scientific reports

OPEN

Description of optochin-resistant *Streptococcus pneumoniae* due to an uncommon mutation in the *atpA* gene and comparison with previously identified *atpC* mutants from Brazil

Aline R. V. Souza[1,4], Sandrine E. C. M. de Pina[1], Natália S. Costa[1], Felipe P. G. Neves[2], Vânia L. C. Merquior[3], José Mauro Peralta[1], Tatiana C. A. Pinto[1] & Lúcia M. Teixeira[1✉]

Optochin susceptibility testing is a major assay used for presumptive identification of *Streptococcus pneumoniae*. Still, atypical optochin-resistant (Opt[r]) pneumococci have been reported and this phenotype has been attributed to nucleotide substitutions in the genes coding for the F_0F_1ATPase. While substitutions in the *atp*C gene (c-subunit of ATPase) are more common and better characterized, data on mutations in the *atp*A (a-subunit) are still limited. We have characterized five Opt[r] isolates presenting alterations in the *atp*A (Trp206Cys in four isolates and Trp206Ser in one isolate), constituting the first report of such mutations in Brazil. Most of the Opt[r] isolates consisted of heterogeneous populations. Except for Opt MICs and the nucleotide changes in the *atp*A gene, Opt[r] and Opt[s] subpopulations originating from the same culture had identical characteristics. In addition, we compared phenotypic and genetic characteristics of these *atp*A mutants with those of *atp*C mutants previously identified in Brazil. No structural alterations were detected among predicted proteins, regardless of mutations in the coding gene, suggesting that, despite the occurrence of mutations, protein structures tend to be highly conserved, ensuring their functionalities. Phylogenetic analysis revealed that atypical Opt[r] strains are true pneumococci and Opt resistance does not represent any apparent selective advantage for clinical isolates.

Streptococcus pneumoniae is a leading cause of invasive diseases among children and the elderly associated with considerable mortality and economic burden[1]. Although infant mortality rates have been declining since 2000 due to broader access to pneumococcal vaccines, pneumococcal pneumonia remains as a major cause of child mortality[2]. Thus, pneumococcal infections require fast and accurate diagnosis. Conventional laboratory identification of *S. pneumoniae* usually relies on one major characteristic of this species: the susceptibility to optochin (Opt). Opt susceptibility testing can distinguish the human pathogen *S. pneumoniae* from other alpha-hemolytic species, such as *Streptococcus mitis* and *Streptococcus pseudopneumoniae*[3].

Nevertheless, isolation of Opt-resistant (Opt[r]) pneumococcal has been reported in different geographical areas[4–11], drawing attention to the potential for misidentification of this relevant pathogen. Point mutations in the *atp*C gene are usually attributed to this atypical phenotype; the *atp*C gene codes for the c-subunit of the bacterial F_0F_1ATPase, the molecular target of optochin[4–6,8,9,11–13]. Alterations in other subunits of this molecule are rare, and only two reports on Opt[r] clinical strains of *S. pneumoniae* presenting mutations in the *atp*A gene (coding for the a-subunit of the bacterial F_0F_1ATPase) are available to date[5,13].

[1]Instituto de Microbiologia Paulo de Goes, Universidade Federal Do Rio de Janeiro, Rio de Janeiro, RJ 21941-902, Brazil. [2]Instituto Biomédico, Universidade Federal Fluminense, Niterói, RJ 24210-130, Brazil. [3]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ 20551-030, Brazil. [4]Present address: Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-902, Brazil. ✉email: lmt2@micro.ufrj.br

Our group has reported the occurrence of previously recognized as well as novel mutations in the *atp*C gene among a set of 26 Opt^r pneumococcal isolates in Brazil[6,9]. In the present study, we describe five additional Opt^r isolates presenting alterations in the *atp*A gene, constituting the first report of such unusual mutations in pneumococcal isolates from our country. In addition, we extended the spectrum of methodologies used and compared phenotypic and genetic characteristics of all Opt^r pneumococcal strains described in Brazil up to now, including both *atp*C and *atp*A mutants.

Results

Strain identification and characterization of Opt resistance phenotypes.
All five isolates described in the study had the following characteristics: they were gram-positive catalase-negative cocci; presented alpha-hemolysis on sheep blood agar plates; generated positive results in the bile solubility and in the latex agglutination tests; harbored the *lytA*, *ply* and *psaA* genes; and were resistant to Opt.

Opt susceptibility testing, under both CO_2-enriched or conventional atmospheres, revealed the occurrence of two phenotypes among the five Opt^r isolates. The first was represented by one isolate displaying no inhibition zone, consisting of a homogeneous Opt^r population. The second phenotype was represented by the other four isolates, showing inhibition zones > 14 mm but with colonies within, consisting of heterogeneous populations comprised by Opt^r and Opt-susceptible (Opt^s) subpopulations. Opt MICs ranged from 16 to 32 µg ml among the five Opt^r strains evaluated (Fig. 1). The single Opt ^{r} homogeneous population had an Opt MIC of 32 µg/ml. Opt^r subpopulations derived from heterogeneous populations exhibited MICs between 16 and 32 µg/ml (Fig. 1); while Opt^s counterparts presented Opt MICs of 1 µg/ml. Opt MICs of Opt^s subpopulations were identical to those detected among optochin-susceptible reference strains of *S. pneumoniae* ATCC BAA-255 and ATCC 49619 (1 µg/ml). Opt MICs of reference strains of *S. pseudopneumoniae* ATCC BAA-960 and *S. mitis* ATCC 49456 were 8 µg/ml and 256 µg/ml, respectively.

In addition to the five Opt^r strains included in the present study, the 26 Opt^r isolates previously reported in Brazil[6,9] were also subjected to identification by MALDI-TOF MS. All 31 strains were identified as *S. pneumoniae,* with scores between 2.089 and 2.452. Of note, Opt^r and Opt^s subpopulations derived from heterogeneous populations were also analyzed separately, and in all cases both subpopulations were identified as pneumococcus with scores higher than 2.0. However, reference strains *S. mitis* ATCC 49456 and *S. pseudopneumoniae* ATCC BAA-960 were also identified as *S. pneumoniae* by MALDI-TOF MS, with scores of 1.938 and 2.213, respectively.

Sequencing of atpA gene.
Sequencing of *atp*A genes revealed single-base substitutions leading to amino acid modifications in codon 206 in all five strains, comprising four isolates with a Trp**206**Cys substitution and one isolate with a Trp**206**Ser substitution (Figs. 1 and 2A,B). Interestingly, the single strain with a Trp**206**Ser substitution was also the single strain homogenously resistant to optochin. All the other four showing the Trp-**206**Cys modification consisted on heterogeneous populations. Of note, while Opt^r subpopulations derived from heterogeneous populations presented this alteration in codon 206, Opt^s counterparts had *atp*A gene sequences identical to those of optochin-susceptible reference strains of *S. pneumoniae* (ATCC BAA-255 and ATCC 49619).

