Serotypes and antibiotic resistance of non-invasive *Streptococcus pneumoniae* circulating in pediatric hospitals in Moscow, Russia

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**S U M M A R Y**

Background: Pneumococcal infections remain a major medical problem associated with high morbidity and mortality. Moreover, the resistance of *Streptococcus pneumoniae* to conventional antibiotics is constantly growing. The implementation of pneumococcal conjugate vaccines (PCVs) in the last decade has dramatically reduced the incidence of the vaccine type-associated invasive pneumococcal diseases in many countries. However, information on the seroepidemiology of *S. pneumoniae* in Russia is limited.

Methods: We report the results of serotyping and antibiotic susceptibility testing performed on 863 non-invasive pneumococcal isolates collected prospectively in 2009–2013 from children (median age 3.5 years) who sought medical care at five pediatric hospitals in Moscow. The isolates were recovered from the nasopharynx (71.2%), middle ear fluid (14.3%), and lower respiratory tract specimens (13.6%).

Results: In total, we identified 45 different serotypes. The six leading serotypes (prevalence >5%) included 19F (21.7%), 6B (12.8%), 23F (10.1%), 14 (9.0%), 6A (8.4%), and 3 (7.5%). Serotype 19A isolates had a prevalence of 2.3%. The proportion of PCV-13 serotypes was 78%; the coverage by PCV-7 was 58.2% and was similar to that of PCV-10 (59.8%). The rate of multidrug-resistant pneumococci (i.e., resistant to ≥3 antimicrobials) was 22%. The majority of the multidrug-resistant isolates were serotype 6B, 14, 19A, and 19F. Penicillin non-susceptibility was displayed by 28% of the isolates. The resistance rate to erythromycin was 26%. Among the examined erythromycin-resistant strains, 54% had the *erm(B)* gene and 13% had the *mef* gene as a single resistance determinant, whereas both determinants were found in 31% of these strains.

Conclusions: Our data predict a good coverage of the circulating *S. pneumoniae* by the PCVs and could be useful for evaluating the serotype distribution in support of the introduction of PCV in Russia. In addition, the antimicrobial resistance rate of *S. pneumoniae* in Russia is substantial, and the emergence of pneumococcal strains with a dual macrolide resistance mechanism is alarming.

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1. Introduction

*Streptococcus pneumoniae* (pneumococcus) is a common bacterial pathogen responsible for various infections, especially in children. The severity of pneumococcal illness varies from self-limiting mucosal infections to life-threatening invasive diseases like bacteremia and meningitis. *S. pneumoniae* is the leading cause of pediatric community-acquired pneumonia and acute otitis media (AOM), which have a substantial burden on healthcare resources worldwide.\(^1\)\(^-\)\(^4\) Pneumococcal infections contribute to a large number of medical care visits and antibiotic prescriptions in children. This coincides with the growing antimicrobial resistance of *S. pneumoniae* to a wide range of antibiotics that are used to treat pneumococcal infections.\(^5\)\(^-\)\(^7\)

The real burden of pediatric pneumococcal infections in the Russian Federation is difficult to estimate precisely because the surveillance system is underdeveloped. A low rate of blood and
cerebrospinal fluid (CSF) culture in conjunction with the common practice of parenteral antibiotic administration in children suspected to have bacteremia or meningitis before any laboratory investigations, preclude the assessment of the true incidence of invasive pneumococcal disease (IPD) in these patients. In addition, only a few laboratories in the country are able to isolate, identify, and serotype S. pneumoniae reliably. Thus, figures on the incidence of IPD in the Russian Federation are mainly based on expert evaluations. These data estimate the following incidence of IPD per 100 000 children under 6 years of age per year: pneumococcal meningitis, 0.22–0.53; pneumococcal bacteremia, 58.5–130; pneumococcal community-acquired pneumonia, 490–1300.1,8–10 In addition, the incidence of severe hospitalized pneumococcal AOM has been assessed as being 97.4–122.1 per 100 000.8

Pneumococcal capsular polysaccharide represents the principal virulence factor that protects the bacteria from phagocytosis and generates the specific antibody immune response. More than 90 variants of capsular polysaccharide, i.e. pneumococcal serotypes, have been described so far. Not all serotypes are equally pathogenic, and the majority of pneumococcal infection is associated with a limited number of serotypes.11 A subset of capsular polysaccharides from clinically important serotypes is included in the pneumococcal polysaccharide conjugate vaccine (PCV). The introduction of PCVs into national immunization programs has been shown to substantially decrease the incidence of IPD caused by vaccine-type pneumococci in many countries.2,12–16

