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ORIGINAL ARTICLE

Molecular characterization of non-vaccine *Streptococcus pneumoniae* serotypes 11A, 15 B/C and 23A recovered from invasive isolates in Colombia

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Introduction: A total of 192 invasive *Streptococcus pneumoniae* isolates, from serotypes 11A, 15B/C and 23A (not included in the conjugated vaccines), were collected in Colombia between 1994 and 2014 as part of the activities of the Network surveillance system for the causative agents of pneumonia and meningitis (SIREVA II).
Objective: To determine the molecular characteristics of invasive *S. pneumoniae* isolates from serotypes 11A, 15B/C and 23A in Colombia from 1994 to 2014.
Materials and methods: The molecular characterization of the isolates was carried out through Pulse-Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST).
Results: Serotype 11A showed one clonal group represented by ST62. Serotype 15B/C was composed of three groups associated with Netherlands^{15B}-37 ST199 (28.75%), ST8495 (18.75%), and SLV (Single-Locus Variant) of ST193 (21.25%). Isolates from serotype 23A were gathered in three clonal groups, with 70.21% closely related to ST42, 17.02% to Colombia^{23F}-ST338, and 6.38% to Netherlands^{15B}-37 ST199.
Conclusion: Clones Colombia^{23F}-ST338 and Netherlands^{15B}-ST199 covered more serotypes than those previously found by other authors, including serotype 23A. These analyses reveal the importance of capsular switching in the spreading of successful clones among non-vaccine serotypes causing invasive pneumococcal disease.

Key words: Vaccines, conjugate; pneumonia; meningitis; pneumococcal infections; multilocus sequence typing; electrophoresis, gel, pulsed-field.

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Caracterización molecular de los serotipos no vacunales 11A, 15 B/C y 23A de *Streptococcus pneumoniae* recuperados de aislamientos invasivos en Colombia

Introducción. En Colombia se recolectaron 192 aislamientos invasivos de *Streptococcus pneumoniae* de los serotipos 11A, 15B/C y 23A (no incluidos en las vacunas conjugadas) entre 1994 y 2014, como parte de las actividades del Sistema de Redes de Vigilancia de los Agentes Responsables de Neumonías y Meningitis Bacterianas (SIREVA II).
Objetivo. Determinar las características moleculares de aislamientos invasivos de los serotipos 11A, 15B/C y 23A de *S. pneumoniae* recolectados en Colombia entre 1994 y 2014.
Materiales y métodos. La caracterización molecular de los aislamientos se hizo mediante electroforesis en gel de campo pulsado (*Pulse-Field Gel Electrophoresis*, PFGE) y por tipificación de secuencias multilocus (*Multilocus Sequence Typing*, MLST).
Resultados. El serotipo 11A mostró un grupo clonal representado por el ST62, en tanto que el serotipo 15B/C se distribuyó en tres grupos asociados con los clones Netherlands^{15B}-37 ST199 (28,75 %), ST8495 (18,75 %) y SLV (variante en un solo locus) de ST193 (21,25 %). Los aislamientos con serotipo 23A se agruparon en tres grupos clonales; 70,21 % de ellos estaban estrechamente relacionados con el ST42, 17,02 % con el Colombia^{23F}-ST338, y 6,38 % con el Netherlands^{15B}-37 ST199.
Conclusión. Los clones Colombia^{23F}-ST338 y Netherlands^{15B}-ST199 encontrados en este estudio abarcaron más serotipos de los reportados previamente por otros autores, incluido el serotipo 23A. Estos análisis revelan la importancia de la conmutación (*switching*) capsular en la expansión de clones exitosos entre los serotipos no vacunales como causa de enfermedad invasiva neumocócica.

Palabras clave: vacunas conjugadas; neumonía; meningitis; infecciones neumocócicas; tipificación de secuencias multilocus; electroforesis en gel de campo pulsado.

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Paola Andrea Palacios, Carolina Duarte, Olga Sanabria and Jaime Moreno: Research design
Paola Andrea Palacios: Research and writing of the paper
All authors read and approved the final paper.

Streptococcus pneumoniae is an important cause of morbidity and mortality in diseases such as otitis, pneumonia, sepsis, and meningitis (1). Many of the 96 serotypes of *S. pneumoniae* rarely cause serious illnesses, but a small number of them cause the great majority of invasive pneumococcal diseases (IPD) (2,3). In the context of surveillance, serotypes recovered from IPDs have been used to guide the development of the pneumococcal conjugate vaccines (PCV) for prevention of infections in developed countries. However, *S. pneumoniae* population biology is not well understood, and neither is the relative disease potential of different serotypes (4).

