Serotype and genotype distributions of pneumococcal carriage isolates recovered from Brazilian children attending day-care centres

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Pneumococcal nasopharyngeal carriage isolates recovered from Brazilian children attending day-care centres in 2005 were assessed for serotype, genotype and penicillin susceptibility phenotype. As 124 of the 253 isolates (49%) were characterized previously with respect to serotype and penicillin susceptibility, the primary objectives were to examine clonal associations and penicillin susceptibility within major serotypes and to assess the suitability of conventional multiplex PCR for deducing carriage serotypes within this population. Using a combination of PCR-based serotyping and the Quellung reaction, serotypes were identified for 81% (205/253) of the isolates, with serogroups or types 14, 6, 23F, 19F and 18 being predominant. Included within the 205 isolates successfully serotyped by PCR were 28 isolates that had become non-viable. Forty-eight isolates were non-typable using both the PCR method and the Quellung reaction. Penicillin non-susceptibility was observed within 16 of the 18 multilocus sequence types detected. Thus, this study provides further evidence from a diverse collection of pneumococcal clones that PCR-based serotype deduction is useful for providing supportive evidence for pneumococcal conjugate vaccine implementation.

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INTRODUCTION

The human nasopharynx (NP) provides a reservoir for pneumococcal strains that cause disease (de Lencastre & Tomaz, 2002), and elucidating capsular serotype distribution within carriage is useful for monitoring vaccine suitability and vaccination impact. In addition, the determination of penicillin-susceptibility profiles among NP isolates potentially provides some assessment of recent β -lactam antibiotic usage within a given region as these drugs directly select for the emergence of detectable penicillin non-susceptibility in pneumococcal populations (Dowson *et al.*, 1994).

Pneumococci are traditionally serotyped by the Quellung reaction; however, sequential multiplexed PCR approaches based on serotype-specific DNA sequences provide a viable and straightforward alternative (Pai *et al.*, 2006; Carvalho *et al.*, 2010). Multilocus sequence typing (MLST) and

penicillin-susceptibility testing allow the global tracking of specific pneumococcal clones as causes of disease or carriage and the detection of emerging resistance to β -lactam antibiotics. Here, we describe the distribution of serotypes and penicillin-resistance phenotypes and genotypes among pneumococcal NP carriage isolates previously recovered from healthy Brazilian children, and show that multiplex PCR provides a useful alternative for revealing serotype distribution among these diverse strains.

METHODS

A collection of 217 viable *Streptococcus pneumoniae* nasopharyngeal swab specimen isolates were recovered from 686 stored isolates originally collected from 1192 healthy children ≤ 5 years old attending 62 day-care centres in the municipality of Goiânia, Brazil, in 2005 (Franco *et al.*, 2010). The remaining 469 of these isolates were not viable after storage. From the latter, we randomly selected 36 stored cultures for PCR-based serotype determination. Of the 253 isolates (217 viable and 36 non-viable) examined in our study at the Centers for Disease Control and Prevention (CDC), serotype and

Abbreviations: MLST, multilocus sequence typing; NP, nasopharynx; NT, non-typable; ST, sequence type.

resistance properties of 124 (49%) have been described previously (Franco *et al.*, 2010; data compared in Results).

All testing described here was performed in the CDC *Streptococcus* Laboratory. Pneumococci were identified by colony morphology, Gram staining, optochin test and bile solubility test (Franco *et al.*, 2010). Susceptibility for penicillin was assessed using an oxacillin disc test (≥ 19 mm) and MIC methods. The MIC breakpoints for penicillin were ≤ 0.06 $\mu\text{g ml}^{-1}$ (susceptible), 0.12–1.0 $\mu\text{g ml}^{-1}$ (intermediate resistance) and ≥ 2.0 $\mu\text{g ml}^{-1}$ (resistant) (CLSI, 2007).