Three PCVs (7-v, 10-v, and 13-valent) are licensed in the Russian Federation, but none has yet been implemented in the national immunization program. To estimate the prophylactic potential of the PCVs on pneumococcal infections, it is important to know how close they match the set of circulating serotypes. The actual local data on pneumococcal serotypes are limited and fragmented, and only a few reports from Russia have been published in the international literature in the last decade.17–19 In the present article, we report serotyping and antibiotic susceptibility testing data for a large collection of S. pneumoniae clinical non-invasive isolates obtained prospectively in a number of pediatric hospitals in Moscow. The vast majority of isolates were recovered from non-sterile respiratory specimens and middle ear fluid (MEF) from children who sought medical care suffering primarily from acute febrile respiratory infections and AOM.

2. Materials and methods

This prospective study included all isolates of S. pneumoniae that were recovered from specimens collected at five pediatric hospitals located in Moscow during March 2009 to April 2013. The pneumococci were isolated from various biological materials collected from patients (median age 3.5 years, interquartile range 0.08–6.0 years) with fever and symptoms of an acute respiratory infection presumably of bacterial etiology, with AOM, and with chronic lung disease. S. pneumoniae was isolated from several specimen types, including nasopharyngeal swabs, lower respiratory tract specimens (sputum, tracheal, or bronchial aspirates), MEF obtained from children with AOM by tympanocentesis or by swabbing the ear discharge in the case of spontaneous draining, and some others. Biological material was collected using an eSWAB kit (Copan, Italy). Signed informed consent was obtained from the parents or legal representatives of enrolled children before sampling.

All specimens were delivered to the laboratory of the Scientific Center for Children’s Health (Moscow) and processed there within 24 h after sampling. The specimens were plated on blood agar medium with 5% sheep blood and 2% horse serum and incubated at 37 °C with 5% CO2 for 24–48 h. S. pneumoniae was identified by optochin test and latex agglutination with the Slidex pneumo-kit (bioMérieux, France). Serotyping was performed by pool antisera for latex agglutination and factor/type antisera in the Quellung reaction using Staten Serum Institut products (SSI, Copenhagen, Denmark). Isolates that agglutinated none of the pool sera (A to I and P to T) were considered non-typeable.

Antibiotic susceptibility testing was done using the disk diffusion method with disks from Bio-Rad (USA). Oxacillin-resistant isolates were further tested for penicillin susceptibility by E-test strips (Oxoid, UK). The penicillin minimum inhibitory concentration (MIC) category interpretations were based on updated standards (European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2012). Intermediate and resistant isolates were collectively grouped as non-susceptible. A PCR was used to detect the erm(B) and mef determinants of macrolide resistance, as described previously.15

The statistical analysis was performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). Contingency table analysis for comparing serotype distributions was done by Chi-square or Fisher’s exact test, where appropriate. Proportions of serotypes were compared by means of the Z-criterion. The tests were considered statistically significant at p < 0.05.

3. Results

In total, 863 pneumococcal isolates were collected. The majority of strains were obtained from nasopharyngeal swabs (n = 617, 71.2%). One hundred eighteen isolates (13.6%) were recovered from sputum and tracheal/bronchial aspirates (lower respiratory tract specimens), and MEF provided 124 (14.3%) isolates. In addition, four strains were isolated from other loci (three from the vagina and one from the eye).

3.1. Serotype distribution

Serotyping was performed for 835 isolates; 10 (1.2%) isolates were not preserved for typing and 18 (2.1%) strains were non-typeable. Thus, the serotype was determined in 835 (96.8%) pneumococcal isolates.

In total, 45 different serotypes were identified. Six major serotypes accounted for 69.5% of the distribution and included serotype 19F (21.7%), 6B (12.8%), 23F (10.1%), 14 (9.0%), 6A (8.4%), and 3 (7.5%) isolates (Table 1).

