

PHENOTYPES AND GENES OF RESISTANCE OF PNEUMOCOCCI TO PENICILLIN ISOLATED FROM CHILDREN

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Abstract: In recent decades, the increase of *Streptococcus pneumoniae* strains resistant to beta-lactams, to other classes of antimicrobial drugs and especially to penicillin (penicillin-resistant pneumococcus – PRP) has further complicated the treatment of pneumococcal infection. Penicillin resistance in pneumococci is due to the development of altered penicillin-binding proteins (PBPs) in the bacterial cell wall. PBPs are known as six different variants (PBP1a, 1b, 2x, 2a, 2b and 3).

Aim: to compare the presence and types of genes responsible for penicillin resistance in *Streptococcus pneumoniae* isolates with the minimal inhibitory concentrations (MIC) of penicillin as well as their correlation within the period of childhood.

Material and methods: A total of 45 pneumococci obtained from nasal swabs and tracheal aspirates of children treated at the University Paediatric Clinic in Skopje were examined. According to age, the children were grouped as 1–3, 4–6 and 7–10 years. The oxacillin test (1 µg) was used as a rapid screening test for the detection of PRP. MIC of penicillin were determined using the agar dilution method and interpreted according to NCCLS as resistant (if MIC are > 2 µg/ml), intermediate resistant (between 0,12–1,0 µg/ml) and susceptible (< 0,06 µg/ml). The genes *pbp2b* and *pbp 2x*, which are the genes mainly responsible for the onset of PRP, were detected using polymerase chain reaction (PCR).

Results: the oxacillin test showed that 38 pneumococci were resistant and 7 susceptible to penicillin. MIC of penicillin showed that 7 strains were resistant, 33 strains were intermediate resistant (12, 18, and 3 with MIC of 0.5 µg/ml, 0.25 µg/ml and 0.12 µg/ml, respectively) and 5 susceptible. According to MIC, of the total 40 resistant/intermediate resistant pneumococci, in 22 genes *pbp2b* and/or *pbp2x*, were confir-

med (3 resistant strains with both genes; 7 intermediate resistant and 3 resistant strains with *pbp2x* genes; whereas 8 intermediate resistance and 1 susceptible strain with *pbp2b*). In a total of 11 strains (10 intermediate resistant and one resistant according to MIC), *pbp2b* and/or *pbp2x* genes were not detected, and their resistance is probably due to some other mechanisms or other genes that code PBP. The largest number of the examined pneumococci (32) were isolated from children aged 1–3 years and in 18 of them either *pbp2b* or *pbp2x* genes were detected.

Conclusion: the oxacillin test is not suitable for discriminating the intermediate resistant and resistant pneumococci, while it is relevant for the detection of susceptible strains. Penicillin resistance of pneumococci that were causes of infection in children was on a lower level (15.5% resistant strains with MIC 1–2 µg/ml and 73.3% intermediate resistant strains with MIC 0.12–1 µg/ml). *Pbp2b* and/or *pbp2x* genes were detected in 22 of the examined strains and all of them except one were intermediate resistant or resistant. The *Pbp2b* gene is mostly present in the intermediate resistant strains and because it was detected in one susceptible strain, this gene is responsible for a low level of resistance. The *pbp2x* gene was detected in all the resistant strains and that is why we could conclude that it was coding the high level of resistance. *Streptococcus pneumoniae* was predominantly isolated from the age group 1–3 years where the PRP were not significant (Chi square; $p > 0.05$).

Key words: *Streptococcus pneumoniae*, Penicillin resistance, Minimal Inhibitory Concentration (MIC), Genes of Resistance.

Introduction

Streptococcus pneumoniae is an important human pathogen that colonizes the upper respiratory tract and causes life-threatening invasive diseases (Hotomi *et al.*, 2002). Young children are the most common carriers and pneumococcal infections at that age are a result of the inadequate immunological response to pneumococcal capsular polysaccharides. The effectiveness of penicillin therapy has been compromised by the increasing prevalence of penicillin-resistant pneumococci (PRP), especially in European countries such as Spain, France and Hungary, where it has reached up to 71%. In some parts of the USA, resistance to penicillin has reached 44%, whereas in Asia it is from 70–78%. The increase of *S. pneumoniae* strains resistant to beta-lactam antibiotics and other classes of antimicrobial drugs (macrolides, tetracyclines, and quinolones) has further complicated the treatment of pneumococcal infection. The appearance of PRP is often associated with irrational antibiotic therapy in certain countries (Opavski *et al.*, 1999; Song *et al.*, 2004).

