Phenotypic and Molecular Characterization of Optochin-Resistant *Streptococcus pneumoniae* Isolates from Brazil, with Description of Five Novel Mutations in the *atpC* Gene

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Optochin (Opt) susceptibility is used largely for the identification of *Streptococcus pneumoniae* in diagnostic laboratories. Opt-resistant (Opt^r) *S. pneumoniae* isolates have been reported, however, indicating the potential for misidentification of this important pathogen. Point mutations in the *atpC* gene have been associated with the emergence of Opt^r *S. pneumoniae*, but data on the characterization of such atypical variants of *S. pneumoniae* are still limited. The present report describes the results of a polyphasic approach to identifying and characterizing 26 Opt^r *S. pneumoniae* isolates recovered from patients or carriers living in Brazil. Sixteen isolates consisted of heterogeneous populations, and 10 isolates were homogeneously Opt^r. The isolates had different serotypes and antimicrobial susceptibility profiles. They also presented diverse genetic characteristics, as indicated by pulsed-field gel electrophoresis (PFGE), multilocus variable-number tandem-repeat analysis (MLVA), and *pspA* gene typing. Except for Opt MICs (4- to 64-fold higher among Opt^r variants), Opt^r and Opt-susceptible (Opt^s) subpopulations originating from the same culture had identical characteristics. Sequencing of the *atpC* gene of the Opt^r variants revealed 13 different nucleotide changes distributed among eight different codons. Changes in codon 49 were the most frequent, suggesting that this might be a hot spot for optochin resistance-conferring mutations. On the other hand, five novel types of mutations in the *atpC* gene (Met13Ile, Gly18Ser, Gly20Ala, Ala31Val, and Ala49Gly) were identified. *In silico* prediction modeling indicated that the *atpC* gene mutations corresponded to alterations in the transmembrane region of the ATPase, leading to a higher hydrophobicity profile in α -helix 1 and to a lower hydrophobicity profile in α -helix 2.

Streptococcus pneumoniae remains as one of the most important agents of infectious diseases worldwide and a leading cause of invasive and noninvasive infections in children and in the elderly (1). Nasopharyngeal carriage is considered the main reservoir of *S. pneumoniae* in nature and is a condition of paramount importance for the continuous circulation of this bacterial species among human populations. Due to the significant morbidity and mortality rates associated with pneumococcal diseases, rapid and accurate diagnosis is essential and frequently relies on the isolation and identification of the etiological agent. For this purpose, optochin (Opt) susceptibility testing has been widely used as the primary or even the only test for the presumptive identification of pneumococci, due to the ability to differentiate *S. pneumoniae* from the other alpha-hemolytic streptococci (2, 3, 4).

Nevertheless, 30 years after the introduction of the Opt susceptibility test as a diagnostic tool (5), the first optochin-resistant (Opt^r) *S. pneumoniae* isolate was described (6). Since then, the isolation of Opt^r *S. pneumoniae* from a variety of clinical sources has sporadically been reported from different locations, with an apparent increasing incidence in the last decade (2, 3, 7, 8, 9, 10, 11, 12). The occurrence of such an atypical variant is a potential cause of misidentification of this important agent, raising questions about the accuracy of laboratory diagnosis when a single identification procedure based on Opt susceptibility testing is used.

Previous studies have shown that single point mutations in the c subunit (Table 1), and less commonly in the a subunit, of the *atpCAB* operon that codes for the molecular target of optochin,

the transmembrane F₀F₁ ATPase, are present in Opt^r *S. pneumoniae* strains and may be associated with this unusual phenotype (2, 7, 9, 10, 12, 13, 14). Although still uncertain, it has been suggested that the use of antimalarial chemotherapy in areas where malaria is endemic (14), as well as exposure to subinhibitory concentrations of penicillin (15), may be related to the emergence of such mutations.

Studies concerning the phenotypic and genetic characteristics of atypical *S. pneumoniae* variants expressing resistance to Opt are still limited and may contribute to a better understanding of the mechanisms involved in the emergence, as well as the biological and epidemiological impacts, of such resistance traits. The present report describes the results of a polyphasic approach applied to characterize 26 Opt^r *S. pneumoniae* isolates recovered from patients and nasopharyngeal carriers living in Brazil. The study encompassed extensive phenotypic and genotypic characterization of Opt^r *S. pneumoniae* isolates presenting a variety of *atpC* gene

Received 8 May 2013 Returned for modification 15 June 2013

Accepted 15 July 2013

Published ahead of print 24 July 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.01168-13>.

