Ciencia Latina
Revista Multidisciplinar

Genotypic analysis and antimicrobial resistance of invasive *Streptococcus pneumoniae* serotype 14 by Pulsed-Field Gel Electrophoresis in children under 5 years of age in Paraguay

María Eugenia León[1],
maruleonayala@hotmail.com

Aníbal Kawabata[1],
bhatayazz2204@hotmail.com

Helena Ortíz[1],
flavia.arce@gmail.com

Gustavo Chamorro[1].
gacortesi@gmail.com
[1]Respiratory and Meningeal Diseases Section,
Central Public Health Laboratory
Asunción, Paraguay.

ABSTRACT

Introduction. *Streptococcus pneumoniae* serotype 14 is an important cause of invasive pneumococcal diseases throughout the world. It often presents resistance to a variety of antimicrobial agents, resulting in difficulties in treatment. Limited data are available on *Streptococcus pneumoniae* serotype 14 in the pediatric population in Paraguay, therefore it`s important monitoring of serotypes and antimicrobial resistance causing pneumococcal disease is important.

Objective: To evaluate the clonal relationships of isolates of invasive *S. pneumoniae* serotype 14 recovered from children under five years of age and to associate *S. pneumoniae* strains according to their antibiotic resistance.

Materials and methods. Forty-seven isolates with susceptibility data to penicillin, ceftriaxone, erythromycin, trimethoprim sulfamethoxazole, chloramphenicol, vancomycin and tetracycline, isolated from children under five years of age, were studied. The data was generated from strains collected in 2011-2013. The restriction pattern of the DNA was determined by pulsed field gel electrophoresis, using the enzyme *SmaI*. The genetic similarity between the isolates and the clone was established according to the Tenover criteria.

Results. Serotype 14 presented resistance to erythromycin 29.8%, trimethoprim-sulfamethoxazole 65.9% and tetracycline 42.5%, finding 11 isolates genetically related to the international clone England ^{14}-9 with resistance to erythromycin.

Conclusion. These results provide information about the circulating clones in order to monitor the antibiotic resistance patterns. There is a need for better data on drug resistance in developing countries for characterizes the magnitude of the problem, provide effective interventions (conjugate vaccines), and antibiotic use control programs.

Keywords: *Streptococcus pneumoniae*, pulsed field gel electrophoresis, PFGE, antimicrobial resistance.

Artículo recibido: 27 marzo 2021
Aceptado para publicación: 30 abril 2021
Correspondencia: bhatayazz2204@hotmail.com
Conflictos de Interés: Ninguna que declarar

Análisis genotípico y resistencia antimicrobiana de Streptococcus pneumoniae serotipo 14 invasivo por electroforesis en gel de campo pulsado en niños menores de 5 años en Paraguay

RESUMEN

Introducción. El serotipo 14 de Streptococcus pneumoniae es una causa importante de enfermedades neumocócicas invasivas en todo el mundo. A menudo presenta resistencia a una variedad de agentes antimicrobianos, lo que genera dificultades en el tratamiento. Se dispone de datos limitados sobre Streptococcus pneumoniae serotipo 14 en la población pediátrica de Paraguay, por lo que es importante el monitoreo importante de los serotipos y la resistencia a los antimicrobianos que causan la enfermedad neumocócica.

Objetivo: Evaluar las relaciones clonales de aislamientos de S. pneumoniae invasivo serotipo 14 recuperados de niños menores de cinco años y asociar cepas de S. pneumoniae según su resistencia a antibióticos.

Materiales y métodos. Se estudiaron 47 aislamientos con datos de susceptibilidad a penicilina, ceftriaxona, eritromicina, trimetoprim sulfametoxazol, cloranfenicol, vancomicina y tetraciclina, aislados de niños menores de cinco años. Los datos se generaron a partir de cepas recolectadas en 2011-2013. El patrón de restricción del ADN se determinó mediante electroforesis en gel de campo pulsado, utilizando la enzima SmaI. La similitud genética entre los aislados y el clon se estableció de acuerdo con los criterios de Tenover.

Resultados. El serotipo 14 presentó resistencia a eritromicina 29,8%, trimetoprim-sulfametoxazol 65,9% y tetraciclina 42,5%, encontrándose 11 aislados genéticamente relacionados con el clon internacional England 14-9 con resistencia a eritromicina.

Conclusión. Estos resultados proporcionan información sobre los clones circulantes para controlar los patrones de resistencia a los antibióticos. Es necesario contar con mejores datos sobre la resistencia a los medicamentos en los países en desarrollo para caracterizar la magnitud del problema, proporcionar intervenciones efectivas (vacunas conjugadas) y programas de control del uso de antibióticos.

Palabras clave: Streptococcus pneumoniae, electroforesis en gel de campo pulsado, PFGE, resistencia a los antimicrobianos.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is the causative agent of serious invasive diseases such as pneumonia, meningitis and bacteremia and of non-invasive diseases such as otitis and sinusitis (1,2). Pneumococcal disease continues to be one of the leading causes of death among diseases prevented by vaccination according to the World Health Organization (WHO) (3). It is a pathogen associated with high morbidity and mortality worldwide, particularly in children and in countries with less access to diagnosis and treatment and whose importance is related to the burden of the disease, especially in the pediatric population, with the associated risks to the development of antimicrobial resistance and its therapeutic consequences (4). The polysaccharide capsule is considered the main determinant of virulence, protecting bacteria from phagocytosis (5). Isolates lacking the capsule almost never cause invasive disease. Based on structural differences in their capsular polysaccharides, 100 pneumococcal serotypes have been identified to date (6) but only a few are responsible for most invasive infections (7).

Some serotypes are more frequently associated with invasive diseases (8). Serotype 14 is one of the prevalent serotypes before the introduction of vaccines, which in addition to having a good capacity to colonize the nasopharynx of children, causes invasive pneumococcal diseases worldwide and often expresses resistance to a variety of antimicrobial agents, which include penicillin, erythromycin and ceftriaxone, which makes treatment difficult (9,10). The overall increase in penicillin resistance and multidrug resistance in pneumococci appears, in large part, as a result of the spread of highly resistant pneumococcal clones. For decades, pneumococci resistant to penicillin, erythromycin, and trimethoprim-sulfamethoxazole have spread rapidly worldwide. Resistances to tetracycline and chloramphenicol have also been identified, with rates that vary by region and population (11).

