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ORIGINAL RESEARCHES

Characterization of *Streptococcus Pneumoniae* Strains Causing Invasive Infections Using Whole-Genome Sequencing

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Purpose: antigenic and genetic characterization of *Streptococcus pneumoniae* strains isolated from patients with invasive forms of pneumococcal infection using data of *high-throughput sequencing.*
Materials and Methods. The study was performed on 46 *S. pneumoniae* strains isolated during the *PEHASus* multicenter studies in 2015–2018. Sequencing was performed using Illumina protocols and equipment. The SPAdes, SeroBA, PneumoCaT software were used for data processing, as well as BIGSdb software (PubMLST.org).
Results and Discussion. Whole-genome sequences of strains were identified; the information was entered into the PubMLST database (id: 51080–51125). Ten (21%) strains were found to have serotype 3. Five (11%) strains belonged to serotype 19F and five to serogroup 6; two of them belonged to serotype 6A; one strain had 6B and 1 had 6BE serotype; 1 strain showed discordant result (6A or 6BE). Serotype 15B was identified in 3 (6.5%) strains. Serotypes 7F, 8, 9V, 14, 22F, 23F and 28A were identified in two strains each; serotypes 1, 4, 9N, 10C, 12F, 18C, 35F, 37 and 38 were found once. The proportion of strains with serotypes included in PCV13 and PPV23 vaccines was 65% and 80%, respectively. 36 sequence types were found in strains; out of them, 6 sequence types were found for the first time. A dominant sequence type or clone complexes could not be identified using multilocus sequence typing except for serotype 3 strains. The failure to identify clone complexes is consistent with the data of previous studies that demonstrated the absence of a pronounced clone structure of *S. pneumoniae* associated with pneumococcal meningitis in Russia.
Conclusion. The information about serotypes of *S. pneumoniae* causing invasive infections together with epidemiologic data about strain sources and vaccination allows us to evaluate the effectiveness of pneumococcal vaccines and provide information for improving the PCR-based routine serotyping.

Keywords: *Streptococcus pneumoniae; invasive pneumococcal infection; high-throughput sequencing; serotyping; multilocus sequence typing; whole genome sequencing.*

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Характеристика штаммов *Streptococcus pneumoniae*, выделенных от больных инвазивными пневмококковыми инфекциями, с использованием высокопроизводительного секвенирования

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Цель работы — характеристика антигенных и генетических свойств штаммов *Streptococcus pneumoniae*, выделенных от больных инвазивными формами пневмококковой инфекции, на основании данных высокопроизводительного секвенирования.

Материалы и методы. Исследовано 46 штаммов *S. pneumoniae*, выделенных при проведении многоцентровых исследований «ПеГАС» в течение 2015–2018 гг. Секвенирование проводилось с использованием реагентов и оборудования фирмы «Illumina». При обработке данных использовались программы «SPAdes» (Россия), «SeroBA» и «PneumoCaT», а также программные возможности PubMLST.org.

Результаты и обсуждение. Определены полногеномные последовательности штаммов, информация внесена в базу данных PubMLST (id: 51080–51125). У 10 (21%) штаммов найден серотип 3. По 5 (11%) штаммов принадлежали серотипу 19F и серогруппе 6, из которых у 2 определен серотип 6A, по 1 — 6B и 6BE, и у 1 — дискордантный результат (6A или 6BE). У 3 (6,5%) штаммов найден серотип 15B. Двукратно найдены серотипы 7F, 8, 9V, 14, 22F, 23F и 28A, однократно — 1, 4, 9N, 10C, 12F, 18C, 35F, 37 и 38. Доля штаммов с серотипами, входящими в состав PCV13, составляет 65%, и в состав PPV23 — 80%. У штаммов найдено 36 сиквенс-типов, из которых 6 — впервые. Мультилокусное секвенирование-типирование не позволяет выявить преобладающий сиквенс-тип или определить клональные комплексы, за исключением штаммов серотипа 3. Невозможность обозначить клональные комплексы согласуется с полученными ранее данными об отсутствии выраженной клональной структуры *S. pneumoniae*, ассоциированных с пневмококковыми менингитами на территории России.

