Serotypes and antimicrobial resistance of \( S. \) \( pneu\)moniae nasopharyngeal carriage in children from Cyprus: A country with relatively low coverage with the seven-valent pneumococcal conjugate vaccine

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ABSTRACT

\( S. \) \( pneu\)moniae may cause serious invasive infections mainly in children and elderly adults leading to significant morbidity and mortality. This report describes the circulating serotypes and antimicrobial resistance of \( S. \) \( pneu\)moniae colonizing the nasopharynx of Cypriot children in 2007-2008 when the immunization coverage of children was still relatively low. The study focused on children between 6 months to 5 years of age in the Nicosia district. A nasopharyngeal specimen was obtained from 402 children who visited public immunization centers, public outpatient departments and the offices of private sector practicing pediatricians. The percentage of carriage was 35.3%. Intermediate and full resistance to penicillin was estimated at 39.4% and 1.4%, respectively. Intermediate and full resistance to erythromycin was estimated at 1.4% and 39.6%, respectively. Resistance to clindamycin was found to be 30.8%. MLSb was the dominant phenotype of resistance (77.2%). Multi-resistance was found amongst 24.1% of strains. Most frequent colonizing serotypes were 15B, 6B, 23A, 23B, 19F. The two vaccine serotypes 6B and 19F were highly resistant to both penicillin and erythromycin and were also multi-resistant at 27% and 50%, respectively. The percentages of fully immunized, partially immunized and non immunized children with the PCV7 were 24.6%, 14.3%, and 61.1%, respectively. Fully immunized children carried fewer vaccine serotypes in comparison with the non immunized children \((p = .05)\). Some vaccine serotypes were among those more frequently colonizing children and exhibit significant resistance. An increase in vaccination coverage and prudent use of antimicrobials could have a significant impact on resistance.

Key Words: Pneumococcus, Serotypes, Vaccine, Anti-microbial resistance, Children, Colonization, Cyprus

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1. INTRODUCTION

*S. pneumoniae* is the causative agent of a variety of infections ranging from relatively mild otitis media to life threatening bacteremia, pneumonia and meningitis in children. Disease caused by the pneumococcus is responsible for about 1 million deaths annually in children younger than 5 years of age. A significant percentage of those surviving pneumococcal meningitis may suffer significant sequelae such as neurosensory deafness, and other neurological disorders.

The nasopharynx is the ecological niche of *S. pneumoniae*. Exchange of genetic material between *S. pneumoniae* and other commensal streptococci in the nasopharynx may lead to the emergence of resistance. Colonization of the nasopharynx is an essential step before pneumococcal invasion and disease development. In addition, the nasopharynx could facilitate the transmission of the bacterium to other individuals in the community. Therefore, colonization of the nasopharynx usually reflects strain circulation within the community. During past decades, global resistance of *S. pneumoniae* to many classes of antimicrobials has emerged; an event mainly attributed to the overuse of antibiotics.

The conjugate seven-valent vaccine (PCV7) against pneumococcus has proved very successful in reducing the burden of invasive disease caused by vaccine serotypes as well as eliminating vaccine serotypes from nasopharynx. This has led to a significant reduction in invasive disease caused by the pneumococcus in children as well as overall protection of older age groups due to the herd immunity effect. A significant decrease in invasive infections caused by non susceptible strains to penicillin as well as macrolides has also been reported. However, despite the dramatic decrease in invasive infections caused by vaccine serotypes a small but significant increase has been noted in colonization and to a lesser extent in invasive disease by types not included in the vaccine in different countries.

Only one previous study has recently been reported from Cyprus on the epidemiology of *S. pneumoniae* in a single district. In addition, very limited data are available from EARS-net on the resistance profiles of invasive strains. In Cyprus the seven-valent conjugate vaccine was first introduced in 2004 but only within the private sector. Therefore, parents had to pay privately in order to have their children vaccinated thus leading to a relatively low vaccine coverage until 2010 when the PCV 10-valent vaccine was introduced in the public sector and was fully subsidized by the state. The same year (2010) the 10-valent vaccine was also introduced by the paediatricians in the private sector. In 2011, the 13-valent vaccine was also introduced in the private sector.

