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Genetic profiles and antimicrobial resistance of *Streptococcus pneumoniae* non-PCV10 serotype isolates recovered from meningitis cases in Salvador, Brazil

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In 2010, the 10-valent pneumococcal conjugate vaccine (PCV10) was introduced into the Brazilian childhood vaccination programme. Concerns have been raised that non-vaccine serotypes could increase in prevalence and reduce the benefits of vaccination; therefore, we examined non-PCV10 isolates recovered from meningitis during pre- (January 2008–May 2010) and post-vaccine (June 2010–December 2012) periods. Surveillance for pneumococcal meningitis was established at the Reference Hospital of Infectious Diseases in Salvador, Brazil. Serotypes were determined by multiplex PCR and/or Quellung reaction. Antimicrobial susceptibility testing was conducted by E-test and broth microdilution. Genotyping used PFGE and multi-locus sequence typing. A total of 148 cases of meningitis were identified from January 2008 to December 2012, 77 (52 %) of which were due to non-PCV10 isolates, with 50 (52.1 %) from pre-vaccine and 27 (52 %) from post-vaccine periods. In the post-vaccine period, the non-PCV10 serotypes 12F (*n*=6; 22.2 %), 10A (*n*=3; 11.1 %), 15B (*n*=2; 7.4 %) and 18B (*n*=2; 7.4 %) were the most prevalent. Forty-three isolates (55.8 %) were non-susceptible to one or more antibiotics. Non-susceptibility to penicillin was observed among serotypes 19A (three isolates), 9N (one isolate) and 12F (one isolate). PFGE and multi-locus sequence typing results demonstrated a wide genetic diversity among the isolates. During the early period following PCV10 introduction, no obvious emergence of a particular serotype was evident among non-PCV10 strains. This study underscores the importance of monitoring any changes among non-PCV10 cases after the introduction of PCV10.

INTRODUCTION

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, causing diseases that range in severity from meningitis, septicaemia and pneumonia, to sinusitis and acute otitis media (WHO, 2012). Almost all isolates that cause these infections are encapsulated, and to date, more than 94 different pneumococcal serotypes have been distinguished expressing structurally and antigenically different capsular polysaccharides (Geno *et al.*, 2015). The capsule is the target of all licensed vaccines (Feldman & Anderson, 2014).

The licensure and subsequent widespread use of pneumococcal conjugate vaccines have decreased the overall incidence of invasive pneumococcal disease in many countries, in part by reducing carriage of vaccine-type strains and

Abbreviations: CSF, cerebrospinal fluid; MLST, multi-locus sequence typing; PCV10, 10-valent pneumococcal conjugate vaccine; ST, sequence type.

inducing herd immunity (Millar *et al.*, 2008; Hammitt *et al.*, 2014). However, concerns have been raised that non-vaccine serotypes could increase in prevalence and reduce the benefits of vaccination (Pilishvili *et al.*, 2010).

The overall effect of the use of conjugate vaccines can include serotype replacement and/or the capsular switching phenomenon. The contribution of each of these effects in geographical areas where a conjugate vaccine is introduced is difficult to predict; therefore, careful monitoring of the epidemiology and understanding of the dynamics of the pneumococcal population are required to assess the initial and long-term benefits of vaccination in each region. In 2010, Brazil introduced the 10-valent non-typable *Haemophilus influenzae* protein D conjugate vaccine (PCV10 or PHiD-CV; GSK Biologicals) into its routine national immunization programme, in a three-dose scheme (2, 4 and 6 months) plus a booster at 12 months. This vaccine contains antigens designed to protect against the serotypes 4, 9V, 14, 19F, 23F, 18C and 6B, in addition to serotypes 1, 5 and 7F (Prymula & Schuerman, 2009).

Since 1996, hospital-based surveillance for pneumococcal antibiotic resistance and capsular serotypes associated with pneumococcal meningitis has been conducted in Salvador, Bahia (de O Menezes *et al.*, 2011; dos Santos *et al.*, 2015; Leite *et al.*, 2016). Here, we describe the genetic diversity and antimicrobial susceptibility of *S. pneumoniae* non-PCV10 serotype isolates recovered from patients with meningitis before (January 2008–May 2010) and after (June 2010–December 2012) the introduction of PCV10 in Salvador, Bahia.

METHODS

Study population and surveillance system. In 1996, an active surveillance system for pneumococcal meningitis cases was initiated at the Hospital Couto Maia, a reference public hospital for infectious diseases with 120 beds, which functions as the medical attendance centre for meningitis in the metropolitan region of the Salvador municipality (3 573 973 inhabitants, 2010 IBGE census). The state health department requires all suspected meningitis cases from the region to attend this hospital, and more than 90 % of meningitis reports from the region are reported at this unit (DATASUS, 2015). Our technicians reviewed the daily clinical laboratory records at the hospital to identify all *S. pneumoniae* isolates obtained from symptomatic patients with meningitis with positive cerebrospinal fluid (CSF) and fulfilling the criteria for bacterial meningitis ($\geq$100 leukocytes mm^{-3}). The pneumococcal isolates identified in this hospital were sent to the Instituto Gonçalo Moniz, Salvador, Brazil, for confirmation.

