Accepted Manuscript

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PII: S2213-7165(17)30150-9
DOI: http://dx.doi.org/doi:10.1016/j.jgar.2017.08.004
Reference: JGAR 474

To appear in:

Received date: 29-6-2017
Revised date: 28-7-2017
Accepted date: 5-8-2017

Please cite this article as: Paulina A. Hawkins, Patrick E. Akpaka, Michele Nurse-Lucas, Rebecca Gladstone, Stephen D. Bentley, Robert F. Breiman, Lesley McGee, William H. Swanston, Antimicrobial resistance determinants and susceptibility profiles of pneumococcal isolates recovered in Trinidad and Tobago. (2010), http://dx.doi.org/10.1016/j.jgar.2017.08.004

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Antimicrobial resistance determinants and susceptibility profiles of pneumococcal isolates recovered in Trinidad and Tobago.

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Highlights

- A WGS-based approach can accurately and reliably predict antimicrobial phenotypes.
- The observed rates of non-susceptibility against cotrimoxazole and erythromycin among these isolates were lower than what has been reported for other countries in the region. In contrast, the proportion of \(\beta\)-lactam non-susceptibility was higher among these isolates.
- Multidrug resistance remains low, but appears to be expanding clonally after the introduction of pneumococcal vaccines, driven by the 19F-CC156 and 19A/F-CC236 lineages.
ABSTRACT

Introduction: In Latin America and the Caribbean, pneumococcal infections were estimated to account for 12,000-18,000 deaths, 327,000 cases of pneumonia, 4,000 cases of meningitis and 1,229 cases of sepsis each year in children under five years old. Resistance of pneumococci to antimicrobial agents has evolved into a worldwide health problem in the last few decades. Objective: The aim of this study was to determine the antimicrobial susceptibility profiles of 98 pneumococcal isolates collected in Trinidad and Tobago and associated genetic determinants. Methods: Whole genome sequences were obtained from 98 pneumococcal isolates recovered at several regional hospitals, including 83 invasive and 15 non-invasive strains, recovered before (n=25) and after (n=73) the introduction of two pneumococcal conjugate vaccines. A bioinformatics pipeline was used to identify core genomic and accessory elements that conferred antimicrobial resistance phenotypes, including β-lactam non-susceptibility. Results and discussion: Forty-one (41.8%) isolates were predicted as resistant to at least one antimicrobial class, including 13 (13.3%) isolates resistant to at least three classes. The most common serotypes associated with antimicrobial resistance were 23F (n=10), 19F (n=8), 6B (n=6), and 14 (n=5). The most common serotypes associated with penicillin non-susceptibility were 19F (n=7) and 14 (n=5). Thirty-nine (39.8%) isolates were positive for PI-1 or PI-2 type pili: 30 (76.9%) were PI-1+, 4 (10.3%) were PI-2+, and 5 (12.8%) were positive for both PI-1 and PI-2. Of the 13 isolates with multidrug resistance, 10 belonged to globally distributed clones PMEN3 and PMEN14 and were isolated in the post-PCV period, suggesting a clonal expansion.

Keywords: S. pneumoniae, antimicrobial resistance, whole genome sequencing.
1. INTRODUCTION

Infections caused by *Streptococcus pneumoniae* include diseases such as meningitis, bacteremia, and pneumonia as well as less severe conditions such as sinusitis and otitis media. The World Health Organization (WHO) estimated that pneumococcal infections caused 476,000 (5%) deaths globally among HIV-negative children under five years of age during 2008 (1). In Latin America and the Caribbean, pneumococcal infections were estimated to account for 12,000-18,000 deaths, 327,000 cases of pneumonia, 4,000 cases of meningitis and 1,229 cases of sepsis each year in children under five years old (2).

The first pneumococcal conjugate vaccine covered 7 serotypes (PCV7: 14, 6B, 19F, 23F, 4, 9V, 18C) and was licensed in 2000, followed by PCV10 (PCV7 serotypes plus 1, 5, and 7F) in 2009, and PCV13 (PCV10 serotypes plus 3, 6A, and 19A) in 2010. In the Caribbean, as well as other regions, vaccine uptake has been variable. PCV7 was introduced into the national immunization program (NIP) in Trinidad and Tobago in March 2010, for infants at risk of pneumococcal disease, mainly those with immune deficiencies and other chronic diseases. Prior to 2010, PCV7 was only available in the private sector (3). PCV10 was introduced into the NIP in 2011, for all children aged <2 years (3-5), and was replaced by PCV13 in August 2015. Vaccine coverage with PCV10 was reported to be 95% as of December 2014 (6).