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doi:10.1128/JCM.01168-13

TABLE 1 Mutations in the *atpC* gene described to date among optochin-resistant *Streptococcus pneumoniae* isolates

Target codon (amino acid alteration) ^a	No. of strains reported	Country of origin (reference or source)
13 (Met→Ile)	1	Brazil (this study)
14 (Gly→Ser)	2	Brazil (this study); France (7)
18 (Gly→Ser)	2	Brazil (this study)
20 (Gly→Ser)	3	Brazil (this study); United States (2)
20 (Gly→Ala)	1	Brazil (this study)
23 (Met→Ile)	4	Brazil (9; this study); United States (2)
31 (Ala→Val)	1	Brazil (this study)
44 (Met→Ile)	1	Japan (12)
45 (Phe→Leu)	2	Brazil (9; this study)
45 (Phe→Val)	1	Brazil (this study)
47 (Gly→Val)	1	Argentina (10)
48 (Val→Phe)	3	Japan (12)
49 (Ala→Ser)	10	Argentina (10); Brazil (9; this study); Japan (12)
49 (Ala→Thr)	10	Argentina (10); Brazil (this study); Japan (12); United States (2)
49 (Ala→Gly)	1	Brazil (this study)
50 (Phe→Leu)	1	Japan (12)

^a Substitutions detected first and only in Brazil are in bold type.

mutations, including the characterization of five novel types of mutations associated with resistance to Opt. Additionally, *in silico* prediction modeling was performed to evaluate the impact of mutations on the structure and hydrophobicity profile of the c subunit of ATPase.

MATERIALS AND METHODS

Bacterial strains and identification tests. Twenty-six Opt^r *S. pneumoniae* strains were included in the present study. Among them, four (Sp 910, Sp 913, Sp 917, and Sp 1008) were previously characterized by use of a variety of conventional and genetic tests (9). These isolates were included in the present study for evaluation of additional characteristics (determination of Opt MIC, multilocus variable-number tandem-repeat analysis [MLVA] type, and PspA family). All of the isolates were recovered during surveillance studies performed by our group or were received from different health institutions for confirmation of the identification. Isolates obtained from diseased individuals were recovered from clinical samples taken as part of standard patient care procedures and they do not need ethics approval for their use. Isolates from carriage studies were recovered from clinical samples collected as approved by the ethics committees of the institutions involved. The isolates were subjected to phenotypic identification tests, including observation of colonial morphology and type of hemolytic activity on blood agar plates, cellular characteristics as observed after Gram staining, and tests of optochin susceptibility, bile solubility, and latex agglutination. PCR tests for detection of the *lytA*, *ply*, and *psaA* genes were also performed. *S. pneumoniae* strains ATCC 49619 and ATCC BAA-255 (R6) and *S. mitis* strain SS-429 were included as reference strains in all the assays.

Optochin susceptibility testing. Opt susceptibility was determined by disk diffusion testing according to standard procedures (4). Optochin disks (BBL Taxo P disks; BD, Sparks, MD) were applied to the surface of 5% sheep blood agar plates (Plast Labor, Rio de Janeiro, Brazil) streaked with the isolate being tested. After overnight incubation at 35°C under both 5% CO₂ and conventional atmospheres (16), growth inhibition zones around the disks were measured. Isolates displaying inhibition zones of ≥14 mm in diameter were identified as susceptible, while strains showing zones of <14 mm or zones of ≥14 mm but containing colonies inside were considered resistant. The optochin (Sigma Chemical Co., St.

Louis, MO) MIC values were determined by the agar dilution method (2). Briefly, bacterial suspensions were prepared in 0.9% saline (Sigma) from overnight cultures and adjusted to achieve the 0.5 McFarland turbidity standard. Suspensions were inoculated onto Mueller-Hinton agar containing 5% sheep blood (Plast Labor, Rio de Janeiro, RJ, Brazil) and various concentrations of optochin (0.5 to 256 µg/ml), followed by incubation at 35°C for 20 to 24 h under a 5% CO₂ atmosphere. The MIC was defined as the lowest concentration of optochin that inhibited visible growth of the isolate. *S. pneumoniae* ATCC 49619 and R6 were included as susceptible controls, while *S. mitis* SS-429 was included as a resistant control in the Opt susceptibility tests.