In Paraguay, serotype 14 is the prevalent serotype in invasive diseases in children under five years of age. In 2011 a prevalence of 50.9% was observed, 36.4% in 2012 and 20.9% in 2013. In cases of meningitis, 54.8% were resistant to penicillin, 4% to Ceftriaxone, 21.4% to erythromycin and 59.5% to trimethoprim-sulfamethoxazole. In cases of non-meningeal infections, antimicrobial resistance is lower with 0.4%

resistance to penicillin, 0.03% to ceftriaxone, 1.7% to erythromycin and 4.9% to trimethoprim-sulfamethoxazole (12-14).

The pulsed field gel electrophoresis (PFGE) technique has been widely applied and considered one of the main tools for epidemiological and surveillance studies (15,16). Due to its ability to differentiate isolates of the same species and relate them to endemic clones, it has also been one of the most frequent methods used for molecular typing of *S. pneumoniae* (17-19).

The objective of this study was to determine the clonal relationships of invasive isolates of *S. pneumoniae* serotype 14, recovered from children under five years of age and to associate strains of *S. pneumoniae* according to their antibiotic resistance.

Limited data are available on *Streptococcus pneumoniae* serotype 14 in the pediatric population in Paraguay, thus, there is a clear need for close monitoring of serotypes and antimicrobial resistance causing pneumococcal disease to contribute public health actions.

MATERIALS AND METHODS

Study population and samples

A descriptive, retrospective cross-sectional study that included strains of *S. pneumoniae* serotype 14 isolated in the period from January 1, 2011 to December 31, 2013.

A total of 47 invasive isolates of *S. pneumoniae* serotype 14 recovered from invasive disease in children under 5 years of age were studied during the period 2011 to 2013, through the surveillance program of the SIREVA II project of the Pan American Health Organization (PAHO), which were referred to the Central Laboratory of Public Health by laboratories of the health services of the country (Pediatric General Hospital, Institute of Social Security, Institute of Tropical Medicine, National Hospital of Itauguá, Regional Hospital Eastern City, San Lorenzo Maternal and Child Center, Institute of Respiratory and Environmental Diseases, Trauma Hospital, General Hospital Worker Neighborhood, Sanatorium Migone, Italian Sanatorium and Diaz Gill Laboratory).

Isolation and susceptibility

The isolates were identified by colony morphology, bile solubility tests, and optochin sensitivity. The isolates had Quellung capsular reaction serotype data (Statens Serum Institut, Copenhagen, Denmark). The antimicrobial susceptibility to penicillin (PEN) and ceftriaxone (CRO) were determined by Minimal inhibitory Concentration (MIC)

and for erythromycin (ERY), chloramphenicol (CHL), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET), and vancomycin (VA) using the method disk diffusion (Kirby-Bauer), in accordance with regionally standardized techniques (20) and in accordance with the recommendations of the National Committee of Standards for the Clinical Laboratory (Clinical Laboratory Standards Institute, CLSI) (21).

Control strains of *S. pneumoniae* ATCC 700669 (clone Spain^{23F}-1 ST81 with resistance to PEN, CHL, TET), ATCC 700671 (clone Spain^{9V}-3, ST156 with resistance to PEN, SXT), ATCC 700676 (clone England^{14}-9, ST9, resistant to ERY), ATCC 700677 (clone Slovakia^{14}-10, ST20, resistant to PEN, ERY and TET), ATCC 700902 (clone Spain^{14}-5, ST18 resistant to TET and CHL) and ATCC 49619 were included.

The strains were conserved in 20.0% skim milk powder (Oxoid, United Kingdom) with glycerol at ultra temperature -80ºC.

PFGE

Briefly, isolates were grown in supplemented Todd-Hewitt broth until they reached the log phase of growth. The cells were embedded in low melting point agarose discs (Bio-Rad) and digested with the restriction enzyme *SmaI* (Promega, United Kingdom) at a concentration of 20 U/mL; restriction fragments were separated on 1% agarose gels (Bio-Rad, USA) by pulsed field electrophoresis using the CHEFF II kit (Bio-Rad, USA).

Electrophoresis was carried out using a 1% PFGE agarose gel in a CHEF-DR III (Biorad System, USA) origin for 15 hours at 14ºC with 5 to 35 s of linear ramp at 6 V / cm and 120 ºC. The gels were stained with ethidium bromide and visualized with ultraviolet light. The PFGE genotype was defined as isolates showing> 90% identity in the dendrogram created using GelCompare II (Applied Maths, Kortrijik, Belgium).

DATA ANALYSIS

The data were collected and entered into a database in an Excel 2007 spreadsheet and analyzed using the statistical program Stata11.0 (Stata Statistical Software: Release 11. College Station, TX: StataCorp LP). The quantitative variables were expressed in measures of central tendency and measures of dispersion.

The distribution profiles of the PFGE electrophoretic run were compared taking into account the Tenover criteria (22), to determine the possible clonal groups (clusters) or distribution patterns of the *Streptococcus pneumoniae* strains (Serotype 14).

The electrophoretic patterns obtained were analyzed with the Gel Compare II software version 5.1 (Applied Maths, Kortrijik, Belgium), applying the Dice coefficient and the UPGMA method, with 1.5% tolerance and 1.5% optimization parameters for the analysis. It was carried out according to the protocol of Soares *et al.* (23) and the modifications of Vela *et al.* (24) as well as the elaboration of the homology dendrogram of the electrophoretic patterns.

Ethical aspects

This study was approved by the Research Ethics Committee of the Central Public Health Laboratory (CEI), resolution Nº. 35/280214. No informed consent statement was obtained because the study is observational, retrospective, with minimal or no risk for the subjects. The confidentiality of the data provided to the person was respected at all times and only the researchers were able to access the referred data.

