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Carriage and distribution of serotypes *Streptococcus pneumoniae* in healthy vaccinated children for the year 2021 in Bulgaria

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Abstract

Streptococcus pneumoniae is one of the common causes of disease in children like pneumonia, otitis and meningitis. Pneumococci colonize asymptomatically the nasopharynx and could cause severe life-threatening illnesses. Since 2010, the 10-valent pneumococcal conjugate vaccine (PCV-10) has been included in Bulgaria for routine immunization of children from 6 weeks to 2 years. For the period 2011-2019 the coverage with it is considered to be 90%.

The aim of the present study is to determine the frequency of carriers and common serotypes of *S. pneumoniae* from nasopharyngeal isolates of children in 2021, more than ten years after the introduction of PCV10 in Bulgaria.

Nasopharyngeal swabs were taken from 220 vaccinated children, aged 5 months to 5 years old, collected from January 2021 to December 2021. Molecular detection methods were used – polymerase chain reaction (PCR) for detection and typing. By amplification of the *lytA* gene *S. pneumoniae* was detected in the taken nasopharyngeal isolates, and by amplification of the *cpsA* gene capsule abundance was determined. Typing was performed by PCR and allelic hybridization.

The results show a total of 73 positive for *Streptococcus pneumoniae* samples (33.2%). The most common serogroup/serotypes determined in carriers was 19B/19C (15%) followed by serotypes/serogroups – 15B/15C (8%), 23B (8%) and 10F/10C (7, 1%). In conclusion from the presented data, we estimate a high percentage of carriers in Bulgarian children, characterized mainly by non-vaccine serotypes in the pneumococcal populations.

Keywords: *Streptococcus pneumoniae*; Nasopharyngeal Carriage; PCR Typing; Pneumococcal Conjugate Vaccine

1. Introduction

Streptococcus pneumoniae (pneumococcus) is one of the most common bacterial pathogens causing invasive diseases in children worldwide [1]. Its most important virulence factor is the polysaccharide capsule variations in its structure determine the microorganism's serotype [1]. The species belongs to the facultative anaerobic Gram-positive cocci of the genus *Streptococcus*, family *Streptococcaceae* [1,3].

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Invasive pneumococcal diseases remain a global health problem, even in developed countries [4]. *S. pneumoniae* is a human pathogen that colonizes asymptomatically the nasopharynx and this is considered a prerequisite for the development of severe, life-threatening diseases such as pneumonia, sepsis and meningitis [5].

Asymptomatic carriage is high in children (over 30%), especially in those under five years of age that plays a key role in the transmission of pneumococci to the majority of the human population [6]. Predisposing factors for asymptomatic carriage are attendance at kindergarten or school, number of children in the family, household crowding and low hygiene [7]. Pneumococci are mainly transmitted from children to adults and about 10% of asymptomatic carriers are observed in adults [1].

Pneumococcal carriage is the precursor of pneumococcal diseases and the only source of spread of the microorganism among humans [8]. *S. pneumoniae* is transmitted by airborne droplets or by direct contact with one or more carriers. Colonization in children is higher during the autumn-winter season when colds and viral infections of the upper respiratory tract are common and are accompanied by nasal secretions [8,9].

Following the introduction of pneumococcal conjugate vaccines (PCVs), a reduction in asymptomatic carriers for this pathogen has been observed, especially in vaccine serotypes [10]. More than 100 pneumococcal serotypes based on capsular polysaccharide have been identified to date. They differ in structure as well as in incidence and manifestation of the disease and resistance to antibiotics. Many factors, such as the introduction of new conjugate vaccines, the use of antibiotics, etc., can affect the replacement of serotypes [11].

In 2010 The Bulgarian Ministry of Health approved and introduced compulsory vaccination of children with the ten valent pneumococcal vaccine – PCV10 that contains the PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, 23F) plus additional serotypes 1, 5, and 7F. In Europe the thirteen valent vaccine – PCV13 is also administered and it covers serotypes 3, 6A and 19A [12]. For the period 2011-2019 in Bulgaria the PCV10 coverage was considered to be 90%. Immunizations are performed in four doses administered on the 2nd, 3rd, 4th and 12th month after birth according to the scheme (3 + 1). Vaccines reduced the incidence and mortality from pneumococcal diseases caused by vaccine serotypes in vaccinated children [13]. As a result of collective immunity, vaccine serotype diseases are also declined among unvaccinated individuals in the population [14]. Vaccine use significantly reduces invasive pneumococcal diseases caused by vaccine serotypes, but replacement of these serotypes with non-vaccine ones has also been observed [14-16].

