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Antimicrobial Susceptibility Patterns of the Most Important Respiratory Pathogens

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Abstract

The present study focuses on antimicrobial resistance among the most important respiratory pathogens. The study population were children with respiratory tract infections (RTIs), treated in ambulatory and hospital settings at the Institute for respiratory diseases in children –Skopje, in the period from 01.04.2016 to 30.09.2016. Standard microbiological procedures were used for isolation and identification of bacteria. Disc diffusion test was used for antibiotic susceptibility testing. Oxacillin-resistant pneumococcal isolates were subjected to MIC determination by gradient E-test. Of all respiratory pathogens, *Streptococcus pneumoniae* was the most frequently isolated with 35% (362/1027). Ampicillin non-susceptibility rate (MIC>2mg/l) was 28.2%. Macrolide-resistant were 46.7%, with cMLSb as dominant phenotype (80.15%). The rate of MRSA was 34.9% (73/209) and gentamycin resistance in *Staphylococcus aureus* in 90.7% was connected with MRSA. 26.5% were macrolide-resistant with iMLSb as dominant phenotype. Ampicillin resistance rates among *Moraxella catarrhalis* and *Haemophilus influenzae* were 100% and 34.9%, respectively. There was no resistance to β-lactams or to macrolides among *Streptococcus β haemolyticus*. Tracing bacterial resistance profiles plays a critical role in preparing guidelines for appropriate antimicrobial treatment.

Key words *antibiotic resistance, Streptococcus pneumoniae, Staphylococcus aureus, Moraxella catarrhalis, Haemophilus influenza*

Резюме

Настоящото изследване се фокусира върху антимикробната резистентност сред най-важните респираторни патогени. В проучването са включени деца с инфекции на дихателните пътища (ИДП), лекувани амбулаторно и в болнични условия в Института за детски респираторни заболявания, Скопие за периода от 01.04.2016 до 30.09.2016. За изолиране и идентифициране на бактериите са използвани стандартни микробиологични процедури, а за определяне на чувствителността към антибиотици е приложен тест за дискова дифузия. Чрез градиент Е-тест е установена минималната инхибираща концентрация (МИК) по отношение на резистентните на оксацилин пневмококови изолати. От всички респираторни патогени най-често се изолира *Streptococcus pneumoniae* - 35% (362/1027). Чувствителните към ампицилин изолати (при МИК> 2мг/л) са 28.2%. Макролидната резистентност е 46.7%, като cMLSb е доминиращ фенотип (80,15%). Степента на метицилин-резистентни *стафилококи (*MRSA) е 34.9% (73/209), а резистентността към гентамицин при тях е 90.7% и е свързана с MRSA. Утойчиви на макролидни антибиотици с iMLSb като доминиращ фенотип са 26.5%. Степента на резистентност към ампицилин сред *Moraxella catarrhalis* и *Haemophilus influenzae* е съответно 100% и 34.9%. Сред *Streptococcus* β *haemolyticus* няма резистентни към β-лактамните и макролидните антибиотици. Определянето на профила на бактериална резистентност играе съществена роля в изготвянето на указания за подходящо лечение при бактериални инфекции.

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Introduction

The increase in antibiotic resistance is of great concern nowadays. The treatment of respiratory tract infections - i.e. those that affect the sinus cavities (sinusitis), ears (otitis media), bronchi (bronchitis), and lungs (pneumonia) is significantly impacted by resistance, as respiratory conditions account for >70% of the visits in which antibiotics are prescribed (Hersh *et al.,* 2011). The most common pathogens implicated in these infections are *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus* and *Streptococcus β- haemolyticus.* In order to determine the most effective antibiotic for an infection due to these pathogens, appropriate antimicrobial susceptibility test results are of great importance. Antibiotics are effective when they interfere with the basic metabolic functions of susceptible pathogens. Macrolides and beta-lactams are favorably used antibiotics in the pediatric population, and this study was aimed to analyze the antimicrobial resistance among respiratory pathogens, especially to emphasize the resistance patterns to macrolides and ampicillin.