RESULTS

In 2011, 29/59 (49.6%) cases of IPD were registered by serotype 14, in 2012 16/52 (30.7%) and in 2013 9/45 (20.0%). The general prevalence during the three years of study was 34.6%.

A total of 47 strains of *S. pneumoniae* isolated from invasive disease were studied in children under 5 years of age in Paraguay during the year 2011-2013. About half (55.3%) of the strains were isolated from female patients, 29.8% from children under 1 year, 72.3% of the cases corresponded to acute bacterial pneumonia and 80.8% were blood samples. **Table 1**.

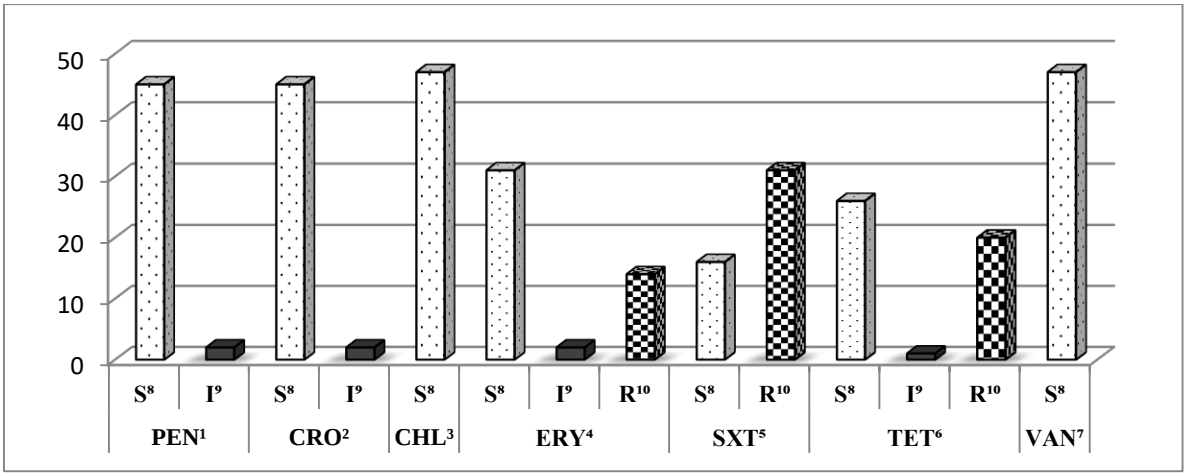
Table 1. *Socio-demographic characteristics of Streptococcus pneumoniae serotype 14 isolates from invasive disease in Paraguay (2011-2013) (n = 47).*

Socio-demographic characteristics	***S.pneumoniae* isolates**	**%**
Gender		
Female	26	55.3%
Male	21	45.7%
Age		
< 1 year	14	29.8%
1 year	9	19.1%
2 years	13	27.7%
3 years	7	14.9%
4 years	4	8.5%
Diagnosis		

Meningitis	3	6.4%
Bacterial Pneumonia	34	72.3%
Community acquired pneumonia	6	12.8%
Sepsis	4	8.5%
Samples		
Cerebrospinal fluid	3	6.4%
Pleural fluid	6	12.8%
Blood	38	80.8%

The isolates studied from meningitis (MBA) 100% (3/3) were susceptible to penicillin G (PEN), ceftriaxone (CRO), chloramphenicol (CHL), erythromycin (ERY), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET) and Vancomycin (VA). Isolates from bacterial pneumonia (NBA or NAC) 1/40 intermediate sensitivity to PEN and CRO (2.5%), 40/40 sensitive to CHL (100.0%), 12/40 resistant to ERY (30.0%), 29/40 resistant to SXT (72.5%), 19/40 resistant to TET (47.5%) and 40/40 sensitive to VA (100.0%). In sepsis isolates, 1/4 intermediate sensitivity to PEN (25.0%) and CTX, 4/4 sensitive to CHL (100.0%) and VA, 2/4 resistant to ERY and SXT (50.0%) and 1/3 resistant to TET (33.3%). **Table 2**. A general resistance to ERY of 29.8%, SXT 65.9% and TET 42.5% was found.

Table 2. *Susceptibility profile of S. pneumoniae isolated in children under 5 years of age in Paraguay (2011-2013).*