In silico prediction of c- and a-subunit 3D models.
The 3D structure of the a-subunit of F_0F_1ATPase predicted in silico from the *atp*A nucleotide sequences of the five Opt^r *S. pneumoniae* strains included in the study were identical to the models generated from Opt^s reference strains (*S. pneumoniae* ATCC BAA-255 and ATCC 49619), consisting of five transmembrane α-helices (Fig. 3A). Likewise, 3D models of the c-subunit, predicted from the *atp*C gene sequences of 26 Opt^r strains previously reported[6,9] and retrieved from GenBank database, were indistinguishable from the ones of Opt^s reference strains (*S. pneumoniae* ATCC BAA-255 and ATCC 49619), consisting of two transmembrane α-helices (Fig. 3B). The structures were also identical between Opt^r and Opt^s subpopulations derived from heterogeneous populations.

Phenotypic and molecular characterization of Opt^r strains.
Opt^r and Opt^s subpopulations derived from heterogeneous populations showed identical results regarding capsular type, antimicrobial susceptibility profile, ST, PFGE, MLVA and PI-1 genes profiles.

The five Opt^r pneumococcal isolates included in this study belonged to serotypes 6A, 6C, 18C, 19A and 19F. All strains were susceptible to antimicrobial agents tested, except for sulfamethoxazole/trimethoprim (Fig. 1).

Within the five *atp*A mutants, different PFGE patterns (Fig. 1), STs (193,733, 763, 3930 and 5847) (Figs. 1 and 5) and MLVA profiles (Fig. 4) were detected. When compared to the 26 Opt^r pneumococcal isolates previously described in Brazil, only three STs and two MLVA profiles were shared by two or more strains (Figs. 1, 4 and 5). It is also noteworthy that ten (32.25%) of all 31 Opt^r pneumococcal isolates analyzed belonged to internationally disseminated clones recognized by the PMEN (Fig. 5). Of note, there was no correlation between the type of *atp*C or *atp*A mutation with serotype, PFGE pattern, ST or MLVA type (Figs. 1, 4 and 5).

PI-1 genes were found in five (16.1%) of all 31 Opt^r isolates analyzed; of those, one had a mutation in the *atp*A gene while four had mutations in the *atp*C gene (Fig. 1). In addition, most of the PI-1 positive strains were related to PMEN clones.

Phylogenetic analysis.
Phylogenetic analysis revealed that all 31 Opt^r pneumococcal strains, including both *atp*A and *atp*C mutants, were clustered within a monophyletic group, with 100% of probability in an analysis using a bootstrap of 1000 (Fig. 6). This analysis also included typical *S. pneumoniae*, representative of Opt^s strains. On the other hand, *S. mitis*, *S. pseudopneumoniae* and *S. oralis* were clustered in different and more distantly related groups.
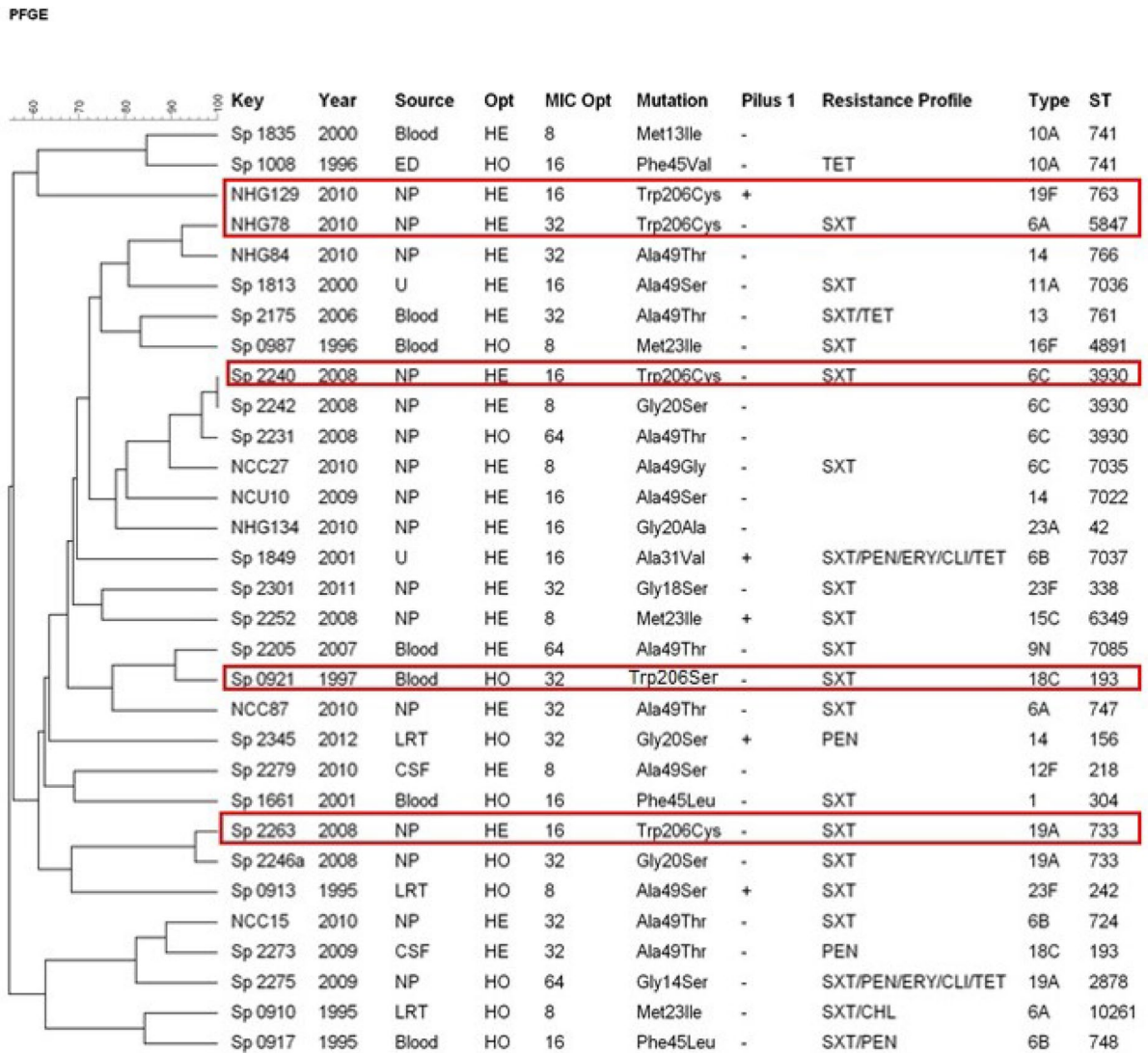
PFGE

