Increase of Denmark14-230 clone as a cause of pneumococcal infection
in Portugal within a background of diverse serotype 19A lineages

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Running title: Streptococcus pneumoniae capsular type 19A

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Abstract

Pneumococci of serotype 19A are increasingly found as causes of infection in various geographic regions. We have characterized the 19A isolates (n=288) found among pneumococci responsible for infections (n=1,925) and recovered from asymptomatic carriage (n=1,973) in Portugal between 2001 and 2006. We show that, in spite of the existence of 19A clones that have a higher invasive disease potential or an enhanced colonization capacity, the lineage increasing as a cause of infections in Portugal is a multiresistant clone, competent at both. The expanding Denmark^{14-230} clone found in Portugal is disseminated in other Mediterranean countries where it is also increasingly responsible for invasive infections in both children and adults. The lineages driving the rise of serotype 19A in infection in Asia and the US (ST320 and ST199) are either absent or account for a small proportion of isolates in Portugal. These data highlight the importance of locally circulating clones having the ability to compete in the nasopharyngeal niche for the emergence of the 19A lineages which are an increasing cause of infection in various geographic regions.
Introduction

In the United States, following the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7), a significant decline in the number of invasive pneumococcal infections caused by vaccine types was observed. This phenomenon occurred not only among children targeted for vaccination but also in other age groups, including the elderly who are known to have a high burden of pneumococcal disease. This indirect herd effect is in fact responsible for most of the observed reduction in disease (9). Vaccination has also been shown to have a broader impact on pneumococcal populations, demonstrated by a reduction of otitis media caused by vaccine serotypes (14), a reduction of the incidence of antimicrobial resistant pneumococcal invasive infections (20) and a declining proportion of vaccine serotypes asymptptomatically carried in the nasopharynx of children (12, 16).

Recent studies from European countries such as Spain and Portugal, where the vaccine is available but is not part of the National Vaccination Plan, have shown that even relatively modest vaccination coverage rates can have a profound effect on the serotypes responsible for invasive infections in children and adults (2, 4, 27).

In addition, all the above studies documented an increase in the number of cases caused by non-vaccine serotypes and, in particular, the emergence of serotype 19A as an increasingly important cause of invasive infections in all age groups (2, 27, 28).

Although vaccination with PCV7 has been frequently implicated as the cause for the increase in serotype 19A (7), recent reports documented the same trend in geographic areas where PCV7 had not been available (11, 19). Taken together, these observations suggest that vaccination may have simply reinforced or accelerated an ongoing temporal trend. In particular, in South Korea, the rise of serotype 19A in the absence of PCV7 was tentatively explained by the emergence of a multidrug resistant lineage of sequence type (ST) ST320. Antibiotic pressure was likely to be a major factor in selecting for this lineage (19).
A study on the recent increase of serotype 19A in the USA identified several lineages currently in circulation among which the preexisting clonal complexes CC199 and CC320 were found to be expanding (28). Capsular switch in STs usually associated with vaccine serotypes and the appearance of multiple drug resistant clonal complexes were also implicated in the rise of serotype 19A (26, 28). The detailed analysis of the capsular loci of some of these lineages showed that concurrent acquisition of the entire capsular locus and the flanking \textit{pbp} genes occurred (7), confirming the emergence of “vaccine escape recombinant” strains. On the other hand, a recent study conducted among the Alaskan native population found that the genetic diversity of circulating serotype 19A isolates was reduced. In this setting the increase in serotype 19A was due to expansion of a single CC, CC172 (35). In contrast, in Europe CC230 has been consistently identified as a major serotype 19A lineage causing invasive infections before vaccine introduction. This clonal complex has increased in the era of PCV7 both in the children (22), as well as in adults (4).

We have previously shown that in Portugal, where the vaccine is available since 2001, the conditional relative risk of invasive infection by serotype 19A increased significantly in the post-vaccine period in both children and adults (2). In the same study we reported that isolates expressing this serotype were frequently associated with antimicrobial resistance (2). In Portugal CC230 was one of the lineages expressing serotype 19A before vaccination (33). In this study, we compare the clonal composition of the population expressing serotype 19A responsible for invasive infections in both adults and children to that recovered from children from both asymptomatic carriers and non-invasive infections in Portugal.
Materials and Methods

Bacterial isolates. Three *S. pneumoniae* collections were examined for the presence of isolates expressing serotype 19A. Each collection represented a different source: isolates responsible for invasive infections, isolates causing non-invasive infections, and isolates recovered in asymptomatic carriage studies.

