4
ИЮЛЬ–АВГУСТ
Том 101
2024

ЖМЭИ

100 ЛЕТ

ЖУРНАЛ МИКРОБИОЛОГИИ ЭПИДЕМИОЛОГИИ и ИММУНОБИОЛОГИИ

ISSN 2686-7613 (Online)
ISSN 0372-9311 (Print)

JOURNAL

OF MICROBIOLOGY EPIDEMIOLOGY AND IMMUNOBIOLOGY

4
JULY – AUGUST
Volume 101
2024

УЧРЕДИТЕЛИ:
ФБУН ЦНИИ ЭПИДЕМИОЛОГИИ РОСПОТРЕБНАДЗОРА
ВСЕРОССИЙСКОЕ НАУЧНО-ПРАКТИЧЕСКОЕ ОБЩЕСТВО ЭПИДЕМИОЛОГОВ, МИКРОБИОЛОГОВ И ПАРАЗИТОЛОГОВ

Журнал микробиологии, эпидемиологии и иммунобиологии

(Zhurnal mikrobiologii, èpidemiologii i immunobiologii)

Двухмесячный научно-практический журнал

Основан в 1924 г.

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В соответствии с рекомендациями ВАК (письмо ВАК от 06.12.2022 № 02-1198), журнал относится к категории К1 как издание, входящее в базы данных SCOPUS и RSCI.

4

ИЮЛЬ – АВГУСТ

ТОМ 101

2024

Журнал зарегистрирован Федеральной службой по надзору в сфере связи, информационных технологий и массовых коммуникаций.

Свидетельство ПИ № ФС77-75442

ISSN 0372-9311 (Print)
ISSN 2686-7613 (Online)

DOI prefix: 10.36233

Журнал открытого доступа, не берущий плату за публикации.

Используется сервис CrossMark для поддержания контента журнала в актуальном состоянии и информирования читателей об изменениях в опубликованных статьях.

При публикации статьи на русском и английском языках статья размещается под одним DOI. Переводы публикуются на сайте журнала.

Журнал представлен в международных базах данных и информационно-справочных системах:
RSCI; RUSMED; SCOPUS; DOAJ; Ulrich's Periodicals Directory, ROAD; EBSCO Publishing (на платформе EBSCOhost); ROAD; HYPERLINK; OPENALEX; FATCAT; ZEITSCHRIFTEN DATENBANK; CrossRef; Dimensions.

ПОДПИСКА:
ГК «Урал-пресс», индекс: 71436.
Тел.: +7(343) 262-65-43.
E-mail: info@ural-press.ru.

Полные тексты статей журнала доступны на сайтах:
https://microbiol.crie.ru;
https://www.elibrary.ru;
https://www.cyberleninka.ru;
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Тел.: +7(495) 974-96-46.
E-mail: crie@pcr.ru

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111123, Москва, ул. Новогиреевская, д. 3А.
Тел.: +7(925)011-87-79.
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Заведующая редакцией:
Устинкова Ольга Владимировна

Журнал размещает рекламу в соответствии с ФЗ РФ от 13.03.2006 № 38-ФЗ «О рекламе» и рекламной политикой.

К публикации принимаются только статьи, подготовленные в соответствии с правилами для авторов (https://microbiol.crie.ru).

Направляя статью в редакцию, авторы принимают условия договора публичной оферты (https://microbiol.crie.ru).

Подписано в печать 29.06.2024.
Формат 60×90 1/8. Тираж 158 экз.
Усл.-печ. л. 17,5.

Отпечатано в «Объединенный полиграфический комплекс».
115114, Москва, Дербеневская набережная, 7с2.
E-mail: info@opk.bz. www.opk.bz

16+

FOUNDERS:
Central Research Institute for Epidemiology
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JOURNAL of MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY

(Zhurnal mikrobiologii, èpidemiologii i immunobiologii)

Bimonthly scientific and practical journal

Founded in 1924

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1.5.11. Microbiology (medical and biological sciences);
3.2.2. Epidemiology (medical and biological sciences);
3.2.7. Clinical immunology, Allergology (medical and sciences).
In accordance with the recommendations of the Higher Attestation Commission (letter of the Higher Attestation Commission dated December 06, 2022 No. 02-1198), the Journal belongs to the K1 category, as a publication included in the SCOPUS and RSCI databases.

4

JULY–AUGUST

VOLUME 101

2024

The journal is registered by the Federal Service for Supervision of Communications, Information Technology and Mass Media. Certificate of registration PI no. FS77-75442

ISSN 0372-9311 (Print)
ISSN 2686-7613 (Online)

DOI prefix: 10.36233

The journal is an Platinum Open Access peer-reviewed scholarly journal, which does not charge author fees.

The CrossMark service is used.

Some articles are translated into English under the decision of the Editorial Board. When publishing an article in Russian and English, the translated article is placed under the same DOI on the Journal's website.

The Journal is indexed by the following abstracting and indexing services:
RSCI; RUSMED; SCOPUS; DOAJ; Ulrich's Periodicals Directory, ROAD; EBSCO Publishing (на платформе EBSCOhost); ROAD; HYPERLINK; OPENALEX; FATCAT; ZEITSCHRIFTEN DATENBANK; CrossRef; Dimensions.

Index for subscription to the printed version of the journal:

Ural Press: 71436.
Tel.: +7(343) 262-65-43.
E-mail: info@ural-press.ru.

Full texts of issues of the journal are available:
https://microbiol.crie.ru;
https://www.elibrary.ru;
https://www.cyberleninka.ru;
https://www.rucont.ru;
https://www.ebsco.com;
https://www.doaj.org;
http://www.elsevier.com

FOUNDERS:
Central Research Institute for Epidemiology
www.crie.ru

Russian Scientific and Practical Society of Epidemiologists, Microbiologists and Parasitologists
http://npoemp.ru

PUBLISHER:
Central Research Institute for Epidemiology, 111123, 3A, Novogireevskaya St., Moscow, Russian Federation.
Phone/fax: +7(495) 974-96-46.
E-mail: crie@pcr.ru

EDITORIAL OFFICE:
5A, Maly Kazenny per., Moscow, 105064, Russia.
Tel.: +7(925)011-87-79.
E-mail: jmei@crie.ru

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The Editorial Board is not responsible for the advertising content.

The materials that do not meet the requirements of the journal (https://microbiol.crie.ru) are rejected without further consideration.

When the author submits an article to the Editorial Board, he/she accepts the terms and conditions of the public offer agreement (https://microbiol.crie.ru).

Signed to the press on June 29, 2024.

Print format 60×90 1/8.

Circulation 158 copies.

Printed at the Ob'yedinennyy poligraficheskiy kompleks Ltd.115114, 7C2, Derbenevskaya emb., Moscow, Russian Federation.

E-mail: info@opk.bz. www.opk.bz

СОДЕРЖАНИЕ

ОРИГИНАЛЬНЫЕ ИССЛЕДОВАНИЯ

ОБЗОРЫ

* Статья опубликована на русском и английском языках на сайте журнала: https://www.microbiol.crie.ru.

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* The article is published in Russian and English on the journal's website: https://www.microbiol.crie.ru.

ORIGINAL RESEARCHES

Original Study Article
https://doi.org/10.36233/0372-9311-554

Genomic surveillance of SARS-CoV-2 in Russia: insights from the VGARus platform

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Abstract

Introduction. In response to the COVID-19 pandemic in the Russian Federation, comprehensive response measures were taken. One of these measures was the development of a viral genome aggregation platform (VGARus) to monitor virus variability.

The **aim** of this paper is to describe the role of the VGARus platform in tracking genetic variation in SARS-CoV-2.

Materials and methods. VGARus utilizes sequencing data and bioinformatics tools to monitor genetic variations in SARS-CoV-2. The viral genomes were aligned using NextClade, which also translated them into amino acids and identified mutations. The viral variability over time was analyzed by counting the number of amino acid changes compared to the reference sequence.

Results. The analysis of data within VGARus enabled the identification of new virus variants, contributing to improved diagnostic tests and vaccine development. The platform allowed for the prediction of epidemiologic trends, facilitating a rapid response to changes in the epidemiologic situation. For example, using VGARus, an increase in COVID-19 incidence was accurately predicted in the summer of 2022 and early 2023, which were associated with the emergence of Omicron subvariants BA.5 and XBB. Data from the platform helps validate the effectiveness of primers and DNA probes to ensure high diagnostic accuracy and reduce the risk of false negatives.

Conclusion. VGARus demonstrates the growing role of genomic surveillance in combating COVID-19 and improving preparedness for future infectious disease outbreaks. The platform is a powerful tool for generating evidence-based solutions to combat a pandemic and mitigate its health, economic and societal impacts. It provides the ability to promptly obtain information on the epidemiologic situation in a particular region of the Russian Federation, use genomic data for phylogenetic analysis, compare the mutational spectrum of SARS-CoV-2 sequences with foreign samples. VGARus data allow for both retrospective analysis and predictive hypotheses. For example, we can clearly see the dynamics of the change of different virus variants: sequences belonging to the Alpha, Beta, Delta, Omicron lineages and many less common ones, clearly form the upsurges of morbidity, the interaction of which is reflected in the epidemiological picture. It is also currently being expanded to monitor other pathogens, increasing its public health relevance.

Keywords: *genomic epidemiology, molecular epidemiology, SARS-CoV-2, next generation sequencing, VGARus platform, genomic surveillance*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Central Research Institute for Epidemiology (protocol No. 111, December 22, 2020).

Acknowledgement. We express our sincere gratitude to everyone who took part in the creation, maintenance and filling of the VGARus platform with genomic data.

Funding source. The sequencing and data analysis efforts at the Institute were supported by funding from the "Sanitary Shield" federal project, subsidies provided under an order from the Russian Federation Government, and the Institute's internal financial resources.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4): ONLINE FIRST
DOI: https://doi.org/10.36233/0372-9311-554
ORIGINAL RESEARCHES

For citation: Kotov I.A., Agletdinov M.R., Roev G.V., Pimkina E.V., Nadtoka M.I., Peresadina A.V., Bukharina A.Yu., Svetlichny D.V., Goncharov S.E., Vykhodtseva A.V., Borisova N.I., Lysenkov V.G., Chanyshev M.D., Agabalaev D.N., Saenko V.V., Cherkashina A.S., Semenenko T.A., Dubodelov D.V., Khafizov K.F., Akimkin V.G. Genomic surveillance of SARS-CoV-2 in Russia: insights from the VGARus platform. *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):435–447.
DOI: https://doi.org/10.36233/0372-9311-554
EDN: https://www.elibrary.ru/irjxcx

Оригинальное исследование
https://doi.org/10.36233/0372-9311-554

Геномный надзор за SARS-CoV-2 в Российской Федерации: возможности платформы VGARus

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Аннотация

Введение. В ответ на пандемию COVID-19 в России были приняты комплексные меры реагирования. Одной из них стала разработка платформы агрегации вирусных геномов (VGARus) для мониторинга изменчивости вируса.

Цель работы — описать роль VGARus в отслеживании генетических изменений SARS-CoV-2.

Материалы и методы. Выравнивание вирусных геномов, последующую трансляцию в аминокислоты и поиск мутаций производили с помощью программы NextClade. С целью анализа геномной изменчивости подсчитывали число аминокислотных изменений относительно референсной последовательности.

Результаты. Анализ данных VGARus позволил идентифицировать новые варианты вируса, что способствовало улучшению диагностических тестов и может помочь в разработке вакцин. Платформа предоставила возможность прогнозировать эпидемиологические тенденции и оперативно реагировать на изменения эпидемиологической ситуации. Например, с использованием VGARus был точно предсказан рост заболеваемости COVID-19 летом 2022 г. и в начале 2023 г., связанный с появлением субвариантов Omicron BA.5 и XBB. Данные платформы помогают проверять эффективность праймеров и ДНК-зондов, что обеспечивает высокую точность диагностики и снижает риск ложноотрицательных результатов.

Заключение. VGARus демонстрирует растущую роль геномного эпиднадзора в борьбе с COVID-19 и повышение готовности к будущим вспышкам инфекционных заболеваний. Платформа является мощным инструментом для формирования научно обоснованных решений по борьбе с пандемией и смягчению её последствий для здоровья населения, экономики и общества. Она предоставляет возможность оперативно получать информацию об эпидемиологической обстановке в конкретном регионе России, использовать геномные данные для проведения филогенетического анализа, сравнивать мутационный спектр последовательностей SARS-CoV-2 с зарубежными образцами. Данные VGARus позволяют проводить ретроспективный анализ и выдвигать гипотезы прогностического характера. Так, явно можно увидеть динамику смены различных вариантов вируса: последовательности, принадлежащие линиям Alpha, Beta, Delta, Omicron и многим менее распространённым, отчётливо формируют подъёмы заболеваемости, которые отражаются на эпидемиологической ситуации. В данный момент платформа расширяется для мониторинга изменчивости других патогенов, что увеличивает её значимость для общественного здравоохранения.

Ключевые слова: *геномная эпидемиология, молекулярная эпидемиология, SARS-CoV-2, секвенирование следующего поколения, платформа VGARus, геномный надзор*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Этическим комитетом ЦНИИ Эпидемиологии (протокол № 111 от 22.12.2020).

Благодарность. Мы выражаем искреннюю благодарность всем, кто принимал участие в создании, обслуживании и наполнении геномными данными базы VGARus.

Источник финансирования. Работы по секвенированию и анализу данных в ЦНИИ Эпидемиологии Роспотребнадзора проводились при финансовой поддержке федерального проекта «Санитарный щит», субсидий, выделенных по распоряжению Правительства РФ, а также за счёт внутренних финансовых ресурсов ЦНИИ Эпидемиологии.

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

Для цитирования: Котов И.А., Аглетдинов М.Р., Роев Г.В., Пимкина Е.В., Надтока М.И., Пересадина А.В., Бухарина А.Ю., Светличный Д.В., Гончаров С.Е., Выходцева А.В., Борисова Н.И., Лысенков В.Г., Чанышев М.Д., Агабалаев Д.Н., Саенко В.В., Черкашина А.С., Семененко Т.А., Дубоделов Д.В., Хафизов К.Ф., Акимкин В.Г. Геномный надзор за SARS-CoV-2 в Российской Федерации: возможности платформы VGARus. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):435–447.
DOI: https://doi.org/10.36233/0372-9311-554
EDN: https://www.elibrary.ru/irjxcx

Introduction

The advent of high-throughput sequencing technologies, also known as next-generation sequencing (NGS), has led to a significant reduction in the cost of genomic sequencing experiments over the past 15 years. NGS is increasingly utilized across various biological and medical fields, including virology, where its application in studying viral genomes has become widespread [1–5]. Furthermore, contemporary bioinformatics tools have expanded the capabilities for the development and analysis of databases containing the genomes of pathogens responsible for various infectious diseases [6–8]. Genomic epidemiology has emerged as a crucial component in epidemic control. It enables the examination of genetic alterations in the genomes of pathogens, the identification and classification of distinct lineages, and the evaluation of their pathogenic potential and transmissibility [9–15]. These studies are very important for the development of new diagnostic kits, the creation of modern and effective vaccines, the formulation of optimal epidemic response strategies, and the prediction of disease incidence.

A striking example of the application of molecular genetic monitoring is the detailed study of a new coronavirus infection during the COVID-19 pandemic [16]. By analyzing the genomes of SARS-CoV-2, associations were established between different virus variants and the characteristics of the course of the epidemic. This approach allows for accurate monitoring, understanding the relationship between genetic variants and their ability to cause disease, and implementing targeted measures to prevent the spread of infection.

At the onset of the COVID-19 pandemic, Professor Edward Holmes from the University of Sydney, representing a research team led by Yong-Zheng Zhang from Fudan University in Shanghai, published the nucleotide sequence of the SARS-CoV-2 genome. This information was posted on the Virological.org platform[1], which allowed the international scientific community to begin taking immediate action to counter the spread of the pathogen, among which were the development of new diagnostic tests and subsequent vaccine development [17, 18]. As the pandemic progressed, countries that had typically relied less on their own genomic data began to conduct extensive sequencing experiments. The knowledge gained was used to develop strategic plans to contain the spread of infection [19]. The widespread use of SARS-CoV-2 genome sequencing led to a significant increase in the number of new sequences uploaded to international databases. The well-known database is GISAID (https://www.gisaid.org) with more than 16 million sequences from more than 200 countries [8].