Among the nasopharyngeal specimens, 44 different serotypes were recovered, whereas only 18 different serotypes were isolated from the MEF specimens. Serotype prevalence in nasopharyngeal, lower respiratory tract, and MEF specimens compared by the Chi-square test was different (Chi-square = 118, p = 0.018), however a paired statistical analysis showed that the proportion of serotypes varied significantly only between nasopharyngeal and MEF specimens (Chi-square = 64, p = 0.015). Serotypes 3 (12.3% vs. 6.8%, Z = 2.13, p = 0.033) and 19A (6.6% vs. 1.6%, Z = 3.42, p = 0.001) strains were more prevalent among MEF isolates than among the other specimen types. In contrast, no serotype 11A isolates were recovered from MEF, and the proportion of this serotype was significantly higher in the nasopharyngeal and lower respiratory tract specimens (0% vs. 3.5%, Z = –2.11, p = 0.039). The prevalence of all remaining serotypes was not statistically different between the specimen sources.

The four S. pneumoniae strains recovered from the other loci had serotypes 11A, 19F, 23F (vaginal isolates) and 6A (a conjunctival isolate).

Considering marginal differences in the serotype distribution, the pneumococci from all sources were collectively designated as ‘non-invasive isolates’ for further analysis.
The serotype distribution displayed some age-dependent variation (Figure 1). Serotype proportions in three age groups (≤24 months, 25–48 months, and >48 months) were significantly different (Chi-square = 63, p = 0.002), but these differences were related to a limited number of serotypes. The prevalence of serotype 19F isolates was higher among children aged ≤48 months than in the older children (25% vs. 16%, Z = 3.27, p = 0.001), whereas serotype 3 (5% vs. 12%, Z = −3.44, p = 0.001) and 37 (0.2% vs. 3%, Z = −3.63, p < 0.001) isolates prevailed in children aged >48 months. The proportion of the remaining major serotypes was similar in all age groups with no statistically significant differences (Figure 1). In addition, pneumococci of rare serotypes with a prevalence <1% in the overall distribution (the ‘Other’ category in Figure 1) collectively had a higher rate in children aged ≥48 months than in younger children (13% vs. 6%, Z = 3.47, p = 0.001).

3.2. Antibiotic susceptibility of S. pneumoniae

Susceptibility to antibiotics was examined in 835 (96.8%) pneumococcal isolates (Table 2). The prevalence of resistant strains was above 20% for all tested antimicrobials. The highest resistance rate (57%) was observed for trimethoprim/sulfamethoxazole. About 30% of pneumococcal strains were resistant to oxacillin. To elucidate the beta-lactam susceptibility pattern further, penicillin susceptibility was analyzed in 189 out of 243 (78%) of the oxacillin-resistant isolates. Thirteen of 189 (7%) penicillin-susceptible pneumococci had a MIC ≥0.06 μg/ml. The remaining isolates (93%, 176/189) were not susceptible to penicillin, having MICs >0.06 μg/ml. Among these isolates, the proportion of strains with a penicillin MIC ≥2 μg/ml was 10% (18/176). Thus, the rate of penicillin non-susceptibility was estimated as 28%. Penicillin non-susceptibility was found predominantly in serotype 14, 19A, 19F, and 23F isolates.

Resistance to erythromycin was found in 26% of pneumococcal isolates. Examination of the molecular mechanisms underlying the macrolde resistance was performed in 157 out of 218 (72%)

### Table 2

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All typed 835 96.8% Non-typeable 18 2.1 Not typed 10 1.2 Overall 863 100.0

PCV, polysaccharide conjugate vaccine.

* Other are serotypes with a prevalence <1% in the distribution. These were 26 non-PCV serotypes (n): 7C (1), 8 (4), 9A (1), 10B (1), 11D (1), 12B (1), 12F (2), 13 (2), 15A (3), 16F (5), 17F (6), 18A (1), 20 (1), 21 (1), 22F (3), 23B (3), 28A (2), 28F (1), 33F (3), 34 (6), 35B (2), 35C (5), 35F (7), 38 (1), 39 (2), 42 (2).
classes with only comprised of non-PCV susceptible 3.1%. These antibiotic pneumococcal serotypes, sulfamethoxazole. serotypes, Table V Number Serotypea Included MDR 6A Inclusion n = (66) 14 n = (75) 15B n = (29) 18C n = (19) 19A n = (19) 19F n = (177) 23A n = (17) 23F n = (80) TMP/SMX, trimethoprim/sulfamethoxazole; MDR, multidrug-resistant. a Included are 11 serotypes that had a prevalence <2% and represented 82.7% of the distribution. b Multidrug-resistant isolates, i.e. resistant to three or four tested antimicrobials. Other serotypes that had MDR phenotypes included (n MDR/n total): 8 (1/4), 9N (2/12), 9V (3/12), 15A (1/3), 35C (1/5).

