

# ANTIMICROBIAL SUSCEPTIBILITY OF *S. PNEUMONIAE* STRAINS ISOLATED FROM CHILDREN WITH NASOPHARYNGEAL CARRIAGE

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## ABSTRACT

*Streptococcus pneumoniae* colonises the nasopharynx of children and could cause life-threatening diseases. As a result of the implementation of conjugate vaccines worldwide the spread of vaccine serotypes has decreased. In Bulgaria PCV10 was introduced in 2010 followed by changes in the invasive clones carrying resistance genes. The aim of our study is to determine the serotype distribution and resistance patterns of isolates from children carriers after vaccination. A total of 834 children were tested for *S. pneumoniae* and 21% showed positive culture results. All isolates were genotyped with PCR. We found that 85% of the positive samples are from children attending kindergartens and schools. The most frequent serotypes/serogroups were 6C (20%) and 24B/F (11.5%), followed by 3 (8.6%), 11A/D (8%), 35F (6.9%), 19A (6.3%), 23A (6.3%) and 15A/F (6.3%). The susceptibility to  $\beta$ -lactams was high and there were strains showing intermediate susceptibility to benzylpenicillin. This study found 76 (44%) MDR strains non-susceptible to at least 3 antibiotic

classes and the most common resistance pattern was erythromycin-clindamycin-tetracycline.

## KEYWORDS:

*S. pneumoniae*, resistance, pneumococcal serotypes, carriage

## INTRODUCTION

*Streptococcus pneumoniae* is a bacterial agent that causes severe life-threatening diseases among children such as bacteremic pneumonia, sepsis and meningitis (1, 2). Asymptomatic carriage of pneumococci is known to be a prerequisite for non-invasive and invasive pneumococcal disease (3). Children under 5 years are considered the major reservoir of infection since they are readily colonised by *S. pneumoniae* (2). Pneumococcal infections are a leading cause of hospitalisations and require common use of antibiotic treatment. Antimicrobial resistance among pneumococcal serotypes poses a serious clinical issue worldwide because it decreases the effectiveness of treatment and therefore increases the mortality risk (4).

Pneumococcal disease is successfully prevented through national programs that include mandatory pneumococcal conjugate vaccines (PCVs) for children. Vaccination of the vulnerable parts of the community prevents infections and spread of vaccine serotypes which indirectly reduces the development of resistance (5). In Bulgaria the 10-valent vaccine (PCV10) was introduced in the National Immunisation Calendar in 2010 and applied with first dose scheduled at 2 months and a booster dose at 12 months of age (6). PCV10 includes the polysaccharide of 10 serotypes (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F) conjugated with protein to induce immune response at early age (7). Pneumococcal serotypes are distinguished by the polysaccharide capsules which are genetically determined from the *cps* locus (8). The expression of a specific capsule enables the survival of pneumococci outside the host and is related to the invasive potential of different serotypes (9). Since the introduction of PCV10 in Bulgaria vaccine serotypes are not found among meningitis cases of vaccinated children that shows the effectiveness of the prevention strategy (10, 11). However, the non-vaccine serotypes are becoming a severe threat in terms of invasive potential. Serotype 3 and 19A are associated with most of the meningitis cases in our

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country and these serotypes are also associated with resistance profiles (6, 10, 11).

Monitoring serotype distribution of *S. pneumoniae* and antimicrobial susceptibility patterns in pneumococcal carriers from several European countries shows decrease in antimicrobial tolerance in vaccinated cases compared to non-vaccinated (12). However serotypes not included in the vaccine also pose a problem with low susceptibility to various antimicrobials. Epidemiological research should be conducted in order to facilitate the informed choice of the primary physicians for the most effective antibiotic treatment of pneumococcal infection region-wise (13).

## MATERIAL AND METHODS

### Sample collection

Nasopharyngeal secretions from 834 healthy children aged 6 months to 8 years were collected and analysed in the period from February 2017 to March 2019. The children mainly resided in their own home or attended children's collectives in Sofia region. The children were vaccinated with at least one dose of PCV10. The transnasal nasopharyngeal samples were collected with flexible, sterile, dry swab in eSwab transport medium (Copan, Italy) and transported within 8 hours to the National Reference Laboratory (NRL) Molecular Microbiology at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia.

