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Optochin Resistance among *Streptococcus pneumoniae* Strains Colonizing Healthy Children in Portugal▽

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Two percent of 1,973 pneumococcus strains isolated from carriers since 2001 in Portugal were found to be optochin resistant. These strains belonged to eight serotypes (and some were nontypeable), and they had diverse genetic backgrounds. Novel optochin-resistant lineages were detected over time, suggesting that there was a continuous, although sporadic, emergence of optochin resistance.

The accurate identification of pneumococcus isolates has traditionally relied on observations of typical colony morphology, α-hemolysis on sheep blood agar, and optochin (ethylhydrocupreine hydrochloride) susceptibility (16). Bile solubility tests, although very sensitive and simple to perform, are far from being widely used as routine tests in clinical microbiology laboratories (1, 5). Other identification methods based on the detection of specific DNA sequences have been recently proposed (2, 5, 19).

Optochin-resistant pneumococcal strains were first reported in Finland in 1987 (10), and since then, sporadic reports of isolates from diverse geographic areas have appeared in the literature (1, 3, 9, 12, 14, 15). In particular, Aguiar et al. have recently reported the emergence of optochin-resistant pneumococci in Portugal, which accounted for 3.2% of all clinical isolates recovered from 30 laboratories across the country (1). These observations prompted us to retrospectively review the detection of optochin resistance among isolates that were colonizing asymptomatic Portuguese carriers and that were recovered in studies conducted since 2001.

Between January and March of 2001, 2002, 2003, and 2006, a total of 717, 834, 766, and 571 nasopharyngeal samples, respectively, were obtained from children attending day-care centers in Lisbon and Oeiras, Portugal, by following previously described procedures (8, 13). The children's ages ranged from 4 months to 6 years. Pneumococcal isolation rates, antibiotypes, and molecular characterizations of antimicrobial-resistant pneumococci isolated in 2001, 2002, and 2003 have been described previously (11).

Pneumococci were isolated based on selective growth on gentamicin blood agar plates, optochin susceptibility, colony morphology, and α-hemolysis (16). Bile solubility tests were performed for isolates with reduced susceptibility to optochin that appeared to be pneumococci based on the other phenotypic observations (16). In particular, optochin susceptibility was performed by disk diffusion, using commercially available optochin discs (5 μg; 6 mm; Oxoid, Hampshire, England) applied onto blood agar plates (Trypticase soy agar supplemented with 5% sheep blood) that had been inoculated with a 0.5 McFarland standard suspension of the culture to be tested. Plates were incubated overnight at 37°C in a 5% CO_2-enriched atmosphere. Isolates were considered to be resistant to optochin if they displayed inhibition zones smaller than 14 mm or larger than 14 mm but containing colonies inside the halo (16).

Antimicrobial susceptibility testing was assayed by the Kirby-Bauer disk diffusion method for susceptibility to erythromycin, clindamycin, tetracycline, chloramphenicol, sulfamethoxazole-trimethoprim, and levofloxacin according to CLSI guidelines (6) and by Etest (AB Biodisk, Solna, Sweden) for penicillin and ceftriaxone. Results were interpreted following CLSI criteria (6).

Capsular typing was done by multiplex PCR (4). For those isolates whose serotype could not be determined by this technique, the Quellung reaction was performed using specific antisera (Statens Serum Institute, Copenhagen, Denmark) (18).

Pulsed-field gel electrophoresis (PFGE) of macrorestriction DNA fragments was done after SmaI digestion, and a dendrogram was generated using Bionumerics Software (Applied Maths, Gent, Belgium) (17).

A total of 1,973 pneumococcal isolates were obtained during the four surveillance periods. Of these, 42 (2.1%) were optochin resistant and bile soluble. The prevalence of optochin resistance ranged from 1.3 to 3.2% depending on the year of isolation (Table 1).

Two optochin resistance phenotypes were observed: 13 isolates had halos that were ≥14 mm, with subpopulations inside the inhibition zone, and 29 were uniformly optochin resistant. These phenotypes have also been described by Pikis et al. (15).

TABLE 1. Origin of optochin-resistant pneumococcus strains[a]

Year	Total no. of pneumococcus isolates	No. of Opt^r isolates (%)	No. of DCC with Opt^r isolates/total no. of DCC
2001	465	15 (3.2)	7/11
2002	559	10 (1.8)	6/14
2003	557	12 (2.2)	6/14
2006	392	5 (1.3)	4/12

[a] Opt^r, optochin-resistant; DCC, day-care centers.