Microbiological procedures. Pneumococcal strains were identified by standard methods, including Gram stain, colony morphology on agar media with 5 % sheep blood, optochin susceptibility (5 µg Oxoid disks) and bile solubility (Werno & Murdoch, 2008). Capsular serotypes were determined by multiplex PCR (Dias *et al.*, 2007; CDC, 2014). Quellung reaction tests were performed by the *Streptococcus* Laboratory at the Centers for Disease Control and Prevention, Atlanta, for all serotypes not resolved by PCR assay, as well as for 10 % of the serotypes resolved by PCR, as a quality control measure. Antimicrobial susceptibility to penicillin, cefotaxime, clindamycin, chloramphenicol, erythromycin, levofloxacin, tetracycline, trimethoprim-sulfamethoxazole and vancomycin (Sigma-Aldrich) was assessed by broth microdilution according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). The reference strain *S. pneumoniae* ATCC 49619 was used for quality control. All isolates with MIC values $\geq$0.12 µg ml^{-1} were defined as pneumococcal non-susceptible to penicillin.

For comparison purposes, non-PCV10 serotypes were separated into two periods: (1) pre-vaccine period (January 2008 to June 2010), with 50 (52.1 %) isolates, and (2) post-vaccine (July 2010 to December 2012), with 27 (52 %) isolates.

Molecular characterization. Non-PCV10 isolates were characterized by PFGE. Chromosomal digests generated by *Sma*I were prepared and analysed as described elsewhere (McEllistrem *et al.*, 2000). Fragments were separated by PFGE in 2 % agarose gels using the CHEF-DRII apparatus (Bio-Rad Laboratories) with pulse times of 2–30 s for 19 h at 14 °C and 6 V cm^{-1}. Restriction profiles were analysed using the GelCompar II software package (version 4.0; Applied Maths, Bionumerics) to compare the band patterns. PFGE patterns were clustered by the unweighted pair-group method using average linkages, and a dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient, with an optimization of 1.0 % and a tolerance of 1.5 %. PFGE patterns were defined as isolates with a similarity of $\geq$80 % in the dendrogram. According to PFGE clustering analysis, based on similarity profiles, a random sample of all isolates showing high relatedness ($\geq$80 %) from the pre- (26/50 isolates) and post-PCV10 (14/27 isolates) periods was selected for multi-locus sequence typing (MLST) analysis, according to an adaption of the method described by Enright & Spratt (1998) (detailed at http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm).

Data analysis. Epidemiological and laboratory information was entered into a standard database and analysed using Epi-Info version 3.5.1 (Centers for Disease Control and Prevention). We calculated annual incidence rates (cases per 100 000 population) by dividing the number of cases among residents of the Salvador municipality by the estimated population, using 2010 census bureau data to calculate rates for 2008 through 2012 (IBGE, 2010). Fisher's exact or chi-square test was used to compare differences between proportions for dichotomous variables. All *P*-values were based on two-sided tests, and $P<0.05$ was considered statistically significant.

Ethical approval. Informed consent procedures were applied to all patients and/or guardians of patients included in this study, which was approved by the National Committee for Research Ethics (CONEP) and the FIOCRUZ Institutional Review Board, Brazilian Ministry of Health (no. 044/2013). All patients or legal guardians gave written informed consent prior to enrolment of subjects in the study.

RESULTS

Overall patient characteristics

A total of 148 cases of pneumococcal meningitis were identified from January 2008 to December 2012, 77/148 (52 %) of which were due to non-PCV10 isolates, with 50/96 (52.1 %) from pre-vaccine and 27/52 (52 %) from post-vaccine periods (Table 1). The total non-PCV10 cases consisted of 32.5 % women and 67.5 % men (Table 1). Of the 71 patients for whom age information was available, eight (16.5 %) were in children aged $\leq$5 years that occurred only in the pre-PCV10 period (P=0.04). The majority of cases were diagnosed among adults aged between 19 and 49 years (41.7 and 60.9 % from the pre- and post-vaccine periods,

Table 1. Characteristics of patients with pneumococcal meningitis caused by non-PCV10 pneumococci before and after vaccination