### Culture and antibiotic susceptibility testing

Samples were processed within 2 hours of arrival in the laboratory and incubated on blood agar plates (Columbia CNA Agar with 5% Sheep Blood) for 24 h at 37°C in an atmosphere with elevated concentration of CO<sub>2</sub>. The identification of *S. pneumoniae* was based on evaluation of cultural characteristics of colonies as well as microscopy – alpha-haemolytic, Gram-positive, catalase-negative cocci, sensitive to optochin and bile salts. The susceptibility of the isolates to antibiotic preparations was tested by the Bauer-Kirby disc-diffusion method, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (14). The following antibiotic discs were used: oxacillin (screening), tetracycline, erythromycin, clindamycin, vancomycin, teicoplanin, linezolid, norfloxacin, trimethoprim/sulfamethoxazole. Multidrug-resistant strains (MDR) were determined after

detection of non-susceptibility to at least 3 different antibiotic classes:  $\beta$ -lactams (benzylpenicillin), tetracyclines, macrolides, lincosamides, glycopeptides, oxazolidinones or sulfonamides.

Initial screening with 1  $\mu$ g oxacillin disc was performed for the detection of  $\beta$ -lactam resistance. Minimum inhibitory concentration (MIC) for benzylpenicillin was tested for strains with oxacillin zone diameter less than 20 mm. MIC for benzylpenicillin was determined by microdilution in Mueller-Hinton broth + 5% lysed horse blood and 20 mg/L  $\beta$ -NAD (MH-F broth) according to ISO 20776-1 standard. The inoculums contained 5x10<sup>5</sup> CFU/ml and were incubated in sealed panels (MIKROLATEST® MIC plates) at 35  $\pm$  1°C for 18  $\pm$  2h. MIC was defined as the lowest concentration of an antibiotic that completely inhibited visible growth. The exponential gradient of antibiotic in the broth was a quantitative measurement of its *in vitro* activity. Test plates for MIC determination contained 8 concentrations of benzylpenicillin. The interpretation of the MIC allowed the definition of microorganisms as sensitive, with reduced sensitivity (intermediate, low susceptibility) or resistant, using the criteria for non-meningitis isolates according to EUCAST v9.0.

### DNA extraction and typing

DNA from all strains was isolated using 5% Chelex 100 with Proteinase K 20 mg/ml method as previously described (11). The detection of pneumococcal DNA was performed with conventional PCR and every reaction was checked for amplification with an internal control for the gene *cpsA*. The primers used in the reactions are described in CDC protocols grouped in 10 multiplex PCR assays for pneumococcal serotype deduction of 70 serotypes (15).

### Statistical analysis

The statistical software used for calculations, including Fisher's exact test or  $\chi^2$ , was Statistical Package for Social Science (SPSS V22.0).  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

For the period 2017-2019 all 834 samples were cultured and a total of 174 isolated strains (21% of all tested samples) were identified as *S. pneumoniae*. All *S. pneumoniae* strains were sensitive to optochin and bile salts. No statistically significant difference was found between males (n=418) and females

(n=416) and culture-positive samples ( $p = 0.76$ ). This could be explained by the same colonisation density of *S. pneumoniae* for both sexes in different age groups.

Studies of pneumococcal carriage in children worldwide confirm that visiting kindergartens and nurseries is a major risk factor for colonisation. The high risk is explained by “overcrowding” in the rooms of the children’s centres, bad hygiene habits of children at those age groups and risk of viral diseases. All of the indicated factors explain the high levels of colonisation reaching up to 90% in some studies (16). In this respect, we found that 85% of the positive samples are from children attending kindergartens and schools. There was a correlation between the high probability of a culture-positive result and children attending kindergartens and schools ( $p=0.000067$ ). Our findings correspond with data from neighbouring countries such as Turkey, where similar levels of *S. pneumoniae* isolates were found from children carriers – 21.9% (17). In

Greece children with a median age of 12 months were examined and overall 33.1% were carriers of *S. pneumoniae*; furthermore, 34.1% of children with positive results attended day centres (18). In Romania 25.25% of the examined children were found to be carriers, whereas the levels among infants were relatively lower – 16.7%, compared to 29.4% among 3- to 5-year-old children (19).

The sensitivity of *S. pneumoniae* strains to the antibiotics tested in the study was as follows: 100% to vancomycin, teicoplanin and linezolid, 96.5% to norfloxacin (screening), 93% to sulfamethoxazole/trimethoprim, 85.6% to oxacillin (screening), 58.6% to tetracycline, 50% to clindamycin, and 49% to erythromycin. MDR strains non-susceptible to at least 3 antibiotic classes were 76 in total – 44% of the isolated strains. All isolated MDR strains were erythromycin-resistant and all clindamycin-resistant strains were also resistant to erythromycin (Table 1). Genotyping defined the distribution of resistant isolates by serotypes shown in Table 1.