The aim of the study is to determine the role of the VGARus platform and its data for analyzing genomic sequences of SARS-CoV-2 virus collected in Russia.

Materials and methods

Before starting this study, informed consent was obtained from patients, and the protocol was approved by the ethical committee of the Central Research Institute of Epidemiology (protocol No. 111 of 22.12.2020). Biological material was obtained by taking nasopharyngeal swabs from patients with COVID-19 symptoms. The samples were collected from different regions of Russia, with most of them coming from Moscow and the Moscow region. The presence of SARS-CoV-2 RNA was confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR). RIBO-prep kit (AmpliSense, Russia) was used for RNA isolation, and REVERTA-L reagent kit (AmpliSense, Russia) was used for reverse transcription.

High-throughput sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v2 (PE 150 + 150 or PE 250 + 250 cycles) or MiSeq Reagent Kit v3 (PE 300 + 300 cycles), Illumina NextSeq 2000 using NextSeq 1000/2000 P2 reagents v3 (300 cycles), MinION using Midnight Kit (Oxford Nanopore Technologies Oxford, UK), DNBSEQ-G50 using ATOPlex RNA Library Prep Set (MGI Tech, Shenzhen, China). The Sanger method was used to sequencing fragments of the spike protein gene, but this information was barely utilized in the analysis. In addition, nucleotide sequence data from the GISAID database were used in case of their geographi-

[1] Novel 2019 Coronavirus Genome.
URL: https://virological.org/t/novel-2019-coronavirus-genome/319

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cal affiliation to Russia. The program "Pangolin" [19], as well as internal tools and scripts, were used to classify different variants of SARS-CoV-2.

In total, more than 82,000 complete SARS-CoV-2 genomes with the date of biomaterial collection from 01.01.2020 to 31.12.2023 were used. Only genomic sequences that met the specified quality criteria were selected. The selected genomes were aligned to the reference sequence NC_045512.2 using the NextClade tool and then translated into amino acid sequences. A specialized script written in Python was used to count the number of amino acid changes compared to the reference.

Results

Development and creation of the Russian viral genome aggregation platform VGARus

In 2021, the VGARus platform (Virus Genome Aggregator of Russia; registration date 06.07.2023, No. 2023622263) was developed and established at the Central Research Institute of Epidemiology (CRIE) of Rospotrebnadzor in accordance with the decree of the Government of the Russian Federation. The key tasks of this platform are collection of data on viral genomes, centralized analysis of genetic diversity and temporal dynamics of identified SARS-CoV-2 variants in Russia. A scientific consortium was established, comprising institutions from Rospotrebnadzor, the Ministry of Health of the Russian Federation, various scientific institutes, and other organizations. Currently, more than 150 organizations are members of the consortium, many of which are actively conducting extensive genomic sequencing of SARS-CoV-2 and uploading the obtained sequences to the VGARus database for further analysis. In addition, the Republic of Armenia and the Republic of Belarus are participating in the project, which makes it possible to track pathogen variability in neighboring countries with active transport connections.

The process of monitoring viral genome variability involves the following steps (**Figure 1**):

- the sequencing laboratory receives biological material from diagnostic laboratories, including those affiliated with hospitals. The quality of these samples is preliminarily assessed, typically through PCR analysis, to determine viral load and assess the sample's suitability for next-generation sequencing (NGS);
- the laboratory that provided the biological material must enter the relevant metadata into the VGARus platform (information on sex, age, vaccination status of the patient, date of collection of the biological material, region of collection, etc.);
- a specialized sequencing laboratory conducts the essential sample preparation followed by the sequencing of viral genomes;
- primary bioinformatics analysis is performed, which includes data quality control, genome assembly (usually by alignment to a reference genome) and sequence validity check (assessment of genome coverage);
- uploaded genomic information is validated and processed automatically using the "Pangolin" program for complete genomes [19, 20] and the "V-TRACE" program (developed by CRIE) for fragment sequencing results. Samples with genomes that fail quality control are marked as invalid on the platform, and a corresponding notification is sent to the originating laboratory.

All SARS-CoV-2 genomic sequences in the country, obtained through routine epidemiologic monitoring, are registered in the VGARus database. The system supports both manual uploading and uploading via specialized APIs, facilitating the addition of large volumes of sequences. Each sample entry in the system includes not only the nucleotide sequence but also associated technical data. Upon registration in the database, the sample is automatically assigned an internal identifier, and the SARS-CoV-2 genome sequence is appended to the sample information field. The technical information encompasses data on the organizations involved in sample collection and laboratory processing, the dates of sample receipt, registration in the system, and sequence upload.

The S-protein SARS-CoV-2 plays a key role in virus variant identification. This is due to its role in vi-

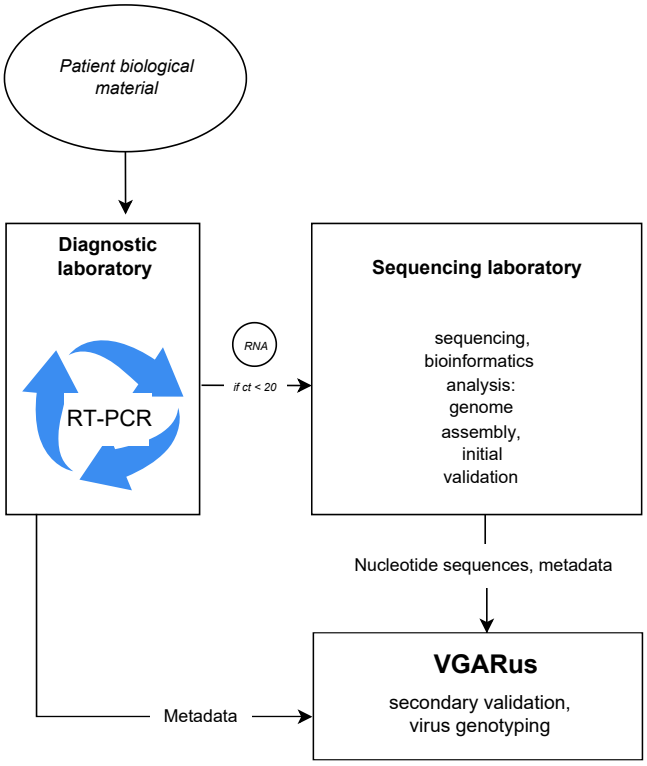

Fig. 1. The stages of the process of monitoring the variability of viral genomes.

rion entry into host cells and high mutation frequency/variability of the sequence [21]. However, attempting to establish a virus variant solely on the basis of mutations in the S-protein gene may lead to incomplete or inconsistent results [22]. Given the complexity of viral evolution, in which individual mutations may affect virus functions differently and interact in ways that may alter the overall effect, bioinformaticians at the Central Research Institute for Virus Evolution have developed the V-TRACE algorithm to address this problem. The algorithm identifies mutations in the S-protein gene of SARS-CoV-2, after which a plausibility measure of whether the sequence under study belongs to different virus lineages is estimated.

Temporal dynamics and evolutionary trajectories of SARS-CoV-2 variants

The VGARus platform is a valuable resource for tracking the dynamics of the COVID-19 pandemic in Russia and studying its peculiarities. In particular, systematically collected sequence information allows us to study the genomic diversity of the virus.

In 2020, a substantial diversity of SARS-CoV-2 lineages was observed [23]. These lineages did not exhibit significant advantages over one another, resulting in none of the variants becoming dominant. In December 2020, about a year after the new coronavirus began to spread worldwide, the UK authorities informed the World Health Organization of the discovery of a new SARS-CoV-2 lineage, named VOC-202012/01. It had numerous mutations in its genome and was originally named "British" but was later renamed Alpha to avoid naming variants by country. Among the mutations found in the S-protein gene, the most important were N501Y, P681H and Δ69-70 [24, 25]. These mutations affected the ability of the virus to infect cells and evade the host immune response and, as a consequence, allowed it to spread more efficiently. This variant was detected in Russia in late 2020 and persisted into early 2021, coinciding with a sharp increase in the number of cases.

The Beta variant was identified shortly thereafter, but it had a much lower prevalence than Alpha. In the spring of 2021, the Delta variant appeared and quickly became dominant, leading to a significant increase in the incidence and hospitalization rate [26]. After a period of relatively favorable epidemiological conditions, the Omicron variant appeared in December 2021 (**Figure 2**), which led to a marked increase in the number of cases in Russia. However, the incidence of the disease declined just as quickly.

Despite a period of low numbers of COVID-19 cases in the spring of 2022, the emergence of Omicron subvariants BA.4 and BA.5 caused an increase in incidence that lasted until the end of October (**Figure 3**). In late 2022 and early 2023, highly contagious variants such as BQ.1* emerged. Such shifts in dominant lineages well illustrate the ever-changing and complex nature of SARS-CoV-2 evolution. Notably, in early 2023, modified versions of pre-existing lineages returned to the virus population, notably Omicron BA.2, presented as recombinant forms of XBB*. In November 2023, a variant of coronavirus BA.2.86, unofficially named Pirola, began to spread rapidly in several countries, including Russia. It was notable for the large number of accumulated changes in the genome compared to earlier lineages and by the end of 2023 had become the predominant virus lineage, and in early 2024 its JN.1 sublineage was almost completely dominant in most countries of the world.

The pathogen variability described above underscores the importance of ongoing epidemiologic monitoring and sequencing of virus genomes for the timely detection of new variants or changes in viral population structure. Rapid identification of such changes can help in the development of public health strategies and in controlling the spread of these variants.

Comparative analysis of Figure 2 and Figure 3 reveals a trend wherein each new significant virus lineage becomes dominant once it reaches a threshold of 50% of the total population size, typically within 1.5 to 3.0 months. Additionally, the period during which an individual lineage remains dominant ranges from 3 months to 1 year.

We then attempted to explain the dynamics of COVID-19 incidence in Russia and to explore the possibility of predicting the rate of spread of a particular virus sublineage on the basis of SARS-CoV-2 sequence data. Our main hypothesis is that specific mutations in the virus genome significantly affect the incidence rate. However, the reported incidence rate undoubtedly depends on other critical factors such as population immunization rates, PCR testing coverage, and seasonal factors, whose exact contribution is difficult to estimate. Therefore, these factors were not used for the analysis.

The period from May 2020 to December 2023 was divided into 21-day intervals. The time period studied was limited to December 2023, allowing for a detailed examination of trends at that time. However, the subsequent emergence of the BA.2.86 variant has shown that predicting future trends can be extremely difficult. Given the key role that missense mutations play in viral transmission rates, nucleotide sequences were aligned to the reference genome and translated into amino acid sequences using NextClade [27]. The number of amino acid changes compared to the reference sequence was chosen as a metric of viral variability. For example, a rapid increase in the number of frequently occurring changes may indicate an active mutational process or the importation of a new lineage into the study region. The higher the mutational activity of a virus, the more likely it is that some subset of acquired mutations can affect the properties of the virus, such as its transmissibility.

The amino acid changes compared to the reference sequence from May 2020 to December 2023 are

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-554

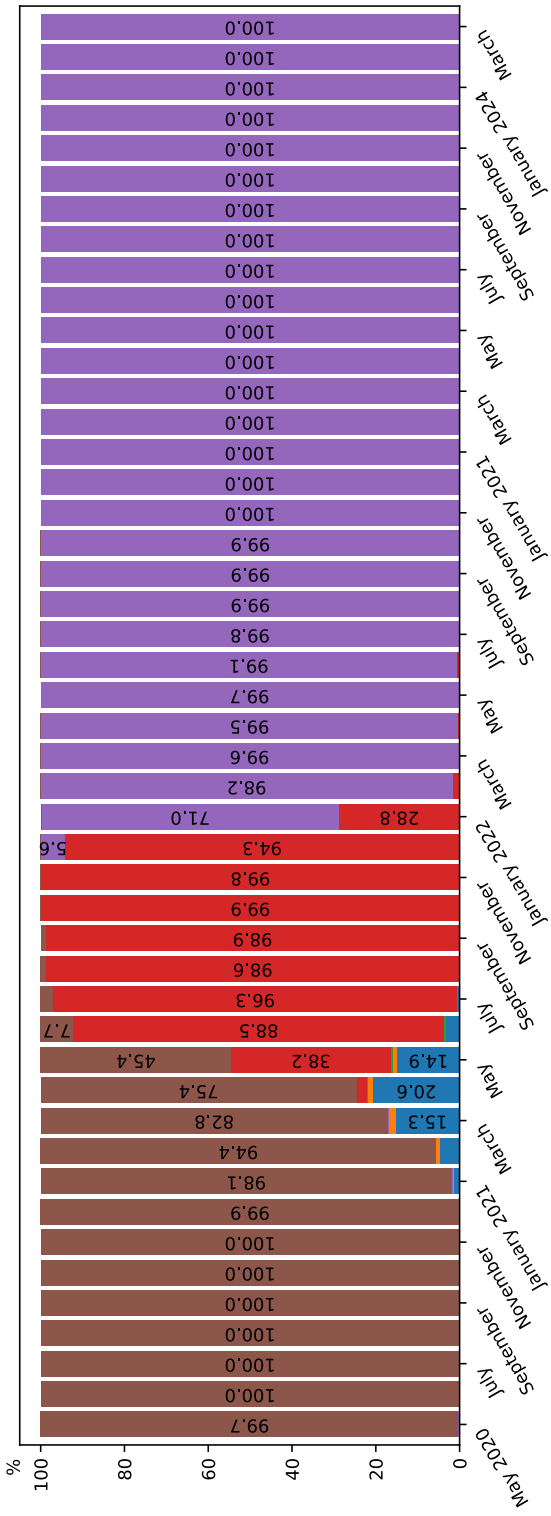

Fig. 2. A diagram illustrating the occurrence of significant SARS-CoV-2 variants of concern in the Russian Federation throughout 2020 until the March of 2024.

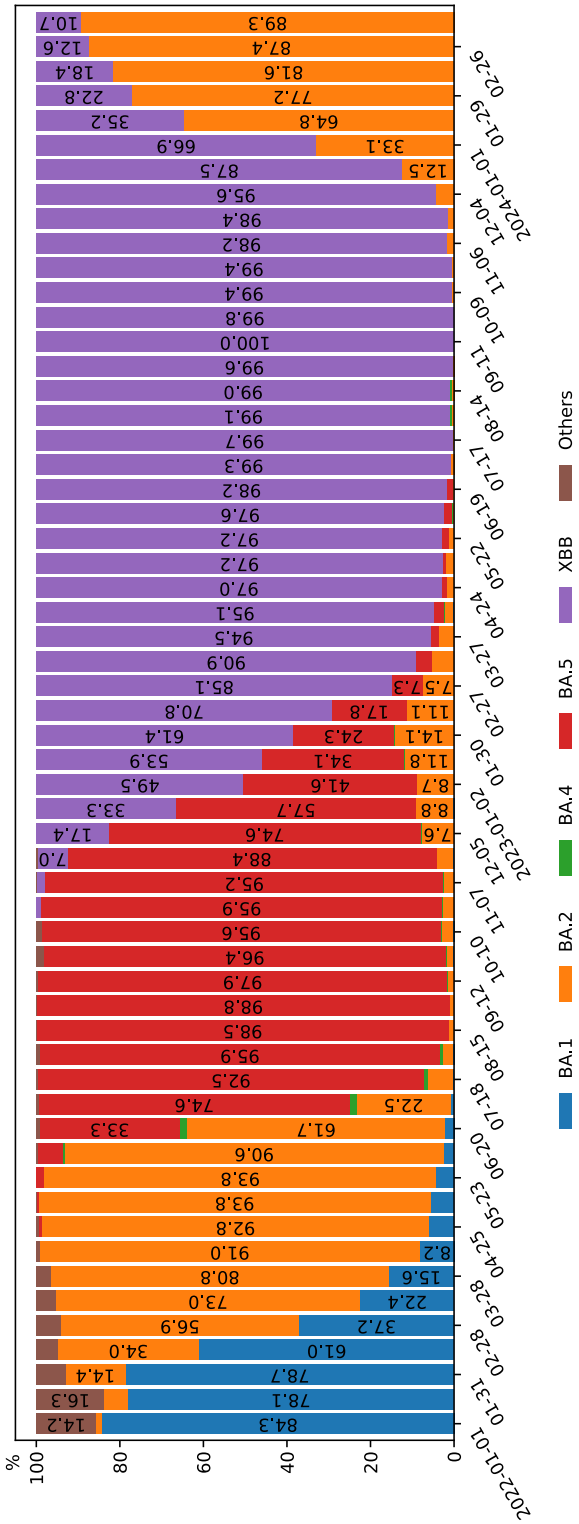

Fig. 3. Chart showing the frequency of occurrence of various Omicron sublineages from early 2022 to March of 2024.
The rise in the prevalence of the BA.2 sublineage by the end of 2023 is attributed to the BA.2.86 variant.

represented by the black line in **Figure 4**. For each interval, only mutations with a frequency of at least 50% are plotted. This approach enables the evaluation of mutations that significantly affect the adaptability of a viral variant or are inherited alongside such mutations. The graph clearly highlights three distinct intervals during which the number of frequent mutations in the SARS-CoV-2 genome increased. These intervals correspond to June 2021, February 2022, and January 2023, aligning with the widespread distribution of the Delta, Omicron, and XBB variants in Russia, respectively.

