Molecular characterization of *Streptococcus pneumoniae* serotype 1 invasive isolates in Colombia

Carolina Duarte,¹ Olga Sanabria,¹ and Jaime Moreno¹

**Objective.** To determine the genetic relationship between *Streptococcus pneumoniae* serotype 1 Colombian isolates recovered from invasive disease between 1994 and 2011 and recognized serotype 1 international clones.

**Methods.** A total of 135 *S. pneumoniae* serotype 1 isolates with epidemiological and antimicrobial susceptibility data (Clinical and Laboratory Standards Institute, 2012) were studied. The genetic relationship with recognized international clones was established by pulsed-field gel electrophoresis (PFGE) with *Sma*I restriction enzyme. Multilocus sequence typing (MLST) was standardized to determine the sequence type (ST) in seven isolates representing different clonal groups. Control and reference strains R6, and clones Sweden¹ ST217, Sweden¹ ST304, Sweden¹ ST306, and USA¹ ST615, were used.

**Results.** PFGE revealed that 89.7% of the isolates were associated with Sweden¹ ST306, 3.7% were associated with Sweden¹ ST304, and 6.6% were not clonally related. Using MLST, ST306 was confirmed in six isolates and ST304 in one.

**Conclusions.** In contrast to Brazil and the United States, where clones Sweden¹ ST304 and ST227 prevail, invasive disease caused by *S. pneumoniae* serotype 1 in Colombia is principally associated with the dispersion of isolates related to clone Sweden¹ ST306.

**Key words** *Streptococcus pneumoniae*; molecular epidemiology; electrophoresis, gel, pulsed-field; multilocus sequence typing; Colombia.
The aim of this study was to determine the clonal distribution of *S. pneumoniae* serotype 1 Colombian isolates recovered from invasive disease between 1994 and 2011 by establishing their genetic relationship with recognized international clones.

**MATERIALS AND METHODS**

**Type of study**

This research consisted of a descriptive study indicating the molecular characteristics of invasive isolates of *S. pneumoniae* serotype 1 in Colombia between 1994 and 2011.

**Isolates**

Out of 479 isolates of *S. pneumoniae* serotype 1 recovered between 1994 and 2011, obtained from national surveillance in Colombia and sent to the Microbiology Study Group (Grupo de Microbiología) at the Instituto Nacional de Salud in Bogotá, a representative sample of 135 isolates was selected for study. The inclusion criteria were available data on epidemiology, and antimicrobial susceptibility to penicillin, ceftaxitoxan, vancomycin, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole (SXT), which was determined by the disk diffusion method (Kirby-Bauer) and broth microdilution, according to the standards of the Clinical and Laboratory Standards Institute (CLSI; 2012) (19, 20).

The representative sample size was calculated based on an assumed prevalence of clonal isolates of 50%, with an absolute error of 10% and 95% confidence intervals (CIs). The data analysis was conducted using Epi Info™ software version 6.1 (Centers for Disease Control and Prevention, Atlanta, USA). A sample of 121 isolates susceptible to antibiotics used clinically was selected at random and per study year; 14 isolates with resistance to some antimicrobials were also included.

The 135 isolates studied were recovered from blood (69.6%), cerebral spinal fluid (19.2%), pleural liquid (10.4%), and other (0.8%). Disease diagnoses included pneumonia (56.3%), meningitis (22.2%), sepsis (17.0%), and other (4.5%). The patients were children less than 4 years old (33.6%), 4 to 6 years old (16.4%), and 6 to 14 years old (17%), and adults (33%) (see supplementary material S1).

**Molecular typing**

PFGE was carried out according to the protocol of Vela et al. (21), using the control and reference strain R6 (donated by Alexander Tomasz of The Rockefeller University, New York, USA), and the clones Sweden1 ST217, Sweden1 ST306, Sweden1 ST304, and USA1 ST615 (donated by the National Centre for Streptococcus (NCS) in Alberta, Canada, and the Instituto Adolfo Lutz in São Paulo, Brazil). The band patterns of each isolate obtained by PFGE were classified according to Tenover’s criteria (22) and analyzed with Bionumerics Gel-Compare (Applied Maths, Austin, TX, 1998–2005).

The sequence type (ST) was determined in seven isolates representing different clonal groups. MLST was performed according to the protocol of Enright et al. (23), with changes in the concentrations of magnesium chloride (MgCl₂) (3.6 mM) and DNA (200 ng/µl). The DNA fragments were purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and were sequenced in each direction, using the primers used for amplification, on an ABI PRISM® 310 automatic sequencer (Applied Biosystems, Cheshire, UK). Software available on the MLST website (http://www.mlst.net/) was used to analyze genetic profiles (24).

**Statistical analysis**

A descriptive analysis of the results was performed. Variables were described using relative frequencies or absolute frequencies, depending on their level of measurement. Data were consigned and verified with Epi Info™ version 6.1 (25).