PEN[1] Penicillin G

CRO[2] Ceftriaxone

CHL[3] Chloranphenicol

ERY[4] Erythromycin

SXT[5] Trimethoprim sulfamethoxazole

TET[6] Tetracycline

VA[7] Vancomycin

S[8] Susceptible

I[9] Intermediate

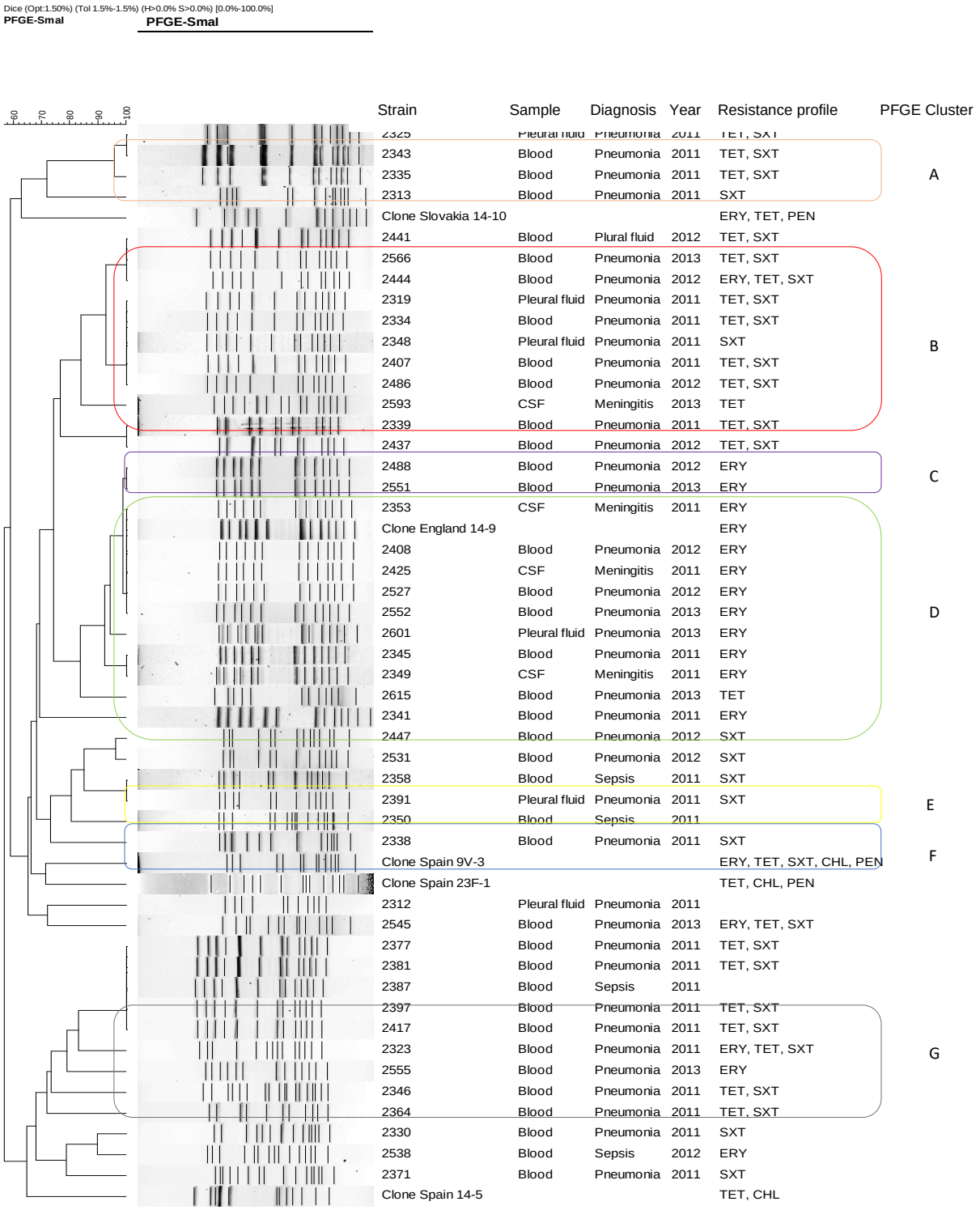
R[10] Resistant

The analysis of the restricted products obtained by digesting the genome of the *Streptococcus pneumoniae* strains (Serotype 14) with SmaI, allowed the identification of 22 different patterns of bands (pulsetypes). The percentage of similarity ranged from 50.0% to 100.0% between isolates. Seven main clonal groups were identified, comprising A with 3 isolates (6.4%), B with 8 (17.0%), C with 2 isolates (4.2%), D with 11 isolates (23.4%), E with 2 isolates (4.2 %), F with 2 isolates (4.2 %), G with 5 isolates (10.6%) and 14 isolates were not genetically related.

Clonal groups A, B, C, E, F and G presented resistance related to TET and SXT. However, group E and F showed resistance only to SXT. In clonal group D all isolates show resistance to ERY. Figure 1. The isolates of the 7 clonal groups correspond to different health services in the country.

.

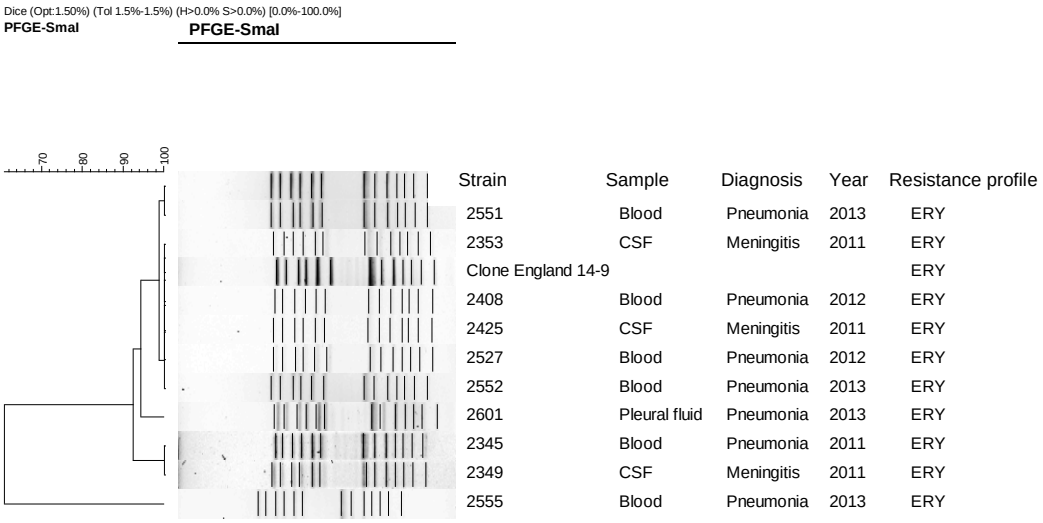
Figure 1. *Dendrogram of Streptococcus pneumoniae serotype 14 strains by PFGE. Paraguay. 2011-2013*



Eleven isolates of *S. pneumoniae* with antibiotic resistance to macrolides (ERY) were analyzed separately, resulting in a clonal group that according to Tenover's criteria are genetically indistinguishable from the England^{14}-9 clone (ATCC 700676) (> 90% genetic relationship with clone England^{14}-9 (10 *S. pneumoniae* isolates and 1 isolate with <90% similarity). **Figure 2**.

The only two isolates with intermediate resistance to the antibiotics PEN and CRO and resistance to SXT, whose electrophoretic profiles or pulsetypes show a difference of > 7 restriction fragments, were compared using PFGE, finding that they are not related to each other.

