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Streptococcus pneumoniae Serotypes 9 and 14 Circulating in Brazil over a 23-Year Period Prior to Introduction of the 10-Valent Pneumococcal Conjugate Vaccine: Role of International Clones in the Evolution of Antimicrobial Resistance and Description of a Novel Genotype

Tatiana C. A. Pinto,[a] Fabíola C. O. Kegele,[b] Cícero A. G. Dias,[c] Rosana R. Barros,[d] José M. Peralta,[a] Vânia L. C. Merquior,[e] Maria da Gloria Carvalho,[f] Sopio Chochua,[f] Paulina Hawkins,[f,g] Lesley McGee,[f] Lucia M. Teixeira[a]

Instituto de Microbiologia Paulo de Goes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil[a]; Instituto Fernandes Figueira, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil[b]; Departamento de Microbiologia e Parasitologia, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil[c]; Instituto Biomédico, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil[d]; Departamento de Microbiologia, Imunologia e Parasitologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil[e]; Respiratory Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[f]; Rollins School of Public Health, Emory University, Atlanta, Georgia, USA[g]

Antimicrobial-resistant pneumococcal strains have been detected worldwide since the 1960s. In Brazil, the first penicillin-nonsusceptible pneumococci (PNSP) were reported in the 1980s, and their emergence and dissemination have been mainly attributed to serogroup 9 and serotype 14 strains, especially those highly related to recognized international clones. In the present study, antimicrobial susceptibility testing and multilocus sequence typing were performed on 315 pneumococcal isolates belonging to serogroup 9 ($n = 99$) or serotype 14 ($n = 216$), recovered from patients or asymptomatic carriers between 1988 and 2011 in Brazil, in order to trace changes in antimicrobial resistance and genotypes prior to the full introduction of the pneumococcal conjugate vaccine in the country. Over the 23-year study period, the PNSP levels increased, and four clonal complexes (CC156, CC66, CC15, and CC5401) have played important roles in the evolution and dissemination of pneumococcal isolates belonging to serogroup 9 and serotype 14, as well as in the emergence of antimicrobial resistance, in the pre-pneumococcal-vaccination era. The earliest PNSP strains detected in this study belonged to serotype 9N/ST66 and were single locus variants of the international clone Tennessee^{14}-18 ST67 (CC66). The first serotype 14 PNSP isolates were identified in 1990 and were related to the England^{14}-9 ST9 (CC15) clone. Serotype 14 PNSP variants of the Spain^{9V}-3 ST156 clone with elevated penicillin MICs and nonsusceptibility to other beta-lactams were detected in 1995 and showed an increasing trend over the years. The results also indicated that introduction of ST156 in our region was preceded by the emergence of trimethoprim-sulfamethoxazole resistance and by the dissemination of ST162. In addition to the presence of successful international clones, a novel regional serotype 14 genotype (CC5401) has emerged in 1996.

Streptococcus pneumoniae is a major human pathogen associated with invasive and noninvasive infections such as meningitis, pneumonia, and acute otitis media. In addition, this microorganism circulates among the human populations by colonizing the upper respiratory tract of asymptomatic carriers, which represent its major reservoir (1, 2).

Antimicrobial-resistant pneumococcal strains, including penicillin-nonsusceptible pneumococci (PNSP), have been detected since the 1960s (3). Although more than 90 capsular types have been reported (1, 2), antibiotic-resistant isolates usually belong to a restricted number of serotypes. Among them, serogroup 9 and serotype 14 are highly prevalent in both invasive pneumococcal disease and nasopharyngeal carriage (1, 4, 5).

In Brazil, emergence of PNSP has been mainly attributed to the expansion of serogroup 9 and serotype 14 isolates, especially those belonging to successful and widespread clones described by the Pneumococcal Molecular Epidemiology Network (PMEN; http://www.pneumogen.net/pmen/), such as Spain^{9V}-3 ST156 (6–13). Moreover, a pneumococcal conjugate vaccine (10-valent scheme, PCV10) was gradually introduced in the Brazilian National Immunization Program for children under 1 year old in 2010 (14), necessitating a more focused and continuous surveillance in the region.

In the present study, antimicrobial susceptibility testing and multilocus sequence typing (MLST) were performed on 315 pneumococcal isolates restricted to serogroup 9 or serotype 14, recovered from patients or asymptomatic carriers between 1988

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Address correspondence to Lucia M. Teixeira, lmt2@micro.ufrj.br.

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and 2011 in Brazil, in order to trace changes in antimicrobial resistance and genotypes prior to the full introduction of the pneumococcal conjugate vaccine in the country.

MATERIALS AND METHODS

Bacterial strains, identification tests, and determination of capsular types. A total of 315 pneumococcal isolates was included in the present study, comprising 216 serotype 14, 3 serotype 9A, 1 serotype 9L, 35 serotype 9N, and 60 serotype 9V isolates (see Table S1 in the supplemental material).

Serotype 14 isolates were obtained between 1989 and 2011 in six different states of Brazil (Espírito Santo, Paraíba, Rio de Janeiro, Rio Grande do Sul, Santa Catarina, and São Paulo), representing the northeastern, southern, and southeastern regions of the country. Most of the isolates ($n = 162$) were recovered from patients with infections (bacteremia, meningitis, pericarditis, peritonitis, pneumonia, ocular infection, or otitis media), and 23 isolates were obtained from nasopharyngeal carriers.

Serotype 9V isolates were obtained from 1990 to 2009 in three different states (Rio de Janeiro, Rio Grande do Sul and São Paulo) of the South and Southeast regions. Forty-eight strains were recovered from cases of infection (bacteremia, meningitis, pneumonia, or ocular infection) and 10 from carriage.