Macrolides inhibit bacterial protein synthesis by binding to the large 50S ribosomal subunit and disrupting protein elongation by causing the dissociation of the peptidyl-tRNA (Poehlsgaard and Stephen, 2005). Though distinct in chemical structure, the lincosamide and streptogramin class of antibiotics have overlapping binding sites with macrolides and have similar mechanisms of action. Modification of the target site because of *e*rythromycin *r*ibosomal *m*ethylase (*erm*) family genes, whose gene product dimethylates the target site of the 23S rRNA, is the main mechanism of macrolide resistance in Europe (Reinert *et al*., 2005). Ribosomal methylation confers resistance to macrolides, lincosamides, and streptogramin B, which is characterized as the MLS_B phenotype. This type of resistance could be constitutive and inducible - when the methylation occurs only in the presence of inducible macrolides (14- and 15-membered ring macrolides) (Montanari *et al*., 2001; Kaieda *et al*., 2004). This phenotype of resistance provides high-level resistance to macrolides (erythromycin MICs usually ≥256 μg/ml). Another mechanism of resistance is active efflux of the drug. Macrolide efflux in *S. pneumoniae* has been the most common cause of macrolide resistance in North America (Schroeder and Stephens, 2016). *S. pneumoniae* with *mef* (E)/*mel* (genes whose products act as transmembrane efflux pump) have been shown to have an M phenotype, which is resistant to 14- and 15-membered macrolides but susceptible to lincosamides and streptogramin B. This type of resistance displays low-level resistance (MICs 1–8 μg/ml) to erythromycin (Schroeder and Stephens, 2016).

Resistance to *β*-lactam antibiotics is mediated by the production of either *β*-lactamase, which hydrolyzes and inactivates the drug, or of an altered target or targets, that is, penicillin-binding proteins (PBPs). Staphylococcal *β*-lactamases are narrow-spectrum penicillinases with relatively poor activity against semi-synthetic antistaphylococcal penicillins. Resistance to ampicillin in *H. influenzae* and *M. catarrhalis* is mainly because of the production of β-lactamases. These enzymes are inhibited by the inhibitor of β-lactamases, such as clavulanic acid. The target of *β*-lactam antibiotics are the enzymes transpeptidases also called penicillin-binding proteins (PBPs). In widely divergent gram-positive and gram-negative species, changes in one or more of the peptodoglican transpeptidase penicillin-binding proteins (PBP) have been correlated with decreased susceptibility to multiple β-lactam antibiotics. The penicillin-binding protein targets in penicillin-resistant strains of *S. pneumoniae* are a modified, low-binding-affinity version of the native PBPs. The level of resistance is determined by how many and to what extent the targets are modified. In contrast, methicillin resistance in staphylococci is due to the expression of PBP 2a, a low-affinity PBP, for which there is no homologue in methicillin-susceptible strains (Munita and Arias, 2016).

Material and Methods

The study population were children with respiratory tract infections (RTIs), treated in ambulatory and hospital settings at the Institute for respiratory diseases in children –Skopje, in the period from 01.04.2016 to 30.09.2016. Standard microbiological procedures were used for processing the microbiological samples, such as sputa or pharyngeal aspirations, ear swabs, nasal swabs, bronchial aspiration, throat swabs, and haemocultures. Identification of bacteria was done using standard microbiological procedures, and antimicrobial susceptibility testing was done using disk diffusion method. All susceptibility tests were performed according to the methodology proposed by the European Committee on Antimicrobial Susceptibility Testing (Matuschek *et al*., 2014). Interpretation of the zones of inhibition around antibiotic disks, and ampicillin MIK values were according to EU-

CAST- breakpoint tables for interpretation of MICs and zone diameters (EUCAST, 2016). Isolates of *S. pneumoniae, M. catarrhalis, H. influenzae, S. aureus* and *S. β - haemolyticus* were taken into account in this research.

Detection of resistance mechanisms

In order to detect inducibile clindamicin resistance, disks containing erythromycin (15μg) and clindamycin (2μg) were placed 30mm apart (from center to center). Detected antagonism of clindamicyn activity by a macrolide agent (D shape of inhibition) meant positive inducible clindamicyn resistance. *H. influenzae* and *M. catarrhalis* isolates were not tested to clindamycin susceptibility, as this is not recommended by EUCAST.

All isolates of *H. influenzae* with a zone of inhibition around 1u penicillin- containing disk less than 11mm were reported as ampicillin- resistant and were tested for β-lactamases production. For this purpose, nitrocefin test was used. Nitrocefin is a chromogenic cephalosporin and, as theamid bond in β-lactam ring is hydrolyzed by a β-lactamses, nitrocefin changes its color from yellow to red.