From the beginning of the antibiotic era to the mid-1970s, *Streptococcus pneumoniae* remained uniformly susceptible to all classes of antibiotics that were active against it, with the exception of tetracycline. In the ensuing decades, resistance of pneumococci to a variety of antimicrobials has evolved into a worldwide health problem (7). A 2004 report by the SENTRY surveillance program
showed that penicillin non-susceptibility rates were as high as 25% among pneumococcal isolates, with penicillin-non-susceptible isolates presenting higher rates of multidrug resistance (8).

In the Caribbean region, infections due to penicillin-resistant pneumococci have been reported in hospitals and community settings, but limited data are available to estimate patterns of drug resistance (9). The aim of this study was to determine the antimicrobial susceptibility profiles of 98 pneumococcal isolates collected in Trinidad and Tobago from invasive and non-invasive sites, and their associated genetic determinants.

2. MATERIALS AND METHODS

Ninety-eight pneumococcal isolates recovered at several regional hospitals in Trinidad and Tobago during the period were included in the study. These isolates included 83 invasive and 15 non-invasive strains. All pneumococcal isolates (n = 73) obtained from routine clinical specimens at the five major public hospitals in Trinidad and Tobago, during the period 2011 to 2013, were included in this study. A number of historical pneumococcal isolates (n = 25) from clinical specimens that were collected between 1997 and 2010 were also included in the analysis; most of the strains were collected from three regional hospitals, prior to the start of the SIREVA (Sistema Regional de Vacunas) project in Trinidad and Tobago. Serotyping and multilocus sequence typing (MLST) results from these 98 isolates have been previously published (10). Minimum inhibitory concentrations (MIC) were determined using broth microdilution, as specified by the Clinical and Laboratory Standards Institute guidelines (11). Penicillin susceptibility, intermediate resistance, and resistance were defined as MIC ≤0.06, 0.12–1.0, and ≥2.0 mg/L, respectively. Cefotaxime and/or ceftriaxone susceptibility, intermediate resistance, and resistance were defined as MIC ≤0.5, 1.0, and ≥2.0 mg/L, respectively. For amoxicillin, susceptibility, intermediate resistance, and resistance were defined as MIC ≤0.12, 0.25–1, and ≥2.0 mg/L, respectively. For
meropenem, susceptibility, intermediate resistance, resistance and high-level resistance were defined at \( \leq 0.25, 0.5, 1.0, \) and \( \geq 2.0 \), respectively. For previously unreported PBP types, MIC values against penicillin and cefotaxime were determined using E-tests (Biomérieux, Marcy l’Etoile, France).

*S. pneumoniae* strains were cultured on Trypticase soy agar (TSA) supplemented with 5% sheep blood and incubated overnight at 37°C in 5% CO2. Genomic DNA was then extracted manually using a modified QIAamp DNA mini kit protocol (Qiagen, Inc., Valencia, CA). Whole genome sequencing was performed at the Sanger Institute using the Illumina HiSeq 2500 system, as part of the Global Pneumococcal Sequencing project (www.pneumogen.net), and the resulting data submitted to the European Nucleotide Archive (accession numbers in Table S1). Sequences were analyzed using the CDC’s *Streptococcus* laboratory pneumococcal typing pipeline (12-13) to identify core genomic alterations and accessory elements that confer antimicrobial resistance phenotypes, as well as pilus genes (https://github.com/BenJamesMetcalf/Spn_Scripts_Reference). Non-susceptibility to \( \beta \)-lactams was predicted based on three key penicillin-binding protein (PBP) transpeptidase domain sequences, or PBP types (13-14), which correlate with recorded MIC values for each of the six \( \beta \)-lactams: penicillin, amoxicillin, ceftriaxone, cefotaxime, cefuroxime, and meropenem (http://www.cdc.gov/streplab/mic-tables.html). Contingency tables and a chi-squared test (or a Fisher’s exact test) were used to determine significance of associations (at \( \alpha=0.05 \)).

### 3. RESULTS AND DISCUSSION

#### 3.1 Antimicrobial resistance

The capability of a WGS-based approach to accurately and reliably predict antimicrobial phenotypes has been previously shown to be an adequate substitute for broth dilution susceptibility testing (13-14). By a WGS-based assessment of resistance, 34 (34.7%) isolates in this study were predicted to be non-susceptible to cotrimoxazole, 18 (18.4%) resistant to erythromycin, 17 (17.3%) non-susceptible to \( \beta \)-
lactams, 9 (9.2%) resistant to tetracycline, 2 (2.0%) resistant to chloramphenicol, and 1 (1.0%) resistant to rifampin. These results were in agreement with the MIC values determined by broth microdilution (Table 1). Overall, 41 (41.8%) isolates were predicted as resistant to at least one antimicrobial class, including 13 (13.3%) isolates resistant to at least three classes (multidrug resistant, MDR). Before PCV7/10 introduction, 28% (n=7) of the isolates were resistant to at least one antimicrobial class; this proportion increased to 46.6% (n=34) in the post-PCV period (p=0.06). The proportion of MDR isolates increased from 4% (n=1) to 16.4% (n=12) after PCV7/10 introduction (p=0.06).

