AKADÉMIAI KIADÓ

Acta Microbiologica et Immunologica Hungarica

69 (2022) 3, 177–184

DOI:
10.1556/030.2022.01816

RESEARCH ARTICLE

Association of pili with widespread multidrug-resistant genetic lineages of non-invasive pediatric *Streptococcus pneumoniae* isolates

ALEXANDRA SASHOVA ALEXANDROVA[1*], DANIELA ROSENOVA PENCHEVA[2], LENA PETROVA SETCHANOVA[1] and RAINA TSVETANOVA GERGOVA[1]

[1] Department of Medical Microbiology, Medical Faculty, Medical University of Sofia, 1, G.Sofiiski Boul., 1431-Sofia, Bulgaria

[2] Department of Medical Chemistry and Biochemistry, Molecular Medicine Center, Medical Faculty, Medical University of Sofia, 2, Zdrave str., 1431-Sofia, Bulgaria

Received: July 1, 2022 • Accepted: August 30, 2022
Published online: September 12, 2022

ABSTRACT

The study aimed to evaluate the presence of pili in non-invasive pediatric pneumococcal isolates and to elucidate possible links with genetic lineages, serotypes, and antimicrobial resistance. We examined 147 *Streptococcus pneumoniae* isolates from children with respiratory tract infections and acute otitis media. Serotyping was performed by latex agglutination and capsule swelling reaction. Serogroup 6 was subjected to PCR-serotyping. Minimum inhibitory concentrations were determined according to EUCAST breakpoints. PCRs for *rlrA* and *pitB* genes were performed to detect a presence of type 1 and type 2 pili. MLST was conducted to define the clonal structure of the piliated strains. Almost all children (96.5%) were vaccinated with the pneumococcal conjugate vaccine PCV10. We detected 76.8% non-PCV10 – serotypes (NVTs) and 14.3% PCV10 serotypes. The predominant serotypes were NVTs: 19A (14.3%), 6C (12.2%), 3 (9.5%), 15A (7.5%) and 6A (6.8%). PI-1 was detected among 10.9% non-PCV10 serotypes 6A, 6C, and 19A and 6.1% PCV10 serotypes 19F and 23F. Type 2 pili were not found in the studied population. High levels of antimicrobial nonsusceptibility to erythromycin (58.5%), oral penicillin (55.8%), clindamycin (46.9%), trimethoprim-sulfamethoxazole (45.6%), tetracycline (39.5%) and ceftriaxone (16.3%) were revealed. The multidrug-resistant strains (MDR) were 55.1%. MLST represented 18 STs and three CCs among the piliated pneumococci: CC386, CC320, and CC81. More than half of the piliated strains (56.0%) belonged to successfully circulating international clones. PI-1 was associated mainly with MDR 6A, 6C, 19A, 19F, and 23F isolates from the widespread CC386, CC320, and CC81.

KEYWORDS

Streptococcus pneumoniae, pilus, serotype, clonality, antimicrobial resistance

INTRODUCTION

Streptococcus pneumoniae is a major cause of mild respiratory tract mucosal infections such as otitis media, sinusitis, bronchitis, and rhinopharyngitis to more severe diseases such as pneumonia, septicemia, and meningitis. The rate of invasive pneumococcal diseases has significantly decreased after the introduction of the 10-valent conjugate vaccine (PCV10) in the Bulgarian Immunization Program in 2010, but the non-invasive pneumococcal infections (NIPD) continue to affect a large number of children under the age of five [1–3].

The development of pneumococcal respiratory infection is preceded by nasopharyngeal colonization. The pneumococcus is a highly adapted commensal that successfully competes

*Corresponding author.
Tel.: + 359 2 91 72 719.
E-mail: alex.alexandrova1@gmail.com

with other microbial agents and colonizes the mucosa of the upper respiratory tract. Transmission, colonization, and invasion depend on the remarkable ability of *S. pneumoniae* to avoid host inflammatory and immune responses.

S. pneumoniae penetrate and survive intracellularly using a combination of virulence factors such as polysaccharide capsule, pneumolysin, adhesins PavA and PavB, pneumococcal surface protein A (PspA), pyruvate oxidase SpxB, IgA1 protease, metalloprotease ZmpB, pili and others [4–6].

The mobile genetic element encoding a pilus-like structure in pneumococci was one of the recently discovered genetic regions associated with virulence factors in *S.pneumoniae*. The pili have a significant role in the adhesion and colonization of the host and are involved in the biofilm formation, characteristic mainly of bacteria that form the oral microflora [7, 8].

The pneumococcal pili are highly immunogenic structures under the selective pressure of the host's immune responses. The expression of pilus is heterogeneous, with the coexistence of two subpopulations expressing different types of pili encoded by two pathogenic islets: PI-1 and PI-2 [8, 9].

Type 1 pili in *S. pneumoniae* are encoded by a rlrA-islet of pathogenicity, referred to as PI-1, which represents a 14.2 kb region composed of seven genes. The genes in the PI-1 islet encode a transcriptional regulator (RlrA), three LPXTG surface proteins recognizing adhesive molecules – MSCRAMMs (RrgA, RrgB, and RrgC), and three sortases (SrtB, SrtC, and SrtD), which are involved in the polymerization of pili [9–11].