In this study, we present findings from a carriage study among children up to 5 years of age conducted in 2007–2008. The circulating serotypes and antimicrobial resistance of *S. pneumoniae* from nasopharynx in association with the PCV7 vaccination coverage are described.

2. MATERIALS AND METHODS

2.1 Study design

The study was conducted in the Nicosia district–Capital of Cyprus, which is the largest district of Cyprus, between November 2007 and May 2008. The population of Nicosia district at the end of 2007 was 301,103 persons representing 38.8% of the total population of the Republic of Cyprus.

The target population of the study included a non-randomized sample of children aged 6 months to 5 years old who visited the government immunization centers in urban and rural areas, the outpatient department of Archbishop Makarios III Hospital (the major public children’s hospital in the country and the only one based in the Nicosia District), as well as the offices of four paediatricians working in the private sector having the highest number of children visits. All eligible children who presented for vaccination at the government immunization centers and the outpatient’s departments every Monday and Tuesday between 9 a.m. and 12 noon were included in the study. The study was approved by the National Bioethics Committee of the Republic of Cyprus. For each child participating in the study, written informed consent was taken from the parents or legal guardians.

Epidemiological and demographic data were collected through standardized questionnaires which were addressed to the parents or legal guardians at the time the specimen was obtained. Information was gathered on gender, date of birth, address, nationality of the parents, exposure to smoking, previous or current breastfeeding, number of siblings, attendance of day care centers, PCV7 vaccine status, state of illness and recent use of antibiotics. Children suffering from a bacterial infection or receiving antibiotics on the day of the specimen were excluded from the study. A specimen was taken only from one child from each family to avoid potential bias.

2.2 Microbiological culture and antimicrobial susceptibility

One nasopharyngeal specimen was taken from each participating child using sterile Rayon swabs on flexible wire (Medical Wire and Equipment Co. Ltd., Corsham, Wilts., England) and were plated within two hours onto blood agar plates supplemented with 5% (v/v) defibrinated horse blood and chocolate agar plates and incubated in 5% CO₂ for 24 hours at 35°C. *S. pneumoniae* strains were identified by optochin sensitivity and bile solubility.
Susceptibility testing was performed on Mueller-Hinton agar supplemented with 5%(v/v) defibrinated horse blood as follows: all strains were tested by the disk diffusion method for oxacillin, cefotaxime, erythromycin, clindamycin, trimethoprim – sulfamethoxazole, tetracycline, vancomycin, linezolid and levofloxacin sensitivity and by assessment of penicillin MIC for all isolates. MIC was measured by the Etest method (AB Biodisk, Solna, Sweden).116 MIC determinations were also undertaken for all antibiotics where S. pneumoniae strains were non susceptible by the disk method. Interpretation of susceptibility was based upon the Clinical and Laboratory Standards Institute (CLSI) guidance 2008 which is still valid until today.117 Based on this new guidance, for a non-meningitis infection (e.g. pneumonia, otitis media, bacteraemia), if penicillin is given intravenously, a sensitive strain to penicillin has an MIC of < 2 µg/ml, intermediate if MIC is 4 µg/ml and fully resistant if MIC is ≥ 8 µg/ml. If the drug is given orally a sensitive isolate to penicillin, has an MIC < 0.06 µg/ml, an intermediate resistant isolate has an MIC between 0.12-1 µg/ml and a fully resistant isolate has an MIC of ≥ 2 µg/ml.117 Multidrug resistant strains are defined as the strains resistant to at least 3 different classes of antimicrobial agents.

The phenotypes of macrolide resistance were also detected.118 Phenotype M confers low level resistance to 14 and 15 ring macrolides but not to lincosamides or streptogramins. Strains were serotyped by the latex agglutination method using commercially available antisera (Statens Serum Institute, Copenhagen, Denmark) as described elsewhere.119

2.3 Statistical analysis

The statistical package SPSS 20 was used for statistical analyses. Statistical significance between different proportions was assessed by the chi-square test. A p value of < .05 was considered statistically significant.