Serotype 9N strains were isolated between 1988 and 2008 in three different states (Rio de Janeiro, Rio Grande do Sul and São Paulo) of the South and Southeast regions. All of them were recovered from infections (bacteremia, meningitis, or pneumonia).

Serotype 9A isolates were obtained in 1992, 1995, or 1999 in two different states (Rio de Janeiro and São Paulo) of the southeast region and were representative of bacteremia, meningitis, or carriage (one isolate each). The single serotype 9L strain was isolated in 1992 in São Paulo (southeast region) from blood.

Information on the clinical source was not available for 38 isolates, including 31 of serotype 14, two of serotype 9V and five of serotype 9N.

All the isolates were recovered during surveillance studies performed by our group, or were received from various health institutions for species confirmation and capsular typing. Isolates obtained from diseased individuals, including children and adults, were recovered from clinical samples taken as part of standard patient care procedures and did not require ethical approval for their use. Isolates from carriage studies, including both children and adults, were recovered from clinical samples collected as approved by the ethics committees of the institutions involved. The isolates were subjected to (i) phenotypic identification tests, including observation of colony morphology and hemolysis on blood agar plates, (ii) cellular characteristics as observed after Gram stain, and (iii) optochin susceptibility and bile solubility, determined according to standard procedures (15). The capsular types were determined either by multiplex PCR (16) or by the standard Quellung reaction (17) with antisera prepared by the Streptococcus Laboratory at the Centers for Disease Control and Prevention (CDC).

Antimicrobial susceptibility was evaluated according to Clinical and Laboratory Standards Institute recommendations and interpretative criteria (18, 19). Antimicrobials tested by the agar diffusion method included chloramphenicol, clindamycin, erythromycin, levofloxacin, oxacillin, rifampin, trimethoprim-sulfamethoxazole, tetracycline, and vancomycin (Oxoid, Basingstoke, United Kingdom). Penicillin, amoxicillin, cefotaxime, ceftriaxone, meropenem, erythromycin, tetracycline, and clindamycin MICs were determined by using a custom commercial panel (Sensititre; Trek Diagnostic Systems, Cleveland, OH). *S. pneumoniae* ATCC 49619 was used for quality control. Pneumococcal isolates presenting penicillin MICs of ≥0.12 μg/ml were considered nonsusceptible to penicillin.

The macrolide-lincosamide-streptogramin B (MLS_B) phenotype was assessed by the double-disk diffusion test (18, 19). The presence of the macrolide resistance determinants *ermA*, *ermB*, and *mef* and the tetracycline resistance genes *tetK*, *tetL*, tetM, and *tetO* was investigated by PCR using previously described protocols (20, 21) and an automated Veriti 96-well thermal cycler (Applied Biosystems, Inc., Carlsbad, CA). DNAs for all PCRs were obtained by using the Chelex 100 resin (Bio-Rad, Hercules, CA) as described earlier (22).

MLST. Multilocus sequence typing (MLST) was performed as described elsewhere (23) with modified primers (http://www.cdc.gov/streplab/alt-MLST-primers.html). Internal fragments from seven housekeeping genes—*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*—were amplified in a GeneAmp PCR System 9700 thermal cycler (Life Technologies, Carlsbad, CA). The fragments were purified using ExoSAP-IT (USB Affymetrix, Cleveland, OH), and the sequences were obtained using an ABI 3130 genetic analyzer (Life Technologies). Editing and alignment of the sequences were performed with the BioEdit software version 7.0.9.0. Alleles and sequence types (STs) were assigned by using the available database (http://pubmlst.org/spneumoniae/), where new alleles and STs were also deposited.

Allelic profiles obtained in the present study were analyzed using the software BioNumerics v7.1 (Applied Maths, Ghent, Belgium), and minimum-spanning trees were constructed to predict clonal complexes (CCs). CCs were named according to the most common ST in the group, and comprised single-locus variants (SLVs) and double-locus variants (DLVs) of the predicted founder. Isolates that could not be assigned to any of the CCs due to the lack of close relationship were designated singletons.

Statistical analysis. The Simpson's index of diversity (SID), with 95% confidence intervals (95% CIs), was used to estimate the level of genetic diversity generated by MLST, while the adjusted Wallace coefficient (AW), with 95% CIs, was used to estimate the level of congruence among MLST, serotyping, and antimicrobial susceptibility testing. Both SID and AW indexes were calculated with the Comparing Partitions tool (http://www.comparingpartitions.info).

Also, the distribution of antimicrobial resistance and clonal complexes over the years included in the present study was evaluated with one-way analysis of variance and/or linear regression tests using the software GraphPad Prism v5.0. *P* values of <0.05 were considered significant.

RESULTS

Antimicrobial susceptibility. A total of 48.9% (154/315) of the serogroup 9 and serotype 14 pneumococcal strains included in the present study presented penicillin MICs ranging from 0.12 to 16 μg/ml and thus were considered nonsusceptible to penicillin. Half of the PNSP (77 isolates) presented MICs of >1 μg/ml. PNSP isolates were recovered from both patients and asymptomatic carriers during the period of 23 years (from 1988 to 2011) and presented an increasing tendency over time ($P = 0.0009$; Fig. 1). Regarding the distribution among specific serotypes, 58.8% (127/216) of serotype 14 isolates were PNSP, while 33.3% (20/60) and 20% (7/35) of serotypes 9V and 9N, respectively, were PNSP. Serotype 9N represented the earliest PNSP isolates detected in the present study (recovered in 1988), followed by serotype 14 in 1990 and serotype 9V in 1996. Highly penicillin-resistant strains (MICs ranging from 4 to 16 μg/ml) were first detected in 1996 and were restricted to serotypes 14 and 9V. Also, a number of PNSP isolates (20/154; 13%) were nonsusceptible to amoxicillin (the MICs ranged from 4 to >8 μg/ml).