Mouse models of pneumococcal pneumonia and bacteremia have been studied to confirm the essential role of pilus in pneumococcal virulence and host inflammatory response [7–9]. Investigations revealed the major adhesin RrgA in PI-1 that mediates pharyngeal colonization in mice models and affects the ability of pneumococci to adhere to human lung epithelial cells. Strains that lack RrgA have a significantly reduced ability to attach to nasopharyngeal epithelial cells [8, 9].

Studies presented immunizations of mice with pilus antigens induce protection against lethal pneumococcal strains [12]. Such data suggested that vaccination with pilus protein subunits offers the same protection as vaccination with heat-killed bacteria. Some studies establish the pilus as a potential candidate for a pneumococcal protein vaccine [7, 13].

PI-1 is not uniformly common among the different 101 pneumococcal serotypes [14]. Analysis of a global collection of *S. pneumoniae* strains reported that the incidence of PI-1 was 30% overall and was observed mainly among antibiotic-resistant pneumococci, indicating a correlation between the presence of a pathogenic islet and the genotype of isolated strains [13, 15, 16].

The pathogenicity islet PI-2 is about 6.5 kb and includes five genes: *pitA*, encoding pilus subunits, *pitB*, encoding the backbone protein, sortase genes *srtG1* and *srtG2*, and *sipA*, encoding the proteolytic enzyme peptidase [17]. The reported prevalence of PI-2 varies from 0% to 21% in invasive diseases, otitis media, and carriers [8, 15].

Currently, there are not enough studies reporting the prevalence of pili in pneumococcal strains carried by the Bulgarian population.

The present study aims to investigate the prevalence of pili among pneumococcal isolates recovered from children with non-invasive diseases and to elucidate a possible correlation between the presence of pili, serotype, antimicrobial resistance, and clonal structure of the examined isolates.

MATERIALS AND METHODS

Patients and specimen collection

We examined a collection of 147 *S. pneumoniae* isolates recovered from nasopharyngeal swabs of children aged 1 month to 9 years with upper respiratory tract infections (URTIs), lower respiratory tract infections (LRTIs), and acute otitis media (AOM). The strains were collected for a period of three years (2019–2021) voluntarily by microbiological laboratories in Sofia, Bulgaria. Clinical and demographic data on the age and diagnosis of patients were described. The diagnosis was confirmed by an otorhinolaryngologist or pediatrician. The vaccine status of the patients was determined based on their age at the date of isolation of the pneumococcal strain. The PCV10 immunization in Bulgaria is performed with two vaccines at 2 and 4 months of age and a booster dose at 12 months of age. Children born after the date of introduction of the vaccine in the Bulgarian immunization program (April 2010) who received ≥2 doses of PCV10 were defined as vaccinated against PCV10. The rate of PCV10 coverage is very high (>90%) among age-appropriate children according to the national epidemiological data.

The pneumococcal strains were stored frozen at −70 °C in double concentrated skim milk (Sigma Aldrich Solutions, Germany). For the experiments the bacteria were grown on blood agar (Oxoid, United Kingdom) and cultured at 37°C and 5%CO_2 overnight. All pneumococcal strains were identified by their sensitivity to optochin and bile salts. After incubation, a thermo-extraction of DNA for the culture isolates was performed. The DNA lysates were stored at −20 °C until use.

Detection of type 1 and type 2 pili

The bacterial DNA was obtained by thermo-extraction of an overnight culture in Todd Hewitt and Yeast extract broth (Sigma-Aldrich Solutions, Germany). PCRs targeting genes for PI-1 and PI-2 were performed as described previously [8, 9]. A specific region of the *rlrA* gene was amplified to detect PI-1 by the Aquiar method [9]. The *pitB* gene was used to detect the presence of PI-2 according to the method described by Bagnoli et al. [8]. To verify the absence of type 1 and type 2 pili additional PCRs were performed with the primer pairs suggested by Aquiar et al. and Bagnoli et al. [8, 9]. The reaction mixture comprising PrimeTaq premix (GenetBio, Korea) and primers purchased from Alpha DNA, Canada.

Serotyping

Serogrouping of *S. pneumoniae* was performed using the latex agglutination method (Pneumotest-Latex kit, Statens Serum Institute-SSI, Copenhagen, Denmark). Serotyping was performed with the capsular Neufeld Quellung method using some common factor antisera provided by the SSI. The non-typeable (NT) strains were tested by both methods, as well as for specific amplification of the lytA gene to distinguish *S.pneumoniae* from related species [18]. All serogroup 6 isolates were subjected to PCR serotyping [19]. We performed PCR to simultaneously detect 6A and 6C, because of their very high similarity in the cps loci. The presence of 6A/6C was proven by the 149-bp amplification product of the *wciP* gene. PCR amplification of the *wciNβ* gene (359 bp) was used to resolve 6C. The isolates of serotype 6B were tested by PCR with primers, which amplify a part of the *wciP* gene (155-bp product).

