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Molecular and biological characterization of *Streptococcus pneumoniae* isolates from patients with pneumococcal meningitis

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Abstract

The aim of the study is to provide key characteristics of *Streptococcus pneumoniae* isolates circulating in Russia in 2015–2020 and isolated from pneumococcal meningitis patients based on high-throughput sequencing data, including global pneumococcal sequence clusters, serotypes, virulence factors and genetic determinants of resistance, in comparison with clinical data on antimicrobial susceptibility.

Materials and methods. We studied 68 invasive *S. pneumoniae* isolates from blood and cerebrospinal fluid of patients with bacterial meningitis in different regions of Russia in 2015–2020. Species identification was performed taking into account the morphology of colonies on blood agar, the presence of α-hemolysis, negative catalase reaction, sensitivity to optoquine, and positive latex-agglutination results. The sensitivity of isolates to antimicrobials was determined by microdilution in broth, and sensitivity categories were determined based on borderline values of minimum inhibitory concentrations (MICs). Whole genome sequencing of *S. pneumoniae* isolates, analysis of isolates for penicillin-binding protein signature, determination of global pneumococcal sequence clusters, MLST alleles, serotypes, sequence types and acquired resistance genes (*mefA*, *ermB*, *tetM*, *folA/P*, *cat*), identification of virulence genes were carried out.

Results. Twenty-eight GPSCs, 45 sequence types and 27 serotypes were identified. The coverage rates of PPV-23 and PCV-13 were 78% and 59%, respectively. Serotypes 3 (18%), 19F (9%), 23F (7%) and 15B (6%) were predominant. The GPSC12 lineage (serotype 3) was predominant (43%). Lineages expressing vaccine serotypes GPSC1(19F), GPSC6(14), GPSC13(6A), GPSC904(14) and GPSC10(19F) exhibited multiple antimicrobial resistance, including penicillin resistance. The resistant lineages expressing non-vaccine serotypes were GPSC230 (13) and GPSC177 (35F). In most cases, genotypic and phenotypic resistance to penicillin (increased MICs of β-lactams correlated with types of penicillin-binding proteins), erythromycin (*ermB*, *mefA*, *ermB*/*mefA*), clindamycin (*ermB*) and tetracycline (*tetM*), and trimethoprim-sulfamethoxazole (*folA*, *folP*) was found to be consistent. The virulence genes *cbpG*, *lytA*, *pce*/*cbpE*, *pavA*, *pfbA*, *ply*, *hysA*, *nanA* and *cps4A* were detected in all isolates. Zinc metalloproteinase C was detected in 13% of isolates.

Conclusion. A high diversity of serotypes and lineages among pneumococcal isolates from meningitis patients was revealed. Out of the 68 *S. pneumoniae* isolates from patients with bacterial meningitis, more than 17% belonged to non-vaccine serotypes. The results of phenotypic and genotypic antimicrobial resistance comparison were characterized by good concordance, which indicates the necessity for further study of the possibility of using whole-genome sequencing as a diagnostic tool to identify resistance mechanisms in clinical isolates of pneumococci.

Keywords: *Streptococcus pneumoniae, invasive pneumococcal infections, whole-genome sequencing, multilocus sequencing-typing; antimicrobial resistance, penicillin-binding proteins, global pneumococcal sequence cluster, serotypes*

Ethics approval. The study was conducted with the informed consent of the patients. The study protocol was approved by the Independent Interdisciplinary Committee for the Ethical Review of Clinical Trials (Protocol No. 1, January 17, 2020).

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Оригинальное исследование
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Молекулярно-биологическая характеристика изолятов *Streptococcus pneumoniae*, выделенных от больных пневмококковым менингитом

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Аннотация

Цель работы — дать ключевые характеристики изолятов *Streptococcus pneumoniae,* циркулирующих на территории России в 2015–2020 гг. и выделенных от больных пневмококковым менингитом, на основании данных высокопроизводительного секвенирования, включая глобальные кластеры пневмококковых последовательностей, серотипы, факторы вирулентности и генетические детерминанты резистентности, в сравнении с клиническими данными по чувствительности к антимикробным препаратам (АМП).

Материалы и методы. Исследовано 68 инвазивных изолятов *S. pneumoniae,* выделенных из крови и ликвора пациентов с бактериальным менингитом в разных регионах России в 2015–2020 гг. Видовую идентификацию проводили с учётом морфологии колоний на кровяном агаре, наличия α-гемолиза, отрицательной каталазной реакции, чувствительности к оптохину, положительных результатов латекс-агглютинации. Чувствительность изолятов к АМП определяли методом микроразведений в бульоне, категории чувствительности — на основании пограничных значений минимальных подавляющих концентраций (МПК). Проводили полногеномное секвенирование изолятов *S. pneumoniae*, анализ изолятов на сигнатуру пенициллинсвязывающих белков, определение глобальных кластеров пневмококковых последовательностей, аллелей MLST, серотипов, сиквенс-типов и генов приобретённой резистентности (*mefA, ermB, tetM*, *folA/P, cat*), идентифицировали гены вирулентности.