Key	Year	Source	Opt	MIC Opt	Mutation	Pilus 1	Resistance Profile	Type	ST
Sp 1835	2000	Blood	HE	8	Met13Ile	-		10A	741
Sp 1008	1996	ED	HO	16	Phe45Val	-	TET	10A	741
NHG129	2010	NP	HE	16	Trp206Cys	+		19F	763
NHG78	2010	NP	HE	32	Trp206Cys	-	SXT	6A	5847
NHG84	2010	NP	HE	32	Ala49Thr	-		14	766
Sp 1813	2000	U	HE	16	Ala49Ser	-	SXT	11A	7036
Sp 2175	2006	Blood	HE	32	Ala49Thr	-	SXT/TET	13	761
Sp 0987	1996	Blood	HO	8	Met23Ile	-	SXT	16F	4891
Sp 2240	2008	NP	HE	16	Trp206Cys	-	SXT	6C	3930
Sp 2242	2008	NP	HE	8	Gly20Ser	-		6C	3930
Sp 2231	2008	NP	HO	64	Ala49Thr	-		6C	3930
NCC27	2010	NP	HE	8	Ala49Gly	-	SXT	6C	7035
NCU10	2009	NP	HE	16	Ala49Ser	-		14	7022
NHG134	2010	NP	HE	16	Gly20Ala	-		23A	42
Sp 1849	2001	U	HE	16	Ala31Val	+	SXT/PEN/ERY/CLI/TET	6B	7037
Sp 2301	2011	NP	HE	32	Gly18Ser	-	SXT	23F	338
Sp 2252	2008	NP	HE	8	Met23Ile	+	SXT	15C	6349
Sp 2205	2007	Blood	HE	64	Ala49Thr	-	SXT	9N	7085
Sp 0921	1997	Blood	HO	32	Trp206Ser	-	SXT	18C	193
NCC87	2010	NP	HE	32	Ala49Thr	-	SXT	6A	747
Sp 2345	2012	LRT	HO	32	Gly20Ser	+	PEN	14	156
Sp 2279	2010	CSF	HE	8	Ala49Ser	-		12F	218
Sp 1661	2001	Blood	HO	16	Phe45Leu	-	SXT	1	304
Sp 2263	2008	NP	HE	16	Trp206Cys	-	SXT	19A	733
Sp 2246a	2008	NP	HO	32	Gly20Ser	-	SXT	19A	733
Sp 0913	1995	LRT	HO	8	Ala49Ser	+	SXT	23F	242
NCC15	2010	NP	HE	32	Ala49Thr	-	SXT	6B	724
Sp 2273	2009	CSF	HE	32	Ala49Thr	-	PEN	18C	193
Sp 2275	2009	NP	HO	64	Gly14Ser	-	SXT/PEN/ERY/CLI/TET	19A	2878
Sp 0910	1995	LRT	HO	8	Met23Ile	-	SXT/CHL	6A	10261
Sp 0917	1995	Blood	HO	16	Phe45Leu	-	SXT/PEN	6B	748

Figure 1. UPGMA dendrogram generated by using BioNumerics v.7.6 based on PFGE profiles of 31 optochin-resistant *Streptococcus pneumoniae* strains isolated in Brazil. The five a*tp*A gene mutants described in this work are marked in red. *Ala* alanine, *CLI* clindamycin, *CHL* chloramphenicol, *CSF* cerebrospinal fluid, *Cys* cysteine, *ED* eye discharge, *ERY* erythromycin, *Gly* glycine, *HE* heterogeneous, *HO* homogeneous, *Ile* isoleucine, *Leu* leucine, *LRT* low respiratory tract, *Met* methionine, *MIC* minimum inhibitory concentration, *NP* nasopharyngeal carriage, *Opt* optochin, *PEN* penicillin, *Phe* phenylalanine, *Ser* serine, *ST* sequence type, *SXT* sulfamethoxazole-trimethoprim, *TET* tetracycline, *Thr* threonine, *Trp* tryptophan, *Val* valine, *U* unknown.

Discussion

During the last decades, the occurrence of atypical optochin-resistant pneumococcal strains has been sporadically reported[4–10,12–14], reinforcing the need of constant evaluation of traditional identification schemes for this relevant pathogen.

Different studies have described the occurrence of two phenotypes within collections of Opt^r *S. pneumoniae* isolates: a homogeneous type in which the isolate is fully resistant to optochin, and a heterogeneous type comprised by Opt^r and Opt^s subpopulations[5–7,9,12,13]. The distribution of each phenotype, however, seems to vary according to the geographical region. In Brazil, as well as in the United States[5], the heterogeneous phenotype has been more commonly reported. Among the 26 Opt^r isolates previously reported from Brazil[6,9], 16 (61.5%) were composed by heterogeneous populations (Fig. 1). On the other hand, in Portugal, homogenous Opt^r populations have been more frequently observed[7]. Of note, the only difference found between subpopulations derived from heterogeneous Opt^r populations, in this and previous studies, is the mutation in *atp*A or *atp*C gene. These observations suggest that an originally Opt^s population might at some point undergo a point mutation resulting in two different types of cells that, then, compose a heterogeneous Opt^r population. However, it remains unknown why in some strains these subpopulations tend to coexist while in others a homogeneous Opt^r phenotype occurs.

MALDI-TOF MS has gained large interest in the clinical microbiology setting and it has already been introduced in the routine of several clinical laboratories worldwide[15,16]. This methodology has been widely used
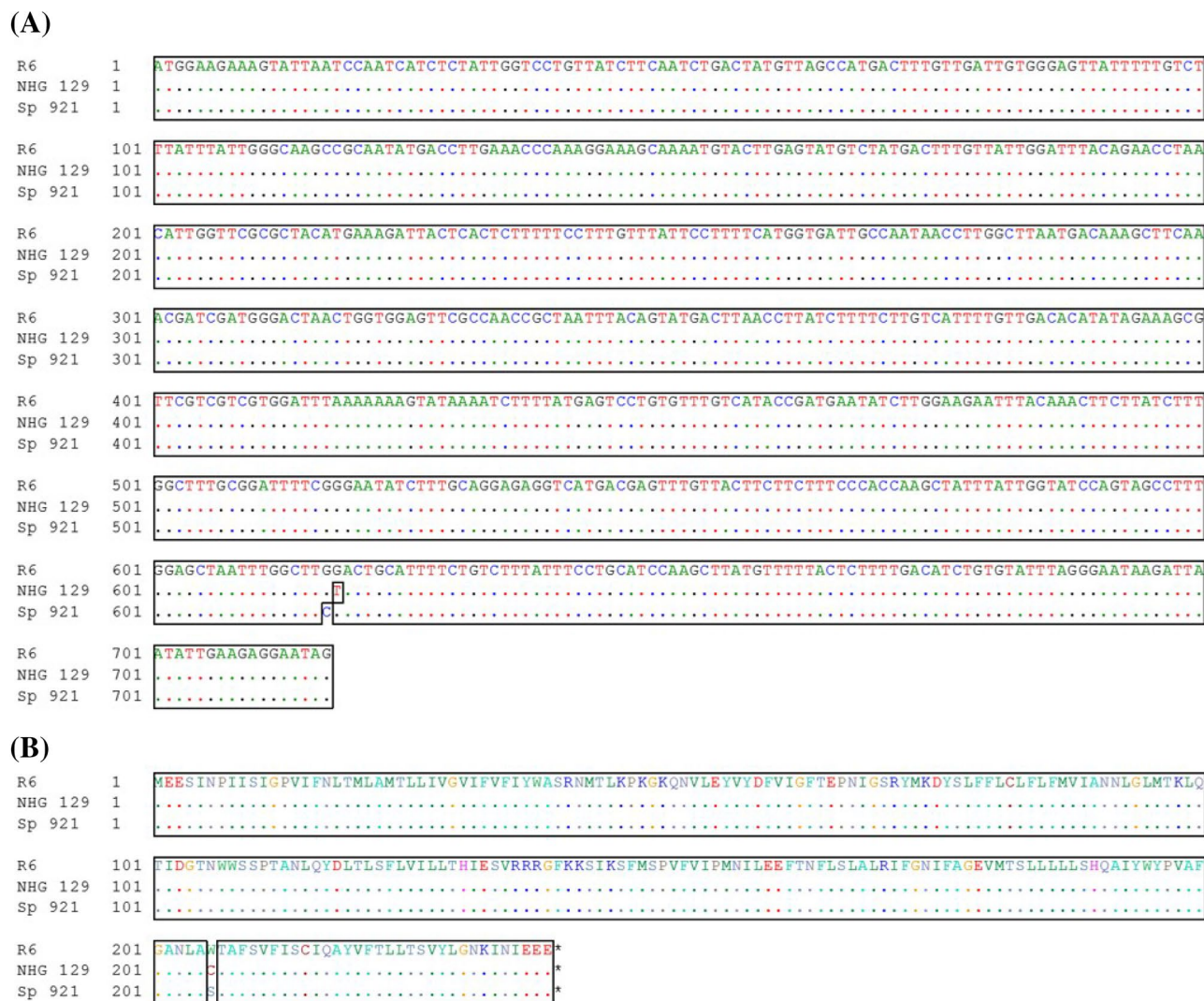
3

