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Characterization of In Vitro-Generated and Clinical Optochin-Resistant Strains of *Streptococcus pneumoniae* Isolated from Argentina▽

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Optochin susceptibility is a key test used for pneumococcal diagnosis, but optochin-resistant (Opt^r) pneumococci have been reported in the last 2 decades. In this work, we characterized eight Opt^r clinical strains which presented a new mutation, G47V, a predominant A49S mutation (recently reported in Brazil) and A49T. These mutations were found in the *c* subunit of the F_0F_1 ATPase encoded by the *atpC* gene, and W206C was found in the *a* subunit encoded by the *atpA* gene. The Opt^r clinical isolates were analyzed by BOX PCR, multilocus sequence typing, and serotype and antimicrobial resistance profiles, and they showed no epidemiological relationship. To characterize the Opt^r mutations that could emerge among clinical strains, we studied a pool of spontaneous Opt^r colonies obtained in vitro from the virulent D39 strain. We compared the *atpAC* mutations of these Opt^r pneumococci (with or without passage through C57BL/6 mice) with those described in the clinical isolates. This analysis revealed three new mutations, G47V and L26M in the *c* subunit and L184S in the *a* subunit. Most of the mutations identified in the laboratory-generated Opt^r strains were also found in clinical strains, with the exception of the L26M and L184S mutations, and we suppose that both mutations could emerge among invasive strains in the future. Considering that *atpAC* are essential genes, we propose that all spontaneous mutations that confer in vitro optochin resistance would not present severe physiological alterations in *S. pneumoniae* and may be carried by circulating pneumococcal strains.

Streptococcus pneumoniae is one of the most important pathogens in children and in elderly populations, being the most common cause of invasive bacterial infections such as pneumonia, bacteremia, and meningitis. The laboratory characterization of *S. pneumoniae* is based on phenotypic tests such as optochin susceptibility, bile solubility, the Quellung reaction, and genotypic tests performed in specialized centers. The optochin susceptibility test is critical in the identification of *S. pneumoniae*, and it has been used for decades in bacteriological laboratories. However, optochin-resistant (Opt^r) strains have been reported since 1987 in the United States, Spain, and Israel and more recently in Portugal and Brazil (1, 2, 7, 9, 16, 17, 23–25, 28), thus complicating the pneumococcal diagnosis. When additional tests are not applied, these Opt^r strains are probably misidentified and overlooked, resulting in inappropriate antimicrobial therapies for patients. Despite several clinical reports describing Opt^r isolates, there are only a few mutants characterized at the molecular level. It was reported that point mutations in the *atpAC* genes, which encode subunits of F_0F_1 ATPase, conferred optochin resistance on *S. pneumoniae* (5, 10, 29).

In this work, our objectives were to characterize the Opt^r strains isolated in our country, to compare the *atpAC* mutations with those identified in Opt^r strains isolated in other geographical regions, and to investigate a putative correlation between the spontaneous Opt^r mutants recovered from optochin agar plates with those isolated from invasive infections.

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MATERIALS AND METHODS

Bacterial strains. From a total of 1,375 pneumococcal strains identified by the National Institute of Infectious Diseases (INEI, Buenos Aires, Argentina) from November 1995 to July 2004, eight uniformly Opt^r isolates were identified as presumed pneumococci. They were recovered from ordinarily sterile body sites of children from different geographic areas of Argentina. The following reference strains were used: *S. pneumoniae* D39 NCTC 7466 (capsulated virulent strain, serotype 2), *S. pneumoniae* R6 ATCC BAA-255 (uncapsulated derivate of D39), *S. pneumoniae* ATCC 49619 (capsulated strain, serotype 19F), *Streptococcus sanguinis* ATCC 10556, *Streptococcus salivarius* ATCC 7073, and *Streptococcus mitis* ATCC 49456.

Pneumococcal identification, serotyping, and antibiotic susceptibility. The identification and antibiotic susceptibility testing of pneumococcal isolates were performed by standard microbiological methods, i.e., colony morphology, optochin susceptibility, and bile solubility, as previously described (29). Antimicrobial susceptibility testing was done by agar dilution and disk diffusion in accordance with the CLSI (formerly the National Committee for Clinical and Laboratory Standards) protocols (4). Serotyping was performed by using the Quellung reaction with antisera produced by the Statens Serum Institut (Copenhagen, Denmark).