Antimicrobial susceptibility testing

Susceptibility testing was performed by the broth microdilution method using the Sensitre custom plate format (TREK diagnostic systems), Plate code: STP6F, and according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints and criteria for non-meningitis isolates [20]. The antibiotics for which the Minimum Inhibitory Concentrations (MICs) were determined were: Moxifloxacin, Levofloxacin, Tetracycline, Cefuroxime, Ceftriaxone, Cefotaxime, Daptomycin, Chloramphenicol, Penicillin, Meropenem, Ertapenem, Amoxicillin/Clavulanic acid 2:1 ratio, Linezolid, Clindamycin, Cefepime, Tigecycline, Azithromycin, Erythromycin, Trimethorpim/sulfamethoxazole, Vancomycin. The strains were classified as non-susceptible to Penicillin at MICs for Benzylpenicillin (MIC $\geq$ 0.12 mg L^{-1}). Penicillin-resistant pneumococci were strains with MICs of (>2 mg L^{-1}). The breakpoints for non-susceptible or resistant isolates to Ceftriaxone/Cefuroxime, iv are with (MIC $\geq$ 0.5 mg L^{-1}) and (MIC $\geq$ 2.0 mg L^{-1}), respectively.

S. pneumoniae ATCC 49619 was used as a control strain in susceptibility testing. Multidrug resistance (MDR) has been reported in evidence of non-susceptibility to at least three classes of antimicrobial agents.

Multilocus sequence typing (MLST)

MLST analysis was carried out on all piliated *S.pneumoniae* strains by the method described by Enright [14]. Briefly, the internal fragments of seven housekeeping genes were amplified by PCR, sequenced, and compared to the pneumococcal MLST database (http://pubmlst.org/spneumoniae) to identify the allelic profiles and sequence types (STs). Clusters of related STs were grouped into clonal complexes (CCs) by use of PHYLOViZ (https://online.phyloviz.net/). Sequence types (STs) sharing six (Single locus variants, SLVs) or five (double locus variants, DLVs) identical alleles were assigned to the same CC, named after the predominant ST in the group.

We compared the relatedness of the STs to reference clones from Pneumococcal Molecular Epidemiology Network (PMEN) and in the global data set – www.pneumogen.net/ Global Pneumococcal Sequencing Project (GPS) and Pathogenwatch – https://pathogen.watch/. GPS is a worldwide genomic surveillance network of *S. pneumoniae* isolates. It includes a large number of pneumococcal genomes clustered into lineages named Global Pneumococcal Sequence Clusters (GPSCs). It used whole genome sequencing to study pneumococcal serotype, sequence type, antibiotic sensitivity, and measuring for invasiveness using odds ratios that relate prevalence in invasive pneumococcal disease to a carriage [21].

RESULTS

Demographic and clinical data of patients

We analyzed 147 non-invasive *S. pneumoniae* strains recovered from children (1 month – 9 years). The distribution in age groups was: 51 children (31.4%) from 1 month to 2 years, 96 children (68.6%) aged 2–9 years.

The children with applied PCV10 vaccine were142 (96.5%). The unvaccinated patients were five children who did not receive a full vaccination course (only one applied dose of PCV10) or were under the eligible age for PCV10 immunization.

The most common manifestation of the NIPD cases among the studied patients was AOM (n = 87). The patients with URTI were (n = 39), followed by children diagnosed with LRTI (n = 21), of which bronchitis cases (n = 13) and pneumonia cases (n = 8).

Serotyping

We identified 26 different serotypes and four NT strains tested with Pneumotest-kit sera (SSI, Denmark). Five strains revealed a positive reaction with only one serum and participation in more than one serogroup or serotype by the latex agglutination method.

We found 17 non-PCV10 serotypes/serogroups (n = 113, 76.9%) and eight PCV10 serotypes/serogroups (n = 21, 14.3%). The remaining 6.1% of the strains showed affiliation to several serotypes/serogroups.

The most common serotypes in the studied collection were non-PCV10 serotypes. The predominant serotypes in descending order were 19A (14.3%), 6C (12.2%), 3 (9.5%), 15A (7.5%), 6A (6.8%) and 23B (4.8%). A vaccine serotype that showed high prevalence levels was 19F (6.1%).

Among the five unvaccinated children, we found non – PCV10 3 (n = 1), 6C (n = 1), 11 (n = 1), serogroup 13/28 (n = 1) and VT 18C (n = 1).

The serotype and pilus distribution among the studied non-invasive *S.pneumoniae* isolates is shown in Fig. 1.

Antimicrobial susceptibility

Non-susceptibility to Benzylpenicillin (MIC values> 0.12 g mL^{-1} for non-meningitic isolates) was found in 56 strains (38.1%). High resistance to Benzylpenicillin was revealed in