Characteristics	Post-PCV10 period, *n*=50 (100 %)	Pre-PCV10 period, *n*=27 (100 %)	*P*
Demographic data			
Male gender	34 (68 %)	18 (69 %)	1.00
Age groups (years), median (range)	26 (10.5–46.5)	36 (27–53)	0.07
≤5	8 (16.5 %)	0 (0)	0.04
6–18	9 (18.8 %)	3 (13 %)	0.58
19–49	20 (41.7 %)	14 (60.9 %)	0.14
≥50	11 (22.9 %)	6 (26.1 %)	0.79
Clinical information			
Neurological status at admission*	33 (66 %)	18 (69 %)	0.96
Days of symptoms, median (range)	3 (1–4.5)	2 (1–3)	0.29
Days of hospitalization, median (range)	14 (11–20)	22 (15–32)	0.03
ICU admission (days), median (range)	13 (6–24)	10 (7–21)	0.78
Mortality	18 %	15 %	0.97
CSF information			
White cell count (cells mm^{-3}), median (range)	4150 (627–10 000)	3840 (1100–10 000)	0.91
Protein (mg dl^{-1}), median (range)	300 (300–500)	500 (350–565)	0.03
Glucose (mg dl^{-1}), median (range)	20 (20–40)	20 (20–32)	0.60
Cases non-susceptible to:			
Penicillin	4 (8 %)	1 (3.7 %)	0.53
Tetracycline	13 (26 %)	3 (11.1 %)	0.13
Trimethoprim-sulfamethoxazole	19 (38 %)	11 (47.7 %)	0.82
Erythromycin	0 (0)	1 (3.7 %)	0.35
Acute illness preceding meningitis (%)			
Pneumonia	6 (12 %)	0 (0)	0.65
Acute otitis media	4 (8 %)	2 (7.7 %)	0.65
Head trauma	6 (12 %)	1 (3.9 %)	1.00
Upper respiratory tract infection	12 (24 %)	3 (11.5 %)	0.19

ICU, intensive care unit (21 patients were admitted).
*Coma or altered mental status.

respectively) and ≥50 years (22.9 and 26.1 %, from the pre- and post-vaccine periods, respectively). Differences in the number of days of hospitalization (14 vs 22; P=0.04) and in the CSF protein content (300 vs 500 mg dl^{-1}; P=0.03) were observed between the pre- and the post-vaccine periods. No differences were observed with respect to antibiotic resistance or acute illness preceding meningitis.

The annual incidence of pneumococcal meningitis (PCV10 and non-PCV10 cases) decreased from 0.9/100 000 inhabitants (30 cases) in 2008 to 0.36/100 000 (12 cases) inhabitants in 2012 (P<0.05). Fig. 1 shows the annual incidence of pneumococcal meningitis stratified by PCV10 and non-PCV10 cases during the study period. In general, there was a decrease in the incidence of both PCV10 and non-PCV10 cases during the study period. In particular, we observed a decrease in the incidence of non-PCV10 cases from 0.69/100 000 inhabitants in 2008 to 0.21/100 000 inhabitants in 2012 (P<0.76). For PCV10 cases, the incidence reduced from 0.57/100 000 inhabitants in 2010 to 0.21/100 000 inhabitants in 2012 (P<1.0) after the introduction of the PCV10 vaccine in June 2010.

Serotype distribution and antimicrobial susceptibility

Over the study period, 28 different serotypes were found: six occurred only in the post-vaccine period (10F, 11A, 21, 22F, 23B and 24F), whereas ten serotypes (6A, 6C, 17F, 18A, 19A, 20, 23A, 28A, 35F and 38) were identified only in the pre-vaccine period. Twelve serotypes were found in both periods (3, 7C, 8, 9N, 10A, 12F, 13, 15B, 16F, 18B, 34 and 35B). In the pre-vaccine period, the most frequent non-PCV10 serotypes were as follows: 3 (*n*=6; 12 %), 19A (*n*=4; 8 %) and 6A (*n*=4; 8 %). In the post-vaccine period, non-PCV10 serotypes 12F (*n*=6; 22.2 %), 10A (*n*=3; 11.1 %), 15B (*n*=2; 7.4 %) and 18B (*n*=2; 7.4 %) were the most prevalent. A slight increase in the number of 12F serotype cases was detected in the post-vaccine period (Fig. 2).

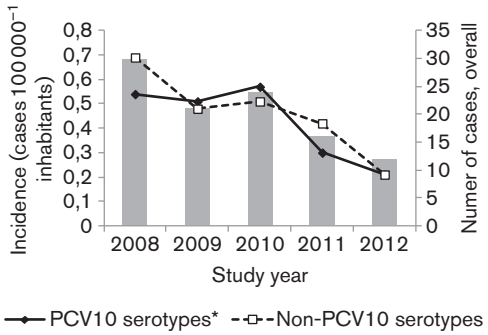

Fig. 1. Incidence and number of cases of pneumococcal meningitis in the metropolitan region of Salvador, Brazil, stratified by PCV10 and non-PCV10 serotypes, 2008–2012.