BOX PCR assay. DNA was purified with a kit (Wizard genomic DNA purification kit; Promega Corporation, Madison, WI) in accordance with the instructions of the manufacturer. The BOX PCR was carried out with primer BOXA1R as previously described (14).

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TABLE 1. Phenotypic and molecular characterization of the Opt^r clinical strains used in this study

Strain or species	Sample type[f]	Yr of isolation	Place of isolation[h]	Serotype	ST[c]	Antibiotic resistance[d]	Optochin MIC[e]	Amino acid change	Bile solubility	*lytA* PCR	*ply* PCR	*psaA* PCR	*cpsAB* PCR	*sodA* sequence homology
R6[a]	NA[g]	NA	NA	NA	NA	None	2	NA	+	+	+	+	−	*S. pneumoniae*
M379[b]	AF	1994	Cba	1	615	None	64	A49T	+	+	+	+	+	*S. pneumoniae*
M1056[b]	Blood	1995	Cba	23F	81	CHL, SXT, PEN^I	32	G47V	+	+	+	+	+	*S. pneumoniae*
M1059[b]	CSF	1995	Cba	5	289	SXT	16	A49S	+	+	+	+	+	*S. pneumoniae*
M1228[b]	Blood	1997	Cba	5	289	SXT	64	W206C	+	+	+	+	+	*S. pneumoniae*
M2002[b]	PF	1998	Cba	14	557	SXT, PEN^r	64	A49S	+	+	+	+	+	*S. pneumoniae*
M2026[b]	CSF	1998	BsAs	18C	3274	ERY, SXT	16	A49S	+	+	+	+	+	*S. pneumoniae*
M4035[b]	CSF	2000	SdeE	27	557	None	64	A49S	+	+	+	+	+	*S. pneumoniae*
M4078[b]	PF	2000	LaR	19F	81	SXT, CLI, ERY, TET, PEN^I	16	A49S	+	+	+	+	+	*S. pneumoniae*
S. mitis[a]	NA	NA	NA	NA	NA	ND[i]	NA	NA	−	−	−	−	−	ND
S. sanguinis[a]	NA	NA	NA	NA	NA	ND	NA	NA	−	−	−	−	−	ND
S. salivarius[a]	NA	NA	NA	NA	NA	ND	NA	NA	−	−	−	−	−	ND

[a] ATCC control strain.
[b] Opt^r clinical isolate identified as *S. pneumoniae*.
[c] Sequence types from the MLST analysis.
[d] CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; PEN^I, intermediate penicillin resistance; PEN^r, penicillin resistance, SXT, sulfamethoxazole-trimethoprim; TET, tetracycline.
[e] Values are in milligrams per milliliter.
[f] AF, abdominal fluid; CSF, cerebrospinal fluid; PF, pleural fluid.
[g] NA, not applicable.
[h] Cba, Córdoba; BsAs, Buenos Aires; SdeE, Santiago del Estero; LaR, La Rioja.
[i] ND, not determined.

PCR assays. PCR amplification of the *ply* (pneumolysin), *psaA* (pneumococcal surface antigen A), *lytA* (autolysin), *sodA* (superoxide dismutase), *atpABC* (subunits of F_0F_1 ATPase), and *cpsAB* (capsular) genes was carried out with primer pairs Fply/Rply (31), FpsaA/RpsaA (22), FlytA (5′-GGCACGGATCCGATGGAAATTAATGTGAGTAAATTAAG-3′)/RlytA (5′-CCGGGATCCAGTTTTACTGTAATCAAGCCATCTGG-3′), FsodA/RsodA (30), Fatpa (5′-AATACATGGAACGAGAAGAAAAGG-3′)/Ratpa (5′-TGCATCAGTTACTCCTTTCTATTCC-3′), Fatpb (5′-TCTTTATTTCCTGCATCCAAGC-3′)/Ratpb (5′-GCGACTTGCTTGATTTGAGTC-3′), Fatpc (5′-CGAAAAGTGGATCAACAACTATCC-3′)/Ratpc (5′-TGGGTTTCAAGGTCATATTGC-3′), and cpsS1B/cpsA3 (15). All of the PCR experiments were performed with an automated thermal cycler (Bio-Rad Gene Cycler). The cycling conditions were as follows: 95°C for 3 min; 35 cycles of denaturation (95°C) for 30 s, annealing (56°C) for 90 s, and extension (72°C) for 90 s; and postcycling incubation for 10 min at 72°C. The PCR products corresponding to the *sodA* and *atpAC* genes were sequenced in duplicate in both senses with the same primers used for amplification. DNA sequencing was performed at Macrogen Inc. (Seoul, Korea).