It is essential to monitor changes in colonizing serotypes to determine the long-term effects of vaccine use. The gold standard in the diagnosis and research of *S. pneumoniae* is the isolation and cultivation of the microorganisms. The culture study was followed by serotyping of each strain by the Quellung method, with antisera for each capsule serotype. This method is extremely expensive and time consuming, and at the same time is not suitable for the detection of simultaneous transmission of several serotypes [17].

Modern methods for molecular genetic typing of pneumococci are based on amplification of serotype-specific genes encoding the synthesis of capsular polysaccharides. The specific PCR amplifies parts of the genome sites located in the *cps* locus, which determines capsule synthesis. Molecular methods are fast, specific and effective for typing both invasive strains and isolates in pneumococcal carriers [18,19]. The Centers for Disease Control and Prevention (CDC) recommends multiplex PCR protocols, with sequential responses for rapid typing of a large number of pneumococcal isolates. Researchers have focused mainly on multiplex PCR schemes, finding multiple serotypes in a single amplification reaction [19,20].

The aim of the present study was to determine the prevalence of *S. pneumoniae* serotypes in healthy children vaccinated with PCV10 using standard cultivation methods and molecular methods for identification and typing.

2. Material and methods

The study aimed at vaccinated, healthy children aged 5 months to 5 years that were vaccinated with at least one dose of PCV10. The research was conducted from January 2021 to December 2021.

2.1. Samples

The nasopharyngeal swabs were collected after the informed consent of the parents. Materials were taken with two swabs - one with eSwab transport medium (Copan, Italy) for microbiological culture on culture medium and one dry sterile swab for DNA isolation. They were transported to the NRL Molecular Microbiology Laboratory, NCIPD, and Sofia, Bulgaria. A total of 220 children were tested for the presence of *S. pneumoniae*: 103 (46.8%) girls and 117 (53.2%). No

additional biological samples were taken and the participating children remained anonymous, only data on year and month of birth, gender were used.

2.2. Culture detection

The collected materials were cultured on Columbia CNA Agar with 5% sheep blood, with an optochin disk to distinguish pneumococci from the rest of the microbial flora, 35 ° C ± 2 ° C for 24 hours, in an aerobic atmosphere enriched with carbon dioxide. In the presence of *S. pneumoniae*, one colony with typical pneumococcal morphology was isolated and subcultured, after which the pure culture was used for DNA extraction (Qiagen kit) and stored.

2.3. Detection by molecular methods

For direct isolation of DNA from swabs with nasopharyngeal secretions, a protocol with ion exchange resin Chellex 5% was used, according to the standard protocol of NRL Molecular Microbiology, NCIPD, and Sofia. Samples were screened by the *lytA* and *cpsA* genes by real-time PCR [15]. Positive samples for both genes were typed by PCR and subsequent allelic hybridization for 76 serotypes/serogroups. *LytA-only* positive samples were considered to be capsule-free (non-encapsulated) and pneumococcal typing was not performed.

2.4. Typing of *S. pneumoniae* isolates

After DNA extraction the strains pneumococci were typed by conventional and real-time PCR and subsequent allelic hybridization with commercial Pneumostrip kit. DNA was isolated from each nasopharyngeal swab in parallel with the culture for direct screening and detection of pneumococci for two genes, according to the CDC protocol [20].