In Colombia, the seven-valent pneumococcal conjugate vaccine (PCV7) was added to the childhood immunization program in 2009, covering serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. Subsequently, in 2010, the PCV10, which includes the seven serotypes of PCV7 and serotypes 1, 5 and 7F, was introduced (5). The benefits of introducing PCVs have been demonstrated by the high efficacy against vaccine serotypes (VT) in invasive diseases and in carriage (6). However, the main concern after their implementation has been the increase of the non-vaccine serotypes (NVT) in IPD cases, as reported in several studies and updated information from surveillance programs (7,8).

This concern can be attributed to "serotype replacement", defined by the expansion of preexisting NVT pneumococci as a response to vaccine pressure (9), and to "serotype switching", a change of serotype in a single clone by alteration or exchange of its cps locus (10). Additionally, these effects are not completely independent because capsular switch variants can subsequently expand within a population (11).

Long-term surveillance has become essential to understand induced changes in NVT carriage and disease, to evaluate the safety and effectiveness of pneumococcal vaccines (12), and to identify the prevalent serotypes causing invasive diseases. Surveillance programs results suggest that the prevalence of pneumococci of various serotypes not included in the conjugated vaccines is rapidly increasing worldwide (e.g., 11, 12, 15, 22F, 23A, 23B, 33F, 24, 34, and 35B) (13,14).

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This study focused on the non-PCV13 serotypes 11A, 23A, 15B/C, that have dominated in carriage after vaccine introduction among other NVTs (15). The surveillance on these serotypes in IPD isolates is crucial given that they can act as the main source for pneumococcal transmission, influencing the significant increase of non-PCV13 strains in invasive diseases (15).

In fact, an important increase of these serotypes in IPD cases has been reported in England (11A and 23A) (16), the United States (15B/C and 23A) (17-19), Spain (23A) (20) Denmark (15B) (21), and Israel (15B) (22) after the introduction of the PCV13.

The analysis of information obtained from patients and isolates has become an important tool to understand the dynamics of NVT before and after vaccine introduction in a population. In Colombia, the surveillance of capsular types and antibiotic resistance from IPD started in 1994, through the SIREVA II program coordinated by the Pan American Health Organization (PAHO) (23). The main objective of this study was the molecular characterization by PFGE and MLST of invasive 11A, 15B/C and 23A non vaccine serotypes recovered between 1994 and 2014 in Colombia.

Materials and methods

Bacterial isolates and patient data

We recovered 192 invasive isolates of serotypes 11A (n=65), 15B/C (n=80) and 23A (n=47) from children and adults, which are kept at the *Grupo de Microbiología* from the *Instituto Nacional de Salud* strain collection as part of the SIREVA II surveillance activities from 1994 to 2014. Currently this surveillance covers 27 regions of the country (5). An IPD case was defined as the isolation of *S. pneumoniae* from a normally sterile body site such as blood, cerebrospinal fluid or other normally sterile body fluid (5).

Isolate typing was carried out by the Quellung reaction using antisera from the Statens Serum Institut (Copenhagen, Denmark), while antimicrobial susceptibility to penicillin (P), ceftriaxone (CRO), trimethoprim-sulfamethoxazole (SXT), chloramphenicol (CLO), tetracycline (TET), erythromycin (E) and vancomycin (V) was determined by the broth microdilution method following the Clinical and Laboratory Standards Institute guidelines. The nonmeningeal breakpoints were interpreted according to these guidelines too (24,25).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed on all isolates following Vela, *et al.,* protocol (26). Genomic DNA was digested with *Sma* I (Promega). Strain R6 and λ ladder marker (New England Biolabs) were used as molecular weight standards. *Streptococcus pneumoniae* clones were included in the PFGE analyses to verify their clonal relation with the isolates (Netherlands^{15B}-37, Tennessee^{23F}-4, Colombia^{23F}-26, S. Africa^{19A} -13, Hungrary^{19A} -6, Spain^{9V} -3, Spain^{23F} -1, Spain^{6B} -2). Band patterns from each isolate were analyzed with the GelCompare software in order to obtain the dendrograms.

Multilocus sequence typing

Multi-Locus Sequence Typing (MLST) was carried out as described previously (27), and nine isolates were selected according to the results obtained by PFGE. For each clonal group, one isolate was chosen in order to determine the sequence type. PCR products were sequenced on each strand and genetic profiles were analyzed using the software available at the pneumococcal MLST website (http://spneumoniae.mlst.net).

Results

Bacterial isolates and patient data

From 1994 to 2014 the frequency of invasive isolates from serotypes 11A, 15B/C, and 23A changed from 1.9% to 9.5% (figure 1). The isolates were recovered from less than 2-year-old patients (20.83%), 2 to 4 years old (8.33%), 5 to 14 years old (10.93%), 15 to 50 years old (28.12%), and more than 50 years old (26.04%). No particular behavior or population preference was found among the three serotypes in the different age groups. The age information was not available in 5.7% of the cases. The main clinical condition caused by the three NVTs was meningitis, followed by bacteremia and pneumonia, as detailed in table 1.

