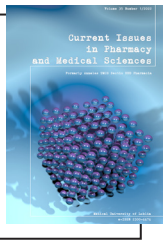
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Penicillin-binding protein genotyping of penicillin-nonsusceptible *Streptococcus pneumoniae* isolates from the nasopharynx of healthy preschool children

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ABSTRACT

Streptococcus pneumoniae is one of the most frequent bacterial identified causes of community-acquired pneumonia, otitis media and meningitis. It is, as well, a common cause of bacteraemia's significant morbidity and mortality. Beta-lactam antibiotics (BLAs) are the first line of empirical treatment for pneumococcal infections. The targets of BLAs are penicillin-binding proteins (PBPs), the modifications of which are one of the reasons why pneumococci are non-susceptible to BLAs. In our work, a total of 39 *Streptococcus pneumoniae* isolates were obtained from 176 healthy children, both vaccinated and non-vaccinated. The isolates were tested for antimicrobial susceptibility, and their penicillin-binding proteins (PBPs) were typed by the restriction fragment length of the polymorphism analysis of their *pbp* genes. The most frequent serotypes among the penicillin non-susceptible *Streptococcus pneumoniae* (PNSSP) isolates were 23B, 35B and 19F. Restriction enzyme analyses of *pbp1*a, *pbp2*b, and *pbp2*x genes revealed 5, 3 and 3 different patterns, respectively, and a total of 4 different PBPs profiles of PNSSP isolates belonging to serotypes not included in pneumococcal conjugate vaccines were demonstrated. We conclude that the level of resistance should be monitored constantly to ascertain the effect of current pneumococcal conjugate vaccines, as well as to recognize new circumstances developing in Poland, as well as the possibility of multiple, independent imports of resistant strains from abroad.

INTRODUCTION

Streptococcus pneumoniae is one of the most common bacterial pathogens of the human respiratory tract. The highest risk of pneumococcal disease is observed in young children under 2 years and in adults over 65 years of age [1]. Nasopharyngeal carriage is one of the main reservoirs of pneumococci and a major factor in the horizontal transmission of pneumococcal strains [2]. *S. pneumoniae* can migrate to the lungs and the middle ear, causing diseases such as pneumonia and otitis media (non-invasive pneumococcal diseases, NIPD) and can also penetrate to the blood and brain, causing bacteraemia and meningitis (severe invasive pneumococcal diseases, IPD) [3].

The main antimicrobial drugs used in the empirical treatment of *pneumococci* infections are β-lactam antibiotics (BLAs). The targets of BLAs are penicillin-binding proteins (PBPs), which are essential enzymes in the biosynthesis of peptidoglycan, the major component of the bacterial cell wall. BLAs work by covalently binding to serine at the active site of PBPs via the β-lactam ring, thereby disrupting the synthesis of bacterial cell walls and ultimately leading to bacterial cell death. The active sites of PBPs in pneumococci contain the three conserved sequences SXXK, SXN and KT (S) G. The SXXK motif serine is the rest of the active site and reacts with BLAs [4]. Changes in PBP active site motifs and their neighboring sequences via substitutions lead to reduction of their reactivity for BLA attachment to the binding site, resulting in antibiotic resistance [5].

Of the six known PBPs in *S. pneumoniae,* PBP2x, PBP2b and PBP1a play major roles in BLA resistance in clinical isolates, whereas changes in other PBPs have been described occasionally. Sequencing has revealed that the genes responsible for the synthesis of PBPs can consist of fragments of other species through horizontal gene transfer. These are called mosaic genes [5]. Modifications in PBP1a and PBP2b account for *S. pneumoniae* resistance to penicillins

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and carbapenems, and since the extended spectrum of cephalosporins does not interact with PBP2b, only PBP2x and PBP1a play a role in resistance to these groups of antibiotics [5].