Oxacillin-susceptible *S. pneumoniae* (zone of inhibition around 1μg oxacillin disk ≥ 20mm) indicates strains with MIC for benzilpenicillin ≤ 0.06mg/L. Only these strains were reported as susceptible to phenoxymethylpenicillin (oral penicillin). When the oxacillin zone diameter was < 20mm but ≥8mm, these strains were reported as susceptible to ampicillin; for the strains with oxacillin zone diameter <8mm, MIK values for ampicillin were determined by gradient E-test and all strains with MIC ≤2mg/L were reported as susceptible. Methicillin resistance in *S. aureus* was determined by cefoxitin(30μg) disk-diffusion, as this method reliably predicts methicillin resistance, mostly due to *mec* A or *mec* C genes (EUCAST, 2016).

Results

During the six-month study period (from 01.04.2016 to 30.09.2016) the total number of isolated respiratory pathogens was 1027. The most frequently isolated was *S. pneumoniae* with 35% (362/1027), followed by *M. catarrhalis, S. aureus, H. influenzae* and *S. β haemolyticus* with frequency of isolation as 21% (219/1027), 20% (209/1027), 13% (129/1027) and 11%(108/1027), respectively. During the study period, only two different strains were obtained from haemocultures – in one case it was *S. pneumoniae,* and in another *H. influenzae*.

The frequency of isolation of *S. pneumoniae* by clinical samples, such as: nasal swabs, sputa, ear swabs, bronchial aspiration and haemocultures, were 70.4%, 28.2%, 0.8%, 0.3% and 0.3%, respectively. Following EUCAST recommendations, oxacillin (1μg) screening demonstrated: oxacillin susceptibility in 103 pneumococcal isolates, which were reported as phenoxymethylpenicillin- and ampicillin-susceptible; 45 isolates were with oxacillin zone diameter between 8mm and 20 mm and reported as ampicillin-susceptible; 214 were with oxacillin zone diameter <8mm and 112 of them, according to ampicillin MIC values, were reported as ampicillin- susceptible. Therefore, of all tested pneumococcal isolates, the ampicillin- susceptible was 71.8% (260/362).

For cefixim, cefalexin, cefadroxil and aminoglicosydes there were no breakpoints and susceptibility testing was not performed. All 362 isolates of *S. pneumoniae* were tested for susceptibility to erythromycin and the macrolide resistance rate was 46.68% (169/362). The phenotypes of macrolide-resistance in *S. pneumoniae* are shown in Fig. 1.

Of the 209 *S. aureus* isolates tested, 176 were obtained from nasal swabs, of which 31.2% (55/176) were found as methicillin-resistant. The remaining strains were obtained from sputa and total methicillin resistance rate was 34.9%.

Gentamycin resistance rate was 26.9% (43/160), and in 90.7% gentamycin-resistant strains were methicillin-resistant at the same time. Macrolide-resistant were 26.7% (48/180), and the phenotypes of macrolide resistance in *S. aureus* are given in Fig. 1. *H. influenzae* was isolated from nasal swabs, sputa, epipharynx and haemoculture in 49.6%, 45%, 4.6% and 0.8%, respectively. Of all *H. influenzae* isolates tested, ampicillin-resistant were

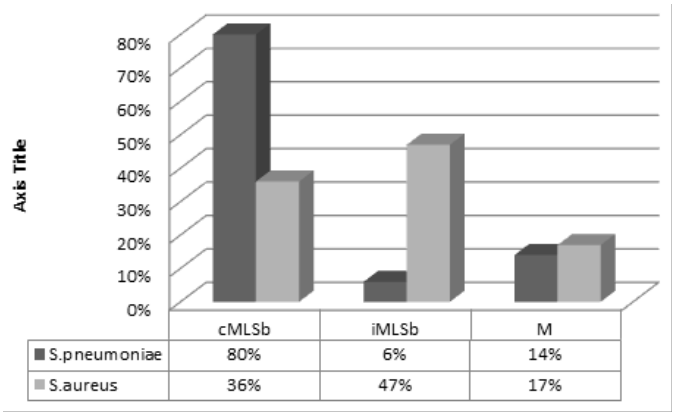

Fig. 1. Proportion of phenotypes of macrolide resistance among *S. pneumoniae* and *S. aureus*

34.9% (45/129). Resistance other than the production of β-lactamases was not detected. All ampicillin-resistant strains were susceptible to the ampiciliin/clavulanc acid combination. Macrolides were reported in 100% as intermediate resistant.

The great proportion of *M. catarrhalis* was isolated from nasal swabs 91.3% (200/219) and in 8.7% (19/219) from sputa. All isolated strains were reported as ampicillin-resistant and susceptible to ampicillin/clavulanic acid combination.

Among the *S. β-haemolyticus* isolates, there was no resistance to penicillin, cephalosporin antibiotics and macrolides. Sulfamethoxazole/trimethoprim resistance rate was 91.8% (45/49).