Among the 154 PNSP isolates, 43.5% ($n = 67$) and 33.8% ($n = 52$) were also nonsusceptible to cefotaxime and ceftriaxone, respectively (MICs ≥ 1 μg/ml). In addition, 29.2% (45/154) and 17.5% (27/154) were intermediate (MICs of 0.5 μg/ml) and resistant (MICs ≥ 1 μg/ml) to meropenem, respectively. Amoxicillin-resistant isolates were also nonsusceptible to cefotaxime, ceftriaxone, and meropenem; such strains belonged to serotypes 14 and 9V, and their occurrence increased over time ($P = 0.0014$; Fig. 1).

Nonsusceptibility to erythromycin was observed in 9.2% (29/

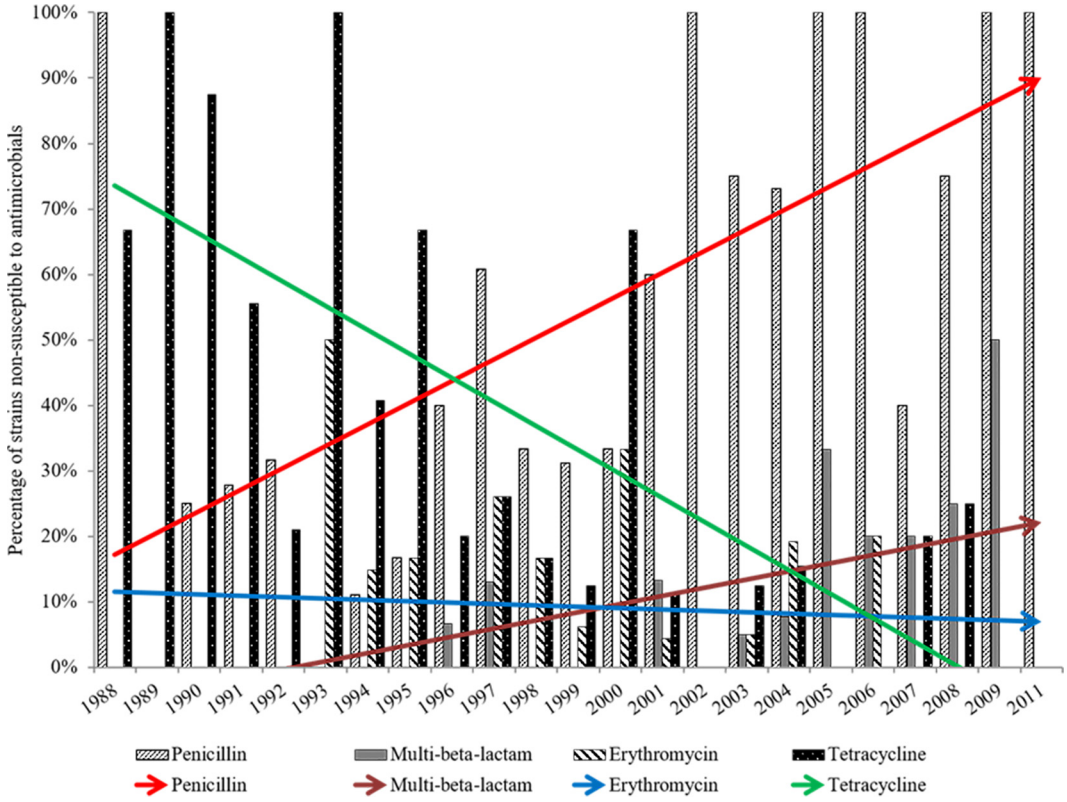

FIG 1 Distribution of serogroup 9 and serotype 14 pneumococcal isolates presenting nonsusceptibility to penicillin, as well as to other beta-lactams, erythromycin, and tetracycline over a 23-year period (from 1988 to 2011) prior to the introduction of PCV10 in Brazil.

315) of the pneumococcal isolates, with MICs ranging from 0.5 to >256 μg/ml. They included isolates recovered between 1993 and 2006, and no significant increasing or decreasing trend was observed over the years ($P = 0.06201$; Fig. 1). While 12.5% (27/216) of serotype 14 strains were erythromycin resistant, of which 74% (20/27) were also resistant to clindamycin (MICs 2-to 64 μg/ml), only 3.3% (2/60) of serotype 9V strains were nonsusceptible to this drug, with low MICs (0.64 μg/ml) and susceptibility to clindamycin. Erythromycin resistance was due to the presence of either the *mef* gene (9/29; 31%) or the *ermB* gene (18/29; 62.1%). Two isolates (6.9%) carried both *mef* and *ermB* genes. All the *mef*-positive isolates had the M phenotype and were associated with lower erythromycin MIC levels (between 0.5 and 8 μg/ml). All the *ermB*-positive strains showed resistance to clindamycin, the constitutive MLS_B phenotype, and were associated with higher erythromycin MIC levels ranging from 1.5 to >256 μg/ml. The isolates harboring both genes (*mef*- and *ermB*-positive isolates) were resistant to clindamycin and had the constitutive MLS_B phenotype and erythromycin MICs of 4 μg/ml. A total of 34.5% (10/29) of the erythromycin-resistant isolates were nonsusceptible to penicillin (penicillin MICs between 0.16 and 4 μg/ml).

