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Antigenic and genetic characterization of *Streptococcus pneumoniae* strains isolated from patients with invasive and non-invasive pneumococcal infections by using high-throughput sequencing

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Abstract

The **objective** of this study was to characterize and compare antigenic and genetic characteristics of *Streptococcus pneumoniae* strains isolated from patients with invasive and non-invasive pneumococcal infections (PIs) by using the data of *high-throughput sequencing*.

Materials and methods. A total of 158 *S. pneumoniae* strains were studied. All of them were isolated during different stages of the PEHASus multicenter study performed in 2015–2020. The data analysis was based on the information about whole-genome sequences of 46 strains isolated during the above study. Real-time PCR methods and *high-throughput sequencing* (the Illumina platform) were used for identification of serotypes. The SeroBA, PneumoCaT software and PubMLST.org website resources were used in the data processing.

Results and discussion. The serotypes of all the studied strains were identified. A number of discrepancies among serotypes in serogroup 6 and one discordant result were revealed by the analysis of whole-genome sequences using 2 programs. The PCR methods were effectively used to characterize serotypes in 87% and 69% of the pathogens of invasive and non-invasive PIs, respectively. The serotypes contained in PCV13 accounted for 59% and 37%, while PPV23 serotypes accounted for 78% and 53% of the strains isolated from patients with invasive and non-invasive PIs, respectively. The data analysis was unable to identify either the dominant sequence type (a total of 81 sequence types have been identified) or clonal complexes, except for serotype 3 strains, thus demonstrating consistency with the data from previous studies suggesting the absence of a well-represented clonal structure of *S. pneumoniae* associated with pneumococcal meningitis in Russia.

Conclusion. The obtained data made it possible to identify the distribution of the circulating serotypes and genetic characteristics of the strains isolated from PI patients, thus being instrumental for assessment of the effectiveness of the existing polyvalent vaccines and providing information for improvement of the PCR-based methods of serotyping.

Keywords: *Streptococcus pneumoniae*, invasive pneumococcal infections, non-invasive pneumococcal infections, high-throughput sequencing, serotyping, real-time PCR, multilocus sequence typing

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Smolensk State Medical University (No. 213, October 11, 2018).

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Антигенная и генетическая характеристика штаммов *Streptococcus pneumoniae*, выделенных от больных инвазивными и неинвазивными пневмококковыми инфекциями, с использованием высокопроизводительного секвенирования

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Аннотация

Цель работы заключалась в характеристике и сопоставлении данных об антигенных и генетических свойствах полученных с помощью высокопроизводительного секвенирования штаммов *Streptococcus pneumoniae*, выделенных от больных инвазивными и неинвазивными формами пневмококковой инфекции (ПИ).

Материалы и методы. Исследовано 158 штаммов *S. pneumoniae*, выделенных при проведении различных этапов многоцентрового исследования «ПеГАС» в 2015–2020 гг. При анализе данных использовалась информация о полногеномных последовательностях 46 штаммов, выделенных ранее в том же исследовании. Для определения серотипов применены методики ПЦР в режиме реального времени и высокопроизводительное секвенирование (платформа «Illumina»). При обработке данных использовались программы «SeroBA», «PneumoCaT» и программные возможности интернет-ресурса PubMLST.org.

Результаты и обсуждение. Определены серотипы всех штаммов, включённых в исследование. Найден ряд несовпадений серотипов внутри серогруппы 6 и один дискордантный результат при анализе полногеномных последовательностей двумя программами. Предлагаемые ПЦР-подходы позволяют охарактеризовать серотип у 87% возбудителей инвазивных и 69% неинвазивных форм ПИ. Доля штаммов с серотипами, входящими в состав PCV13, составляет 59 и 37%, в состав PPV23 — 78 и 53% для штаммов, выделенных от больных инвазивными и неинвазивными ПИ соответственно. Анализ данных не позволяет выявить преобладающий сиквенс-тип (всего найден 81 сиквенс-тип) или определить клональные комплексы, за исключением штаммов серотипа 3, что согласуется с полученными ранее данными об отсутствии выраженной клональной структуры *S. pneumoniae*, ассоциированных с пневмококковыми менингитами, на территории России.

Заключение. Получены данные, позволяющие определить распределение циркулирующих серотипов и генетические характеристики штаммов, выделенных от больных ПИ, что даёт возможность оценить эффективность существующих поливалентных вакцин и предоставляет информацию для коррекции основанных на ПЦР способов серотипирования.