(A)

(B)

Figure 2. Alignment of representative *atp*A gene sequences observed among optochin-resistant (Opt[r]) *Streptococcus pneumoniae* isolates. (**A**) Nucleotide sequences of the *atp*A gene representing the two different base-substitutions observed among the five Opt[r] isolates included in the present study. (**B**) Deduced amino acid sequences of the a-subunit of ATPase representing, respectively, the two different base-substitutions mentioned above. Optochin-susceptible reference strain *S. pneumoniae* R6 was included for comparative purposes. Alterations are outlined with black boxes.

for identification of a number of bacterial species, but the lack of discrimination among streptococcal species belonging to the mitis group is well documented[15,17]. Although alternative MALDI-TOF MS-based protocols for accurate differentiation of mitis group streptococci have been proposed, the majority depend on the analysis of mass spectra by trained staff, being unfeasible for application in many clinical laboratories, which usually rely on results generated by automated processing of spectra by dedicated softwares[16,18]. Therefore, when using MALDI-TOF MS for identification of *S. pneumoniae* in the clinical microbiology laboratory, it is mandatory to perform a complementary test, such as bile solubility or optochin susceptibility testing[19]. In this regard, laboratory personnel should be aware of the possible occurrence of atypical optochin-resistant pneumococcus, and preferentially perform an additional test if the isolate shows resistance to optochin.

Opt MICs found in this study, ranging between 16 and 32 μg/ml, were in agreement with MICs (8–64 μg/ml) observed among the 26 Opt[r] Brazilian isolates previously described[6,9] (Fig. 1), and also with those obtained for isolates from other locations[5,7]. Comparison of Opt[r] and Opt[s] MICs in heterogeneous populations revealed similar results to earlier studies, in which MICs of Opt[r] subpopulations were 4–64 fold higher than those of Opt[s] counterparts[5,9]. Moreover, no correlation between Opt MIC levels and Opt[r] phenotype was detected since similar MIC levels were observed in both homogenous and heterogeneous populations. Likewise, no correlation between Opt MIC levels and type of mutation was observed, as previously reported[5,9,10].

To the best of our knowledge, this is the first study to describe *atp*A mutants among clinical pneumococcal isolates in Brazil and also represents the largest and more extensively characterized collection of Opt[r] *S. pneumoniae* clinical isolates reported in the literature. Earlier, only two Opt[r] *S. pneumoniae* clinical isolates presenting mutations in *atp*A gene had been described[5,13]. The first was recovered from an individual's nasopharynx in the
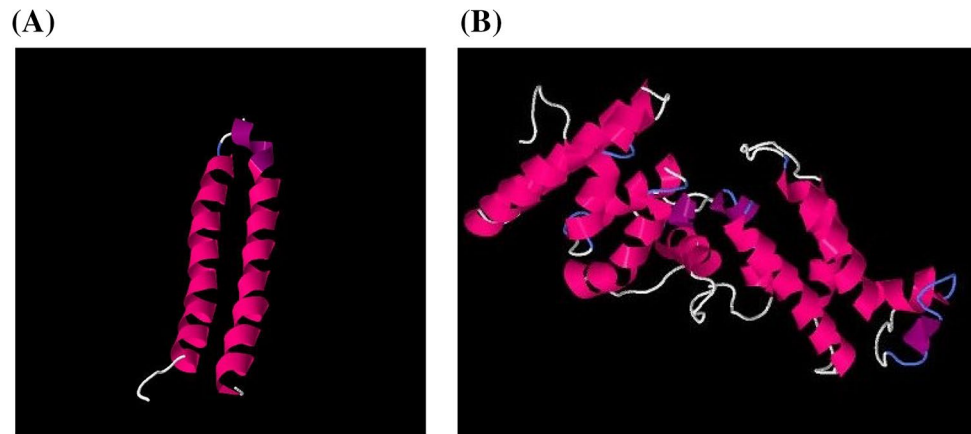
Figure 3. In silico predicted 3D models for c- (**A**) and a-subunits (**B**) of F0F1ATPase of *Streptococcus pneumoniae* created with Bioserf v.2.0. Alpha helices are colored in pink. All 31 optochin-resistant *S. pneumoniae* strains analyzed and the reference strains ATCC BAA-255 and ATCC 49619 generated identical models for both subunits.
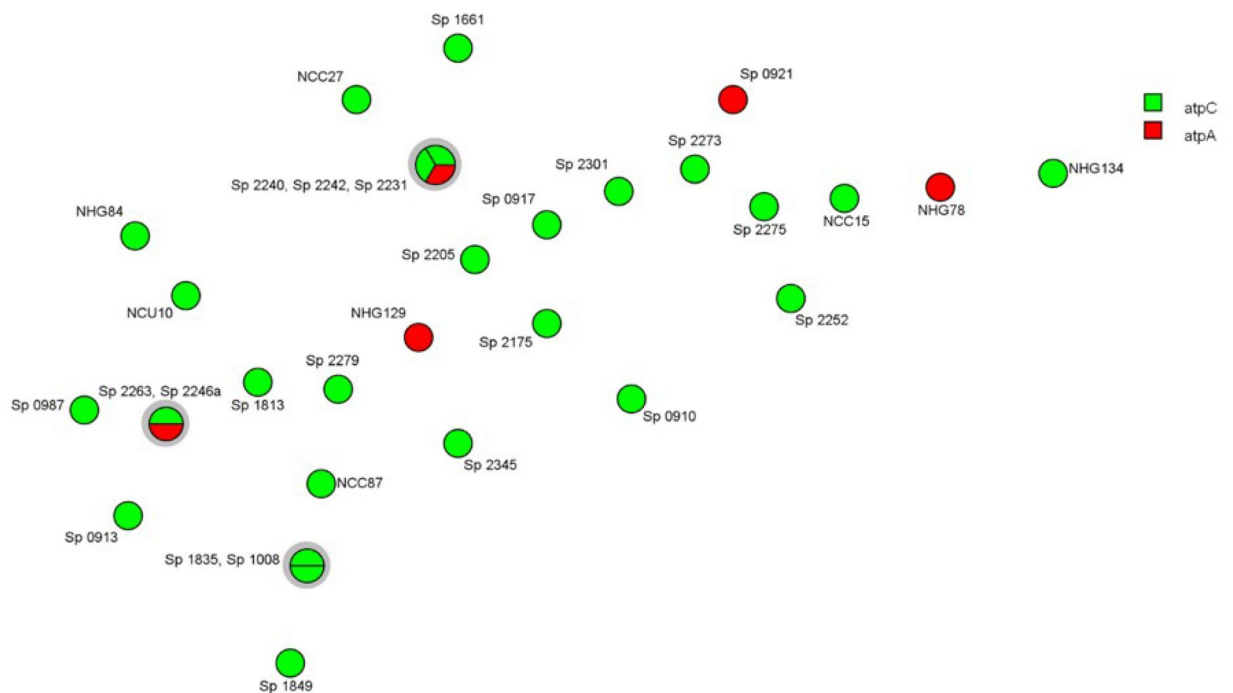