Заключение. С учетом эпидемиологических данных об источниках штаммов и информации о прививочном статусе полученные результаты позволяют оценить эффективность существующих пневмококковых вакцин в отношении инвазивных форм пневмококковых инфекций и предоставляют информацию для расширения возможностей основанных на ПЦР способов серотипирования.

Ключевые слова: *Streptococcus pneumoniae; инвазивные пневмококковые инфекции; высокопроизводительное секвенирование; серотипирование; мультилокусное секвенирование-типирование.*

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

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Introduction

Streptococcus pneumoniae bacteria are the most common cause of pneumococcal infections (PI), which are divided into noninvasive and invasive [1]. The most frequently diagnosed invasive PI forms are purulent bacterial meningitis, bacteremic pneumonia and sepsis. The most frequently used methods of intraspecific characterization of *S. pneumoniae* are antigenic characterization of capsular polysaccharide — identification of serogroups or serotypes, the number of which exceeds 90, and genetic characterization using multilocus sequence typing (MLST) [2, 3]. Diagnostics of invasive PI and characterization of the corresponding viruses are not only a top-priority clinical task implying targeted therapy, but also an important component of the epidemiological surveillance, as they make it possible to estimate the contribution of particular pathogens to the overall PI incidence and to develop preventive measures, the main one being vaccination [1]. At present, in Russia the widely used vaccines are 13-valent pneumococcal conjugate vaccine (PCV13, Prevenar 13®) and a 23-valent pneumococcal polysaccharide vaccine (PPV23, PNEUMOVAX®23).

S. pneumoniae serotypes can be identified by serological methods — a quellung reaction or a latex agglutination test, using, for example, factor antisera or a Pneumotest-Latex reagent kit (Statens Serum Institut, Denmark). As nucleotide sequences of genes (cps-locus) encoding the synthesis and assembly of capsular polysaccharides are known [4], there is a possibility of identifying serogroups and serotypes using the PCR method and primers for amplification of serotype-specific targets in the *S. pneumoniae* genome. More specifically, researchers tend to use most frequently the approaches recommended by the US Centers for Disease Control and Prevention [5] for identifying 40 serotype-specific targets; the approaches are based on studies of R. Pai et al. [6]. The Central Research Institute of Epidemiology developed and uses a multiplex procedure for identifying 16 serotypes using real-time PCR (this method identifies all serotypes included in PCV13) [7]. The prospective data on the serogroup composition of pathogens can also determine the tactics of laboratory-based identification of antigenic characteristics of currently circulating etiologic agents of PI .

The microbiological monitoring of strains causing different PI forms with the help of the MLST

method is an important practical task aimed at the identification of genetic characteristics of circulating strains and timely identification of resistant pathogens or strains with increased virulent properties resulting from recombination or being imported [2]. The main advantage of MLST over other molecular and biological typing methods is that data can be integrated in a collection of databases available on the PubMLST.org website [3].

Serological and PCR-based methods can be not efficient enough to to characterize the diversity of existing etiological agents for PI, which have to continuously adapt in response to herd immunity. At the same time, the whole-genome analysis makes it possible to obtain comprehensive data on antigenic and genetic characteristics of pathogens; these data, among other things, can be used in developing and improving the existing PCR-based approaches to identification of serotypes. Thus, the **objective** of our study was to provide antigenic and genetic characterization of *S. pneumoniae* strains associated with invasive forms of PI by using the data of high-throughput sequencing.