Infection isolates. Since 1999 the Portuguese Surveillance Group for the Study of Respiratory Pathogens has monitored invasive and non-invasive pneumococcal infections in Portugal. This is a laboratory based surveillance system, in which 30 microbiology laboratories throughout Portugal are asked to identify all cases of pneumococcal infection and to send the isolates to a central laboratory for characterization. A case of invasive disease is defined by an isolate of *S. pneumoniae* recovered from a normally sterile body site.

A collection of 1,480 *S. pneumoniae* isolates responsible for invasive pneumococcal infections during the period of 2001-2006 was characterized. Among this collection, 122 strains were isolated from children <2 years, 102 strains were isolated from children with ages ≥2 and <6 years, 52 strains were obtained from children and adolescents with ages between ≥6 and <18 years, and 1,204 strains were obtained from adults (≥18 years). The collection of non-invasive pneumococci (mainly recovered from lower respiratory products and associated with a diagnosis of pneumonia) was composed of 445 isolates all obtained from children <6 years.

The results of serotyping and antimicrobial resistance of the isolates responsible for invasive disease up to 2005 were reported previously (2, 34) and so was the genetic analysis of the isolates recovered up to 2002 (33).

Carriage isolates. In 2001, 2002, 2003, and 2006 surveys of pneumococcal carriage were conducted in healthy children (aged 6 months to 6 years) attending day care centers (DCC) in the Lisbon area. Between 2001 and 2003, in the months of January to March, 2,314 nasopharyngeal swabs were obtained from children attending 13 DCCs.
In 2006, in the same months, 571 nasopharyngeal swabs were recovered from children attending 11 DCCs. A total of 1,973 pneumococcal isolates were obtained. A description of resistant strains recovered between 2001 and 2003 was published previously (23, 37) and so were the results of serotyping and antimicrobial resistance of the isolates recovered in 2006 (31).

**Serotyping and antimicrobial susceptibility testing.** Serotyping was performed by the standard capsular reaction test using the chessboard system (36) and specific sera (Statens Serum Institut, Copenhagen, Denmark). E-test strips (AB Biodisk®, Solna, Sweden) were used to determine the MICs for penicillin as previously described (25), and the Clinical and Laboratory Standards Institute (CLSI) recommended breakpoints (10) were used to interpret MIC values. Isolates were further characterized by determining their susceptibility to co-trimoxazole, levofloxacin, erythromycin, clindamycin, tetracycline and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria (10).

**Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST).** PFGE profiling was performed for all serotype 19A isolates included in this study. Preparation of chromosomal DNA, digestion with SmaI, separation by PFGE, and analysis of patterns were performed as previously described (33). MLST analysis (13) was undertaken for a third of the isolates of each PFGE cluster with ≥ 4 isolates. The isolates within each PFGE cluster were selected to represent the three collections analyzed and, as much as possible, all the years studied. Lineage assignment was done using goeBURST (15) and the complete *S. pneumoniae* database available at spneumoniae.mlst.net.

**Statistical analysis.** Simpson’s index of diversity (SID) was used to measure the population diversity (8). Clustering comparison coefficients, Adjusted Rand (AR) and Wallace (W), were used to compare two sets of partitions (8, 29).
Statistical association between PFGE clones and population type or antimicrobial resistance were characterized by odds ratios (OR) with 95% Wald confidence intervals (CI) (3). For null OR, 95% CI were computed through the Fisher method implemented in the epitools for the R language. OR significance was tested with the chi-square statistic. The resulting p-values were corrected for multiple testing by controlling the False Discovery Rate (FDR) under or equal to 0.05 through the linear procedure of Benjamini and Hochberg (5).

The ORs for enhanced invasive potential of the main serotype 19A PFGE clusters identified were calculated using the number of invasive and carriage isolates by reference to all other serotype 19A isolates from these two sources. The ORs measuring the association of particular PFGE clusters with resistance were calculated by reference to the resistance found in all other PFGE clusters expressing serotype 19A identified in the study.