Figure 4. Minimum Spanning Tree generated by using BioNumerics v.7.6 and showing the genetic relationship among 31 optochin-resistant *Streptococcus pneumoniae* strains isolated in Brazil, according to the MLVA profiles. Each node represents one MLVA profile, and nodes are divided proportionaly to the number of isolates included in each MLVA profile. Clonal complexes (strains sharing five or more identical alleles) are shaded in grey. Absence of lines between nodes indicate they show three or more different alleles and are, therefore, genetically unrelated. Strains were differentiated by color according to the location of mutation (*atp*C or *atp*A; see legend).

United States, presented a heterogeneous phenotype, a Trp**206**Ser substitution, an Opt MIC of 32 µg/ml, and belonged to serotype 6B[5]. The second was recovered from a patient's blood in Argentina, showed a homogeneous phenotype, a Trp**206**Cys modification, an Opt MIC of 64 µg/ml, and belonged to serotype 5[13].

Our results reinforce the observation that mutations in the *atp*A gene are not correlated with specific clinical source or serotype, but also highlight that substitutions in this gene, regardless of any other characteristic, are always in the same position, the codon 206. This is quite different from what has been observed within *atp*C mutants, among which substitutions have been identified in 12 different codons of the c-subunit[4–6,8–10,13,14]. Still, despite the higher variability, around 50% of the 48 *atp*C mutants described to date show alterations in the same location, the codon 49, which has been suggested as a hot-spot for this type of mutation. Codon 206 in the a-subunit could act as a hot-spot as well, but this should be established as more *atp*A mutants are identified.
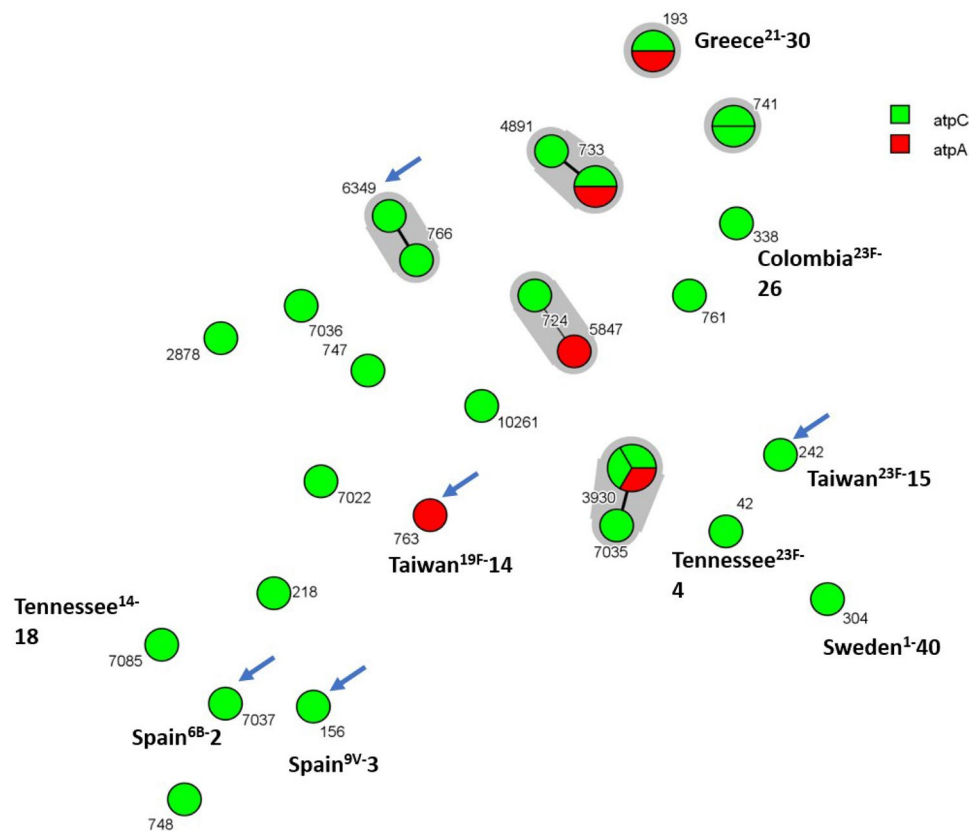
Figure 5. Minimum Spanning Tree generated by using BioNumericsv.7.6 and displaying the genetic relatedness inferred by MLST among 31 optochin-resistant *Streptococcus pneumoniae* strains isolated in Brazil. Each node represents one sequence type (ST), and nodes are divided proportionaly to the number of isolates included in the same ST. Internationally disseminated clones recognized by the PMEN and related to strains analyzed in the study are indicated. Clonal complexes (strains sharing five or more identical alleles) are shaded in grey. Absence of lines between nodes indicate they show three or more different alleles and are, therefore, genetically unrelated. Strains were differentiated by color according to the location of mutation (*atp*C or *atp*A; see legend). Strains harboring pilus type 1 genes are highlighted with a blue arrow.

In silico predicted 3D models of a- and c-subunits indicate that, despite the occurrence of alterations at the primary level, 3D models of c- and a-subunits tend to be highly conserved, regardless of optochin susceptibility profile, which could probably confer the maintenance of enzyme functional activity and, consequently, the survival of bacteria. Although no structural alterations on predicted proteins were detected in the present study, it has been previously observed that mutations in the c-subunit led to inability of these bacteria to survive in acidic conditions, including survival inside of macrophages[20]. Mutations in the a-subunit of F0F1 ATPase, on the other hand, presented no implications regarding acid tolerance[20]. Nevertheless, additional studies are needed to clarify the impact of *atp*A and *atp*C mutations on pneumococcal fitness.

The variability of PFGE profiles, STs and MLVA types detected in the study indicate that optochin resistance is not a clonal-specific characteristic, in agreement with previous findings[7,9]. However, PMEN clones previously associated with the presence of PI-1[21] and known to be circulating in Brazil in recent years[9,22–24] were found among Opt[r] strains, suggesting that highly successful clones can exhibit the atypical Opt[r] phenotype, increasing the concern on possible consequences of the misidentification of these variants.

Phylogenetic analysis clearly shows that atypical Opt[r] *S. pneumoniae* and typical pneumococci have evolved from the same ancestral (Fig. 6), while evolution that resulted in different streptococcal species occurred by an independent route. These data, in conjunction with the other results of this study, strongly suggest that Opt[r] isolates are authentic *S. pneumoniae* that have diverged from typical strains by point mutations in the the *atp*C or *atp*A genes.