Ключевые слова: *Streptococcus pneumoniae*, инвазивные пневмококковые инфекции, неинвазивные пневмококковые инфекции, высокопроизводительное секвенирование, серотипирование, ПЦР в режиме реального времени, мультилокусное секвенирование-типирование

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Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

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

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Introduction

Streptococcus pneumoniae is one of the most common human pathogens that can cause variously localized infections. The clinical spectrum of pneumococcal infections (PIs) ranges from invasive to non-invasive infections [1]. In invasive infections, the pathogen is isolated from normally sterile sites, such as blood or cerebrospinal fluid. In non-invasive infections, including non-bacteremic pneumonia, the pathogen can be isolated from the lower respiratory tract. Therefore, microbial cultures from patients with invasive and non-invasive PIs will be hereinafter referred to as “invasive strains” (ISs) and “non-invasive strains” (NISs), respectively.

The microbiological monitoring is a critical component of PI epidemiological surveillance, giving special attention to antigenic and genetic characteristics of PI pathogens, and to the data on antibiotic sensitivity. The analysis of a pathogen’s antigenic properties is used to identify serotypes and, consequently, to assess the effectiveness of the existing polyvalent vaccines. In Russia, the commonly used vaccines are the 13-valent pneumococcal conjugate vaccine (PCV13, Prevenar 13) and the 23-valent pneumococcal polysaccharide vaccine (PPV23, Pneumovax 23). The identification of the PI pathogens’ serotypes is essential for planning preventive immunization measures and for assessment of their effectiveness for people involved in the epidemic process.

While the antibiotic sensitivity is generally assessed by using standard microbiological methods, following the EUCAST recommendations [2, 3], as in most cases the variety of resistance mechanisms [1] prevents using molecular methods, the efficiency of the latter methods used for identification of serogroups and serotypes (the key elements in the antigenic characterization of *S. pneumoniae*) has been demonstrated in multiple Russian and foreign studies [4–7]. The identification of genetic characteristics of *S. pneumoniae* strains by using multilocus sequence typing (MLST) or other approaches based on the analysis of whole-genome data provides efficient tools for describing the clonal structure of microorganisms involved in the epidemic process, for evaluating the recombination potential of a bacterial population and for analyzing the evolutionary processes leading to emergence of new, potentially virulent or antibiotic-resistant strains [1, 8–10].

Antigenic and genetic characteristics can be identified by using molecular and biological methods such as real-time PCR (RT-PCR) and sequencing. High-throughput sequencing gives a comprehensive insight into microorganisms, underlying the analysis of their whole-genome sequences: sequences of *cps*-locus genes for identification of serotypes and data on primary sequences of core genome loci, the analysis of which has the highest discriminative power for identification of genetic relationships among strains and for identification of the clonal structure of a bacterial population.

Despite its multiple advantages for serotype identification, RT-PCR has certain limitations, as pathogens go through continuous adaptation, trying to survive under pressure of herd immunity, including the immunity acquired through preventive vaccination using polyvalent vaccines. At least 100 serotypes of *S. pneumoniae* have been identified to date, most of them being associated with invasive PIs [1, 11], thus proving the importance of studying strains, which are not typeable with standard serological or PCR-based methods, and the urgency of improving the existing laboratory techniques, for example, by using additional serotype-specific targets. The resulting information about epidemiological features of circulating pathogens is required for development of vaccination programs and for monitoring of their effectiveness.

Thus, the **objective** of this study was to characterize *S. pneumoniae* strains isolated from patients with invasive and non-invasive PIs by using high-throughput sequencing, to analyze and compare their antigenic and genetic properties in the microbiological monitoring context.

Materials and methods

The study was performed on 22 invasive (isolated from blood or cerebrospinal fluid) and 90 non-invasive (isolated from sputum samples of patients with community-acquired pneumococcal pneumonia) strains of *S. pneumoniae*. All the strains were isolated during different stages of the *PEHASus* multicenter study performed in 2015–2020 [2]. Most of the strains characterized in this study as well as ISs also isolated during the *PEHASus* studies and described earlier [5] were isolated in 2019 ($n = 58$) and 2020 ($n = 47$).