For the 217 viable isolates, crude DNA extraction and sequential multiplex PCR for 29 serotypes commonly associated with pneumococcal disease were performed as described previously (Pai *et al.*, 2006). For the 36 non-viable isolates available for analyses, 500 μl of the resuspended stored specimen was centrifuged and also subjected to crude DNA extraction. The multiplex reactions used for this work (29 individual reactions) were redistributed to target common invasive and carriage serotypes observed in Latin America (Laval *et al.*, 2006; Dias *et al.*, 2007). All multiplex reactions included corresponding serotype controls and the internal pneumococcal identification control *cpsA*. For viable isolates, the Quellung reaction, employing CDC typing sera, was used for final serotype resolution from isolates PCR typed as 6A/6B/6C/6D, 22A/22F and serogroup 18. A *wciN6*^C-specific PCR was performed to identify potential serotype

6C and 6D isolates, as described previously (Carvalho *et al.*, 2009; Bratcher *et al.*, 2010). Classical serotyping, including the Quellung reaction, for assigning the specific serotype was used to assess viable isolates that were non-serotypable by PCR.

MLST was performed as described on the global pneumococcal MLST database (<http://spneumoniae.mlst.net>) with modifications (Moore *et al.*, 2008) on 55 viable isolates, representing 12 detected serotypes and a random selection of non-typable (NT) isolates. These isolates were all reconfirmed for penicillin-susceptibility phenotypes using the broth dilution method (CLSI, 2007).

RESULTS AND DISCUSSION

Capsular serotypes or serogroups were assigned for 177/217 (81.6%) of the viable isolates and for 28/36 (77.8%) of the non-viable stored isolates (Table 1). Forty-eight of the 253 isolates (19.0%) were NT using PCR, including 40 viable isolates that were also NT using the classical Quellung methodology for all known serotypes. The *cpsA* positive control was detected in only 12 (25%) of the 48 NT isolates. Serotype distribution was similar to that in the

Table 1. Serotype, penicillin MICs and genotype (ST) distribution of pneumococci isolated from the NP of healthy children ≤ 5 years old attending day-care centres in Brazil

Serotype	No. isolates (%)	No. isolates genotyped	Genotype (no. isolates)	<i>aroE-gdh-gki-recP-spi-xpt-ddl</i>	Penicillin MIC ($\mu\text{g ml}^{-1}$) (no. isolates)
14*	107 (42.3)	10	156 (7) 66 (3)	007-011-010-001-006-008-001 002-008-002-004-001-001-001	0.032 (1), 0.125 (1), 2.0 (5) 0.015 (1), 0.125 (1), 1.0 (1)
6A	20 (7.9)	6	726 (5) 4747 (1)†	001-005-006-012-013-020-018 001-005-006-016-034-008-018	0.032 (1), 0.125 (1), 0.5 (1), 2.0 (2) 0.015 (1)
23F	18 (7.1)	10	285 (4) 4024 (6)	004-004-002-004-038-001-001 007-011-010-001-006-008-008	0.0032 (1), 1.0 (1), 2.0 (1), 4.0 (1) 0.032 (2), 0.125 (2), 0.5 (1), 2.0 (1)
6A/6B	12 (4.7)	ND	ND		
19F	9 (3.5)	5	1203 (5)	010-041-047-063-006-014-002	0.032 (2), 0.125 (2), 0.25 (1)
2	7 (2.8)	3	595 (3)	007-005-001-001-010-007-015	0.032 (1), 0.125 (1), 1.0 (1)
18C	7 (2.8)	1	193 (1)	008-010-002-016-001-026-001	0.25 (1)
6C	6 (2.4)	3	2777 (3)	007-013-008-006-001-001-008	0.125 (1), 2.0 (2)
19A	5 (2.0)	5	810 (2) 2880 (3)	007-005-009-001-013-001-008 002-005-009-012-014-003-006	0.125 (1), 0.25 (1) 0.032 (1), 0.125 (2)
6B	4 (1.6)	2	748 (1) 751 (1)	007-010-001-002-006-001-018 007-047-029-037-006-001-080	0.25 (1) 0.25 (1)
4	3 (1.2)	ND	ND		
10A	2 (0.8)	ND	ND		
15A	2 (0.8)	2	1058 (1) 2216 (1)	002-013-002-001-004-001-001 002-136-002-015-006-001-001	0.125 (1) 1.0 (1)
9V	1 (0.4)	ND	ND		
11A	1 (0.4)	1	62 (1)	002-005-029-012-016-003-014	0.125 (1)
18B	1 (0.4)	ND	ND		0.25 (1)
NT‡	48 (18.9)	7	2315 (5) 4746 (2)†	008-037-009-012-002-028-224 008-037-009-012-002-028-018	0 0.015 (1), 0.064 (1), 0.125 (2), 0.25 (1) 0.032 (1), 2.0 (1)
Total	253 (100)	55			

ND, Not done (non-viable stored isolates).