Temporal trends in proportions were evaluated with the Cochran-Armitage test (1). Chi-square statistic or the Fisher exact tests were also used to evaluate associations between population type and antimicrobial resistance. The p values were considered significant if lower than 0.05.
Results

Proportion of isolates expressing serotype 19A.

From the collections of pneumococcal isolates responsible for infections, 178 isolates presented serotype 19A. This set was composed of 121 isolates (of which 45 were from children less than 6 years) recovered from normally sterile sites and 57 isolates obtained from non-invasive infections (all from children less than 6 years). Among the collection recovered from asymptomatic carriers, 110 isolates presented serotype 19A. Among all pneumococcal isolates responsible for infection, the proportion of isolates expressing serotype 19A increased over time, being 4.1% (n= 8) in 2001 and 10.2% (n=48) in 2006 (p<10^{-3}) reflecting an increase of serotype 19A isolates in both invasive and non-invasive isolates. Although an increase in serotype 19A isolates was also noted among asymptomatic carriers, 4.3% (n=20) in 2001 to 6.5% (n= 27) in 2006, it did not reach significance (p= 0.124).

Antimicrobial susceptibility.

The proportion of resistant isolates is summarized in Table 1. Although there were differences in the proportion of resistant isolates in each of the three populations analyzed, none were statistically supported.

Notwithstanding, when considering the proportion of multidrug resistant isolates (resistance to three or more different classes of antimicrobials), it was higher among invasive isolates (49%) than among non-invasive isolates (36%) and carriage isolates (30%) (p=0.004).

Genotypic analysis and evaluation of invasive disease potential.

The three populations of serotype 19A were compared genotypically using a combination of PFGE and MLST. All isolates were analyzed by PFGE and representatives of the PFGE clusters were characterized by MLST (n= 95). Overall, serotype 19A strains were distributed through 23 different PFGE clusters (Figure 1) and 26 distinct sequence types (STs) were detected. PFGE cluster and ST demonstrated a
good correlation. In fact, the W between ST and PFGE cluster was 0.772 [95% CI, 0.678 to 0.865] indicating that there is a high probability that two strains that have the same ST will also belong to the same PFGE cluster. This value is even higher if one considers the clonal complexes defined by the eBURST rules (W=0.833, 95% CI 0.744 to 0.922, i.e. four out of five pairs of isolates belonging to the same clonal complex will also be classified in the same PFGE cluster). In spite of these high Wallace values a few STs were detected in several PFGE groups, such as ST193 and ST994.

Notwithstanding, the Wallace values indicated that most isolates belonging to the same genetic lineage, as defined by eBURST, would be classified in the same PFGE cluster allowing us to compare our data based on PFGE analysis with other reports where only MLST data was available.

The number of invasive isolates of serotype 19A recovered from children (<6 yrs, n=45) was smaller than the number of isolates recovered from other age groups (n=76). This reduced number of isolates could compromise the statistical efficiency of the tests comparing invasive isolates to other isolate sources. On the other hand, if it could be demonstrated that the distribution of clones was indistinguishable between children (<6 yrs) and older individuals, all invasive isolates could be regarded as representing a single population, greatly enhancing the power of the comparisons. In fact, we could not show a difference in the distribution of the PFGE clusters between the two age groups (Fisher’s exact test, p=0.775), indicating that all serotype 19A invasive isolates constitute an homogeneous population independent of the age group considered. We therefore used of the entire collection of invasive isolates in the comparisons with other isolate sources.

All three isolates sources presented a high genetic diversity as determined by PFGE cluster analysis, with high and undistinguishable SID values (Table 2). Analysis of the distribution of isolates between PFGE clusters showed that most included isolates of the three distinct sources. This is highlighted by an AR of 0.044 indicating a low overall
congruence between PFGE cluster and isolate source. In spite of the absence of a strong correlation between the source of the isolates and the PFGE clusters, some PFGE clusters were not uniformly distributed in the three populations (Table 2).

To identify serotype 19A PFGE clusters associated with carriage, the OR for the main clusters were determined based on the number of isolates of that particular cluster found in carriage and in invasive disease with reference to all other isolates from these two sources. This approach identified two PFGE clusters associated with carriage – PFGE cluster 14 (OR=0.30 [95% CI 0.12 to 0.73]) and PFGE cluster 15 (OR=0.16 [95% CI 0.06 to 0.36]), both significant after FDR correction (p=0.030 and p<10^{-4}, respectively).