Multilocus sequence typing (MLST). MLST was performed as described at http://www.mlst.net. The sequences of the internal fragments from the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* genes were amplified by PCR with primers described previously (8). DNA sequencing was performed by Macrogen Inc. (Seoul, Korea), and DNA sequences were edited with Bioedit software (11). Alleles and sequence types (STs) were assigned by using the database available at the above-mentioned MLST website.

Transformation assays. *S. pneumoniae* strain R6 was genetically transformed by a procedure described previously (6). Transformants were selected on Mueller-Hinton agar plates containing 6 mg/liter optochin (Sigma, St. Louis, MO) and supplemented with 5% defibrinated sheep blood.

Mutation frequency assays. Determination of mutation frequencies was performed as previously described (12), with modifications, after freezing the stocks at −80°C. Ten milliliters of brain heart infusion (BHI) was inoculated with 0.1 ml from the stock starter cultures, and the cultures were incubated at 37°C for about 4 h until they reached an optical density at 620 nm of 0.4. The frequency of mutation to optochin resistance was determined by spreading 0.4 ml of each culture on BHI agar plates containing 6 mg/liter optochin.

Selection of in vitro-generated Opt^r strains. Ten milliliters of BHI was inoculated with 0.1 ml of the stock starter cultures of strain D39. These cultures were incubated at 37°C until they reached an optical density at 620 nm of 0.4, and 0.4 ml was plated onto BHI agar containing 6 mg/liter optochin. The Opt^r colonies were recovered from these plates, which were incubated at 37°C for 16 h.

Passage of Opt^r pneumococci by mice. The experimental protocols and all of the tests performed were reviewed and approved by the Animal Care and Use Committee at our institution. Three female C57BL/6 mice, 4 to 5 weeks old, were inoculated intraperitoneally under isoflurane (Sigma Co., St Louis, MO) anesthesia with 1×10^5 CFU in 0.1 ml (50 mM glucose in phosphate-buffered saline) obtained from a pool of in vitro-generated Opt^r mutants. The mice were sacrificed by CO_2 asphyxiation after 2 days, and Opt^r strains were recovered from a pool of the three homogenized livers by plating onto BHI agar containing 6 mg/liter optochin.

Nucleotide sequence accession numbers. The nucleotide sequence data obtained in this study were deposited in the GenBank database under accession numbers EU179872 to -78 and EU256631 (Opt^r clinical strains) and EU256624 to -30 and EU256632 to -36 (in vitro-generated Opt^r strains). The new *gdh* allele and its corresponding ST were deposited in the *S. pneumoniae* MLST database (http://spneumoniae.mlst.net).

RESULTS

Characterization of the Opt^r clinical strains isolated from Argentina. Eight Opt^r clinical strains were isolated from patients with invasive diseases and characterized in our laboratory. All of the atypical Opt^r streptococci presented colony morphology, alpha-hemolysis, and bile solubility characteristics that were typical of pneumococci. To confirm pneumococcal identification, a battery of genetic tests was applied, such as PCR amplification of the *cpsAB* (15), *ply* (31), *lytA*, and *psaA* genes (19) and *sodA* partial sequence analysis (13, 30). All Opt^r strains showed positive PCR results for the genes analyzed (Table 1), and the *sodA* sequences confirmed that the isolates were pneumococci. To assess a possible clonal relationship of Opt^r strains isolated from the same geographical area over a 9-year period, we analyzed all of the Opt^r strains by MLST and BOX PCR. The MLST analyses revealed only five STs, and one of them turned out to be new (Table 1). However, the BOX PCR analysis showed eight patterns (Fig. 1), with more than two different DNA bands among them, correlating with serotypes and antimicrobial resistance profiles among these strains (Table 1), indicating no epidemiological relationship. To identify *atp* mutations that conferred optochin resistance,