Of 77 non-PCV10 isolates characterized, 43 (55.8 %) were non-susceptible to one or more antibiotics. All isolates were susceptible to cefotaxime, clindamycin, chloramphenicol, levofloxacin and vancomycin. A total of 28 (36.4 %) and 16 (20.8 %) isolates were resistant to trimethoprim-sulfamethoxazole and tetracycline, respectively. Serotypes 19A (three isolates), 9N (one isolate) and 12F (one isolate) were not susceptible to penicillin. One isolate (serotype 21) from the post-vaccine period was erythromycin resistant (Fig. 3).

PFGE and MLST analysis

We identified 45 isolates grouped into 19 PFGE profiles with $\geq$80 % similarity. Among those profiles, 14/19 (73.7 %) and 5/19 (26.3 %) occurred in the pre- and post-vaccine periods, respectively. Overall, 32/77 (41.5 %) of isolates were not clustered due to exhibiting <80 % similarity in PFGE, with higher variability in the post-vaccine period (17/27; 63 %). Sequence types (STs) 80 (n=3 isolates), 193 (n=2 isolates) and 8376 (n=2 isolates; serotype 12F) were found in both pre- and post-vaccine periods. The two 12F isolates (ST8376) were susceptible and resistant to penicillin in the pre- and post-vaccine periods, respectively. Seven STs were newly assigned in our study, of which five were from the pre-vaccine period (ST9678, ST9679, ST9680, ST9070 and ST9071), one from the post-vaccine period (ST9681) and one was detected in both periods (ST8376). Furthermore, in the pre-vaccine period, two serotypes (3 and 35B) were found to belong to the same ST (ST180), while in the post-vaccine period, serotypes 12F and 21 (resistant to trimethoprim-sulfamethoxazole and erythromycin) showed identical PFGE profile and the same ST (ST218).

DISCUSSION

In Brazil, the introduction of PCV10 to the childhood immunization schedule in 2010 resulted in several changes in the epidemiology of cases of pneumococcal meningitis; in particular, we observed an overall decrease in the number of cases. During the years following its introduction, vaccination coverage rates improved dramatically, with 38 035 doses administer in 2011 (73 % of the target population) and 42 415 doses in 2012 (81.5 % of the target population) – levels which are considered satisfactory (DATASUS, 2015).

We did not observe any post-PCV10 emergence of a particular serotype in this study. The PCV10 vaccine was only recently introduced to our region, and 2010 was a year of transition. Furthermore, comparison of the number of 12F, 10A, 15B and 18B serotype isolates identified in the post-vaccine period to those obtained in past years indicated a normal fluctuation of these serotypes over the years, with no evidence of specific emergence of any particular serotype (data not shown). Fluctuations in the prevalence of a particular serotype may occur naturally in the population of pneumococci, even in the absence of the selective pressure imposed by the use of conjugate vaccines (Finland & Barnes, 1977; Lagos *et al.*, 2008; Ruckinger *et al.*, 2008). Continued surveillance over a longer period is needed to fully assess the impact of vaccine introduction in our region on the pneumococcal population.

In this study, only four isolates of serotype 19A were identified in the pre-vaccine period. These findings are in agreement with those of dos Santos *et al.* (2013) in São Paulo, which did not show an increased incidence of this serotype

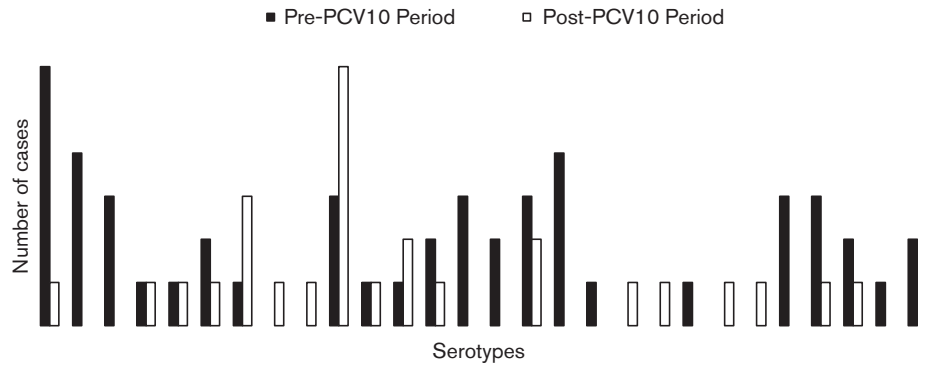

Fig. 2. Distribution of non-vaccine serotypes among isolates from pneumococcal meningitis cases in the metropolitan area of Salvador, 2010–2012, stratified by pre-vaccine and post-vaccine periods (n=77).