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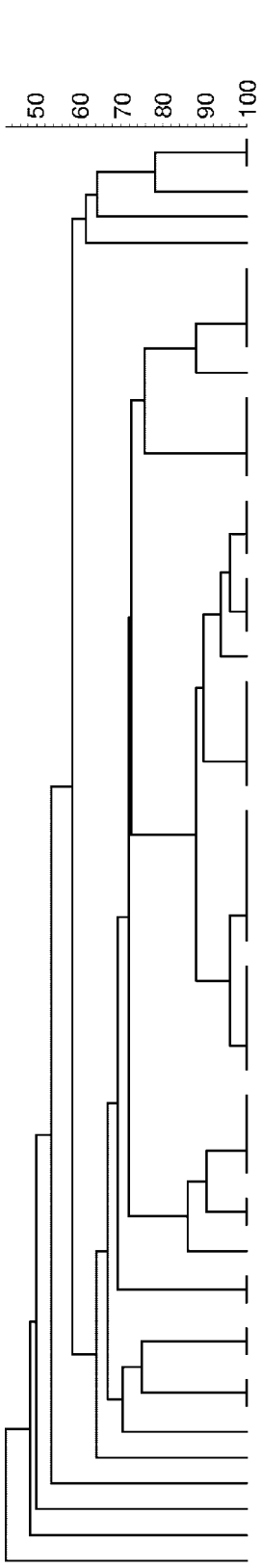

Strain	Isolate	Serotype	Antimicrobial resistance profile	Year	Day care center
PT5081	I	16F		2003	5
PT5081	O	16F		2003	5
PT187		6B	Ery,Da,Tet	2001	21
PT5108		23A		2003	16
PT5472b		NT	PG,Ery	2006	20
PT3082	I	19F		2002	21
PT3082	O	19F		2002	21
PT4602b	I	NT	PG,Ery,Da,Tet,SXT	2003	20
PT4602b	O	NT	PG,Ery,Da,Tet,SXT	2003	20
PT4624	O	10A		2003	20
PT323	I	14	PG,SXT	2001	16
PT323	O	14	PG,SXT	2001	16
PT329	I	14	PG,SXT	2001	16
PT329	O	14	PG,SXT	2001	16
PT4624	I	3	SXT	2003	20
PT2919		3	SXT	2002	17
PT5123		3	SXT	2003	16
PT2913	I	3	SXT	2002	17
PT2913	O	3	SXT	2002	17
PT4612		3	SXT	2003	20
PT5125		3	SXT	2003	16
PT29	I	3		2001	20
PT29	O	3		2001	20
PT2939		3		2002	20
PT2967		3		2002	20
PT4601b		3		2003	20
PT3095	O	3		2002	21
PT499		3		2001	5
PT802		3		2001	26
PT808		3		2001	26
PT819		3		2001	26
PT3412		3		2002	5
PT270		3		2001	19
PT272		3		2001	19
PT286		3		2001	19
PT4868		3		2003	25
PT4872		3		2003	25
PT3536	I	6B	Ery,Da,Tet,SXT,Chl	2002	31
PT3536	O	6B	Ery,Da,Tet,SXT,Chl	2002	31
PT3559	I	6B	Ery,Da,Tet,SXT,Chl	2002	31
PT3559	O	6B	Ery,Da,Tet,SXT,Chl	2002	31
PT61	I	19F	Ery,Da,Tet	2001	17
PT61	O	19F	Ery,Da,Tet	2001	17
PT453		6B	Ery,Da,Tet,SXT,Chl	2001	5
PT3095	I	3		2002	21
PT3293		3		2002	25
PT377	I	10A		2001	16
PT377	O	10A		2001	16
PT367	I	20		2001	16
PT367	O	20		2001	16
PT4812		NT	SXT	2003	31
PT5600		NT	PG,Ery,Da	2006	16
PT5234		NT	Ery,Tet,SXT	2006	16
PT4720		NT	Ery,Da,Tet,SXT	2003	18
PT5312		NT	Ery	2006	32
PT5468		NT	Ery,SXT	2006	20

FIG. 1. Dendrogram of optochin-resistant pneumococci. I, subpopulation isolated from the inhibition zone close to the optochin disk; O, subpopulation isolated from the farthest zone from the halo; NT, nontypeable; PG, penicillin; Ery, erythromycin; Da, clindamycin; Tet, tetracycline; SXT, trimethoprim-sulfamethoxazole; Chl, chloramphenicol.