Figure 2. *Analysis of macrolide-resistant S. pneumoniae clones compared to clone England 14-9 (n = 11).*



DISCUSSION

In Paraguay, the prevalence of serotype 14 during the 2011-2013 period was 34.6%. According to the SIREVA II surveillance published by PAHO (12), the frequency of serotype 14 in 2011 in Argentina was 22.1%, Brazil 19.2% and Chile 31.9%. In Latin America, the most frequent serotype isolated in childhood disease was serotype 14 in 34.1% of the samples (95% CI: 29.4–38.9) in a study period of 2000-2016 (25). Serotype 14 was also found to be one of the most frequent in several studies conducted in countries such as the United States, China, Spain, and England (26-29).

The PCV10 vaccine was incorporated into the vaccination scheme of the Ministry of Public Health and Social Welfare of Paraguay in 2012, with serotype 14 found in a high frequency during the study period, mainly in children under 5 years of age (30). Several

studies conducted with isolates of *S. pneumoniae* have suggested that serotype 14 is among the most important capsular types that drive the emergence of resistance to penicillin and other antibiotics (31-33).

The resistance found in this study was ERY 29.8%, SXT 65.9% and TET 42.5%, in addition to 2 isolates with intermediate sensitivity to PEN and CRO. Other authors in Brazil evaluated the susceptibility profile of *S. pneumoniae* isolated from invasive disease over a period of 16 years, finding the highest frequency of resistance (4.5%) in serotype 14 and in another study the highest rate of no susceptibility to SXT (51.2%), non-penicillin-sensitive pneumococci accounted for 27.3% of the isolates (MIC 0.12 to 4 μg / mL). Non-susceptibility to penicillin was strongly associated with serotypes 14 and 23F (34, 35). In Colombia, serotype 14 was the most prevalent in children under 2 years of age and between 2 and 5 years of age. In contrast, serotype 14 was related to penicillin resistance in 60.0%. Resistance to ERY increased from 7.6% (2009) to 9.8% (2010) (p = 0.17), associated with serotypes 6A (21.1%), 19A (20.3%), 6B (19.5%), 14 (13.0%) and other serotypes (n = 12) (26.1%) (36). Penicillin-resistant strains of *S. pneumoniae* serotype 14 have also been seen in countries such as Korea, Japan, and Morocco (37-39).

Taking into account that nasopharyngeal colonization is the gateway to pneumococcal disease and that the distribution of antimicrobial resistance patterns in nasopharyngeal *S. pneumoniae* carrier strains can predict resistance rates in invasive strains, in Russia high rates of resistance to penicillin accompanied by ERY and clindamycin were observed, and this may be due to an expansion of the endemic multi-resistance clone ST143 with serotype 14 (40). Another study carried out in 9 European countries showed the highest proportion of resistance to ceftazidime and penicillin among strains of serotype 14 (13 of 16, 81.3%) (41).

Molecular typing studies have identified multi-drug resistant *S. pneumoniae* clones, some of which are globally dispersed. Internationally disseminated antimicrobial resistant clones are Spain^{14}–5 (ST18), England^{14}–9 (ST9), CSR^{14}–10 (ST20), Tennessee^{14}–18 (ST67), Denmark^{14}–32 (ST230) and Holland^{14}–35 (ST124) according to the Pneumococcal Molecular Epidemiology Network (PMEN) (http://www.sph.emory.edu/PMEN/index.html).

In this study we detected a correlation between clonal groups of PFGE and resistance to antibiotics. The typing of the 47 strains of *S. pneumoniae* belonging to serotype 14 revealed 5 clonal groups, where most of the strains belonged to group B with 13 isolates with resistance profile to TET and SXT. Clonal groups B and C presented resistance related to TET and SXT, clonal group D showed resistance to ERY and clonal group E presented resistance to SXT.

Previous studies demonstrated the presence of 3 of these global clones: England^{14}-9, Columbia^{23F}-26 and Spain^{9V}-3 within the group of resistant PEN isolates. Resistance to ERY was found mainly among PEN sensitive isolates where most were assigned to clone England^{14}-9 (ST-15), a clone related to isolates serotype 14 (42). In Brazil, four important clonal complexes (CC) were shown to be responsible for the emergence and spread of antimicrobial resistance among S. pneumoniae strains of serotypes 14 and 9, three are related to PMEN clones, including CC156 (related to Spain9V-3), CC66 (related to Tennessee14-18) and CC15 (related to England14-9), while the fourth, CC5401, is characterized as a regional clone (43).

SXT is one of the antibiotics used for the treatment of *S. pneumoniae* infections (44). A group of researchers in Thailand found the highest rate of resistance to SXT (78.94%) of *S. pneumoniae* isolated from children (45).

Resistance to macrolides is observed with great variations between the range of <10 to> 90% according to geographic regions (46). In agreement with the study by Corso et al. Regarding invasive infections, the emergence of ERY-resistant *S. pneumoniae* was mainly due to England14-9, Poland 6B-20 and Spain 9V-3 international clones (47).

A clonal group resulted from 11 isolates of *S. pneumoniae* with resistance to macrolides, specifically to ERY, in which 5 of them obtained 100% clonality with the clone England14-9, other 5 isolates obtained >90% clonality and 1 isolation <90%. However, to confirm the results of this international comparison, it is required to use the multilocus sequence typing technique (MLST) with the isolates. The England14-9 clonal complex generally belongs to serotype 14 and is typically resistant to ERY, but sensitive to TET and CHL (9). In a study carried out in Brazil, they found 2 strains of *S. pneumoniae* belonging to serotype 14 resistant to erythromycin, cloned with the clone England14-9 but sensitive to PEN (48). Canadian *S. pneumoniae* isolates studied between 2007 and 2013 were related (at least five out of seven MLST allele numbers in

common) to Spain23F-1 (ST81), Spain9V-3 (ST156), England14-9 (ST9), Taiwan19F -14 (ST236) and Sweden 15A-25 (ST63) (49).

The high rate of resistance can be caused by the indiscriminate use of antibiotics, the presence of a particular genotype, vaccination rates, and the presence of multi-resistant clones (50). The increase in resistance of *S. pneumoniae* to antibiotics such as penicillin, macrolides and trimethoprim-sulfamethoxazole is a worldwide concern, therefore it is relevant to control this resistance through strategies such as surveillance of circulating clones in order to monitor the resistance patterns and thus allow the proper use of antimicrobials that reduce the number of patients with invasive diseases.