**RESULTS**

Among the 135 isolates of *S. pneumoniae* serotype 1 studied, only two genetic lineages (types E and F) were identified by the PFGE patterns. Type E comprised 121 isolates (89.7%), with 15 subtypes (E1–E15) genetically related to the clone Sweden1 ST306. The subtype E1 was observed in 100 of the 135 isolates (74.1%), with a genetic similarity of 98.0%. The subtypes E2–E15 were observed in 21 isolates (15.5%), with a genetic similarity between 83.0% and 92.8%. Type F comprised five isolates (3.7%; 5/135) genetically related to the clone Sweden1 ST304, with a genetic similarity between 82.8% and 92.8%. Nine (6.6%) of the remaining isolates were not clonally related. MLST confirmed the clonal relationship initially identified by PFGE in six isolates related to Sweden1 ST306 and in one related to Sweden1 ST304. In these isolates neither single nor double locus variants were observed, and MLST did not provide additional information for differentiating isolates within a clonal group based on differences in electrophoretic patterns (Figure 1).

The results indicate the establishment and permanence of clone Sweden1 ST306 during all surveillance years, with an increase from 1999 to 2011. The isolates related to clone Sweden1 ST304 were more frequent in the first years of surveillance (Figure 2).

At total of 51.0% of the isolates (69/135) were recovered from children less than 6 years old, and 84% (58/69) of those were genetically related to the clone Sweden1 ST306; 49% of all isolates (66/135) were recovered from patients more than 6 years old, of which 95.4% (63/66) were related genetically to clone Sweden1 ST306.

Among the 135 isolates of *S. pneumoniae* studied, 56.3% (76/135) were recovered from patients with a diagnosis of pneumonia, and of those 88.1% (67/76) were related genetically to clone Sweden1 ST306. A total of 22.2% of isolates (30/135) were recovered from patients with meningitis, of which 90% (27/30) were related to the same clone.

Of the 14 isolates with resistance to some antibiotics used clinically, nine were genetically related to clone Sweden1 ST306 and three to clone Sweden1 ST304. Of the isolates related to clone Sweden1 ST306, seven isolates were resistant to SXT, one was resistant to tetracycline, and one was resistant to erythromycin. The isolates related to clone Sweden1 ST304 were resistant to tetracycline.

**DISCUSSION**

Laboratory-based surveillance for invasive pneumococcal disease has been carried out in Colombia since 1994, through the national surveillance program conducted by the Microbiology group of the National Institute of Health.
in Bogotá (part of the SIREVA II project coordinated by the Pan American Health Organization (PAHO)). This type of surveillance has identified the frequency of serotypes, resistance to antibiotics, and movement of international clones in Colombia and other Latin American countries (26).

Previous studies in Colombia have established the presence and dispersion of non-penicillin-susceptible clones of *S. pneumoniae* (Spain<sup>ST5</sup> ST156, with capsular variant 14; Spain<sup>ST4</sup> ST90; Spain<sup>ST81</sup>; and Colombia<sup>ST338</sup> ST338 (21)), and clone Colombia<sup>ST289</sup>, which is penicillin-susceptible but resistant to tetracycline and chloramphenicol (27–29). This study determined the establishment of clone Sweden<sup>ST306</sup>, associated with invasive disease (mostly pneumonia). The allelic profile and ST of isolates characterized by MLST were included in the MLST website’s centralized database (http://spneumoniae.mlst.net/earth/maps/). The MLST website provides data that can be transmitted electronically via the Internet, allowing for more adequate surveillance worldwide (24).

In a study conducted among children in 10 Latin American countries between 2000 and 2005, *S. pneumoniae* serotype 1 ranked third in frequency (7.5%) primarily associated with pneumonia, with a 3% reduction in susceptibility to penicillin, and additional variations were detected in the prevalence of this serotype, with increases at the beginning and end of the study period, revealing a cyclic pattern, in Argentina, Chile, Colombia, and Uruguay (6). To date, Colombia is the only Latin American country where the presence and dispersion of isolates related to clone Sweden<sup>ST306</sup> (30, 31) has been determined. This clonal group, which is fully susceptible to antibiotics, was initially identified in Sweden (32) and later found in Canada, Denmark, France, Germany, Netherlands, Norway, Poland, Spain, and the United States, and is always associated with invasive disease (16). Since the introduction of the heptavalent conjugate vaccine in Portugal and Spain, an increase in this clonal group among healthy carriers and in invasive disease has been reported (33, 34). Some authors have suggested an association between the use of the seven-valent conjugate vaccine and the increase in invasive pneumococcal disease caused by non-vaccine serotypes such as serotype 1 (31, 33, 34).

Moreover, Le Hello et al. reported outbreaks in South Pacific countries associated with the same clonal group (35). It is noteworthy that most of the molecularly characterized outbreaks have been associated with the highly virulent clonal complex Sweden<sup>ST217</sup> (14), which was not been identified in this study.