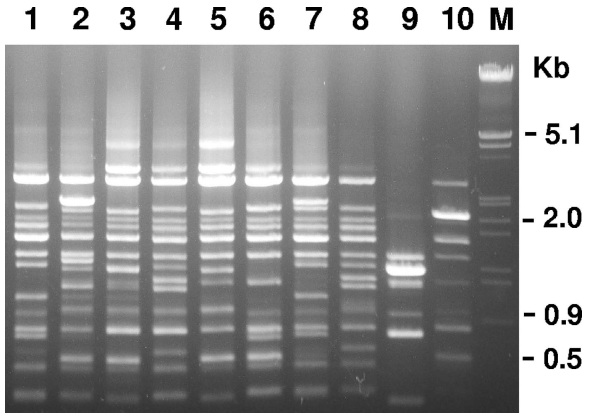

FIG. 1. BOX PCR DNA profiles of eight Opt^r clinical streptococci. Lane M, DNA molecular size marker (λ HindIII/EcoRI). Isolates: lane 1, M4078; lane 2, M2026; lane 3, M4035; lane 4, M1228; lane 5, M2002; lane 6, M379; lane 7, M1056; lane 8, M1059; lane 9, *S. mitis*; lane 10, *S. pneumoniae* ATCC 49619 (control strain).

we compared the DNA sequences of the *atpABC* genes amplified from all of the Opt^r strains. We found mainly substitutions in the *atpC* genes, with one strain displaying modifications in *atpA* that correspond to the W206C substitution in the *a* subunit of the F_0F_1 ATPase, which was described in clinical strains (29). Regarding the *c* subunit, six mutations were localized at position 49. In five of them, alanine was changed to serine (A49S), and only one clinical strain showed replacement of alanine to threonine (A49T). Another mutation was found at position 47, glycine for valine (G47V), was reported only in a laboratory-generated strain (27), and this is the first report of its isolation from clinical strains. The capacity of these *atpAC* mutations to confer optochin resistance was demonstrated by transformation of the wild-type R6 strain with the respective PCR products. Three Opt^r colonies were selected from each transformation, and their *atpAC* sequences were analyzed. All of the *atpAC* mutations were coincident with those found in the original strains. Also, when the optochin MICs for Opt^r transformants were tested, they were similar to those for the original strains, as expected (data not shown).

Characterization of in vitro-generated Opt^r strains. Another purpose of this work was to investigate a putative correlation between the spontaneous Opt^r mutants recovered from optochin agar plates with those isolated from patients with invasive infections. To characterize spontaneous *atpABC* mutations, 20 Opt^r colonies were randomly picked from a pool of Opt^r mutants generated in vitro from virulent strain D39. To identify the different mutations that confer optochin resistance, the *atpABC* genes from these mutants were individually amplified and only those PCR products capable of conferring optochin resistance on the R6 strain were sequenced. We found five mutations in the *c* subunit previously reported in clinical isolates, G14S, F45V, A49S, A49T, and F50L (5, 7, 10) (Table 2). As a control, we also analyzed the *atpAC* genes from 12 optochin-sensitive strains but we could not find any mutation, and the PCR products were transformed into strain R6 and the transformation frequency in optochin plates was similar to that of the wild-type strain.