eythromycin-resistant strains that were subjected to PCR for erm(B) and mef genes. As a single determinant of macrolide resistance, 54% (85/157) of strains had the erm(B) gene and 13% (20/157) had the mef gene. Both erm(B) and mef genes were found in 31% (48/157) of the examined strains. Four erythromycin-resistant strains had neither erm(B) nor mef genes.

A multidrug resistance phenotype (MDR; resistance to ≥3 classes of antibiotic) was found in 22% of the isolates. Notably, antibiotic resistance was attributed to a limited number of the pneumococcal serotypes. A high resistance rate was mainly found in four serotypes: 6B, 14, 19A, and 19F (Table 2). Among these serotypes, serotype 19A demonstrated the most resistant profile, with resistance rates varying from 90% (17/19) to oxacillin to 32% (6/19) to clindamycin. The majority of the 19A pneumococci (63%, 12/19) had an MDR phenotype.

Serotype 3 largely preserved susceptibility to the tested antibiotics. One oxacillin-resistant strain of this serotype had a penicillin MIC <0.06 μg/ml, thus being penicillin-susceptible, and only 5% (3/60) of strains were resistant to trimethoprim/sulfamethoxazole. None of the serotype 3 isolates had an MDR phenotype. The majority of the serotype 23F isolates were susceptible to oxacillin, erythromycin, and clindamycin; the rate of MDR phenotypes in the serotype 23F strains was 6% (5/80).

In addition, several rare serotypes had an MDR phenotype. These included serotype 8 (one out of four isolates were MDR strains), 9N (2/12), 9V (3/12), 15A (1/3), and 35C (1/5). No other serotypes were MDR.

3.3. PCV coverage

The overall prevalence of PCV-7 serotypes in the recovered isolates was 58.2% (Table 1). Six additional serotypes included in PCV-13 increased coverage by 20% up to 78%, mainly due to serotypes 3, 6A, and 19A. No serotype 5 strain was identified in the examined collection. In comparison to PCV-7, the 10-valent PCV having three more serotypes (1, 5, and 7F) covered an additional 1.6% of the distribution. Importantly, PCV coverage was even higher in children at the age of ≤24 months, who are at the highest risk of developing IPD. The coverage rate in this age group was 62.8% for PCV-7, 64.5% for PCV-10, and 81.2% for PCV-13.

Non-PCV serotypes had a prevalence of 22%. Among these, isolates from serogroup 15 accounted for 5% and serotype 11A for 3.1% of the overall distribution (Table 1). The remaining rare 26 non-PCV serotypes (proportion of each one <1% in the total distribution; listed in the footnotes to Table 1) collectively comprised 8%.

The PCVs covered the majority of serotypes that had MDR phenotypes (PCV-7 and PCV-10, 87.7%; PCV-13, 97.2%). In addition, most of the penicillin-non-susceptible isolates had PCV-related serotypes.

4. Discussion

The results of this study describe the serotype assortment and antibiotic susceptibility of non-invasive S. pneumoniae isolated from pediatric patients. Serotype 3, 6A, 6B, 14, 19F, and 23F isolates were predominant, covering two-thirds of the distribution, with serotype 19F having the largest proportion at >20%. Comparison of the serotype distributions obtained from different specimen sources showed statistically significant differences in the prevalence of several serotypes between the respiratory specimens and MEF. Serotype diversity was much higher in the respiratory samples than in MEF (44 vs. 18 different serotypes). The recovery of serotype 3 and 19A isolates from MEF was higher than from other specimen sources. In contrast, serotype 11A was found exclusively in the respiratory specimens and not in MEF. This serotype is one of the typical carriage serotypes that are rarely associated with pneumococcal disease,11,15,20 although some carriage serotypes may have the potential to cause IPD, which has become apparent under PCV pressure. For instance in the USA, the recovery of serotype 22F, 23A, and 35B isolates in IPD has increased substantially since the introduction of PCV-7.15

