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ORIGINAL ARTICLE

Phenotypic and genotypic characterization of serogroup 6 *Streptococcus pneumoniae* isolates collected during 10-valent pneumococcal conjugate vaccine era in Bulgaria

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ABSTRACT

Serogroup 6 remains common in the pneumococcal-conjugated vaccine era in Bulgaria; therefore, we investigated its clonal and serotype dynamics. The antibiotic susceptibilities were assessed by broth microdilution. Strains identified as serogroup 6 with latex agglutination method were subjected to serotype-specific PCRs. Erythromycin-resistant strains were analyzed by PCR for presence of *ermB* and *mefE* genes. MLST was performed to define clonal composition of the sequence types (STs). Serogroup 6 was represented by 40 (13.3%) from 301 invasive and non-invasive *Streptococcus pneumoniae* isolates. Molecular serotyping revealed new emerging serotype 6C (6.6%), not detected in pre-vaccine era. Among unvaccinated patients, mostly we observed serotypes 6A (57.1%) and 6B (28.6%). Serotype 6C was distinctive for vaccinated children (64%), followed by 6A (24%). Penicillin and ceftriaxone non-susceptible serogroup 6 strains were 65% and 5%, respectively; erythromycin- and clindamycin-resistant were 70.0% and 52.5%, respectively. Multidrug-resistant strains were 57.5%. Prevalent genetic determinant for macrolide resistance was *ermB* gene (75%). MLST revealed 17 STs into 5 clonal complexes and 7 singletons. Predominant genetic lineage was CC386, represented by MDR-6C non-invasive strains. Serotype 6B, principally responsible for invasive diseases in the pre-vaccine era, retreated this position to serotype 6A.

KEYWORDS

Streptococcus pneumoniae, serogroup 6, clonal composition

INTRODUCTION

The widespread use of pneumococcal-conjugated vaccines has decreased the incidence of invasive diseases in children, reduced the carriage of vaccine-type strains, and also conferred indirect herd immunity to other age groups [1, 2].

In 2010, the 10-valent pneumococcal-conjugated vaccine (PCV10, Synflorix, GSK, Brendfort, UK) was introduced for universal vaccination into Bulgarian National Pediatric Immunization Program with over 90% coverage among targeted age groups in the period 2011–2017. Prior to PCV10 implementation, PCV7 was not used in our country.

Before the introduction of PCV10, one of the most isolated serotypes in Bulgaria was 6B, following serotypes 19F and 3. Serogroup 6 is represented only by serotype 6B in PCV7 and PCV10 and an additional serotype 6A in PCV13.

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Serogroup 6 has been recognized worldwide as an important cause of invasive and non-invasive pneumococcal diseases. Initially, it was composed of the serotypes 6A and 6B, but in recent years, additional types as 6C and less frequent 6D, 6E, 6F, 6G, and 6H have been reported [3–6]. This confirms that vaccination is often accompanied by a rise in the frequency of non-vaccine serotypes (NVTs) [7, 8].

The ancestral origin of the new serotype 6C is still unknown. Serotypes 6A and 6B are with very high similarity in capsular loci, which only differ consistently in one nucleotide of the *wciP* gene. Similarly, both serotypes 6A and 6C harbored *wciN*β gene, but serotype 6C has a glucose residue in place of a galactose residue in the 6A cps repeating unit, which results in a glucosyl transferase in 6C and galactosyl transferase in 6A [9, 10].

In the post-vaccine era, 10 years after the introduction of PCV, serogroup 6 is still represented at high rates in our country.

The aim of this study was to examine the antimicrobial susceptibility profiles, clonal composition, and changes in capsular serotypes in serogroup 6 *Streptococcus pneumoniae* isolates collected after the introduction of PCV in Bulgaria.

MATERIALS AND METHODS

Patients and specimen collection

In the period January 2011–March 2019, we collect 301 invasive and non-invasive pneumococcal isolates from patients at different ages (0–84 years of age) on a voluntary basis from the Department of Medical Microbiology, Medical University – Sofia and microbiological laboratories throughout Bulgaria (Sofia, Plovdiv, and Pleven).