Poland was one of the last European countries to introduce (in 2017) compulsory vaccinations starting with children from the 2nd months of life against pneumococcal colonization and diseases. Moreover, only a few publications, with limited information, have addressed the genetic diversity of *S. pneumoniae* in detail in Poland [6-8]. In the present study, the susceptibility to BLAs and the genetic characteristics of PBP in penicillin non-susceptible *S. pneumoniae* (PNSSP) isolates colonizing the nasopharynx in healthy, vaccinated and non-vaccinated children in Poland, is assessed.

MATERIALS AND METHODS

Bacterial isolates

A total of 39 *S. pneumoniae* isolates were obtained from the nasopharynx of 176 children between 1 and 6 years old attending three day care centres or visiting the Department of Paediatric Otolaryngology, Phoniatrics and Audiology, Medical University of Lublin (South-eastern Poland), over the winter and spring seasons in the period between March and June, 2020. The bacteriological methods and serotyping have been described previously [9].

LABORATORY PROCEDURES

Susceptibility of the isolates to oxacillin was determined by the disk diffusion method of Bauer and Kirby, on Mueller-Hinton agar with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD (β-Nicotinamide Adenine Dinucleotide). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing recommendations. Isolates exhibiting a zone of > 20 mm around a 1 µg oxacillin disk were reported as penicillin susceptible *S. pneumoniae* (PSSP). Isolates exhibiting a zone of ≤ 20 mm were further tested via E-test (AB Biodisk, Sweden), following the manufacturer's instruction, to determine minimal inhibitory concentration (MIC) for benzylpenicillin. Accordingly, isolates with MIC ≤ 0.064 mg/L were considered as fully susceptible to benzylpenicillin, while isolates with MIC > 0.064 mg/L were called PNSSP.

Susceptibility to amoxicillin (AMC), ampicillin (AM), cefuroxime (CXM), cefotaxime (CT), ceftriaxone (XT) for PNSSP isolates was tested by the E-test (AB Biodisk, Sweden). The results were interpreted according to EUCAST v 13.0 [https://www.eucast.org/]. Quality control was conducted using *S. pneumoniae* ATCC 49619.

Genotyping of *pbp1a*, *pbp2b* AND *pbp2x* genes

DNA from pneumococcal cultures was extracted using Genomic DNA purification with QIAamp DNA Mini Kit (Qiagen, Germantown, USA) according to the manufacturer's instructions. REDTaq ReadyMix (Sigma-Aldrich) was used in standard PCR for the amplification of *pbp1*a, *pbp2*b and *pbp2*x genes, with the primers described elsewhere [10].

PCR products were digested with the 10U of restriction enzyme *Hinf*I (Thermo Scientific), according to the manufacturer's instructions and Coffey et al. as described [10]. The digest PCR products were separated on 2.5% (wt/vol) agarose gel with 5 ml SimplifySafe (Euryx) at 120 V for 2 h. Gels were scanned by using Quantum Vilber Lourmat transilluminator. The restriction fragment length patterns for each gene were grouped by visual judgment.

RESULTS

Serotyping and antibiotic resistance patterns of the 39 isolates obtained from 176 healthy children were determined previously [9]. The pneumococcal isolates were susceptible to all tested antimicrobial agents to 43.6%. These isolates belonged to serotype 23A (5 isolates), serotypes 23B (4 isolates), 35F/47F (3 isolates), 10A (2 isolates), and 6A, 15B, 35B, (1 isolate per each serotype). Among all of the isolates, 20.5% showed non-susceptibility to penicillin 3 (7.7%), 2 (5.1%), 2 (5.1%), and 1 (2.6%) belonged to serotypes 35B, 23B, 19F and 19A, respectively. Figure 1 presents the serotype distribution of the 39 *S. pneumoniae* isolated from healthy preschool children vaccinated (n=28 isolates) and non-vaccinated (n=11 isolates) with PCV. Among pneumococcal isolates acquired from vaccinated children, 21.4% (6/28 isolates) were PNSSP, whereas among isolates from the unvaccinated group, 18.2% (2/11 strains) were PNSSP (Figure 1).