Resistance to tetracycline was detected in 26% (82/315) of the pneumococcal isolates analyzed, with MICs between 4 and 64 μg/ml, and included serotypes 14 (52 isolates), 9N (25 isolates), 9V (3 isolates), 9A (1 isolate), and 9L (1 isolate). These isolates were recovered between 1988 and 2008, and their prevalence strongly decreased over time ($P < 0.0001$; Fig. 1). Tetracycline resistance was associated with the presence of the *tetM* gene in all tetracycline-resistant isolates. In addition to *tetM*, five isolates also had the *tetO* gene, while three isolates also carried the *tetK* gene, and one isolate harbored all three genes. A total of 14.6% (12/82) of the tetracycline-resistant strains were nonsusceptible to penicillin.

Eighteen isolates were resistant to both tetracycline and erythromycin. They represented 62.1% (18/29) of the erythromycin-resistant isolates and 21.9% (18/82) of the tetracycline-resistant strains. Concomitant resistance to tetracycline and erythromycin was associated with the presence of both the *ermB* and *tetM* genes in 17 isolates and the *mef*, *tetM*, and *tetO* in the remaining isolate.

More than 80% (258/315) of all pneumococcal strains included in the present study were resistant to trimethoprim-sulfamethoxazole, including isolates of serotype 14 (188 isolates), serotype 9V (58 isolates), serotype 9N (10 isolates), and serotype 9A (2 isolates). Among these isolates, 53.5% (138/258) were also PNSP. Furthermore, resistance to trimethoprim-sulfamethoxazole was observed in 96.5% (28/29) and 64.2% (52/81) of the erythromycin- and tetracycline-resistant strains, respectively. The rates of trimethoprim-sulfamethoxazole resistance increased with time, with an index of 63.6% between 1989 and 1995 and an index of 93.1% from 1996 to 2011.

All pneumococcal strains analyzed were susceptible to vancomycin and levofloxacin. Three strains, including one of serotype 14 and two belonging to serotype 9N, were resistant to chloramphenicol, and three other isolates, including two of serotype 14 and one of serotype 9N, were resistant to rifampin. A total of 12.1% (38/315) of the pneumococcal strains analyzed were mul-