Among this collection of 301 *S. pneumoniae* isolates, 121 were invasive strains isolated from cerebrospinal fluids (CSFs), blood, and pleural fluids. A case of invasive pneumococcal disease (IPD) was defined as the recovery of an isolate of *S. pneumoniae* from a normally sterile site. The rest were non-invasive isolates ($n = 180$), obtained from the nasopharynx, middle-ear fluid (MEF), the conjunctiva, and sputum. Non-invasive pneumococcal disease (NIPD) was defined as *S. pneumoniae* strains causing infection detected in the ear, eye, tracheal aspirate specimens, or nasopharynx; no invasive (sterile sites) isolates were collected from the same patient. From children diagnosed with acute otitis media (AOM), one MEF sample or one nasopharyngeal sample per child was collected. The AOM episodes were confirmed by an otorhinolaryngologist or pediatrician. In the cases of persistent or recurrent AOM, MEFs were collected. AOM episodes diagnosed in children without perforation were also included, in which nasopharyngeal samples were taken through the nose.

Among 301 invasive and non-invasive *S. pneumoniae* isolates collected during the post-vaccine period, serogroup 6 constituted 13.3% (40/301) out of all pneumococcal strains.

Serogroup 6, which was the object of this study, consisted of 8 invasive strains isolated from CSF ($n = 6$) and blood ($n = 2$) and 32 respiratory strains isolated from the nasopharynx ($n = 23$), MEF ($n = 4$), sputum ($n = 2$), and eye ($n = 3$).

All pneumococcal strains were confirmed with both methods – optochin susceptibility test and bile solubility.

Data on demographic characteristics, source of isolate, clinical diagnosis, and vaccination status were collected for all received isolates (Table I).

PCV status was determined on the basis of patients' age at the date when the pneumococcalstrain was isolated. Children born April 1, 2010 or thereafter and those who had received ≥3 doses of PCV10 were defined as PCV10-vaccinated. The coverage rate of PCV10 was very high (>90%) among age-eligible children according to the national epidemiological data. The remaining children and adults were defined as an unvaccinated population.

Antimicrobial susceptibility testing

The antibiotic susceptibilities (MICs) were determined by the broth microdilution method on microtiter plates (Sensititre, Trek Diagnostic Systems Ltd., UK). STR6F MIC plate was inoculated for *S. pneumoniae* strains. Antibiotic susceptibilities were defined according to the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [11]. Pneumococcal non-meningitis isolates were classified as penicillin-non-susceptible (PNSP) with minimal inhibitory concentration (MIC) values for benzylpenicillin (MIC ≥ 0.1 mg/L) and ampicillin/ceftriaxone/cefuroxime, iv (MIC ≥ 1.0 mg/L), while penicillin-resistant pneumococci were isolates having a MIC of (>2 mg/L) according to EUCAST 2018 breakpoints. For meningitis isolates, all strains that have shown a penicillin MIC of ≥0.12 mg/L have been interpreted as resistant. *S. pneumoniae* ATCC 49619 was used as a control strain for the susceptibility test. Multidrug resistance (MDR) was defined by non-susceptibility to at least three or more classes antimicrobial agents.

PCR detection of erythromycin resistance genes

Polymerase chain reaction (PCR) method was applied to all erythromycin-resistant strains to disclose the macrolide resistance determinants *ermB* and *mefE*. The PCR conditions and primers have complied with the protocol described by Sutcliffe et al. [12].

Serotyping

Serotyping was first performed by capsular swelling reaction using commercial serogroup 6 and serotype specific factor antisera for determination of serotypes 6A and 6B provided by the Staten Serum Institute, Copenhagen, Denmark. All isolates identified to serogroup 6 were then subjected to PCR serotyping. To confirm the phenotypically determined serotypes and to identify the most recent serotypes, three PCR reactions were used.