We also considered the ability of the in vitro-generated Opt^r strains to survive in mice. Consequently, three C57B/6 mice were inoculated intraperitoneally with a pool of Opt^r colonies (approximately 400 randomly selected) generated in vitro from virulent strain D39 as mentioned before. The mice were sacrificed after 2 days, and Opt^r strains were recovered from the liver. Then, 20 Opt^r strains were picked at random and *atpABC* mutations were analyzed as described before. We identified different substitutions in the *c* subunit, such as G20S, M23I, A49T, and W206C in the *a* subunit, which were previously characterized in clinical isolates (6, 10, 29). We also found G47V, at the same position as the laboratory-generated G47A mutation (27) which was detected in our clinical strains and recovered after the mouse passage. Furthermore, we identified two new mutations, L26M in the *c* subunit and L184S in the *a* subunit (Table 2). The ability of all *atpAC* mutations to confer

TABLE 2. Comparison of amino acid modifications in the *a* and *c* subunits of the F_0F_1 ATPase

Subunit	Laboratory-generated Opt^r isolates of strain D39		Opt^r clinical isolates		Reference(s)
	Recovered in vitro[a]	Recovered in vivo[a]	Our strain[b]	Other reported strains	
c					
	G14S (4)			G14S	This work, 4
		G20S (5)		G20S	This work, 20, 25
		M23I (4)		M23I	6, 20, 25
		L26M (1)			This work
	F45V (3)			F45V	This work, 6
		G47V (4)	G47V (1)		This work
				V48F	8
	A49S (6)		A49S (5)	A49S	This work, 6
	A49T (3)	A49T (2)	A49T (1)	A49T	This work, 8, 25
	F50L (4)			F50L	This work, 8
a					
		L184S (3)			This work
		W206C (1)	W206C (1)	W206C	This work, 20

[a] A total of 20 Opt^r strains with single mutations were analyzed, and the frequency of each mutation is indicated in parentheses.
[b] A total of eight Opt^r clinical strains with single mutations were analyzed, and the frequency of each mutation is indicated in parentheses.

optochin resistance was confirmed by the amplification of individual genes, transformation of the R6 strain, and selection on optochin plates as described in Materials and Methods. In these cases, we obtained transformation frequencies of 1.6×10^{-4} to 3.2×10^{-4}, in contrast to the frequency of mutation of strain R6 to optochin resistance (6.4×10^{-8}). Although we recovered similar numbers of different *atpAC* mutations from the laboratory-generated Opt^r strains recovered before (5/11) and after (7/11) mouse passage, this animal model allowed us to obtain a different mutation profile that was not detect in our first screening. These Opt^r mutants were probably favored in this particular model. However, 9 of 11 mutations identified from laboratory-generated Opt^r strains (recovered before and after mouse passage) were also detected in invasive pneumococcal infections (Table 2), showing a clear correlation between the two populations.

DISCUSSION

Optochin is a quinine derivative that was used in 1912 for the treatment of pneumococcal diseases, but pneumococci isolated from treated patients showed optochin resistance (20). This finding is considered the first report of antimicrobial resistance developed in vivo. The optochin assay to identify the pneumococcus was described in 1915 (21), but it was only in the 1950s that Bowers and Jeffries proposed this test for routine pneumococcal diagnosis (3). Over the last 20 years, several publications have described Opt^r strains (7, 16, 17, 24, 28, 32), but the genetic basis of optochin resistance was elucidated by de la Campa's laboratory (10).

To date, only 13 Opt^r clinical strains have been characterized at the molecular level and nine *atpAC* mutations have been reported. In this work, we have characterized eight invasive Opt^r pneumococci, 20 Opt^r strains generated in vitro, and 20 Opt^r strains from the same pool after passage through mice. We have identified three new modifications from a total of 11 different *atpAC* mutations analyzed (Table 2). Concerning our Opt^r clinical mutants, mainly modifications were shown at position 49 in the *c* subunit of the F_0F_1 ATPase, with not only a new replacement of alanine with serine but also a substitution of threonine, which were reported previously (7, 10, 29). In both cases, the nonpolar hydrophobic alanine was replaced by polar uncharged amino acids (serine or threonine) in the α-helix 2 domain. This polarity change might interfere with the binding of optochin to the *c* subunit. In the same domain in clinical strains, we identified G47V, which had previously only been described as a laboratory-generated mutation (27).