**Table 1.** Distribution of serotypes/serogroups of *S. pneumoniae* by resistance patterns.

Serotype/ serogroup	Number of isolates	PEN	ERY	CLI	TET	SXT	MDR
6C	35	1	30	28	27	4	29
24B/F	20	2	18	18	14	1	16
3	15	1	4	4	2	-	2
11A/D	14	-	5	5	4	-	3
35F	12	-	-	-	1	-	-
19A	11	4	5	5	4	-	5
23A	11	1	8	8	6	1	6
15A/F	11	-	6	6	6	3	6
15B/C	9	-	1	1	2	1	1
23B	5	-	1	1	1	-	-
35B	5	3	4	4	-	-	3
6A	4	-	3	3	1	2	2
18A	4	-	-	-	-	-	-
4	3	-	1	1	1	-	1
10F/C	3	-	-	-	-	-	-
21	3	-	-	-	2	-	-
33F/A	3	-	2	2	1	-	1
10A	2	-	-	-	-	-	-
5	1	-	-	-	-	-	-
17F	1	-	-	-	-	-	-
19F	1	-	-	-	-	-	-
31	1	1	1	1	-	-	1
Total number (%)	174 (100)	13 (7.5)	89 (51)	87 (50)	72 (41.4)	12 (7)	76 (44)

\* PEN – benzylpenicillin (penicillin G), ERY – erythromycin, CLI – clindamycin, TET – tetracycline, SXT – sulfamethoxazole/trimethoprim

The sensitivity to  $\beta$ -lactams was tested according to the chart presented in EUCASTv9. The initial screening with oxacillin showed 14.4% non-susceptibility, followed by MIC testing for benzylpenicillin. High-level penicillin resistance was not found in the study, however intermediate susceptibility was determined (MIC<sub>50</sub>, 0.06 to 2 mg/L) for 13 strains (7.5% of all isolates) (Fig. 1). In the period 1991-1993, before the introduction of PCV10 in Bulgaria, resistance to penicillin in clinical isolates was 24.3%. Moreover, 40% of strains isolated from asymptomatic carriers among children were penicillin-resistant and more than half of all isolates were multidrug-resistant (20). The situation with *S. pneumoniae* resistance appears to be stable in Europe, with few countries reporting increasing or decreasing trends during the period 2015-2018. There are large inter-country variations among European countries, for example, penicillin-non-susceptibility of non-invasive pneumococcal isolates varies from 1.7% in Norway to 83% in Romania. Due to the geographical diversity of the resistance found in *S. pneumoniae* strains which depends on the local antimicrobial policy, there is a need of epidemiological studies in each region (21). A study from 9 European countries including Austria, Belgium, Croatia, France, Hungary, Spain, Sweden, the Netherlands and the United Kingdom, examined nasal swabs from 200 healthy persons older than 4 years (except for UK) with no history of antibiotic therapy or hospitalisation in the previous 3 months. A large variation was found in the serotype distribution among the participating countries, as well as difference in antimicrobial resistance including multidrug resistance. The highest rate of resistance to ceftazidime and penicillin was observed among strains from serotype 14. Serotype 14 was the most frequent serotype showing resistance to penicillin, followed by serotype 19A and 15A. This might be due to differences in the use of antimicrobial agents in the participating countries (12). High rates of resistance to macrolides and penicillin were observed in Romania, especially in serotypes covered by PCV13. Antibiotic resistance rates among nasopharyngeal isolates in Hungary were reported as 22% for erythromycin and 17% for clindamycin and tetracycline; 21% had intermediate resistance to penicillin.

Nasopharyngeal isolates in Ukraine showed resistance to ciprofloxacin (100%), trimethoprim/sulfamethoxazole (48%), erythromycin (33%), azithromycin (33%), amoxicillin/clavulanic acid (R and I, 33%), penicillin (20%) and cefuroxime (12%). Between 60% and 80% of isolates from invasive disease cases in Poland were susceptible to penicillin across the different age groups (22).  $\beta$ -lactam antibiotics are the first choice in the treatment of pneumococcal disease. Other widely used alternatives are macrolides because of the good tolerance and wide range of action (23). The widespread use of macrolides limits their effectiveness (24). For 2017 Bulgaria was ranked among the European countries with the highest rate of macrolide resistance in *S. pneumoniae* strains. For most countries, macrolide non-susceptibility has been more frequent than penicillin non-susceptibility (25). Another class of antibiotics to which pneumococci were reported as resistant are the tetracyclines. A frequent resistotype found among carriers in China was erythromycin-clindamycin-tetracycline that differs from the clinical resistotypes. In our study we found a high level of resistance to erythromycin with the most frequent resistotype in 85.5% of MDR strains being the erythromycin-clindamycin-tetracycline which corresponds to the results observed in China (26).