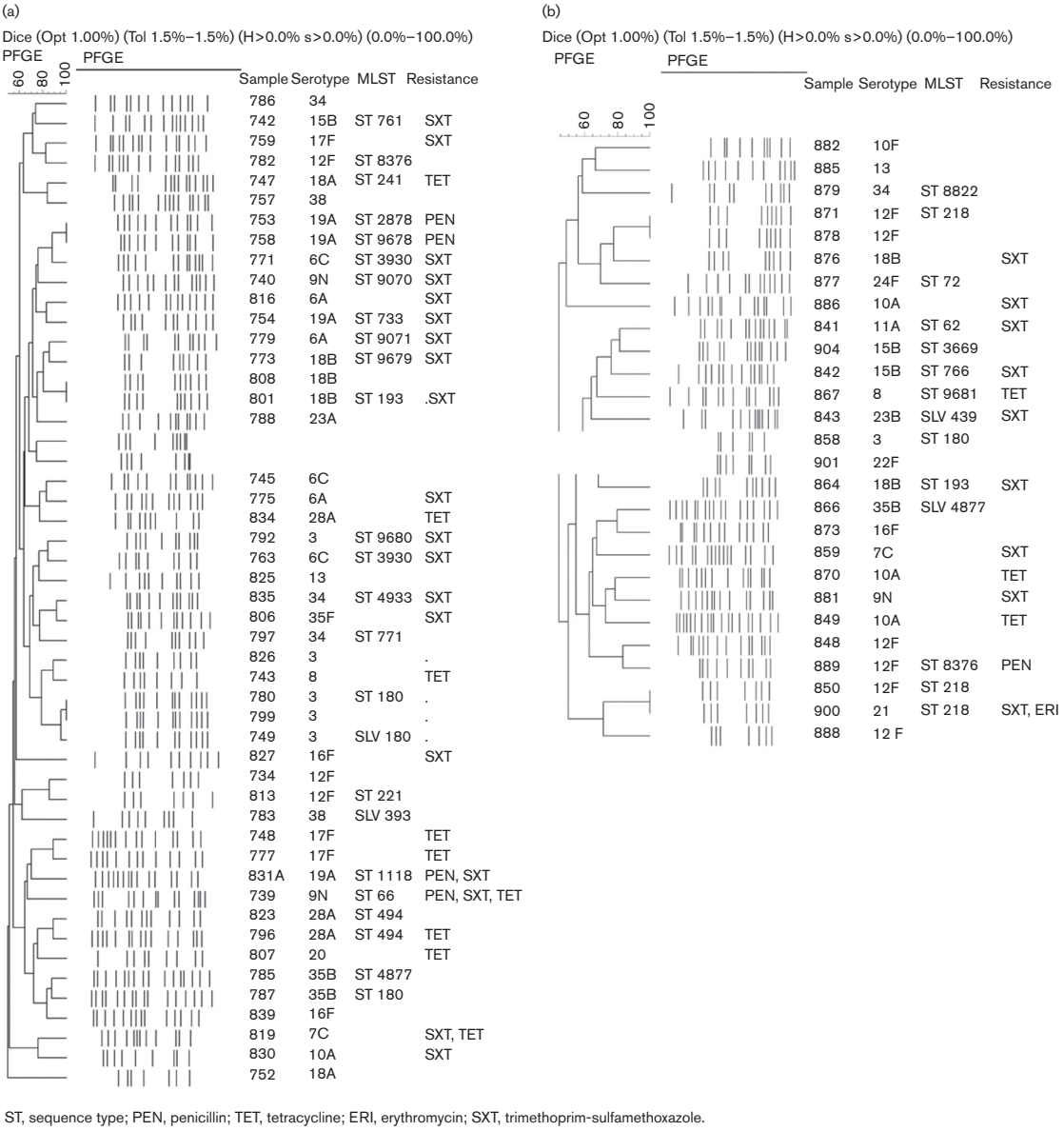

Fig. 3. Dendrogram of PFGE molecular profiles of non-PVC10 serotypes isolated from pneumococcal meningitis in the metropolitan area of Salvador. (a) Pre-vaccine period (n=50). (b) Post-vaccine period (n=27).

in that city during the post-vaccine period (dos Santos *et al.*, 2013). The incidence of serotype 19A has remained low and stable in Latin America and the Caribbean for 20 years (Castaneda *et al.*, 2012), and a recent study in Brazil demonstrated the specific effectiveness of PCV10 against serotype 19A (Domingues *et al.*, 2014). Regarding antimicrobial susceptibility, three of four serotype 19A isolates identified in the pre-vaccine period were penicillin non-susceptible. In the USA, after PCV7 vaccination, serotype 19A emerged as an important cause of invasive pneumococcal disease, and the most common 19A clones were associated with increased multi-resistance to penicillin and other antimicrobials (Pilishvili *et al.*, 2010).