Thirty-seven PBP allele combinations (PBP types) were identified among these isolates, 11 of them novel; 8 (21.6%) of these combinations (3 of them novel) were associated with non-susceptibility to penicillin (MIC $\geq$0.12 µg/ml). Identifying new allele combinations was expected, as the database used for analyses mostly contains isolates from the United States. Of the 17 isolates that were predicted as non-susceptible to $\beta$-lactams, all were predicted as non-susceptible to penicillin (PNS); nine were predicted as non-susceptible to ceftriaxone, cefuroxime, cefotaxime, and meropenem (in addition to penicillin), and three isolates were predicted as non-susceptible to all six of the $\beta$-lactams tested.

Of the 34 isolates predicted as non-susceptible to cotrimoxazole, all contained 1-2 codon insertions within the folIP gene (intermediate phenotype, MIC 1-2µg/ml), while 19 of them (55.9%) also contained the I100L substitution in folA (resistant phenotype, MIC $\geq$4µg/ml). Of the 18 isolates predicted as resistant to erythromycin: 11 (61.1%) were positive for mef alone, 2 (11.1%) for ermB alone, and 5 (27.8%) for ermB plus mef; these seven isolates containing ermB were also predicted as resistant to clindamycin. In addition, nine isolates were positive for tetM and two isolates for the cat gene. One isolate contained a change in the rpoB gene (H499Y), and was resistant to rifampin by broth microdilution (MIC >2µg/ml).
The observed rate of non-susceptibility against cotrimoxazole was lower than what has been reported for other countries in the region (15), like Venezuela (100%) and the Dominican Republic (65%); the rate of resistance against erythromycin was similarly lower (vs 45% in Venezuela and 20% in Dominican Republic). In contrast, the proportion of β-lactam non-susceptibility was higher among these isolates than among isolates from the Dominican Republic (9.6%), but similar to that observed among isolates recovered in Venezuela (18.2%).

The most common serotypes associated with antimicrobial resistance were 23F (n=10), 19F (n=8), 6B (n=6), and 14 (n=5). The most common serotypes associated with PNS were 19F (n=7) and 14 (n=5). The 13 isolates with resistance against multiple drugs belonged to only four different CCs/STs and four serotypes, mostly CC156 (global clone PMEN3) and CC236 (global clone PMEN14), suggesting a clonal expansion after PCV7/10 introduction (Table 2).

3.2 Pilus genes

In *S. pneumoniae*, pili are encoded by two different pathogenicity islets, type 1 (PI-1) and type 2 (PI-2). The Pilus Islet-1, particularly the RrgA subunit, has been shown to not only contribute to adherence and virulence, but to also stimulate the host inflammatory response (16). The Pilus Islet-2 has also been shown to contribute to adherence, but in a less effective manner than PI-1 (17). Overall, 39 (39.8%) isolates were positive for PI-1 or PI-2 type pili (inferred by detection of rrgA or pitB pilus subunit genes): 30 (76.9%) of them were solely PI-1+, 4 (10.3%) were PI-2+, and 5 (12.8%) were positive for both PI-1 and PI-2.
Consistent with previous reports (18), we observed that the presence of PI-1 or both of the pilus loci was associated with certain clonal complexes (CCs) and serotypes (Table 3), and in consequence, with antimicrobial susceptibility profiles. Eleven of the 30 (28.9%) PI-1-only isolates belonged to CC156 (PMEN3) and serotypes 9V, 14, and 19F; all serotype 9V isolates were susceptible to all antibiotics tested, all serotype 14 were PNS, and all 19F isolates were MDR. In addition, 7 (23.3%) PI-1-only isolates belonged to ST138 (5 of them serotype 6B) and 5 (16.7%) to CC145 (all serotype 6B); the ST138 isolates were susceptible to all drugs, while three of the CC145 isolates were non-susceptible to cotrimoxazole (intermediate phenotype). All five PI-1+PI-2 isolates belonged to CC236 (PMEN14) and serotypes 19A/19F and were MDR. Three of the four (75.0%) PI-2-only isolates belonged to CC62 (serotype 11A) and the remaining one to ST191 (serotype 7F); all four were susceptible to all antimicrobials tested.