In conclusion, our results suggest that optochin resistance in *S. pneumoniae* is not related to a specific phenotypic or genetic characteristic, but is rather due to the random occurrence of mutations in the *atp*A or *atp*C genes. Even though mutations in a- or c-subunits of F_0F_1 ATPase can result in amino acid changes, their predicted 3D structures remain unaltered, ensuring survival of these strains. Phylogenetic analysis revealed that atypical Opt[r] strains are true pneumococci and Opt resistance does not represent any apparent selective advantage for clinical strains.
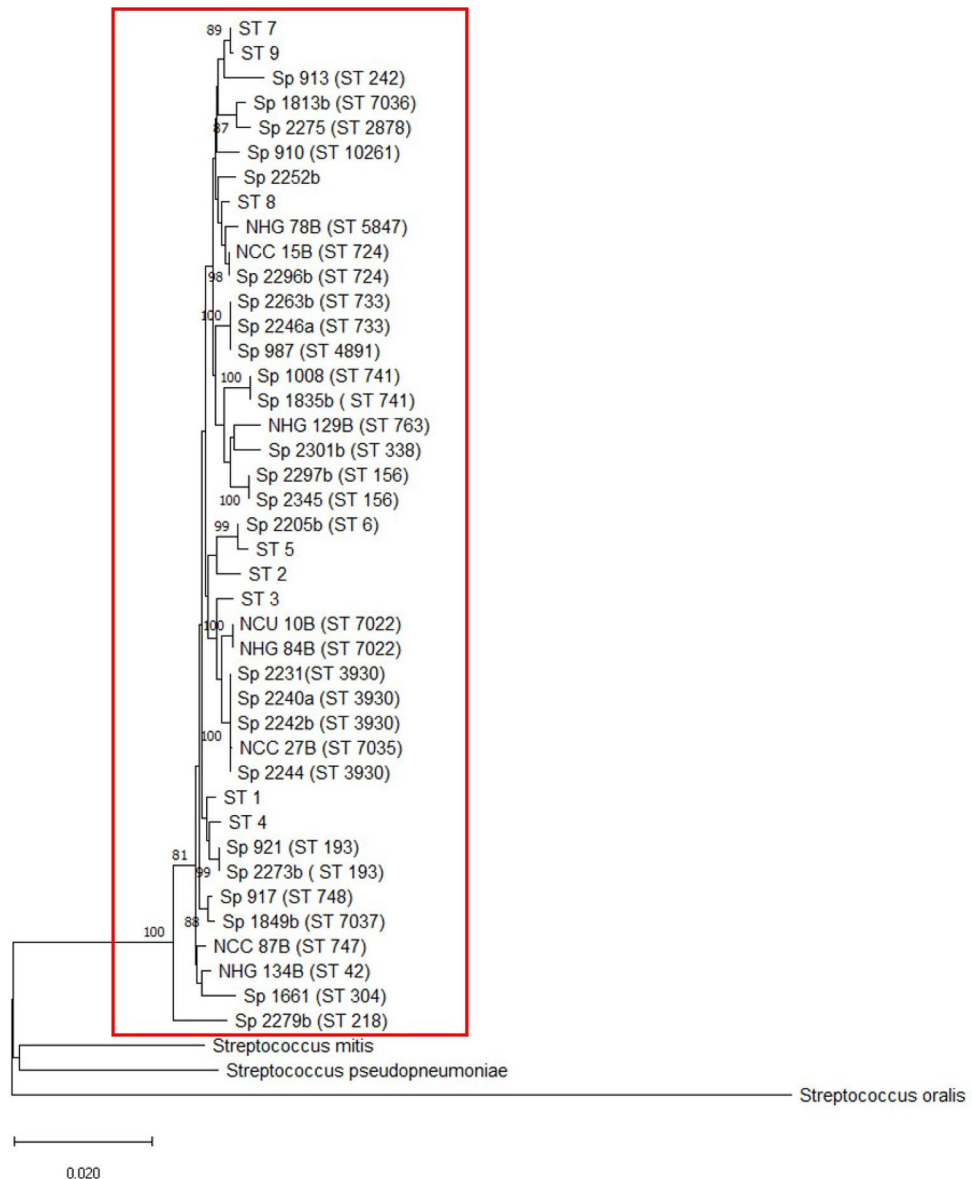
Figure 6. MLSA tree generated from the concatenated sequences of six housekeeping genes (*aro*E, *gdh*, *gki*, *rec*P, *spi* and *xpt*) showing the phylogenetic relationship among optochin-resistant *Streptococcus pneumoniae* (31 isolates), typical *S. pneumoniae* (8 isolates), *Streptococcus pseudopneumoniae* (strain 9230), *Streptococcus mitis* strain (SS691) and *Streptococcus oralis* (strain SS527). Scale bar indicates the nucleotide subtitution per site. Highlighted with a red box is the cluster comprising all 39 *S. pneumoniae* sequences analyzed, including the typical and the optochin-resistant strains.

Methods

Bacterial strains and identification tests. Five Opt^r *S. pneumoniae* isolates (Sp 921, Sp 2240, Sp 2263, NHG78 and NHG129) recovered from individuals living in Brazil were included in the present study, based on the finding that they showed no alterations in the *atp*C gene during initial screening tests, carried out as previously described[9]. Four isolates were obtained during nasopharyngeal carriage surveillance studies performed as approved by the ethics committees of the institutions involved. One isolate (Sp 921) was recovered from a blood culture taken as part of the standard patient care procedures, so a specific ethical approval was not needed. The isolates were subjected to conventional phenotypic identification tests, including observation of colony morphology and type of hemolytic activity on sheep blood agar plates; cellular characteristics as observed after Gram stain; Opt susceptibility, bile-solubility, and latex agglutination tests[3]. Identification was also performed by MALDI-TOF MS, using a Microflex LT equipment (Bruker Daltonics, Bremen, Germany), according to the manufacturer recommendations. For comparative purposes, a collection of 26 Opt^r pneumococcal isolates presenting mutations in the *atp*C gene, previously identified by our group[6,9], was also included for further characterization by MALDI-TOF, MLSA, MLST, detection of PI-1 genes and in silico prediction of c-subunit 3D

model. Reference strains of *S. pneumoniae* (ATCC BAA-255 and ATCC 49619), *S. pseudopneumoniae* (ATCC BAA-960) and *S. mitis* (ATCC49456) were included in all identification tests.

Optochin susceptibility testing. Opt susceptibility was determined by disk diffusion testing according to standard procedures[3]. Optochin disks (BBL Taxo P Discs, BD, Sparks, MD, USA) were applied to the surface of 5% sheep blood agar plates (Plast Labor, Rio de Janeiro, RJ, Brazil) streaked with the isolates being tested. After overnight incubation at 36 °C, under both 5% CO_2 and conventional atmospheres[25], growth inhibition zones around the disks were measured. Isolates displaying inhibition zones ≥ 14 mm in diameter were identified as susceptible, while strains were considered resistant when showing zones < 14 mm or zones ≥ 14 mm but with colonies within. This last case was classified as heterogeneous population, composed by both Opt[r] and Opt-susceptible (Opt[s]) subpopulations; all subsequent experiments were carried out separately for both subpopulations after obtaining pure cultures of each one. Additionally, optochin (Sigma Chemical Co., St. Louis, MO, USA) minimal inhibitory concentrations (MICs) were also determined, by the agar dilution method as previously described[9].