### Table 1. In vitro susceptibilities of Streptococcus pneumoniae isolates

| Antibiotic                          | Sensitive | Intermediate | Resistant |
|------------------------------------|-----------|--------------|-----------|
| Penicillin                         | 84 (59.2) | 56 (39.4)    | 2 (1.4)   |
| Ceftriaxone                        | 120 (86.3)| 11 (7.9)     | 8 (5.8)   |
| Erythromycin                       | 85 (59.0) | 2 (1.4)      | 57 (39.6) |
| Clindamycin                        | 99 (69.2) | 0            | 44 (30.8) |
| Tetracycline                       | 95 (67.4) | 1 (0.7)      | 45 (31.9) |
| Trimethoprim-sulfamethoxazole      | 45 (30.8) | 16 (11.0)    | 85 (58.2) |
| Linezolid                          | 143 (100) | 0            | 0         |
| Vancomycin                         | 143 (100) | 0            | 0         |
| Levofloxacin                       | 143 (100) | 0            | 0         |
| Multi-resistant*                   |           |              | 34 (24.1) |

*Multi-resistance is defined as resistance to 3 or more classes of antimicrobial agents

### RESULTS

A total of 402 children participated in the study representing 2.4% of the total pediatric population of the same age in the Nicosia district. About half (n = 207, 51.5%) were male. A third of the participating children (n = 130, 32.3%) were younger than one year of age, about a third (n = 123, 30.6%) were between one and two years, and (n = 149, 37.1%) were between 2 and 5 years. The majority of children had both parents of Cypriot origin (n = 274, 68.1%), and 3 out of 4 children (n = 304, 75.6%) were either on breastfeeding at the time of specimen collection or had breastfed in the past. The majority of children (n = 220, 54.7%) stayed during the morning hours at their home supervised by an adult family member. A number of children (n = 143, 35.6%) attended 19 different day care centers, while 33 (8.2%) children were cared for during the morning hours by an adult who also cared for a small number of other children. The majority of samples (n = 242, 60.3%) were collected from children visiting the public sector centers and 160 samples (39.7%) were obtained from the offices of private paediatricians. About a third of the children in the study (n = 142, 35.3%) were colonized by S. pneumoniae.

The susceptibility patterns of the strains to various antibiotics are presented in Table 1. Resistance to penicillin presented, was interpreted based on the new 2008 definitions when penicillin is given orally for a non meningitis site of infection.117 Intermediate resistance to penicillin was quite high (39.4%) and full resistance was detected in 1.4% of the strains. On the other hand, in cases of non-meningitis infection, if treatment is given intravenously, full resistance to penicillin was zero, while intermediate resistance was limited to 1.4% of the strains. According to the same definitions, in cases of meningitis, 40.8% of the strains were resistant to penicillin. The percentage of multidrug resistant strains was also high (24.1%) but no resistance was detected to vancomycin, linezolid or levofloxacin.
Resistance phenotypes to macrolides appear in Table 2. The M phenotype was expressed in 22.8% of strains resistant to macrolides and the MIC were low (0.38 µg/ml–8 µg/ml). MLBs phenotype was detected in 77.2% of cases and conferred high level of resistance to both erythromycin (MIC = 3 – ≥256 µg/ml) and clindamycin (MIC = 12 – ≥ 256 µg/ml).

Table 2. Resistant phenotypes to the macrolides and serotype

| Phenotype | Number of isolates (%) | Range of MIC (µg/ml) | Serotypes (number of isolates) |
|-----------|------------------------|----------------------|-------------------------------|
|           |                        | Erythromycin        | Clindamycin                   |
| M         | 13 (22.8)              | 0.38-8              | 0.047-0.250                   |
|           |                        |                     | 6A (1), 6B (5), 9A (1), 11C (1), 17F (1), 19B (1), 19F (1) |
| MLSb      | 44 (77.2)              | 3-256+              | 12-256+                       |
|           |                        |                     | 6B (4), 7F (1), 10A (2), 14 (1), 15A (5), 15B (11), 15C (4), 15F (2), 19A (2), 19B (3), 19F (5), 23B (1) |

3.1 Serotypes and sensitivity of the pneumococcal strains

Colonizing strains belonged to 33 different serotypes. The most frequent serotypes are documented in Table 3. Two of these serotypes namely 6B and 19F are included in the 7-valent conjugate pneumococcal vaccine and are among the most frequent serotypes colonizing the children population in our sample [6B (12.7%) and 19F (6.3%)].

