An International Serotype 3 Clone Causing Pediatric Noninvasive Infections in Israel, Costa Rica, and Lithuania

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Background: Serotype 3 is known for its ability to cause invasive diseases worldwide. In the United States, after introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), the prevalence of a serotype 3 clone (Netherlands3-31/ST180) increased. The present study was aimed to evaluate the importance of serotype 3 clones in noninvasive infections in Israel, Costa Rica, and Lithuania.

Methods: Molecular typing and antibiotic resistance were performed on 77 serotype 3 strains recovered from pediatric noninvasive infections during 2003–2005, and on 50 carried strains from healthy carriers.

Results: Serotype 3 ranked second among isolates from noninvasive infections in Costa Rica and Lithuania, and seventh among the Israeli isolates. Pulsed field gel electrophoresis (PFGE) analysis revealed the presence of 1 major cluster (64/77, 83%); this cluster comprised 60/64 fully susceptible strains that corresponded to the Netherlands3-31/ST180 clone, and 4/64 multidrug-resistant strains, all from Lithuania, that corresponded to ST505, a double locus variant of ST180. Two additional fully susceptible clones, ST458 (11/77, 14%) and ST1116 (2/77, 3%), were found among the Israeli and Costa Rican strains, respectively. The same PFGE clusters identified among noninvasive infections were found among 50 isolates from carriers, with the same molecular characteristics.

Conclusions: Serotype 3 accounts for a large proportion of mucosal disease in children, even before the introduction of PCV7. The data presented here describe for the first time the importance of a multidrug-resistant serotype 3 clone, ST505, in noninvasive infections.

Key Words: Streptococcus pneumoniae, serotype 3, antibiotic resistant, international clones

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S. pneumoniae is a major pathogen involved in invasive disease and in noninvasive infections such as acute otitis media (AOM) and acute conjunctivitis. Several studies have shown that the serotype is the primary determinant in disease potential of S. pneumoniae, and that a limited number of serotypes cause most diseases.1–3

A multicenter study conducted in the United States demonstrated that the proportion of cases of AOM caused by serotype 3 clone sequence type (ST)-180 (www.mlst.net) increased from 3% in 1999 to 11% in 2002,4 after the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in the United States. We have also assessed the disease potential of individual serotypes among a pediatric population in Israel before the introduction of a PCV7 vaccination program, by relating their prevalence among isolates from different anatomic sites to nasopharyngeal carriage in healthy children residing in the same community during the same period, and found a significant positive association between serotype 3 isolates and cases of AOM and acute conjunctivitis.5 The present study evaluated the importance of serotype 3 clones in noninvasive infections caused by S. pneumoniae. To this end, the following objectives were addressed: (1) assessment of the prevalence of serotype 3 strains isolated from children with AOM, acute conjunctivitis, and sinusitis in Israel, Costa Rica, and Lithuania; (2) determination of the clonality of these isolates, and (3) comparison of the clones that were isolated from patients with noninvasive infections (AOM, acute conjunctivitis, and sinusitis) with those isolated from the nasopharynx (NP) of healthy children.

MATERIALS AND METHODS

Databases. Three databases from Israel, Costa Rica, and Lithuania, containing information on pneumococci isolated during 2003–2005 from the NP and oropharynx (OP) (carried isolates), and from the middle ear fluid (MEF), conjunctiva, and sinuses (noninvasive infections) were used. PCV7 had not been introduced in Israel and Lithuania at the time the isolates were collected. In Costa Rica, Prevenar was introduced to the private market in 2004, and approximately 15% of the pediatric population receives the vaccine. However, none of the Costa Rican patients from whom serotype 3 was isolated received the PCV7. The database from Israel consti-
tuted 2788 *S. pneumoniae* strains recovered from children aged 0–18 years: 1041 from NP and 1747 from noninvasive infections, including 1463 from MEF and 284 from conjunctiva; the database from Costa Rica constituted 359 strains from children aged 0–4 years: 174 from NP, 62 from OP, and 123 from noninvasive infections, all from MEF; the database from Lithuania constituted 156 strains recovered from children aged 0–18 years: 106 from NP and 50 from noninvasive infections, including 7 from MEF, 39 from sinuses, and 4 from conjunctiva.

Isolates were confirmed as *S. pneumoniae* by inhibition with optochin and by a positive slide agglutination test (Phadebact; Pharmacia Diagnostics, Uppsala, Sweden). One *S. pneumoniae* colony per culture was subcultured, harvested, and kept frozen at −70°C for further testing.