Materials and Methods

The study was performed on 46 *S. pneumoniae* strains isolated from blood (n = 10) and cerebrospinal fluid (n = 36) of patients with invasive forms of PI; the strains were isolated during the multicenter studies PEHASus [8] in 2015–2018. The strains were transported to the central laboratory (the Institute of Antimicrobial Chemotherapy of the Smolensk State Medical University and the Ministry of Health of the Russian Federation) on a Dorset egg medium. The central laboratory conducted species-specific identification of the strains. The strains were cultured on blood agar plates (BioMedia, Russia); their identification by microbiological methods (evaluation of the colony morphology, presence of α-hemolysis, negative catalase test results, the optochin susceptibility test) was confirmed by a latex agglutination test conducted with a Slidex Pneumo-Kit (bioMerieux). For species-specific identification of strains, we also used time-of-flight mass spectrometry using reagents and equipment from Bruker Daltonics. All strains were stored at –70°C, in tubes filled with trypticase soy broth (bioMerieux) with the addition of 30% sterile glycerol (Sigma).

DNAs were isolated by using DNeasy Blood & Tissue Kits (Qiaqen). The sequencing was performed at the Department of Molecular Diagnostics and Epidemiology of the Central Research Institute of Epidemiology. The concentration of the obtained DNA samples was measured using Qubit 2.0 fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific); 40 ng of genomic DNA were used for sample preparation. The same preparation was performed using Nextera protocol (Illumina). The indexed whole genome libraries were pooled in equimolar ratios; each set of pooled libraries was purified and size-selected using SpeedBeads Magnetic Carboxylate Modified Particles (GE Healthcare). The quality of pools was checked with a High Sensitivity DNA Kit (Agilent). High-throughput sequencing performed at HiSeq 1500 instrument using HiSeq PE Rapid Cluster Kit v2 and HiSeq Rapid SBS Kit v2 kits (Illumina).

The SPAdes version 3.13 software (Russia) was used to assemble whole-genome nucleotide sequences [9]. To identify *S. pneumoniae* serotypes SeroBA [10] and PneumoCaT applications were used [11]. Alleles and sequence types were determined in accordance with the MLST scheme for *S. pneumoniae* bacteria [2]. Bioinformatic online resource PubMLST.org was used for the processing of the results of sequencing and MLST analysis [3]. By the completion of the study, the PubMLST database [12] stored the typing results for nearly 48,000 isolates, including more than 14,000 whole-genome sequences of *S. pneumoniae*, 19 of them being received from sequencing of Russian isolates associated mainly with noninvasive forms of PI.

Results

Whole-genome nucleotide sequences were determined for all strains included in this study. The detailed information about the strains and their phenotypic characteristics: serotype, antibiotic susceptibility (for 38 strains) and data about the source (year, region, age, PI form) was entered into the PubMLST database [12]; identification (id) numbers were assigned to the strains: 51080–51125. The PubMLST database also stores the information about the assessment of the quality of the assembly of whole-genome sequences (N50, L50, N90 and other parameters) of the sequenced strains.

The serotype for all studied strains was established through the whole-genome data analysis conducted by using two algorithms [10, 11].. Ten (21%) strains belonged to serotype 3. Five (11%) strains belonged to serotype 19F and 5 strains belonged to serogroup 6; 2 of them belonged to serotype 6A, two (1 and 1) belonged to 6B and 6BE, respectively; and one strain (id-51089) demonstrated the discordant result: 6A or 6BE. Three (6.5%) strains belonged to serotype 15B. Serotypes 7F, 8, 9V, 14, 22F, 23F and 28A were found twice each; serotypes 1, 4, 9N, 10C, 12F, 18C, 35F, 37 and 38 were found once.

Denomination of 7 alleles was completed for all the strains and sequence types were identified [2]. Six sequence types never described previously were identified: ST-15247–15250 (formed by allele combinations unknown before), ST-15251 and ST-15252 (have newly discovered alleles - aroE-510 and xpt-924, respectively, in the allelic profile).