Susceptibility to antibiotics of the most frequent serotypes colonizing the children is presented in Table 4. Some of these serotypes carry high resistance to both penicillin and erythromycin. A percentage of strains belonging to some of the serotypes are multi-resistant. The two serotypes 6B and 19F, included in the PCV7 were also found to exhibit resistance to both penicillin and erythromycin and other classes of antimicrobials at a higher percentage.

Table 3. Most frequent serotypes colonizing children

| Serotype | No of isolates | % |
|----------|----------------|---|
| 15B      | 18             | 14.3 |
| 6B       | 16             | 12.7 |
| 23A      | 9              | 7.1 |
| 23B      | 9              | 7.1 |
| 19F      | 8              | 6.3 |
| 15C      | 7              | 5.6 |
| 7B       | 5              | 4.0 |

Table 4. Antimicrobial susceptibilities of prevalent serotypes*

| Resistance                        | 15B (n = 16) | 6B (n = 15) | 23B (n = 9) | 23A (n = 9) | 19F (n = 8) | 15C (n = 7) | 7B (n = 5) |
|-----------------------------------|--------------|-------------|-------------|-------------|-------------|-------------|------------|
| Intermediate and full resistance to penicillin (%) | 69           | 66.7        | 0.1         | 0           | 87.5        | 28.6        | 0          |
| Intermediate and full resistance to erythromycin (%) | 62           | 53.3        | 0           | 0           | 87.5        | 57.1        | 0          |
| Multi-resistant** (%)             | 56           | 27          | 0           | 0           | 50          | 42.9        | 0          |

* PCV7 serotypes in red color; **Multi-resistance is defined as the resistance to 3 or more classes of antimicrobial agents.

4. DISCUSSION

In this study we describe the epidemiology of S. pneumoniae colonizing the nasopharynx of healthy children in the Nicosia district. The circulating serotypes and susceptibility to antimicrobials are presented. To our knowledge, this is the second report describing the serotypes and resistance of S. pneumoniae colonizing Cypriot children. Our sample included children from a relatively large number of different day care centers as well as from young children staying at home, thereby avoiding a cluster effect. [20]

S. pneumoniae colonizing Cypriot children showed high percentage of resistance to antimicrobials (see Table 1). Based on 2008,[17] definitions of resistance to penicillin for non-meningitis infections in case the drug is given orally, intermediate resistance to penicillin was found in 39.4% of strains and full resistance in 1.4%. These findings were in accordance with other published studies performed during the same time period in countries of Southern Europe.[21–23] In a Spanish study conducted in 2005-2006, intermediate resistance to penicillin was found to be 28% and full resis-
It has been reported in various studies that the percentage of antimicrobial resistance in strains colonizing the nasopharynx is always higher than the resistance of invasive strains. In a study conducted in Greece, non-susceptibility to penicillin (resistant and intermediate) was 43.2% in strains from colonized children and only 17.1% in strains isolated from invasive infections.

Data on susceptibility patterns of invasive strains in Cyprus were limited and originated mainly from the European Antimicrobial Resistance surveillance network (EARS-net). Between 2006 and 2009 laboratories from public hospitals in Cyprus submitted a total of 53 strains to the EARS network. Intermediate resistance to penicillin ranged from 7.7% to 26.7% vs. 39.4% in our study and full resistance ranged from 6.7% to 30.8% vs. 1.4% in our carriage study.

High percentage of resistance to erythromycin has also been detected in our study. High resistance to macrolides was also reported from other countries such as Greece, where the percentage of full resistance varied from 30.5% to 40.6%. At the same time, the percentage of non-susceptible pneumococci to erythromycin in Spain was 37%, whilst, between 2006 and 2008, in a similar study in Belgium it was 24%. The M phenotype of resistance confers low level resistance only to the 14- and 15-ring macrolides and was detected in 22.8% of the strains isolated in our study (see Table 3). This phenotype is associated with an efflux pump mechanism of resistance usually codified by the mef genes (mefA, mefE). The second phenotype MLSb usually confers high level resistance to all macrolides, lincosamides (e.g. clindamycin) and the streptogramins. This phenotype is associated with a target modification mechanism of resistance usually mediated by the ermB gene-encoded methylase. The MLSb phenotype was more frequently detected in 77.2% of strains in our study. Phenotype M is more frequent in the USA, Canada, UK, Germany, Norway and Greece. In other countries such as Spain, France, Italy and Cyprus phenotype MLSb is most frequently encountered.