Table I. Phenotypic and genotypic data of serogroup 6 *S. pneumoniae* isolates recovered in Bulgaria during post-PCV10 era (2011–2019)

Serotype	Lab no.	Patients' age	Vaccine[a]	Specimen	Diagnosis	Antibiotic R pattern	MIC (mg/L)		Macrolide R genotype	ST	CC[b]
							P	CTX			
6A	173 HID	7 years	No	CSF	BM	Susceptible	≤0.03	≤0.12	–	488	CC490
	15 OI	5 years	Yes	Nph.	AOM	E, C, and Sxt	0.06	≤0.12	*mef*E	490	CC490
	1119 HID	2 years and 9 months	No	Nph.	URTI	E, C, and Sxt	≤0.03	≤0.12	*mefE*	13460	CC490
	257 PI	30 days	No	MEF	AOM	P, E, C, and Sxt	0.12	≤0.12	*mefE*	490	CC490
	1126 Pd	59 years	No	CSF	BM	E and Sxt	0.06	0.01	*mef*E	490	CC490
	134 OI	2 years 6 months	Yes	Nph.	AOM	E, C, and Sxt	0.06	≤0.12	*ermB*	13460	CC490
	565 Pd	2 years	Yes	CSF	BM	P, E, C, and Sxt	0.25	0.125	*mef*E	3614	CC3614
	9542 Pd	2 years	Yes	Nph.	URTI	P and Sxt	0.25	0.03	–	3614	CC3614
	1-XII	1 year	Yes	Nph.	AOM	P, E, Cli, C, and Sxt	2.0	1.0	*ermB*	135	CC3614
	394 Pd	1 year	No	CSF	BM	Susceptible	≤0.03	≤0.12	–	2467	CC3614
	182 OI	1 year and 10 months	Yes	MEF	AOM	P, E, Cli, C, and Sxt	0.25	≤0.12	*ermB*	395	CC395
	867 CM	8 years	Yes	Nph	Carriage	E, Cli, T, C, and Sxt	0.016	0.023	*ermB*	13569	CC395
	3177 PI	1 year	Yes	Eye	Conjunctivitis	Sxt	≤0.03	≤0.12	–	600	CC600
	936 CM	4 years	Yes	Nph.	URTI	P, E, Cli, T	0.12	≤0.12	*ermB*	386	CC386
	3381 PI	59 years	No	CSF	BM	P and Sxt	0.12	≤0.12	–	3510	Singleton[c]
6B	14 OI	6 years and 10 months	No	Nph.	AOM	E, Cli, C, T, and Sxt	0.06	≤0.12	*ermB*	273	CC395
	18-XI	1 year and 3 months	Yes	Nph.	AOM	Susceptible	≤0.03	≤0.12	–	395	CC395
	1933 Pd	84 years	No	CSF	BM	P and Sxt	0.25	0.03	–	3614	CC3614
	1235 HID	2 years	No	MEF	AOM	P, E, Cli, T, and Sxt	0.5	0.25	*ermB*	149	Singleton
	146 AH	59 years	No	Sputum	CAP	P, E, Cli, and T	0.12	≤0.12	*ermB*	2922	Singleton

(Continued)

Table I. Phenotypic and genotypic data of serogroup 6 *S. pneumoniae* isolates recovered in Bulgaria during post-PCV10 era (2011–2019) *(Continued)*

