristics of isolates belonging to serotype 19A and 22F: specimen, age of the patient, MLST, MLVA, and resistance profile

MLST, Multi Locus Sequence Type; MLVA, Multiple Locus Variable Tandem Repeat Number; ST, Sequence Type. PEN, Penicillin; CRO, Ceftriaxone; MERO, Meropenem; ERY, Erythromycin; TET, Tetracycline; SXT, Trimethoprim-Sulfamethoxazol.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Number</th>
<th>Specimen</th>
<th>Age</th>
<th>MLST</th>
<th>MLVA</th>
<th>Minimal inhibitory concentrations (MIC) (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19A</td>
<td>062–13</td>
<td>Blood</td>
<td>61</td>
<td>ST320</td>
<td>MT19A.2†</td>
<td>2* 2* 0.25 &gt;256* 4* 4*</td>
</tr>
<tr>
<td></td>
<td>063–13</td>
<td>Blood</td>
<td>75</td>
<td>ST320</td>
<td>MT19A.3†</td>
<td>2* 2* 0.5* &gt;256* 16* 16*</td>
</tr>
<tr>
<td></td>
<td>014–14</td>
<td>Blood</td>
<td>72</td>
<td>ST9678</td>
<td>MT19A.4</td>
<td>0.06 0.06 ≤0.03 0.03 0.25 0.25</td>
</tr>
<tr>
<td></td>
<td>016–14</td>
<td>Blood</td>
<td>50</td>
<td>ST320</td>
<td>MT19A.5†</td>
<td>1* 1* 0.25 4* 0.12 4*</td>
</tr>
<tr>
<td></td>
<td>017–14</td>
<td>Blood</td>
<td>78</td>
<td>ST320</td>
<td>MT19A.6†</td>
<td>1* 1* 0.5* &gt;256* 4* 4*</td>
</tr>
<tr>
<td></td>
<td>030–14</td>
<td>Blood</td>
<td>60</td>
<td>ST320</td>
<td>MT19A.1†</td>
<td>2* 2* 0.06 2* 0.12 2</td>
</tr>
<tr>
<td></td>
<td>037–14</td>
<td>Blood</td>
<td>57</td>
<td>ST320</td>
<td>MT19A.7†</td>
<td>4* 2* 0.25 1* 0.12 1</td>
</tr>
<tr>
<td></td>
<td>041–14</td>
<td>Blood</td>
<td>63</td>
<td>ST320</td>
<td>MT19A.8†</td>
<td>2* 2* 0.5* &gt;256* 4* 2</td>
</tr>
<tr>
<td></td>
<td>049–14</td>
<td>Blood</td>
<td>61</td>
<td>ST733</td>
<td>MT19A.9</td>
<td>≤0.03 0.06 ≤0.03 0.03 0.12 2</td>
</tr>
<tr>
<td></td>
<td>056–14</td>
<td>Blood</td>
<td>57</td>
<td>ST320</td>
<td>MT19A.1†</td>
<td>1* 1* 0.5* 2* 0.12 2</td>
</tr>
<tr>
<td></td>
<td>057–14</td>
<td>CSF</td>
<td>57</td>
<td>ST320</td>
<td>MT19A.1†</td>
<td>1* 1* 0.5* 2* 0.12 2</td>
</tr>
<tr>
<td></td>
<td>060–14</td>
<td>Blood</td>
<td>69</td>
<td>ST2260</td>
<td>MT19A.10</td>
<td>0.06 0.06 ≤0.03 0.06 0.12 2</td>
</tr>
<tr>
<td>22F</td>
<td>030–15</td>
<td>CSF</td>
<td>77</td>
<td>ST320</td>
<td>MT19A.11</td>
<td>≤0.03 ≤0.03 ≤0.03 0.06 0.25 0.12</td>
</tr>
<tr>
<td></td>
<td>031–13</td>
<td>CSF</td>
<td>66</td>
<td>–</td>
<td>MT22F.2</td>
<td>0.012 0.023 0.002 0.016 2 0.064</td>
</tr>
<tr>
<td></td>
<td>045–13</td>
<td>Blood</td>
<td>83</td>
<td>ST6403</td>
<td>MT22F.3</td>
<td>0.016 0.032 0.006 0.016 0.094 0.125</td>
</tr>
<tr>
<td></td>
<td>051–13</td>
<td>Blood</td>
<td>57</td>
<td>ST6403</td>
<td>MT22F.4</td>
<td>0.023 0.032 0.064 0.023 0.19 0.094</td>
</tr>
<tr>
<td></td>
<td>040–14</td>
<td>Blood</td>
<td>58</td>
<td>–</td>
<td>MT22F.5§</td>
<td>0.023 0.047 0.006 0.016 0.094 0.094</td>
</tr>
<tr>
<td></td>
<td>050–14</td>
<td>Blood</td>
<td>65</td>
<td>ST6403</td>
<td>MT22F.1</td>
<td>0.016 0.047 0.012 0.047 0.094 0.094</td>
</tr>
<tr>
<td></td>
<td>051–14</td>
<td>Pleural</td>
<td>65</td>
<td>ST6403</td>
<td>MT22F.1</td>
<td>0.016 0.047 0.012 0.032 0.094 0.094</td>
</tr>
<tr>
<td></td>
<td>064–14</td>
<td>Blood</td>
<td>87</td>
<td>–</td>
<td>MT22F.6§</td>
<td>0.016 0.064 0.008 0.032 0.19 0.125</td>
</tr>
<tr>
<td></td>
<td>021–15</td>
<td>Blood</td>
<td>60</td>
<td>ST6403</td>
<td>MT22F.7</td>
<td>0.032 0.023 0.032 0.032 0.19 0.094</td>
</tr>
<tr>
<td></td>
<td>032–15</td>
<td>Blood</td>
<td>76</td>
<td>ST6403</td>
<td>MT22F.8</td>
<td>0.023 0.016 0.006 0.064 0.12 0.064</td>
</tr>
<tr>
<td></td>
<td>047–15</td>
<td>Blood</td>
<td>62</td>
<td>–</td>
<td>MT22F.9§</td>
<td>0.023 0.016 0.003 0.032 0.19 0.064</td>
</tr>
</tbody>
</table>