Sequencing of *atp*A gene. DNAs for all PCR reactions were obtained by using the Chelex 100 resin (Bio-Rad, Hercules, CA, USA) as described earlier[9], followed by amplification of the *atp*A gene[11]. Products were purified with ExoSAP-IT (USB Affymetrix, Cleveland, OH, USA) and sequenced using ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were aligned and analyzed with Bioedit software v7.0.9.0[26], using *atp*A nucleotide sequences of Opt[s] reference strains (*S. pneumoniae* ATCC BAA-255 and ATCC 49619) as standards.

In silico prediction of c- and a-subunit 3D models. Using the amino acid sequences translated from the nucleotide sequences obtained in this study, predicted 3D models of the a-subunit of F_0F_1 ATPase were designed by using the online tool Bioserf v2.0, available at PSIPRED Protein Sequence Analysis Workbench[27,28]. Predicted 3D models of the c-subunit were generated using the *atp*C gene sequences of 26 Opt[r] strains previously reported[6,9] retrieved from the GenBank database (accession numbers KC513927 to KC513948); *atp*C and *atp*A nucleotide sequences of Opt[s] reference strains (*S. pneumoniae* ATCC BAA-255 and ATCC 49619) were included for comparative purposes.

Determination of capsular types. Capsular types were determined by either PCR[29] or the standard Quellung reaction[30].

Antimicrobial susceptibility testing. Susceptibility to antimicrobial agents was evaluated by the agar diffusion test according to CLSI recommendations and interpretative criteria[31]. The following agents were tested: chloramphenicol, clindamycin, erythromycin, levofloxacin, oxacillin, rifampicin, tetracycline, sulfamethoxazole-trimethoprim and vancomycin (all from Oxoid, Basingstoke, Hampshire, United Kingdom). *S. pneumoniae* ATCC 49619 was used for quality control.

Detection of virulence-associated genes. The presence of virulence genes *ply* (coding for pneumolysin), *lyt*A (coding for autolysin), *psa*A (coding for pneumococcal surface antigen A)[32–34], and those coding for pilus type 1 (PI-1)[21] was investigated by PCR using previously described primers and an automated Veriti 96-well thermal cycler (Applied Biosystems Inc, Carlsbad, CA, USA).

Evaluation of genetic diversity. Genetic diversity was assessed by Pulsed-Field Gel Electrophoresis (PFGE)[9], Multiple Locus VNTR Analysis (MLVA)[35] and Multilocus Sequence Typing (MLST)[36–38] with modified primers[39], according to earlier recommendations.

Phylogenetic analysis. Phylogenetic analysis was performed by Multilocus Sequence Analysis (MLSA)[40]. Concatenates of six *housekeeping* genes (*aro*E, *gdh*, *gki*, *rec*P, *spi*, *xpt*) were generated with an online tool[41] and a phylogenetic tree was built using Mega X software[42], using the following parameters: Neighbor Joining algorithm, with bootstrap of 1000, Kimura 2p model and uniform rates of substitutions. There were also included in the analysis nucleotide sequences of typical *S. pneumoniae* strains belonging to STs 1, 2, 3, 4, 5, 7 and 9 (obtained from PubMLST)[37] and sequences of other *Streptococcus* species (retrieved from GenBank database) including *S. mitis* strain S691 (accession numbers EU075887.1, EU075743.1, EU075784.1, EU075814.1, EU075851.1 and EU075674.1 for genes *aro*E, *gdh*, *gki*, *rec*P, *spi* and *xpt* respectively), *S. pseudopneumoniae* strain 9230 (accession numbers KC491132.1, KC491108.1, KC491058.1, KC491034.1, KC490998.1 and KC490972.1 for genes *aro*E, *gdh*, *gki*, *rec*P, *spi* and *xpt* respectively) and *Streptococcus oralis* strain S527 (accession numbers EU076013.1, EU076236.1, EU075980.1, EU076137.1, EU076186.1 and EU075945.1 for genes *aro*E, *gdh*, *gki*, *rec*P, *spi* and *xpt* respectively).

Nucleotide sequence accession numbers. The *atp*A gene sequences of the five mutated isolates reported in the present study were deposited in GenBank database under accession numbers KR012497 to KR012501.