Bile-solubility testing. Bile-solubility tube testing was performed as previously described (4). Briefly, heavy bacterial suspensions were prepared in 0.9% saline (Sigma) from overnight cultures. The suspensions were placed into 2 tubes, and an equal volume of 2% deoxycholic acid (Sigma) solution was added to one tube (test tube) while an equal volume of 0.9% saline was added to the other (control tube). Tubes were incubated at 35°C for up to 2 h. Complete visual clearing of the turbidity in the deoxycholate-containing tube, but not in the saline control tube, indicated a positive test.

Latex agglutination tests. The isolates were tested for the presence of capsular polysaccharide antigens by latex agglutination testing using the Slidex pneumo-Kit according to the manufacturer's instructions (bio-Mérieux, Marcy l'Etoile, France).

Determination of capsular type. The capsular types were determined by either multiplex PCR using the Latin America scheme (17) or the standard Quellung reaction with type-specific antisera prepared at the Centers for Disease Control and Prevention (CDC), as described previously (18).

Antimicrobial susceptibility testing. Antimicrobial susceptibility was evaluated by the agar diffusion method according to the CLSI recommendations and interpretative criteria (19, 20). Antimicrobials tested included chloramphenicol, clindamycin, erythromycin, levofloxacin, oxacillin, rifampin, trimethoprim-sulfamethoxazole, tetracycline, and vancomycin (Oxoid, Basingstoke, Hampshire, United Kingdom). Penicillin MICs were determined by using Etest strips (AB Biodisk, Solna, Stockholm, Sweden). *S. pneumoniae* ATCC 49619 was used for quality control.

Preparation of bacterial DNA. DNAs for all PCRs were obtained by using the Chelex 100 resin (Bio-Rad, Hercules, CA, USA) method. Briefly, a loopful of overnight cultures was suspended in a solution containing 5% Chelex and proteinase K 20 mg/ml (Invitrogen, Life Technologies, Carlsbad, CA). After incubation at 56°C for 1 h, followed by incubation at 95°C for 10 min, the solutions were centrifuged and the supernatant was used as the template for all the subsequent PCRs.

Detection of resistance- and virulence-associated genes. We investigated the presence of the macrolide-resistance determinants *ermA*, *ermB*, and *mef* (21) and the tetracycline-resistance genes *tetK*, *tetL*, *tetM*, and *tetO* (22), as well as the virulence-associated genes *ply* (coding for pneumolysin), *lytA* (coding for autolysin), and *psaA* (coding for pneumococcal surface antigen A) (23, 24), by PCR using protocols previously described and an automated Veriti 96-well thermal cycler (Applied Biosystems, Inc., Carlsbad, CA).

Pulsed-field gel electrophoresis analysis. Genomic DNA was prepared in agarose plugs as described by Teixeira et al. (25), with the following modifications: bacteria were grown in blood agar plates for 8 to 10 h and plugs were prepared with low-melting-temperature agarose at 2.5% (NuSieve GTG agarose; FMC Bioproducts, Rockland, ME) and were washed 8 times before restriction. After macrorestriction using SmaI (New England BioLabs, Ipswich, MA), the fragments were separated in a CHEF-DR III system (Bio-Rad) using the parameters recommended by the Pneumococcal Molecular Epidemiology Network (PMEN) (<http://www.sph.emory.edu/PMEN/index.html>). The restriction profiles were analyzed by using BioNumerics software version 6.6 (Applied Maths, Ghent, East Flanders, Belgium). A dendrogram was created using the unweighted pair group method with arithmetic mean (UPGMA) and the

FIG 1 Genetic relationship among optochin-resistant *Streptococcus pneumoniae* (Opt^r Sp) isolates included in the study as evaluated by PFGE analysis. The 80% cutoff considered for the delineation of clonal groups is illustrated by a red dotted line. ^aStrain identification; those highlighted in red are reference strains of internationally disseminated clones that were genetically related to some of the Opt^r *S. pneumoniae* strains. ^bSources of isolation: NP, nasopharynx; U, unknown; LRT, lower respiratory tract; CSF, cerebrospinal fluid; ED, eye discharge. ^cOptochin susceptibility phenotype: HE, heterogeneous population; HO, homogeneous population. ^dpspA gene family; NT, nontypeable. ^eMIC of optochin expressed in μg/ml. ^fMutations in the *atpC* gene, including codon number and the respective deduced amino acid substitution. ^gSXT, trimethoprim-sulfamethoxazole; PEN, penicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol.