Beta-lactamase production has never been observed in the genus pneumococcus, in contrast to staphylococci or enterococci. Penicillin resistance

in *S. pneumoniae* is a complex process that involves alterations on the penicillin target site, the penicillin-binding proteins (PBPs). The PBPs are multiple proteins with extracytoplasmic domains that function during the later stages of murein biosynthesis. *S. pneumoniae* possess six PBPs, termed PBP1a, 1b, 2x, 2a, 2b and 3. The relatively small change of the genes that participate in the encoding of these proteins causes lower affinity to beta-lactams. Resistance to beta-lactams occurs most frequently as a result of changes in amino acids near the active site of the three different PBPs 1a, 2x and 2b, resulting in a decreased affinity of PBPs to beta-lactams. Each penicillin-binding protein has a different affinity for different beta-lactam antibiotics and reduced susceptibility to cephalosporin is a result of a change in the PBPs 1a and 2b (Granger *et al.*, 2006; Opavski *et al.*, 1999; Zighelboim *et al.*, 1981).

Standard susceptibility tests are not specific enough for the detection of pneumococcal resistance to penicillin and to other different groups of antibiotics.

The oxacillin test is a screening test that can directly detect resistant PRP. Agar dilution methods are more precise, but not practical for routine examination. the polymerase chain reaction (PCR) is a more rapid molecular method which offers fast (in a few hours) and specific detection of the resistance genes, as well as elucidation of resistance mechanisms (Jalal *et al.*, 1997; Zettler *et al.*, 2004).

Aim

The aim of this study was: to compare the presence and types of genes responsible for penicillin resistance in *Streptococcus pneumoniae* isolates with the minimal inhibitory concentrations (MIC) of penicillin as well as their correlation within the period of childhood.

Material and method

Forty-five strains of *S. pneumoniae* were isolated from nasal swabs and tracheal aspirates of children treated at the University Paediatric Clinic in Skopje in the period from 2005 to 2006. The children were divided into three groups according to age: 1–3 years, 4–6 years and 7–10 years. The samples were analyzed at the Institute of Microbiology and Parasitology, Medical Faculty, Ss Cyril and Methodius University, Skopje using standard bacteriological techniques: cultivation on blood agar plates, incubation for 24 hours at 37°C, the optochin test (5µg) for identification, a screening test with an oxacillin disk

(1 µg) for the quick detection of PRP. Because of the rapid lysis, pneumococci were kept in skimmed milk (Merck) at 70° until the examination.

Determination of MIC to penicillin: in order to determine MIC (minimal inhibitory concentrations) of penicillin, the agar dilution method on a Mueller Hinton agar with 5% sheep's blood and inoculums of 0.5 McFarland turbidity (10^8 CFU/ml) was used. MICs were interpreted according to the guidelines of NCCLS (National Committee for Clinical Laboratory Standards): resistant (R) pneumococci had a MIC > 2 µg/ml; intermediate resistant (IR) from 0.12 to 1 µg/ml and susceptible (S) < 0.06 µg/ml.

Polymerase chain reaction (PCR): The procedure of DNA preparation for penicillin-resistance genes detection was done by bacterial lysis: a suspension of 10^8 CFU/ml pneumococci in 100 µl sterile distilled water was heated at 95°C for 15 min, cooled at 4°C and centrifuged (5–10 min; 6000 rpm), The obtained DNA was kept at 4°C for at least six months.

Contents of the 50 µl mixture for PCR: 5 µl 10x buffer; 4 µl 25 mM MgCl₂; 1,25 µl 10 mM dNTP (dATP; dCTP; dGTP; dTTP); 1 µl primer I; 1 µl primer II; 0.5 µl Tag DNA polymerase; 32,25 µl H₂O; 5 µl DNA.