Studies of the immunogenicity of the PCV10 vaccine show that, although it is active against serotype 6A, its activity is approximately 33–50 % lower than that observed with PCV7. Therefore, it is not yet clear whether PCV10 induces cross-protection against serotype 6A (Vesikari *et al.*, 2011; Farkouh *et al.*, 2012). In this study, serotype 6A was found only in the pre-vaccine period.

Of the 77 isolates characterized in this study, 43 (55.8 %) exhibited resistance to at least one antimicrobial, and 13.5 % of the isolates (n=7) showed resistance to two or more antibiotics. Multi-drug resistance to antibiotics in *S. pneumoniae* is undoubtedly a cause for concern.

However, in the USA, the introduction of PCV13 in 2010 led to a reduction in resistant infections (Moore *et al.*, 2015).

The evidence of non-susceptibility to penicillin in the single serotype 12F isolate of the post-vaccination period suggests that this serotype should be followed in the epidemiological surveillance system in our region, especially if this lineage becomes established as the most prevalent over time. A prospective study with a longer period of monitoring may provide conclusive information. The resistance to trimethoprim-sulfamethoxazole (39 %) and tetracycline (20.8 %) observed in this study is consistent with previous work in our region (de O Menezes *et al.*, 2011) and with another national study (Brandileone *et al.*, 2006), and may be partly associated with the previous use of antimicrobials. We found resistance to erythromycin in only one isolate (serotype 21) of the post-vaccine period, in contrast to observations elsewhere; in fact, in the USA, macrolide resistance is overtaking penicillin resistance due to the removal of PCV13 strains (especially 19A), leaving serotypes like 33F, which are primarily macrolide resistant (Metcalf *et al.*, 2015).

Genotypic characterization of the *S. pneumoniae* isolates by PFGE showed great genetic diversity among the non-PCV10 serotypes in the post-vaccine period (63 %). In the pre-vaccine period, one 9N isolate was identified as belonging to ST66. It is important to note that this ST, associated with serotype 14, is considered one of the leading and most prevalent clones associated with invasive disease in Salvador and other regions of Brazil (Brandileone *et al.*, 1997; Reis *et al.*, 2008). ST180 (serotype 3), ST193 (serotype 18B) and ST218 (serotype 12F) identified in our study are related to the globally disseminated clones Netherlands^{3}-31, Greece^{21}-30 and Denmark^{12F}-34, respectively, as described by the Pneumococcal Molecular Epidemiology Network (http://web1.sph.emory.edu/PMEN/). This molecular surveillance also encountered similar PFGE profiles (relatedness $\geq$80 %) exhibiting different capsular serotypes, suggesting the occurrence of capsular switching events in this epidemiological setting. However, this finding requires careful interpretation and further investigation using a more complete molecular approach.

As pointed out by Afonso *et al.* (2013), any study that uses a temporal method to determine the initial effects of a vaccine can be subject to variation depending on vaccination coverage and the natural lag period between the start of a vaccination programme and protection in a population (Afonso *et al.*, 2013). This study was conducted in the pre- and early post-implementation stages of the PCV10 vaccine programme in a large urban centre, and any conclusions that can be drawn from the results obtained here should be considered preliminary observations of the distribution and molecular characteristics of non-PCV10 serotypes in our region in the post-vaccine period. The scenario that we describe here could change as the use of the PCV10 vaccine is expanded and a larger number of isolates can be studied. The limitations of this study include the small number of isolates and the fact that the data may have been collected too early in the vaccination programme to detect replacement strains. Furthermore, the majority of cases were diagnosed in adults (63.4 %), a group to which the vaccine does not apply. The effects of the eventual replacement of PCV10 would need an even longer period to be detected.

It is important to note the evidence of an isolate from the pre-vaccine period expressing capsular types 35B and ST180, which had not been described before in the MLST database (http://spneumoniae.mlst.net/). Similarly, ST218 has never previously been identified in serotype 21 (resistant to trimethoprim-sulfamethoxazole and erythromycin), which had the same PFGE profile as serotype 12F in our study.

In conclusion, our study demonstrates that, during the early period following PCV10 introduction, no obvious emergence of a particular serotype was evident among non-PCV10 strains. Further studies are necessary to confirm the occurrence of capsular switching events in those serotypes presenting the same PFGE profile and the same ST. The results of our study provide the basis for future analyses regarding the effects of vaccination in the years following PCV10 introduction and for monitoring changes in population biology associated with vaccine introduction.

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REFERENCES

Afonso, E. T., Minamisava, R., Bierrenbach, A. L., Escalante, J. J. C., Alencar, A. P., Domingues, C. M., Morais-Neto, O. L., Toscano, C. M. & Andrade, A. L. (2013). Effect of 10-valent pneumococcal vaccine on pneumonia among children, Brazil. *Emerg Infect Dis* **19**, 589–597.