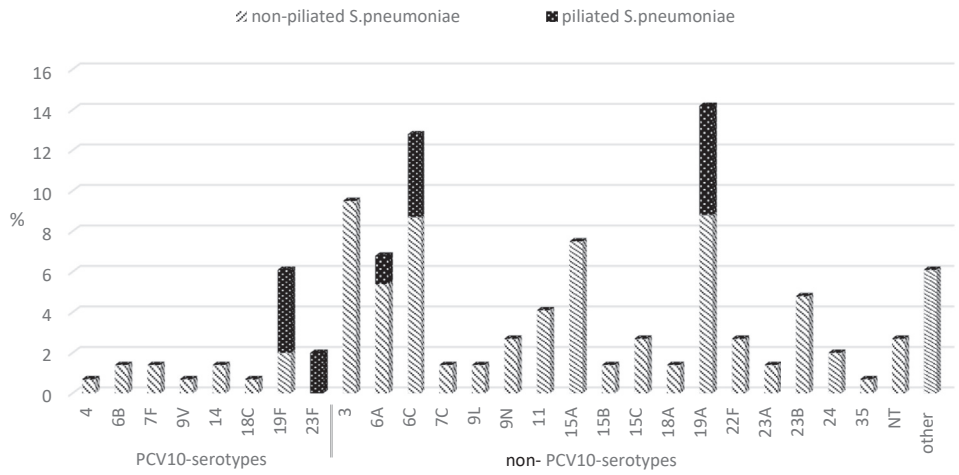

Fig. 1. Serotype and pilis distribution among 147 non-invasive *S. pneumoniae* isolates recovered from PCV10-vaccinated children
Notes: PCV10- Pneumococcal conjugate vaccine contains serotypes: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. Other – Expected to be serotypes/serogroups (strains were positive with one of the pooled sera only): 16,36,37 (n = 2, 1.3%); 25,38,43–46,48 (n = 2, 1.3%); 13,28 – (n = 1, 0.7%). NT – Non-typeable strains with Pneumotest-kit sera, SSI, Denmark

19 pneumococci (17.7%) with MIC values ranging between 2 and 8 g mL^{-1}.

Overall non-susceptible and resistant strains to ceftriaxone were 16.3%, and ten of these strains showed a MIC> 2 g mL^{-1}.

Resistance to erythromycin and clindamycin was observed in 58.5% and 46.9% of the strains, respectively. The resistant pneumococci to tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole were 39.5%, 12.9%, and 45.6%, respectively. More than half of the strains (55.1%) were MDR. The most commonly isolated resistance profile (40.8%) among all MDR strains was penicillin, erythromycin, tetracycline, clindamycin, and sulfamethoxazole-trimethoprim resistance.

The widespread serotypes among benzylpenicillin non-susceptible pneumococci were 6C (19.7%), 19A (18.5%) and 15A (12.2%).

The highly resistant strains to benzylpenicillin and ceftriaxone (MIC>2 mg L^{-1}) were from serotypes 19A and 19F.

Serotypes 6C (18.8%), 19A (16.9%), and 23A (10.4%) were the most common among the erythromycin-resistant strains.

The MDR serotypes in our study were 6C (18.6%), 19A (17.7%), 15A (11.4%), 19F (10.1%) and 23A (8.9%).

The antimicrobial non-susceptibility rates of the examined pneumococcal isolates are shown in Fig. 2.

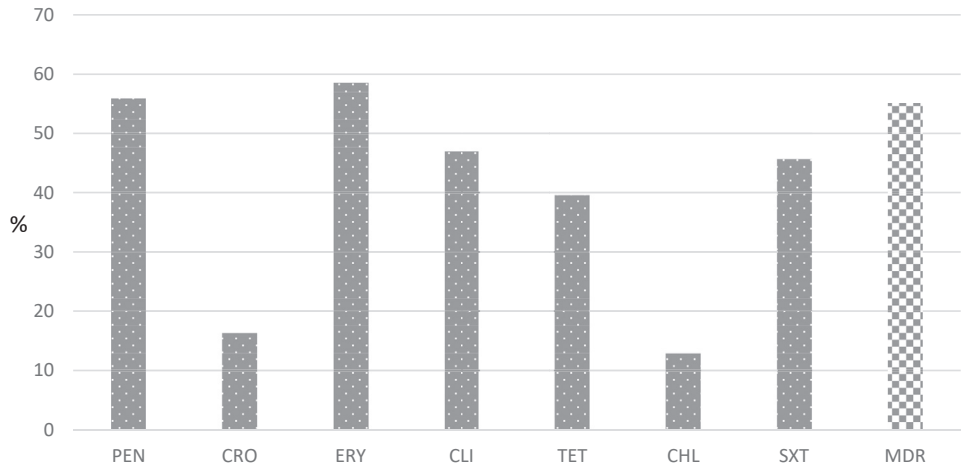

Fig. 2. Antimicrobial non-susceptibility in 147 *S. pneumoniae* isolates recovered from children with non-invasive pneumococcal diseases.
Notes: Minimal inhibitory concentrations (MICs) are set according to EUCAST (European Committee for Antimicrobial Susceptibility Testing) breakpoints values for indications other than meningitis. (2022). PEN – benzylpenicillin, CRO – ceftriaxone, ERY – erythromycin, CLI – clindamycin, TET – tetracycline, CHL – chloramphenicol, SXT – trimethoprim-sulfamethoxazole. MDR – Multidrug resistance to three or more classes of antimicrobial agents

Detection of type 1 and type 2 pili

PI-1 was detected in a total of 25 strains (17.0%). Most of the piliated strains were found among non-PCV10 serotypes (64.0%): 6A ($n = 2$), 6C ($n = 6$) and 19A ($n = 8$) and 36.0% of the strains were from vaccinal serotypes 19F ($n = 6$) and 23F ($n = 3$). PI-2 was not detected in the studied population.

The piliated strains recovered from children under 2 years of age ($n = 9$) were 36.0% ($n = 9$). In children aged 2–9 years, the strains that possessed type 1 pili were 64.0% ($n = 16$)

Piliated strains were found mainly in patients diagnosed with AOM ($n = 12$), followed by those with URTI ($n = 9$) and LRTI ($n = 4$).

A significant part of the piliated strains showed multidrug resistance ($n = 20$, 80.0%).

The distribution of PI-1 according to the serotype and the antimicrobial resistance of the strains are given in Table 1.