3. Results and discussion

All positive children were 73 (33.2%): 43 (58.9%) boys and 30 (41%) girls; only 17 samples from them (7.7%) were culture positive – results are combined in Tabl.1. Twenty seven (37%) of the children were found to be carriers of just one pneumococcal serotype. Whereas 28 (38.4%) were found to be positive for more serotypes: 15 children with two serotypes (20.5%), 9 children with three serotypes (12.3%), 3 children with four serotypes (4.1%) and one child with five serotypes (1.4%) at the same time. The isolates positive for *lytA* but negative for *cpsA* genes – 9 (12.3%) were considered non-encapsulated. The positive for both genes without a determined serotype/serogroup were also 9 (12.3%) suggesting they contained pneumococci of serotypes not targeted by the molecular methods used. The total counts of results from the 73 positive children were 113. The positive samples distributed by age groups were: 0% in the group under 1 year (0/17), 10% in the group of 1- 2 years (7/17), 37% in children from 3 to 4 years. (27/68), 53% of children were 5 to 6 years of age (39/118). In total, 27 different serotypes/serogroups were detected represented in Tabl.1. Predominantly serogroup 19 was fond in 20.3% of samples with serotypes – 19A (4.4%), 19B and 19C (15% detected together), 19F (0.9%). Serotype 19A was found to be presented by highly resistant strains causing invasive diseases abundant in the Bulgarian population before and after PCV10 [14,15]. Serotype 19F was included early in vaccines – PCV7 associated with antimicrobial resistance and invasive disease [17]. Serotypes 19B and 19C have polysaccharide capsules having N-acetyl group they are associated with carriage, specific binding of M-ficolin related to the innate immune system and complement activation [21]. The next most abundant serotypes are 15B and 15C with 23B that are known to increase in prevalence following the clinical use of pneumococcal conjugate vaccines and in abundance in invasive disease [16,17]. Both serotypes 3 and 19 are known to be “replacing serotypes” in invasive disease globally after PCV7 and locally after PCV10 in Bulgaria [10,15]. We assume carriage for vaccine serotype 9V but it is genetically detected together with non-vaccine 9A (PCV10) for vaccine serotype abundance we detected 9V, 14, 19F – 2,7%. Replacing serotypes detected in post PCV10 period in Bulgaria from meningitis cases were also found in the current study – 10B, 11A/11D, 15A/15F, 24A/24B/23F, 17F, 10A, 15B/15C differently distributed in percentages shown in Table 1 [15].

Multiple serotype carriage was found in children with respiratory infections and in about 30% of healthy children in Europe, competition between strains in colonization is also described [17]. Previous studies have shown that competition for nutrients and space occurs between pneumococcal strains during colonization. Our results show that the isolates contained up to five co-colonizing serotypes – 6C, 10B, 15B/15C, 23A and 23B. This phenomenon is considered mediated by bacteriocin production [22] and thus the specimens with four serotypes are just 3 and with three serotypes are 9, but those with two serotypes are 15 with most frequent serotypes again 19B/19C.

Table 1 Distribution of results from serotyping of positive samples

Serotype/serogroup	Number of positive samples	% positive samples	Included in PCVs
2	1	0.9	Not
3	1	0.9	PCV13
6A	3	2.7	PCV13
6C	5	4.4	Not
6D	1	0.9	not
7B	1	0.9	not
7C /40	6	5.3	not
9A/9V	1	0.9	PCV10, PCV13
10A	1	0.9	not
10B	5	4.4	not
10F/10C	8	7.1	not
11A/11D	1	0.9	not
12A/46	1	0.9	not
14	1	0.9	PCV10, PCV13
15A/15F	4	3.5	not
15B/15C	9	8.0	not
19A	5	4.4	PCV13
19B/19C	17	15.0	not
19F	1	0.9	PCV10, PCV13
20	1	0.9	not
21	2	1.8	not
23A	7	6.2	not
23B	9	8.0	not
24A	1	0.9	Not
24B/24F	1	0.9	Not
31	1	0.9	Not
41A/41F	1	0.9	Not
Nontypable	9	8.0	Not
non-encapsulated	9	8.0	not

4. Conclusion

In conclusion, our study confirms high carriage frequency of *S. pneumoniae* in healthy children comparable to global data. The genetic methods used have proven at least three times more sensitive than the culture methods for detecting the microorganism in the nasopharynx. After the introduction of PCV10 in Bulgaria the serotype replacement of pneumococcal population from non-vaccine strains increases, especially with the serotypes 19B/19C, 23B, 15B/15C. It is essential to continue monitoring changes in colonizing serotypes to determine the effects and efficiency of PCVs used in the country.

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Compliance with ethical standards

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Disclosure of conflict of interest

All authors declare no conflict of interest. The study was reviewed and approved by the institutional review board (IRB) 00006384 and informed consent was obtained from the patients.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study

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