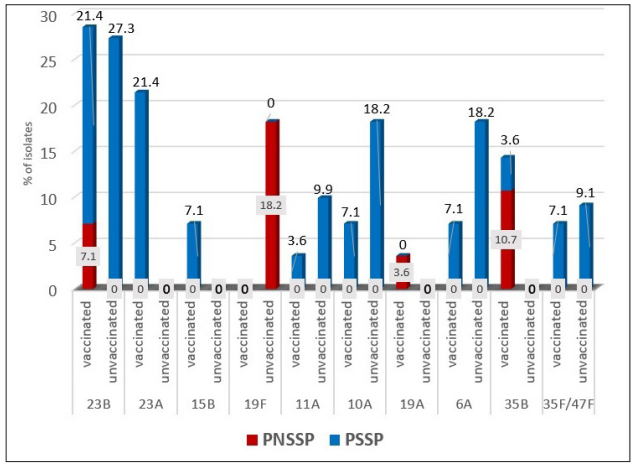
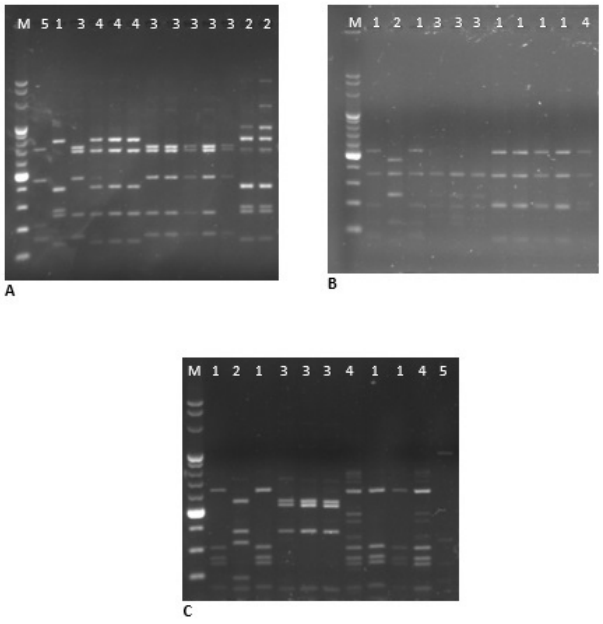


Figure 1. Serotype distribution of the 39 *S. pneumoniae* isolated from healthy preschool children vaccinated (n=28 isolates) and non-vaccinated (n=11 isolates) with PCV

To study the diversity of PBP1a, PBP2b, and PBP2x of the Polish PNSSP isolates, the PCR products of their respective genes from 8 of the PNSSP isolates and 10 penicillin-susceptible isolates (PSSP) were digested with the enzyme *Hinf*I. PCR fragments showed 5 different patterns for each *pbp1*a, *pbp2*b, and *pbp2*x. A total of 8 different *pbp* profiles were distinguished among the 18 isolates by the combination of the restriction patterns of the three *pbp* genes (Figure 2). Three profiles were identified more than once, and all of the restriction patterns were designated by roman numerals I to VIII (Table 1).



(A) Profile of pbp1a gene, lanes 2-13 correspond to RFLP profiles; (B) profile of pbp2b gene, lanes 2-11 correspond to RFLP profiles; (C) Profile of pbp2x gene, lanes 1-11 correspond to RFLP profiles. Lines M correspond to DNA size standards (GeneRuler™ 100bp DNA Ladder Plus, Thermo Scientific)