References

1. Shiri, T. *et al.* Pneumococcal disease: A systematic review of health utilities, resource use, costs, and economic evaluations of interventions. *Value Health.* **22**, 1329–1344 (2019).
2. UN IGME (United Nations Inter-agency Group for Child Mortality Estimation). *Levels & trends in child mortality report 2020* at https://childmortality.org/wp-content/uploads/2020/09/UNICEF-2020-Child-Mortality-Report.pdf (2020).
3. Spellerberg B & Brandt C. *Streptococcus* in *Manual of Clinical Microbiology* (ed. Carroll KC *et al.*) 383–402 (ASM Press, 2015).
4. Cogné, N., Claverys, J. P., Denis, F. & Martin, C. A novel mutation in the α-helix 1 of the C subunit of the F1/F0 ATPase responsible for optochin resistance of a *Streptococcus pneumoniae* clinical isolate. *Diagn. Microbiol. Infect. Dis.* **38**, 119–121 (2000).
5. Pikis, A., Campos, J. M., Rodriguez, W. J. & Keith, J. M. Optochin resistance in *Streptococcus pneumoniae*: Mechanism, significance and clinical implications. *J. Infect. Dis.* **184**, 582–590 (2001).
6. Dias, C. A. *et al.* Diversity of mutations in the *atpC* gene coding for the c subunit of F_0F_1ATPase in clinical isolates of optochin-resistant *Streptococcus pneumoniae* from Brazil. *J. Clin. Microbiol.* **45**, 3065–3067 (2007).
7. Nunes, S., Sá-Leão, R. & De Lencastre, H. Optochin resistance among *Streptococcus pneumoniae* colonizing healthy children in Portugal. *J. Clin. Microbiol.* **46**, 321–324 (2008).
8. Nagata, M. *et al.* Emergence of optochin resistance among *Streptococcus pneumoniae* in Japan. *Open J. Med. Microbio.l* **2**, 8–15 (2012).
9. Pinto, T. C. *et al.* Phenotypic and molecular characterization of optochin- resistant *Streptococcus pneumoniae* isolates from Brazil, with description of five novel mutations in the *atp*C gene. *J. Clin. Microbiol.* **51**, 3242–3259 (2013).
10. Raddaoui, A., Tanfous, F. B., Achour, W., Baaboura, R. & Hassen, A. B. Description of a novel mutation in the *atp*C gene in optochin-resistant *Streptococcus pneumoniae* strains isolates from Tunisia. *Int. J. Antimicrob. Agents* **51**, 803–805 (2018).
11. Fenoll, A., Muñoz, R., Garcia, E. & de la Campa, A. G. Molecular basis of the optochin-sensitive phenotype of pneumococcus: Characterization of the genes encoding the F0 complex of the *Streptococcus pneumoniae* and *Streptococcus oralis* H(+)- ATPases. *Mol. Microbiol.* **12**, 587–598 (1994).
12. Martín-Galiano, A. J., Gorgojo, B., Kunin, C. M. & De La Campa, A. G. Mefloquine and new related compounds target the F0 complex of the F0F1 ATPase of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **46**, 1680–1687 (2002).
13. Cortes, P. R., Orio, A. G., Regueira, M., Piñas, G. E. & Echenique, J. Characterization of in vitro-generated and clinical optochin-resistant strains of *Streptococcus pneumoniae* isolated from Argentina. *J. Clin. Microbiol.* **46**, 1930–1934 (2008).
14. Ikryannikova, L. N. *et al.* The mystery of the fourth clone: comparative genomic analysis of four non-typeable *Streptococcus pneumoniae* strains with different susceptibilities to optochin. *Eur. J. Clin. Microbiol. Infect. Dis.* **35**, 119–130 (2016).
15. Isaksson, J. *et al.* Comparison of species identification of endocarditis associated viridans streptococci using *rnp*B genotyping and 2 MALDI-TOF systems. *Diagn. Microbiol. Infect. Dis.* **81**, 240–245 (2015).
16. Marín, M. *et al.* Accurate differentiation of *Streptococcus pneumoniae* from other species within the *Streptococcus mitis* group by peak analysis using MALDI-TOF MS. *Front. Microbiol.* **8**, 698 (2017).
17. Ikryannikova, L. N. *et al.* Discrimination between *Streptococcus pneumoniae* and *Streptococcus mitis* based on sorting of their MALDI mass spectra. *Clin. Microbiol. Infect.* **19**, 1066–1071 (2013).
18. Yahiaoui, R. Y., Goessens, W. H., Stobberingh, E. E. & Verbon, A. Differentiation between *Streptococcus pneumoniae* and other viridans group streptococci by matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Clin. Microbiol. Infect.* **26**, 1088 (2020).
19. Burckhardt, I., Panitz, J., Burckhardt, F. & Zimmermann, S. Identification of *Streptococcus pneumoniae*: development of a standardized protocol for optochin susceptibility testing using total lab automation. *Biomed. Res. Int.* **2017**, 4174168 (2017).
20. Cortes, P. R., Piñas, G. E., Cian, M. B., Yandar, N. & Echenique, J. Stress-triggered signaling affecting survival or suicide of *Streptococcus pneumonia*. *Int. J. Med. Microbiol.* **305**, 157–169 (2015).
21. Moschioni, M. *et al. Streptococcus pneumoniae* contains 3 *rlr*A pilus variants that are clonally related. *J. Infect. Dis.* **197**, 888–896 (2008).
22. Barroso, D.E *et al.* B-Lactam resistance, serotype distribution, and genotypes of meningitis causing *Streptococcus pneumoniae*, Rio de Janeiro, Brazil. *Pediatr. Infect. Dis. J.* **31**, 30–36 (2012).
23. Pinto, T. C. *et al. 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. *Antimicrob. Agents Chemother.* **60**, 6664–6672 (2016).
24. Neves, F. P. G. *et al.* Population structure of *Streptococcus pneumoniae* colonizing children before and after universal use of pneumococcal conjugate vaccines in Brazil: emergence and expansion of the MDR serotype 6C-CC386 lineage. *J. Antimicrob. Chemother.* **73**, 1206–1212 (2018).
25. Arbique, J. C. *et al.* Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. *J. Clin. Microbiol.* **42**, 4686–4696 (2004).
26. Hall, L. M. C. Application of molecular typing to the epidemiology of *Streptococcus pneumoniae*. *J. Clin. Pathol.* **51**, 270–274 (1998).
27. Buchan, D. W., Minneci, F., Nugent, T. C. O., Bryson, K. & Jones, D. T. Scalable web services for the PSIPRED protein analysis workbench. *Nucleic Acids Res.* **41**, 349–357 (2013).
28. Jones, D.T., Buchan, D., Cozzetto, D. & Bryson, K. *The PSIPRED protein sequence analysis workbench.* http://bioinf.cs.ucl.ac.uk/psipred (2016).
29. Dias, C. A., Teixeira, L. M., Carvalho, M. G. & Beall, B. Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children. *J. Med. Microbiol.* **56**, 1185–1188 (2007).
30. Sorensen, U. B. Typing of pneumococci by using 12 pooled antisera. *J. Clin. Microbiol.* **31**, 2097–2100 (1993).
31. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing.* 29thl supplement M100-S29 (2019).
32. Toikka, P., Nikkari, S., Ruuskanen, O., Leinonen, M. & Mertsola, J. Pneumolysin PCR-based diagnosis of invasive pneumococcal infection in children. *J. Clin. Microbiol.* **37**, 633–637 (1999).
33. Andrade, A. L. *et al.* Non-typeable *Streptococcus pneumoniae* carriage isolates genetically similar to invasive and carriage isolates expressing capsular type 14 in Brazilian infants. *J. Infect.* **61**, 314–322 (2010).
34. Morrison, K. E. *et al.* Confirmation of *psa*A in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification and diagnosis. *J. Clin. Microbiol.* **38**, 434–437 (2000).
35. Costa, N. S. *et al.* MLVA Typing of *Streptococcus pneumoniae* isolates with emphasis on serotypes 14, 9N and 9V: comparison of previously described panels and proposal of a novel 7 VNTR loci-based simplified scheme. *PLoS ONE* **11**, e0158651 (2016).
36. Enright, M. C. & Spratt, B. G. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: Identification of clones associated with serious invasive disease. *Microbiology* **144**, 3049–3060 (1998).
37. Jolley, K. A., Bray, J. E. & Maiden, M. C. J. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open. Res.* **3**, 124. https://doi.org/10.12688/wellcomeopenres.14826.1 (2018).
38. Jolley, K.A., Bray J.E. & Maiden M.C.J. *Public databases for molecular typing and microbial genome diversity* https://pubmlst.org/organisms/streptococcus-pneumoniae (2018).
39. Centers for Disease Control and Prevention. *Streptococcus Laboratory.* https://www.cdc.gov/streplab/pneumococcus/resources.html (2020).

40. Hanage, W. P. *et al.* Invasiveness of serotypes and clones of *Streptococcus pneumoniae* among children in Finland. *Infect. Immun.* **73**, 431–435 (2005).
41. Hanage, W. P. *et al.* Using multilocus sequence data to define the pneumococcus. *J. Bacteriol.* **187**, 6223–6230 (2005).
42. Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).

Acknowledgements

Author contributions

All authors have contributed substantially to the work reported. Conceptualization, L.M.T., F.P.G.N., V.L.C.M., and T.C.A.P.; methodology, A.R.V.S., N.S.C., S.E.C.M.P. and T.C.A.P.; formal analysis, A.R.V.S., L.M.T. and T.C.A.P.; investigation, L.M.T., F.P.G.N. and T.C.A.P.; resources, V.L.C.M., J.M.P. and L.M.T.; data curation, A.R.V.S, T.C.A.P. and L.M.T.; writing-original draft preparation, A.R.V.S, F.P.G.N., T.C.A.P; writing-review and editing, L.M.T, F.P.G.N., V.L.C.M., J.M.P, T.C.A.P and L.M.T.; supervision, V.L.C.M., J.M.P. and L.M.T.; funding acquisition, V.L.C.M., J.M.P. and L.M.T. All authors have read and agreed to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to L.M.T.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.