## CONCLUSION

Attending kindergartens and other children's centres was found as the major risk factor for *S. pneumoniae* colonisation. Children asymptotically carry pneumococci susceptible to  $\beta$ -lactams which could be associated with the estimated high vaccine coverage in Bulgaria. The results show high rates of macrolide resistance among isolates from carriers and co-resistance to lincosamides which corresponds to data reported for invasive pneumococci in our country. MDR strains were represented with a specific resistotype found also in other studies on *S. pneumoniae* isolates from carriers.

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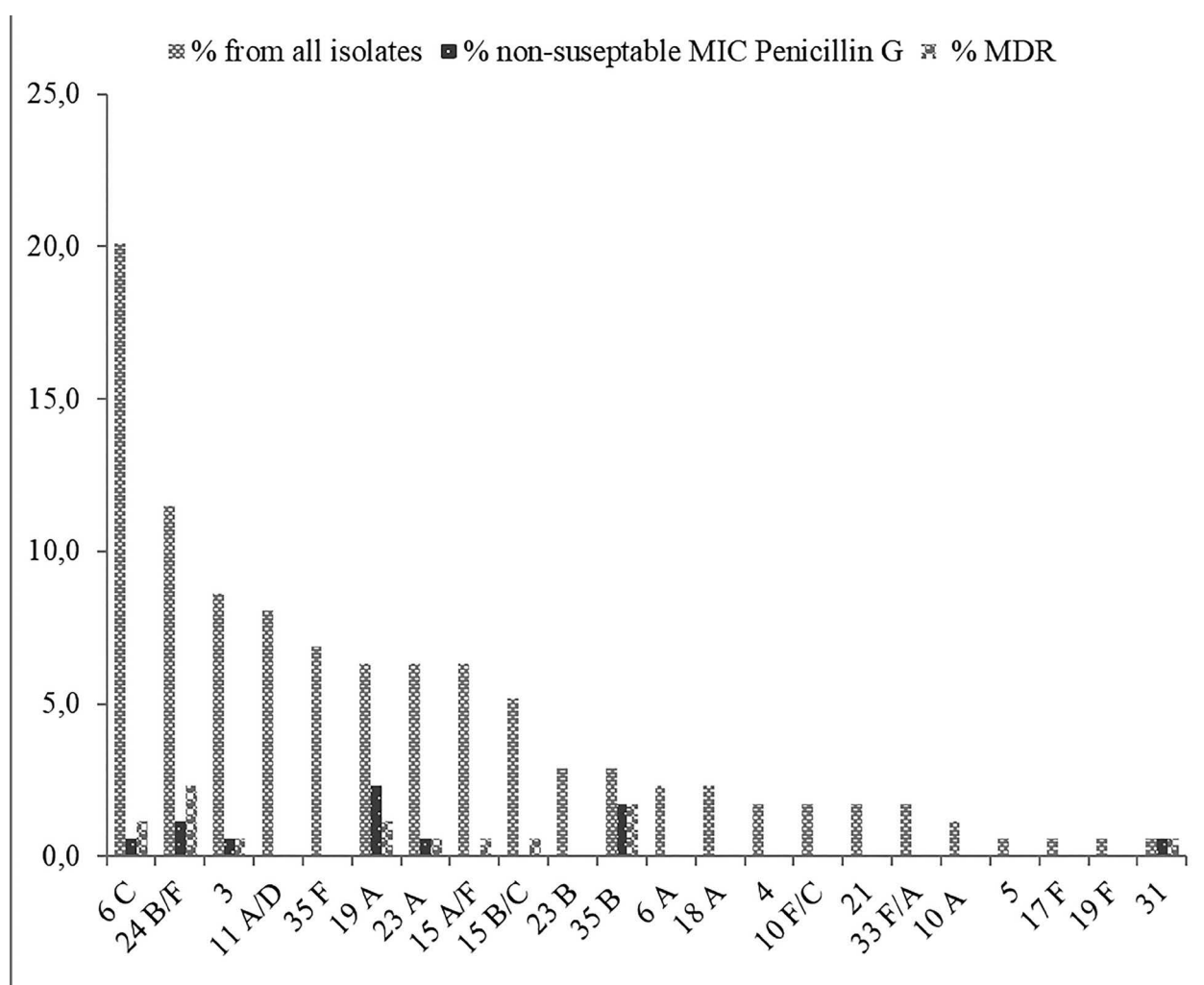


Figure 1.

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