The analysis of in vitro-generated Opt^r mutants also revealed that F45V was recovered in vitro, was absent in our clinical isolates, but was recently reported as a new mutation in Brazil (7). On the other hand, the A49T and A49S mutations have been previously described (7, 10) and also detected in our clinical and in vitro-generated Opt^r strains. The A49S mutation represents a privileged mutant (62%) among our invasive Opt^r pneumococci, and coincidently, this modification was recently detected in Brazil in two of four Opt^r strains analyzed (7). The G14S and F50L mutations were recovered in vitro; we could not detect them among our clinical isolates, but they had been described in other clinical strains (5, 10).

The passage of Opt^r pneumococci through mice showed an enrichment of some Opt^r mutants that were surely underrepresented in the sampling of the assay in vitro but were more competent at surviving in mice. In this group, we found two new Opt^r mutations, L26M and G47V. The latter was also detected in our clinical strains, being similar to the G47A mutation generated by exposure to mefloquine (18). In addition, we detected G20S and M23I, which have been described by other authors in clinical isolates (6, 29). Among these Opt^r mutants recovered after mouse passage, we found W206C (29) and L184S, both mutations localized in a leucine-rich region in the α-helix 5 domain of the *a* subunit of the F_0F_1 ATPase. The unique modification previously localized near L184S was L186P, which was also generated in vitro and recovered by mefloquine resistance and also presented cross-resistance to optochin (18).

Because the *atpAC* genes encoding the *a* and *c* subunits of the F_0F_1 ATPase have been showed to be essential in *S. pneumoniae*, the *atpAC* mutations should not produce severe physiological alterations in pneumococci. The comparison of in vitro-generated and Opt^r clinical mutations revealed that most of spontaneous mutants recovered from optochin agar plates were isolated from invasive infections, indicating that these mutations did not alter virulence.

However, we identified two new mutations, L26M and L184S, which were recovered after mouse passage but have still not been identified in clinical strains. We suppose that both of these mutations could emerge among invasive strains in the future.

Considering serotype, antimicrobial resistance, MLST, and BOX PCR profiles, the clinical strains characterized in this work showed no epidemiological relationship, in agreement with previous reports (7, 25). However, we found a clonal origin of two strains isolated 2 years apart. The origin of Opt^r strains is unknown. It has been proposed that antimalarial treatment with quinine and mefloquine could favor their selection and dissemination (29). In this case, the optochin resistance level of *S. pneumoniae* isolates in regions where malaria is endemic should be elevated; however, this situation has not been reported to date. On the basis of our results, we propose that the presence of Opt^r strains is due to spontaneous mutants that maintain their virulence, which are not selected by any antimicrobial agent, and they should have a rate of turnover into the circulating pneumococcal population similar to that of the optochin-sensitive strains.

Currently, the optochin test is critical in the identification of alpha-hemolytic colonies. The nonutilization of an additional test for Opt^r strains has led to the misidentification of *S. pneumoniae* as *S. viridans* and, consequently, to inadequate therapies with unpredictable results for the infected patients. Bile solubility is an additional test, but only a few laboratories in Argentina use it for pneumococcal diagnosis. Bile-insoluble strains have been reported (26), but an association with optochin resistance has not been found. In agreement with Pikis et al. (29), we suggest routinely performing the bile solubility test, particularly in cases in which the clinical data are inconsistent with the clinical prognosis. Alternatively, several molecular tests have been proposed to characterize Opt^r strains. All of the Opt^r clinical strains analyzed were positive for bile solubility and for amplification of the *lytA*, *ply*, *psaA*, *cpsAB*,

and *sodA* genes, and the *sodA* sequences were compatible with *S. pneumoniae* (26).

We believe that Opt^r strains have been overlooked in recent years, and our purpose in this work was to alert diagnostic laboratories that Opt^r strains may be appearing at our work benches more frequently than expected. Recently, Nunes et al. (25) reported a prevalence of approximately 2.1% from a total of 1,973 pneumococcal strains isolated during a period of 6 years in Portugal. For that reason, optochin resistance must be carefully considered and screened for in pneumococcal diagnosis.

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