**Antibiotic Susceptibility Testing.** Susceptibility of isolates to penicillin, erythromycin, tetracycline, chloramphenicol, clindamycin, and trimethoprim-sulfamethoxazole (TMP/SMX) was performed by the disc-diffusion method following the NCCLS recommendations. Isolates exhibiting an inhibition zone with a radius ≥19 mm around a 1-µg oxacillin disc were further tested for susceptibility to penicillin by E-test (PDM Epsilometer; AB biodisk, Solna, Sweden) following the manufacturer’s instructions. Isolates with a penicillin MIC <0.1 µg/mL were considered susceptible to penicillin, and those with an MIC ≥0.1 µg/mL were considered nonsusceptible. Isolates with resistance to ≥3 antibiotic classes were considered multidrug-resistant (MDR).

**Serogrouping and Serotyping.** Serogrouping and serotyping were performed by means of the quellung reaction using sera provided by Statens Serum Institute of Copenhagen, Denmark.

**Pulsed Field Gel Electrophoresis.** Chromosomal DNA fragments, generated by *Sma*I digestion, were prepared and analyzed as described elsewhere. A CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA) was used for running the gels. Running conditions were 23 hours at 11.3°C at 200 V ramped with initial forward time of 5 seconds and final forward time of 35 seconds. Interpretation of strains relatedness on the basis of PFGE pattern was according to current consensus.

**Multilocus Sequence Typing.** Pneumococcal isolates were unambiguously characterized by multilocus sequence typing (MLST) as described by Enright and Spratt. The sequences (alleles) at each locus were compared with those at the MLST website (www.mlst.net) and were assigned allele numbers if they corresponded to sequences already submitted to the pneumococcal MLST database. The allelic profiles of isolates (the allele numbers at the 7 loci) were compared with those at the MLST website, and sequence types (STs) were assigned.

**RESULTS**

**Prevalence of Serotype 3 Strains.** The distribution of *S. pneumoniae* serotypes in children with AOM, acute conjunctivitis, and sinusitis (noninvasive infections) was examined in each of the 3 databases (Table 1). Among the 1747 pneumococci that were isolated from noninvasive infections of Israeli children, the most common capsular type was 14 (225, 13%) followed by serotypes 3 and 19F (202, 12%), 19A (168, 10%), 23F (165, 9%), 6A and 6B (128 each, 7%), nontypable (107, 6%), and 3 (85, 5%). Among the 123 *S. pneumoniae* isolated from noninvasive infections in Costa Rican children, serotype 19F was most common (21, 17%), followed by serotypes 3 and 14 (18 each, 15%), 6B (15, 12%), 23F (11, 9%), 11A (7, 6%), 15B/C (5, 4%), and 9V (4, 3%). Serotype 19F was also the most common among the 50 noninvasive strains from Lithuanian children (8, 16%), followed by serotypes 3 and 6B (7 each, 14%), 6A (5, 10%), nontypable (4, 8%), 23F and 19A (3 each, 6%), 15A, 18C, and 37 (2 each, 4%). Thus, serotype 3 ranked second among both Costa Rican and Lithuanian noninvasive specimens, and seventh in the Israeli specimens.

Of the non-PCV7 serotypes, the most common serotype among the Costa Rican and Lithuanian noninvasive infection strains was serotype 3, which comprised 15% and 14%, respectively. This serotype ranked fourth among the non-PCV7 serotypes of the Israeli strains accounting for 5% of the noninvasive infections.

**Molecular Analysis of Serotype 3.** All the serotype 3 strains recovered from noninvasive infections of Cost Rican and Lithuanian children, and representative isolates from Israeli children were analyzed by PFGE (Fig. 1, available online only); these included 18 MEF organisms from Costa Rican children, 6 organisms from the sinuses, and 1 from MEF of Lithuanian children, and 46 from MEF and 6 from the conjunctiva of Israeli children (total of 77 isolates from noninvasive infections).

<table>
<thead>
<tr>
<th>Rank</th>
<th>Israel N = 1747</th>
<th>Costa Rica N = 123</th>
<th>Lithuania N = 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>19F</td>
<td>19F</td>
</tr>
<tr>
<td>2</td>
<td>19F</td>
<td>19F</td>
<td>19F</td>
</tr>
<tr>
<td>3</td>
<td>19A</td>
<td>6B</td>
<td>6A</td>
</tr>
<tr>
<td>4</td>
<td>23F</td>
<td>23F</td>
<td>9V Nontypable³</td>
</tr>
<tr>
<td>5</td>
<td>6A, 6B</td>
<td>11A</td>
<td>23F, 19A</td>
</tr>
<tr>
<td>6</td>
<td>Nontypable³</td>
<td>15B/C</td>
<td>15A, 18C, 37</td>
</tr>
<tr>
<td>7</td>
<td>7, 9V</td>
<td>3, 7C, 7P, 8, 9N, 15C, 38</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers are the percentage of isolates of the indicated serotype.*