*Minimal Inhibitory Concentrations (MIC) corresponding to non-susceptibility to β-lactams (higher MIC≥1 µg ml⁻¹ to penicillin and ceftriaxone, and>0.25 µg ml⁻¹ to meropenem) or resistance to other agents (MIC≥1 µg ml⁻¹ to erythromycin, ≥4 µg ml⁻¹ to tetracycline and ≥4/76 µg ml⁻¹ to trimethoprim-sulfamethoxazol).

**Intermediate resistance to tetracycline.
†Isolates belonging to the same clonal group (CG), with ST 320.
‡Isolates belonging to the same clonal group (CG), with ST 6403.
§Isolates belonging to the same clonal group (CG), with ST not available.

In a recent study conducted after the introduction of conjugate vaccine among children in Japan, pneumococci obtained from a population constituted exclusively by adults with invasive diseases, investigators observed a similar pattern compared to our findings, with an predominance in the prevalence of the same non-PCV10 serotypes: 19A and 22F [35]. A study by dos Santos et al. conducted in children with IPD noticed a clear reduction trend in the incidence of vaccine serotypes after the introduction of PCV10 in Brazil [18]. On another side, the incidence of pneumococcal diseases in adults evaluated in studies conducted in Southern Brazil two years after vaccine introduction, showed that vaccine serotypes remain among the most frequent serotypes for this group and a slight increase of the frequency of serotypes not included in PCV10, as serotype 19A [36, 37]. One of the hypotheses is the use of PCV10 in children does not seem to affect the carrier state of non-PCV10 serotypes [38]. Another hypothesis reported in the literature is that herd immunity effects generally require 5–6 years post vaccine implementation to become evident [39].