Sixty two percent of the three NVT isolates showed susceptibility to all the antibiotics tested. Trimethoprim/sulfamethoxazole resistance was mainly observed in isolates from serotypes 11A and 15B/C, while tetracycline resistance predominated in serotypes 15B/C and 23A (as described in table 2). Most of the isolates that presented erythromycin resistance belonged to the serotype 23A (table 2). All erythromycin-resistant isolates within the serotype 23A were also resistant to tetracycline. Multidrug resistance to three or more classes of antibiotics was observed in 3.1% of all the isolates: 11A: P + CLO + SXT + TE (n=1), 15B/C: P + CE + SXT (n=1) and 23A: P + TE + SXT (n=1), CLO + SXT + E + TE (n=1), P + E + TE (n=2).

Molecular characterization

PFGE results indicated that the different serotypes expressing the same sequence type also shared the same clonal group. Band patterns in dendrograms were very similar. For example, in the 23A PFGE analysis, we found not only serotype 23A isolates within the ST199 clonal group, but also serotypes 15C and 19A. However, the antibiotic resistance profile inside the clonal groups is not the same for all the isolates, and in the case of the ST199 it can vary from antibiotic susceptibility (15C) to penicillin and erythromycin resistance (19A).

PFGE results also revealed that 89.23% of 11A isolates were closely related in only one clonal group. This group was associated with the ST62, while 10.8% of the isolates were genetically unrelated. Serotype 15B/C presented three clonal groups, and 28.75% of these isolates were related to the

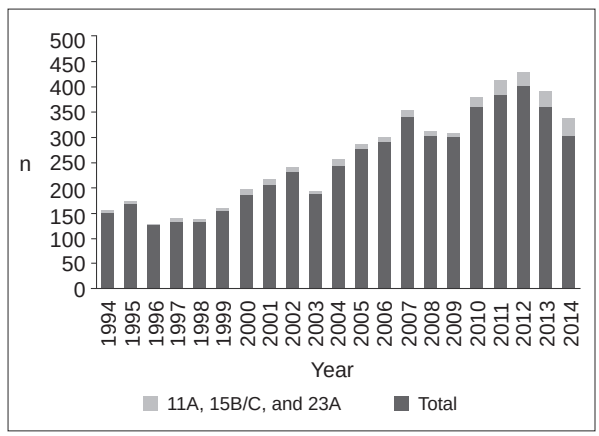

Figure 1. Annual frequency of invasive *S. pneumoniae* isolates from non-vaccine serotypes 11A, 15B/C and 23A, compared with the frequency of total invasive isolates recovered in Colombia from 1994 to 2014 (http://www.ins.gov.co)

Table 1. Distribution by diagnosis of invasive *S. pneumoniae* isolates, serotypes 11A, 15B/C and 23A, during study period

Diagnosis	11A		15B/C		23A	
	n	%	n	%	n	%
Meningitis	16	24.6	36	44.4	16	34.8
Bacteremia	16	24.6	16	19.7	10	21.7
Pneumonia	11	16.9	11	13.5	10	21.7
Not available	22	33.8	17	22.2	11	21.7
Total	65		80		47	

Table 2. Resistance of *S. pneumoniae* isolates from non-vaccine serotypes 11A, 15B/C and 23A (1994-2014 invasive pneumococcal diseases surveillance)

Antibiotic	Number of isolates					
	11A		15B/C		23A	
	n	%	n	%	n	%
Trimethoprim/sulfamethoxazole	13	20.0	14	17.3	2	4.3
Tetracycline	8	12.3	17	21.1	24	52.2
Penicillin	2	3	7	8.6	6	13.0
Chloramphenicol	2	3	0	0	1	2.2
Erythromycin	2	3	0	0	8	17.4
Ceftriaxone	0	0	1	1.2	0	0
Non-antibiotic resistance	46	70.7	51	62.9	22	47.8

Netherlands^{15B}-37 ST199, 21.25% to the single-locus variant of ST193, 18.75% to the ST8495, and 31.25% were genetically unrelated. Serotype 23A presented three clonal groups, and the largest group gathered 70.2% of the isolates associated with the ST42 (the double locus variant of Tennessee^{23F}-4 clone), while 17.02% of isolates from this serotype were closely related to the Colombia^{23F}-ST338, 6.38 % to the Netherlands^{15B}-37 ST199, and 6.38% were genetically unrelated.

Antibiotic resistance profiles per clonal group showed similarities among isolates. Nevertheless, susceptible and resistant isolates to one, two or more antibiotics were found in the same clonal group in the three NVT analyses.

Discussion

The widespread use of conjugate vaccines has been very effective in reducing IPD cases. However, disease increase caused by NVT ("serotype replacement") has subsequently offset some of these reductions (7,8).