One potential limitation of the method described above is that it cannot effectively reflect the dynamics of change when new and old dominant lineages have different characteristic mutations, but their absolute number differs only slightly. A simple counting of mutations in such a case will lead to the erroneous conclusion that genetic evolution does not occur. To solve this problem, the qualitative composition of mutations was analyzed.

Sets of amino acid changes with a frequency greater than 50% were considered as separate sets for each time interval. Differences between them for adjacent time intervals were evaluated and used to measure the genetic variability of the virus. In this case, both the appearance and disappearance of a mutation with a frequency of at least 50% compared to the previous period was considered a change (Figures 2, 4). This auxiliary strategy demonstrated its reliability. Thanks to its application, we observed qualitative changes in the set of frequent mutations in the population caused by the transition from lineage BA.1 to BA.2. At the same time, fluctuations in the absolute number of common mutations were minimal. Both data sets described above are consistent with the World Health Organization data on the incidence of SARS-CoV-2 in Russia (Figures 3, 4).

Increases in disease incidence are often preceded by significant changes in the pathogen genome, as was the case in the summer of 2021 with the appearance of the Delta variant, in December 2021 with Omicron (BA.1/BA.2), in July 2022 with Omicron (BA.5), and in early 2023 with XBB (**Figure 5**). However, seasonal factors also play an important role.

We investigated the dynamics of mutation frequencies in the SARS-CoV-2 genome in Russia (**Figure 5**). It was noted that during the spread of the new dominant lineage at the above-mentioned time points, the frequency of mutations characteristic of them demonstrated a rapid S-shaped growth. When the new lineage began to dominate in the country, the frequency of mutations characteristic of the replaced lineage decreased along a similar S-shaped trajectory. In addition to the general picture, local trends can be observed on the mutation frequency distribution graph.

The frequency distribution of mutations also reveals two distinct trends on the right side of the graph. A more precise analysis of the mutations and sequences comprising these trends shows that both groups of lineages include XBB.1.9.1, FL.24, FL.1.5.1, XBB.1.16, XBB.1.16.11, and XBB.1.16.17. The upper trend, situated in the high-frequency range (70–90%) and indicated by the green dashed line, is formed by mutations common to all these lineages, such as S:G252V and ORF1b:S959P. Meanwhile, in the low-frequency range, the trend indicated by the dashed line is formed by mutations found in subgroups of the same lineages, such as S:E180V, which is present only in XBB.1.16, XBB.1.16.11, and XBB.1.16.17, and ORF1a:G1819S, found in the remaining lineages.

Studying the spreading dynamics of these lineages with time can potentially help predict the evolution of SARS-CoV-2. For this reason, the frequency dynamics for several of the discussed sublineages from June

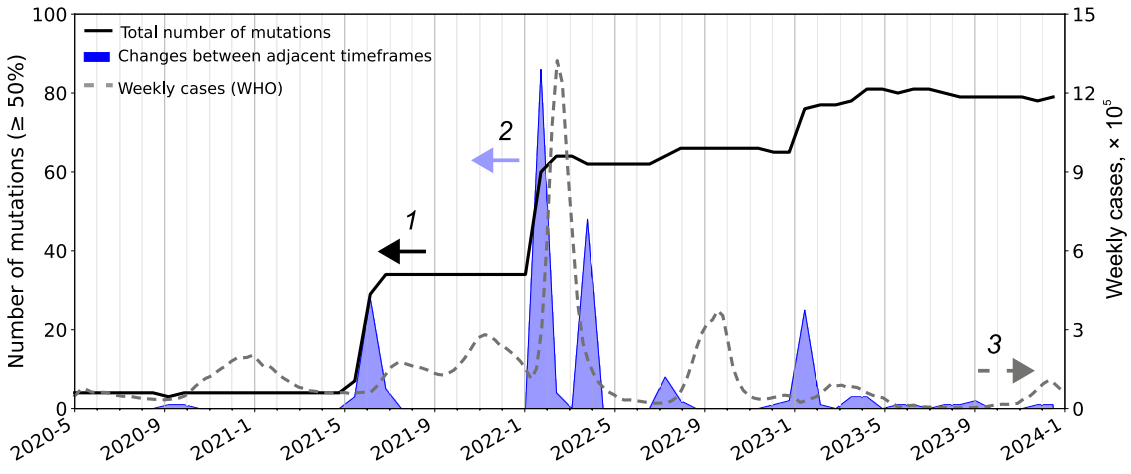

Fig. 4. Changes in amino acid sequences compared to the reference sequence from May 2020 to December 2023.

1 — number of amino acid substitutions relative to the reference variant (NC_045512.2) with a frequency of 50% or higher;
2 — number of changes in the set of mutations with a frequency of at least 50% compared to the previous period;
3 — temporal dynamics of COVID-19 cases in Russia according to WHO data.

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
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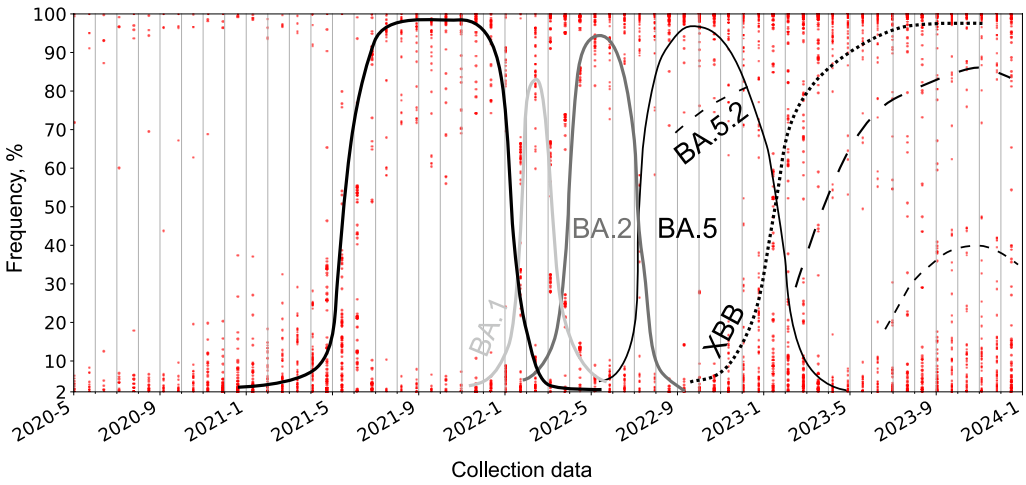

Fig. 5. Dynamics of the frequency distribution of amino acid mutations.

The lines indicate trends associated with changes in the dominant virus lineage. Each point represents a mutation plotted on the timeline, with its position indicating the frequency of occurrence at the time of sample collection. The major lineages leading to changes in mutation frequencies are shown. Local trends are highlighted with dashed lines.

through December 2023 are presented in **Figure 6**. Lineages XBB.1.9.1 and XBB.1.16 were intentionally excluded from this analysis due to the decreasing or static nature of their frequency trend during the period under consideration. The former line significantly decreased its prevalence to almost zero, while the latter line did not change its frequency, remaining between 6–16%. Consequently, they are not considered potentially future dominant lineages.

However, after this period, the BA.2.86* ("Pirola") lineage became dominant shortly after XBB, which could not have been predicted from data obtained by mid-December 2023, when this lineage was found in only a few samples. **Figure 6** also shows that most of the lineages previously considered potentially dominant, although becoming more common, reach a frequency of only about 16% in a few months. Meanwhile, earlier observations in this study suggest that a dominant line typically reaches 50% frequency within 1.5–3.0 months of emergence.

These data suggest that a future potentially dominant lineage must possess a certain minimum spreading rate; otherwise, it is likely to be displaced by others. This hypothesis aligns with the periodic nature of lineage changes. Additionally, such events complicate the prediction of the pandemic's trajectory, particularly in determining the dominant lineage in the near future and its impact on public health. Finally, the rapid emergence and spread of an entirely new variant can render all previous predictions irrelevant.

Data on the distribution of mutations in the genome were also obtained. **Figure 7** highlights mutations that reached a frequency of at least 50% at the considered time points. Many of these mutations were found in the S-protein gene, which aligns with the findings of other research groups [28]. It is notable that each new dominant lineage introduces progressively fewer new mutations in this gene. While it is challenging to interpret this observation unambiguously at present, it may suggest the degree of relatedness between the lineages

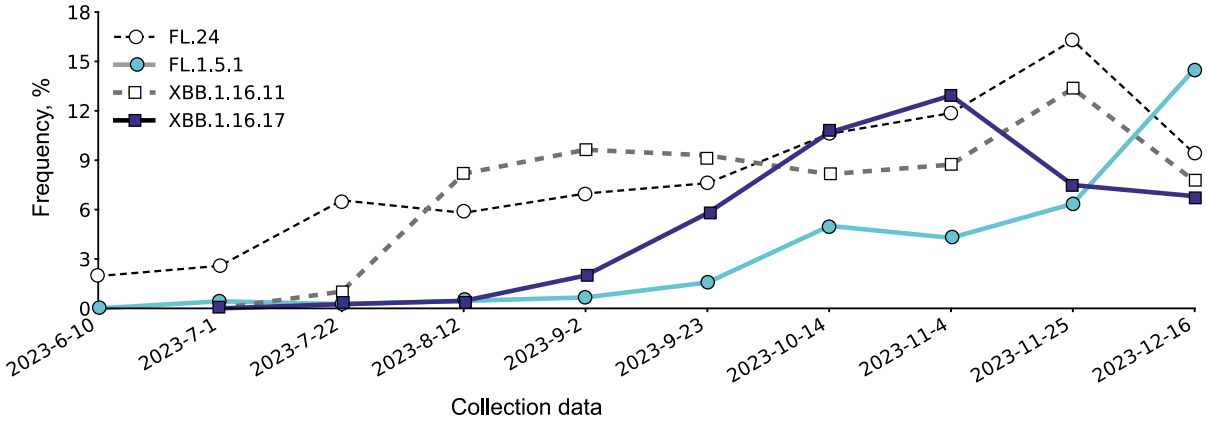

Fig. 6. Prevalence of SARS-CoV-2 from June 2023 through mid-December 2023.

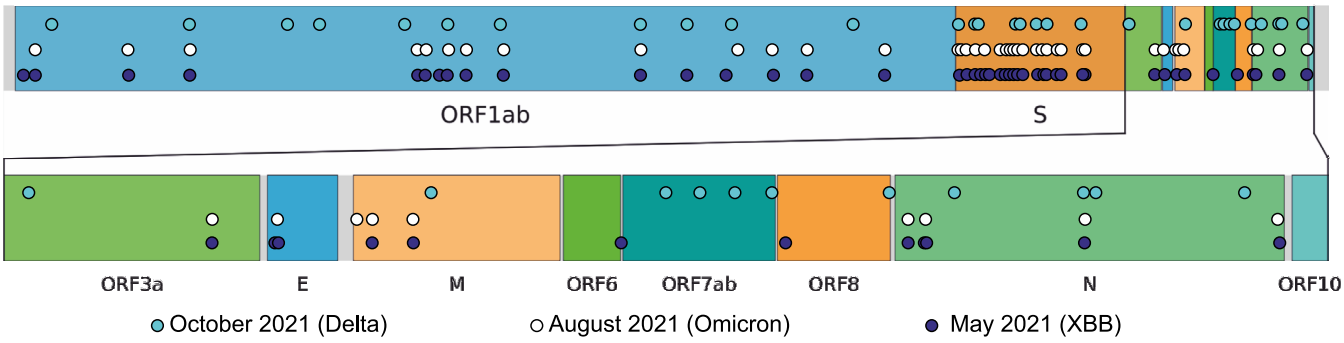

Fig. 7. Distribution of mutations across the SARS-CoV-2 genome presented in at least 50% of the samples for October 2021, August 2022, and May 2023.
For each timeframe dominant strain is presented.

or that the gene is approaching its optimal structure for maximizing affinity to the human ACE2 receptor.

Discussion

The development of the Russian viral genome aggregation platform VGARus has become an important aspect in the fight against the COVID-19 pandemic in the country. This database contains over 320,000 SARS-CoV-2 genome sequences, including approximately 200,000 complete genomes. VGARus supports many essential functions, such as the identification of novel virus variants, the creation of effective diagnostic tools, and the formulation of public health policies [12–15].

VGARus has significantly contributed to the understanding of the spatial and temporal dynamics of the COVID-19 pandemic. By providing detailed information on the time and location of each genomic sample, the platform allows visualizing the distribution of specific viral variants in Russia and their evolution over time. This detailed knowledge provides an important advantage in predicting epidemiologic trends in the coming months. For example, using VGARus, we accurately predicted an increase in COVID-19 incidence in the summer of 2022 and early 2023, associated with the emergence of Omicron BA.5 and XBB subvariants, respectively. These predictive capabilities enable public health authorities to respond rapidly to changing epidemiologic situations.

In general, the results presented above on the frequency of occurrence of various SARS-CoV-2 variants in Russia are consistent with the data of other studies in different countries. For example, B. Xiao et al. used data from the GISAID platform [29]. They considered sequences obtained in the period from January 2020 to May 20, 2023 in the USA, UK, India, South Africa, Brazil, and Russia. During this period, the GISAID platform collected about 79,000 genomes from Russia.

The overall picture of the evolution and spread of the virus in different countries is similar, but there are significant regional differences. For example, in the USA and the UK, the peak proportion of the Alpha variant was about 65% and 100%, respectively. At the same time, in Russia, the estimate of its peak prevalence was about 40%, according to the foreign study, and 20% in the current work. This difference can be explained by a wider geography of our study than the one based only on GISAID data.

The Delta variant arrived in Russia in approximately the same period as in the other countries studied — April–May 2021. Exceptions included India, where this variant appeared in March 2021, and Brazil, where the spread of the strain began only in July 2021. As in most of the other countries studied, the Gamma variant was practically undetectable in Russia, while it was dominant in Brazil and was registered in the USA with a peak frequency of about 10%.

The frequency dynamics of the BA.1 and BA.2 sublineages were nearly identical in Russia and all the countries studied, except for India. In India, BA.1 did not become dominant, remaining below 40%, while BA.2 spread earlier and remained dominant for almost 8 months. A unique characteristic of Russia and India was the almost complete absence of BA.4, which was found in other countries with peak frequencies ranging from 20% to 60%.

The presented comparison demonstrates that individual virus variants may begin their spread in different regions of the planet with a difference of several months, and the time of their maximum prevalence may vary considerably. The data on the frequency dynamics of SARS-CoV-2 variants are consistent with the findings on the timing of their spread and dominance made in this study.

In this study, we also demonstrated the main capabilities of the VGARus platform, presented the dynamics of variant diversity and mutations of SARS-CoV-2 in Russia, and examined in detail the scenario preceding the change of the dominant lineage in December 2023. The results of this part of the study revealed certain limitations in predicting the dominant lineage in the near future. These limitations, caused by the ra-

DOI: https://doi.org/10.36233/0372-9311-554

pid emergence of new lineages subsequently becoming dominant, represent a significant challenge for existing prediction tools [30, 31]. Nevertheless, we remain optimistic that future approaches will effectively address this factor. This work has, however, highlighted some general trends in the course of a pandemic, with the periodic nature of the change in the dominant variant being particularly crucial.