Amongst invasive isolates submitted from Cyprus to the EARS–net between 2006 and 2009, resistance to erythromycin ranged from 20.0% to 30.8%. These percentages, as expected, were lower than the levels of resistance in our carriage study (39.6%).

In 2008, the rates of non-susceptible invasive strains of pneumococcus to penicillin in countries such as the UK, Norway, and Sweden were estimated at 5.2%, 2.4%, 2.0%, respectively. In contrast, percentages of non-susceptibility in southern Europe such as Spain, Italy and Cyprus were found to be 22.8%, 10.2% and 42.9%, respectively. A similar picture is seen for the percentages of non susceptible invasive strains of pneumococcus to erythromycin. In 2008, EARS-net presented data where UK, Norway and Sweden had levels of non susceptible pneumococcus to erythromycin at 6.3%, 7.2% and 5.6%, respectively, whilst in the southern European countries such as Spain, Italy and Cyprus the levels were 21.9%, 28.6% and 26.0%, respectively.

A very important factor associated with antimicrobial resistance is the consumption of antibiotics in a specific country. The European Surveillance of Antimicrobial Consumption Network (ESAC) monitors annual consumption of antimicrobials in the countries of Europe. In 2008, Cyprus, Greece and other southern European countries had high consumption of antibiotics in comparison to countries of Northern Europe. This pattern of antibiotic consumption has not changed as of today.

In general, the introduction of pneumococcal conjugate vaccines has led to a decrease of the incidence of invasive disease by S. pneumoniae resistant to antibiotics. In parallel the carriage of resistant Streptococcus pneumoniae has also decreased. This is mainly due to the fact that some of the vaccine serotypes are characterized by high percentage of resistance to antimicrobials. This was evident in our study. In Table 4, two of the most frequent serotypes colonizing Cypriot children, specifically 6B and 19F are vaccine serotypes and demonstrated a high percentage of resistance to both penicillin and erythromycin and also to other classes of antimicrobials. At the time of our study, the pediatric population in our sample had a low coverage with PCV7 vaccine. Specifically, 24.6% of children participating in this study were fully vaccinated, 14.3% were partially vaccinated and 61.1% were not vaccinated. These findings are in accordance with findings from the tri-annual survey performed by the Unit for Communicable Diseases of the Ministry of Health (MOH) 2009. In the 2009 survey, it was found that only 37.5% of children were fully vaccinated, 42% were partially vaccinated and 20.5% were not vaccinated.

Even with this low PCV7 coverage, it was evident that fully vaccinated children were colonized by lower percentage of vaccine serotypes in comparison to those children that had not been vaccinated. Based on these findings, it appeared that full vaccination with PCV 7 would cover 22.2% of the serotypes that circulated within this population, PCV 10 would cover 23.8% and PCV13 would cover for 28.6% of circulating serotypes.
Our study has some limitations. The sample size was based upon convenience sampling methodology therefore the representativeness for the whole population of the Nicosia district was not complete. Furthermore, 60.3% of the children that participated in the study originated from the public immunization centers and the others from the private sector. As the PCV7 vaccine was not subsidized by the state at the time of the study, children visiting the public sector had lower vaccine coverage in comparison with children from the private sector. However, the fact that children participating in the study came from a very large number of day care centers and more than half of them were children not attending day care centers contributes to a more representative sample.

In summary, we found high percentages of non susceptibility of S. pneumoniae to antimicrobials used in every day practice in Cyprus such as penicillin and macrolides. At the time this study was performed there was a high consumption of antibiotics as the stress exerted by antibiotic overuse may limit the benefits of vaccination. Cyprus remains a European country with one of the highest consumptions of antimicrobials. A continuous and multidisciplinary campaign should be instituted towards decreasing antibiotic use and increasing vaccine coverage in order to have a significant effect towards decreasing bacterial resistance to antimicrobials.

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CONFLICTS OF INTEREST DISCLOSURE
The authors declare that they have no conflict of interest.

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