The transportation and storage, microbiological methods, species-level identification methods, DNA isolation techniques, whole-genome sequencing and genome-assembly procedures were described in previous works [2, 5]. All the strains were studied additionally by using RT-PCR methods for identification of 16 serotypes [4] and by using the specially designed techniques for identification of serotypes 12F, 15BC, 22FA, and 8. The RT-PCR reaction mixtures contained a set of 4 serotype-specific oligonucleotides corresponding to the groups shown in **Table 1**. The serotype identification based on the whole-genome sequencing data was performed with SeroBA [6] and PnemoCaT programs [7].

The nucleotide sequences were deposited, the sequencing results, including assignment of alleles and sequence types, were processed and the MLST data were analyzed with BURST and Genome Comparator tools on PubMLST.org¹ [9]. At the end of the study, the database contained information about more than 37 thousand genomes of *S. pneumoniae*, including

¹ PubMLST. *Streptococcus pneumoniae* MLST Databases.
URL: <https://pubmlst.org/organisms/streptococcus-pneumoniae>

Table 1. Serotype groups identified in invasive and non-invasive strains

Serotype groups		Invasive strains (n = 68)	Non-invasive strains (n = 90)	χ^2 (p-value)*
Methods RT-PCR	3, 6AB, 9VA, 19F	26 (38%)	28 (31%)	0,59 (0,44)
	1, 4, 14, 23F	11 (16%)	9 (10%)	0,84 (0,36)
	9NL, 11AD, 15AF, 18	6 (9%)	11 (12%)	0,18 (0,67)
	2, 5, 7AF, 19A	3 (4%)	0	0,08**
	8, 12F, 15BC, 22FA	13 (19%)	14 (16%)	0,14 (0,71)
	Total	59 (87%)	62 (69%)	5,94 (0,015)
Included in vaccines	PCV13	40 (59%)	33 (37%)	6,78 (0,009)
	PPSV23	53 (78%)	48 (53%)	9,13 (0,0025)

Note. * χ^2 of Pearson with Yates correction; **Fisher's exact test, p-value.

288 whole-genome sequences of Russian isolates, most of which were characterized in the earlier works [5, 10]. As we previously studied the sample containing 46 ISs isolated during the *PEHASus* study, we used the pooled sample of ISs (n = 68), which included previously described strains, to compare the results of antigenic and genetic characterization of ISs and NISs [5].

Results

The whole-genome nucleotide sequences of the studied strains, the data on serotypes and antibiotic sensitivity (for most of the strains) as well as the information about sources of strains were deposited in the PubMLST database under the following accession numbers: ISs — 73010, 73011, 73013–73015, 73017–73033, NISs — 142542, 142543, 142546–142569, 142572–142574, 142578, 142579, 142581, 142583–142604, 142606–142610, 142612–142625, 142627–142643. Allelic profiles and the respective sequence types were identified for all of the strains, some of them being described for the first time.

The analysis of whole-genome data by using two programs [6, 7] helped assign the studied strains to serotypes and serogroups. NISs demonstrated discrepancies during the identification of serotypes belonging to serogroups 6 (only serotypes B or C, A or B and D or C) in 5 strains, 15 (B or C) in 3 strains, and 35 (A or C) in 1 strain; one discordant result was obtained in isolate with id142633, where the serotype was identified controversially (35A or 42). No discordance between the serotype identification *in silico* and with RT-PCR methods was found.

Discussion

Antigenic characterization

ISs and NISs had 28 and 33 variants of serotypes, respectively (42 were non-repeating). The most frequent serotypes found in ISs (more than in 5%) were serotypes 3 (18%), 19F (9%) and 23F (7%), while in

NISs, these were serotypes 3 (11%), 19F (10%), 15C (8%) and 11A (8%), 23F (7%) and 23A (6%). Table 1 shows the data on frequencies of group-assigned serotypes for both groups of strains. The serotype groups have the respective serotype-specific targets detectable by RT-PCR methods and capsular antigens used for polyvalent vaccines PCV13 and PPSV23. As seen from Table 1, the strains with the serotype detectable with the RT-PCR method applicable to 16 serotypes [4] and including all serotypes of vaccine PCV13 account for 67% for ISs and 53% for NISs. These percentages are almost identical to the percentage of the serotypes (65%) obtained during the studies of pathogens causing pneumococcal meningitis and circulating in Moscow during the reference period (2016–2019) [12] and are lower (79%) than those circulating before (2007–2010) [4]. The decrease in the proportion of serotypes present in vaccine PCV13 can be explained both by the changing range of the antigenic diversity of *S. pneumoniae* due to vaccination and by the territorial diversity of the pathogens included in this study and the *PEHASus* study [2]. The additionally detected serotype-specific targets 12F, 15BC, 22FA, and 8 increased the proportion of identified serotypes to 87 and 69%, demonstrating significant difference between the samples. The RT-PCR method designed in 2014 and its algorithm [4] were not well suited for ISs and NISs in the studied sample collected in several Russian regions and circulating primarily in 2019–2020. The best option used for RT-PCR-based identification of serotypes should include additional serotype-specific targets 12F, 15BC, 22FA, and 8, while the detection of serotypes 2, 5, 7AF, and 19A can be omitted.