In addition to its role in tracking the course of epidemics, VGARus has practical implications, such as the development and assessment of diagnostic tests. For example, researchers at the Central Research Institute of Epidemiology regularly use VGARus data to test the effectiveness of primers and DNA probes used in test kits. Thus, the platform provides information for the development and processing of oligonucleotides for diagnostic test systems, information about the variants circulating in the country, about the variability of the pathogen sequence in the annealing site of primers and probes [32].

The capabilities of the VGARus platform have now been extended beyond SARS-CoV-2 research. The platform is currently being expanded to include data on additional pathogens, such as the viruses causing hepatitis, influenza, varicella, measles, and others. This multi-pathogen functionality will serve as a valuable tool for virologists and infectious disease specialists, enabling enhanced monitoring of the spread of various diseases and facilitating timely public health interventions.

Overall, VGARus is a significant achievement of the joint efforts of numerous scientific institutes of Rospotrebnadzor and other agencies [12]. Its implementation has expanded our understanding of SARS-CoV-2 and contributed to the study and control of the COVID-19 pandemic. VGARus emphasizes the critical importance of epidemiological monitoring in controlling infectious disease outbreaks and the significance of collaborative efforts in addressing global health crises.

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The article was submitted 24.06.2024;
accepted for publication 10.08.2024;

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Статья поступила в редакцию 24.06.2024;
принята к публикации 10.08.2024;
опубликована 29.08.2024

Original Study Article
https://doi.org/10.36233/0372-9311-525

Anthrax in the Russian Federation in 2023 or in other words, «the same old story»

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Abstract

Introduction. The current anthrax situation in Russia is characterized by instability. In 2023, there was an increase in the number of infection outbreaks compared to the long-term average (for five years).

The aim of the study is to assess the epizootological and epidemiological situation regarding anthrax in the Russian Federation in 2023 and the reasons for its deterioration, and to analyze data from genomic epidemiological surveillance of this infection.

Materials and methods. The information of the territorial bodies of Rospotrebnadzor on the investigation of anthrax outbreaks, reference materials about anthrax stationary hazardous areas and anthrax burials were used. The phylogenetic position of the identified *Bacíllus anthracis* strains and genomes structure were determined based on whole-genome sequencing data.

Results. In 2023 anthrax outbreaks were registered in the Chuvash Republic — Chuvashia (1), the Tyva Republic (1), Tambov (1), Ryazan (1) and Voronezh (3) regions. 14 farm animals and 19 people fell ill. The infection of animals not vaccinated against anthrax, as well as vaccinated long before contact with the source of infection, occurred mainly during grazing in the territories of old (unregistered) anthrax soil foci. Human disease is caused by contact with sick animals during care, forced slaughter, cutting, transportation of carcasses and meat, cooking processing of contaminated meat and offal, and consumption of insufficiently heat-treated liver. 17 patients were diagnosed with a cutaneous form of anthrax, while 2 had an oropharyngeal form combined with a cutaneous form of the disease.

In all cases, the genome structure typical of the *B. anthracis* species has been established. The phylogenetic relationship of *B. anthracis* isolates with *B. anthracis* strains previously isolated in Russia is shown.

Conclusion. The reason for the trouble in anthrax in 2023 was a number of violations of veterinary and sanitary-epidemiological regulations against the background of the presence of soil foci of infection. Stabilization of the situation can be achieved only in full range of regulated preventive measures are constantly implementated. The results of molecular genetic typing of B. anthracis strains isolated during the epidemiologic investigation of seven anthrax outbreaks in the Russian Federation in 2023 allow us to conclude that they are of local origin and have a genome structure typical of the species. Genetic analysis of the isolated strains demonstrated the effectiveness of the developed wgSNP typing system in the epidemiologic investigation of outbreaks.

Keywords: anthrax, soil focus, outbreak, *Bacillus anthracis*, whole genome sequencing

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Local Ethics Committee of the Stavropol Research Anti-Plague Institute (protocol No. 109, May 19, 2022).

Funding source. This study was not supported by any external sources of funding.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Kulichenko A.N., Ryazanova A.G., Logvin F.V., Eremenko E.I., Aksenova L.Yu., Pisarenko S.V., Semenova O.V., Gerasimenko D.K., Kovalev D.A., Golovinskaya T.M., Bobrysheva O.V., Pechkovskii G.A., Oleynikova K.A., Nikitina A.V. Anthrax in the Russian Federation in 2023 or in other words, «the same old story». *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):448–461.
DOI: https://doi.org/10.36233/0372-9311-525
EDN: https://www.elibrary.ru/anetvp

Оригинальное исследование
https://doi.org/10.36233/0372-9311-525

Сибирская язва в Российской Федерации в 2023 году, или «старая сказка о главном»

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Аннотация

Введение. Современная ситуация по сибирской язве (СЯ) в России характеризуется неустойчивостью. В 2023 г. отмечено увеличение числа вспышек инфекции по сравнению со средним многолетним показателем (за 5 лет).

Цель работы — оценка эпизоотолого-эпидемиологической ситуации по СЯ, сложившейся в России в 2023 г., и причин её ухудшения, анализ данных геномного эпидемиологического надзора за этой инфекцией.

Материалы и методы. Использовали информацию территориальных органов Роспотребнадзора о расследовании вспышек СЯ, справочные материалы о стационарно неблагополучных по СЯ пунктах и сибиреязвенных захоронениях. Филогенетическое положение идентифицированных штаммов *Bacíllus anthracis* и структуру геномов определяли на основе данных полногеномного секвенирования.

Результаты. В 2023 г. вспышки СЯ зарегистрированы в Чувашской Республике (1), Республике Тыва (1), Тамбовской (1), Рязанской (1) и Воронежской (3) областях. Заболело 14 сельскохозяйственных животных и 19 человек. Заражение животных, не вакцинированных против СЯ, а также привитых задолго до контакта с источником инфекции, происходило преимущественно при выпасе на территориях старых (неучтённых) почвенных очагов СЯ. Заболевание людей обусловлено контактом с больными животными при уходе, вынужденном убое, разделке, транспортировке туш и мяса, кулинарной обработке заражённого мяса и субпродуктов, употреблением в пищу недостаточно термически обработанного ливера. У 17 заболевших диагностирована кожная форма СЯ, у 2 — орофарингеальная форма в сочетании с кожной формой болезни. Во всех случаях установлена типичная для вида *B. anthracis* структура геномов. Показана филогенетическая связь изолятов со штаммами *B. anthracis*, ранее выделенными в России.

Заключение. Причиной неблагополучия по СЯ в 2023 г. стал ряд нарушений ветеринарного и санитарно-эпидемиологического нормирования на фоне наличия почвенных очагов инфекции. Стабилизация обстановки может быть достигнута только при постоянной реализации в полном объёме комплекса регламентированных профилактических мероприятий. Результаты молекулярно-генетического типирования штаммов *B. anthracis,* выделенных в ходе эпидемиологического расследования 7 вспышек СЯ на территории России в 2023 г., позволяют сделать вывод об их местном происхождении и типичной для вида структуре генома. Генетический анализ изолированных штаммов показал эффективность применения разработанной системы wgSNP-типирования при эпидемиологическом расследовании вспышек.

Ключевые слова: *сибирская язва, почвенный очаг, Bacillus anthracis, полногеномное секвенирование*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Ставропольского государственного медицинского университета (заключение № 109 от 19.05.2022).

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.

Для цитирования: Куличенко А.Н., Рязанова А.Г., Логвин Ф.В., Еременко Е.И., Аксенова Л.Ю., Писаренко С.В., Семёнова О.В., Герасименко Д.К., Ковалев Д.А., Головинская Т.М., Бобрышева О.В., Печковский Г.А., Олейникова К.А., Никитина А.В. Сибирская язва в Российской Федерации в 2023 году, или «старая сказка о главном». *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):448–461.
DOI: https://doi.org/10.36233/0372-9311-525
EDN: https://www.elibrary.ru/anetvp

DOI: https://doi.org/10.36233/0372-9311-525

Introduction

Anthrax is a particularly dangerous zoonotic disease that is practically ubiquitous in the world [1, 2]. The danger of the disease is due to the ability of its causative agent, *Bacillus anthracis*, to form highly resistant to environmental factors spores, which, once in the soil, remain viable for decades, forming persistent soil foci of infection.

In the past, anthrax of susceptible animals and humans in Russia was widespread. Since 1900, more than 70,000 outbreaks of animal and human anthrax have been recorded in Russia on the territory of more than 35,000 stationary anthrax-affected sites [3]. Thus, in the 1920s, the number of cases among animals was measured in tens of thousands (142,800 animals in 1920–1923), among humans — in thousands (9,037 for the same period), and in some years more than 10,000 human cases per year were registered (46,326 cases in 1924–1926) [4]. Thanks to the introduction of mass prophylactic immunization of farm animals and contingents at high risk of infection through occupational contact with livestock and livestock products since the 1950s, a striking reduction in the incidence of both animal and human diseases was achieved [5].

The current situation on anthrax in Russia is characterized by instability. While in 2018–2022 there were sporadic cases in animals (1-3 per year) and humans (2–5 per year), and in 2017 anthrax was not observed at all [6–8], then in 2016, the largest epizootic was registered in the Yamalo-Nenets Autonomous District with the disease in more than 2650 reindeer and 36 people with 1 fatal case [9].

In 2023, Russia again recorded a worsening of the situation on anthrax, which makes it necessary to analyze the causes that led to the unfavorable situation on this infection.

Currently, due to the development of sequencing technologies, a large amount of data on the genomic sequences of microorganisms has been accumulated. The method of whole genome sequencing is used to study the structure of genome sequences, phylogenetic analysis of isolates, and is successfully used in the process of epidemiological investigation of outbreaks of infectious diseases, including anthrax [10-13]. In this regard, the active improvement and implementation of genomic epidemiologic surveillance methods seems to be particularly relevant.

The aim of this study is to assess the epizootological and epidemiological situation of anthrax in Russia in 2023 and the reasons for its deterioration, as well as to analyze the data of genomic epidemiological surveillance of this infection.

Materials and methods

Information from the Rospotrebnadzor offices in the Chuvash Republic, Republic of Tyva, Tambov, Ryazan, and Voronezh regions on the investigation of anthrax outbreaks in the respective subjects in 2023 was used in this work. In order to conduct a retrospective analysis of the anthrax situation in these territories, we used official reference materials on stationary anthrax-affected sites [3], anthrax burial sites [14–16], as well as databases of stationary anthrax-affected sites and anthrax burial sites created in 2023, based on updated information (database "Inpatient anthrax-affected sites in the Russian Federation", certificate of state registration from 01.08.2024 No. 2024623389).

All subjects gave informed voluntary consent to participate in these studies (according to the Federal Law "On Fundamentals of Health Protection of Citizens in the Russian Federation" of 21.11.2011 No. 323-FZ, ed. of 30.12.2021). Clinical trials were approved by the local ethical committee of the Federal State Budgetary Educational Institution of Higher Professional Education "Stavropol State Medical University" of the Ministry of Health of Russia (conclusion of the local ethical committee from 19.05.2022 № 109).

All manipulations with laboratory animals were performed according to the "European Convention for the Protection of Vertebrate Animals Used for Experiments or Other Scientific Purposes" (Strasbourg, 18.03.1986 ETS No. 123).

Laboratory diagnosis of anthrax and identification of 32 cultures of *B. anthracis* isolated in 2023 from clinical material (7), animal material (17) and environmental objects (8) were performed in accordance with the methodological guidelines "Laboratory Diagnosis and Detection of Anthrax Pathogen"[1].

B. anthracis genomes were sequenced using the DNBSEQG50RS high-throughput sequencing platform (BGI). Genotyping of 1169 *B. anthracis* strains (302 strains from the collection of the Stavropol Anti-Plague Institute of Rospotrebnadzor, 867 strain genome sequences from the international GenBank database) on the basis of wg-SNPs was performed using Parsnp [17]. The obtained SNP profiles were used to construct phylogenetic reconstruction using the maximum likelihood method according to the Tamura-Nei model [18] in the Mega10 software. To visualize the phylogenetic tree, the FigTree program (Tree Figure Drawing Tool v. 1.4.3) was used [19].

The genome sequences of *B. anthracis* strains from the collection of the Stavropol Plague Control Institute of Rospotrebnadzor are presented in the "Nucleotide sequences of complete genomes of *B. anthracis* strains isolated in Russia and neighboring countries" electronic database (certificate of state registration No. 2022620144 dated 18.01.2022), in the electronic database of the Stavropol Plague Control Institute of Rospotrebnadzor 'Full genome sequences of *B. anthra-*

[1] Methodological guidelines MG 4.2.2413-08 "Laboratory diagnosis and detection of anthrax pathogen" (approved by the Head of Rospotrebnadzor on 29.07.2008).

cis strains' (reg. No. B.ab-R-1–B.ab-R-302) and can be provided on request (stavnipchi@mail.ru).

Results

From March to September 2023, 7 outbreaks of anthrax were registered in Russia in 5 subjects of 3 federal districts of the Russian Federation: Volga, Siberian and Central (**Table**).

The first outbreak of anthrax in Russia in 2023 was recorded in March in the Chuvash Republic. In the private farm of a resident of the village of Starye Aktashevo, Tsivilsky District, a bull calf was slaughtered without anyone notifying veterinary specialists, after which 1 of 4 participants, the most active in the slaughter, became ill. In the process of culinary processing of by-products from cattle the spouse of the owner of the sick steer became infected.

It was found that the animal was not registered and was not vaccinated against anthrax. During the epidemiologic investigation, the owner of the cattle sold meat and skin of the steer to unknown persons on the highway, which were timely seized and destroyed during the epidemiologic investigation of the outbreak.

The clinical diagnosis of cutaneous anthrax in the diseased was confirmed by specialists of the Reference Center for monitoring of the anthrax pathogen (hereinafter referred to as the Reference Center), operating on the basis of the Stavropol Plague Control Institute of Rospotrebnadzor, and the Center for Hygiene and Epidemiology in the Chuvash Republic (Chuvashia) by polymerase chain reaction (PCR), based on the detection of DNA of the anthrax pathogen in skin samples, as well as positive results of additional immunological methods: detection of specific anti-anthrax antibodies by indirect fluorescent antibody method, allergy test with anthrax allergen *in vitro* using flow cytometry. *B. anthracis* culture was isolated from the source of infection — bovine meat.

The second outbreak of anthrax infection was recorded in June in Barun-Khemchiksky district of the Republic of Tyva with the disease in 2 unvaccinated horses, one of which was subjected to forced slaughter and the other fell ill. A total of 5 people fell ill; 2 of the ill persons who carried out unauthorized forced slaughter of a sick horse at a private shepherd's camp near the village of Bizhiktig-Khaya. The skin manifestations of the infection in them were preceded by the formation of inflammatory foci in the oropharynx. On the basis of clinical data, positive PCR detection of *B. anthracis* DNA both in swabs from the pharynx and in samples of skin affects, the patients were diagnosed with the oropharyngeal form of anthrax combined with the cutaneous form of the disease. Thanks to timely diagnosis and immediate start of intensive therapy of the prognostically unfavorable oropharyngeal form of the infection, fatal outcomes were avoided.

Due to the shipment of contaminated meat for sale to a butcher shop in Ak-Dovurak, 3 people became ill with cutaneous anthrax, which was diagnosed on the basis of positive PCR results: the meat transporter, the son of the shop clerk and a female customer who purchased ground beef from the shop. It was found that the meat of cattle, from which the minced beef was made, was contaminated during storage in the refrigerator in the neighborhood with infected horse meat delivered from the outbreak in the village of Bizhiktig-Khaya, during laboratory examination of which *B. anthracis* culture was isolated in the Tuva anti-plague station of Rospotrebnadzor.

An epizootic outbreak with disease in one head of cattle was detected in June in Bondarsky district of Tambov region – in a pasture located 3 km from the village of Shacha Molokanskaya, Mitropolsky rural council. This is the only outbreak of anthrax among livestock in 2023 that did not result in epidemic complications. No samples were sent to the Reference Center for research.