Optochin resistance was confirmed for all isolates by picking one sample from the closest growth to the optochin disk and another from the farthest zone from the halo. For all 13 isolates with subpopulations, PFGE profiles were obtained for the two subpopulations. In all samples but two (PT3095 and PT4624), identical PFGE patterns were observed (Fig. 1). For the 29 isolates displaying uniform resistance to optochin, a single culture was grown for DNA extraction and PFGE profiling.

Fifty percent of optochin-resistant isolates were susceptible to all antimicrobial agents tested, and 21% were multidrug resistant (defined as resistance to three or more antimicrobial agents). Fifty percent were of serotype 3. Other serotypes detected were 6B (4 strains), 10A (2 strains), 14 (2 strains), 19F (2 strains), 16F (1 strain), 20 (1 strain), and 23A (1 strain). Eight strains were nontypeable.

Seventeen PFGE clusters were identified, indicating genetic variability among the collection as was suggested by the serotyping results (Fig. 1). In particular, novel optochin-resistant genetic backgrounds were detected for all years surveyed. Furthermore, among optochin-resistant strains of serotypes 3, 6A, 10A, and 19F and among nontypeable isolates, more than one genetic background was identified and, specifically, all eight nontypeable isolates had unique genetic backgrounds. Among the clusters detected, the largest one was represented by 19 of the 21 serotype 3 optochin-resistant isolates.

Of interest, all optochin-resistant strains, with the exception of the single isolates of serotypes 20 and 23A, had genetic backgrounds that were also detected among optochin-susceptible pneumococci circulating in Portuguese day-care centers (data not shown). In addition, all clusters with two or more isolates included strains recovered from at least two day-care centers in different years (Fig. 1).

Finally, optochin-resistant strains of serotypes 3, 6B, 14, and 19F were found to be members of internationally disseminated clones: Netherlands^{3} ST180 (19 isolates), Poland^{6B} ST315 (1 isolate), Greece^{6B} ST273 (3 isolates), Spain^{9V} ST156 (2 isolates of serotype 14), and Portugal^{19F} ST177 (1 isolate).

Our study shows that optochin-resistant strains were already present in Portugal in 2001 and continue to circulate and emerge among asymptomatic carriers. We are unable to conclude whether optochin-resistant strains were already in the community before 2001, since at that time, pneumococcus-like cultures exhibiting an optochin resistance phenotype were not further characterized or preserved in our laboratory. Full resistance to optochin was the most abundant phenotype (69%) in our study, in contrast to the study by Aguiar et al. (1), which reported that all optochin-resistant clinical isolates were a mixture of subpopulations. These observations suggest that different mechanisms leading to optochin resistance are disseminated in Portugal. At present, these mechanisms remain uncharacterized.

We found that optochin-resistant colonizing strains from Portugal are associated with several different serotypes and genetic backgrounds, including internationally disseminated clones. They were present in several day-care centers, suggesting they were not confined geographically. Similar conclusions were reached by Aguiar et al. (1), although the two collections, colonizing versus disease isolates, included mostly different serotypes and genetic backgrounds. In our study, 86% of the optochin-resistant strains had capsular types not targeted by the 7-valent pneumococcal conjugate vaccine.

The majority of optochin-resistant colonizing isolates had genetic backgrounds that were also detected among optochin-susceptible pneumococci, and novel optochin-resistant genetic backgrounds were detected in all years, suggesting that there was a continuous, although sporadic, emergence of optochin resistance. The driving forces for such selection are currently unknown, but compounds similar to optochin, such as quinine and mefloquine, are used for treatment of and prophylaxis against malaria (7). Contacts between Portugal and African countries with endemic malaria are frequent due to tourism and immigration. Whether optochin resistance mechanisms in pneumococcus isolates from healthy children in Portugal can be linked to this flow has not been investigated.

Still, clonal expansion of a serotype 3 clone accounted for 50% of all optochin-resistant isolates and, additionally, represented 16% of all serotype 3 isolates recovered during the 4 years of surveillance (regardless of their optochin susceptibility pattern).

In summary, optochin-resistant pneumococci were detected from asymptomatic carriers in Portugal since 2001 but might have been present before. Optochin resistance is associated with different clones, most of which express serotypes that are not included in the current 7-valent pneumococcal conjugate vaccine. Therefore, optochin susceptibility should be complemented with other pneumococcal identification tests, such as bile solubility tests or PCR-based techniques, when suspected pneumococcal cultures exhibiting resistance to optochin are isolated. Accurate identification of pneumococci is important not only for the diagnosis and treatment of infections but also for colonization studies, such as those aimed to evaluate the impact of pneumococcal conjugate vaccines.

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