Funding information

This work was supported by grants from the Mercosur Structural Convergence Fund (FOCEM) - Mercosur, FOCEM agreement N° 03/11 Project "Research, Education and Biotechnologies Applied to Health (COF 03/11).

Conflicts of interest

The authors declare that there no conflicts of interest.

Authors and contributors

Nombre	ORCID	Roles
León Ayala María Eugenia	0000-0001-7416-249X	Conceptualization and Methodology Investigation and Supervision Validation and Writing – Review and Editing
Kawabata Anibal	0000-0002-3960-435X	Conceptualization and Methodology Data Curation and Formal Analysis Investigation and Validation Writing - Review & Editing
Helena Ortiz	0000-0001-5617-1528	Supervision
Chamorro Cortesi Gustavo	0000-0003-3959-1533	Supervision and Project Administration Writing –Review and Editing

REFERENCES

Croney CM, Nahm MH, Juhn SK, Briles DE, Crain MJ. Invasive and noninvasive *Streptococcus pneumoniae* capsule and surface protein diversity following the use of a conjugate vaccine. Clin Vaccine Immunol. 2013; 20(11):1711-1718. doi:10.1128/CVI.00381-13

Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med. 2013; 3 (7): a010215. doi:10.1101/cshperspect.a010215.

World Health Organization. 2014. Global immunization data. World Health Organization, Geneva, Switzerland: http://www.who.int/immunization/monitoring_surveillance/global_immunization_data.pdf?ua=1 Accessed 21 September 2020.

Michelin L, Weber FM, Scolari BW, Menezes BK, Gullo MC. Mortality and costs of pneumococcal pneumonia in adults: a cross-sectional study. J. bras. pneumol. [Internet]. 2019 [cited 2020 Sep 22] ; 45(6): e20180374. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1806-37132019000600204&lng=en. Epub Oct 17, 2019. https://doi.org/10.1590/1806-3713/e20180374.

Agudelo B. Tratamiento de la neumonía por *Streptococcus pneumoniae* y consideraciones de resistencia. Neumol Pediatr. 2013; 8 (2): 86-90.

Ganaie F, Saad JS, McGee L, van Tonder AJ, Bentley SD, Lo SW, et al. A New Pneumococcal Capsule Type, 10D, is the 100th Serotype and Has a Large cps Fragment from an Oral Streptococcus. mBio. 2020; 11:e00937-20. https://doi.org/10.1128/mBio.00937-20.

Ktari S, Jmal I, Mroua M, Maalej S, Ben Ayed EN, Mnif B, et al. Serotype distribution and antibiotic susceptibility of *Streptococcus pneumoniae* strains in the south of Tunisia: A five-year study (2012–2016) of pediatric and adult populations. International Journal of Infectious Diseases 2017; 65: 110-117. https://doi.org/10.1016/j.ijid.2017.10.015.

del Amo E, Brotons P, Monsonis M, Trivióo M, Ióigo M, Selva L, et al. High invasiveness of pneumococcal serotypes included in the new generation of

conjugate vaccines. Clinical Microbiology and Infection. 2014; 20 (7): 684-689. https://doi.org/10.1111/1469-0691.12422.

Pinto TCA, Kegele FCO, Dias CAG, Barros RR, Peralta JM, Merquior VLC, et al. *Streptococcus pneumoniae* Serotypes 9 and 14 Circulating in Brazil over a 23-Year Period Prior to Introduction of the 10-Valent Pneumococcal Conjugate Vaccine: Role of International Clones in the Evolution of Antimicrobial Resistance and Description of a Novel Genotype. Antimicrobial Agents and Chemotherapy. 2016; 60 (11): 6664-72; DOI: 10.1128/AAC.00673-16.

Toda H, Satoh K, Komatsu M, Fukuda S, Nakamura T, Jikimoto T, et al. Laboratory surveillance of antimicrobial resistance and multidrug resistance among *Streptococcus pneumoniae* isolated in the Kinki region of Japan, 2001–2015. J Infect Chemother. 2018; 24: 171–176. https://doi.org/10.1016/j.jiac.2017.12.010.

Kim L, McGee L, Tomczyk S, Beall B. Biological and Epidemiological Features of Antibiotic-Resistant *Streptococcus pneumoniae* in Pre- and Post-Conjugate Vaccine Eras: a United States Perspective. Clin Microbiol Rev. 2016; 29(3): 525-552. doi:10.1128/CMR.00058-15.

Organización Panamericana de la Salud. Informe Regional de SIREVA II, 2011. Datos por país y por grupos de edad sobre las características de los aislamientos de *Streptococcus pneumoniae*, *Haemophilus influenzae* y *Neisseria meningitidis* en procesos invasores. Documentos técnicos. Regulaciones Sanitarias Internacionales, Alerta y Respuesta y Enfermedades Epidémicas (HSD/IR). Washington, D.C.: OPS; 2012. https://www.paho.org/hq/index.php?option=com_content&view=article&id=5536:2011-sireva-ii&Itemid=3966&lang=pt.

Organización Panamericana de la Salud. Informe Regional de SIREVA II, 2012. Datos por país y por grupos de edad sobre las características de los aislamientos de *Streptococcus pneumoniae*, *Haemophilus influenzae* y *Neisseria meningitidis* en procesos invasores. Documentos técnicos. Regulaciones Sanitarias Internacionales, Alerta y Respuesta y Enfermedades Epidémicas (HSD/IR). Washington, D.C.: OPS; 2013. https://www.paho.org/hq/index.php?option=com_content&view=article&id=5536:2011-sireva-ii&Itemid=3966&lang=pt.

Organización Panamericana de la Salud. Informe Regional de SIREVA II, 2013. Datos por país y por grupos de edad sobre las características de los aislamientos de *Streptococcus pneumoniae*, *Haemophilus influenzae* y *Neisseria meningitidis* en procesos invasores. Documentos técnicos. Regulaciones Sanitarias Internacionales, Alerta y Respuesta y Enfermedades Epidémicas (HSD/IR). Washington, D.C.: OPS; 2014. https://www.paho.org/hq/index.php?option=com_content&view=article&id=5536:2011-sireva-ii&Itemid=3966&lang=pt.

