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**Clonal distribution, antimicrobial resistance, and pilus islets in *S.pneumoniae* isolates recovered from PCV10-vaccinated children with suppurative AOM in Bulgaria (2015-2020), BULGARIA**

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**Title page**

# Clonal distribution, antimicrobial resistance, and pilus islets in *S.pneumoniae* isolates recovered from PCV10-vaccinated children with suppurative AOM in Bulgaria (2015-2020), BULGARIA

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**Clonal distribution, antimicrobial resistance, and pilus islets in *S.pneumoniae* isolates recovered from PCV10-vaccinated children with suppurative AOM in Bulgaria (2015-2020)**


## Summary

*Streptococcus pneumoniae* is still a leading bacterial pathogen of acute otitis media (AOM), despite the available pneumococcal conjugate vaccines (PCVs). We conducted a study on the population structure, antibiotic nonsusceptibility, serotype distribution, and presence of pilus in middle ear fluids - *S. pneumoniae* isolates recovered from PCV10-vaccinated children with suppurative AOM in Bulgaria. Non-susceptibility was revealed in 68.75% (n=33) of the isolates. Multidrug-resistance (MDR) has been detected in 60.4%. The dual macrolide resistance mechanism was predominant. Most widespread were non-PCV10 serotypes 3 (27.1%, n=13), 19A (25.0%, n=12), and VT 19F (23.0%, n=11). A total of 64.6% were non-PCV10-serotypes. Presence of Pilus type I was observed mostly in PCV10-serotypes. We disclosed a strong association between CCs, serotype, and antimicrobial resistance. The MLST revealed the presence of four CCs: CC320 (39.6%), CC505 (12.5%), CC1377 8.3%), and CC230 (8.3%), respectively. The most abundant CC320 comprised MDR 19A and 19F isolates. CC230 clustered MDR isolates from serotype 19A, 6C, and 14. CC505 and CC1377 covered serotype 3 susceptible isolates. The vaccine-induced changes and trends in antimicrobial resistance and clonality must be an object of systematic investigations.

*Streptococcus pneumoniae* is a vaccine-preventable agent due to available pneumococcal conjugate vaccines (PCVs), but still in many countries is a leading bacterial pathogen of acute otitis media (AOM).

PCV10 was the first pneumococcal vaccine implemented in the Bulgarian immunization program in 2010. This reduced the incidence of invasive pneumococcal diseases and affected the cases of AOM, but cost dynamic changes in the serotype distribution. Some investigations demonstrated vaccine types have almost disappeared in children (1). Other factors as selective antibiotic pressure and dissemination of multidrug-resistant clones resulted in complicated therapy.

The successful dissemination of epidemic clones has been linked to capsular types, that differ by invasiveness, disease severity, antibiotic resistance profiles, carriage of a pilus islet, and various virulence factors (2).

We conducted a study on pneumococcal suppurative AOM cases complicated by tympanic membrane perforation and otorrhea of the spontaneous rupture of the tympanic membrane during the PCV10-era in our country. We aimed at the population structure, antibiotic nonsusceptibility, serotype distribution, and presence of pilus in pediatric *S. pneumoniae* isolates.

The studied isolates were recovered from PCV10-vaccinated children at age up to 10 years. Following the PCV10-implementation in our country, the criteria was for children born after April 2010. The Bulgarian pneumococcal vaccination schedule is carried out with two vaccines at 2 and 4 months of age and re-immunization with one vaccine at 12 months of age.

The patient collection was not consecutive through the study period 2015-2020. The isolates were collected voluntarily from three laboratories in Sofia (n=109) and two in Plovdiv (n=9), and Pleven (n=7). All cases of suppurative AOM were confirmed by otorhinolaryngologists, who collected

middle ear fluids (MEF)/otorrhea specimens from children with severe AOM cases, in which either required tympanocentesis or presentation with spontaneous otorrhea. Only one episode of AOM was included per patient. It was obtained a consent form from the parents of the patients. Clinical and demographic data (age, date of admission in a hospital, diagnosis, vaccine status) were collected.*S. pneumoniae* isolates were identified according to susceptibility to optochin and bile-solubility. Antibiotic susceptibilities were tested by microdilution minimal inhibitory concentrations (MICs) (3). Multidrug resistance (MDR) was defined as nonsusceptibility to three or more classes of antimicrobials.