Serotype	Lab no.	Patients' age	Vaccine[a]	Specimen	Diagnosis	Antibiotic R pattern	MIC (mg/L)		Macrolide R genotype	ST	CC[b]
							P	CTX			
6C	30 QI	4 years	Yes	Nph.	AOM	P, E, Cli, and T	0.12	≤0.12	*ermB*	386	CC386
	68 XI	5 years and 5 months	Yes	Nph.	AOM	P, E, Cli, and T	0.12	≤0.12	*ermB*	386	CC386
	104 XII	3 years and 6 months	Yes	Nph.	AOM	P, E, Cli, and T	0.12	0.06	*ermB*	386	CC386
	1209 CM	2 years	Yes	Nph.	URTI	P, E, Cli, and T	0.12	0.06	*ermB*	386	CC386
	476033 PI	1 year	Yes	Eye	Conjunctivitis	P, E, Cli, and T	0.25	0.06	*ermB*	386	CC386
	304 CM	2 years and 7 months	Yes	Nph.	Carriage	P, E, Cli, and T	0.25	0.06	*ermB*	386	CC386
	179 MH	2 years and 2 months	Yes	Nph.	AOM	P, E, Cli, T, and Sxt	0.12	0.06	*ermB*	386	CC386
	180 MH	4 years and 2 months	Yes	Nph.	AOM	P, E, Cli, and T	0.12	0.06	*ermB*	386	CC386
	182 MH	3 years	Yes	Nph.	AOM	P, E, Cli, and Sxt	0.12	0.03	*ermB*	386	CC386
	4031 CM	2 years	Yes	Nph.	URTI	P, E, Cli, and T	0.12	≤0.12	*ermB*	4310	CC 386
	4110 CM	4 months	No	Nph.	Bronchiolitis ac.	P, E, Cli, and T	0.25	0.06	*ermB*	1876	CC3614
	4815 Vn	71 years	No	Blood	Pneumoniae	Susceptible	≤0.03	≤0.12	–	1135	CC3614
	546 CM	6 months	Yes	Nph.	Carriage	P	0.25	0.125	–	3614	CC3614
	3254 CM	13 years	No	Nph.	Carriage	E, Cli, and T	0.06	≤0.12	*ermB*	2924	CC395
	498 AH	13 years	No	Sputum	Bronchiectasis	P, E, Cli, and Sxt	2.0	0.75	*ermB*	8	CC395
	33 QI	3 years	Yes	Nph.	AOM	Susceptible	≤0.03	≤0.12	–	1205	CC600
	1224 PI	1 year	Yes	MEF	AOM	P, T, and Sxt	0.12	≤0.12	–	8630	Singleton
	2523 PI	1 year	Yes	Eye	Conjunctivitis	P, E, C, and T	0.12	≤0.12	*mef*E	367	Singleton
	3967 CM	31 years	No	Blood	Bacteriemia	Susceptible	0.008	0.006	–	1804	Singleton
	1559 Pd	2 years	Yes	Nph.	CAP	P, E, Cli, T, and Sxt	2.0	1.0	*ermB*	5740	Singleton

Note: Pneumococcal isolates were classified as penicillin – nonsusceptible (benzylpenicillin MIC ≥ 0.1 mg/L and ampicillin/ceftriaxone/cefuroxime, iv – nonsusceptible (MIC ≥ 1.0 mg/L) according to EUCAST breakpoints, 2018. Abbreviations: CSF: cerebrospinal fluid; Nph: nasopharynx; MEF: middle year fluid; BM: bacterial meningitis; AOM: acute otitis media; URTI: upper respiratory tract infection; CAP: community acquired pneumonia, P: penicillin; E: erythromycin; Cli: clindamycin; Sxt: trimethoprim – sulfametoxasole; T: tetracycline; C: chloramphenicol; MIC: minimal inhibitory concentration; ST: sequence type; CC: clonal complex.[a]Application of PCV10 according to age. All children born April 1, 2010 or thereafter were eligible for vaccination. The remaining children and adults were defined as an unvaccinated population.[b]Defined using eBurst algorithm. A cut-off point of 5 identical loci to the predictor founder was used to determine a CC, named after the predominant ST in the group.[c]ST without defined CC, but belonging to a known eBurst group.

We performed PCR for simultaneously detecting 6A and 6C, because of their very high similarity in the cps loci. The presence of 6A/6C was proven by 149-bp amplification product of *wciP* gene. PCR amplification of *wciN*β gene (359 bp) was used to resolve 6C. The isolates of serotype 6B were tested by PCR with primers, which amplify a part of *wciP* gene (155-bp product). The primers used in this study are previously published by Jin et al. [13] and Park et al. [14]. PCR buffers and DNA polymerase were supplied by GenetBio, Korea and all DNA primers were obtained from Alpha DNA, Canada. Gene amplification was performed using a GenePro-thermal cycler (Bioer, China) as follows: 94 °C for 15 s, 35 cycles of 95 °C for 30 s, 60 °C for 60 s, 72 °C for 60 s, and 72 °C for 10 min. Electrophoresis on 1.5% agarose gels was used to distinguish PCR products.

Multilocus sequence typing (MLST)

MLST was performed as previously described [15]. Seven housekeeping genes were sequenced and compared to the pneumococcal MLST database (http://pubmlst.org/spneumoniae) to identify the alleles and respective sequence types (STs). PHYLOViZ (https://online.phyloviz.net/) was used to define clonal complexes (CCs). STs sharing five (double locus variants – DLVs) or six (single locus variants – SLVs) identical alleles were assigned to the same CC, named after the predominant ST in the group. STs not assigned to any CC were designed singletons (Figure 1).