Результаты. Выявлены 28 GPSC, 45 сиквенс-типов и 27 серотипов. Степень охвата ППВ-23 и ПКВ-13 составила 78 и 59% соответственно. Доминировали серотипы 3 (18%), 19F (9%), 23F (7%) и 15B (6%). Преобладала (43%) линия GPSC12 (серотип 3). Линии, экспрессирующие вакцинные серотипы GPSC1(19F), GPSC6(14), GPSC13(6A), GPSC904(14) и GPSC10(19F), обладали множественной антимикробной резистентностью, включая резистентность к пенициллину. Резистентные линии, экспрессирующие невакцинные серотипы, — GPSC230 (13) и GPSC177 (35F). В большинстве случаев установлено соответствие генотипической и фенотипической резистентности к пенициллину (повышенные МПК β-лактамов коррелировали с типами пенициллинсвязывающих белков), эритромицину (*ermB, mefA, ermB/mefA*), клиндамицину (*ermB*) и тетрациклину (*tetM*) и триметоприму-сульфаметоксазолу (*folA, folP)*. У всех изолятов обнаружены гены вирулентности *cbpG, lytA, pce/cbpE, pavA, pfbA, ply, hysA, nanA* и *cps4A*. Цинковая металлопротеиназа С обнаружена у 13% изолятов.

Заключение. Выявлено высокое разнообразие серотипов и линий среди изолятов пневмококков, выделенных у больных менингитом. *И*з 68 изолятов *S. pneumoniae*, выделенных у пациентов с бактериальным менингитом, более 17% относились к невакцинным серотипам. Результаты сопоставления фенотипической и генотипической антимикробной резистентности характеризовались хорошей конкордантностью, что указывает на необходимость дальнейшего изучения возможности использования полногеномного секвенирования в качестве диагностического инструмента для выявления механизмов резистентности у клинических изолятов пневмококков.

Ключевые слова: *Streptococcus pneumoniae, инвазивные пневмококковые инфекции, полногеномное секвенирование, мультилокусное секвенирование-типирование, резистентность к антимикробным препаратам, пенициллинсвязывающие белки, глобальный кластер пневмококковых последовательностей, серотипы*

Этическое утверждение. Исследование проводилось при добровольном информированном письменном согласии пациентов. Протокол исследования одобрен Независимым междисциплинарным комитетом по этической экспертизе клинических исследований (протокол № 1 от 17.01.2020).

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Streptococcus pneumoniae is a human respiratory pathogen and a major cause of morbidity and mortality worldwide. *S. pneumoniae* is the 4th most common cause of fatal infections, such as septicemia and meningitis, and is estimated by the World Health Organization (WHO) to cause 1.6 million deaths, of which 0.7–1.0 million occur in children under 5 years of age, mostly in developing countries [1–3]. Surface capsular polysaccharides of *S. pneumoniae* are one of the most important virulence factors and the basis of pneumococcal serotyping. Currently, more than 100 serotypes of *S. pneumoniae* are known [4]. Due to its ability to acquire exogenous DNA, pneumococcus can switch serotypes and acquire antibiotic resistance genes [5]. Uncontrolled use of antimicrobials, selective pressure of pneumococcal vaccines, high level of genetic recombination of *S. pneumoniae* inevitably lead to changes in pneumococcal population: emergence of new non-vaccine serotypes, emergence of isolates with multiple antimicrobial resistance, change in virulence profile. Due to the current situation in the world, in 2024 WHO included macrolide-resistant *S. pneumoniae* in the updated list of priority bacterial pathogens of intermediate level in the world[1].

Currently, whole-genome sequencing technologies allow obtaining information on genetic changes, serotypes, sequencing types determined both by the classical 7-locus scheme and by the core genome MLST, virulence profile, antimicrobial resistance status of pneumococci, which is important for epidemiologic surveillance [6–10].

The aim of the study: to provide key characteristics of *S. pneumoniae* isolates from pneumococcal meningitis patients circulating in Russia in 2015–2020, based on high-throughput sequencing data, including global pneumococcal sequence clusters, serotypes, virulence factors and genetic determinants of resistance, in comparison with clinical data on susceptibility to antimicrobials.