The use of conjugate vaccine could explain the incidence of non-PCV10 serotypes, especially the predominance of serotype 19A, which represents a main concern worldwide and even more for the Brazilian elderly community, in which the impact of the PCV10 has not been fully investigated [11, 12, 14, 40, 41]. Comparing our study to previous data generated by a recent study conducted in South Brazil, serotype 19A demonstrated a consistent dominance four years after the introduction of PCV10 [14]. Besides that, it is important to highlight that serotype 14, which was a predominant vaccine serotype in prior studies [36, 37], showed a different pattern by its complete disappearance.
Considering meningitis breakpoints, the prevalence of isolates with MIC $\geq 0.12 \, \mu g \, ml^{-1}$ to penicillin was 31.6%, which could limit the use of this agent in the treatment of meningitis. However, several studies evidenced a high susceptibility of $\beta$-lactams (penicillin and ceftriaxone, considering non-meningitis breakpoints) [37, 42, 43]. The guidelines of the Brazilian Society of Pneumology and Tisiology recommend the therapy combination ($\beta$-lactam associated with a macrolide or quinolone) or the monotherapy with extended coverage for atypical pathogens (quinolone or macrolide) as a scheme of empiric therapy for community acquired pneumonia [44]. Higher prevalence of resistance for some specific antimicrobials tested, such as erythromycin, was found. It is concerning that almost 20.0% of the isolates from our study are non-susceptible to erythromycin, a drug included in the empirical treatment of pneumonias according to the Brazilian guidelines. The prevalence of resistance to multiple classes of antimicrobials showed in the present study a particular association with serotype 19A that was also reported by other authors [11, 12, 14, 36, 37, 45].

Genotyping using MLVA and MLST showed a clonal dissemination of serotype 19A, associated with ST320. All isolates genotypically characterised as ST320 were grouped in a single clonal MLVA complex, but one has a distinct susceptibility profile when compared to the other ST320 isolates. In addition, the ST320 is a well-described and characterised double locus variant (DLV) of ST236, the PMEN clone Taiwan19F-14, associated with high antimicrobial resistance that emerged after the introduction of PCV7 worldwide [46]. Serotype 22F was the second capsular type frequently found, being characterised as ST6403, which was previously found in serotype 22F in two carriage isolates from the Midwest Brazil and also has been described in our population (Southern Brazil) in previous study [36, 47]. In contrast to another study performed in 2007–2012 with 325 isolates from the same region of Southern Brazil in which only one isolate (0.31%) belonged to serotype 22F (ST6403) [36], the present study demonstrated that this particular serotype is among the most frequent, 10/102 (9.8%), mostly characterised as ST6403 (Table 2). This ST

Fig. 2. Minimum spanning tree from MLVA results of isolates from invasive diseases obtained during the post-vaccination period from 2013 to 2015.
is a single locus variant of ST698 described previously in Australia, United Kingdom (UK), Brazil and United States of America (USA) [47, 48]. In the UK, the ST698 was reported as a major clone, while in Brazil and the USA it was considered as relatively rare among serotype 22F isolates [36, 48–50]. Isolates belonging to this serotype (22F) were found and widely susceptible to antimicrobial agents tested for our study. Genotyping by MLVA was less correlated with MICs for 22F than 19A; all isolates of serotype 22F which were submitted to MLST belonged to the same ST6403 and were grouped in the same CG.

We observed that serotypes 19A and 22F were the most frequent serotype among the non-PCV10 serotypes. Our data can be considered as a baseline for comparing future emergence of non-PCV10 serotypes, especially serotype 19A related to ST320 presenting a resistance to antimicrobial agents. Nevertheless, isolates of serotype 22F were observed as ST6403 and were susceptible to the antibiotics agents tested. Carriage studies to investigate the presence of non-vaccine serotypes and vaccine types in the elderly population in addition to children would further contribute to understanding the dynamics of pneumococcal disease. Overall, we consider that the surveillance of bacterial resistance and the distribution of prevalent serotypes should be monitored continuously over time.

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Conflicts of interest
The authors declare that there are no conflicts interest.

Ethical statement
The original project was approved by the Research Ethics Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), and registered by the number 1.115.418

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