Statistical analysis

The $\chi 2$ and/or Fisher's exact tests were used for the analysis of categorical data, and statistical significance was set at $p \leq 0.05$.

RESULTS

Demographic and clinical data of patients

Serogroup 6 was represented by 40 (13.3%) from all 301 invasive and non-invasive *S. pneumoniae* isolates collected after PCV10 implementing in our routine immunization program. The invasive strains isolated from patients with meningitis and bacteremia were ($n = 8$) 20%. The prevailed group of pneumococci ($n = 16$) was from patients with AOM (40%). The rest of the patients ($n = 16$) were with upper respiratory tract infections, bronchiolitis, bronchiectasis, community-acquired pneumonia and conjunctivitis (Table I). The higher proportion of the strains ($n = 31$) was isolated from children of age 0–7 years (77.5%), among them 54.8% were from patients less than 2 years of age. Five strains were from ≥59-year-old patients and the rest four patients were from middle-age group (8–31 years of age). Out of the 31 children, 25 had received PCV10 vaccination (80.6%).

Antimicrobial non-susceptibility

Using the meningitis criteria and the MIC values for benzylpenicillin (MIC ≥ 0.1 mg/L), 65% of the serogroup 6 isolates were classified as PNSP and 5% were ceftriaxone non-susceptible (Table I). Among the meningitis isolates ($n = 6$), we observed three penicillin-resistant pneumococci having a MIC of ≥0.12 mg/L, the others were susceptible to all tested antimicrobial drugs.

We found also high rates for erythromycin and clindamycin resistance of 70.0% and 52.5%, respectively. Tetracycline-resistant strains were 47.5%. Chloramphenicol resistance was observed in 25% of the strains. The resistance to trimethoprim–sulfamethoxazole reached 50% among the serogroup 6 isolates. Six isolates were susceptible to all tested classes of antimicrobial drugs. The MDR serogroup 6 isolates were 70%. The non-susceptibility rates to antimicrobial agents of all 40 serogroup 6 *S. pneumoniae* isolates are shown in Figure 2.

Serotyping

All of the isolates belonging to serogroup 6 were identified as serotype 6A ($n = 35$) and 6B ($n = 5$) by Quellung test using serotype-specific factor antisera provided by Statens Serum Institute (Copenhagen, Denmark). The results from serotype-specific PCR's disclosed in fact three serotypes in serogroup 6: 6A ($n = 15$), 6B ($n = 5$), and 6C ($n = 20$). They were 37.5%, 12.5%, and 50%, respectively.

The distribution among serotypes in children ≤7 years of age was 54.8% for serotypes 6C, 35.5% for serotype 6A, and only 3 strains from serotype 6B, including two patients without an applied vaccine. In adults who are ≥59 years of age, we observed heterogeneous data: equal proportions for 6A and 6B and one isolate from serotype 6C.

We determined three 6C and one 6A isolate from the rest four patients, which were non-vaccinated (13–31 years of age), born after April 1, 2010.

The results showed prevalence for serotype 6A among all invasive isolates ($n = 4/8$), followed by 6C ($n = 2/8$) and only one isolate from a patient with meningitis at 84 years of age from serotype 6B. All patients with meningitis were non-vaccinated and the isolated pneumococcal strains were from serotype 6A. The isolated pneumococcus from blood specimens was from serotype 6C from unvaccinated patients.

In the group of AOM isolates ($n = 16$), the predominant serotype was 6C (50%), followed by serotype 6A (31.3%) and serotype 6B (18.7%). The widespread serotype isolated from patients with respiratory tract infections and conjunctivitis was serotype 6C (62.5%), which was not encountered in the pre-vaccine era in our country (1995–2010). The rest respiratory isolates were from serotype 6A (31.2%) and only one was from serotype 6B recovered in an old unvaccinated patient.

In the group of unvaccinated children ($n = 7$), four strains were from serotype 6A, followed by two 6B strains and only one 6C strain. In comparison to group of vaccinated children ($n = 25$), we discovered an emerging serotype 6C ($n = 16$)

with 64.0% distribution, less frequent 6A ($n = 6$) 24%, and only one 6B isolate.