A cluster with enhanced invasive disease potential was also identified – PFGE cluster 13 (OR=7.02 [95% CI 1.55 to 65.18], p=0.030). Since the MLST analysis of PFGE cluster 13 revealed mostly isolates representing ST193, we repeated the analysis grouping together all PFGE clusters where ST193 was found (PFGE clusters 8, 12 and 13) and the result was also significant (OR=8.76 [95% CI 2.54 to 46.80], p<10^{-4}).

**Temporal fluctuations of PFGE clusters.**

To probe temporal variations in the proportion of isolates in each PFGE cluster, the Cochran-Armitage test was used. This analysis was performed for the major PFGE clusters (n\(\geq\)10) found among isolates causing infection, and also considering as a single genetic lineage all PFGE clusters associated with ST994 (n= 24) and as another all those associated with ST193 (n= 35). The test revealed that among serotype 19A isolates responsible for infections PFGE cluster 14 decreased during the study period (p<10^{-4}), while PFGE cluster 17 became more abundant (p= 0.002).

**PFGE cluster and antibiotic resistance.**

The relationships between PFGE cluster and antimicrobial resistance were determined by odds ratios for each antimicrobial and the significant values are presented in Table 3. PFGE cluster 17 stood out in the analysis since it was associated with resistance to all antimicrobial classes with the exception of chloramphenicol.
Discussion

Several studies have demonstrated that after conjugate vaccine introduction significant changes occur in the distribution of the serotypes responsible for invasive infections. These are characterized by a sharp decrease in the number of infections caused by vaccine types and an increase in the number of infections by non-vaccine serotypes, even if only incremental (2, 4, 18, 27, 39). Pneumococcal serotypes not represented in vaccine formulations are generally considered to be less virulent (38). Yet, the lower incidence of invasive infections caused by non-vaccine serotypes could be due, not to reduced virulence, but to a lower transmissibility or to a weaker competitive capacity for nasopharyngeal colonization. If the later was the case, then the removal by vaccination of the serotypes commonly found in the nasopharynx would allow the establishment of non-vaccine serotypes that could then emerge as important causes of invasive infections (21). In this scenario, all other things being equal, one could expect that the more virulent clones found among non-vaccine serotypes would increase in prevalence in invasive infections. Another hypothesis for vaccine selection of non-vaccine serotypes would be the emergence of capsular transformants (30). These would retain the successful genotypes previously found among vaccine serotypes but would express a capsular polysaccharide not targeted by the vaccine (7).

In line with other studies, an increased proportion of infections caused by serotype 19A isolates has been noted in Portugal, despite non-universal vaccination [(2) and this study]. If occupation of a vacant colonization niche would be the sole driver behind the increase of serotype 19A infection, then an increase in 19A colonization would be expected. Although we did observe an increase in serotype 19A among carriage isolates, it was not statistically significant and may therefore not offer a full explanation for the rise of serotype 19A in infection. A possible explanation for the more modest increase
of serotype 19A in carriage may be that other serotypes may be equally adapted at exploiting the vacant niche left by the reduction of vaccine serotypes in colonization.

Each of the three serotype 19A populations analyzed was highly diverse and several genetic lineages were identified, most of which were found among all three populations. This finding is in contrast with other studies that included isolates expressing all serotypes, which found a genetically more diverse population among carriage isolates (17, 32). A higher diversity among carriage isolates is frequently interpreted as supporting the hypothesis that only a few lineages are capable of causing invasive infections. However, this conclusion may be conditioned by the larger number of serotypes found among carriage isolates. Studies of other pneumococcal populations homogeneous for their serotype are needed to clarify if the similar genetic diversity found among 19A isolates causing infections and those carried asymptotically is an unusual characteristic of serotype 19A or a more general property.

Previous studies have suggested that capsular serotype may be more important than genotype in the ability of pneumococci to cause invasive disease (6). Of interest, among all 19A lineages identified, two groups established opposing relationships with the human host – one was associated with asymptomatic carriage (PFGE clusters 14 and 15 – representing ST1151 and ST416 and closely related STs, respectively) while the other was associated with invasive disease (PFGE cluster 13 – representing mostly ST193).