*Three isolates tested as serotype 14 by Quellung reaction consistently yielded the mixed multiplex PCR result of serotype 14 and serotype 6A/6B, despite repeated isolation attempts to resolve this.

†New STs discovered during this work.

‡NT, Non-typable by conventional multiplex PCR and Quellung reaction.

recently published overlapping study (Franco *et al.*, 2010) in that serotypes/serogroups 14, 23F, 19A, 6 and 19F were the most common serotypes. However, we did find discrepancies among nine (7.3 %) of the 124 results that were tested in both studies. Three isolates that were found previously to be NT were serotypes 14 (two isolates) and 6B (one isolate). The other six discrepancies comprised serotypes 19A (previously 19F), 19F (previously 19A), 14 (previously 23F), 6C (previously 6B) and two NT isolates (previously 6A and 6B). Although the previously published study (Franco *et al.*, 2010) reported the detection of 46 different serotypes, among our smaller sampling we detected only 16 (in addition to NT isolates). It is possible that our study was biased due to the fact that we primarily characterized viable isolates, which were in the minority among the collection. It is also possible that there were differences in serotyping methodologies. As described in Methods, 217 of the 253 isolates that we characterized were from an overall collection of 686 stored isolates, 469 (68.4 %) of which did not maintain viability during storage. It is conceivable that much of the original serotype diversity reflected in the original study was lost, as we have noted profound differences in the abilities of different pneumococcal strains to maintain viability under different environmental conditions.

Using the first multiplex reaction for serotypes 6A/6B/6C/(6D), 9V/(9A), 14, 19A and 23F (note that serotypes rarely encountered in carriage or disease are in parentheses), serotypes or serogroups were deduced for 171 (67.6 %) of the isolates; and using the second reaction for serotypes 3, 7F/(7A), 10A, 15B/15C, 18C/(18A)/(18B)/(18F) and 19F, serotypes or serogroups were deduced for an additional 19 (7.5 %) isolates. Sequential PCRs 3–6 [reaction 3 comprised 1, 5, 9N/(9L), 11A/(11D) and 17F; reaction 4 comprised 4, 7C/(7B)/(40), 12F/(12A)/(44)/(46), 23A and 38/(25A)/(25F); reaction 5 comprised 2, 8, 20, 22F/(22A), 31 and 34; reaction 6 comprised 15A/(15F), 16F, 33F/(33A)/37, 35B and 35F/(47F)] added an additional 15 isolates that were PCR serotyped, giving a total of 205 (81.0 %) of the 253 isolates. The *cpsA* pneumococcal identification control was not detected in four serotype 14 isolates. Using the Quellung reaction, we verified that these strains expressed serotype 14. The most frequent types or serogroups recovered were serotype 14, serogroup 6, serotype 23F, serotype 19F and serogroup 18, accounting for 183 (72.3 %) of the isolates. The absence of the *cpsA* amplicon positive control was much more common among NT isolates, occurring in 75 % (36/48) of the NT isolates. Remarkably, none of the 40 viable Quellung NT isolates corresponded to a 'PCR serotype'. We have found that most invasive NT isolates yield a PCR-derived serotype, indicating that non-typeability among invasive isolates could possibly reflect loss of capsule expression after recovery from invasive infections.

Mixed carriage of two to three serotypes was detected in 17 children, involving the combinations 14 + 6A/6B + 15A (two children), 14 + 6A/6B + 19A (one), 14 + 6A/6B + 18C