A short version of a cumulative antibiogram is given in Table 1.

Discussion

Respiratory tract infections are the reason for excessive and often inappropriate prescribing of antibiotics, which has undoubtedly contributed to increased resistance (Hersh *et al*., 2011). Data have shown a direct correlation between the use of antibiotics and resistance (Granizio *et al*., 2000). Countries with a higher consumption of antibiotics show higher rates of resistance (Coossens *et al*., 2005). *S. pneumoniae* is the most common community-acquired pathogen causing infections in young children (CDC, 2001). This state correlates with our findings, as *S. pneumoniae* has been the most frequently isolated respiratory pathogen.

The present study demonstrated high-level macrolide resistance among pneumococcal isolates, where approximately 47% of all tested isolates were macrolide-resistant. In the literature, the proportion of resistant isolates ranges from less than 10% to higher than 80% in different countries (Farrell *et al*., 2008). The dominant phenotype of resistance was cMLS with high MIK values for erythromycin, which implies clinically significant type of resistance (Nuermberger and Bishai, 2004). This finding correlates with the situation in Europe, where *erm* (B) that implies MLSb phenotype, dominates (Montanari *et al.,* 2003). Bergman and co-workers found a statistically significant association between macrolide resistance in *S. pneumoniae* and total macrolide consumption, and between macrolide resistance and azithromycin consumption (Bergman *et al*., 2006). If this statement is taken into consideration together with the resistance rates from the present study, it can be concluded that unnecessary use of macrolides, especially azithromycin, should be avoided.

Data from the literature suggest that respiratory tract infections caused by isolates of *S. pneumoniae* that were considered to be ampicillin intermediately resistant (MIC≤ 2μg/L) should respond well to treatment with a β–lactam agent used in appropriate doses (File *at al*., 2014). The majority of pneumococcal isolates in the present study were with such ampicillin susceptibility pattern. Ampicillin resistance among *H. influenzae* isolates was 35%, which is relatively high compared with data from other countries (Hoban and Felmingham, 2002). A mechanism of resistance other than production of β-lactamases was not detected in this study.

Nearly 85% of all isolated *S. aures* strains were obtained from nasal swabs and more than 30% of them were reported as MRSA. That im-

Table 1. Cumulative antibiogram (period 01.04.2016 – 30.09.2016)

Institute for respiratory diseases in children, Skopje Cumulative antibiogram (period 01.04.2016 – 30.09.2016)										
Organism	№ of strain	Susceptible [%]								CIP[1]
		AMP	AMC	CFM	CPD	E	CD	GM	MOX	
S. pneumoniae[1]	362	71	71	-	100	53	67	-	100	-
M. catarrhalis	219	0	100	100	-	95	-	-	98	98
H. influenzae	129	65	100	100	100	0	-	-	100	100
S. aureus	209	2	65	-	65	74	90	-	-	92

[1]*S. pneumoniae* (non meningitis); AMP – ampicillin, AMC – amoxicillin–clavulanic acid, CFM – cefixim, CPD – cefpodoxim, E – erythromycin, CD – clindamycin, GM – gentamycin, MOX – moxifloxacin , CIP – ciprofloxacin

plies high nasal MRSA colonization rates in the child population. The fact that colonization precedes infection is supported by studies that have demonstrated that nasal *S. aureus* isolates are often identical to strains later causing clinical infections (Wertheim *et al*., 2004). In some regions, community acquired- MRSA isolates account for 75% of community associated *S. aureus* infection in children (Kaplan *et al*., 2005).

Data from the study imply a high prevalence of antibiotic resistance among respiratory pathogens and rational use of antibiotics seems to be of great importance in fighting antimicrobial resistance. The recommendations for providing cumulative antibiograms as periodic reports should be implemented as a reasonable option for monitoring emerging trends in resistance at the local level (Hindler and Stelling, 2007). Cumulative antibiograms should help the physician select the most appropriate antibiotic for empirical treatment of respiratory infections.

References

Centers for Disease Control and Prevention. (2001). Active Bacterial Core Surveillance. Division of Bacterial and Mycotic Diseases.

Bergman, M., S. Huikko, P. Huovinen, P. Paakkari, H. Seppälä, Finnish Study Group for Antimicrobial Resistance (FiRe Network) (2006). Macrolide and azithromycin use are linked to increased macrolide resistance in *Streptococcus pneumonia. Antimicrob. Agents Chemother*. **50:** 3646–3650.