The Erythromycin-resistant strains (ERSP) were tested for macrolide resistance determinants *ermB* and *mefE* (4).

Serogrouping and serotyping were performed using latex agglutination method and capsular swelling reaction (SSI, Denmark). Serogroup 6 strains were subjected to serotype-specific PCR's (5).

Presence or absence of pilus islets - PI type I and PI type IIwas confirmed by PCRs, described by Aquiar and Bagnoli. (6,7).

Multilocus sequence typing (MLST) was carried out for seven housekeeping genes to identify the alleles and sequence types (STs) (http://pubmlst.org/spneumoniae) (8). Clusters of related STs shared five of more than five alleles: SLVs (single locus variants) and DLVs (double locus variants) were grouped into clonal complexes (CCs) by use of PHYLOViZ (https://online.phyloviz.net/). CCs are named after the predominant ST. STs not assigned to any CC were designed singletons.

A total of 48 were the culture-positive MEF (n=45) and otorrhea (n=3) specimens.

The patients were at one month to nine years of age. All were PCV10-vaccinated according to the age with at least one dose, except one child at age 1 month.

The non-susceptible strains to all tested antimicrobials were 68.7%. Penicillin non-susceptibility was disclosed in 66.7%. MDR has been detected in 60.4%, where 89.6% were resistant to five antimicrobial agents. The ERSP were 56.2%. Susceptible to all tested antimicrobials were 31.2%. Table 1.

Almost all 19F and 19A isolates were MDR. The serotype 3 isolates were susceptible to all tested antimicrobials.

The dual macrolide resistance mechanism (*ermB* plus *mefE* gene) was predominant out of the ERSP- isolates (67.8%). Two strains harbored only *mefE* gene, and 25.0% possessed only *ermB* gene.

The serotype distribution revealed a leading position for non-PCV10 serotypes 3 (27.1%), and 19A (25.0%), followed by vaccinal serotype (VT) 19F (23.0%). A total of 64.6% were non-vaccinal PCV10-serotypes (NVTs). The rest VTs and NVTs were distributed in similar proportions of 12.5%.

Fig.1

We observed the presence of Pilus type I in seven strains (14.6%) from serotype 19F(n=4), and one isolate of serotypes 14, 15C, and 19A. All isolates were negative for the presence of Pilus type II.

The MLST disclosed twenty-seven STs and four CCs, comprising 68.7% of the examined isolates. The distribution of the CCs was: CC320 (39.6%), CC505 (12.5%), CC1377(8.3%), and CC230 (8.3%). The singletons were 27.1%. The first three leading positions were for ST320 (25.0%), ST505 (12.5%), and ST11157 (6.2%). The most abundant CC320 clustered STs related to PMEN

clone Taiwan$^{19F}$-14 and comprised MDR 19A and 19F isolates. The 19A isolates (66.7%) participated as DLVs of Taiwan$^{19F}$-14. All 19F isolates show genetic relatedness to clone Taiwan$^{19F}$-14. CC230 is named after reference clone Denmark$^{14}$-32/ST230. It comprised MDR isolates from serotype 19A (16.7%), and two isolates from serotypes 6C and 14. The serotype 3 susceptible isolates belonged to CC505(46.1%), and CC1377(30.8%).

The other STs were genetically heterogeneous singletons from various serotypes. We noted relatedness to clones England$^{14}$-9 and Portugal$^{6A}$-14 in serotypes 6A and 14 isolates.

In Bulgaria, AOM continues to be a problem due to the increased number of NVTs, as well as high antimicrobial resistance. Compared to a pre-vaccine period in Bulgaria, we observed a significant expansion of NVTs among the MEF-isolates (9).

Among the studied pediatric population, the most widespread were non-PVC10 serotypes 3 and 19A, and VT 19F. Serotype 3 remains epidemiologically important globally even after the introduction of PCV13 in many countries. Investigations about the dynamics of the serotype 3 international clone ST180 are reported from North America, Eastern, and Western Europe. (10,11)

Possible explanation for the prevalence of serotype 3 in our study is not only the fact, it is a non-vaccinal PCV10-serotype, but some reports about increased recombination rates in serotype 3 clades, which lead to more diverse antigenic profile and phenotypic variations (10). There is evidence about variable protein antigens of serotype 3 strains involved in the expression of the comCDE operon, which plays an important role in competence, survival, and virulence of pneumococci (12).