The existence of several lineages within serotype 19A with opposing properties and their different prevalence in various geographic regions, may explain why some studies identified this serotype as having enhanced invasive disease potential (32) while others did not (6).

Neither the lineage defined by ST193 (including all PFGE clusters presenting this ST) nor the one defined by ST416 and its single-locus variants increased over time among infection isolates. Instead, two other genetic lineages did show temporal variations. The
lineage identified by PFGE cluster 14 – representing mostly ST1151 and ST2732, single-locus variants of each other and apparently only detected in Portugal – which was significantly associated with carriage and susceptibility to most antimicrobials declined as a cause of infection during the study period. On the other hand, the lineage characterized by PFGE cluster 17 associated with resistance to most antimicrobials, including β-lactams and macrolides (antibiotics of choice for the treatment of pneumococcal infections), increased significantly over time as a cause of infection. In fact, this lineage was the most abundant in all collections. A comparison based on PFGE profiles identified members of cluster 17 as closely related to clone Denmark14-. ST230 (24). MLST analysis of representative isolates of this PFGE cluster identified ST276 (a single-locus variant of ST230) as the dominant ST and most isolates (30 of 32) belonged to CC230 corroborating that isolates in this PFGE cluster represent mostly the Denmark14- ST230 clone.

Studies characterizing invasive isolates in both France (22) and Spain (4) have also identified representatives of clone Denmark14- ST230, in particular ST276, as major causes of 19A invasive disease in both children and adults. A study from Israel also identified ST276 as an important cause of acute otitis media in recent years (11). The later is a particularly interesting study, since it associated the presence of ST276 to high antibiotic consumption, a situation that may be mirrored in Portugal.

Studies from two other non-European countries – Korea and the US – have also analyzed in great detail the genetic diversity of the pneumococcal population expressing serotype 19A (19, 26). The study from Korea revealed a 19A population with limited diversity (only 4 STs) while that of the US was much more diverse (73 STs), but the major clonal complex found in Korea (n=53/58), CC320, was also a major lineage in the US (n=111/528). In contrast, in Portugal, ST320 was not detected and the most frequent lineage in the US (ST199) was found in a single isolate, in a medium sized
PFGE cluster (PFGE cluster 4). The most frequent lineage in our study, PFGE cluster 17 – representing the Denmark\textsuperscript{14} ST230 clone – (n= 93/288 or 32%), was a minor lineage in the US (accounting for 3% of the 19A isolates) and was not found in Korea. These data highlight the genetic diversity and geographic differences behind the increase in serotype 19A isolates and suggest that the locally circulating clones, as well as other selective pressures such as antibiotic consumption and vaccination, may play an equally important role in the emergence of serotype 19A as a major cause of pneumococcal infections.

Although this study has limitations, we consider that these do not affect our conclusions. First, isolates causing infections were recovered from centers geographically dispersed in Portugal, whereas pneumococcal carriage isolates were collected only in the Lisbon area. Our previous studies with isolates causing invasive infections did not identify any significant regional asymmetries (2, 33) so we do not expect these to exist in colonization. Moreover, the three major clones found in colonization, together accounting for close to 80% of colonization isolates, were found in six DCCs each, indicating that these clones are widely disseminated and do not correspond to local clusters. Secondly, no colonization isolates were available from 2004 and 2005 since in these years no sampling was performed. This did not allow the analysis of temporal variations in the clonal composition of the colonization population, but a qualitative evaluation indicated there was a strong parallel with the changes detected in the infection isolates. The major clones (n≥10) found in carriage were present in all years studied, indicating no sudden variations in clonal distribution. These two observations reassured us that we were effectively sampling the carriage population. Thirdly, we included in our comparison isolates recovered from invasive infections in both adults and children, whereas the carriage isolates were recovered from children only. Our choice was guided by the smaller number of isolates analyzed if we
had excluded those of adults that would have resulted in much lower statistical
efficiency and by the observation that there were no differences in clonal composition
among invasive isolates when stratified by age group. In fact, asymptomatic carriage in
adults is low and children are considered the major reservoir of pneumococci (16).

Further confirmation of the pivotal role of children in adult infections was obtained
from the recent observation that children vaccination had a major impact in
pneumococcal infections in adults (2, 4, 9).