(one), 14 + 6A/6B + 23F (one), 14 + 6A (six), 14 + 6B (one), 14 + 6C (one), 14 + 18C (one), 14 + 9L/9N (one), 14 + 6A/6B (one) and 19F + 6A/6B (one). Three of the six 6A/6B isolates were *wciN6*^{CD} negative but were non-viable, so could not be resolved into component serotypes 6A and 6B using our PCR assay. Curiously, three viable isolates were found to be PCR positive for 6A/6B and PCR negative for *wciN6*^{CD}, yet were also PCR positive for serotype 14. These three isolates were Quellung positive for serotype 14, yet Quellung negative for serogroup 6, despite repeated restreaking and testing of multiple single colonies by both the Quellung reaction and PCR. Presumably, these three isolates reflect the expression of a functional *cps14* locus and the presence of non-functional serogroup 6-specific capsular biosynthetic locus sequences. We have not encountered this result previously among thousands of diverse isolates that have been serotyped in parallel using both the Quellung reaction and PCR (unpublished data). A mix of serotypable and non-serotypable pneumococci were found in three children: 14 + NT (two isolates) and 6A/6B + NT (one non-viable isolate). It was interesting that serotype 14 and serogroup 6 were over-represented among the 20 mixed-culture specimens (18 and 15, respectively).

Our strategy was to perform MLST within each serotype encountered that exhibited any penicillin non-susceptibility whenever possible. We targeted representative penicillin-sensitive (MIC 0.015–0.064 µg ml⁻¹), penicillin-intermediate (MIC 0.125–1.0 µg ml⁻¹) and penicillin-resistant (MIC ≥ 2 µg ml⁻¹) isolates within each serotype whenever possible. Thirteen (23.6 %) of 55 genotyped isolates were penicillin resistant (MIC ≥ 2 µg ml⁻¹) and 28 (50.9 %) isolates had intermediate penicillin resistance (MIC 0.125–1.0 µg ml⁻¹). Among these, we found 18 multilocus sequence types (STs) (Table 1), two of which had not been identified previously. In our experience, we have found that pneumococcal strains of the same ST and serotype generally share a narrow range of penicillin MIC values (Gertz *et al.*, 2003). It was therefore interesting in this study to reproducibly observe a broad range of penicillin MICs (exhibiting up to 66-fold differences) within 11 of the 12 ST/serotype combinations represented by multiple isolates (Table 1). The observation of intermediate penicillin resistance within STs 62, 66 and 193 is possibly reflective of high antibiotic use within this community, as these STs are normally associated with basally penicillin-sensitive strains (Gertz *et al.*, 2003; Beall *et al.*, 2006). Several of these STs or ST/serotype combinations were interesting in that they have been documented exclusively from Brazil and other Latin American countries within the global pneumococcal MLST database (<http://spneumoniae.mlst.net>; unless specific references are provided regarding MLST data presented here, the source of the information provided is the global database). These included STs 726, 748, 810, 1751, 2777, 2880, 4746 and 4747 (this work or previously recorded work). ST/serotype associations unique to Brazil or other South American countries include ST595/serotype 2, ST2777/6C, ST193/18C, ST1058/15A, ST2216/15A, ST810/19A, ST285/22F and ST4024/23F. New STs encountered

during this work included ST4747 and ST4746. Many of the remaining ST/genotype associations depicted in Table 1 also differed from that typically encountered in the USA and elsewhere. For example, among the ten serotype 14 isolates that were genotyped, we found that ST156 was predominant. ST156 is associated primarily with globally disseminated penicillin-resistant serotype 9V (McGee *et al.*, 2001) and is also the most common invasive serotype 9V genotype in the USA (Beall *et al.*, 2006). The remaining serotype 14 isolates were ST66, usually associated with penicillin-susceptible serotype 9N in the USA (Gertz *et al.*, 2003; Beall *et al.*, 2006) and elsewhere. The clonal complex represented by ST156 was also well represented by serotype 23F. Six of the nine serotype 23F isolates were ST4024, a single-locus variant of ST156. It is interesting that ST4024 was initially discovered within a single invasive serotype 19A isolate recovered in the USA from a child in 2006 (Beall *et al.*, 2011).

Eleven serogroup 6 isolates were genotyped, with no inter-serotype genetic relatedness detected between the three detected serotypes (6A, 6B, 6C). We have recently shown that we are capable of resolving all four known serogroup 6 serotypes, including 6D (Mercado *et al.*, 2011), using our specific factor sera prepared against serotypes 6A, 6B and 6C (Melnick *et al.*, 2010).