Among PNSP ($n = 26$), we noted 61.5% serotype 6C isolates, 27% serotype 6A isolates, and 11.5% from serotype 6B. The erythromycin-resistant strains were principally from serotype 6C (53.6%) and less often from serotype 6A (35.7%) and serotype 6B (10.7%). The highest percent of MDR strains was distinctive for 6C isolates (56%), followed by 6A (28%) and 6B (16%).

PCR – Analysis of macrolide resistance genes from serogroup 6 *S. pneumoniae* isolates

The predominant genetic determinant for macrolide resistance out of all erythromycin-resistant serogroup 6 isolates was *ermB* gene, observed in 20 (75%) strains. *MefE* gene was determined in the other 25% of the strains.

The strains from serotype 6C strains possessed mostly *ermB* genes in their genome (75%) and two 6C strain carried *mefE* gene. Efflux mechanism was prevailing for serotype 6A strains (41.7%) and *ermB* gene was represented by 16.7% 6A pneumococci. All macrolide-resistant 6B strains possessed *ermB* genes.

Multilocus sequence typing (MLST)

Among the isolates of serogroup 6, MLST revealed 17 STs distributed into 5 CCs named after with the predominant ST and 7 singletons (Figure 1).

CC490 ($n = 6$) was represent by 3 STs: ST490, ST488, and ST13460. All strains from CC490 were from serotype 6A, isolated from patients with meningitis and AOM. Except one strain susceptible to all antimicrobial agents, all other isolates from CC490 were erythromycin-resistant, principally harbored *mefE* gene (80%).

CC3614 was the most diverse, distributed in 5 STs and 8 strains from serotype 6C ($n = 4$), serotype 6A ($n = 3$), and serotype 6B ($n = 1$), isolated from patients with invasive and non-invasive diseases. The PNSP isolates in CC3614 were 75%, including 37.5% MDR strains.

CC386 was the most common among the studied population of serogroup 6 ($n = 11$). All of the strains, except one from ST4310, were from ST386 ($n = 10$). These two STs were SLVs and were associated with international clone Poland^{6B}-20/ST315 from Pneumococcal Molecular Epidemiology Network (PMEN). All pneumococci from CC386 were from serotype 6C isolated from patients less than 7 years of age with non-invasive infections, except one strain from serotype 6A. The isolates from CC386 were MDR strains. The responsible genetic determinant for macrolide resistance in CC386 was represented only by *ermB* gene.

CC395 was shared between PCV10 NVTs 6A and 6C and two isolates from vaccine serotype (VT) 6B. It consisted of five STs: ST395, ST273, ST13569, ST2924, and ST8, which were DLVs. ST395 was DLV of PMEN clone Portugal^{6A}-41. ST273 was identical to PMEN clone Greece^{6B}-22. All representatives to this CC395 were also MDR, except one. We detected *ermB* gene out of all erythromycin-resistant strains from this genetic lineage.

The next clonal lineage was CC600, with ST600 and ST1205 represented by two *S. pneumoniae* strains isolated from children with conjunctivitis and AOM. The strains were susceptible and resistant only to trimethoprim–sulfamethoxazole, respectively.

We disclosed seven singletons among serogroup 6: ST8630, DLVs of PMEN clone Denmark^{14}-32, ST1804, ST3510, ST149, ST5740, ST367, and ST2922, which are SLV of England^{14}-9. Out of all singletons, we observe 85.7% PNSP, including 71.4% MDR strains.

Figure 1. Clonal composition of serogroup 6 serotypes among *S. pneumoniae* isolates collected during PCV10-vaccine era in Bulgaria (2011–2019). *Note:* Each circle represents an ST. Circle sizes are proportional to the number of isolates within the ST. Solid, gray lines connect STs that are SLVs and thin, light lines connect STs that are DLVs according to the PHYLOVIZ tree cut-off: 2, NLV 2 rule reached (https://online.phyloviz.net/). STs that are linked belong to the same CC, named after with the predominant ST in the CC. Text in dark gray indicates PMEN-related clones. STs not assigned to any CC were designed singletons