MLST. The MLST analysis revealed 18 different STs and three CCs among the piliated *S.pneumoniae* strains: CC386, CC320, and CC81. The widespread CC320 ($n = 9$, 36.0%) comprised highly resistant 19A and 19F isolates to more than five classes of antimicrobial agents. All of them are SLVs and DLVs of reference PMEN clone Taiwan^{19F}-14.

CC386 comprised four MDR 6A and 6C DLVs of Poland^{6B}-20. CC81 was recognized in two 23F isolates from clone Spain^{23F}-1/81 with multidrug resistance and resistance to Penicillin, respectively.

More than half of the piliated strains ($n = 14$, 56.0%) revealed relatedness to successfully circulating international PMEN clones, and 18 isolates (72.0%) were with known GPSC types in the global database of pneumococcal genomes.

All GPSC types of the STs found in the database of available pneumococcal genomes and the relatedness with reference PMEN clones are described in Table 1.

DISCUSSION

Our study aimed at piliated non-invasive *S. pneumoniae* strains recovered from PCV10-vaccinated children and their phenotypic and genotypic characteristics. Pneumococcal pilus was discovered about 15 years ago, although *S. pneumoniae* is an object of thorough research and was first isolated from Pasteur in 1881.

The role of the pilus for adhesion to the extracellular matrix proteins of the host and evasion of mucosal clearance is unequivocal and discussed in many studies [4, 7, 10]. Pilus is also involved in tissue tropism, biofilm formation, modulation of innate immune responses, and their contribution to virulence.

The currently available vaccines provide protection against a limited number of serotypes and their distribution and characteristics require careful and continuous monitoring and evaluation of vaccine efficacy. Following the abundance of data on serotype switching events and vaccine escape, some studies have investigated pilus as a future candidate for an alternative protein vaccine. The idea for a pneumococcal vaccine with pilus protein subunits needs confirmation that these structures are widespread among pneumococcal populations.

In our investigation, we found a 17.0% prevalence of PI-1 genes among the non-invasive pneumococcal isolates. PI-2 islet was not detected, even in strains with multidrug antimicrobial resistance. Different studies declared a prevalence of PI-1 compared to PI-2. Type 1 pili are more widespread both in invasive and non-invasive isolates [13,15–17]. The presence of pathogenic PI-1 was associated with certain serotypes. Some genomic studies have shown that PI-1 is not present in all pneumococcal isolates, suggesting that it may have been acquired by horizontal gene transfer [7, 11, 31] and found in more competitive strains. There has been a greater prevalence of piliated strains among non-vaccinal serotypes [22, 23]. Strains from serotypes 6A, 6C, 19A, 19F, and 23F possessed type 1 pili. It is assumed that the presence of pili gives a selective advantage to the strains, which is confirmed by the predominant prevalence of these serotypes among the studied population.

The routine PCV10 immunization affected the serotype dynamics of the pneumococcal strains. Non-vaccinal serotypes, which were rarer or not isolated in pre-vaccinal periods in our country, showed increases in the current study [24, 25]. In our investigation, the isolates with non-PCV10 serotypes predominated by 76.9% versus 14.3% PCV10-isolates. All common serotypes were non-PCV10: 19A (14.3%), 6C (12.2%), 3 (9.5%), 15A (7.5%) and 6A (6.8%). Among the vaccinal serotypes, serotype 19F dominated with 6.1%, but its spread has been significantly reduced in the post-vaccinal period. Both serotypes 19F and 19A successfully circulated in the early post-vaccination period, but serotype 19A has taken advantage in recent years [24]. Before the implementation of PCV10, one of the most isolated serotypes in Bulgaria was 6B, following serotypes 19F and 3 [24]. Initially, serogroup 6 was composed of serotypes 6A and 6B, but in the last years, additional types as 6C and less frequent 6D, 6E, 6F, 6G, and 6H have been reported [25]. In our study, almost all of the representatives from serogroup 6 belonged to serotype 6C, which was not detected in the pre-vaccine era [25]. Serotype 15A was rarely detected, but in recent years raise its frequency and confirms vaccination is accompanied by mostly non-vaccinal serotypes. Investigations from Hungary and Sweden reported increases in the prevalence of serogroup 15 and mostly of serotype 15A [26, 27]. Serotype 3 was leading in the pre-vaccinal periods too. Many reports declared its diverse antigenic profile and phenotypic variations [28], which helps serotype 3 to sustain and plays an important role in the serotype distribution and pathogenicity through the years.

The studied strains displayed increased levels of antibiotic resistance significantly complicates the therapy of NIPD infections. High rates of antimicrobial non-susceptibility in descending order to erythromycin (58.5%), oral penicillin (55.8%), clindamycin (46.9%), trimethoprim-sulfamethoxazole (45.6%), tetracycline (39.5%), ceftriaxone (16.3%) was

Table 1. Clonal and phenotypic characteristics of type 1 pili among *S. pneumoniae* isolates recovered from children under 10 years of age with non-invasive pneumococcal diseases