Figure 2. PCR-RFLP profiles of the different pbp genes digested by HinfI

Table 1. RFLP profiles of pbp genes and penicillin MIC values of *S. pneumoniae* isolates from healthy preschool children

pbp profile	Allele at			No. of isolates	MIC range (mg/L)	Susceptibility categories
	pbp1a	pbp2b	pbp2x			
I	4	3	3	3	0.25-0.5	I
II	2	2	2	2	2.0	R
III	1	2	2	1	1.0	I
IV	5	1	1	2	0.128-0.25	I
V	3	1	1	4	≤ 0.064	S
VI	3	5	4	2	≤ 0.064	S
VII	3	5	5	2	≤ 0.064	S
VIII	3	1	4	2	≤ 0.064	S

S-susceptible; R-resistant; I-susceptible, increased exposure

Of the PNSSP isolates, 6 were susceptible with increased exposure to penicillin, while 2 were resistant to higher levels of penicillin (MICs ≥ 2 mg/L). Resistance to cephalosporins tested was higher in penicillin-resistant isolates than in penicillin susceptible ,with increased exposure isolates (Table 2). One of the penicillin-resistant isolates was also resistant to cefotaxime. The largest group among PNSSP isolates (profile I) comprised three isolates belonging to serotype 35B, and the penicillin MIC range from 0.25 to 0.5 mg/L (Table 2). Profile II included two serotype 19F isolates isolated from unvaccinated children, and the penicillin MIC value was 2.0 mg/L. Profiles III and IV included isolates presenting 19A and 23B serotypes, respectively. The penicillin MIC range was 1.0 mg/L (III), and 0.128-0.25 mg/L (IV). Isolates belonging to I, III-VI profiles were obtained from vaccinated children.

The tested PSSP isolates were divided into 4 RFLP profiles designated by the next roman numerals, V to VIII. The largest group among PSSP isolates (profile V) consists of isolates belonging to serotype 23B. Profiles VI and VIII were presented by the isolate with serotypes 6A and 23B. They differed only in the *pbp2*x profile.

DISCUSSION

Considering that *S. pneumoniae* prevalence reports focused mainly on the studies carried out on clinical isolates and invasive strains of pneumococci [6,11-13], our research study is the first of its kind in Poland as it concerns PNSSP and PBP profiles of *Streptococcus pneumoniae* isolated from healthy children in the PCV era. The present study results have revealed that the PNSSP rate in a carriage was 4.5% (8/176 children), of which 25% were resistant to penicillin. Serotyping of the isolates revealed that in the vaccination era, Polish PNSSP strains belonged to serotypes 23B, 35B, 19F and 19A [9], and 75% (6/8) of these isolates belonged to serotypes 23B and 35B not included in PCVs. These data correspond to previous findings in other European countries and in the USA that reveal that pneumococcal vaccination caused an increase in the prevalence of non-vaccine serogroups among penicillin-resistant *S. pneumoniae* colonizing young and healthy children [13-15].

In *S. pneumoniae*, penicillin resistance is achieved by inter- or intra-specific recombinational exchanges leading to altered forms of PBPs [10,16,17], and, hence, different patterns of the *pbp1*a, *pbp2*b and *pbp2*x genes could be established in different recombinational patterns. Various restriction enzymes for RFLP genotyping of the PBP protein genes can be found in literature: *pbp1*a digested with *Sty*I plus *Dde*1, while *pbp2*b is digested with *Hae*III [18]. In our work, one restriction enzyme (*Hinf*1) was used for all three *pbp* tested genes, in accordance with the data of other authors [19,20]. It is possible that if other enzymes or their combinations were used, we would have obtained a greater number of restriction patterns. This would enable greater genotypic diversity of the studied genes.