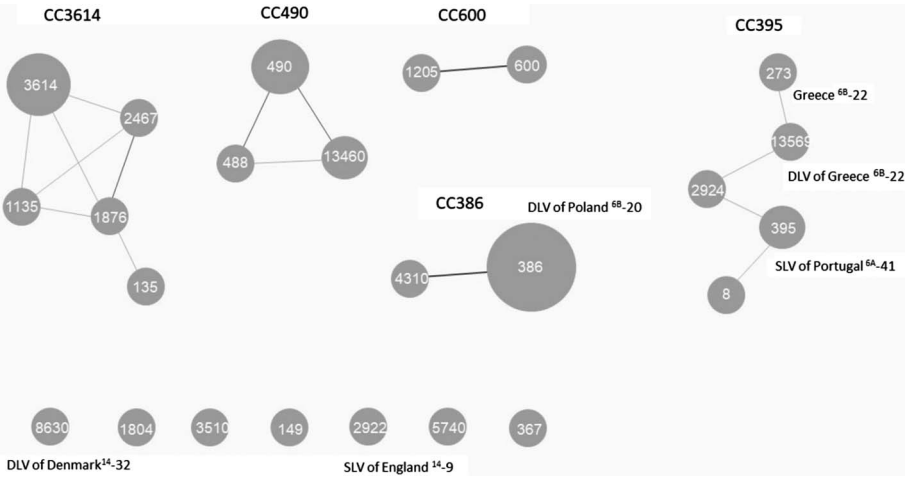
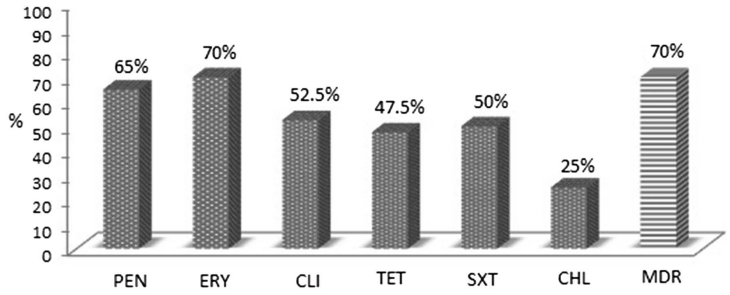


Figure 2. Antimicrobial non-susceptibility among 40 serogroup 6 *S.pneumoniae* isolates. *Note:* The following non-susceptible MIC (mg/L) breakpoints were used: benzylpenicillin (penicillin) ≥ 0.12 according to the EUCAST. For other antimicrobials, both categories intermediate and resistant isolates were summarized [11]. PEN: benzylpenicillin; ERY: erythromycin; CLI: clindamycin; TET: tetracycline; CHL: chloramphenicol; SXT: trimethoprim–sulfamethoxazole; MDR: multidrug-resistant isolates



DISCUSSION

This study shows that in the post-PCV10-vaccine period, serogroup 6 remains common in our country. In the pre-vaccine era, serotype 6B was the most frequent, but it subsequently decreased in both adults and children in the post-vaccine era.

Compared to our previous study, serotype 6B was responsible for 10.5% of the invasive diseases ($n = 222$) for the period 2006–2010 in contrast to only one case of IPD among the studied population ($n = 301$) [16]. A significant decrease in the proportion of serotype 6B ($p = 0.0048$) was found in this study.

In addition, we observed a significant reduction of serotype 6B among MEF isolates from 15.6% to 4.6% ($p = 0.0202$), when we compared results of this study with the pre-vaccine era for the same kind of specimen [17]. We concluded that PCV10 was highly successful in reducing IPD and NIPD caused by VTs in our country.

As expected, a significant increase of NVTs was found in the post-vaccine era. Compared with our findings from the non-vaccine period [18], we noted escalation in the proportions of NVT from serogroup 6 (6A and 6C) from 4.8% to 11.6% ($p < 0.003$). Our findings suggest that after implementation of PCV10 into our National Immunization Program, the majority of the circulating pneumococci among vaccine-eligible children was NVTs.