Parizad EG, Parizad EG, Valizadeh A. The Application of Pulsed Field Gel Electrophoresis in Clinical Studies. J Clin Diagn Res. 2016; 10(1):DE01-DE4. doi:10.7860/JCDR/2016/15718.7043.

Rayner RE, Savill J, Hafner LM, Huygens F. Genotyping *Streptococcus pneumoniae*. Future Microbiology. 2015; 10 (4): 653-664. https://doi.org/10.2217/fmb.14.153.

Donkor E. Molecular typing of the pneumococcus and its application in epidemiology in sub-Saharan Africa. Frontiers in cellular and infection microbiology. 2013; 3 (12): 1-5. doi:10.3389/fcimb.2013.00012.

Palacios PA, Duarte C, Sanabria O, Moreno J. Molecular characterization of non-vaccine *Streptococcus pneumoniae* serotypes 11A, 15 B/C and 23A recovered from invasive isolates in Colombia. Biomédica [Internet]. 2017 Sep [cited 2020 Sep 25]; 37(3): 390-396. Available from: http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0120-41572017000300390&lng=en. http://dx.doi.org/10.7705/biomedica.v34i2.3223.

Zhang J, Hu D-K, Gao C-Y, Shen W-W, Luo X-H, Yuet J, et al. Homology analysis of 51 penicillin-intermediate *Streptococcus pneumoniae* isolates from Wenzhou City, China. Journal of International Medical Research. 2020; 48(6) 1–9. https://doi.org/10.1177/0300060520919061.

Organización Panamericana de la Salud e Instituto Nacional de Salud de Colombia. Programa de vigilancia de los serotipos y resistencia antimicrobiana de Streptococcus pneumoniae y Haemophilus influenzae. Manual de procedimientos del proyecto. SIREVA II, 2004, p. 41-99. Fecha de consulta: 9 de diciembre de 2011. Disponible en: http://

new.paho.org/hq/index.php?option=com_content&view=cate gory&layout=blog&id=3609&Itemid=3953&lang=fr. 9.

Clinical Laboratory Standards Institute. MIC testing. Supplemental tables, (M2-A9). Wayne, PA: Clinical Laboratory Standards Institute. 2013.

Tenover FC, Arbeit RD, Gaering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial isolate typing. J Clin Microb 1995; 33:2233-9.

Soares S, Kristionsson KG, Musser JM, Tomasz A. Evidence for introduction of a multiresistant clone of serotype 6B *Streptococcus pneumoniae* from Spain to Iceland in late 1980s. J Infect Dis 1993;168:158-63.

Vela MC, Fonseca N, Di Fabio JL, Castañeda E. Presence of international multiresistant clones of *Streptococcus pneumoniae* in Colombia. Microb Drug Resist 2001; 7:153-64.

Bardach A E, Rey-Ares L, Calderon Cahua M, Ciapponi A, Cafferata ML, Cormick G, Gentile A. Burden of culture-confirmed pediatric pneumococcal pneumonia in Latin America and the Caribbean: a systematic review and meta-analysis. Value Health Reg Issues. 2017; 14:41–52. doi:10.1016/j.vhri.2017.04.004.

Richter SS, Heilmann KP, Dohrn CL, et al. Pneumococcal serotypes before and after introduction of conjugate vaccines, United States, 1999-2011. Emerging Infectious Diseases. 2013; 19(7): 1074-1083. DOI: 10.3201/eid1907.121830.

Ma X, Zhao R, Ma Z, Yao K, Yu S, Zheng Y et al. Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* isolates causing invasive diseases from Shenzhen Children's Hospital. PLoS One. 2013; 8(6): e67507. doi:10.1371/journal.pone.0067507.

Ludwig G, Garcia-Garcia S, Lanaspa M, Ciruela P, Esteva C, Fernandez de Sevilla M, et al. (2020) Serotype and clonal distribution dynamics of invasive pneumococcal strains after PCV13 introduction (2011-2016): Surveillance data from 23 sites in Catalonia, Spain. PLoS ONE 15(2): e0228612. https://doi.org/10.1371/journal.pone.0228612.

Pichon B, Ladhani SN, Slack MP, Segonds-Pichon A, Andrews NJ, Waight PA, Miller E, George R. Changes in molecular epidemiology of *Streptococcus pneumoniae* causing meningitis following introduction of pneumococcal conjugate vaccination

in England and Wales. J Clin Microbiol. 2013; 51(3):820-7. doi: 10.1128/JCM.01917-12.

León ME, Kawabata A, Nagai M, Rojas L, Zárate N, Irala J, et al. Frecuencia de *Streptococcus pneumoniae* aislados de enfermedad invasiva en Paraguay, serotipos y perfil de sensibilidad (2010- 2018). Mem. Inst. Investig. Cienc. Salud. 2020; 18(1): 38-46.

Abreu-Capote M, Dickinson-Meneses F, Pías-Solis L, Rodríguez-Ortega M, Toraño-Peraza G, Varcárcel-Sánchez M. Serotipos y resistencia antimicrobiana de aislamientos meníngeos de *Streptococcus pneumoniae*. Cuba, 2007-2012. Vaccimonitor [Internet]. 2014 Dic [citado 2020 Sep 28] ; 23(3): 117-123. Disponible en: http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S1025-028X2014000300006&lng=es.

Caierão J, Hawkins P, Sant'anna FH, da Cunha GR, d'Azevedo PA, McGee L, et al. Serotypes and Genotypes of Invasive *Streptococcus pneumoniae* Before and After PCV10 Implementation in Southern Brazil. PLoS ONE. 2014; 9(10): e111129. https://doi.org/10.1371/journal.pone.0111129.

Grenón SL, Salvi Grabulosa MC, Regueira MM, Fossati MS, von Specht MH. Meningitis neumocócica en niños menores de 15 años. Dieciséis años de vigilancia epidemiológica en Misiones, Argentina. 2014; 46 (1): 14-23. https://doi.org/10.1016/S0325-7541(14)70042-2.