The mutations in the *pbp2*b gene are closely associated with the resistance to penicillin [20], whereas the mutations in the *pbp2*x gene are closely associated with the resistance

Table 2. MIC values for β-lactams and pbp profiles of non-penicillin-susceptible *S. pneumoniae* isolates from healthy preschool children

Isolate		PEN	Aminopenicillins		Cephalosporins			*pbp* RFLP profile		
Id	serotype		AM	AMC	CXM	CT	XT	1a	2b	2x
23	23B	0.25	0.064	0.064	0.25	0.128	0.128	5	1	1
81	23B	0.128	0.032	0.032	0.25	0.128	0.128	5	1	1
84	35B	0.5	1.0	2.0	3.0	0.5	0.25	4	3	3
K3	35B	0.25	2.0	1.0	4.0	0.38	0.25	4	3	3
K34	35B	0.5	0.75	0.75	1.0	0.25	0.19	4	3	3
69	19A	1.0	3.0	2.0	6.0	1.0	0.5	1	2	2
K6	19F	2.0	3.0	4.0	12.0	1.0	0.5	2	2	2
K7	19F	2.0	4.0	3.0	16.0	2.0	1.0	2	2	2

PEN - Penicillin; AM - ampicillin; AMC - amoxicillin; CXM - cefuroxime; CT - cefotaxime; XT - ceftriaxone

to cephalosporins [21,22]. Adding mutations in the *pbp1a* gene will have caused a high resistance to β-lactams [12]. Other studies have shown that modifications in the active sites of PBP2x and PBP2b only brought about low levels of β-lactam resistance resulting from alterations within the primary resistance PBPs, PBP2x and PBP2b. PBP2x modifications affect low-level resistance to all or most β-lactams, whereas PBP2b mutations influence mainly penicillin resistance, however, these lack resistance to cephalosporins. In contrast, PBP2x alterations primarily affect expanded-spectrum cephalosporin resistance, whereas both PBP2x and PBP2b confer resistance to penicillins [12].

Considering the fact that PNSSP isolates in this study showed the MIC difference range of 0.13 to 2.0 mg/L, this might indicate that mechanisms other than PBPs modifications are influencing the resistance to BLAs. Similar conclusions were reached by Sogstad *et al*. [19], based on PBP analysis with an indication of the PBP2x determinant. Consistent with other studies [10,23], we have found reduced profile variation in the PBP genes among the majority of β-lactam-sensitive isolates.

PNSSP isolates showed diversity among PBP genes, indicating independent mutation of PBP genes, or horizontal transfer of PBP genes between strains [24]. The overall data was consistent with the likelihood that all or most of the penicillin-resistant isolates described here carried mosaic *pbp2*b and *pbp2*x gene sequences that originated from past recombination events with other streptococcal species. In this study, the penicillin-resistant isolates had the same RFLP profile of *pbp* genes. They belonged to serotype 19F and came from unvaccinated children. It is likely that they are derived from a clone present in the population for a long time. The remaining PNSSP isolates with non-vaccine serotypes 35B and 23B had lower MIC values. That may suggest the beginning of the phenomenon of vaccine serotypes replacement after pneumococcal vaccination. More frequent episodes of colonization by strains with non-vaccine serotypes may sequentially result in increasing recombinational exchanges between oral streptococci and pneumococci. Relation between serotypes and *pbp* profiles is probably due to close linkage and the position of the *cps* loci between the *pbp1*a gene in upstream and the *pbp2*x gene in downstream [25,26]. This configuration could endorse a co-transfer and a genetic congruence between serotype and *pbp* genotypes.

CONCLUSIONS

We have found that *pbp* genotypes were closely related to serotypes. The PBP genotyping revealed four different profiles of PNSSP isolates of pneumococcal isolates belonging to serotypes not included in PCVs. Thus, we believe that the level of resistance should be monitored constantly to explore the effect of current pneumococcal conjugate vaccines, as well as regarding new circumstances in Poland and the risk of multiple, independent imports of resistant strains from abroad. As long as the occurrence of resistant pneumococci in the neighboring countries around Poland is increasing, it is probable that we will have the constant import of resistant strains at a high level. Continuous monitoring for resistance among *S. pneumoniae* and knowledge of the characteristics of the resistant strains in Poland are important to be able to detect an eventual change in the situation as well as to rapidly control a possible clonal spread within the country.

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