Brandileone, M. C., Vieira, V. S., Casagrande, S. T., Zanella, R. C., Guerra, M. L., Bokermann, S., De Moraes, J. C., Baldacci, E. R., Chamone, C. B. & other authors (1997). Prevalence of serotypes and antimicrobial resistance of *Streptococcus pneumoniae* strains isolated from Brazilian children with invasive infections. Pneumococcal study group in Brazil for the SIREVA project. regional system for vaccines in Latin America. *Microb Drug Resist* **3**, 141–146.

Brandileone, M. C., Casagrande, S. T., Guerra, M. L., Zanella, R. C., Andrade, A. L. & Di Fabio, J. L. (2006). Increase in numbers of beta-lactam-resistant invasive *Streptococcus pneumoniae* in Brazil and the impact of conjugate vaccine coverage. *J Med Microbiol* **55**, 567–574.

Calix, J. J., Dagan, R., Pelton, S. I., Porat, N. & Nahm, M. H. (2012). Differential occurrence of *Streptococcus pneumoniae* serotype 11E between asymptomatic carriage and invasive pneumococcal disease isolates reflects a unique model of pathogen microevolution. *Clin Infect Dis* **54**, 794–799.

Castañeda, E., Agudelo, C. I., De Antonio, R., Rosselli, D., Calderón, C., Ortega-Barria, E. & Colindres, R. E. (2012). *Streptococcus pneumoniae* serotype 19A in Latin America and the Caribbean: a systematic review and meta-analysis, 1990-2010. *BMC Infect Dis* **12**, 124.

CDC. Centers for Disease Control and Prevention (2014). PCR deduction of pneumococcal serotypes. Retrieved 06/06/15, 2015, from. http://www.cdc.gov/streplab/pcr.html

CLSI. Clinical and Laboratory Standards Institute (2014). Performance standards for antimicrobial susceptibility testing; Twenty-fourth informational supplement. *C. D. M100-S24*. Wayne, PA.

DATASUS. Departamento de Informática do Sistema Único de Saúde (2015). Tecnologia da informação a serviço do SUS – Informações da Saúde. Retrieved 06/06/15, 2015, from. http://tabnet.datasus.gov.br/cgi/deftohtm.exe?pni/CNV/CPNIBA.def

de O Menezes, A. P., Campos, L. C., dos Santos, M. S., Azevedo, J., Dos Santos, R. C., Carvalho, M. G., Beall, B. W., Martin, S. W., Salgado, K. & other authors (2011). Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* prior to introduction of the 10-valent pneumococcal conjugate vaccine in Brazil, 2000-2007. *Vaccine* **29**, 1139–1144.

Dias, C. A., Teixeira, L. M., Carvalho, M. G. & Beall, B. (2007). Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children. *J Med Microbiol* **56**, 1185–1188.

Domingues, C. M., Verani, J. R., Montenegro Renoiner, E. I., de Cunto Brandileone, M. C., Flannery, B., de Oliveira, L. H., Santos, J. B., de Moraes, J. C. & Brazilian Pneumococcal Conjugate Vaccine Effectiveness Study Group (2014). Effectiveness of ten-valent pneumococcal conjugate vaccine against invasive pneumococcal disease in Brazil: a matched case-control study. *Lancet Respir Med* **2**, 464–471.

dos Santos, M. S., Azevedo, J., Menezes, A. P., Cordeiro, S. M., Escobar, E. C., Lima, J. B., Campos, L. C., Carvalho, M. G., Reis, M. G. & other authors (2015). Temporal trends and clonal diversity of penicillin non-susceptible pneumococci from meningitis cases from 1996 to 2012, in Salvador, Brazil. *BMC Infect Dis* **15**, 302–312.

dos Santos, S. R., Passadore, L. F., Takagi, E. H., Fujii, C. M., Yoshioka, C. R., Gilio, A. E. & Martinez, M. B. (2013). Serotype distribution of *Streptococcus pneumoniae* isolated from patients with invasive pneumococcal disease in Brazil before and after ten-pneumococcal conjugate vaccine implementation. *Vaccine* **31**, 6150–6154.

Enright, M. C. & Spratt, B. G. (1998). A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* **144**, 3049–3060.

Farkouh, R. A., Klok, R. M., Postma, M. J., Roberts, C. S. & Strutton, D. R. (2012). Cost-effectiveness models of pneumococcal conjugate vaccines: variability and impact of modeling assumptions. *Expert Rev Vaccines* **11**, 1235–1247.

Feldman, C. & Anderson, R. (2014). Review: current and new generation pneumococcal vaccines. *J Infect* **69**, 309–325.

Finland, M. & Barnes, M. W. (1977). Changes in occurrence of capsular serotypes of *Streptococcus pneumoniae* at Boston City hospital during selected years between 1935 and 1974. *J Clin Microbiol* **5**, 154–166.