The prepared mixture, in Eppendorf tubes, was covered with 25 µl mineral oil.

Programme for amplification (30 cycles) in Thermal Cycler was carried out as follows: denaturation of DNA at 94°C 20 sec., anyling of primers at 57°C 20 sec., extension of primers at 72°C 20 sec. The following primers were used in the investigation (Sigma):

	Code	Gene	Sequence (5–3)	Length of the product
I.	X 1	<i>pbp2x</i>	(5' – GTCATGCTGGAGCCTAAATT -3')	277
	X 2	<i>pbp2x</i>	(5' – AACCCGACTAGATAACCACC -3')	
II.	B 1	<i>pbp2b</i>	(5' – ACTCAGGCTTACGGTTCATT -3')	359
	B 2	<i>pbp2b</i>	(5' – ACGAGGAGCCACACGAACAC -3')	

For the process of electrophoresis 1.5–2% agarose gel was used. Amplified products were directly visualized and documented against a 100bp ladder DNA marker.

Results

According to the oxacillin test 38 pneumococci were resistant and 7 susceptible. MIC of penicillin showed that the greatest number [33] of pneumococcal strains were intermediate resistant (18 strains with MIC 0.25 µg/ml;

12 with 0.5 µg/ml; 3 with 0.12 µg/ml). There were 7 resistant isolates (MIC > 2 µg/ml) and 5 strains were susceptible (MIC < 0.06 µg/ml) (Table 1).

Table 1 Табела 1

Oxacillin screening test and Minimal Inhibitory Concentration penicillin to pneumococcal isolates

Оксацилин скрининг тест и минимални инхибиторни концентраци кон пенцилин кај пневмококни изолати

Susceptibility		Number of isolates
	Oxacillin test	MIC
Resistant (inhibition zone < 19 mm) (MIC > 2 µg/ml)	38 (84.4%)	7 (15.5%)
Intermediate resistant (MIC 0,12–1 µg/ml)		33 (73.3%)
Susceptible (inhibition zone > 19 mm) (MIC < 0,06 µg/ml)	7 (15.5%)	5 (11.1%)
Total	45	45

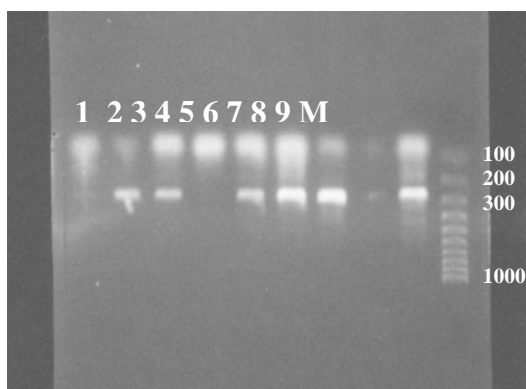


Figure 1 – Agarose gel electrophoresis of PCR – amplified fragments of the pbp2x gene (277 bp); positive: 2, 3, 5, 6, 7, 9

Слика 1 – Агарозен гел од PCR електрофореза амплифицирани фрагменти на pbp2x gene (277 bp); позитивни: 2, 3, 5, 6, 7, 9

Out of a total number of 45 pneumococci, genes *pbp2b* and *pbp2x* were confirmed in 22 strains. According to MIC and the presence of genes, 15 intermediate resistant strains possessed only one of the examined genes. In 3

resistant strains with MIC > 2 µg/ml both *pbp2x* and *pbp2b* genes were detected and in the other 3 with the same MIC only the *pbp2x* gene was present. Only one susceptible strain possessed a resistance gene of the *pbp2b* type.