Dice similarity coefficient, with optimization and position tolerance settings of 0.5% and 1.3%, respectively. Profiles showing more than 80% similarity were considered to belong to the same cluster. Pulsed-field gel electrophoresis (PFGE) profiles of the 43 clones described by the PMEN were also included in the *in silico* analysis for comparative purposes.

Multilocus variable-number tandem-repeat analysis. Eight variable-number tandem repeats (VNTRs) (Spneu15, Spneu17, Spneu25, Spneu33, Spneu36, Spneu37, Spneu39, and Spneu42), selected (N. S. Costa, T. C. A. Pinto, F. C. O. Kegele, V. L. C. Merquior, J. M. Peralta, and L. M. Teixeira, unpublished data) among an initially suggested panel of 18, were amplified by PCR using primers and a protocol described previously (26). MLVA profiles were analyzed by using BioNumerics software version 6.6 (Applied Maths), in order to construct a dendrogram by the UPGMA and a diagram based on the minimum spanning tree (MST) method. Reference strain *S. pneumoniae* R6 was used as the quality control for the PCRs.

pspA gene family typing. The *pspA* genes were detected by PCR using primers and protocols previously described (27). Control strains for *pspA* family 1 (strains BG9739, DBL1, and EF10197), family 2 (strains AC122, BG11703, BG7591, and ATCC 6303), and family 3 (strain BG6380) were also included. Strains were categorized as nontypeable (NT) if negative results were obtained in 3 attempts of amplification.

Sequencing of the *atpC* gene. The gene coding for the c subunit of ATPase was amplified by PCR using primers and protocols previously

described (2, 9, 13). The fragment was purified using ExoSAP-IT (USB Affymetrix, Cleveland, OH) and the sequences were obtained using an ABI 3130 genetic analyzer (Applied Biosystems). Edition and alignment were performed with BioEdit software version 7.0.9.0 (28), as well as the construction of charts based on the Kyte and Doolittle scale mean hydrophobicity profile. Using the amino acid sequences translated from the nucleotide sequences obtained in the study, a predicted model for the c subunit was designed by using the resources available at the Protein Structure Prediction (PSIPRED) server (<http://bioinf.cs.ucl.ac.uk/psipred/>) (29). Opt-susceptible (Opt^s) reference strains (*S. pneumoniae* ATCC 49619 and R6) were also included for comparative purposes.

Nucleotide sequence accession numbers. The *atpC* gene sequences of the 22 isolates originally reported in the present study were deposited in the GenBank database under accession numbers KC513927 to KC513948, as indicated in Fig. 2A.

RESULTS AND DISCUSSION

Identification of the isolates and characterization of Opt resistance. The 26 Opt^r *S. pneumoniae* isolates included in this study were recovered from different clinically significant sources (14 isolates) or from nasopharyngeal secretions of healthy carriers (12 isolates) between 1995 and 2012, as shown in Fig. 1. All of the isolates had the following characteristics. They were Gram-posi-

FIG 2 Genetic relationship among optochin-resistant *Streptococcus pneumoniae* (Opt^r Sp) isolates included in this study as evaluated by MLVA. (A) Dendrogram showing the genetic relatedness, the alleles detected for each VNTR, and the GenBank accession numbers (^a) of the *atpC* gene sequences of the 26 Opt^r *S. pneumoniae* strains. The 80% cutoff considered for the delineation of clonal groups is illustrated by a red dotted line. (B) Diagram constructed by using the minimum spanning tree (MST) method. Node colors represent the different serotypes listed in Fig. 1. Higher levels of genetic relationships between nodes are indicated by darker lines.

tive catalase-negative cocci; presented alpha-hemolysis on blood agar plates; gave positive results in the bile solubility and latex agglutination tests; possessed the *lytA*, *ply*, and *psaA* genes; and were resistant to Opt.