Medeiros MIC, Almeida SCG, Bokermann S, Watanabe E, Guerra MLLS, Andrade D. Suscetibilidade antimicrobiana de *Streptococcus pneumoniae* isolados de pacientes na macrorregião nordeste do estado de São Paulo, Brasil, entre 1998 e 2013. J. Bras. Patol. Med. Lab.2017; 53(3):177-182.

Neves FP, Pinto TC, Corrêa MA, et al. Nasopharyngeal carriage, serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* among children from Brazil before the introduction of the 10-valent conjugate vaccine. BMC Infect Dis. 2013; 13:318. doi:10.1186/1471-2334-13-318.

Parra EL, Ramos V, Sanabria O, Moreno J. Serotype and Genotype Distribution among Invasive *Streptococcus pneumoniae* Isolates in Colombia 2005–2010. PLoS ONE. 2014; 9(1): e84993. doi:10.1371/journal.pone.0084993.

Kim CJ, Song JS, Choi SJ, Song KH, Choe PG, Park WB, et al. Serotype Distribution and Antimicrobial Susceptibilities of Invasive *Streptococcus pneumoniae* Isolates from Adults in Korea from 1997 to 2012. Journal of Korean medical science. 2016; 31(5), 715–723. https://doi.org/10.3346/jkms.2016.31.5.715.

Toda H, Tanaka Y, Satoh K, Komatsu M, Wada Y, Tobe T, et al. Epidemiological and molecular characterization of invasive streptococcus pneumoniae isolated following introduction of 7-valent conjugate vaccine in kinki region, japan, 2008-2013. Journal of Infection and Chemotherapy: Official Journal of the Japan Society of Chemotherapy. 2020; 26(5), 451–458. https://doi.org/10.1016/j.jiac.2019.11.010.

Diawara I, Barguigua A, Katfy K, et al. Molecular characterization of penicillin non-susceptible *Streptococcus pneumoniae* isolated before and after pneumococcal conjugate vaccine implementation in Casablanca, Morocco. Ann Clin Microbiol Antimicrob. 2017; 16(1):23. doi:10.1186/s12941-017-0200-6.

Vorobieva S Jensen V, Furberg AS, Slotved HC, et al. Epidemiological and molecular characterization of *Streptococcus pneumoniae* carriage strains in pre-school children in Arkhangelsk, northern European Russia, prior to the introduction of conjugate pneumococcal vaccines. BMC Infect Dis. 2020; 20(1): 279. doi: 10.1186/s12879-020-04998-5.

Yahiaoui RY, Bootsma HJ, den Heijer CDJ, Pluister GN, Paget WJ, Spreeuwenberg P, et al. Distribution of serotypes and patterns of antimicrobial resistance among commensal *Streptococcus pneumoniae* in nine European countries. BMC Infect Dis. 2018; 18 440. https://doi.org/10.1186/s12879-018-3341-0.

Barroso DE, Godoy D, Castiñeiras TM, Tulenko MM, Rebelo MC, Harrison LH. β-Lactam resistance, serotype distribution, and genotypes of meningitis-causing *Streptococcus pneumoniae*, Rio de Janeiro, Brazil. The Pediatric infectious disease journal. 2012; 31(1), 30–36. https://doi.org/10.1097/INF.0b013e31822f8a92.

Pinto TCA, Costa NS, Pina SECM, Souza ARV, Oliveira LMA, Moura CAB, et al. Virulence-Associated Characteristics of Serotype 14 and Serogroup 9 *Streptococcus pneumoniae* Clones Circulating in Brazil: Association of Penicillin Non-susceptibility With Transparent Colony Phenotype Variants. Frontiers in Microbiology. 2020; 11:2009. https://doi.org/10.3389/fmicb.2020.02009.

Soeters HM, von Gottberg A, Cohen C, Quan V, Klugman KP. Trimethoprim-sulfamethoxazole prophylaxis and antibiotic nonsusceptibility in invasive pneumococcal disease. Antimicrob Agents Chemother. 2012; 56 (3): 1602-1605. DOI: 10.1128/AAC.05813-11.

Thummeepak R, Leerach N, Kunthalert D, Tangchaisuriya U, Thanwisai A, Sutthirat Sitthisak S. High prevalence of multi-drug resistant *Streptococcus pneumoniae* among healthy children in Thailand. Journal of Infection and Public Health. 2015; 8(3): 274-281. https://doi.org/10.1016/j.jiph.2014.11.002.

Schroeder MR. Stephens DS Macrolide resistance in *Streptococcus pneumoniae*. Mini Rev. Front Cell Infect Microbiol 2016; 6:98.

Reijtman V, Gagetti P, Faccone D, Fossati S, Sommerfleck P, Hernández C, et al. Macrolide resistance in *Streptococcus pneumoniae* isolated from Argentinian pediatric patients suffering from acute otitis media. Rev Argent Microbiol. 2013; 45(4):262-266.

Azevedo J, Silva dos Anjos E, Cordeiro SM, dos Santos MS, Escobar EC, Lobo PR, et al. Genetic profiles and antimicrobial resistance of *Streptococcus pneumoniae* non-PCV10 serotype isolates recovered from meningitis cases in Salvador, Brazil. Journal Medical Microbiology. 2016; 65(10): 1164–1170. https://doi.org/10.1099/jmm.0.000346.

Golden AR, Rosenthal M, Fultz B, Nichol KA, Adam HJ, Gilmour MW, et al. Characterization of MDR and XDR *Streptococcus pneumoniae* in Canada, 2007–13, Journal of Antimicrobial Chemotherapy. 2015; 70(8):2199–2202. https://doi.org/10.1093/jac/dkv107.

Chancey ST, Agrawal S, Schroeder MR, Farley MM, Tettelin H, Stephens DS. Composite mobile genetic elements disseminating macrolide resistance in *Streptococcus pneumoniae*. Front Microbiol 2015; 6:26. https://doi.org/10.3389/fmicb.2015.00026.