The proportion of serotypes present in vaccines PCV13 and PPSV23 varies in the studied strain samples, while the proportion of serotypes is significantly higher in the IS sample for both vaccines.

Genetic characterization

During this and earlier studies [5], a total of 81 sequence types were identified, the most frequent be-

Table 2. Sequence types of invasive and non-invasive strains identified in 2 or more cases

Number of strains with matching sequence-type	Invasive strains, 45 sequence types (n = 68)	Non-invasive strains, 54 sequence types (n = 90)
6	180	–
5	–	81, 505
4	6202	1262
3	239, 505, 1025	62, 150, 143, 393, 423, 1025 , 1012, 9659, 11900
2	15, 225, 236, 311, 433 , 1262, 2361, 2991, 3544	42, 239, 433 , 2754, 6202 , 9248, 12493
Once	31 sequence types	35 sequence types

Note. *Sequence types found in both samples are in bold.

ing ST-180 (6%), ST-505 (5%), ST-1025, ST-1262, and ST-6202 (each accounting for 4%), ST-81 and ST-239 (both accounting for 3%). In both samples of strains, the maximum number of sequence types was detected only once. Out of 81 sequence types, 18 types (22%) were found in both samples of strains, 27 (33%) sequence types were found only in ISs, and 36 (44%) sequence types were found only in NISs. Some differences in the distribution and occurrence frequencies of the detected sequence types are shown in **Table 2**.

Although the detected sequence types differ in their composition and number, they did not show any difference in their Simpson's diversity index, which was properly calculated [13] and reached 98.3% and 98.5% for ISs and NISs, respectively. On the one hand, the high values of the diversity index demonstrate the absence of a well-represented clonal structure of the studied pathogens; on the other hand, the BURST-based MLST analysis identifies two groups of genetically related strains with core sequence types ST-311 and ST-505 (if strains with minimum 2 discrepancies in the allelic profile are grouped together). Group ccST-311 includes 9 strains with sequence types ST-36, ST-42, ST-15248, ST-16095, and ST-16358, all of them belonging to serogroup 23 (serotypes A or F). Group ccST-505 includes 20 strains of serotype 3 with sequence types ST-180, ST-2049, ST-15250, and ST-15251; ISs have sequence type ST-180, while NISs have sequence type ST-505 (Table 2). Group ccST-505 corresponds to clonal group CC180 identified in the study [3] and representing one of Russia's most common clonal complexes of *S. pneumoniae*, which were isolated in 1980–2017.

The comparison of the detected sequence types with the sequence types of 81 Russian strains of *S. pneumoniae* (39 sequence types), which were isolated from meningitis patients in 2011–2015 and characterized in the subsequent study [10] revealed 12 matches, while no differences in the number of matches were found in IS and NIS samples. The core genome (cgMLST)-based analysis, which includes the calculation of a genetic distance for 1367 loci, makes it possible to specify individual groups of strains of certain sero-

types (for the most common serotypes: 3, 19F, 11A, 15BC, and 9V), while some groups contain strains belonging to several serogroups (for example, 10A and 6C). The comparison of the findings of two studies and the genetic characterization confirm the previous assumptions regarding the absence of a well-represented clonal structure of *S. pneumoniae* associated with invasive PIs in Russia [5].

The whole-genome sequencing combined with the molecular monitoring of PI pathogens provides timely acquisition of reliable data on changes in the structure of circulating *S. pneumoniae* serotypes, improving the efficiency of planning and the assessment of the effectiveness of preventive immunization measures. The genetic characterization based on whole-genome data is a powerful tool for the intraspecific classification of pathogens, which is required for the extended microbiological monitoring being a constituent part of the PI epidemiological control. The accumulation and analysis of whole-genome data will enhance the insight into basic genetic variations associated with the ability of certain representatives of *S. pneumoniae* to cause invasive PIs.

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