In June, 6 cattle fell in LLC Lenin's Way, Zakharovsky district, Ryazan region, grazing in the summer pasture in the village of Staroye Zimino. As a result of direct contact with sick animals, a cattleman became ill with anthrax. According to the veterinary service of the region, scheduled specific immunization of cattle was carried out in October-November 2022. However, the employees of LLC Lenin's Way, including the sick person, were not vaccinated against anthrax.

The diagnosis of anthrax in 2 cattle was established by the Ryazan Regional Veterinary Laboratory based on the results of bacteriological examination of pathological material samples. In the course of epidemiologic investigation Rospotrebnadzor specialists isolated cultures of anthrax microbe from soil and water samples taken in the places of animal deaths. The clinical diagnosis of cutaneous infection in a patient was confirmed by the detection of *B. anthracis* DNA in a scrape from a skin affect scab.

In 2023, special attention was focused on the situation on anthrax in Voronezh region, where in August–September, 3 outbreaks of infection with 11 people falling ill were recorded in 3 districts of the region.

The first outbreak was detected in August in Paninsky district, where the owner of a private subsidiary farm in Krasnye Kholmy village fell ill after forced slaughter of 1 head of cattle without notification of veterinarians. The animal had not been vaccinated against anthrax. The owner sold the meat to unknown persons, and as a result of search activities it was found that the resellers sold the meat to an entrepreneur of one of the markets in Voronezh, where it was promptly seized almost in its entirety, and remnants of infected meat were found in Semiluki and Novaya Olshanka villages. During material examination by specialists of the Center for Hygiene and Epidemiology in Voronezh region and Stavropol Anti-Plague Institute of Rospotreb-

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-525
ORIGINAL RESEARCHES

Epizootological and epidemiological situation on anthrax in the Russian Federation in 2023

No. in oder	Federal District of the RF	Subject of the RF	Municipal district, locality	Number and type of sick livestock	Number of sick people	Previous outbreak
1	Privolzhsky	Chuvash Republic — Chuvashia	Tsivilsky district, v. Staroe Aktashevo	1 cattle	2	1930
2	Siberian	Tyva Republic	Barun-Khemchiksky district, v. Bizhiktig-Khaya	2 horses	5	2021
3	Central	Tambov region	Bondarsky district, v. Shacha Molokanskaya	1 cattle	-	1959
4	Central	Ryazan region	Zakharovsky district, v. Staroe Zimino	6 cattle	1	1944
5	Central	Voronezh region	Paninsky district, v. Krasnye Holmyi	1 cattle	1	1958
6	Central	Voronezh region	Bogucharsky district, v. Lebedinka	2 cattle	9	1952
7	Central	Voronezh region	Novousmansky district, farm Krylovsky	1 cattle	1	1948
Total	3	7	7	12 cattle, 2 horses)	19	1930–2021

nadzor, *B. anthracis* culture was isolated from samples of cattle skin and meat, DNA of the pathogen was found in samples of pathological skin effects of the patient.

In September, a large outbreak with the disease of 2 cattle and 9 people was registered in Bogucharsky district. Infection of 5 people occurred in the process of forced slaughter of 1 head of cattle, carried out in a peasant (private) farm in Lebedinka village without veterinary inspection, subsequent cutting of the carcass and meat. The second animal fell and was buried on the territory of the farm.

It was found that the cows in the farm were not fully vaccinated, and the diseased animals were not immunized in a planned manner. The employees of the farm were not immunized against anthrax either. During the investigation, it was determined that the owner of the farm transported part of the meat to his own cafe, and sold the rest in an unauthorized place of trade to the owner of another cafe and random persons. As a result, 4 more people became ill from contact with the purchased meat.

In the process of material examination, cultures of the pathogen were isolated from samples of clinical material, meat and cattle hides. Positive PCR results were also obtained in the study of semi-finished meat products from the cafe of the owner of the farm, intended for sale and catering of employees of his farm.

The diagnosis of cutaneous anthrax in patients was also confirmed by the detection of *B. anthracis* DNA in skin samples, specific antibodies and positive results of allergy test.

The third focus of infection was recorded in Novousman district. One farm worker in the Krylovskoye state farm became ill after contact with the carcass of fallen cattle. The clinical diagnosis was confirmed by PCR and immunological methods in the Reference Center.

A total of 32 cultures of the anthrax pathogen were isolated during the outbreaks. Final identification performed at the Reference Center using basic and additional bacteriological tests showed that all isolates were typical virulent cultures of *B. anthracis* with high sensitivity to antibacterial drugs used to treat anthrax in humans (penicillins, carbapenems, tetracyclines, fluoroquinolones, aminoglycosides, rifampicin).

As a result of molecular genetic typing, the cultures isolated in the Chuvash Republic, Ryazan and Voronezh regions were determined to belong to the main genetic clade A, canSNP-group A.Br.008/009 (A.Br.008/011) of the widespread trans-Eurasian subclade, and the isolate isolated in the Republic of Tyva — to canSNP-group B.Br.001/002 of the main genetic clade B.

According to full genome sequencing data, all strains were found to have a typical genome structure and contain a set of virulence genes characteristic of the *B. anthracis* species.

Phylogenetic analysis based on wg-SNP typing of more than 1100 *B. anthracis* strains from the collection of the Stavropol Anti-Plague Institute of Rospotrebnadzor and genomes from the international GenBank database showed that the strain isolated in the Chuvash Republic belongs to the lineage A.Br.118 (STI) (subcluster 2 of the A.Br.125 cluster) and has the closest affinity with *B. anthracis* strains previously isolated during outbreaks of anthrax in the Volgograd region (Bykovsky district, 1985) and Saratov region (Balashovsky district, 2015) (**Fig. 1**).

Cultures from the Paninsky district of Voronezh region and Zakharovsky district of Ryazan region belong to the A.Br.117 (Tsiankovskii) lineage, A.Br.215 cluster, and the strain isolated in the Anninsky district of Voronezh region in 1982 is the closest to them (**Fig. 2**).

The strains isolated in Bogucharsk district of Voronezh region also belong to the A.Br.117 (Tsiankovskii) lineage and cluster with strains isolated in Stavropol Krai (Petrovsky district — 1959, Novoselitsky District — 2001), Volgograd Region (Oktyabrsky District, 2014), Kaluga Region (Kozelsky District, 1989), Republic of Ingushetia (Malgobeksky District, 1968), Ryazan Region (1981), and Republic of Kalmykia (Gorodovikovsky District, 2002) (Fig. 2).

The culture isolated in the Barun-Khemchik District of the Republic of Tyva belongs to the B.Br.013 Asia cluster of the B.Br.012 lineage and has a close phylogenetic relationship with strains isolated in the same region of the republic in 2018 and 2021 (subcluster 1 in **Fig. 3**).

Discussion

By definition, stationary anthrax-affected sites have on their territory soil centers of anthrax, which are anthrax burial sites [20]. However, the number of stationary anthrax-affected sites, let alone outbreaks, is dozens of times greater than the number of registered anthrax burial sites. In the Republic of Tyva, while more than 300 outbreaks have been registered in 154 stationary anthrax-affected sites since 1910, there is information about only 11 burial sites of corpses/ash remains of anthrax-infected animals (of which 7 are carcass incineration sites in 2018–2023). In the Chuvash Republic, where more than 3,600 foci of infection in 1,231 stationary anthrax-affected sites have occurred since 1901, 397 anthrax burial sites were registered as of 2013 [16]; however, in accordance with the resolution of the Cabinet of Ministers of the Chuvash Republic, 345 burial sites were eliminated in 2015, including in the village of Staroye Aktashevo. In the Voronezh region, where 780 stationary anthrax-affected sites have been recorded since 1902, 81 anthrax burial sites were registered [14], but, according to the order of the Voronezh Region Veterinary Department, they were removed from the regional register in 2011. And in the Ryazan region,

DOI: https://doi.org/10.36233/0372-9311-525

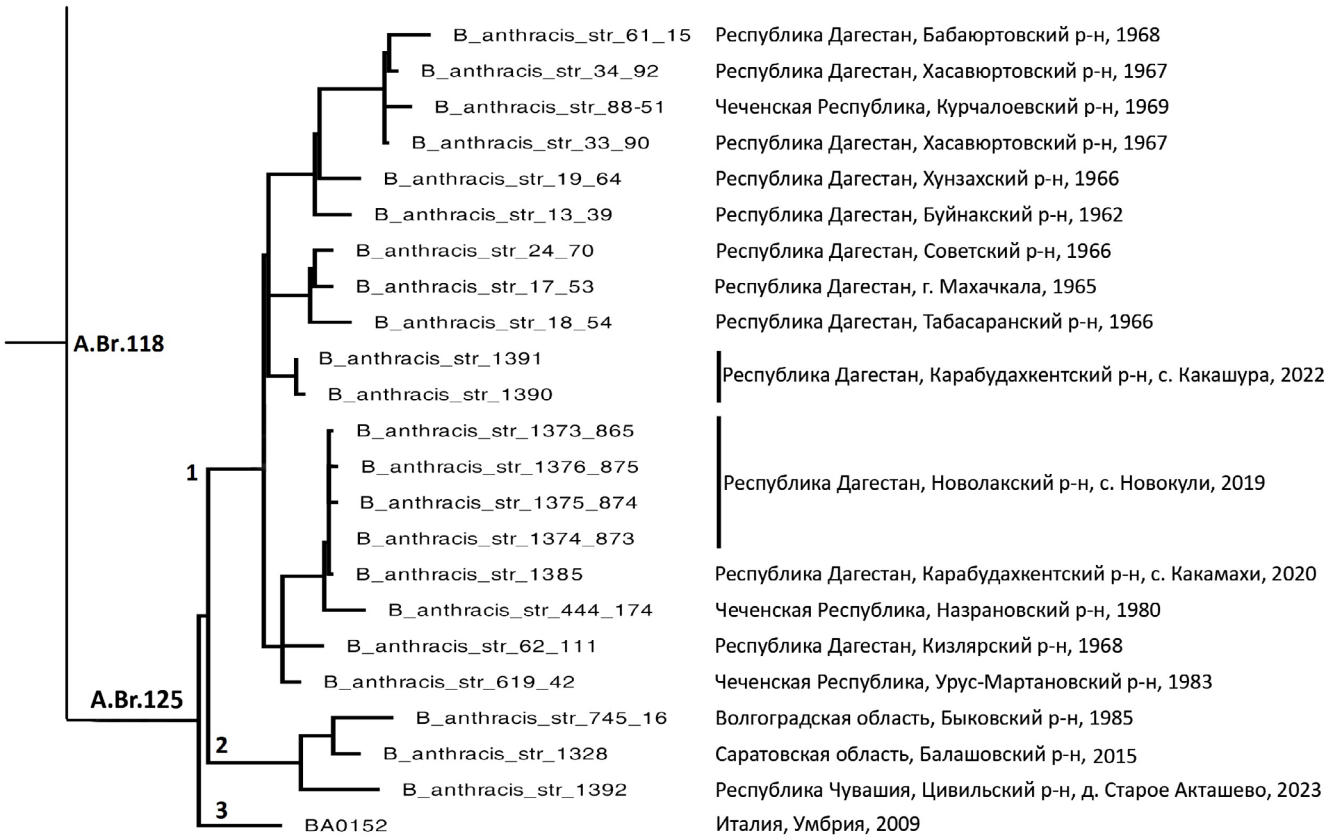

Fig. 1. Phylogenetic position of *B. anthracis* strains isolated in the Chuvash Republic — Chuvashia (2) and the Dagestan Republic (1). Fragment of dendrogram reconstructed on the basis of wg-SNP-analysis by method Maximum Likelihood according to Tamura–Nei model.

in which, since 1901, more than 1900 outbreaks have been noted on the territory of more than 900 stationary anthrax-affected sites, and in the Tambov region, where almost 700 stationary anthrax-affected sites are known to be active more than 1600 times since 1929, not a single anthrax burial site has been accounted for [14]. Thus, on the territory of the constituent entities of the Russian Federation, where outbreaks of anthrax in 2023 have been noted, there is a huge number of unattended animal burial sites that are soil foci of anthrax — unknown, due to the historical absence of their registration or incomplete registration by veterinary services in violation of the veterinary regulatory framework on the need to document and supervise anthrax[2], removed from registration.

Epidemiological investigation of the outbreak and retrospective analysis of the anthrax situation on the territory of the subjects where outbreaks were detected in 2023, conducted in accordance with the regional updated databases of stationary anthrax-affected sites, anthrax, Cadastre information [3], lists of livestock burial grounds [14–16], showed the following.

In the Tsivilsky district of the Chuvash Republic in 1901–1979, 155 cases of anthrax were reported in 72 stationary anthrax-affected sites. The village of Staroye Aktashevo is a stationary anthrax-affected site with manifestations of activity in 1929 and 1930. At a distance of 1 km from the village there is an anthrax burial site organized in 1930. It was found that cattle grazing near the anthrax burial site was impossible due to the presence of persistent snow cover and lack of vegetation in March 2023; it is possible that the animals were infected when consuming fodder prepared earlier on the territory of the anthrax burial site.

Since 1929, anthrax has been reported more than 40 times in the Barun-Khemchik District of the Tyva Republic, but no anthrax has been recorded. The previous outbreak on the territory of this district took place in 2021 in the village of Bizhiktig-Khaya [21]. Previously, the village had not been reported to be infected, but the investigation showed that cattle grazing

[2] Instruction "On measures against anthrax" (approved by the Ministry of Agriculture of the USSR on 28.02.1953); Instruction "On measures against anthrax" (approved by the Main Department of the Ministry of Agriculture of the USSR on 05.06.1981, as amended on 12.11.1982); Sanitary Rules (SP 3.1. 089-96) and Veterinary Rules (VP 13.3.1320-96) "Prevention and control of contagious diseases common to humans and animals", section 6 "Anthrax" (approved by the State Committee for Sanitary and Epidemiological Surveillance of the Russian Federation on 31.05.1996 No. 11, Ministry of Agriculture and Food of the Russian Federation on 18.06.1996 No. 23).

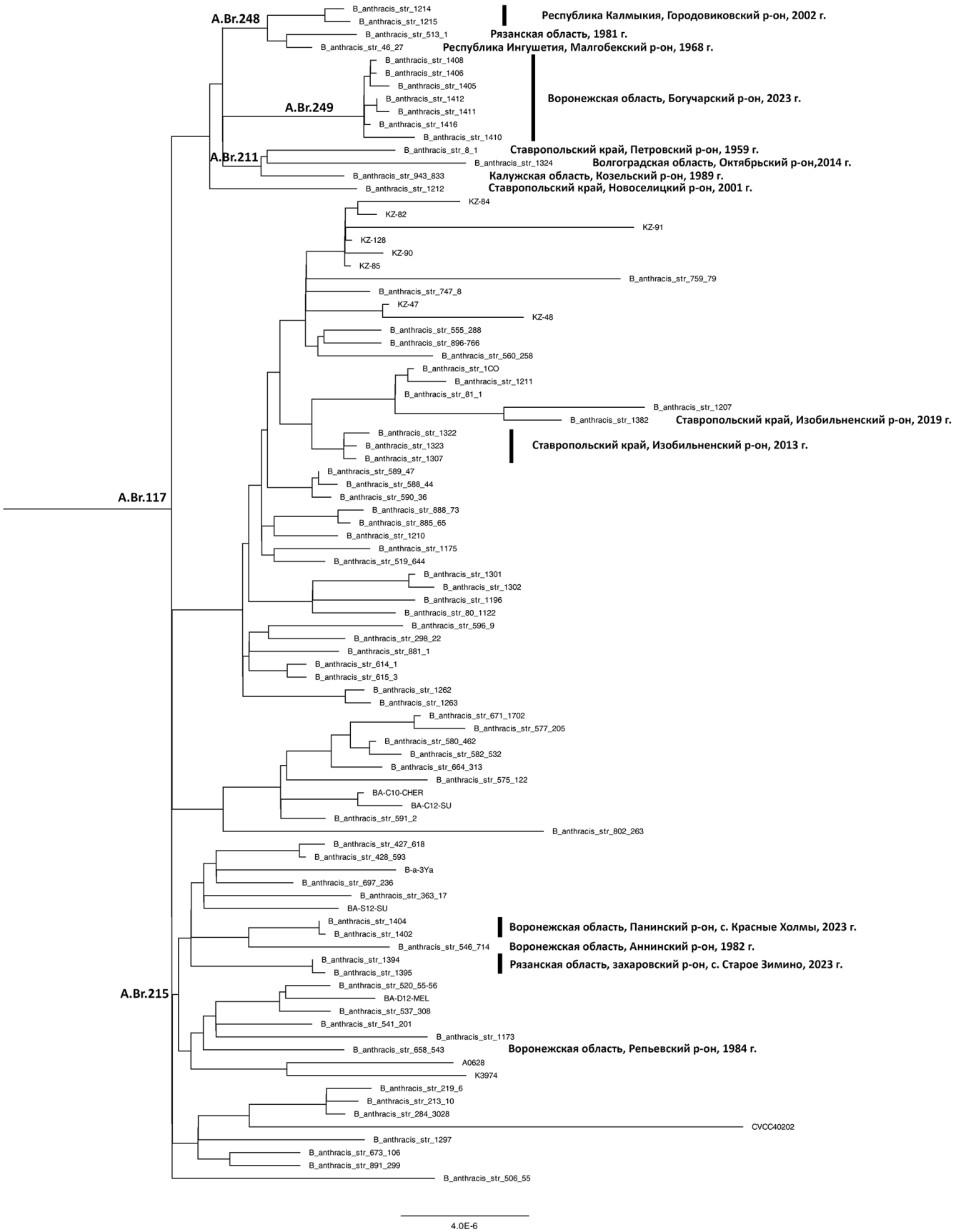

Fig. 2. Results of phylogenetic analysis of B. anthracis strains isolated in the Voronezh and Ryazan regions, Stavropol Territory. Fragment of dendrogram reconstructed on the basis of wg-SNP-analysis by method Maximum Likelihood according to Tamura–Nei model.