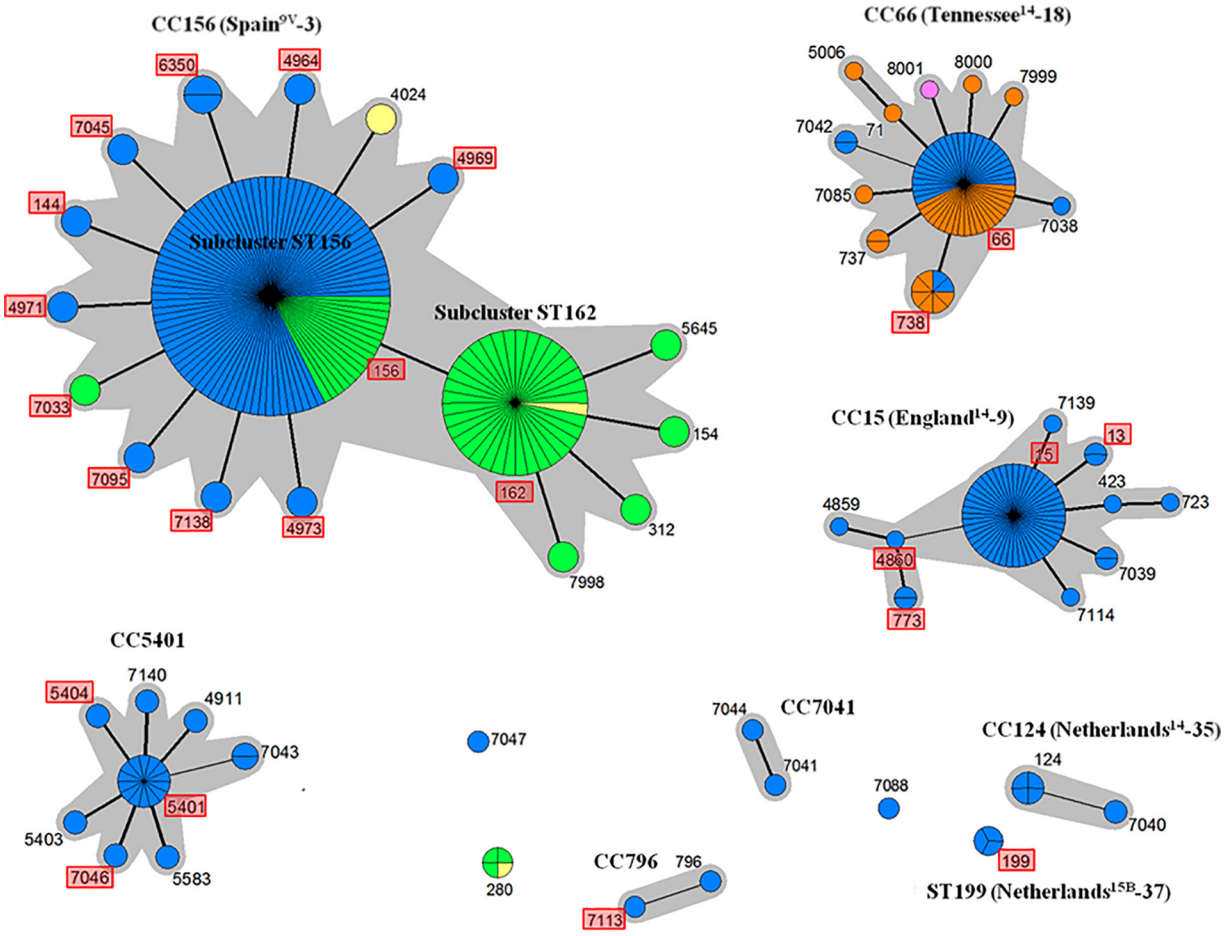

FIG 2 Minimum-spanning tree representing the analysis of the allelic profiles generated by MLST for 315 serogroup 9 and serotype 14 *Streptococcus pneumoniae* strains isolated in Brazil over a 23-year period prior to the introduction of PCV10. Each color represent a serotype: blue, serotype 14; green, serotype 9V; orange, serotype 9N; yellow, serotype 9A; and pink, serotype 9L. Clonal complexes (CC) are outlined by a gray zone, and the PMEN international clone associated is indicated in parentheses. The size of the nodes indicates the number of strains comprised by each ST. STs highlighted in red include penicillin-nonsusceptible strains.

tidrug resistant, since they were resistant to three or more classes of antimicrobial agents.

MLST. The 315 pneumococcal isolates included in this study were classified into 56 STs (see Table S1 in the supplemental material; SID = 0.846, 95% CI = 0.820 to 0.871), distributed in seven CCs and four singletons, some of which were associated with international clones recognized by the PMEN (Fig. 2). The majority of the 56 STs, 37 in all, were described for the first time among strains included in the present study, and they were mainly generated by novel combinations of existing alleles, with only nine new alleles detected (Table 1).

Among the 216 serotype 14 isolates, 41 STs (SID = 0.799, 95% CI = 0.759 to 0.838) distributed among seven CCs and three singletons were observed, while 18 STs distributed in two CCs and one singleton were detected among the 99 serogroup 9 isolates (SID = 0.790, 95% CI = 0.738 to 0.842).

Congruence between ST and serotype was low (AW = 0.462, 95% CI = 0.328 to 0.591), but a higher level of congruence was seen between the ST and the penicillin susceptibility profile (AW = 0.797, 95% CI = 0.756 to 0.838). PNSP isolates showed a lower genetic diversity (SID = 0.574, 95% CI = 0.488 to 0.659) compared to penicillin-susceptible isolates (SID = 0.857, 95% CI = 0.823 to 0.892).

The earliest serotype 9N strains included in the present study (isolated in 1988) were clustered in CC66, which comprised 21.3% (67/315) of all pneumococcal isolates analyzed. This CC included isolates recovered from both patients with infection and asymptomatic carriers, and its distribution over the years did not show any significant trend ($P = 0.2395$; Fig. 3), although the last isolate was detected in 2008.

Nonsusceptibility to penicillin was observed in 47.8% (32/67) of the strains belonging to CC66, and these resistant strains represented 20.8% (32/154) of all PNSP isolates. No erythromycin-resistant strains were observed in this cluster, but 43.3% (29/67) were resistant to tetracycline, which represented 35.4% (29/82) of all tetracycline-resistant strains included in the present study. They carried *tetM* alone or in combination with *tetO* as the tetracycline resistance determinants, and five of them were also PNSP. Moreover, 58.2% (39/67) of the strains were nonsusceptible to trimethoprim-sulfamethoxazole, and four isolates were multidrug resistant, with the predominant profile including resistance to trimethoprim-sulfamethoxazole, penicillin, and tetracycline.

TABLE 1 Novel sequence types detected among serogroup 9 and serotype 14 *Streptococcus pneumoniae* isolates in Brazil in the pre-PCV10 era

Sequence type	Allelic profile[a]						
	aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
4859	1	5	4	6	9	1	8
4860	1	5	4	6	9	3	8
4911	2	13	2	8	17	6	288
4964	7	11	10	1	6	76	1
4969	7	13	10	1	6	8	1
4971	7	15	10	1	6	8	1
4973	7	26	10	1	6	8	1
5006	10	13	2	4	6	1	1
5401	2	13	2	8	17	**341**	288
5403	2	13	2	2	17	**341**	288
5404	5	13	2	8	17	**341**	288
5583	1	13	2	8	17	**341**	288
5645	12	11	10	1	6	8	14
6350	7	11	10	108	6	8	1
7033	7	11	10	1	6	8	18
7038	2	8	2	4	9	1	1
7039	1	5	261	5	5	3	8
7040	7	5	1	8	14	49	6
7041	43	5	4	10	9	3	74
7042	2	8	**269**	4	6	1	20
7043	2	5	2	5	17	**341**	288
7044	43	5	4	10	1	3	74
7045	7	14	10	1	6	8	1
7046	2	13	2	8	17	**341**	18
7047	10	48	4	1	10	1	8
7085	2	8	2	4	6	1	**479**
7088	2	16	4	1	9	**433**	1
7095	7	11	**318**	1	6	8	1
7113	**222**	50	4	1	53	3	18
7114	1	5	**325**	5	5	3	8
7138	7	11	10	1	6	8	**491**
7139	1	5	4	**196**	5	3	8
7140	2	13	2	8	17	**341**	**492**
7998	7	11	10	18	6	8	14
7999	**26**	8	2	4	6	1	1
8000	2	8	2	5	6	1	1
8001	23	8	2	4	6	1	1

[a] Novel alleles are indicated in boldface.