Materials and methods

The study of virulence factors and resistance genes in 68 invasive isolates of *S. pneumoniae* isolated from blood and cerebrospinal fluid of patients diagnosed with bacterial meningitis was performed. All isolates were obtained during different stages of the PeGAS multicenter study in 2015–2020 in different regions of Russia [11]. The isolation and primary identification of isolates were performed in local microbiological laboratories of the centers participating in the study as part of the standard procedure for bacteriological examination of biological material obtained from patients diagnosed with bacterial meningitis and in accordance with MG 4.2.1887-04 "Laboratory diagnosis of meningococcal infection and purulent bacterial meningitis". *S. pneumoniae* isolates were transported to the central laboratory of the Research Institute of Antimicrobial Chemotherapy (RIAC) on modified Dorset medium. The RIAC evaluated the compliance of the sent isolates with the inclusion criteria and performed their identification based on colony morphology on blood agar (NEM), the presence of α-hemolysis, negative catalase reaction, sensitivity to optoquine (Oxoid) and positive results of latex-agglutination using the DrySpot Pneumo kit (Oxoid). All isolates were stored in tubes with trypticase-soy broth (bioMerieux) supplemented with 30% sterile glycerol (Sigma) at –70°C until antimicrobial sensitivity was determined. Contaminated and non-viable isolates were excluded from the study. Sensitivity to antimicrobials was determined by broth microdilution in accordance with the requirements of ISO 20776-1:2020[2], and the sensitivity categories of isolates to antimicrobials were determined based on borderline values of minimum inhibitory concentrations (MIC) in accordance with EUCAST standards[3] and Russian recommendations. To control the quality of sensitivity determination, a reference strain of *S. pneumoniae* ATCC 49619 was tested in parallel with the isolates under study.

Whole-genome sequencing was performed at the Central Research Institute of Epidemiology of Rospotrebnadzor. Sample preparation was performed using the Nextera (Illumina) protocol. High-throughput sequencing was performed on a HiSeq 1500 instrument using HiSeq PE Rapid Cluster Kit v2 and HiSeq Rapid SBS Kit v2 (Illumina). Whole-genome nucleotide sequences were assembled using the SPAdes v. 3.13 program. A detailed description of the sample preparation methodology for whole-genome sequencing was given earlier [12].

The whole genomic nucleotide sequences of the studied isolates, data on serotypes and antibiotic sensitivity, as well as information on the sources of strains were deposited in the PubMLST database[4]: accession numbers: 51080–51125, 73010–73011, 73013–73015, 73017–73033.

Invasive pneumococcal isolates were analyzed for the PBP signature, where the combination of 3

[1] WHO bacterial priority pathogens list, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. URL: https://who.int/publications/i/item/9789240093461

[2] ISO 207761:2019 Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices. Part 1: Broth microdilution reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases.

[3] European Committee on Antimicrobial Susceptibility testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Ver. 14.0, 2024. URL: www. eucast.org/clinical_breakpoints/ (data of access: 01.11.2024).

[4] *Streptococcus pneumoniae* MLST Databases. URL: https://pubmlst.org/spneumoniae/ (data of access: 17.02.2020).

signatures (PBP1A, PBP2B, PBP2X) determines the level of resistance to β-lactams, global pneumococcal sequence clusters were identified, MLST alleles, serotypes, sequence types and acquired resistance genes (*mefA*, *ermB*, *tetM*, *folA/P*, *cat*) using a tool available on Pathogenwatch, a global genomic surveillance platform[5]. Virulence genes were identified using the online AMRseq program[6] and the BacWGSTdb program[7].

The study was non-interventional and did not involve comparison of groups. Descriptive statistics methods were used to present the results, with the determination of the absolute and relative number of observations.

Results

Molecular and biological characterization of invasive S. pneumoniae isolates

We identified 28 global pneumococcal sequence clusters (GPSCs), 45 sequence types and 27 serotypes (**Table 1**).

Analysis of whole genomic data showed that serotypes included in pneumococcal polysaccharide 23-valent vaccine (PPV-23) and pneumococcal conjugate vaccine 13 (PCV-13) were predominant among invasive isolates — 3 (18%), 19F (9%), 23F (7%) and 15B (6%). The coverage rate of PPV-23 was 79% and PCV-13 was 59%. Frequency of isolation of pneumococci of non-vaccine serotypes in meningitis in Russia: 28A and 35F — 3% each, 13, 37, 38, 10C, 15C and 15F — more than 1% (**Figure**).