In summary, we have shown that different genetic lineages expressing serotype 19A
preferentially establish colonization or cause infections in the human host. The lineage
currently expanding in Portugal and other southern European countries as a cause of
infection was not identified as particularly virulent. The availability of an enlarged
nasopharyngeal niche seems to have allowed the expansion of a clone competent at
colonization and infection, which drove the rise in 19A infections. Similarly to what
was found elsewhere, this dominant serotype 19A lineage was resistant to most
antimicrobials. Together with the decline of a successful local clone, that was mostly
susceptible, this observation suggests that antibiotic use may also be an important factor
shaping the pneumococcal population in Portugal and may have been decisive for the
clonal fluctuations among 19A isolates causing infections observed here and in other
Mediterranean countries. Nevertheless, the reasons why the successful multi-resistant
ST320 lineage is not found in Portugal or why the equally resistant ST199 lineage did
not expand to dominate the 19A population, as it did in the US, remain unclear and
point out to the importance of circulating clones and other local selective forces in
driving the expansion of the most successful 19A clones. These data reinforce the
importance of continuous surveillance to understand serotype changes in the era of
pneumococcal conjugate vaccines and highlight important differences between Europe
and the US. Such knowledge may allow us to understand the emergence of non-vaccine
serotypes and guide the design and use of future pneumococcal vaccines.
Acknowledgments

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References


   


   


**Figure 1. Macrorestriction dendrograms and ST information of 19A isolates.** Dice coefficient values (percentages) are indicated in the scale above the dendrogram.

Whenever $\geq 2$ isolates had a macrorestriction pattern with a Dice coefficient $\geq 80\%$ a triangle proportional to the number of isolates is indicated in the dendrogram. The number in bold type beside the triangle indicates the number of the PFGE cluster and the number in parentheses that of the isolates grouped in that cluster. All STs determined for isolates of each PFGE cluster are indicated, and the number in parentheses indicates the number of isolates exhibiting that ST. The distribution of the isolates found in each PFGE cluster ($n \geq 4$) over the study years is presented. The letters in superscript identify STs identifying international spread clones as follows: a, Spain$^{23F}$-ST81; b, Netherlands$^{15B}$-ST199; c, Greece$^{21}$-ST193; d, Denmark$^{14}$-ST230; e, Spain$^{9V}$-ST156. Non-susceptibility to various antimicrobial agents is indicated by superscripts as follows: R, resistance; I, intermediate; CL, chloramphenicol; CM, clindamycin; EM, erythromycin; PV, penicillin; SXT, co-trimoxazole; and TC, tetracycline. The number of isolates sharing the same antimicrobial resistance profiles is indicated in parentheses.
Table 1. Antibiotic resistance of serotype 19A *S. pneumoniae* isolates (n=288)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Invasive (n= 121)</th>
<th>Non-invasive (n= 57)</th>
<th>Carriage (n= 110)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin¹</td>
<td>56 (46.3)ᵇ</td>
<td>23 (40.4)</td>
<td>43 (39.1)</td>
</tr>
<tr>
<td>Erythromycin²</td>
<td>70 (57.9)</td>
<td>23 (40.4)</td>
<td>59 (53.6)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>69 (57.0)</td>
<td>21 (36.8)</td>
<td>58 (52.7)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>71 (58.7)</td>
<td>23 (40.4)</td>
<td>67 (60.9)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>40 (33.1)</td>
<td>20 (35.1)</td>
<td>20 (18.2)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>17 (14.0)</td>
<td>3 (5.5)</td>
<td>9 (8.2)</td>
</tr>
<tr>
<td>Levofloxacinn</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹Isolates non-susceptible to penicillin (MIC≥0.12 μg/ml) are indicated.

²Five isolates were fully resistant (MIC≥2 μg/ml).