For the serotypes found in this study, only serotype 2 was not included among the 29 individual PCR assays that constituted the sequential multiplex assay used (Pai *et al.*, 2006). We have since expanded the number of individual reactions to 40 (see <http://www.cdc.gov/ncidod/biotech/files/pcr-Latin-Amer-clinical-specimens.pdf> for the Latin American sequential multiplex assay), such that the current assay includes serotype 2 and ten additional serotypes that we have found to be well represented in other carriage studies. There were a large number of isolates (48) that were NT using the Quellung reaction and PCR methods. It appears that the majority of these NT isolates are likely to represent genotypes within the large ST344 lineage, as both STs that were detected (ST2315 in five isolates and ST4746 in two isolates) were found to be triple-locus variants of ST344. ST344 is the ancestral strain of a large pneumococcal lineage that has lost its capsular locus, is commonly associated with carriage and is the most commonly implicated pneumococcal genotype in conjunctivitis outbreaks (Hanage *et al.*, 2006).

For serotype 14 and NT isolates, our genotyping results were in general agreement with those described recently for yet another overlapping study that analysed some of the same isolates (Andrade *et al.*, 2010). These authors performed PFGE analysis on 106 carriage isolates comprising 84 serotype 14 isolates and 22 NT isolates. They found that the major cluster of serotype 14 isolates shared similar PFGE patterns to ST156 strains. In addition, they found that the majority of their NT strains shared the same multilocus ST, ST2315, which we also found among five of the seven NT isolates from our study. They did not, however, point out the relatedness of ST2315 within the

major NT lineage with the founder genotype ST344. This is also evident when performing eBURST on the entire known database of 6500 STs (as of 11 May 2011) allowing for ST sets to share five of seven identical alleles for inclusion within the same eBURST group. When doing so, both ST2315 and ST4716 (found among two of our NT isolates) were within the same 82-member group predicted to have originated from the NT ST344 clone (see <http://spneumoniae.mlst.net/eburst/database.asp>).

Although multiple strains of pneumococci often colonize the NP simultaneously, the identification of multiple serotypes from a single specimen is often unsuccessful. The study presented here was limited in that it did not utilize more sensitive methodology recently developed that uses broth enrichment of NP swabs (Carvalho *et al.*, 2010). Broth enrichment was found to improve pneumococcal isolation on solid medium and was especially useful for detection of multiple serotypes within mixed broth cultures without pneumococcal isolation. When this methodology is utilized, it is essential to carry out all of the multiplex reactions, even after encountering positives within earlier reactions. It is extremely difficult to detect mixed serotypes using conventional isolation-based methodology due to the predominant serotype masking the presence of less densely colonizing serotypes. Nonetheless, using conventional Quellung-based serotyping and multiplex PCR on isolates, we succeeded in identifying mixed carriage from 20 NP swabs. PCR-based serotype assignment of 36 non-viable cultures allowed us to assign serotypes to 28 additional isolates, with 8 isolates found to be PCR-nontypable (*cpsA*-positive but lacking serotype-specific amplicons).

In this study, we targeted the most prevalent pneumococcal serotypes reported to be found in Brazil with the PCR serotyping assay. Through the use of the first reaction, we could serotype nearly 70% of the isolates, which greatly streamlined our efforts and reduced costs. The serotype distribution among these carriage isolates in our study was similar to that found during previous invasive (Dias *et al.*, 2007) and carriage (Laval *et al.*, 2006; Franco *et al.*, 2010) studies in Brazil, which collectively predict excellent coverage by the conjugate vaccines PCV7, PCV10 and PCV13. Pneumococcal serotypes found during this study that are covered by PCV7 and PCV10 (14, 6A or 6B, 23F, 19F, 18C, 4 and 9V) represented 181 (71.5%) isolates. When including the additional serotypes (6C and 19A) predicted to be covered by PCV13 (Dinleyici, 2010), this percentage increases to 75.9% (192 isolates). Among PCV7-related types, serotypes 6A, 6C and 19A were prevalent. Recent studies have documented an increase in the incidence of serotype 19A invasive disease (Moore *et al.*, 2008) and, to a relatively small degree, an increase in 6C invasive disease (Park *et al.*, 2008). Our results further support the contention that multiplex PCR is a reliable method for serotyping genetically diverse pneumococcal carriage isolates (or stored non-viable pneumococcal cultures) and should prove useful for evaluating serotype

distribution in support of pneumococcal conjugate vaccine introduction in Brazil.

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