Table 2 – Табела 2

Distribution of resistance genes and MIC of penicillin in 45 pneumococcal isolates

Дистрибуција на гениите на резистенција и МИК кон пеницилин кај 45 пневмококни изолати

Genes	Susceptible (MIC < 0,06 µg/ml)	Intermediate resistant (MIC 0,12–1 µg/ml)	Resistant (MIC > 2 µg/ml)
<i>Pbp2x</i>	0	7	3
<i>Pbp2b</i>	1	8	0
<i>pbp2x/pbp2b</i>	0	0	3
With genes (22)*	1	15	6
No genes (23)*	4	10	1

NS – Not signification, Chi square, $p > 0.05$

Table 3 – Табела 3

Distribution of resistance genes in pneumococcal isolates according to children's ages

Дистрибуција на гениите на резистенција на пневмококните изолати според возрастта на децата

Age (years)	Number of children*	Genes: <i>pbp2x</i> , <i>pbp2b</i> or <i>pbp2x/pbp2b</i> *
1–3	32	18
4–6	7	3
7–10	6	1
Total	45	22

NS – Not significant; Chi square, $p > 0.05$

The largest number of pneumococci (32) was isolated from children aged 1–3 years, followed by 7 isolates from children aged 4–6 years and 6 from children aged 7–10 years. Of 32 children aged 1–3 years, resistance genes were

detected in 18 (56.2%). In the second group of children (4–6 of age, 7 strains) and in the third group (7–10 of age, 6 strains) the number of strains with detected resistance genes was lower (3 and 1, respectively). The comparison of the presence of resistance genes in the group of 1–3 years and all the other (4–10 years old) children showed a higher but statistically not significant prevalence of resistance ($p < 0.005$) (Table 3).

Discussion

In the period between 1967 and 1977 reports were published on sporadic cases of PRP in different geographic areas of the world. Today, the incidence of PRP worldwide is a common clinical and particularly paediatric problem due to the fact that *S. pneumoniae* resistance is constantly increasing. Penicillin resistance has been widely spread with a high variable prevalence among different countries (Opavski *et al.*, 1999; Song *et al.*, 2004). There are no precise data about the presence of PRP in the Republic of Macedonia. The data from susceptibility testing of pneumococci isolated from in – and out-patients, performed at our Institute for penicillin (P) (detected by a disk-diffusion test with penicillin and not with an oxacillin screening test), azithromycin (AZM), ciprofloxacin (CIP) and ceftriaxone (CRO) showed the following: 1996–1998 P 20.8%/3.1%; AZM 9.2%/1.8%; CIP 3.3%/1.9% and CRO 2.4%/0.3%; 1999–2000 P 20.5%/7.2%; AZM 10.1%/4.3%; CIP 1.8%/1.1%; CRO 4.6%/2.7% and 2001–2005 P 37.4%/20.6%; AZM 18.7%/13.3%; CIP 1.9%/1.5; CRO 1.7%/0.4. These data have emphasized the need for testing and determining the pneumococcal susceptibility, especially to groups of antibiotics to which resistance has increased over the last few years (penicillin, azithromycin) (Kotevska *et al.*, 2006).

Antibiotic susceptibility testing of pneumococci, especially to penicillin, is a special laboratory problem. Oxacillin disks are recommended for screening. The agar dilution method for the determination of MIC is a good and precise method, but complex and time-consuming (48–72 hours). Our results show that the oxacillin test and the agar dilution test did not correspond completely.

The majority of studies have pointed out the usefulness of DNA methods in the identification and detection of resistance genes in PRP as well as of resistance genes for other groups of antibiotics. It is possible to obtain relevant information for the type of resistance mechanism when molecular methods are used (Zettler *et al.*, 2004). In our study all the examined strains which were resistant to penicillin (with MIC > 2 $\mu\text{g/ml}$) possessed the *pbp2x* gene, alone or together with the *pbp2b* gene. The *pbp2b* gene was detected in the strains which were intermediate resistant (MIC 0.12 – 1 $\mu\text{g/ml}$) and in one

susceptible strain (MIC < 0.06 µg/ml). Granger *et al.* (2006), Opavski *et al.* (1999) and Zigelboim *et al.* (1981) have concluded that a change in PBP 2b is most frequently associated with a low level of resistance to penicillin whereas a mutation in PBP 2x is important for a high level of resistance.