According to Williams et al., invasive pneumococcal disease caused by *S. pneumoniae* serotype 1 shows different levels of virulence, which may be attributable to the presence and dispersion of different clonal complexes that are characterized by the presence of genomic regions and polymorphisms associated with specific virulence factors for each clonal complex. For this reason, countries with dispersion of isolates related to clone Sweden<sup>ST217</sup> the invasive disease is associated with bacteremia and meningitis with high mortality rates, whereas in countries where the clone Sweden<sup>ST306</sup> circulates, invasive dis-

FIGURE 1. Electrophoretic patterns of seven *Streptococcus pneumoniae* serotype 1 isolates (six related to Sweden<sup>ST304</sup>) obtained through pulsed-field gel electrophoresis (PFGE) and confirmed by multilocus sequence typing (MLST), Colombia, 2011

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tenover’s criteria</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>INS Spn 1277 E7</td>
<td>E1</td>
<td>306</td>
</tr>
<tr>
<td>INS 1807 E441</td>
<td>E1</td>
<td>306</td>
</tr>
<tr>
<td>INS Spn 2060</td>
<td>E1</td>
<td>306</td>
</tr>
<tr>
<td>INS Spn 3547 E1126</td>
<td>E2</td>
<td>306</td>
</tr>
<tr>
<td>INS Spn 1732 E408</td>
<td>E3</td>
<td>306</td>
</tr>
<tr>
<td>INS Spn 1259</td>
<td>E4</td>
<td>306</td>
</tr>
<tr>
<td>INS Spn 1277 E107</td>
<td>F1</td>
<td>304</td>
</tr>
</tbody>
</table>

FIGURE 2. Distribution of isolates related to clones Sweden<sup>ST306</sup> and Sweden<sup>ST304</sup> by surveillance year, Colombia, 1994–2011

The allelic profile and ST of isolates

According to Williams et al., invasive pneumococcal disease caused by *S. pneumoniae* serotype 1 shows different levels of virulence, which may be attributable to the presence and dispersion of different clonal complexes that are characterized by the presence of genomic regions and polymorphisms associated with specific virulence factors for each clonal complex. For this reason, countries with dispersion of isolates related to clone Sweden<sup>ST217</sup> the invasive disease is associated with bacteremia and meningitis with high mortality rates, whereas in countries where the clone Sweden<sup>ST306</sup> circulates, invasive dis-

The allelic profile and ST of isolates
ease is associated with nonlethal pneumonia with or without empyema. The clone Sweden ST306 has a worldwide distribution. This may be related to the apparent predominance in Colombia. However, the lack of molecular characterization of isolates of \textit{S. pneumoniae} serotype 1 in several countries hides actual distribution of this pathogen. In this study, the identification of isolates with resistance to some antibiotics for clinical use genetically related to clone Sweden ST306 is of particular concern as it may add a further advantage to such serotype 1 isolates, leading to their emergence in Colombia, where this clone is prevalent.

In this study, the isolates characterized for MLST did not present single- or double-locus variants. This technique does not provide additional information to differentiate isolates within a clonal group with some differences in electrophoretic patterns. For future studies we recommend the use of the new methodologies with higher discriminatory power, such as MLST (multi-invasive-locus sequence typing) or MLVST (multi-virulence-locus sequence typing), which allow a better understanding of genetic relationships among strains that appear identical but may have individual characteristics useful for differentiation (36, 37).

In conclusion, the increase of invasive disease caused by \textit{S. pneumoniae} serotype 1 in Colombia can be attributed to an increased circulation of isolates genetically related to clone Sweden ST306. This study also confirmed that continuing surveillance is necessary to detect the emergence of pneumococci serotypes and clones so that appropriate control measures of infections caused by pneumococci can be applied within an appropriate time frame.

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Conflicts of interests. None.

REFERENCES

Caracterización molecular de cepas invasoras de Streptococcus pneumoniae serotipo 1 aisladas en Colombia

Objetivo. Determinar la relación genética entre las cepas de Streptococcus pneumoniae serotipo 1 aisladas en Colombia en casos de enfermedad invasora entre 1994 y 2011 y los clones internacionales reconocidos del serotipo 1.

Métodos. Se estudiaron un total de 135 cepas de S. pneumoniae serotipo 1 de las que se tenían datos epidemiológicos y de sensibilidad a los antimicrobianos (Clinical and Laboratory Standards Institute, 2012). Se estableció su relación genética con los clones internacionales reconocidos mediante electroforesis en gel de campo pulsátil (PFGE) utilizando la enzima de restricción Smal. Se estandarizó la tipificación de secuencias multilocus (MLST) para determinar el tipo de secuencia (ST) en siete cepas que representaban diferentes grupos clonales. Se utilizaron la cepa de control y referencia R6 y los clones Sweden1 ST217, Sweden1 ST304, Sweden1 ST306, y USA1 ST615.

Resultados. La PFGE reveló que 89,7% de las cepas se asocian con Sweden1 ST306, 3,7% con Sweden1 ST304, y 6,6% no mostraron clonal. Mediante MLST, se confirmó la relación con ST306 en seis cepas y con ST304 en una.

Conclusiones. A diferencia de Brasil y Estados Unidos, donde prevalecen los clones Sweden1 ST304 y ST227, la enfermedad invasora causada por S. pneumoniae serotipo 1 en Colombia se asocia principalmente con la dispersión de cepas relacionadas con el clon Sweden1 ST306.

Palabras clave. Streptococcus pneumoniae; epidemiología molecular; electroforesis en gel de campo pulsado; tipificación de secuencias multilocus; Colombia.