Optochin resistance among *Streptococcus pneumoniae* colonizing healthy children in Portugal

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Running title: Carriage of optochin resistant pneumococci
ABSTRACT

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

TEXT

Accurate identification of pneumococci has traditionally relied on observation of typical colony morphology, α-haemolysis on sheep blood agar, and optochin (ethylhydrocupreine hydrochloride) susceptibility (16). Bile solubility, although very sensitive and simple to perform, is far from being widely used in the routine of clinical microbiology laboratories (1, 5). Other identification methods based on 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 thirty laboratories across the country (1). These observations prompted us to retrospectively review detection of optochin resistance among isolates colonizing Portuguese asymptomatic carriers recovered in recent 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 centres in Lisbon and Oeiras, Portugal following described procedures (8, 13). Children’ ages ranged from four months to six years old. Pneumococcal isolation rates, antibiotype and molecular characterization of antimicrobial-resistant pneumococci isolated in 2001, 2002, and 2003 have been described (11).

Pneumococci were isolated based on selective growth on gentamicin blood-agar plates, optochin susceptibility, colony morphology, and α-haemolysis (16). Bile solubility was 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 suspension of the culture to be tested. Plates were incubated overnight at 37°C in a 5% CO₂ enriched atmosphere. Isolates were considered to be resistant to optochin if displaying inhibition zones smaller than 14 mm or higher 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 the CLSI guidelines (6) and for penicillin and
ceftriaxone by Etest (AB Biodisk, Solna, Sweden). 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).

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. Its prevalence ranged from 1.3-3.2% depending on the year of isolation (Table 1).

Two optochin resistance phenotypes were observed: 13 isolates had halos ≥14mm with subpopulations inside the inhibition zone, and 29 were uniformly optochin resistant. These phenotypes have also been described by Pikis et al. (15). 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 (Figure 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), 14 (2), 19F (2), 16F (1), 20 (1), and 23A (1). Eight strains were non-typeable.

Seventeen PFGE clusters were identified indicating genetic variability among the collection as also suggested by the serotyping results (Figure 1). In particular, novel optochin-resistant genetic backgrounds were detected in all years surveyed. Furthermore, among optochin-resistant strains of serotypes 3, 6A, 10A, 19F and non-typable, more than one genetic background was identified and, specifically, all eight non-typable 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 serotype 20 and 23A, had genetic backgrounds 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 (Figure 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\(^6\)B ST315 (1 isolate), Greece\(^6\)B ST273 (3 isolates), Spain\(^9\)V ST156 (2 isolates of serotype 14), and Portugal\(^1\)9F 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%) contrasting with the study of 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 also detected among optochin susceptible pneumococci and novel optochin-resistant genetic backgrounds were detected in all years suggesting that, although sporadic, there was a continuous 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 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 pneumococci isolated 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 four 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 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 or PCR based techniques when
suspected pneumococcal cultures exhibiting resistance to optochin are isolated. Accurate
identification of pneumococci is not only important for diagnosis and treatment of infections but
also in colonization studies such as those aimed to evaluate the impact of the pneumococcal
conjugate vaccines.

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REFERENCES


FIGURE 1. Dendrogram of optochin-resistant pneumococci.

Legend of Figure 1: I, subpopulation isolated from the inhibition zone close to the optochin disk; O, subpopulation isolated from the farthest zone from the halo; NT, non-typable; PG, penicillin; Ery, erythromycin; Da, clindamycin; Tet, tetracycline; SXT, trimethoprim-sulfamethoxazole; Chl, chloramphenicol.

TABLE 1. Origin of optochin resistant strains.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total pneumococcal collection</th>
<th>OptR isolates no. (%)</th>
<th>No. of DCC with OptR (out of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>465</td>
<td>15 (3.2%)</td>
<td>7 out of 11</td>
</tr>
<tr>
<td>2002</td>
<td>559</td>
<td>10 (1.8%)</td>
<td>6 out of 14</td>
</tr>
<tr>
<td>2003</td>
<td>557</td>
<td>12 (2.2%)</td>
<td>6 out of 14</td>
</tr>
<tr>
<td>2006</td>
<td>392</td>
<td>5 (1.3%)</td>
<td>4 out of 12</td>
</tr>
</tbody>
</table>

OptR, optochin resistant pneumococci; DCC, day care center.