Penicillin-resistant pneumococci do not produce beta-lactamases. Former investigations have clearly indicated that the three penicillin-binding proteins (PBP 1a, 2x and 2b) are important for the development of resistance to beta-lactams; however, the other two penicillin-binding proteins (PBP 2a and 1b) might also be important in this process. *S. pneumoniae* is a rare bacterium that is naturally highly transformable. During the gene transformation process, the cell takes the free DNA from the surrounding environment and incorporates it, thus changing the genotype. The process can be divided into several phases: the ability of DNA overtaking, DNA binding, DNA transport, DNA integration into the chromosome of the cell-recipient. Analysis of genes that code the synthesis of penicillin-binding protein has shown that they are uniform in strains susceptible to penicillin, whereas isolates resistant to penicillin have many variable genes for penicillin-binding proteins. They are built as "mosaics" and consist of blocks of nucleotides that are identical or similar to isolates of penicillin-resistant strains as well as of blocks where nucleotides vary by 20% in comparison to penicillin-susceptible strains. These "mosaic" genes are most probably created with a recombination between *S. pneumoniae* and genes of related species (e.g. donor for the gene of protein 2b is *S. mitis*, and for 2a is *S. oralis*). This enables the appearance of resistant and virulent pneumococcal isolates (Bogaert *et al.*, 2000).

In our study, *pbp2b* and *pbp2x* genes were not detected in 1 resistant and 18 intermediate resistant pneumococci, which means that the origin of resistance to penicillin in these strains may be due to a mutation of some other genes that code the remaining PBP. In our study intermediate resistant pneumococci were the most frequently found [33], which means the third phase of low level of resistance of pneumococci, where MIC is 0.12 µg/ml and rarely from 1 to 2 µg/ml. However, this transient phase of PBP 1a, 2x and 2b involvement can be followed by a resistance jump, which is one more reason for building a potential strategy in preventing PRP (Opavski *et al.*, 1999).

It is known that during the first year of life colonization with *S. pneumoniae* appears in about 20% of children. Pneumococcal infections are most common in children younger than 5 years. In our study out of 45 isolates of pneumococci, the largest number [32] was found in the age group 1–3 years and in 18 of them *pbp2b* or *pbp2x* genes were confirmed as being responsible for the onset of PRP. Resistant pneumococcal isolates are the most common in the youngest population, which is important in the process of spreading penicillin-resistant clones. The close contact between children in kindergartens

and schools result in young children becoming the commonest carriers of resistant pneumococci (Bogaert *et al.*, 2002; Stratchounski *et al.*, 2006).

PRP prevalence is most frequently associated with the common use of beta-lactam antibiotics. As opposed to adults, children are treated with antibiotics very often. Irrational antibiotic therapy with oral penicillin at low and not-adjusted doses, then the use of oral cephalosporins, may be the reason for PRP. (Hotomi *et al.*, 2002). Data from Germany and Italy have shown that beta-lactams are prescribed the least in these countries, and thus *S. pneumoniae* resistance is insignificant. Furthermore, increased *S. pneumoniae* resistance to penicillin is associated with the common use of oral cephalosporins. In Japan the use of cephalosporins has caused mutations of the *pbp2x* gene (Stephanie *et al.*, 2000).

Statistical analysis (the chi square test) in our study showed no relation between the resistance genes and the ages of the children. Since there was a small number of strains (< 5 per group) the statistical test was not reliable. We plan to continue our study by providing an additional number of examined strains in order to make a more confident statistical test.

Conclusion

The oxacillin test is not suitable for discriminating the intermediate resistant and resistant pneumococci. Penicillin resistance of pneumococci cancer infection in children is at a lower level (15.5% resistant strains with MIC 1-2 µg/ml and 73.3% intermediate resistant strains with MIC 0.12–1 µg/ml). *Pbp2b* and/or *pbp2x* genes were detected in 48.9% of the examined strains and all of them except one were intermediate resistant or resistant. The *Pbp2b* gene is present mostly in the intermediate resistant strains and because it was detected in one susceptible strain, this gene is responsible for a low level of resistance. The *pbp2x* gene was detected in all the resistant strains and that is why we could conclude that it was coding the high level of resistance. *Streptococcus pneumoniae* was isolated predominantly in the 1–3 years age group where the PRP were also predominant but not statistically significant (chi square, $p > 0.05$).