The earliest serotype 14 strains included in the present study, isolated in 1989, belonged to CC15. This CC was represented by 18.7% (59/315) of all strains analyzed and was mainly composed of isolates recovered from infections, being the predominant CC among isolates recovered from patients with meningitis. The distribution of CC15 over the years showed a decreasing tendency ($P = 0.0001$; Fig. 3), with the last strain being detected in 2003.

A total of 32.2% (19/59) of the CC15 strains were resistant to erythromycin, representing the earliest erythromycin-resistant isolates (1993-2001) and 65.5% (19/29) of all erythromycin-resistant strains included in the study. In general, such isolates presented high erythromycin MIC levels and the *ermB* gene, and many were also resistant to tetracycline. A total of 71.2% (42/59) of the strains in this CC were resistant to tetracycline, representing 53.2% (42/79) of all tetracycline-resistant isolates included in the study. Furthermore, only five isolates (8.5%) were nonsusceptible to penicillin, 74.6% (44/59) were resistant to trimethoprim-sulfamethoxazole, and 18 isolates were multidrug resistant, with the predominant profile being resistance to trimethoprim-sulfamethoxazole, erythromycin, and tetracycline.

The most prevalent cluster observed in the present study was CC156 (47.3% of the isolates; 149/315), which comprised isolates recovered from a variety of clinical sources, representing either infection or asymptomatic colonization. After the first detection of CC156 among isolates recovered in 1990, it showed an increasing trend over the years ($P < 0.0001$; Fig. 3). However, this CC was divided in two subclusters (ST156 and ST162), which presented different distributions of serotypes and resistance profiles (Fig. 2). A total of 73.8% (110/149) of the strains belonging to CC156 were nonsusceptible to penicillin. Among these, 98.2% (108/110) corresponded to the subcluster ST156, which was detected from 1995 to 2011. Penicillin MICs ranged from 0.16 to 16 μg/ml, and this subcluster included all the strains that had penicillin MICs of >1 μg/ml and also all the isolates that were nonsusceptible to all of the beta-lactams tested. The subcluster ST162, in turn, was mainly associated with penicillin susceptibility and was detected from 1990 to 2004.

Ten erythromycin-resistant isolates, also resistant to penicillin, belonged to CC156. They were the most recently isolated (2001-2006) erythromycin-resistant strains detected in the present study, and most of them presented low erythromycin MIC levels and the *mef* gene. Two isolates were resistant to tetracycline (*tetM* gene), 95.3% (142/149) of the strains were resistant to trimethoprim-sulfamethoxazole, and 10 isolates were multidrug resistant, with the predominant profile being resistance to trimethoprim-sulfamethoxazole, penicillin, and erythromycin.

A novel CC was also detected in the present study. CC5401, which included 7% (22/315) of the strains, comprised only new STs. Isolates were exclusively recovered from patients with serious infections since 1996, and the prevalence of CC5401 remained similar over the years, presenting no significant increasing or decreasing trend ($P = 0.9657$; Fig. 3). A total of 22.7% (5/22) of these strains were nonsusceptible to penicillin, with low MICs (0.12 to 0.19 μg/ml). No erythromycin-resistant isolates were observed in this cluster, and only one isolate was resistant to tetracycline (*tetM* gene). In contrast, all of the strains were resistant to trimethoprim-sulfamethoxazole.

Other, less-prevalent genotypes observed in the present study included CC124 (associated with the international clone Netherlands[14]-35 ST124), CC796, and CC7041. Nine isolates (2.8% of total) were represented by four singletons, including ST280, ST199 (international clone Netherlands[15B]-37), ST7047, and ST7088 (Fig. 2). The distribution of STs according to serotypes, strain sources, and penicillin susceptibility is given in Table S1 in the supplemental material.

DISCUSSION

Antimicrobial resistance rates among pneumococcal isolates can vary according to the clinical condition, patient age, geographical region, and serotype of the pneumococcal strain. In this context, several studies performed with pneumococcal isolates from Brazil have suggested that serogroup 9 and serotype 14 are among the most important capsular types driving the emergence of PNSP in our region (6–13). In the present study, nearly half of the serogroup 9 and 14 isolates were PNSP, confirming that nonsusceptibility to penicillin is very common among these serotypes in Brazil. In addition, we observed increasing PNSP rates ($P = 0.0009$)