Of the 28 GPSCs, 20 GPSCs were of vaccine serotypes and 6 GPSCs were of non-vaccine serotypes. Two lineages, GPSC212 (12F, 15F) and GPSC229 (15B, 15C) expressed both vaccine and non-vaccine serotypes. The GPSC12 lineage expressed only vaccine serotype 3 and accounted for more than 42%. In some cases, the same serotype was associated with different lineages. The second most frequent serotype 19F was expressed by lineages GPSC1, 10, 44 and a new lineage whose GPSC is undefined, serotype 23F by GPSC7 and GPSC49, and serotype 15B by GPSC11 and GPSC229. Non-vaccine serotypes were expressed by the lineages GPSC 365 (serotype 28A), GPSC177 (serotype 35F), GPSC212 (serotype 15F), GPSC229 (serotype 15C), GPSC123 (serotype 37), GPSC38 (serotype 38), and GPSC230 (serotype 13).

Antimicrobial sensitivity of invasive isolates of S. pneumoniae to antimicrobials

Analysis of sensitivity to antimicrobials showed that 6 (9%) invasive isolates were resistant to penicillin

[5] A Global Platform for Genomic Surveillance. URL: https://pathogen.watch
[6] AMRseq. URL: https://amrseq.net/ru/
[7] BacWGSTdb. URL: http://bacdb.cn/BacWGSTdb/index.php

Table 1. Global clusters of pneumococcal sequences associated with sequence types and serotypes

GPSC	Sequence types	Serotype	Number of isolates, *n* (%)
1	236	19F	2 (7)
2	15249	1	1 (4)
3	1012	11A	1 (4)
6	3418, 143	6E(6B),14	2 (7)
7	311, 152248, 16095, 311	23F	4 (14)
10	230	19F	1 (4)
11	1262	15B	2 (7)
12	505, 180, 15251, 15250, 2049	3	12 (43)
13	12493, 473	6A, 6B	2 (7)
16	66, 16098	9N	2 (7)
19	433	22F	2 (7)
32	3244, 11901,2824, 3544	7F, 8	4 (14)
38	393	38	1 (4)
43	239	9V	3 (11)
44	179	19F	1 (4)
49	440	23F	1 (4)
68	15252, 3187	18C	2 (7)
76	490	6A	1 (4)
98	1480	8	1 (4)
123	447	37	1 (4)
162	2361	4	2 (7)
177	2991	35F	2 (7)
212	6202	12F, 15F	4 (14)
229	1025	15B, 15C	3 (11)
230	2754	13	1 (4)
365	225	28A	2 (7)
376	9247	6E(6B)	1 (4)
904	782	14	1 (4)
Not assigned	15	19F	2 (7)
Not assigned	15247	10C	1 (4)
Not assigned	5205	8	1 (4)
Not assigned	13459	10A	1 (4)
Not assigned	16099	4	1 (4)

Note. Not assigned — global pneumococcal sequence cluster number is not assigned.

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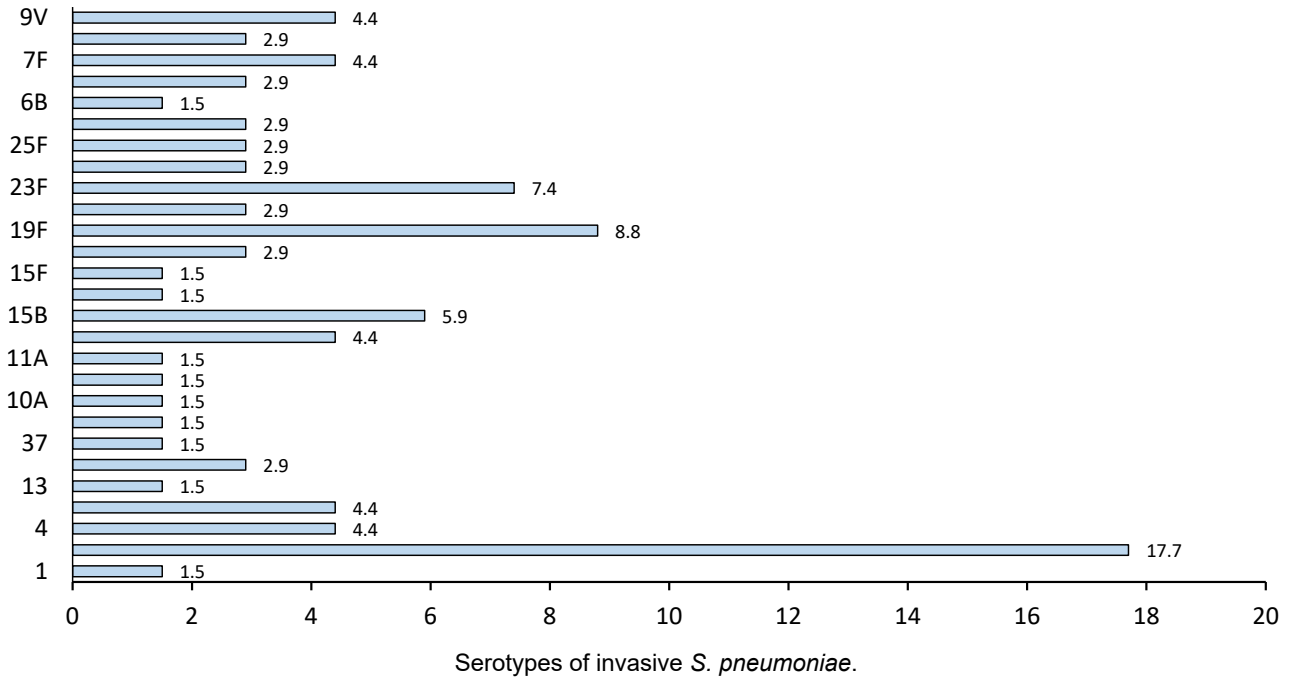