³Nontypable, refers to strains reacting negatively with all pooled sera and with omni serum.
The PFGE patterns of the serotype 3 strains recovered from noninvasive infections revealed the presence of 1 major cluster (64/77, 83%), arbitrarily designated as A. Most of the isolates belonging to this cluster (60/64, 94%) were fully susceptible to penicillin, erythromycin, tetracycline, chloramphenicol, clindamycin, and TMP/SMX. On the other hand, 4 isolates (4/64, 6%) recovered from children with sinusitis, all from Lithuania, were MDR being resistant to erythromycin, tetracycline, and chloramphenicol. The 4 MDR strains had a PFGE profile with an additional band at about 340 kilobases compared with the susceptible, cluster A, strains (Fig. 1). Three representative strains from noninvasive infections with clusters A-PFGE pattern, including 2 MEF isolates from Israel and Costa Rica and 1 MDR isolate recovered from the sinus of a Lithuanian child, were further characterized by MLST (Table 2). The MLST data revealed that the cluster A strains, which were susceptible to all antimicrobial agents tested (60/64), had the same allelic profile as the international Netherlands3-ST180 clone.12 However, the MDR, cluster A strains from Lithuania (4/64) differed from ST180 in 2 alleles, *aroE* and *gdh*, and were designated as ST505.

In addition to cluster A, 2 smaller clusters were also identified: cluster B (11/77, 14%), with 10 isolates from MEF and 1 from conjunctiva, all from Israel, and cluster C (2/77, 3%) with 2 MEF isolates from Costa Rica. MLST of representative isolates from cluster B and cluster C assigned them to ST458 and ST1116, respectively (Table 2). Molecular Analysis of Serotype 3 Carrier Strains. The carriage state is considered to constitute a prerequisite for mucosal infections. Therefore, we examined by PFGE the clonal characteristics of all (50) serotype 3 strains isolated from healthy carriers. The proportion of serotype 3 strains among the carried isolates from Israel, Costa Rica, and Lithuania during the study period was 1.3% (14/1041), 9.7% (23/236), and 12.3% (13/106), respectively (Fig. 2, available online only). The same PFGE clusters identified in the non-invasive infections were found among the carried isolates, with the same molecular characteristics. Cluster A was further divided into 2 major patterns resembling MLST sequence types ST180 and ST505. The carried strains of ST180 were fully susceptible to all antimicrobial agents, whereas those belonging to ST505 showed multidrug resistance to erythromycin, tetracycline, and chloramphenicol. The carried strains belonging to clones ST458 and ST1116 (Table 2, clusters B and C, respectively) were susceptible to all antimicrobial agents.

### DISCUSSION

*S. pneumoniae* of serotype 3 is known for its ability to cause invasive diseases in children and adults worldwide.13 Bacteremia caused by this organism is considered to have the highest mortality rate compared with other serotypes.14 The proportion of invasive disease caused by serotype 3 among children increased between the pre- and postvaccine period, despite an overall decline in the number of invasive infections.15,16 However, data regarding the importance of serotype 3 in noninvasive disease are still limited.2,4,5 The present study evaluated the frequency of serotype 3 strains, recovered from noninvasive disease, in 3 datasets. Serotype 3 ranked second among mucosal isolates from Costa Rica and Lithuania, and seventh among the Israeli isolates. Thus, this highly virulent serotype is emerging as an important pathogen, not only in invasive diseases but also at mucosal sites, even before the implementation of PCV7.

Antibiotic susceptibility testing revealed that 95% (73/77) of the serotype 3 pneumococci recovered from noninvasive infections were fully susceptible to penicillin, erythromycin, tetracycline, chloramphenicol, clindamycin, and TMP/SMX. Only 5% (4/77) of the organisms, all from the Lithuanian dataset, were resistant to erythromycin, tetracycline, and chloramphenicol. Molecular typing by PFGE showed that most isolates belonged to a single cluster, arbitrarily designated as A. Among cluster A, the fully susceptible strains corresponded to the wide spread Netherlands3-ST180 clone, which was previously recovered from the NP and invasive infections in Europe.17 As published in the MLST database, all the strains belonging to this clone were fully susceptible to antibiotics, unlike the clone from Lithuania that showed multidrug resistance. Serotype 3 isolates have not yet been defined as one of the major antibiotic resistant international clones, because most serotype 3 isolates are broadly suscep-