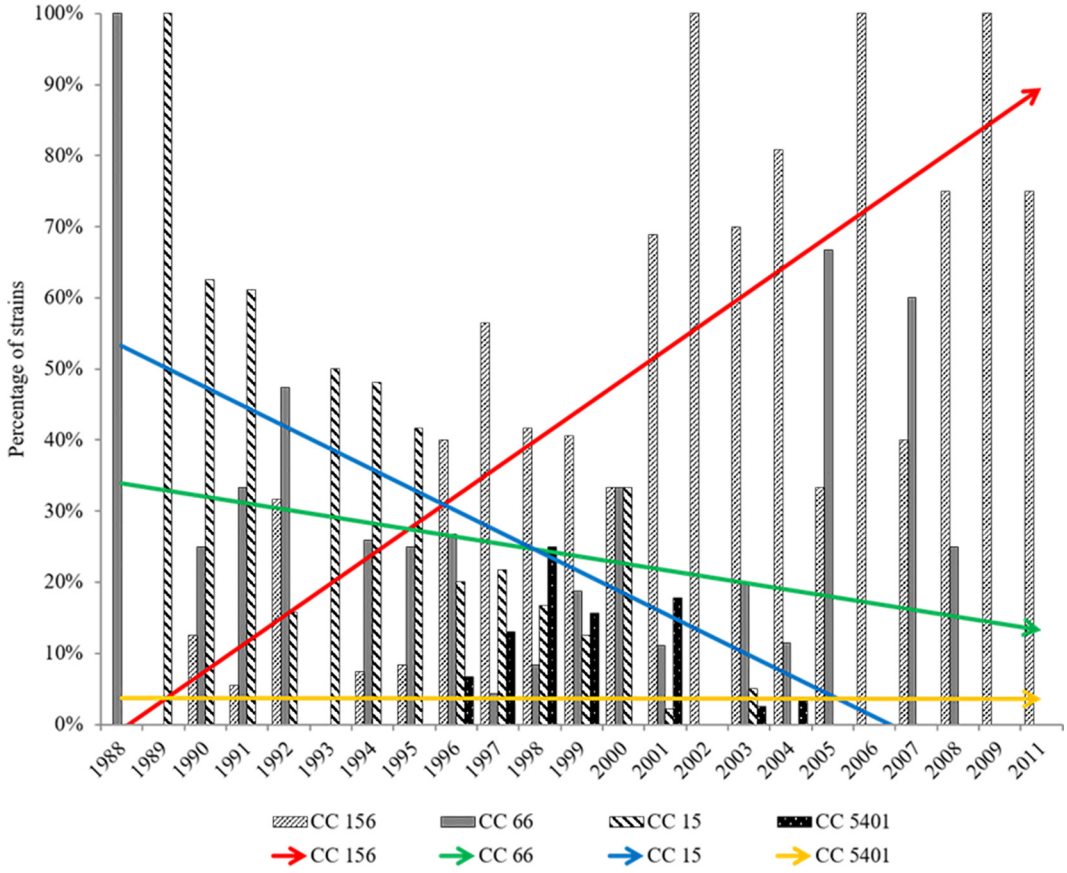

FIG 3 Distribution of the major clonal complexes (CC) determined by MLST among 315 serogroup 9 and serotype 14 *Streptococcus pneumoniae* strains isolated in Brazil over a 23-year period prior to the introduction of PCV10.

and penicillin MIC levels ($P < 0.0001$) over the years. From 1988 to 1999, 31.8% (55/173) of the isolates presented nonsusceptibility to penicillin, with a mean MIC of 1 μg/ml (range, 0.12 to 4 μg/ml). From 2000 to 2011, 69.7% (99/142) of the isolates were PNSP, with an average MIC of 2 μg/ml (range: 0.12 to 16 μg/ml), supporting previous studies that have shown higher PNSP rates and MIC levels among isolates recovered in the 2000s compared to those obtained in the 1990s (7, 9, 10, 12).

Among PNSP isolates included in the present study, CC156 was largely represented, followed by CC66. In Brazil, the emergence of PNSP has been linked to the dispersion of both CC156 and CC66 (7, 12), which, before this study, were known to be circulating in the South and Southeast regions of Brazil since 1998. Spain^{9V}-3 ST156 is a widespread clone that has been circulating around the world for more than 20 years (http://www.pneumogen.net/pmen/) and, although originally described in a serotype 9V strain, it has been widely disseminated in Latin American countries by its serotype 14 variant (24). This genotype is also widely known to be related to penicillin nonsusceptibility and trimethoprim-sulfamethoxazole resistance (http://www.pneumogen.net/pmen/), being among the most predominant PNSP clones in different countries (25, 26). In the present study, although CC156 was detected since 1990 in the form of the subcluster ST162, PNSP emergence was mainly due to appearance of the subcluster ST156 in 1995, when the first strains nonsusceptible to other beta-lactams were also detected. ST162 is thought to be the penicillin-susceptible ancestor of ST156 (26). Moreover, our data show that introduction of ST156 in our setting was accompanied by the emergence of trimethoprim-sulfamethoxazole resistance, since the rates of resistance to this combination increased from 63.6% until 1995 to 93.1% from 1996 to 2011.

CC156 also included all of the isolates resistant to both penicillin and erythromycin, suggesting further evolution of this clone after initial introduction. Erythromycin-resistant PNSP isolates of CC156 have also been detected in other countries (27, 28), and it is speculated that long-acting macrolides, such as clarithromycin and azithromycin, which are being increasingly prescribed worldwide, are associated with selection of coresistance to macrolides, penicillin, and trimethoprim-sulfamethoxazole (29).

CC66, in turn, accounted for a significant part of the PNSP isolates recovered before the appearance of ST156 (from 1988 to 1995), including the earliest PNSP isolates that were recovered from human blood in 1988 and belonged to serotype 9N and ST738 (DLV of the PMEN clone Tennessee^{14}-18 ST67). To date, the only record of ST738 in the MLST database (http://pubmlst.org/spneumoniae/ [accessed 24 March 2016]) is a penicillin-susceptible strain recovered from a patient with meningitis in Brazil in 2000. In contrast to what we found among isolates in the present study, serotype 9N/ST66 strains recovered in the United States, although very common, are usually associated with penicillin susceptibility (30).

ST66 is an SLV of the international clone Tennessee^{14}-18 ST67,

which is an erythromycin-resistant clone disseminated in the United States since the 1990s (http://www.pneumogen.net/pmen/). In the present study, however, CC66 isolates lacked the typical erythromycin resistance marker associated with this clone. Since laterally acquired genes, including resistance determinants, usually accumulate proportionately to the age of clones (31), the lack of erythromycin resistance among isolates of CC66 in the present study could be associated with a slower evolution. In accordance with this observation, it was shown that ST66 isolates belonging to serotype 9N were among the oldest representatives of this cluster (dated from the 1960s), being therefore the predicted ancestor of the clonal group (32).