Farrell, D. J., C. Couturier, W. Hryniewicz (2008). Distribution and antibacterial susceptibility of macrolide resistance genotypes in *Streptococcus pneumoniae*: PROTEKT Year 5 (2003-2004). *Int. J. Antimicrob. Agents* **31:** 245–249.

File, T. M, J. Garau, F. Blasi, C. Chidiac , K. Klugman, H. Lod, JR. Lonks, L. Mandel, J. Ramirez, V. Yu (2014). Guidelines for empiric antimicrobial prescribing in community-acquired pneumonia. CHEST **125:** 1888-1901.

Goossens, H., M. Ferech, R. Vander Stichele, M. Elseviers, ESAC Project Group (2005). Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* **365:** 579-587.

Granizio, J. J., I. AguilaI, J. Casal, C. Garcia-Rey, R. Del-Re, F. Baquera (2000). *Streptococcus pneumoniae* resistant to erythromycin and penicillin in relation to macrolide and β-lactam consumption in Spain (1979-1997). *J. Antimicrob. Chemother.* **46:** 767-773.

Hersh, A. L., D. J. Shapiro, A. T. Pavia, S. S. Shah (2011). Antibiotic prescribing in ambulatory pediatrics in the United States. *Pediatrics* **128:** 1053–1061.

Hindler, J. F, J. Stelling (2007). Analysis and presentation of cumulative antibiograms: a new consensus guideline from the Clinical and Laboratory Standards Institute. *Clin. Infect. Dis.* **44:** 867-873.

Hoban, D., D. Felmingham (2002). The PROTEKT surveillance study: antimicrobial susceptibility of *Haemophilus influenzae and Moraxella catarrhalis* from community-acquired respiratory tract infections. *J. Antimicrob. Chemother*. **50:** 49-59.

Kaieda, S., H. Yano, N. Okitsu, Y. Hosaka, R. Okamoto, M. Inoue, H. Takahashi (2004). Phenotypic differentiation of macrolide resistance among *Streptococcus pneumonia* carrying mefA and/or ermB genes. *Acta Med. Nagasaki* **49:** 19-24.

Kaplan, S L., K. G. Hulten, B. E. Gonzalez, W. A. Hammerman, L. Lamberth, J. Versalovic, E. O. Mason (2005). Three-year surveillance of community-acquired methicillin-resistant *Staphylococcus aureus* in children. *Clin. Infect. Dis.* **40:** 1785-1791.

Matuschek, E., D. F J. Brown, G. Kahlmeter (2014). Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin. Microbiol. Infect*. **20:** O255–O266 10.1111/1469-0691.12373

Montanari, M. P., M. Mingoia, E. Giovanetti, P. E. Varaldo (2001). Differentiation of resistance phenotypes among erythromycin-resistant pneumococci. *J. Clin. MIcrobiol.* **39:** 1311–1315.

Montanari, M. P., M. Mingoia, I. Cochetti, P. E. Varaldo (2003). Phenotypes and genotypes of erythromycin-resistant pneumococci in Italy. *J. Clin. Microbiol*. **41:** 428–431.

Munita, J. M., C. A. Arias (2016). Mechanisms of antibiotic resistance. *Microbiol. Spect.* **4:** 10.1128/microbiolspec. VMBF-0016-2015.

Nuermberger, E., W. R. Bishai (2004). The clinical significance of macrolide resistant *Streptococcus pneumoniae*: It is all relative. *Clin. Infect. Dis.* **38:** 99-103.

Poehlsgaard, J., D.Stephen (2005). The bacterial ribosome as a target for antibiotics. *Nature Rev. Microbiol*. **3:** 870-881.

Reinert, RR., A. Ringelstein, M. van der Linden, M. Y. Cil, A. Al-Lahham, F. J. Schmitz (2005). Molecular epidemiology of macrolide-resistant *Streptococcus pneumoniae* isolates in Europe. *J. Clin. Microbiol.* **43:** 1294-1300.

Schroeder, M. R., D. S. Stephens (2016). Macrolide resistance in *Streptococcus pneumoniae*. *Front. Cell. Infect. Microbiol*. **6:** 98. doi:10.3389/fcimb.2016.00098

The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.1, 2016. http://www.eucast.org.

Wertheim, H. F. L., M. C. Vos, A. Ott, A. van Belkum, A. Voss, J. A. J. W. Kluytmans, P. H. J. van Keulen, C. M. J. E. Vandenbroucke-Grauls, M. H. M. Meester, H. A. Verbrugh (2004). Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet* **364:** 703–705.