Opt susceptibility testing, performed either under CO₂-enriched or conventional atmospheres, revealed the occurrence of two phenotypes among the Opt^r *S. pneumoniae* isolates analyzed (Fig. 1). The prevalent phenotype comprised 16 strains and was expressed as a typical inhibition zone of ≥ 14 mm around the Opt disk with colonies inside, representing a heterogeneous population. The subpopulations were discriminated based on their Opt susceptibility, and the subsequent experiments were carried out separately for both subpopulations. The second phenotype was observed for the remaining 10 Opt^r *S. pneumoniae* strains that had no zone of inhibition around the Opt disk, representing homogeneous resistant populations. The finding of two different Opt^r phenotypes among *S. pneumoniae* isolates has been reported before, among isolates from Portugal (11) and from the United States (2) with different distributions. Among isolates from Portugal, a predominance of homogeneous Opt^r populations was observed, while the majority of the isolates from the United States were reported to be composed of heterogeneous populations, as also observed in the present study. Opt MICs ranged from 8 to 64 μ g/ml for the Opt^r *S. pneumoniae* strains (Fig. 1), which is in agreement with earlier reports on Opt resistance (2, 7, 11). On the other hand, the 16 Opt^s subpopulations derived from heterogeneous cultures, as well as the susceptible reference strains *S. pneumoniae* ATCC 49619 and R6, showed MIC values of 1 to 2 μ g/ml, which were 4- to 64-fold lower than those obtained for their respective Opt^r counterparts. Except for the unusual Opt^r phenotype, all the *S. pneumoniae* strains analyzed showed phenotypic and genetic characteristics of typical pneumococcal isolates, as

they were bile soluble, produced capsular antigens, and carried the *ply*, *lytA*, and *psaA* genes.

Distribution of capsular types. The isolates tested belonged to a variety of capsular types (Fig. 1). Most of the 16 different capsular types (1, 6A, 6B, 6C, 9N, 10A, 11A, 12F, 13, 14, 15C, 16F, 18C, 19A, 23A, and 23F) observed among the isolates included in the present study corresponded to types commonly found in our region (30, 31), reinforcing the concept that serotype distribution among Opt^r *S. pneumoniae* strains is mostly dependent on the epidemiological profile of each area (2, 3, 10, 11, 12). Identical serotypes were observed for the Opt^r and Opt^s subpopulations derived from the same culture. Interestingly, among the eight isolates belonging to serogroup 6, three belonged to the recently described variant 6C (32), which seems to have emerged during the postvaccination era in developed countries (33, 34). Moreover, five of the capsular types identified (1, 6B, 14, 18C, and 23F), comprising a total of 10 strains, are included in the 10-valent pneumococcal conjugate vaccine (PCV10), which was incorporated into the Brazilian Immunization Programme in 2010 (http://portal.saude.gov.br/portal/arquivos/pdf/intro_pneumococica10_val_04_02_10_ver_final.pdf).

Antimicrobial susceptibility profiles. The Opt^r *S. pneumoniae* strains were susceptible to most of the antimicrobial agents tested (Fig. 1), with only two of them presenting a multidrug-resistance profile (including simultaneous resistance to clindamycin, erythromycin, penicillin, tetracycline, and trimethoprim-sulfamethoxazole), suggesting that Opt resistance is not associated with increasing or specific resistance to other drugs. Resistance to both erythromycin and clindamycin was detected in 2 (7.7%) of the strains, and it was associated with the simultaneous presence of the *ermB* and *mef* genes. Resistance to tetracycline was observed in four (15.4%) of the isolates carrying the *tetM* gene. All of the

FIG 4 Hydrophobicity profiles of the c subunit of ATPase of optochin-resistant *Streptococcus pneumoniae* (*Opt^r S. pneumoniae*) isolates. (A) Mean hydrophobicity profiles obtained for seven representative *Opt^r S. pneumoniae* isolates with alterations in six different codons belonging to helix 1 of the c subunit of ATPase. (B) Mean hydrophobicity profiles obtained for five representative *Opt^r S. pneumoniae* isolates with alterations in two different codons belonging to helix 2 of the c subunit of ATPase. Reference strain ATCC 49619 was also included. Codons with alterations are outlined with a vertical black line.

mutations and Opt resistance. The fact that *Opt^r* and *Opt^s* subpopulations were differentiated only on the basis of the Opt MICs and the *atpC* gene sequencing suggests that an originally homogeneous *Opt^s* population might have undergone a point mutation resulting in two different types of cells.