(**Table 2**), 11 (16%) to tetracycline, 5 (7%) to erythromycin, 2 (3%) to clindamycin, and 1 (1%) to respiratory fluoroquinolones. To trimethoprim-sulfamethoxazole 12 (18%) isolates were resistant and 12 (18%) were sensitive at increased exposure. Among the isolates of 28 GPSC lineages, isolates of 5 lineages expressing vaccine serotypes were resistant to 3 or more classes of antimicrobials simultaneously: GPSC1 (serotype 19F), GPSC6 (serotype 14) were resistant to penicillin, tetracycline, trimethoprim-sulfamethoxazole, erythromycin, and clindamycin; GPSC10 (serotype 19F), GPSC904;9 (serotype 14) — to penicillin, tetracycline and trimethoprim-sulfamethoxazole, GPSC6 (serotype 6E(B)) — to penicillin, tetracycline and trimethoprim-sulfamethoxazole, GPSC6 (serotype 6E(B)) — to penicillin, erythromycin, and trimethoprim-sulfamethoxazole. One lineage with vaccine serotypes was resistant to two classes of antibiotics simultaneously: GPSC44 (serotype 19F) to tetracycline and erythromycin. Two lineages expressing non-vaccine serotypes, GPSC177 (serotype 35F) and GPSC230 (serotype 13), were resistant to tetracycline and trimethoprim-sulfamethoxazole.

Genetic determinants of antimicrobial resistance

In clinical isolates of *S. pneumoniae*, resistance to β-lactams is primarily due to variations in amino acid sequences in the transpeptidase domains of penicillin-binding proteins (PBPs): PBP1a, PBP2b and PBP2x, which reduce the affinity of β-lactam antibiotics for these sites. The type of PBPs can predict the levels of resistance to β-lactams [13]. Analysis of the results of whole-genome sequencing of invasive *S. pneumoniae* isolates revealed that the most common type of PBP in sensitive isolates (PBP1a-PBP2b-PBP2x) was 2-0-2 — 29%, 11-4-0 — 4% and 12-0-6 — 3% were less common. All penicillin-resistant isolates had signatures: 13-16-47, 17-15-22, 24-53-77, 36-34-44, and 31-12-18. Several combinations of new PBP types were also identified: new-27-new, 34-11-new. Resistance to macrolides end lincosamides (erythromycin and clindamycin) was due to the presence of the methylase (*ermB*) gene in 5 (7%) isolates, while 1 (1%) isolate had both *ermB* and macrolide efflux pump (*mefA/E*) genes detected simultaneously. Resistance to trimethoprim-sulfamethoxazole was associated with substitution in *folA* I100L and/or insertion of 1 or 2 *foP* codons, whereas isolates categorized as sensitive at higher exposure often had insertion of 1 or 2 codons in *folP*. Chloramphenicol resistance was predicted by the presence of the chloramphenicol acetyltransferase gene (*cat*), while fluoroquinolone resistance was predicted by mutations in *gyrA* and *parC* genes.

Comparison of resistance phenotype and genotype

The concordance of resistance genotype and phenotype was generally high, but in some cases there were discrepancies between genotypic and phenotypic resistance.

Resistance to β-lactams showed good concordance. Six penicillin-resistant pneumococcal isolates had PBP signatures characteristic of resistant pneumococci. Interestingly, all 6 penicillin-resistant isolates had PBP1a(13-36)-PBP2b(12-53)-PBP2x(18-77) signatures, whereas all sensitive isolates had low-numbered PBP signatures. Thus, PBP transpeptidase signatures are reliable indicators of the MICs of various β-lactam antibiotics in clinical isolates of pneumococci and can serve as an alternative to phenotypic sensitivity testing.