Serotype 19F persisted in the post-vaccine era due to the successful circulation of the MDR Taiwan$^{19F}$-14 clone. Other studies have also noted Taiwan$^{19F}$-14 as an important clone in AOM isolates (13,14).

CC230 was disclosed in our geographic area a decade ago and continues to emerge (15).

The examined AOM isolates were resistant to more than five classes of antimicrobial agents and this creates significant difficulties in therapy. CC320 comprises only MDR 19F and 19A isolates. CC505 clustered susceptible serotype 3 isolates. We observed pilus type I mainly with PCV10-capsular vaccine-type strains as serotype 19F strains.

In conclusion, serotypes 3, 19A, and 19F account for the large proportion of the studied pneumococcal AOM isolates recovered from PCV10-vaccinated children. We noted the epidemiological importance of ST320, ST505, and CC320 clones among the suppurative AOM pediatric infections. A good association was observed between the circulating clones, serotypes, and resistance.

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**Conflict of interest**: The authors declare no conflict of interest.

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Figure Legends:

Fig 1. Distribution of serotypes and multidrug-resistance in 48 *S.pneumoniae* middle ear fluids-isolates recovered from PCV10-vaccinated children with suppurative AOM in Bulgaria

Notes: PCV10-serotypes: 6B, 9V, 14, 18C, 19F. Non-PCV10 serotypes: 3, 6A, 6C, 15C, 18A, 19A, 22

MDR – multidrug resistance to three or more classes of antimicrobials

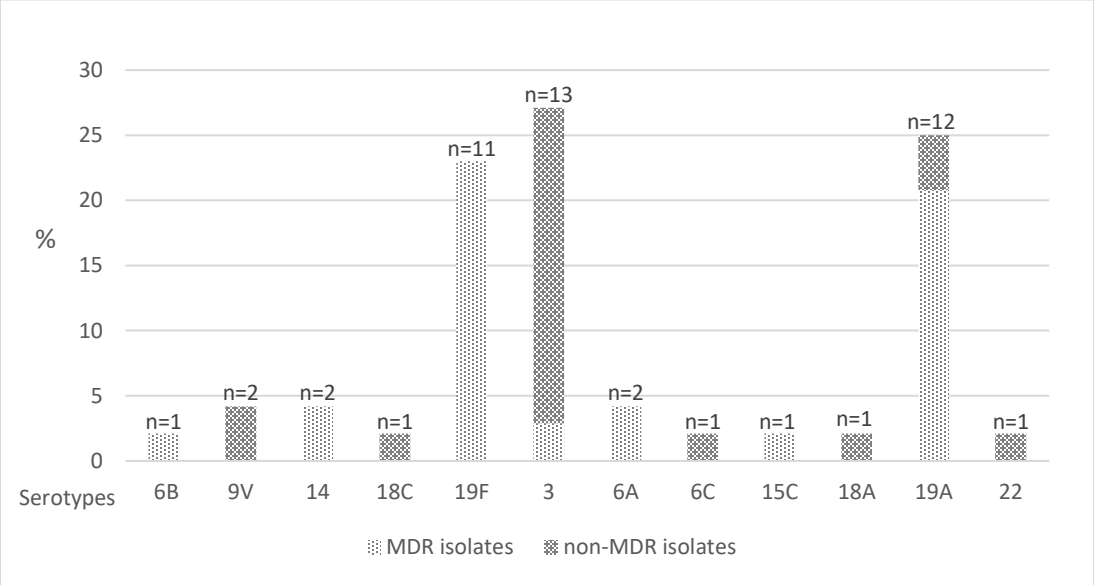
Table 1. Genotypic and phenotypic characteristics of *S.pneumoniae* isolates recovered from children from 1 month to 9 years age with suppurative AOM during PCV10-era in Bulgaria (2015-2020)