The c subunit of the ATPase is known to consist of 66 amino acids distributed in two antiparallel α -helices linked by a conserved cytoplasmic loop (13). Previous studies have shown that mutations leading to Opt resistance usually occur in the transmembrane portion of the α -helices, as it does not interfere with the activity of the whole enzyme (14). Indeed, all the altered codons identified in this study are located in the transmembrane region, according to the predicted model of the subunit (Fig. 3). Eleven strains presented alterations in α -helix 1 of the c subunit (codons 13, 14, 18, 20, 23, or 31) and 15 in α -helix 2 (codons 45 or

49), among which 12 isolates (accounting for 48% of all *Opt^r S. pneumoniae* strains analyzed) had mutations in codon 49 (Table 1 and Fig. 1).

It was noticeable that all the *atpC* gene mutations identified in the present study generated coding sequences, involving amino acid replacement, and no termination codons that would inhibit expression of *atpC* due to early termination were detected. Single base substitutions are likely to occur randomly along the *atpC* gene sequence over time, including synonymous or even deleterious mutations. However, those that are evolutionarily neutral, or may be advantageous, and that lead to phenotypic variation, as illustrated by optochin resistance, are possibly the only ones eventually detected.

The prevalence of mutations in codon 49 (Table 1) is noteworthy, leading to speculation on its role as a hot spot for mutations

associated with resistance to Opt. No peculiar characteristics around codon 49 that could predispose this part of the *atpC* gene to frequent mutations, such as a higher incidence of repeated nucleotides, were observed in the present work. On the other hand, in the predicted folded structure of ATPase, certain amino acids, including those designated by codon 49, are placed next to residues of glutamic or aspartic acid, two nonessential amino acids, that are believed to represent the central portion of α -helix 2 and the binding site for optochin molecules (13). In terms of natural selection and evolution, especially when considering optochin and its analogues as selective pressures, codons producing such amino acids, such as codon 49, could represent preferential positions for preserved nonsynonymous genetic alterations associated with phenotypic advantages.

Although the exact mechanisms leading to Opt resistance remain to be elucidated, it is reasonable to infer that structural alterations in the folding of the protein generated as a result of a mutated *atpC* sequence, rather than the alterations in the amino acid sequences alone, may, at least in part, account for resistance to optochin. It has also been suggested that the alterations probably change the hydrophobicity profile of the molecule, causing the loss of affinity to the drug (14). Indeed, among Opt^r *S. pneumoniae* strains included in the present study, changes in α -helix 1 led, in general, to substitutions for amino acids with higher hydrophobicity profiles (Fig. 4A), while in α -helix 2 the replacements were for amino acids with lower hydrophobicity profiles (Fig. 4B). Additional studies, however, are required to elucidate the direct or indirect consequences of these changes in the interaction with optochin.

Our data indicate that emergence of Opt^r *S. pneumoniae* strains is not due to a clonal spread, since they may possess a diversity of serotypes, phenotypes, and genotypes. Also, it seems to be related to a random mutational event, which is more likely to occur in the c subunit of ATPase, and more frequently in codon 49, near the center of α -helix 2. The occurrence of two types of Opt^r *S. pneumoniae* strains additionally increases the complexity of this phenomenon, although their individual importance or consequences are still unclear. Overall, our results provide additional information and reinforce previous observations about Opt resistance among *S. pneumoniae* strains, contributing to the global and local pool of data on the characteristics of these atypical isolates. Health care-associated professionals should be aware of the occurrence of the unusual Opt^r *S. pneumoniae* strains, especially considering that they can be isolated from a variety of clinical sources, including asymptomatic carriers and patients with invasive and noninvasive infections, leading to incorrect bacterial identification, and ultimately to misinterpretation of carriage studies or imprecise diagnosis and treatment of infectious conditions.

ACKNOWLEDGMENTS

This work was supported in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Ministério da Ciência e Tecnologia (MCT/PRONEX), Brazil.

We thank Maria Cristina C. Brandileone (Instituto Adolfo Lutz, SP, Brazil) for providing most of the control strains used in the *pspA* gene typing experiments, and Lesley McGee (Centers for Disease Control and Prevention, Atlanta, GA) for providing representative strains of the PMEN clones.

The authors declare that they have no competing interests.

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