Table 2. Resistance of pneumococcal lineages GPSC to antimicrobials

GPSC	Serotype	Number	Genotype, resistance, *n* (%)						
			PEN	TET	TS	CL	ERY	CHL	FX
1	19F	2	2 (3)	2 (3)	2 (3)	1 (1)	2 (3)	0	0
2	1	1	0	0	0	0	0	0	0
3	11A	1	0	0	1 (1)	0	0	0	0
6	6E(B),14	2	1 (1)	2 (3)	2 (3)	2 (3)	2 (3)	0	0
7	23F	4	0	0	4 (6)	0	0	0	0
10	19F	1	1 (1)	1 (1)	1 (1)	0	0	0	0
11	15B	2	0	0	2 (3)	0	0	0	0
12	3	12	0	1 (1)	0	0	0	0	1 (1)
13	6A, 6B	2	1 (1)	1 (1)	2 (3)	0	1 (1)	0	0
16	9N	2	0	0	1 (4)	0	0	0	1 (1)
19	22F	2	0	0	0	0	0	0	0
32	7F,8	4	0	0	0	0	0	0	0
38	38	1	0	0	0	0	0	0	0
43	9V	3	0	0	3 (4)	0	0	0	0
44	19F	1	0	1 (1)	1 (1)	1 (1)	1 (1)	0	0
49	23F	1	0	0	1 (1)	0	0	0	0
68	18C	2	0	0	2 (3)	0	0	0	0
76	6A	1	0	0	1 (1)	0	0	0	0
98	8	1	0	0	0	0	0	0	0
123	37	1	0	0	0	0	0	0	0
162	4	2	0	0	0	0	0	0	0
177	35F	2	0	2 (3)	2 (3)	0	0	0	0
212	12F, 15F	4	0	0	0	0	0	0	0
229	15B, 15C	3	0	0	3 (4)	0	0	0	0
230	13	1	0	1 (1)	1 (1)	0	1 (1)	0	0
365	28A	2	0	0	0	0	0	0	0
376	6E(6B)	1	0	0	1 (1)	0	0	1 (4)	0
904;9	14	1	1 (1)	1 (1)	1 (1)	0	0	0	0
Not assigned	10C, 19F, 8, 10A ,4	6	0	2 (3)	2 (3)	0	0	0	0
28	27	68	6 (9)	14 (21)	33 (49)	4 (6)	7 (10)	1 (1)	2 (3)

Note. PEN, penicillin resistance predicted based on PBP1a, PBP2b and PBP2x sequences; TET, tetracycline resistance predicted by the presence of the *tet* M gene; TS, trimethoprim-sulfamethoxazole resistance, associated with substitution in folA I100L and/or insertion of 1 or 2 codons in *folP_aa_insert_57-70*, CL — clindamycin resistance predicted of gene *erm B*; ERY — macrolide resistance predicted by the presence of the methylase gene (*ermB*) and macrolide efflux pump gene (*mefA/E*); CHL — chloramphenicol resistance predicted by the presence of the chloramphenicol acetyltransferase gene (cat); FX — fluoroquinolone resistance predicted by the presence of mutations in the *gyrA*, *parC* genes.

All 11 tetracycline-resistant pneumococci carried *tetM* genes. Three isolates containing *tetM* were categorized as sensitive, which may be due to mutations in *tetM* not considered in our study.

In the case of trimethoprim-sulfamethoxazole, a good correlation between the presence of resistance markers and phenotypic resistance was found for most isolates. Twelve isolates were double mutants with a

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substitution in *folA* I100L and an insertion of 1 or 2 codons in *folP_aa_insert_57-70* and were resistant to trimethoprim-sulfamethoxazole (Table 2). Twelve pneumococcal isolates containing insertions in *folP* had an IPC of 2 mg/L and were categorized as sensitive at increased exposure, and 9 isolates with single mutations in *folP* were categorized as sensitive (IPC ≤ 1 mg/L).

All 5 erythromycin-resistant isolates carried the *ermB* gene, 1 resistant isolate carried both *ermB* and *mefA*, while 2 isolates with the *mefA* gene remained phenotypically sensitive to erythromycin. The 2 clindamycin-resistant isolates carried *ermB* genes, but *ermB* was also detected in 2 phenotypically sensitive isolates.

Due to the fact that there are no criteria for determining the category of sensitivity to chloramphenicol for *S. pneumoniae*, the activity of this drug was assessed based on the epidemiological cut-off value. The MIC of chloramphenicol for all isolates tested was less than 8 mg/L, which corresponds to the wild-type population, but 1 isolate carried *cat* gene.

One isolate was found to be resistant to respiratory fluoroquinolones (levofloxacin and moxifloxacin); it had mutations in the *gyrA* and *parC* genes. At the same time, a mutation in the *parC* gene was detected in 1 isolate among fluoroquinolone-sensitive pneumococci.