In conclusion, this study offered a snapshot of the antimicrobial resistance profiles and genetic determinants of resistance among 98 pneumococcal isolates recovered in Trinidad and Tobago, adding to the limited body of data available for the Caribbean region. The observed rates of resistance were similar to those reported for neighboring Caribbean countries. Multidrug resistance remains low, but appears to be expanding clonally after PCV7/10 introduction, driven by the 19F-CC156 and 19A/F-CC236 lineages. Thus, the introduction of PCV13 will likely have a marked impact on multidrug resistance in Trinidad and Tobago.

**Declarations**

**Funding:** Bill and Melinda Gates Foundation grant # OPP1034556

**Ethical approval:** Not required
Competing interests: None to declare.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

ACKNOWLEDGEMENTS

Funding: Isolates were characterized as part of the Global Pneumococcal Strain Bank established with funding from PATH and currently housed at CDC (https://www.cdc.gov/streplab/global-pneumo-strain-bank.html). Whole genome sequencing was performed as part of the Global Pneumococcal Sequencing project, funded by the Bill and Melinda Gates Foundation (grant number OPP1034556). The funding sources had no involvement in the study design, the collection, analysis or interpretation of data, the writing of the report, or the decision to submit the article for publication.
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1. WHO. Estimated Hib and pneumococcal deaths for children under 5 years of age, 2008. (http://www.who.int/immunization/monitoring_surveillance/burden/estimates/Pneumo_hib/en/)


Table 1. Non-susceptibility predicted by whole genome sequencing vs non-susceptibility determined from observed MIC values.

<table>
<thead>
<tr>
<th>Resistance determinants</th>
<th>Predicted non-susceptible</th>
<th>Observed non-susceptible</th>
<th>Observed MIC Range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ERY²</td>
<td>CLD²</td>
</tr>
<tr>
<td>folA+folP¹</td>
<td>19</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>folP-only¹</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>ermB-only</td>
<td>2</td>
<td>2</td>
<td>&gt;32</td>
</tr>
<tr>
<td>mef-only</td>
<td>11</td>
<td>10³</td>
<td>0.12-16</td>
</tr>
<tr>
<td>ermB+mef</td>
<td>5</td>
<td>5</td>
<td>&gt;32</td>
</tr>
<tr>
<td>tetM</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>cat</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>rpoB (H499Y)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

1. 1-2 codon insertions within the folP gene (at nt 171, 176, 177, 178, 180, 185, 186, or 195) result in an intermediate phenotype (MIC 1-2 µg/ml) against cotrimoxazole; when combined with the folA substitution I100L, they result in a resistant phenotype (MIC ≥4 µg/ml).


3. One isolate was susceptible to erythromycin in spite of being mef+ (MIC 0.12 µg/ml).
Table 2. Clonal complexes and sequence types associated with multidrug resistant isolates.

<table>
<thead>
<tr>
<th>CC/ST</th>
<th>n</th>
<th>Serotype (n)</th>
<th>PBP types (n)</th>
<th>Resistance phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC156 (PMEN3)</td>
<td>4</td>
<td>19F(4)</td>
<td>15:12:36 (3) 121:12:36 (1)</td>
<td>SXT, ERY, PEN, TAX, CFT, CFX, MER</td>
</tr>
<tr>
<td>CC236 (PMEN14)</td>
<td>6</td>
<td>19F(3)</td>
<td>13:16:47 (2) 119:16:47 (1)</td>
<td>SXT, ERY, CLD, TET, PEN, TAX, CFT, CFX, MER</td>
</tr>
<tr>
<td>ST554</td>
<td>2</td>
<td>14</td>
<td>120:16:80 (14)</td>
<td>ERY, CLD, TET, CHL, PEN</td>
</tr>
<tr>
<td>ST490</td>
<td>1</td>
<td>6A</td>
<td>susceptible</td>
<td>SXT, ERY, RIF</td>
</tr>
</tbody>
</table>

Table 3. Association between the presence of pilus loci, sequence type, and serotype.

<table>
<thead>
<tr>
<th>ST/CC</th>
<th>n</th>
<th>PI1</th>
<th>PI2</th>
<th>PI1+PI2</th>
<th>Associated serotypes (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC156</td>
<td>11</td>
<td>11</td>
<td></td>
<td></td>
<td>19F(4), 9V(4), 14(3)</td>
</tr>
<tr>
<td>ST138</td>
<td>10</td>
<td>7</td>
<td></td>
<td></td>
<td>6B(5), 6A(1), 19F(1)</td>
</tr>
<tr>
<td>CC145</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td>6B(5)</td>
</tr>
<tr>
<td>CC236</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td></td>
<td>19A(3), 19F(3)</td>
</tr>
<tr>
<td>CC62</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
<td>11A(3)</td>
</tr>
<tr>
<td>ST191</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td>7F(1)</td>
</tr>
</tbody>
</table>

ST: sequence type, CC: clonal complex, PI1: pilus locus 1, PI2: pilus locus 2.