The PCVs have been shown to exert a profound influence on the epidemiology of S. pneumoniae serotypes. In countries where universal PCV-7 vaccination has been introduced, vaccine-type pneumococci have virtually disappeared.15,20 Considering these data, we compared our current findings with pre-PCV reports because pneumococcal vaccination has not yet been introduced into the national immunization program in Russia. Despite the above indicated relative differences, the composition of the major serotypes in all specimen sources was similar. Therefore for comparison purposes, we regarded isolates recovered from the different specimen sources as one distribution of non-invasive pneumococcal strains. Our results indicated that the assortment and recovery rate of the major serotypes in the present study were similar to those described in the pre-PCV era worldwide, when the leading non-invasive isolates were represented by S. pneumoniae from serogroups 3, 6, 14, 19, and 23.2,12-13 These are the typical pediatric pneumococci that accounted for most IPD in children and had high carriage rates before the implementation of the PCV11,21

The antibiotic resistance rate in the isolated pneumococci was substantial. Given that 93% of oxacillin-resistant strains were
penicillin-non-susceptible pneumococci, the overall prevalence of penicillin-non-susceptibility could be estimated as 28%. Macrolide resistance was above 25%, and 22% of isolates had an MDR phenotype. In earlier studies performed in Russia on nasopharyngeal pneumococcal isolates collected in the late 1990s and in 2001–2002, the prevalence of non-susceptible strains was much lower.\(^\text{1,7,16}\) The rate of the penicillin-non-susceptible S. pneumoniae was below 10%, the proportion of erythromycin-resistant strains was around 5%, and only 2% MDR pneumococci were reported. Resistance to trimethoprim/sulfamethoxazole is similar to what was found in the present study (53–65%). Our findings indicate that in the last decade a significant increase in the prevalence of resistant S. pneumoniae has occurred. This result coincides with the global trend of a progressive increase in the prevalence of resistant pneumococci when penicillin-non-susceptibility has increased from negligible to a rate of 25–50% in many geographic locations.\(^\text{2–7}\)

The same is true for macrolides, for which resistance rates in Russia have increased from 5% in 2001–2002 to 26% today.\(^\text{17–19}\) Our study demonstrated that the predominant mechanism of macrolide resistance was the target site modification mediated by the \(erm(B)\)-encoded methylase found in 54% of the erythromycin-resistant isolates as a single determinant and in 31% in association with the \(mef\)-related efflux pump. In addition, in 13% of the erythromycin-resistant isolates, only \(mef\) was identified. The rates of \(erm(B)\), \(mef\), and their combination were similar to those reported for 2004–2005 isolates from Russia.\(^\text{19}\) In contrast, in a collection from the late 1990s to 2002, no pneumococci carrying both determinants were reported.\(^\text{18}\) Thus, in the last decade, macrolide resistance of S. pneumoniae in Russia has increased significantly and strains combining \(erm(B)\) and \(mef\) determinants have emerged. The emergence of pneumococci with a dual resistance mechanism is alarming because this phenotype has been related to genetic elements from the MDR pneumococcal clonal complexes with the highest resistance.\(^\text{20}\)

In our study, PCV serotypes predominated among recovered pneumococcal isolates, with PCV-13 having the highest coverage, close to 80%. Moreover, the majority of resistant S. pneumoniae, including bacteria with an MDR phenotype, were vaccine-type pneumococci. Although we examined non-invasive pneumococci, these data predict an excellent protective potential of the available PCVs against IPD in Russia because most pediatric pneumococcal diseases in the pre-PCV era have been associated with the serotypes that predominated in the presented collection (see above). In addition, the PCVs may contribute to a reduction in the circulation of the resistant pneumococcal clones, which often have vaccine-related serotypes.

In conclusion, to our knowledge this is the largest study reporting the distribution of non-invasive pneumococcal serotypes from the Russian Federation. These data may be used as a starting point to monitor and evaluate the future impact of the PCVs on the seroepidemiology and antimicrobial resistance of S. pneumoniae in the country after the implementation of PCV in the national immunization program.

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Conflict of interest: NM, TK, LNB have been speakers for Pfizer. All other authors report no conflict of interest.

References