Genetic determinants of pneumococcal virulence

To gain insight into the genetic features contributing to virulence, we examined the presence of the major protein virulence factors of pneumococcus. Choline-binding proteins (CbpG, LytA and Pce/CbpE), PavA and PfbA, known as fibronectin- and plasminogen-binding proteins, as well as hyaluronidase, pneumolysin, neuraminidase, and capsule-associated Cps4A were detected in all invasive pneumococcal isolates (**Table 3**). Zinc metalloproteinase C was detected in 9 (13%) isolates.

Discussion

In our study, pneumococci of serotype 3 prevailed among clinical isolates causing meningitis in Russia. A similar situation is observed in many other countries during the postvaccination period. In Austria, England, Canada, Sweden and Germany, a significant increase in invasive pneumococcal diseases of serotype 3 in adults has been observed over the last 3 years [14]. In Brazil, serotype 3 became the predominant cause of invasive disease in the post-PCV era among adults [15, 16]. The low efficacy of conjugate vaccines against serotype 3 pneumococci is related to the structure of the polysaccharide capsule, which is non-covalently bound to cell wall peptidoglycan [17–21]. It should be noted that isolates of the GPSC12 lineage (serotype 3), remaining the main cause of invasive forms of pneumococcal infection worldwide after the introduction of PCV13, usually retain sensitivity to antimicrobials [22–25]. The low incidence of antimicrobial resistance in serotype 3 isolates may be associated with the high invasiveness of this serotype and the relatively short duration of carriage, which, in turn, reduces the impact of antimicrobials in the treatment of infections of other etiologies [26]. At the same time, a serotype 3 study conducted in England [27] revealed that since 2018, GPSC12 lineage isolates resistant to penicillin, macrolides, chloramphenicol and tetracycline have been emerging [28, 29]. The increasing resistance of serotype 3 isolates indicates the circulation of more antibiotic-resistant clones [30]. In our study, all *S. pneumoniae* serotype 3 isolates were sensitive to penicillin; of 12 isolates isolated from cerebrospinal fluid, only 2 contained resistance genes to fluoroquinolones — *parC* and to tetracycline — *tetM*, while retaining phenotypic sensitivity to these drugs.

Pneumococci of serotype 19F were the second most frequently isolated in meningitis, which may be

Table 3. Characteristics of virulence genes

Virulence gene	Name of the encoded protein	% identity	Number of isolates	
			n	%
cbpG	Choline-binding protein G	99,30	68	100
lytA	Autolysine	98,75	68	100
pce/cbpE	Choline-binding protein E	99,18	68	100
ply	Pneumolysine	99,86	68	100
pavA	Fibronectin-binding protein	99,52	68	100
pfbA	Plasmin and fibronectin-binding protein A	99,72	68	100
hysA	Hyaluronidase	99,16	68	100
nanA	Neuraminidase A	98,77	68	100
cps4A	Capsule synthesis	96,54	68	100
zmpC	Zinc metalloproteinase C	99,96	9	13

due to the peculiarities of the capsular polysaccharide, which is more resistant to the deposition of the C3b component of complement and antibodies on the bacterial walls, which reduces the sensitivity to opsonophagocytosis [31, 32]. Our results indicate that the circulation of serotype 19F is associated with the spread of 3 lineages: GPSC10, GPSC1 and GPSC44. All lineages were characterized by resistance to various antimicrobials, with the GPSC10 and GPSC1 lineages showing multiple antimicrobial resistance. In Canada, 19F serotype dominance was associated with the spread of GPSC1, GPSC4, GPSC9, GPSC10, GPSC18, GPSC44 and GPSC119 lineages [33], in Sweden with the GPSC1 lineage [34], and in Asia, Europe, North America, and South America, 19F serotype was one of the dominant serotypes in the GPSC1 lineage [35]. In South Africa, the dominance of the 19F serotype was associated with the spread of the GPSC1 and GPSC21 lineages, and a high, about 50%, hospital-acquired mortality rate from pneumococcal meningitis was found to be associated with the 19F serotype [35, 36]. The increased incidence of 19F serotype after the introduction of PCV13 in India has been associated with the multidrug-resistant GPSC1 and GPSC10 lineages, with GPSC10 being of particular note as it expressed both vaccine serotypes, including 19F, and non-vaccine serotypes, and thus contributed most to the spread of non-vaccine serotypes among clinical isolates [37]. The GPSC10 lineage is capable of simultaneously expressing a wide range of serotypes, which facilitates its adaptation to selective vaccine pressure. An international dataset of the GPSC10 lineage showed that this lineage expresses 16 serotypes, of which only 6 are included in PCV13. Moreover, the GPSC10 lineage has a relatively high potential to develop invasive forms of infection and a propensity to cause meningitis, regardless of serotype [38]. It was found that it took about 3-5 years for pneumococci of the GPSC10 lineage to spread in France, and in Spain, Argentina, and Israel a rapid change in the serotype composition of this lineage occurred in the post-vaccination period. Thus, together with its transmissibility, GPSC10 should be considered as a high-risk lineage that may eventually reduce the efficacy of vaccines worldwide [39, 40]. In a study conducted by E. Egorova et al. found that in Russia in 2011-2018, serotype 19F isolates belonged to 8 different lineages (GPSC1, GPSC44, GPSC10 GPSC6, GPSC11, GPSC18, GPSC43 and GPSC591), of which the lineages GPSC1, GPSC6 and GPSC10 were characterized by resistance to antimicrobials [41].