| **CC[1]** | **Serotype** | **ST[2] (n of isolates )** | **PMEN[3] clone** | **Antibiotic R[4]-profile** | **MR gene(s)[5]** | **Pilus Islet (n of isolates)** |
|---|---|---|---|---|---|---|
| CC505 | 3 | ST505 (n=6) | | S | | |
| CC1377 | 3 | ST1377 (n=2) | | S | | |
| | 3 | ST378 (n=1) | | S | | |
| | 3 | ST232 (n=1) | | S | | |
| CC230 | 19A | ST230 (n=1) | Denmark$^{14}$-32 | P,E,Cli,T,Sxt | *ermB* | |
| | 19A | ST276 (n=1) | SLV of Denmark$^{14}$-32 | P,E,Cli,T,Sxt | *ermB+mefE* | |
| | 6C | ST8630 (n=1) | DLV of Denmark$^{14}$-32 | P,T,Sxt | | |
| | 14 | ST4782 (n=1) | SLV of Denmark$^{14}$-32 | P,E,Cli,T,Sxt | *ermB* | Pilus Islet-1 (n=1) |
| CC320 | 19A | ST320 (n=8) | DLV of Taiwan$^{19F}$-14 | P,E,Cli,T,Sxt | *ermB+mefE* | Pilus Islet-1 (n=1) |
| | 19F | ST320 (n=4) | DLV of Taiwan$^{19F}$-14 | P,E,Cli,T,C,Sxt | *ermB+mefE* | Pilus Islet-1 (n=2) |
| | 19F | ST11157 (n=3) | DLV of Taiwan$^{19F}$-14 | P,E,Cli,T,Sxt | *ermB+mefE* | Pilus Islet-1 (n=1) |
| | 19F | ST352 (n=2) | SLV of Taiwan$^{19F}$-14 | P,E,Cli,T,Sxt | *ermB* | |
| | 19F | ST271 (n=1) | SLV of Taiwan$^{19F}$-14 | P,E,Cli,T,Sxt | *ermB+mefE* | |
| | 19F | ST1396 (n=1) | DLV of Taiwan$^{19F}$-14 | P,E,Cli,T,Sxt | *ermB+mefE* | Pilus Islet-1 (n=1) |
| singleton | 3 | ST4201 (n=1) | | S | | |
| singleton | 3 | ST180 (n=1) | Netherlands$^{3}$-31 | P,E,Cli,T,C | *ermB* | |
| singleton | 6A | ST395 (n=1) | SLV of Portugal$^{6A}$-41 | P,E,Cli,C,Sxt | *ermB+mefE* | |
| singleton | 6A | ST490 (n=1) | | P,E,C,Sxt | *mefE* | |
| singleton | 6B | ST149 (n=1) | | P,E,Cli,T,Sxt | *ermB* | |
| singleton | 9V | ST3911 (n=1) | | P,Sxt | | |
| singleton | 9V | ST280 (n=1) | | P,Sxt | | |
| singleton | 14 | ST1161 (n=1) | SLV of England$^{14}$-9 | P,E,C,Sxt | *mefE* | |
| singleton | 15C | ST5024 (n=1) | | P,E,Cli,T,Sxt | *ermB* | Pilus Islet-1 (n=1) |
| singleton | 18A | ST241 (n=1) | | T | | |
| singleton | 18C | ST1923 (n=1) | | S | | |
| singleton | 19A | ST199 (n=1) | Netherlands$^{15B}$-37 | S | | |
| singleton | 19A | ST2632 (n=1) | | P,Sxt | | |
| singleton | 22 | ST7181 (n=1) | | S | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Total (n=48) | | | | |

[1]CC - clonal complex; [2]ST - Sequence type; [3]PMEN – Genetic Relatedness to Pneumococcal Molecular Epidemiology Network-clone; SLV-single locus variant; DLV- double locus variant; [4]R - resistance; P - Penicillin, E - Erythromycin, Cli - Clindamycin; T - Tetracycline; C -Chloramphenicol; Sxt - Trimethoprim-Sulfamethoxazole; [5]MR-gene(s)- Macrolide resistance gene(s)

Fig 1. Distribution of serotypes and multidrug-resistance in 48 *S.pneumoniae* middle ear fluids-isolates recovered from PCV10-vaccinated children with suppurative AOM in Bulgaria

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