DOI: https://doi.org/10.36233/0372-9311-525

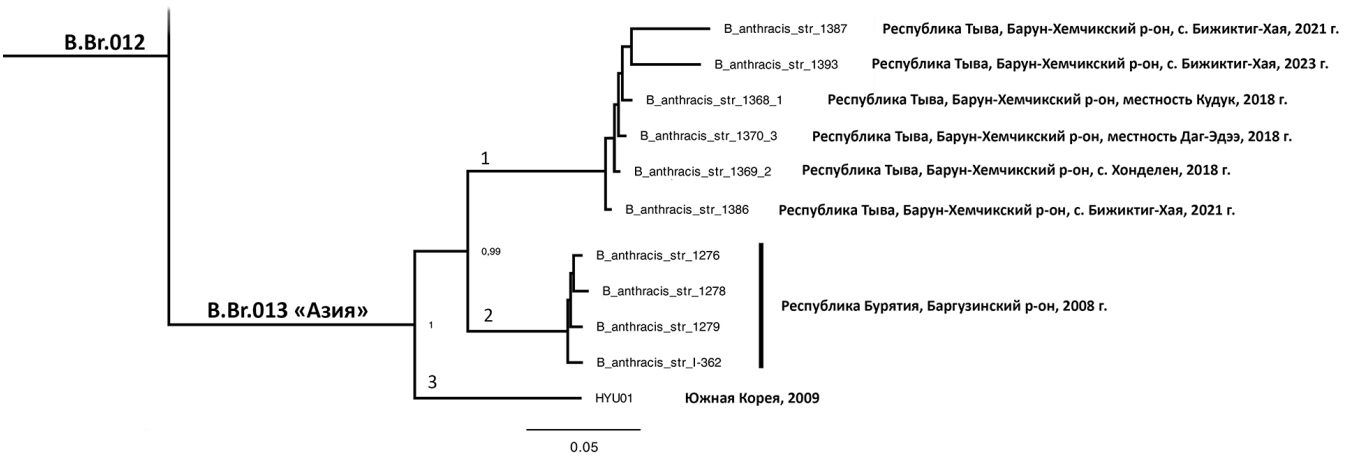

Рис. 3. Филогенетическое положение штаммов *B. anthracis*, выделенных в Республике Тыва (1) и Республике Бурятия (2).

Фрагмент дендрограммы, реконструированной на основе wg-SNP-анализа методом максимального правдоподобия в соответствии с моделью Tamura– Nei.

Fig. 3. Phylogenetic position of B. anthracis strains isolated in the Tyva Republic (1) and the Buryatia Republic (2).

Fragment of dendrogram reconstructed on the basis of wg-SNP-analysis by method Maximum Likelihood according to Tamura–Nei model.

from Bizhiktig-Khaya village and Kyzyl-Mazhalyk village had not been reported. Kyzyl-Mazhalyk, located at a distance of 6 km from the analyzed village, are grazing on a common pasture, and the village of Kyzyl-Mazhalyk. Kyzyl-Mazhalyk is a stationary anthrax-affected site with five times of activity from 1941 to 1989. Thus, infection of livestock both in 2023 and 2021 occurred during grazing on the territory of old soil outbreaks on the territory of the pasture. Prior to that, in 2018, 3 outbreaks were recorded in Barun-Khemchik District (in Khondelen village and two nearby areas – Kuduk, Dag-Edey, and Edegey tract), in which anthrax had been observed many times in the past with the last occurrences in 1950 and 1982, respectively. It was also revealed that the emergence of epizootic foci in 2018, 2021 and 2023 was preceded by heavy rainfall alternating with high ambient temperatures – this gave birth to the term "anthrax weather" [22–24]. This contributed to the release of spores of the anthrax pathogen on the surface of soil foci with subsequent drying and spreading, which facilitated the infection of livestock by aspiration and alimentary routes during grazing.

In Bondarsky district, Tambov region, more than 50 outbreaks of anthrax were recorded in 26 locations in 1933–1974. On the territory of Mitropolsky rural council, which includes the v. Shacha Molokanskaya, anthrax was registered 16 times in 1933-1959, in connection with which it is quite obvious that there are soil foci here – old burials of fallen animals, including in the pasture, where, probably, the infection of cattle occurred.

There are 71 known outbreaks of anthrax in 26 settlements of Zakharievsky District, Ryazan region, in 1904-1980, but there are no recorded cases of anthrax in the district. The village of Staroye Zimino, where the summer pasture of LLC "Lenin's Way" is located, belongs to stationary sites with outbreaks of activity in 1911 and 1944. It was noted that the emergence of the epizootic in the Ryazan region, as well as in the Republic of Tyva, was preceded by heavy precipitation and hot weather, which contributed to the activation of soil foci, resulting in infection during grazing in the territory of the old burial site with unknown localization even of immunized animals.

In Panin district, Voronezh region, 70 outbreaks of infection were recorded in 1938–1984 on the territory of at least 28 counted SNPs. Anthrax in the village of Krasnye Kholmy, the first outbreak focus in the Voronezh region 2023, was reported in 1955 and 1958. In the district, one anthrax was previously registered in the village of Chernavka. Thirty-four stationary anthrax-affected sites have been registered in Bogucharsky District, Voronezh region, in which 74 outbreaks were noted in 1948–1981. No SNPPs are registered in the district. There are no data on the registration of anthrax cases in Lebedinka village, where the second outbreak in the region took place, but outbreaks were registered in other nearby settlements of Pervomaisky rural settlement, which includes this village (Plesnovka village in 1948, Batovka village in 1952). In Novousman district of Voronezh region, where the third outbreak of infection took place, 20 SNPs with activity 42 times in 1941–1997 were recorded, and one stationary anthrax-affected site was registered earlier (Petropavlovka village). According to archival data, the registration of anthrax in 1948 on the territory where the Krylovsky state farm is currently located is not excluded. Thus, infection of animals in Voronezh region also occurred during grazing in the territories of old anthrax-infected soil foci.

A number of violations of the requirements of the legislation in the field of veterinary medicine[3], sanitary and epidemiological well-being of the population[4] and the regulatory and legal acts adopted in accordance with it contributed to the formation of the disease in 2023[5]. First of all – concealment of livestock during registration by owners of private subsidiary farms in the Chuvash Republic, Paninsky district of Voronezh region, the Republic of Tyva and the peasant farm enterprise in Bogucharsky district of Voronezh region, as a result of which farm animals were not covered by specific immunization in a planned manner, which caused the disease of animals in contact with the pathogen. The reasons for the disease of 6 cattle in Ryazan region vaccinated against anthrax are probably related to the fact that routine vaccination was carried out in the fall of 2022, i.e., long before the most dangerous spring-summer season in terms of anthrax infection, and did not ensure the preservation of proper immunity in animals; infection could have been promoted by the entry of a massive dose of the pathogen into the organism, as well as the realization of the vector-borne mechanism of transmission of the sybillivirus microbe from the diseased animal to the others through the bites of blood-sucking insects. There is no information about immunization of farm animals in the foci of Tambov region and Novousman district of Voronezh region; it is obvious that the diseased animals were also not accounted for and, accordingly, were not routinely vaccinated against anthrax.

The next violation, which caused human infection, is the forced slaughter of sick livestock without notifying the veterinary service, whereas the owners were obliged to report within 24 hours by any available means about the case of disease or death of an animal to a veterinary specialist, who determines the order of further actions on site based on the results of the inspection.

Also, the requirements of points 1098–1102 of SanPiN 3.3686-21 on routine vaccination of persons exposed to the risk of occupational infection due to their occupation, which caused the disease in workers of livestock farms in Ryazan region, Bogucharsky and Novousman districts of the Voronezh region.

A gross violation punishable under Article 236 (part 1) of the Criminal Code of the Russian Federation was the sale by owners of farm animals of knowingly contaminated meat from sick animals subjected to forced slaughter and fallen livestock, as a result of contact with which people fell ill in the Republic of Tyva and Bogucharsky district of Voronezh region. Also, a veterinarian of one of the markets in Voronezh city authorized the sale of dangerous products accepted without veterinary accompanying documents from resellers of infected meat from the Paninsky district of Voronezh region.

As a result, the described violations, first of all, of owners of private subsidiary farms and private farms, which initially led to non-coverage of livestock with routine vaccination, caused the formation of epizootological-epidemiological disadvantage for anthrax, epidemiological investigations and implementation of a set of anti-epidemic measures required considerable labor inputs from specialists of Rospotrebnadzor, Rosselkhoznadzor, Ministry of Health of Russia, as well as territorial structures of the Ministry of Internal Affairs of Russia and the Federal Security Service of Russia, which provided substantial assistance in the investigation of outbreaks and large expenditures of budget funds.

Stabilization of the anthrax situation consists in the continuous full implementation of the main regulated prophylactic countermeasures. Since it is not possible to determine the localization of old burial sites, which serve as a permanent risk factor for complication of the situation, the priority is to ensure biological safety of known burial sites (landscaping, veterinary and sanitary control of the condition, prevention of the use of burial sites for economic needs, establishment of sanitary protection zones for anthrax burial sites) and to prevent the de-registration of anthrax burial sites. In order to prevent the formation of new soil foci of infection during the burial of livestock remains obtained by burning with the use of improvised means, which does not always allow to achieve guaranteed destruction of the pathogen, it is advisable to purchase mobile incinerators that ensure the burning of animal carcasses to a safe inorganic ash residue.

The basis for preventing anthrax in animals is to ensure their universal coverage with specific immunization in threatened areas, which can be implemented only if additional measures are taken to ensure complete cattle registration. Strict control of routine vaccination of persons at high occupational risk of infection is necessary. Important aspects of prevention include educating

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[3] Law of the Russian Federation from 14.05.1993 № 4979-1 "On veterinary medicine" (ed. from 25.12.2023).

[4] Federal Law of 30.03.1999 № 52-FZ "On sanitary-epidemiological well-being of the population" (ed. of 24.07.2023).

[5] Federal Law of 27.12.2018 No. 498-FZ "On Responsible Treatment of Animals and on Amendments to Certain Legislative Acts of the Russian Federation"; Sanitary Rules and Norms SanPiN 3.3686-21 "Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases" (approved by Resolution of the Chief State Sanitary Doctor of the Russian Federation of 28.01. 2021 No. 4); Veterinary rules for the implementation of preventive, diagnostic, treatment, restrictive and other measures, establishment and lifting of quarantine and other restrictions aimed at preventing the spread and elimination of anthrax outbreaks (approved by the order of the Ministry of Agriculture of the Russian Federation of 28.01.01. Order of the Ministry of Agriculture of Russia No. 648 of 23.09.2021); Veterinary rules for slaughtering animals (Annex No. 1 to Order of the Ministry of Agriculture of Russia No. 269 of 28.04.2022, as amended on 18.11.2022); Rules for animal registration (approved by Resolution of the Government of the Russian Federation No. 550 of 05.04.2023).

DOI: https://doi.org/10.36233/0372-9311-525

the population about the risk factors of infection and the danger of anthrax, the inadmissibility of concealing the actual number of farm animals on the farm, which entails not including unaccounted livestock in the vaccination plan, forced slaughter of sick animals without veterinary examination, sale of raw materials and livestock products, purchase of meat products in places of unauthorized trade. The use of the algorithm of genetic analysis of isolated strains, which makes it possible to identify modifications of the genome structure and to establish the probable origin of isolates, contributes to the improvement of surveillance of anthrax and the efficiency of epidemiological investigation.

Molecular genetic monitoring as part of microbiological monitoring of infectious agents is an integral component of modern epidemiological surveillance of infectious diseases. The algorithm of wg-SNP-typing of *B. anthracis* strains developed using the data on the genetic structure of *B. anthracis* populations obtained in the course of Reference Center research was used in the investigation of anthrax outbreaks in 2023. The algorithm is intended for solving operational tasks and allows:

- identify atypical, modified, new forms of the pathogen by comparative analysis of the genome structure;
- increase the reliability of determining the origin and possible routes of spread of strains.

This approach was previously used by us in the epidemiologic investigation of anthrax outbreaks accompanied by the formation of epidemic foci, during which cultures of anthrax pathogen were isolated in the Yamalo-Nenets Autonomous District (2016), Stavropol Krai (2019), Republic of Tyva (2018, 2021), Republic of Dagestan (2019, 2020, 2022) [25–28].

Using genomic surveillance data, it was shown that the strains that caused the above-mentioned outbreaks were of local origin. Analysis of the target regions of the strains' genome, primarily pathogenicity factor genes, showed a typical genome structure for the *B. anthracis* species.

Conclusion

The analysis of the situation with anthrax in Russia in 2023 indicates that the reason for the formation of epizootic foci was the contact of unrecorded and, consequently, unvaccinated animals, vaccinated livestock with insufficiently strained level of specific immunity, with the soil of old unsupervised anthrax, as well as, probably, with fodder harvested in the territory of soil foci. A number of violations of veterinary and sanitary-epidemiological regulations, which led to contact with sick animals during care, forced slaughter, cutting and transportation of carcasses, preparation and cooking of infected meat and by-products, consumption of liver of insufficient heat treatment, caused the disease of people, both unvaccinated against anthrax of farm workers and persons not belonging to the contingent of risk of occupational contamination.

The complex of anti-epidemic measures in the course of anthrax outbreaks, carried out in the format of interdepartmental cooperation, allowed timely localization and elimination of infection foci and avoidance of even greater epidemic complications. Genetic analysis of the isolated strains indicated their local origin and absence of modifications in the genome structure, demonstrating the suitability of the developed wg-SNP typing system for epidemiologic investigation of outbreaks.

The results of molecular genetic typing of *B. anthracis* strains isolated during the epidemiologic investigation of seven anthrax outbreaks in the Russian Federation in 2023 allow us to conclude that they are of local origin and have a genome structure typical of the species. Genetic analysis of the isolated strains demonstrated the effectiveness of the developed wgSNP typing system in the epidemiologic investigation of outbreaks.

The results of the investigation of the reasons for the complication of the anthrax situation in Russia in 2023 show that the incomplete implementation of the regulated set of preventive measures can actually lead to a situation that will become a new chapter of the "old tale" about anthrax.