PI-1	Serotype	CC	ST	GPSC type	PMEN clone	MIC (g / mL)							R -profile	Diagnosis
						PEN	CRO	ERY	CLI	TET	CHL	SXT		
(*n* = 2)	6A		490	76	–	0.25	0.12	>256	>256	0.06	>256	>256	MDR	AOM
	6A	CC386 (*n* = 4)	386	47	DLV of Poland^{6B}-20	0.12	0.01	16	0.06	0.06	0.06	>256	MDR	AOM
(*n* = 6)	6C		386	47	DLV of Poland^{6B}-20	0.12	0.06	>256	>256	>256	0.06	0.06	MDR	AOM
	6C		386	47	DLV of Poland^{6B}-20	0.12	0.06	>256	>256	>256	0.06	0.03	MDR	AOM
	6C		386	47		0.25	0.06	>256	>256	>256	0.06	>256	MDR	AOM
	6C		1,205	35	–	0.12	0.03	>256	>256	0.06	0.06	>256	MDR	AOM
	6C		1,714	29	–	0.06	0.03	0.06	0.06	0.03	0.06	0.06	S	URTI
	6C		1,876	13	–	0.25	0.5	0.06	0.06	0.03	0.06	0.06	PEN	URTI
(*n* = 8)	19A		199	4	–	1	0.06	>256	>256	>256	0.06	0.06	MDR	AOM
	19A		733	18	–	0.25	0.01	0.06	0.06	0.06	0.06	>256	PEN, SXT	URTI
	19A		2,260	-	–	0.06	0.01	16	0.06	0.06	0.06	0.06	ERY	URTI
	19A	CC320 (*n* = 9)	271	1	SLV of Taiwan^{19F}-14	1	0.5	>256	>256	>256	0.06	>256	MDR	AOM
	19A		320	1	DLV of Taiwan^{19F}-14	4	2	>256	>256	>256	0.06	>256	MDR	URTI
	19A		320	1	DLV of Taiwan^{19F}-14	4	1	>256	>256	>256	0.06	>256	MDR	AOM
	19A		320	1	DLV of Taiwan^{19F}-14	8	2	>256	>256	>256	0.06	>256	MDR	LRTI
	19A		320	1	DLV of Taiwan^{19F}-14	8	1	>256	>256	>256	0.06	>256	MDR	LRTI
(*n* = 6)	19F		236	1	Taiwan^{19F}-14	2	0.5	>256	>256	>256	0.06	>256	MDR	URTI
	19F		2,476	–	DLV of Taiwan^{19F}-14	4	2	>256	>256	>256	0.06	>256	MDR	AOM
	19F		2,476	–	DLV of Taiwan^{19F}-14	4	2	>256	>256	>256	0.06	>256	MDR	URTI
	19F		3,607	–	DLV of Taiwan^{19F}-14	2	0.5	>256	>256	>256	0.06	>256	MDR	AOM
	19F		686	–	–	4	2	>256	>256	>256	0.06	>256	MDR	LRTI
	19F		2,393	–	–	4	1	>256	>256	>256	0.06	>256	MDR	LRTI
(*n* = 3)	23F	CC81 (*n* = 2)	81	16	Spain^{23F}-1/81	0.25	0.5	0.06	0.06	0.03	0.06	>256	PEN	AOM
	23F		9,695	–	SLV of Spain^{23F}-1/81	2	1	>256	>256	0.06	>256	>256	MDR	URTI
	23F		242	14	–	0.5	0.25	>256	0.06	>256	0.06	0.06	MDR	URTI
Total *n* (*n* =25)													Total MDR (*n* = 20)	

Notes: PI-1 – pilus islet 1; AOM – acute otitis media; URTI – upper respiratory tract infection; LRTI – lower respiratory tract infection.
MDR – Multidrug resistance to more than three classes of antimicrobials. S – antimicrobial susceptibility to all tested antibiotics.
[a]CC – Clonal complex.
[b]ST – Sequence Type.
[c]GPSC type – Global Pneumococcal Sequence Cluster type according to Global Pneumococcal Sequencing Project.
[d]MIC range – minimal inhibitory range (mg L^{-1}) according to EUCAST, 2021. PEN – Benzylpenicillin; CRO – Ceftriaxone; ERY – Erythromycin; CLI – Clindamycin, TET – Tetracycline, CHL – Chloramphenicol, SXT – Sulfamethoxazole-trimethoprim.
[e]PMEN – Pneumococcal Molecular Epidemiology Network.
SLV – single locus variants, DLV- double locus variants.

registered. More than half (55.1%) of the isolates were MDR. The high percentage of MDR non-PCV isolates is a consequence of the distribution of antimicrobial-resistant clones, and the new recombinant antigenic variants that avoid PCV10 [29–32].

Almost all piliated strains were multidrug-resistant. It revealed a clear association between the presence of pili and high antimicrobial resistance. Resistant strains are more likely to have pathogenic pilus islets than susceptible strains. The analysis showed that the presence of pili is not correlated with the isolation sites and the age.

More than half of the piliated strains showed relatedness to international pneumococcal clones. Three successfully circulating CCs were revealed: CC386, CC320, and CC81 which represented mainly MDR strains from serotypes 19A,19F, 6A, 6C, and 23F. CC386 and CC320 significantly expanded in the post-PCV10 period in our geographic area. The increased colonization capacity of these genetic lineages was reported in other European countries, and it may be associated with the presence of PI-1 [33–36]. CC320 was the most prevalent clone. The 19A isolates of the genetic lineage Taiwan^{19F}-14 arise by 19F capsular switching events and expand in the post-vaccine era under the PCV10 selection. CC386 was represented by non-PCV10 6A and 6C MDR isolates, which replaced the most common 6B serotype in the pre-vaccine era. PCV10-serotypes 19F and 23F continue to emerge in highly resistant pneumococci and become a great concern to public health and clinical settings.