³The majority (148/152) of macrolide resistant isolates presented the MLS₉ phenotype, characterized by simultaneous resistance to erythromycin and clindamycin. Only four isolates (2.6%) presented the M phenotype, characterized by resistance to erythromycin only.
Table 2. Diversity and major PFGE clusters of the three 19A populations analyzed

<table>
<thead>
<tr>
<th></th>
<th>Proportion of isolates in PFGE cluster (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SID% [95% CI]&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Carriage</strong></td>
<td>78.8 [74.5 to 83.0]</td>
</tr>
<tr>
<td>(n=110)</td>
<td></td>
</tr>
<tr>
<td><strong>Invasive</strong></td>
<td>82.2 [76.8 to 87.5]</td>
</tr>
<tr>
<td>(n=121)</td>
<td></td>
</tr>
<tr>
<td><strong>Non-invasive</strong></td>
<td>81.4 [73.3 to 89.6]</td>
</tr>
<tr>
<td>(n=57)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Simpson’s index of diversity and corresponding 95% confidence intervals

<sup>b</sup>PFGE clusters including at least 5% of any of the 3 analyzed populations are represented. When the proportion of the population grouped into one of the PFGE clusters is ≥10% the value is highlighted in bold.
<table>
<thead>
<tr>
<th>PFGE Cluster (ST in PFGE cluster)</th>
<th>Penicillin OR [95%CI]</th>
<th>p</th>
<th>Erythromycin OR [95%CI]</th>
<th>p</th>
<th>Chloramphenicol OR [95%CI]</th>
<th>p</th>
<th>Tetracycline OR [95%CI]</th>
<th>p</th>
<th>Co-trimoxazole OR [95%CI]</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (ST1201, [ST81, 634], ST199)</td>
<td>0 [0.00 to 0.13]</td>
<td>&lt;10^{-4}</td>
<td>18.32 [7.09 to 47.31]</td>
<td>&lt;10^{-4}</td>
<td>3.47 [1.48 to 8.13]</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 (ST994)</td>
<td>0 [0.00 to 0.44]</td>
<td>0.002</td>
<td>0 [0.00 to 0.44]</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (ST994, ST4197, ST1151)</td>
<td>0 [0.00 to 0.44]</td>
<td>0.002</td>
<td>0 [0.00 to 0.44]</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 (ST193, ST202)</td>
<td>0.07 [0.01 to 0.55]</td>
<td>0.001</td>
<td>4.85 [1.37 to 17.15]</td>
<td>0.007</td>
<td>29.76 [9.94 to 89.07]</td>
<td>&lt;10^{-4}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 (ST1151, 2732, 9001, ST276)</td>
<td>0.03 [0.01 to 0.14]</td>
<td>&lt;10^{-4}</td>
<td>0 [0.00 to 0.74]</td>
<td>0.021</td>
<td>0.06 [0.02 to 0.18]</td>
<td>&lt;10^{-4}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 (ST416, 4189, 3866, ST9004)</td>
<td>0 [0.00 to 0.07]</td>
<td>&lt;10^{-4}</td>
<td>0 [0.00 to 0.56]</td>
<td>0.008</td>
<td>0.04 [0.01 to 0.29]</td>
<td>&lt;10^{-4}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 (ST276, 230, 2037, 2807, 4196, 9002, 9003, [ST156, 2635])</td>
<td>83.34 [31.46 to 220.74]</td>
<td>&lt;10^{-4}</td>
<td>24.01 [10.52 to 54.82]</td>
<td>&lt;10^{-4}</td>
<td>0.14 [0.03 to 0.59]</td>
<td>0.002</td>
<td>9.70 [4.96 to 18.96]</td>
<td>&lt;10^{-4}</td>
<td>2.34 [1.37 to 3.99]</td>
<td>0.002</td>
</tr>
</tbody>
</table>

a Only PFGE clusters with a significant association with at least one antimicrobial are indicated. Only significant values are shown. OR>1 (in bold) indicates significant association with resistance whereas OR<1 indicates a significant association with susceptibility. Square brackets indicate STs that belong to the same eBURST group.

b If all PFGE clusters representing exclusively ST994 are analyzed together (PFGE clusters 1, 5, 9 and 10), a significant association with susceptibility to co-trimoxazole (OR = 0 [0.00 to 0.40], p=0.002) emerges, in addition to those already identified for PFGE clusters 9 and 10.

c If all PFGE clusters representing exclusively ST193 are analyzed together (PFGE clusters 12 and 13), no qualitative changes occur in relation to those identified for PFGE cluster 13.

d This PFGE cluster was the only one presenting significant associations with both multidrug resistance and simultaneous resistance to penicillin and erythromycin.