Discussion

The methods used by Russian researchers until the present time for identification of *S. pneumoniae* serotypes associated with invasive PI were based on

serological tests or PCR or their combination, had limitations and could not provide full-scale characterization of all studied strains or clinical samples containing DNA of encapsulated (cps-positive) isolates. It can be explained by the limited number of antibodies or serotype-specific targets used for diagnostics and by the occurrence of false-negative results. Both factors affected the identification of the entire antigenic diversity of the pathogens that may cause PI. For example, the PCR-based method [7] used for characterization of 89 samples of cerebrospinal fluid from patients with pneumococcal meningitis obtained in Moscow in 2007–2010 made it possible to identify a serotype in 79% of cases; in the same study, the use of additional serotype-specific targets with alternative primers [5, 6] could not increase significantly the proportion of samples with identified serotype. Application of the same method for 235 strains and specimens obtained from patients with pneumococcal meningitis in 2010–2014 in Russia gave almost the same proportion of successfully identified pathogens — 76% [13]. It is approximately 10% greater than the proportion of strains, whose serotype we would have been able to identify in this study: the application of the method [7] would have resulted in the identified serotypes for 31 (67%) strains.

The distribution and relative ratio of the serotypes of circulating pathogens can vary depending on epidemiological patterns which are influenced by the application of polyvalent vaccines. Among strains characterized in this study the proportion of serotypes that are included in PCV13 and PPV23 vaccines was 65% and 80%, respectively. The decrease in the number of cases of invasive PI caused by *S. pneumoniae* serotypes containing in PCV13, which was observed in 2015–2018, could result from the greater vaccination coverage and, most likely, from the fact that pneumococcal vaccine was included in the National vaccination schedule in 2014. At the same time, although PCV13 and PPV23 vaccines contain serotypes 3, 6 and 19F, these serotypes are found more frequently than others among the characterized strains, like during the previous years [1, 13]. The distinctiveness of the studied collection of the samples is also evidenced by the relatively high proportion of strains with serotype 15B and the presence of strains with serotypes 28A, 37 and 38, which previously were not associated with pneumococcal meningitis in Russia.

Taking into account the epidemiological data on strain sources and the information about a vaccination status, the obtained results make it possible to assess efficiency of the existing pneumococcal vaccines in relation to invasive forms of PI and imply the importance of expanding capabilities of the PCR-based methods of serotyping [7] through using additional serotype-specific targets generally aimed at detection of *S. pneumoniae* serotypes 15B, 8, 22F and 12F.

Total 36 sequence types were found in the characterized collection of strains. The MLST-based test is not able to determine the dominant sequence type or to identify clonal complexes, except for strains of serotype 3, which tend to have a clonal complex combining sequence types ST-180 (5 strains), ST-505 (2 strains) and ST-2049, ST-15250, ST-15251 (each having 1 strain). The comparison of the found sequence types with the sequence types of 108 isolates obtained from patients with pneumococcal meningitis in Russia in previous studies and deposited in PubMLST [12] demonstrated that in both datasets strains with serotype 3 had sequence types ST-180 and ST-505, while the strains of other serotypes had sequence types ST-236, ST-239 and ST-1262; the other sequence types did not match the sequence types found in the previous years. The failure to identify clonal complexes in the studied collection of strains, relatively high occurrence of the newly discovered sequence types (6 out of 36) and the discrepancy between the majority of the detected sequence types and the sequence types found in the studied region in the previous years are consistent with the reported absence of a pronounced clone structure of *S. pneumoniae* associated with pneumococcal meningitis in Russia [1].

Altogether, the results of the whole-genome sequencing provide comprehensive information about antigenic and genetic characteristics of circulating *S. pneumoniae*. Further analysis of the whole-genome data should be focused on the analysis of evolutionary processes and genetic relationships between the characterized strains and strains isolated from other cases of PI, and should be based on the MLST analysis of core genome. The data should be also used for analysis of genetic factors associated with antibiotic resistance and the mechanisms of its development.

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