CONCLUSIONS

In conclusion, we found that the studied non-invasive pneumococcal isolates revealed a PI-1 mainly in multidrug resistant genetic lineages and in largely distributed serotypes. The presence of pili gives a selective advantage to these strains in the microbial competition. The role of PI-1 should be evaluated in further studies and potentially considered in the spread of antibiotic resistant clones.

Conflict of interests: The authors report no declarations of interest.

ACKNOWLEDGEMENTS

This study was supported by Grant No 135/14.06.2022 of the Medical University of Sofia. We acknowledge the microbiological laboratories and Children's Otorhinolaryngology Ward of University Hospital "Queen Joanna – ISUL" for providing *S. pneumoniae* clinical isolates for this study.

REFERENCES

1. Maraqa FN. Pneumococcal infections. Pediatr Rev 2014; 35(7): 299–310.
2. Brooks LRK, Mias GI. *Streptococcus pneumoniae*'s virulence and host immunity: aging, diagnostics, and prevention. Front Immunol 2018; 22(9): 1366.
3. Subramanian K, Henriques-Normark B, Normark S. Emerging concepts in the pathogenesis of the *Streptococcus pneumoniae*: From nasopharyngeal colonizer to intracellular pathogen. Cell Microbiol 2019; 21(11): e13077.
4. Danne C, Dramsi S. Pili of gram-positive bacteria: roles in host colonization. Res Microbiol 2012; 163(9–10): 645–58.
5. Henriques-Normark B, Tuomanen E. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med 2013 PMID: 23818515.
6. Blumental S, Granger-Farbos A, Moïsi J, Soullie B, Leroy P, Njanpop-Lafourcade B, et al. Virulence factors of *Streptococcus pneumoniae*. Comparison between African and French invasive isolates and implication for future vaccines. PLoS One 2015; 27; 10(7): e0133885. Available from: https://doi.org/10.1371/journal.pone.0133885.
7. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, et al. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci USA 2006; 103: 2857–62.
8. Bagnoli F, Moschioni M, Donati C, Dimitrovska V, Ferlenghi I, Facciotti C, et al. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. J Bacteriol 2008; 190: 5480–92.
9. Aguiar S, Serrano I, Pinto F, Melo-Christino J, Ramirez M. The presence of the pilus locus is a clonal property among pneumococcal invasive isolates. BMC Microbiol 2008; 28(8): 41.
10. Nelson AL, Dahlberg S, Falker S, Rounioja S, et al. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. Mol Microbiol 2007; 66(2): 329–40.
11. Figueira M, Moschioni M, De Angelis G, Barocchi M, Sabharwal V, Masignani V, et al. Variation of pneumococcal Pilus-1 expression results in vaccine escape during Experimental Otitis Media [EOM]. PLoS One 2014; 9(1): e83798. Available from: https://doi.org/10.1371/journal.pone.0083798.
12. Turner P, Melchiorre S, Moschioni M, Barocchi M, Turner C, Watthanaworawit W, et al. Assessment of *Streptococcus pneumoniae* pilus islet-1 prevalence in carried and transmitted isolates from mother-infant pairs on the Thailand Burma border. Clin Microbiol Infect 2012; 18(10): 970–5.
13. Kulohoma BW, Gray K, Kamng'ona A, Cornick J, Bentley S, Heyderman R, et al. Piliation of invasive *Streptococcus pneumoniae* isolates in the era before pneumococcal conjugate vaccine introduction in Malawi. Clin Vaccin Immunol 2013; 20: 1729–35.
14. Ganaie F, Maruhn K, Li C, Porambo R, Elverdal P, Abeygunwardana C, et al. Structural, genetic, and serological elucidation of *Streptococcus pneumoniae* serogroup 24 serotypes: discovery of a new serotype, 24C, with a variable capsule structure. J Clin Microbiol 2021; 59(7): e0054021. Available from: https://doi.org/10.1128/JCM.00540-21.
15. Moschioni M, De Angelis G, Melchiorre S, Masignoni V, Leibovitz E, Barocchi M, et al. Prevalence of pilus-encoding islets among acute otitis media *Streptococcus pneumoniae* isolates from Israel. Clin Microbiol Infect 2010; 16: 1501–4.
16. Regev-Yochay G, Hanage WP, Trzcinski K, Rifas-Shiman S, Lee G, Bessolo A, et al. Re-emergence of the type 1 pilus among Streptococcus pneumoniae isolates in Massachusetts, USA. Vaccine 2010; 28(30): 4842–6.