According to the results of the present study, pneumococci of serotype 23F were the third most common serotype responsible for the development of bacterial meningitis in Russia. Four out of 5 isolates of serotype 23F belonged to the GPSC7 lineage, 1 — to the GPSC49 lineage. All isolates of serotype 23F were characterized by resistance to trimethoprim-sulfamethoxazole. In studies conducted in China and Iran, serotype 23F was one of the dominant serotypes isolated from the cerebrospinal fluid of patients [42, 43]. The results of studies performed in the UK showed that serotype 23F isolates were part of the GPSC7 lineage. While in 2002 this lineage was dominated by serotype 23F and had only a small number of serotype 23A, in 2009 isolates of serotype 23B appeared [29]. The GPSC7 lineage was one of the dominant lineages responsible for invasive disease during the PCV13 era in Hong Kong, Israel, Malawi, South Africa, Gambia and the USA [22].

The results of international studies indicate that the post-vaccination period is characterized by a decrease in the proportion of vaccine serotypes and an increase in non-vaccine pneumococcal serotypes among different age groups of the population [44, 45]. Lineages represented by vaccine serotypes in which non-vaccine serotypes were already present persist in the population [15]. It has been observed that these non-vaccine serotypes have a high potential to develop invasive forms of infection [46, 47]. The results of our study indicate that in Russia more than 17% of pneumococcal meningitis cases were caused by pneumococci of non-vaccine serotypes. The relatively high proportion of isolates with serotype 15B (6%) and the presence of isolates with non-vaccine serotypes 28A, 37 and 38, which were not previously associated with pneumococcal meningitis in Russia, are noteworthy. The level of antibiotic resistance was found to be lower in non-vaccine serotypes than in vaccine serotypes and differed depending on the GPSC lineage. In our study, lineages expressing non-vaccine serotypes were sensitive to all tested antimicrobials, except for 2 lineages (Table 2).

Different virulence factors are involved in the process of invasive infection at different stages. According to the results of our study, classical genes encoding virulence factors such as capsule synthesis, pneumococcal surface adhesin, autolysin, fibronectin binding protein and pneumolysin were found in all invasive isolates. An interesting observation was made regarding the *zmpC* gene. It was detected only in 9 invasive pneumococcal isolates of the pneumococcal lines GSPC229 (serotype 15C,15B), GSPC3 (serotype 11A), GSPC162 (serotype 4), GSCP904 (serotype 14) and the new line GSPC (serotype 8). Previously, in a study in the Netherlands, the *zmpC* gene was identified in invasive serotypes 8, 11A and 4 belonging to the GPSC3 lineage. It was found that cases of invasive pneumococcal infections caused by *zmpC*-positive pneumococci were more often accompanied by sepsis [48]. Japanese scientists suggested that the zinc metalloprotease *zmpC* suppresses the virulence of pneumococci by inhibiting bacterial invasion into the central nervous system [49]. Thus, zinc metalloprotease *zmpC* is of particular interest and requires additional research.

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Conclusion

1. 28 global pneumococcal sequence clusters (GPSC) and 45 *S. pneumoniae* sequence-types associated with invasive strains were identified in Russia. Of 68 *S. pneumoniae* isolates from patients with bacterial meningitis, more than 17% belonged to non-vaccine serotypes.

2. Antibiotic resistance of pneumococci of vaccine serotypes was higher than that of non-vaccine serotypes.

3. The emergence of non-vaccine serotype lineages of pneumococcus with determinants of high virulence, including resistance to antibiotics, necessitates further research into the molecular genetic characterization of isolates causing meningitis.

4. The results comparing phenotypic and genotypic antimicrobials were characterized by good concordance, indicating the need to further explore the possibility of using whole-genome sequencing as a diagnostic tool to identify resistance mechanisms in clinical isolates of *S. pneumoniae.*

In conclusion, the characterization of pneumococcal lineages and their genetic variations that influence resistance and invasiveness are highly informative for establishing a global strategy for continuous epidemiological surveillance of the pneumococcal population.

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