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The article was submitted 12.04.2024;
accepted for publication 20.06.2024;
published 29.08.2024

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Статья поступила в редакцию 12.04.2024;
принята к публикации 20.06.2024;
опубликована 29.08.2024

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-518
ORIGINAL RESEARCHES

Original Study Article
https://doi.org/10.36233/0372-9311-518

The molecular-genetic characteristics of uropathogenic *Escherichia coli* isolated from pregnant women with asymptomatic bacteriuria

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Abstract

Introduction. Uropathogenic *Escherichia coli* (UPEC) are the dominant bacterial pathogens of urinary tract infections (UTIs). UPEC belong to different phylogenetic groups and have many virulence factors, the study of which, in conjuction with the assessment of their relationship with clinical forms of UTI, is necessary for a better understanding of the pathogenesis of UTI and the development of new diagnostic algorithms.

Aim: determination of the molecular genetic characteristics of uropathogenic *Escherichia coli* isolated from pregnant women with asymptomatic bacteriuria.

Materials and methods. Clinical isolates of uropathogenic *E. coli* (*n* = 70) from pregnant women with asymptomatic bacteriuria were included in the study. The PCR method was used to determine the belonging to phylogenetic groups and detect 15 virulence markers — genes associated with adhesion (*fimH, papC, sfa, afa, focG*); toxin synthesis (*cnf 1, hlyA, sat, vat, usp*); siderophores (*fyuA, iroN, iuc*); capsular antigen (*kpsMII*). To assess the statistical significance of differences, Fisher's exact test was used. Differences were considered statistically significant at a confidence interval of 95% ($p < 0.05$).

Results. Most of the UPEC isolates belonged to phylogroup B2 (51,4%) and were characterized by the detection of all UPEC-associated virulence factors included in this study; genes associated with adhesion (*sfa, focG*), invasins (*ibeA*), synthesis of toxins (*hlyA, cnf1, vat, usp*) and capsule (*kpsMII*), siderophores (*fyuA, iroN, hlyA*) were detected significantly more frequently ($p < 0.05$). Two or more virulence determinants were detected in 93% of isolates.

Conclusion. The identification of key determinants of virulence and/or a combination of virulence genes can be a prognostic marker for predicting the course of UTI, especially in pregnant women, and will expand diagnostic capabilities taking into account the virulent properties of the uropathogen.

Keywords: *urinary tract infections, asymptomatic bacteriuria, uropathogenic Escherichia coli, phylogenetic characteristics, virulence factors*

Ethics approval. The study was conducted with the informed consent of the patients. The study was approved by the Ethical Committee at the D.O. Ott Research Institute of Obstetrics, Gynaecology and Reproductology (protocol No. 114, December 14, 2021)

Funding source. The work was carried out as part of the fundamental scientific research FGWN-2022-0004 “Optimization of methods for prediction, prevention and treatment of “major obstetric syndromes, as well as delivery strategies in high-risk pregnant women, in order to improve obstetric and perinatal outcomes”.

Conflict of interest. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

For citation: Khusnutdinova T.A., Budilovskaya O.V., Krysanova A.A., Shalepo K.V., Sinyakova A., Savicheva A.M., Kogan I.Yu. The molecular-genetic characteristics of uropathogenic *Escherichia coli* isolated from pregnant women with asymptomatic bacteriuria. *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):462–469.
DOI: https://doi.org/10.36233/0372-9311-518
EDN: https://www.elibrary.ru/hzatlt

Оригинальное исследование
https://doi.org/10.36233/0372-9311-518

Молекулярно-генетическая характеристика уропатогенных *Escherichia coli*, выделенных при бессимптомной бактериурии у беременных

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Аннотация

Введение. Уропатогенные *Escherichia coli* (UPEC) являются доминирующими бактериальными патогенами при инфекциях мочевыводящих путей (ИМП). UPEC относятся к разным филогенетическим группам и обладают множеством факторов вирулентности, изучение которых в совокупности с оценкой их связи с клиническими формами ИМП необходимо для лучшего понимания патогенеза и разработки новых диагностических алгоритмов.

Цель исследования — молекулярно-генетическая характеристика UPEC, выделенных при бессимптомной бактериурии у беременных.

Материалы и методы. В исследование включены клинические изоляты *E. coli* (n = 70), выделенные у беременных с бессимптомной бактериурией (ББУ). Методом полимеразной цепной реакции определяли принадлежность к филогенетическим группам и 15 маркеров вирулентности — гены, ассоциированные с адгезией (*fimH*, *papC*, *sfa, afa*, *focG*), инвазией (ibeA), синтезом токсинов (*cnf1*, *hlyA*, *sat*, *vat*, *usp*), сидерофоров (*fyuA*, *iroN*, *iuc*), капсульного антигена (*kpsMII*). Для оценки статистической значимости различий средних величин применяли точный критерий Фишера. Статистически значимыми считали различия при 95% доверительном интервале (p < 0,05).

Результаты. Большинство изолятов UPEC, выделенных при ББУ, принадлежали к филогруппе B2 (51,4%) и характеризовались детекцией всех ассоциированных с UPEC факторов вирулентности, включённых в настоящее исследование; достоверно чаще были обнаружены гены, ассоциированные с адгезией (*sfa, focG*), синтезом токсинов (*hlyA, cnf1, vat, usp*) и капсул (*kps*), сидерофоры (*fyuA, iroN, hlyA*). Две и более детерминанты вирулентности выявлены у 93% изолятов.

Заключение. Определение ключевых детерминант вирулентности и/или комбинации генов вирулентности может быть прогностическим маркером для прогнозирования течения ИМП, особенно у беременных, и позволит расширить возможности диагностики с учётом вирулентных свойств уропатогена.

Ключевые слова: *инфекция мочевыводящих путей, бессимптомная бактериурия, уропатогенные Escherichia coli, филогенетическая характеристика, факторы вирулентности*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациенток, протокол исследования одобрен локальным этическим комитетом НИИ акушерства, гинекологии и репродуктологии им. Д.О. Отта (протокол № 114 от 14.12.2021).

Источник финансирования. Работа выполнена в рамках фундаментального научного исследования FGWN-2022-0004 «Оптимизация методов предикции, профилактики и лечения «больших акушерских синдромов, а также стратегии родоразрешения у беременных из групп высокого риска, с целью улучшения акушерских и перинатальных исходов».

Конфликт интересов. Спонсор не играл никакой роли в разработке исследования, сборе и анализе данных, принятии решения о публикации или подготовке рукописи.

Для цитирования: Хуснутдинова Т.А., Будиловская О.В., Крысанова А.А., Шалепо К.В., Синякова А.А., Савичева А.М., Коган И.Ю. Молекулярно-генетическая характеристика уропатогенных *Escherichia coli,* выделенных при бессимптомной бактериурии у беременных. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):462–469.
DOI: https://doi.org/10.36233/0372-9311-518
EDN: https://www.elibrary.ru/hzatlt

DOI: https://doi.org/10.36233/0372-9311-518

Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases. They account for up to 150 million cases worldwide each year. According to various authors, up to 50-60% of women experience an episode of UTI at least once in their lifetime [1, 2]. Clinical symptoms associated with UTIs may vary in severity depending on both the virulent properties of the pathogen and the susceptibility of the organism to infection: from asymptomatic course (asymptomatic bacteriuria) to clinically significant cystitis, pyelonephritis, up to severe urosepsis [3]. Asymptomatic bacteriuria represents bacterial colonization of the urinary tract in the absence of clinical manifestations of the disease. During pregnancy, asymptomatic bacteriuria has been considered for many years as a risk factor for pyelonephritis and adverse pregnancy outcomes (preterm labor, low birth weight, etc.) [4, 5]. Administration of antibacterial drugs for the treatment of asymptomatic bacteriuria during pregnancy may be accompanied by undesirable effects: changes in the composition of the intestinal microbiome of the pregnant woman, which subsequently determines the composition of the microbiota of the newborn; impaired development of the child's immune system [6, 7]. Furthermore, antibiotic therapy can lead to the elimination of potentially protective strains of microorganisms that prevent colonization by virulent uropathogens, thus indirectly contributing to the development of symptomatic UTIs.

The dominant bacterial pathogen among UTIs is *Escherichia coli*, a Gram-negative, motile, facultatively anaerobic bacillus belonging to the order Enterobacterales. *E. coli* is part of the commensal microbial population of the human intestine and maintains stability and homeostasis of the lumenal intestinal microbiota through symbiotic interaction with the human body. *E. coli* strains possessing certain virulence factors are able to adapt to new niches and cause a wide range of diseases of intestinal and extraintestinal localization.

E. coli associated with UTIs are known as uropathogenic *E. coli* (UPEC) [8]. UPEC possess a variety of both structural and secreted virulence factors necessary to realize their pathogenic potential in UTIs. The expression of adhesive organelles such as type 1 pili, P- and S-fimbriae allows UPEC to bind to receptors on the surface of epithelial cells in the urinary tract, colonize the uroepithelium and penetrate cells and tissues, and activate the innate immune response. In addition, S-fimbrial adhesins can be expressed by sepsis- and meningitis-associated *E. coli* (neonatal meningitis-associated *E. coli* (NMEC)). Invasins also play a significant role in the pathogenesis of neonatal meningitis, which are found predominantly in NMEC strains. An important pathogenic factor is toxins (hemolysin, cytotoxic necrotizing factor, vacuolizing vehicle toxin, secreted vehicle toxin) that damage cells and disrupt their metabolism. Production of siderophores (iron-transporting proteins) determines the ability of E. coli to capture iron, which increases viability in the urinary tract. The severity of symptomatic manifestations of UTIs is related to the acquisition and expression of virulence genes. In asymptomatic colonization of UTIs, UPEC are unable to express key virulence factors, which is probably a mechanism of adaptation to prolonged bladder persistence [9].

Based on molecular analysis, E. coli strains are divided into phylogenetic groups: A, B1, B2, C, D, E, F and G [10]. UPECs most often belong to phylogroups B2, D and to a lesser extent to groups E and F, whereas commensal strains, considered less virulent, belong predominantly to phylogroups A or B1 [11].

Numerous studies have shown an association between the presence of virulence genes and UPEC phylogroups [12–14]. However, the number of studies aimed at studying the molecular characterization and assessing the genotypic diversity of UPEC strains isolated in different manifestations of UTIs (especially in asymptomatic bacteriuria) is limited. Genetic determinants of virulence as a criterion for assessing and predicting the course of the infectious process are not currently used. Thus, an urgent direction of molecular genetic research is to study the pathogenic potential of UPEC isolated from pregnant women with asymptomatic bacteriuria to determine their clinical significance, as well as to determine the molecular basis of pathogenesis, develop new diagnostic algorithms and effective treatment methods.

The aim of the study was the molecular and genetic characterization of UPEC isolated from pregnant women with asymptomatic bacteriuria.

Materials and methods

Clinical isolates of *E. coli* (n = 70) were isolated from the urine of pregnant women with IBU who were observed by an obstetrician-gynecologist at the D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology in the years 2018–2023.

The study was conducted with voluntary informed consent of the patients, the study protocol was approved by the local ethical committee of the D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology (protocol No. 114 of 14.12.2021).

The diagnosis of asymptomatic bacteriuria was established when the same microorganism was isolated in the amount of $\geq 10^5$ CFU/mL in 2 consecutive urine samples taken at least 24 h apart, in the absence of clinical manifestations of UTI. If more than 1 microorganism was isolated, the sample was excluded from the study. Bacteriologic examination of clinical material was performed using chromogenic nutrient medium for isolation of UTI pathogens (Brilliance UTI Clarity Agar, Oxoid). The identification results were confirmed by mass spectrometry (MALDI-TOF MS, Bruker Dal-

tonics). The cultures were stored in trypticase-soy broth with addition of 30% glycerol at –70ºC.

DNA extraction was performed using the DNA-sorb AM reagent kit (Central Research Institute of Epidemiology).

The affiliation of *E. coli* strains to phylogenetic groups was determined by quadriplex-polymerase chain reaction (PCR) according to O. Clermont et al. [10].

All isolates were tested for 15 virulence markers: genes associated with adhesion (*fimH*, *papC*, *sfa*, *afa*, *focG*); invasion (*ibeA*), toxin synthesis (*cnf1*, *hlyA*, *sat*, *vat*, *usp*), siderophores (*fyuA*, *iroN*, *iuc*), and capsular antigen (*kpsMII*). Previously studied primers were used; PCR primers were synthesized by Syntol LLC [15-21]. For PCR amplification we used the Tersus plus PCR reagent kit (Eurogen) and Tertsik thermocycler (DNA-Technology). The amplicons were separated in 2% agarose gel. Data were visualized and documented using the Infinity gel documentation system (Vilber Lourmat).

Fisher's exact test was used to assess the statistical significance of differences in mean values. Differences with a confidence interval (CI) of 95% ($p < 0.05$) were considered statistically significant.

Results

Phylogenetic analysis of *E. coli* sequences isolated in pregnant women with asymptomatic bacteriuria showed that clinical isolates belonging to phylogroup B2 (51.4%) were significantly more frequent ($p < 0.05$); the remaining isolates belonged to phylogroups D, A, B1, and F (**Table 1**).

Analysis of virulence factors associated with adhesion showed that the *fimH* gene was detected in 97.1% of the strains studied; *rarC*, in 34.3%; *sfa*, in 27.1%; *focG*, in 11.4%; and *afa*, in 2.9%. The *ibeA* gene responsible for endothelial cell invasion was not detected among UPEC isolated in pregnant women with asymptomatic bacteriuria. The most common gene encoding toxin synthesis was *vat* (42.9%); *hlyA*, *cnf1*, and sat genes were detected in 21.4, 22.9, and 32.9% of isolates, respectively. The uropathogen-specific *usp* protein was detected in 57.1% of isolates. Among the genes associated with siderophore production, the *fyuA* gene was detected in 78.6% of the isolates, *iroN* in 48.6%, and *iuc* in 37.1%. The gene encoding capsule antigen synthesis (*kpsMII*) was detected in 65.7% of the studied *E. coli* strains.

Between 1 and 12 virulence genes were present in UPEC clinical isolates. None of the 70 *E. coli* strains contained all 15 virulence markers included in the study. Phylogenetic group A was significantly more frequently represented by isolates with 1 virulence gene (75%); the remaining strains were characterized by a combination of 2 (12.5%) and 5 (12.5%) genes without statistically significant differences. Isolates belonging to phylogenetic group B1 had a combination of 2 (33.3%) and 4 (50%) virulence markers in their genome without statistically significant differences; 1 (16.7%) strain had 1 virulence gene in its genome. Phylogenetic group B2 was characterized by the highest number of genes in various combinations (from 5 to 12); isolates containing combinations of 7 (11.1%), 8 (25%), 9 (13.9%), 10 (25%) and 12 (2.8%) genes in their genome were significantly more frequent. Phylogenetic groups D and F were represented by isolates in which genes encoding virulence factors were present without significant differences in combinations of 2 to 8 and 3 to 8, respectively.

Depending on the types of UPEC pathogenicity factors present, all isolates were divided into 6 clusters (**Table 2**).

Table 1. Belonging to phylogenetic groups of *E. coli* isolated from pregnant women with asymptomatic bacteriuria

Indicator	Phylogroup				
	A	B1	B2	D	F
Number of isolates	8	6	36	14	6
%	11,4	8,6	51,4	20,0	8,6
95% CI	5.1–21.3	3.2–17.7	39.2–63.6	11.4–31.3	3.2–17.7

Table 2. Frequency of detection of pathogenicity factors in uropathogenic *E. coli* isolated from pregnant women with asymptomatic bacteriuria

Pathogenicity factor	*n*	%	95% CI
Adhesion	7	10	4.1–19.5
Adhesion + siderophores	11	15,7	8.1–26.4
Adhesion + toxins	1	1.4	0.04–7.7
Siderophores + capsules	1	1.4	0.04–7.7
Adhesion + siderophores + capsules	2	2.9	0.03–12.6
Adhesion + siderophores + toxins	4	5.7	1.8–14.2
Adhesion + siderophores + toxins + capsules	44	62.9	50.5–74.1

DOI: https://doi.org/10.36233/0372-9311-518

Genetic determinants encoding 1 pathogenicity factor were identified in the genomes of 7 isolates (7%, 95% CI 4.1–19.5). The frequency of isolates containing combinations of 4 factors (62.9%, 95% CI 50.0–74.1) was statistically significantly ($p < 0.0001$) different from isolates characterized by the presence of combinations of genes encoding 2 and 3 pathogenicity factors.