Almost 10% of the strains included in the present study were nonsusceptible to erythromycin, with a higher incidence among serotype 14 isolates. In Brazil, low percentages of erythromycin resistance are typically seen among pneumococcal isolates, which are usually not detected at rates above 10% (11–13, 33). Serotype 14 frequently accounts for an important fraction of the erythromycin-resistant strains in many countries. For example, in Germany, 16.2% of all pneumococcal isolates recovered from invasive diseases were found to be resistant to erythromycin, but when only serotype 14 strains were considered, this resistance rate increased to 69.5% (5).

Erythromycin resistance in the present study was primarily associated with CC15. In contrast to what was observed for CC156, CC15 was associated with high-level erythromycin resistance and penicillin susceptibility, as previously documented in Brazil (12, 33). In Spain, as well, CC15 and CC156 are among the most common CCs comprising macrolide-resistant isolates (34), where the former is most often associated with the simultaneous presence of *ermB* and *tetM* genes in Tn*916*-like transposons. In the present study, CC15 also comprised all the isolates resistant to both erythromycin (*ermB*) and tetracycline (*tetM*).

Although tetracycline is not recommended for treating pneumococcal infections, the tracking of this resistance marker is important for predicting the emergence of other resistance traits (35). Moreover, tetracycline resistance among pneumococcal strains has been commonly reported in many countries, including Brazil (7, 12, 34, 36, 37). Both CC66 and CC15 were associated with tetracycline resistance in the present study. In our setting, this characteristic has been detected among pneumococcal strains since the early 1980s (38, 39), and the first appearance of CC66 and CC15 in this study, in 1988 and 1989, respectively, was therefore preceded by the emergence of tetracycline resistance.

England^{14}-9 ST9, the international clone associated with CC15, is an erythromycin-resistant clone that has been circulating worldwide since the 1990s (http://www.pneumogen.net/pmen/). In this study, in addition to the typical erythromycin resistance marker, CC15 also presented low-level penicillin nonsusceptibility, high rates of tetracycline resistance, and constituted the majority of the multidrug-resistant strains. Moreover, with the appearance of CC15, resistance to trimethoprim-sulfamethoxazole, which was previously uncommon in our setting (38, 39), began to emerge. In the United States, CC15 was one of the main contributors to the emergence of penicillin resistance before PCV introduction (30).

CC156, CC66, and CC15 have a global circulation, demonstrating their evolutionary success regardless of geographical area (12, 40, 41, 42). It is likely that these CCs have been imported, spread, and selected for by the use of antibiotics in Brazil. In contrast, CC5401 seems to have originated and disseminated locally. This CC comprised only new STs, showed no association with PMEN clones, and included strains recovered from infections in the southern part of Brazil, where this CC was the second most prevalent, after CC156. Moreover, although erythromycin and tetracycline resistance were not associated with CC5401, all the strains were resistant to trimethoprim-sulfamethoxazole, and a significant portion (around 20%) was nonsusceptible to penicillin. Based on the MLST database (http://pubmlst.org/spneumoniae/ [accessed 24 March 2016]), only two other SLVs of ST5401, ST7183 and ST7543, have been described. These genotypes are represented by serotype 14 or serotype 35B strains recovered in 2009 from asymptomatic carriers living in Nepal. ST7183 also comprises one serotype 14 strain recovered in Denmark in 1968. The fact that such an old pneumococcal strain shows only one locus difference compared to ST5401 suggests that this might be a close variant of a very successful clone that has been circulating among human populations since before the increased use of antibiotics or the introduction of new vaccination strategies.

A timeline depicting the emergence of antimicrobial resistance patterns and year of the first detection of the major CCs observed among serogroup 9 and serotype 14 *Streptococcus pneumoniae* isolated in Brazil over a 23-year period in the pre-PCV10 era, including their characteristics and trends over time, is shown in Fig. S1 in the supplemental material.

Many of the worldwide-disseminated resistant clones have appeared with different capsular types, suggesting a high genetic versatility that could be useful for escaping serotype-based immunization approaches. This is a special concern now in Brazil, since PCV10 was introduced in the Brazilian Immunization Program (14). Such variants arise commonly through recombination and more rarely by mutation, within and around the capsular biosynthesis locus, and many of them are eventually selected for and preserved in the pneumococcal population (32, 43). Of note in the present study was the detection of an unusual serotype 14 variant of the Netherlands^{15B}-37 ST199 clone. Recently, it was suggested that serotype 19A variants of CC156 have arisen by recombination of the capsular locus with CC199 (32); thus, the serotype 14 variants of CC199 observed in the present study could have been generated by recombination with CC156.

The present study contributes to a better understanding of pneumococcal evolution and epidemiology in Brazil, showing the distribution of antimicrobial resistance patterns and genetic diversity among serotype 14 and serogroup 9 strains recovered from patients and asymptomatic carriers living in Brazil over a 23-year period before pneumococcal vaccination was fully introduced in the country. In addition to describing a unique regional CC associated with invasive pneumococcal disease, the prevalence and circulation of some of the most successful PMEN clones and their association with the emergence and the spread of resistance markers are reported, serving as a comparative baseline. Since nationwide implementation of PCV10 is relatively recent in Brazil, it is of paramount importance to continue tracking the circulation and prevalence of these serotypes and clones, as well as their contribution to the emergence of antimicrobial resistance, in the post-pneumococcal-vaccination era.

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