Like other authors, we found that serotype-specific PCR of some of our isolates previously serotyped as 6A by Quellung test was in fact 6C, due to cross-reaction of both serotypes 6C and 6A [19]. Serotype 6C was not detected before PCV10 implementation. At present, we found a rate of 6.3% for serotype 6C in the PCV10 period. Serotype 6C can be cross-reacted serologically with serotype 6A and is an example of a serotype 6A immunological variant that escaped notice for decades, due to its serological similarity to serotype 6A [20, 21]. It has been frequently found in young children primarily with an association of upper respiratory tract infections. We do not observe serotype 6C accountable for invasive infections in this population age group in our country. Only two clinical cases of adults with IPD caused by 6C pneumococci were recorded. Low prevalence of serotype 6C IPD in children has also been observed in other studies from France, Portugal, and Brazil [22–24]. There are also investigations in which 6C is becoming more common as a causative agent of IPD, which confirms that vaccination is accompanied with serotype dynamics and rise in the frequency of NVTs [25–27].

Serotype 6A was accounted for IPD in both age groups after the introduction of PCV. We observed an increase in the rate of 6A-IPD of 5.3% in the post-vaccine period in contrast to 2.1% in the pre-vaccine period (2006–2010). Although it is not statistically supported (0.1016) at $p < 0.05$, 6A was the most frequent serotype from serogroup 6, responsible for invasive diseases.

A change in serotype frequencies is often coupled with an increase in antibiotic resistance among NVTs [28]. Other investigations reported that serotypes 6A and 6B exhibited significantly higher levels of erythromycin resistance and penicillin resistance [29].

More than half of the studied serogroup 6 isolates were MDR. Intermediately penicillin- and ceftriaxone-resistant isolates were much more common, while fully resistant isolates were rarely encountered. Serotype 6C comprised most MDR isolates. The genetic determinants for macrolide resistance kept the same layout as the non-vaccine period: widespread *ermB* gene, followed by *mef*E gene.

To explore the relationship between antibiotic resistance and CCs, we analyzed which of these CCs are associated with antimicrobial resistance. Genetic lineage CC386 was represented only by MDR strains. We also observed a high proportion of resistant strains in CC395. CC3614 represented mostly PNSP strains. The majority of PNSP isolates expressed low-level resistance (MIC = 0.12 ± 1 mg/L), with the exception of a singleton ST5740 and two others ST135 and ST8, which expressed high-level penicillinresistance (MIC ≥ 2.0 mg/L). CC490 was associated with low levels of macrolide resistance. CC600 included susceptible isolates: fully susceptible and one resistant only to trimethoprim–sulfamethoxazole.

The clonal composition of serogroup 6 was heterogeneous

Serotype 6A was the most diverse, distributed in all 5 CCs, found in this study and 11 STs. Among them, three STs such as ST395, ST386, and ST13569 were closely related SLVs or DLVs to international clones recognized by PMEN. Serotype-switching events were detected in CC395 for ST13569, ST292, and ST8, which were associated with serotypes 6A and 6C and were related to PMEN Greece^{6B}-22 clone. Another supporting evidence of recombination between the cps regions of serotypes 6A and 6B leading to changes from one of the serotypes to the other has been published in several studies [20, 30].

Serotype 6B was represented by two MDR-singletons; one of them is SLV of England^{14}-9, a MDR isolate identical to PMEN clone Greece^{6B}-22/ST273; an PNSP from ST3614, the primary founder of CC3614 and one susceptible isolate from ST395, SLV of Portugal^{6A}-14.

Among serotype 6C, we observed three CCs, such as CC386, DLVs of PMEN Poland 6^{B}-20/ST315, CC3614, and CC600, and almost all singletons found in the studied population: ST8630, ST367, ST5740, ST1804, and ST8. All strains belonging to the most widespread ST386 from this study were resistant both to erythromycin and tetracycline, in support of the evidence ST386 is related to the 6B genetic background of the international Poland^{6B}-ST315 clone, which harbors the Tn6002 transposon [31]. Strains isolated in France and Brazil had been previously related to CC386 [22, 23].

In conclusion, we observed a gradual increasing of NVTs among serogroup 6 after implementation of PCV10 in our country. The prevalent serotype 6C was associated with MDR non-invasive strains. Due to the vaccine pressure, it has been proposed that the associated antibiotic resistance characteristics of 6C would facilitate its emergence. Serotype 6B, which was responsible for all the more IPD cases before introduction of PCV in Bulgaria, retreated and 6A occupy this position in the post-PCV-era. ST386 was the most widely presented in the studied population of serogroup 6, which is represented in other countries in Europe and South America also.

Further studies are necessary to clarify the serotype dynamics and fast changing epidemiological characteristics.

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