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Резиме

**ФЕНОТИПОВИ И ГЕНИ НА РЕЗИСТЕНЦИЈА НА ПНЕВМОКОКИ
КОН ПЕНИЦИЛИН ИЗОЛИРАНИ ОД ДЕЦА**

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Резистенцијата на *Streptococcus pneumoniae* (*S. pneumoniae*) кон бета лактамите, особено кон пеницилин (ПРП) и другите групи на антибиотици, во последниве декади се зголемува и претставува проблем во лекувањето на пневмококните инфекции. Појавата на пеницилин резистентните пневмококи (ПРП) се должи на промена на гените кои ја кодираат синтезата на пеницилин врзувачките протеини (ПВП): 1a, 1b, 2b, 2a, 2x и 3.

Цел: Да се спореди застапеноста и типовите на гени одговорни за појава на резистенција на *Streptococcus pneumoniae* кон пеницилин со минималните инхибиторни концентрации на пеницилин (МИК), како и нивната корелација со возраста на децата.

Материјал и методи: Испитани беа 45 пневмококи добиени од брисеви од нос и трахеални аспирати од деца лекувани на Универзитетската клиника за детски болести. Според возраста децата беа групирани: од 1–3 год.; 4–6 год.; 7–10 год. Пневмококите се идентификуваа со оптохински тест. Оксацилински тест (1 µg) се употребуваше како брз скрининг тест за детекција на ПРП. Со агар дилуционен метод се одредуваа МИК кон пеницилин кои се интерпретираа според NCCLS: резистентните пневмококи имаа МИК > 2 µg/ml., интермедиерно резистентни од 0,12–1,0 µg/ml; и осетливите < 0.06 µg/ml. Со полимераза верижна реакција (PCR) се детектираа гените *pbp2b* или *pbp2x* одговорни за појава на ПРП.

Резултати: Според оксацилинскиот тест резистентни пневмококи кон пеницилин беа 38, а 7 беа осетливи. МИК кон пеницилин покажаа дека 7 соеви беа резистентни, 33 беа интермедиерно резистентни (18 со МИК 0,25 µg/ml.; 12 со МИК 0,5 µg/ml; 3 со 0,12 µg/ml), и 5 осетливи. Од вкупно 40 резистентни/интермедиерно резистентни пневмококи (според МИК) гените *pbp2b* или *pbp2x* беа потврдени кај 22 (7 интермедиерно резистентни; 3 резистентни со присуство на двата гена; 8 интермедиерно резистентни и 1 осетлив со *pbp2b*). Од вкупно 11 соеви (10 интермедиерно резистентни и 1 резистентен според МИК), *pbp2b* и/или *pbp2x* гените не беа детектирани, и нивната резистенција најверојатно е резултат на други механизми или други гени кои ги кодираат ПРП. Најголем број (32) пневмококи беа изолирани од

децата на возраст од 1–3 год. и кај 18 беше детектиран *pbp2b* или *pbp2x* генот.

Заклучок: Оксацилинскиот тест не е погоден за разликување на резистентните од интермедиерно резистентните пневмококи, но е добар за потврдување на осетливите соеви. Резистенцијата кон пеницилин кај пневмококите кои предизвикуваат инфекции кај децата е на ниско ниво (15,5% се резистентни со МИК 1–2 µg/ml и 73,3% интермедиерно резистентни со МИК 0,12–1.0 µg/ml). Кај 22 пневмококи се детектираа *pbp2b* и/или *pbp2x* и сите, освен еден, беа резистентни или интермедиерно резистентни. *Pbp2b* генот е главно присутен кај интермедиерно резистентните и бидејќи тој беше докажан кај една осетлива пневмокока, можеби е одговорен за појавата на ниско ниво на резистенција. *Pbp2x* генот е докажан кај сите резистентни пневмококи и може да се поврзе со појавата на високо ниво на резистенција. *Streptococcus pneumoniae* главно беше изолирана кај децата од 1–3 години, каде што присуството на ПРП не беше статистички значајно (X^2 ; $p > 0.05$).

Клучни зборови: *Streptococcus pneumoniae*, резистенција кон пеницилин, Минимални инхибиторни концентрации (МИК), гени на резистенција.

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