17. Hjalmarsdottir MA, Petursdottir B, Erlendsdottir H, Haraldsson G, Kristinsson K. Prevalence of pilus genes in pneumococci isolated from healthy preschool children in Iceland: association with vaccine serotypes and antibiotic resistance. J Antimicrob Chemother 2015; 70(8): 2203–8.
18. Greve T, Møller JK. Accuracy of using the lytA gene to distinguish *Streptococcus pneumoniae* from related species. J Med Microbiol 2012; 61: 478–82.
19. Jin P, Xiao M, Kong F, Oftadeh S, Zhou F, Liu C, et al. Simple, accurate, serotype-specific PCR assay to differentiate *Streptococcus pneumoniae* serotypes 6A, 6B, and 6C. J Clin Microbiol 2009; 47(8): 2470–4. Available from: https://doi.org/10.1128/JCM.00484-09.
20. European committee on antimicrobial susceptibility testing breakpoint tables for interpretation of MICs and zone diameters version 10.0, valid from 2021-01-01.
21. Gladstone R, Lo S, Lees J, Croucher N, van Tonder A, Corander J, et al. International genomic definition of pneumococcal lineages, to contextualise disease, antibiotic resistance and vaccine impact. EBioMedicine 2019; 43: 338–46. Available from: https://doi.org/10.1016/j.ebiom.2019.04.021.
22. Kriger O, Yochay G. The effect of pneumococcal conjugate vaccine on pneumococcal carriage and invasive disease. Harefuah 2019; 158(5): 316–20.
23. McElligott M, Vickers I, Cafferkey M, Cunney R, Humphreys H. Non-invasive pneumococcal serotypes and antimicrobial susceptibilities in a paediatric hospital in the era of conjugate vaccines. Vaccine 2014; 32(28): 3495–500.
24. Setchanova L, Kostyanev T, Alexandrova A, Mitov I, Nashev D, Kantardjiev T. Microbiological characterization of *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* isolates as primary causes of acute otitis media in Bulgarian children before the introduction of conjugate vaccines. Ann Clin Microbiol Antimicrobials 2013; 12: 6. Available from: https://doi.org/10.1186/1476-0711-12-6.
25. Alexandrova A, Setchanova L, Pencheva D, Mitov I. Phenotypic and genotypic characterization of serogroup 6 *Streptococcus pneumoniae* isolates collected during 10-valent pneumococcal conjugate vaccine era in Bulgaria. Acta Microbiol Immunol Hung 2020; 67(2): 91–9.
26. Kovács E, Sahin-Tóth J, Tóthpál A, Kristóf K, van der Linden M, Tirczka T, et al. Vaccine-driven serotype-rearrangement is seen with latency in clinical isolates: comparison of carried and clinical pneumococcal isolates from the same time period in Hungary. Vaccine 2019 Jan 3; 37(1): 99–108. Available from: https://doi.org/10.1016/j.vaccine.2018.11.026.
27. Yamba Yamba L, Uddén F, Fuursted K, Ahl J, Slotved HC, Riesbeck K. Extensive/multidrug resistant pneumococci detected in clinical respiratory tract samples in Southern Sweden are closely related to international multidrug-resistant lineages. Front Cell Infect Microbiol 2022 Mar 22; 12: 824449. https://doi.org/10.3389/fcimb.2022.824449.
28. Azarian T, Mitchell PK, Georgieva M, Thompson C, Ghoulia A, Pollard A, et al. Global emergence and population dynamics of divergent serotype 3 CC180 pneumococci. Plos Pathog 2018; 14: e1007438.
29. Song JH, Dagan R, Klugman KP, Fritzell B. The relationship between pneumococcal serotypes and antibiotic resistance. Vaccine 2012; 30(17): 2728–37.
30. Kawaguchiya M, Urushibara N, Aung MS, Ito M, Takahashi A, Habadera S, et alet al. High prevalence of antimicrobial resistance in non-vaccine serotypes of non-invasive/colonization isolates of *Streptococcus pneumoniae*: a cross-sectional study eight years after the licensure of conjugate vaccine in Japan. J Infect Public Health 2020; 13(8): 1094–100.
31. Cherazard R, Epstein M, Doan TL, Salim T, Bharti S, Smith M. Antimicrobial resistant *Streptococcus pneumoniae*: prevalence, mechanisms, and clinical implications. Am J Ther 2017; 24(3): e361–9. Available from: https://doi.org/10.1097/MJT.0000000000000551.
32. Regev-Yochay G, Jaber H, Hamdan A, Daana M, Nammouz H, Thalji A, et al. Vaccine escape of piliated *Streptococcus pneumoniae* strains. Vaccine 2016 May 27; 34(25): 2787–92.
33. Dzaraly ND, Muthanna A, Mohd Desa MN, Taib NM, Masri SN, Rahman N, et al. Pilus islets and the clonal spread of piliated *Streptococcus pneumoniae*: a review. Int J Med Microbiol 2020 Oct; 310(7): 151449.
34. Del Grosso M, Camilli R, D'Ambrosio F, Petrucci G, Melchiorre S, Moschioni M, et al. Increase of pneumococcal serotype 19A in Italy is due to expansion of the piliated clone ST416/CC199. J Med Microbiol 2013 Aug; 62(Pt 8): 1220–5.
35. Mayanskiy N, Savinova T, Alyabieva N, Ponomarenko O, Brzhozovskaya E, Lazareva A, et al. Antimicrobial resistance, penicillin-binding protein sequences, and pilus islet carriage in relation to clonal evolution of *Streptococcus pneumoniae* serotype 19A in Russia, 2002–2013. Epidemiol Infect 2017; 145: 1708–19. Available from: https://doi.org/10.1017/S0950268817000541.
36. Zemlickova H, Jakubu V, Fridrichova M, Malisova L, Musilek M, Trojanek M. The association of pili with the emergence and replacement of the major antibiotic resistant pneumococcal clones. J Microbiol Immunol Infect 2020 Oct; 53(5): 690–5.