### TABLE 2. Multilocus Sequence Typing of Pneumococcal Isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year</th>
<th>Source</th>
<th>Country</th>
<th>Serotype</th>
<th>Cluster</th>
<th>Allele Numbers</th>
<th>Sequence Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS-1433</td>
<td>2003</td>
<td>MEF</td>
<td>Israel</td>
<td>3</td>
<td>A</td>
<td>7 15 2 10 6 1</td>
<td>122 180</td>
</tr>
<tr>
<td>CR-41</td>
<td>2004</td>
<td>MEF</td>
<td>Costa Rica</td>
<td>3</td>
<td>A</td>
<td>7 15 2 10 6 1</td>
<td>122 180</td>
</tr>
<tr>
<td>LI-520</td>
<td>2005</td>
<td>NP</td>
<td>Lithuania</td>
<td>3</td>
<td>A</td>
<td>7 15 2 10 6 1</td>
<td>122 180</td>
</tr>
<tr>
<td>LI-736</td>
<td>2005</td>
<td>Sinus</td>
<td>Lithuania</td>
<td>3</td>
<td>A</td>
<td>46 8 2 10 6 1</td>
<td>122 505</td>
</tr>
<tr>
<td>LI-4041</td>
<td>2005</td>
<td>NP</td>
<td>Lithuania</td>
<td>3</td>
<td>A</td>
<td>46 8 2 10 6 1</td>
<td>122 505</td>
</tr>
<tr>
<td>IS-984</td>
<td>2003</td>
<td>MEF</td>
<td>Israel</td>
<td>3</td>
<td>B</td>
<td>2 32 9 47 6 21</td>
<td>17 458</td>
</tr>
<tr>
<td>IS-922</td>
<td>2005</td>
<td>NP</td>
<td>Israel</td>
<td>3</td>
<td>B</td>
<td>2 32 9 47 6 21</td>
<td>17 458</td>
</tr>
<tr>
<td>CR-1739</td>
<td>2004</td>
<td>MEF</td>
<td>Costa Rica</td>
<td>3</td>
<td>C</td>
<td>1 26 28 11 13</td>
<td>14 1116</td>
</tr>
<tr>
<td>CR-23053</td>
<td>2003</td>
<td>NP</td>
<td>Costa Rica</td>
<td>3</td>
<td>C</td>
<td>1 26 28 11 13</td>
<td>14 1116</td>
</tr>
</tbody>
</table>

*aroE* indicates gene encoding shikimate dehydrogenase; *gdh*, gene encoding glucose-6-phosphate dehydrogenase; *gki*, gene encoding glucose kinase; *spi*, gene encoding signal peptide 1; *xpt*, gene encoding xanthine phosphotransferase; *ddl*, gene encoding d-alanine d-alanine ligase; MEF, middle ear fluid; NP, nasopharynx.
tible to antibiotics. However, here we describe a cluster of MDR isolates, ST505, which caused sinusitis in 4 Lithuanian children. An additional smaller cluster from Israel (cluster B) corresponded to ST458. This clone was found in the United Kingdom, Ghana, and Egypt among strains of invasive origin. Another less common cluster from Costa Rica (cluster C) corresponded to ST1116, and was previously found only in another Latin American country (Argentina) in 2001.

The present study has some limitations because it includes only 1 center from each of the 3 countries, and the number of isolates in the Latin American database is limited. However, information on pneumococci isolated from sinusitis cases is rare. The data presented here describe for the first time the importance of a MDR serotype 3 clone (ST505) in cases of sinusitis in Lithuanian children.

Serotype 3 strains are known for their mucoid appearance because of the large amount of capsular polysaccharides. Large amounts of capsule have been reported to have an inhibitory effect on transformation, and therefore may block the uptake of foreign DNA. All the serotype 3 isolates in our study were penicillin susceptible and most of them displayed an identical PFGE profile and clonal type (ST180). The low genetic heterogeneity of these strains is consistent with previous finding showing that serotype 3 strains exhibit low degree of competence.

Overall, serotypes not covered by the PCV7 have increased both in colonization and in AOM. Specifically, the present study shows that serotype 3 accounts for large proportions of noninvasive infections in Israel, Costa Rica, and Lithuania, even before the introduction of PCV7, suggesting that this serotype may cause more infections after the implementation of pneumococcal conjugate vaccination programs. It is suggested that future conjugate vaccines could expand to include serotype 3 because of its high virulence and limited capacity to undergo capsular transformation. One experimental pneumococcal conjugate vaccine (a 13-valent conjugated to CRM197), includes also serotype 3. This investigative vaccine, which is currently undergoing development, would potentially offer broader coverage against pneumococcal disease.

REFERENCES