NVT surveillance is an issue of concern given its relation with invasive diseases affecting less than two-year-old children, i.e., the most vulnerable population (28). Nevertheless, in this study 20.8% of isolates came from less than 2-year-old children, and 26.0% from more than 50-year-old adults. In this case, the less invasive serotypes, acting as "opportunistic pathogens", would be expected to preferentially cause disease in older patients with higher levels of comorbidity. These associations can be considered to predict which pneumococcal vaccine would be recommended according to the population group (29).

PFGE results for serotype 11A revealed a clonal behavior related to ST62 erythromycin resistance, as reported by other authors (30,31). Despite its characteristic erythromycin resistant profile, only 3% of this group of isolates showed resistance to this antibiotic during the susceptibility test (table 2).

Aguinagalde, *et al*., reported that serotype 11A, ST62 invasive isolates are more resistant to phagocytosis than other 11A clones. Therefore, the isolates that are more resistant to neutrophil clearance will have an advantage and persist in the nasopharynx to facilitate its clonal expansion (31). This divergence could have implications for decision-makers making product choices for adult pneumococcal vaccination programs.

The carriage prevalence of this NVT has been reported after the PCV introduction in Hungary (32), Colombia (33), and from invasive diseases in Spain (20) and the United States (30). Even when this serotype is associated with asymptomatic carriage, its importance resides on the significant mortality caused by invasive diseases (30).

Harboe, *et al.,* showed that highly encapsulated and frequently carried serotypes such as 11A might have high mortality rates in healthy people. These rates can be comparable to those of serotypes 19F and 6B, which were successful in the pre-vaccine era (34).

PFGE analyses of serotype 15B/C established that the Netherlands^{15B}-37 ST199 clone comprised the major clonal group of the dendrogram. This sequence type has been found and reported in other serotypes, including 19A, 14 and 15B/C (35). In the United States, it is known as a penicillin susceptible invasive type for serotype 15B/C and an intermediately penicillin resistant type for serotype 19A (36). This clonal complex is successful at population level for its adaptation in carriage, otitis media, and invasive disease. Therefore, the 15B/C isolates can disseminate as the 19A ones and cause pneumococcal diseases (17,37).

The single-locus variant (SLV) of ST193 (Greece^{21}-30), the second most frequent in this study for 15B/C isolates, has been previously associated with serotypes 21 (38) and 19A (38), and with serogroup 15 (37). According to the MLST database, they have been found in the United States, Germany, Italy, and the Kingdom of Saudi Arabia, and they are distributed at least in eight different serotypes (39). During a study of antimicrobial resistance in *S. pneumonia* in Finland, the ST193 predominated among the telithromycin resistant isolates, which were serotyped as 19A (40).

One of the serotype 23A clonal groups was represented by the double locus variant of Tennessee^{23F}-4 clone, known as the ST42. Little information is available about this sequence type, although it is considered the founder of the clonal complex CC42, between the serotypes 23A and 23F (41). Our results indicated that most of the isolates associated with this sequence type showed antibiotic susceptibility or tetracycline resistance.

All the 23A erythromycin-resistant isolates were resistant to tetracycline, and most of them were related to the ST338. In this sense, Ramos, *et al.,* observed that 53.4% of the Colombian erythromycin-resistant isolates presented tetracycline resistance (42). The sequence type ST338 in Colombia has been associated with serotype 19A and 23F (33), and it was originally reported from penicillin-non susceptible invasive isolates in Colombia, Brazil and Iceland between 1989 and 1996 (42,43). However, in this study only three isolates showed penicillin resistance.

Given that this successful clone covers more than one serotype, it would be important to monitor the development of resistant isolates through SIREVA II surveillance, as done in other countries with the successful clone ST199 (44). This ST199 corresponds to the Netherlands^{15B}-37 clone mentioned above, with serotype 15B/C occurring in different capsular types.

Our results suggest that the ST199 now covers the 23A capsular type with three isolates from 2011, 2012 and 2014, as expected from any successful clone. This is only one example of a successful clonal complex that can “camouflage” through capsular switching in NVTs to cause IPD as a response to vaccine pressure.

Here we recovered information from the sequence types ST199 and ST338 that cover more than two serotypes through capsular switching. In some cases, they can act like the predominant clone, probably due to their successful mechanisms for causing invasive diseases through pneumococci replacement (8).

Continuous surveillance of IPD based on susceptibility analyses, PFGE, MLST, Whole-Genome Sequencing, and other techniques, will allow us to follow the dynamics of these clones over time. However, we need to study pneumococcal virulence factors in prevalent clones in order to obtain more information regarding the spread of successful clones like the Colombia^{23F}-ST338 and the Netherlands^{15B} -ST199 among NVTs.

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Conflicts of interest

The authors declare that they have no competing interests.

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