Geno, K. A., Gilbert, G. L., Song, J. Y., Skovsted, I. C., Klugman, K. P., Jones, C., Konradsen, H. B. & Nahm, M. H. (2015). Pneumococcal capsules and their types: past, present, and future. *Clin Microbiol Rev* **28**, 871–899.

Hammitt, L. L., Akech, D. O., Morpeth, S. C., Karani, A., Kihuha, N., Nyongesa, S., Bwanaali, T., Mumbo, E., Kamau, T. & other authors (2014). Population effect of 10-valent pneumococcal conjugate vaccine on nasopharyngeal carriage of *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* in Kilifi, Kenya: findings from cross-sectional carriage studies. *Lancet Glob Health* **2**, e397–405.

IBGE. Instituto Brasileiro de Geografia e Estatística (2010). Resultado do censo populacional de salvador e cidades na área metropolitana, Bahia. Retrieved 10/06/2015.

Lagos, R., Muñoz, A., San Martin, O., Maldonado, A., Hormazabal, J. C., Blackwelder, W. C. & Levine, M. M. (2008). Age- and serotype-specific pediatric invasive pneumococcal disease: insights from systematic surveillance in Santiago, Chile, 1994-2007. *J Infect Dis* **198**, 1809–1817.

Leite, C. R., Azevedo, J., Galvão, V. S., Moreno-Carvalho, O., Reis, J. N. & Nascimento-Carvalho, C. (2016). Clinical and bacteriological characteristics of invasive pneumococcal disease after pneumococcal 10-valent conjugate vaccine implementation in Salvador, Brazil. *Braz J Infect Dis* **20**, 56–60.

McEllistrem, M. C., Stout, J. E. & Harrison, L. H. (2000). Simplified protocol for pulsed-field gel electrophoresis analysis of *Streptococcus pneumoniae*. *J Clin Microbiol* **38**, 351–353.

Metcalf, B. J., Gertz, R. E., Gladstone, R. A., Walker, H., Sherwood, L. K., Jackson, D., Li, Z., Law, C., Hawkins, P. A. & other authors (2015). Strain features and distributions in pneumococci from children with invasive disease before and after 13-valent conjugate vaccine implementation in the USA. *Clin Microbiol Infec* **22**, 60.e9–60.e29.

Millar, E. V., Watt, J. P., Bronsdon, M. A., Dallas, J., Reid, R., Santosham, M. & O'Brien, K. L. (2008). Indirect effect of 7-valent pneumococcal conjugate vaccine on pneumococcal colonization among unvaccinated household members. *Clin Infect Dis* **47**, 989–996.

Moore, M. R., Link-Gelles, R., Schaffner, W., Lynfield, R., Lexau, C., Bennett, N. M., Petit, S., Zansky, S. M., Harrison, L. H. & other authors (2015). Effect of use of 13-valent pneumococcal conjugate vaccine in children on invasive pneumococcal disease in children and adults in the USA: analysis of multisite, population-based surveillance. *Lancet Infect Dis* **15**, 301–309.

Pilishvili, T., Lexau, C., Farley, M. M., Hadler, J., Harrison, L. H., Bennett, N. M., Reingold, A., Thomas, A., Schaffner, W. & other authors (2010). Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* **201**, 32–41.

Prymula, R. & Schuerman, L. (2009). 10-valent pneumococcal nontypeable *Haemophilus influenzae* PD conjugate vaccine: Synflorix. *Expert Rev Vaccines* **8**, 1479–1500.

Reis, J. N., Palma, T., Ribeiro, G. S., Pinheiro, R. M., Ribeiro, C. T., Cordeiro, S. M., da Silva Filho, H. P., Moschioni, M., Thompson, T. A. & other authors (2008). Transmission of *Streptococcus pneumoniae* in an urban slum community. *J Infect* **57**, 204–213.

Rückinger, S., von Kries, R., Reinert, R. R., van der Linden, M. & Siedler, A. (2008). Childhood invasive pneumococcal disease in Germany between 1997 and 2003: variability in incidence and serotype distribution in absence of general pneumococcal conjugate vaccination. *Vaccine* **26**, 3984–3986.

Vesikari, T., Karvonen, A., Korhonen, T., Karppa, T., Sadeharju, K., Fanic, A., Dieussaert, I. & Schuerman, L. (2011). Immunogenicity of 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D conjugate vaccine when administered as catch-up vaccination to children 7 months to 5 years of age. *Pediatr Infect Dis J* **30**, e130–141.

Werno, A. M. & Murdoch, D. R. (2008). Medical microbiology: laboratory diagnosis of invasive pneumococcal disease. *Clin Infect Dis* **46**, 926–932.

World Health Organization – WHO (2012). Pneumococcal vaccines – WHO position paper. Retrieved 02/07/2016 from. http://www.who.int/wer/2012/wer8714/en