The highest number of virulence genes was detected in strains belonging to phylogroup B2; *focG*, *afa*, *cnf1*, and *ibeA* genes were undetectable in phylogroup D; 8 virulence genes were detected in phylogroup F, except for *afa*, *sfa*, *focG*, *cnf1*, *hlyA*, *vat*, and *ibeA* (**Table 3**). The lowest gene diversity was detected in phylogroups A and B1. Virulence genes associated with adhesion (*sfa*, *focG*), encoding toxin synthesis (*hlyA*, *cnf1*, *vat*, *usp*), siderophores (*fyuA*, *iroN*, hlyA) and capsules (*kps*) were statistically significantly more frequent ($p < 0.05$) in isolates of phylogenetic group B2 compared to isolates of other phylogenetic groups. Statistically significant differences were found in the frequency of occurrence of the *kps* gene ($p < 0.01$) in UPEC isolates belonging to phylogenetic group A; *vat* ($p < 0.05$) and *usp* ($p < 0.001$) genes belonging to phylogenetic group D.

Discussion

Studies on the molecular and genetic characterization of uropathogenic *E. coli*, the results of which are currently available for analysis, were based on the assessment of the pathogenic potential of strains isolated in UTIs. Given the significant impact of asymptomatic bacteriuria on the development of pregnancy complications, we conducted a study aimed at investigating *E. coli* strains isolated from patients with this pathology.

Clinical isolates of *E. coli* isolated from pregnant women with asymptomatic bacteriuria belonged to 5 phylogenetic groups (A, B1, B2, D and F). It was shown that the dominant phylogroups were B2 and D (to a lesser extent) with 51.4 and 20%, respectively, which is consistent with the results of other studies [11]. According to the extended classification by O. Clermont (2013), phylogroup F is a subgroup of phylogroup B2, and *E. coli* belonging to this group are also considered uropathogenic. In our study, 8.6% of the isolates in asymptomatic bacteriuria were attributed to this phylogroup. *E. coli* belonging to phylogenetic groups A (11.4%) and B1 (8.6%) were also found to be associated with commensal isolates; this suggests that the main reservoir of *E. coli* that are able to colonize the urinary tract is the intestine.

Adhesion factors play a key role in the pathogenesis of UTIs by facilitating the attachment of *E. coli* to the uroepithelium. Overall, adhesion factors in our study were detected in isolation or in various combinations in 69 (98.6%) isolates, confirming the role of adhesins as a major urovirulence factor. According to numerous studies, the *fimH* gene is the most common adhesion gene encoding type 1 fimbriae, which was confirmed

Table 3. Occurrence of virulence genes in *E. coli* of various phylogenetic groups isolated from pregnant women with asymptomatic bacteriuria

Virulence factors	Gen	Frequency of gene occurrence, *n* (%)				
		A (*n* = 8)	B1 (*n* = 6)	B2 (*n* = 36)	D (*n* = 14)	F (*n* = 6)
Adhesins	*papC*	0	0	15 (41.7%)	5 (35.7%)	4 (66.7%)
	afa	0	0	2 (5.6%)	0	0
	fimH	8 (100.0%)	6 (10.0%)	36 (100.0%)	13 (92.8%)	6 (100.0%)
	sfa	0	0	18^{+***} (50.0%)	1 (7.1%)	0
	focG	0	0	8^{+**} (22.2%)	0	0
Invasins	*ibeA*	0	0	0	0	0
Siderophore	*fyuA*	2 (25.0%)	4 (66,7%)	33^{+**} (91.7%)	12 (85.7%)	4 (66.7%)
	iroN	0	4 (66,7%)	25^{+***} (69.4%)	3 (21.4%)	2 (33.3%)
	iuc	1 (12.5%)	3 (50,0%)	14 (38.9%)	4 (28.5%)	4 (66.7%)
Toxins	*hlyA*	0	0	14^{+***} (38.9%)	1 (7.1%)	0
	cnf1	0	0	16^{+***} (44.4%)	0	0
	sat	1 (12.5%)	0	14 (38.9%)	7 (50.0%)	2 (33.3%)
	vat	0	0	30^{+***} (83.3%)	2^{-*} (14.3%)	0
	usp	0	0	36^{+****} (100.0%)	1^{-***} (7.1%)	3 (50.0%)
Capsules	*kpsMII*	1^{-**} (12.5%)	0	32^{+****} (88.9%)	9 (64.3%)	4 (66.7%)

Note. $^{-}$ — the frequency of gene occurrence in the group is lower than the frequency of occurrence of the same gene in the overall sample; $^{+}$ — the frequency of gene occurrence in the group is higher than the frequency of occurrence of the same gene in the overall sample.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

by the results of our study: the *fimH* gene was detected in the majority of isolates (97.1%). Virulence factors associated with the development of ascending infection (pyelonephritis) play an important role in the pathogenesis of UTIs, especially in pregnant women. The *rarC* gene (pyelonephritis-associated pili) and *afa*-adhesins (associated with the development of gestational pyelonephritis) were found in 34.3 and 2.9% of isolates in our study, respectively. The prevalence of genes encoding fimbrial adhesins *sfa* and *focG*, which are expressed by strains causing meningitis, sepsis and pyelonephritis, was 34.7 and 12.9%, respectively. The results of similar studies on the genetic determinants of virulence of UPEC isolated in asymptomatic bacteriuria show a lower prevalence of *rarC* (12.9% to 20.6%), *sfa* (8.1% to 16.9%), but a higher frequency of *afa* (34.9%) and *focG* (35.1%) genes detected [20, 22, 23].

The urinary tract can be a source of NMEC, which is one of the most common infections with high morbidity and mortality in the neonatal period [24]. The *ibeA* gene, which is one of the important virulence factors of NMEC and is responsible for endothelial cell invasion, was not detected among *E. coli* isolated from pregnant women with asymptomatic bacteriuria.

UPEC is characterized by the presence of a uropathogen-specific protein, bacteriocin-like toxin, associated with the development of pyelonephritis and bacteremia. The appearance of this virulence marker is often associated with an increase in the virulence of the strain and its survival in the urinary tract. The results of our study showed a higher detection rate of the *usp* gene (57.1%) compared to the results of similar studies, where the detection rate of the *usp* gene in UPEC isolated in asymptomatic bacteriuria was 22.6–34.4% [20, 22].

Toxin formation is characteristic of *E. coli* strains responsible for more severe forms of disease (pyelonephritis, urosepsis). The *hlyA* and *cnf1* genes associated with toxin synthesis were found in 68.2 and 63.6% of isolates from pyelonephritis cases; in 19.4 and 25.8% of those isolated in asymptomatic bacteriuria [22]. The results of our study showed a similar pattern — *hlyA* and *cnf1* genes were detected in 21.4 and 22.9% of isolates from pregnant women with asymptomatic bacteriuria. Secreted autotransport toxin (*sat*) is also a virulence factor characteristic of UPEC isolated in pyelonephritis. In our study, the detection rate of *sat* gene was 32.9%, which is significantly higher than that of L. Maniam et al. (7.5%) [20]. The *vat* gene is found in more than half of *E. coli* isolates from patients cystitis and pyelonephritis [25]. In our study, the *vat* gene was detected in 42.9% of *E. coli* isolates isolated in asymptomatic bacteriuria.

The production of siderophores, which play an important role in iron capture, increases the viability of microorganisms within the urethral tract. The presence of these virulence factors in UPEC appears to compensate for the absence of other virulence genes related to adhesion and toxin formation, and thus promotes prolonged colonization in the urinary tract without inducing an inflammatory response in the host. In the study performed, genes responsible for the synthesis of yersinebactin (*fyuA*), salmochelin (*iroN*) and aerobactin (*iuc*) were identified in 78.6%, 48.6% and 37.1% of isolates, respectively.

The capsule has a protective role against the host immune system, which promotes long-term persistence in the urinary tract. We detected the *kpsMII* gene in 65.7% of the isolates from patients with asymptomatic bacteriuria. Similar studies have shown that the frequency of this gene ranged from 38 to 73% [20, 23].

The presence of individual virulence genes in the genome is not sufficient for the realization of uropathogenic potential. Many studies suggest that several virulence factors are present in bacteria at once [22, 26]. Our data also indicate the genetic diversity of UPEC isolated in asymptomatic bacteriuria. The results of quantitative distribution of virulence factors in phylogenetic groups showed that 90% of *E. coli* isolates had two or more virulence markers. *E. coli* belonging to phylogroups B2, D, E, and F associated with UPEC contained more virulence genes than phylogroups A and B1 associated with commensal strains. Furthermore, *E. coli* belonging to phylogenetic group B2 possessed the highest number of genes in various combinations (5 to 12); genes associated with adhesion (*sfa*, *focG*), synthesis of siderophores (*fyuA*, *iroN*, *iuc*), toxins (*hlyA*, *cnf1*, *vat*, *usp*) and capsules (*kpsMII*) were found significantly more frequently ($p < 0.05$).

Numerous factors (immune response, ability to form biofilms) may influence the expression of virulence factors and the ability of UPEC isolates to adapt to persistence in UTIs. When comparing the virulence properties of UPEC isolates in asymptomatic bacteriuria and symptomatic UTIs (cystitis, pyelonephritis) in pregnant women, it was shown that E. coli isolates in asymptomatic bacteriuria and cystitis showed comparable virulence rates [23]. The determination of a large number of virulence genes in the isolate, according to the researchers, may indicate the uropathogenic potential of the pathogen, and the ability to manifest depends on the expression of this gene or a set of genes.

Conclusion

The molecular characterization and genotypic diversity of *E. coli* strains are essential for better understanding of their role in the pathogenesis of UTIs. Identification of key virulence determinants and/or virulence gene combinations may be a marker for predicting the course of UTIs, especially in pregnant women, and will enhance the diagnostic capabilities based on the virulence properties of the uropathogen

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The article was submitted 25.04.2024;
accepted for publication 17.06.2024;
published 29.08.2024

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Статья поступила в редакцию 25.04.2024;
принята к публикации 17.06.2024;
опубликована 29.08.2024

Original Study Article
https://doi.org/10.36233/0372-9311-528

Virulence and tissue tropism of different epidemiologically significant SARS-CoV-2 variants for golden Syrian hamsters

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Abstract

Introduction. Animal models for SARS-CoV-2 infection, reproducing the clinical features of COVID-19 in humans, are important tools for studying the pathogenesis of the disease, transmission of the pathogen and are indispensable for testing antiviral drugs and vaccines.

The aim of the study was to assess the virulence and tissue tropism for golden Syrian hamsters of SARS-CoV-2 strains belonging to different variants of concern: Wuhan-like, Delta, Omicron BA.1.1 and Omicron BA.5.2.

Materials and methods. Hamsters were intranasally infected with different SARS-CoV-2 strains. Virulence and tissue tropism of SARS-CoV-2 strains were assessed by comparing the dynamics of weight, viral load in organs and histopathological changes in lungs in infected and uninfected animals.

Results. The Wuhan-like Dubrovka strain had the greatest virulence for hamsters, which was manifested by the development of severe pneumonia and a delay in weight gain by 14.6%, high virus content in the lungs, nasal passages and brain — 6.2, 5,9 and 3.7 log10 $TCID_{50}$/ml of homogenate, respectively. Presumably, it was the infection of the Wuhan-like virus of the central nervous system that negatively affected the weight and general condition of the animals. When hamsters were infected with viruses belonging to the Delta and Omicron variants, the observed minor weight loss in animals was uninformative, so indicators such as lung histopathology, viral load in the lungs, nasal passages, heart and other organs played a decisive role in assessing the virus pathogenicity. A score assessment of lung histopathology was of particular value in assessing the severity of pneumonia, since it reduced subjectivity in evaluating the results of histological examination and provided a semi-quantitative assessment of the pathological process.

Conclusion. Despite the revealed lower virulence for hamsters of viruses belonging to the Delta and Omicron variants compared to the ancestral Wuhan virus, this animal model for COVID-19 retains its value for conducting preclinical trials of antiviral drugs.

Keywords: *animal model for COVID-19, golden Syrian hamsters, virulence, tissue tropism, epidemiologically significant SARS-CoV-2 variants*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July 2010). The research protocol was approved by the Ethics Committee of the I.I. Mechnikov Research Institute for Vaccines and Sera (protocol No. 2, May 24, 2021).

Funding source. Исследование выполнено за счёт гранта Российского научного фонда № 23-25-00146, https://rscf.ru/project/23-25-00146/. В исследовании использовано научное оборудование центра коллективного пользования «НИИВС им. И.И. Мечникова» — при финансовой поддержке проекта Российской Федерацией в лице Минобнауки России.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Gracheva A.V., Drokov A.O., Smirnova D.I., Khokhlova D.M., Korchevaya E.R., Pankratov A.A., Trunova G.V., Khokhlova V.A., Vorontsova M.S., Leneva I.A., Svitich O.A., Zverev V.V., Faizuloev E.B. Virulence and tissue tropism of different epidemiologically significant SARS-CoV-2 variants for golden Syrian hamsters. *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):470–482.
DOI: https://doi.org/10.36233/0372-9311-528
EDN: https://www.elibrary.ru/jukmca

Оригинальное исследование
https://doi.org/10.36233/0372-9311-528

Вирулентность и тканевая специфичность разных эпидемически значимых вариантов SARS-CoV-2 для золотистых сирийских хомячков

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Аннотация

Введение. Животные модели инфекции SARS-CoV-2, воспроизводящие клинические особенности COVID-19 у человека, являются важными инструментами изучения патогенеза заболевания, трансмиссии возбудителя и незаменимы при испытаниях противовирусных лекарственных препаратов и вакцин.

Целью исследования являлась оценка вирулентности и тканевой специфичности для золотистых сирийских хомячков штаммов SARS-CoV-2, относящихся к разным эпидемически значимым вариантам: Ухань-подобному, Delta, Omicron BA.1.1 и Omicron BA.5.2.

Материалы и методы. Хомячков интраназально заражали разными штаммами SARS-CoV-2. Вирулентность и тканевую специфичность штаммов SARS-CoV-2 оценивали путём сравнения динамики массы, вирусной нагрузки в органах и выраженности патоморфологических изменений в лёгких у заражённых и незаражённых животных.

Результаты. Наибольшей вирулентностью для хомячков обладал Ухань-подобный штамм, что проявлялось в развитии тяжёлой пневмонии и задержке в приросте массы на 14,6%, высоком содержании вируса в лёгких, носовых ходах и головном мозге — 6,2, 5,9 и 3,7 lg $ТЦД_{50}$/мл гомогената соответственно. Предположительно именно поражение Ухань-подобным вирусом центральной нервной системы негативно повлияло на показатели массы и общее состояние животных. При заражении хомячков штаммами, относящимися к вариантам Delta и Omicron, незначительная потеря массы животными была неинформативной, поэтому при оценке патогенности вируса решающую роль играли такие показатели, как гистопатология лёгких, вирусная нагрузка в лёгких, носовых ходах, сердце и других органах. Особую ценность при сравнении тяжести пневмонии имела балльная оценка выраженности патоморфологических изменений в лёгких, поскольку она снижала субъективизм в оценке результатов гистологического исследования и давала полуколичественную оценку патологического процесса.

Заключение. Несмотря на выявленную более низкую вирулентность для хомячков штаммов, относящихся к вариантам Delta и Omicron, по сравнению с родоначальным Уханьским вирусом, данная животная модель COVID-19 сохраняет свою ценность для проведения доклинических испытаний противовирусных препаратов.

Ключевые слова: *животная модель COVID-19, золотистые сирийские хомячки, вирулентность, тканевая специфичность, эпидемически значимые варианты SARS-CoV-2*

Этическое утверждение. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с «Consensus Author Guidelines for Animal Use» (IAVES, 23.07.2010). Протокол исследования одобрен Этическим комитетом НИИВС им. И.И. Мечникова (протокол № 2 от 24.05.2021).

Источник финансирования. Исследование выполнено за счёт гранта Российского научного фонда № 23-25-00146, https://rscf.ru/project/23-25-00146/. В исследовании использовано научное оборудование центра коллективного пользования «НИИВС им. И.И. Мечникова» — при финансовой поддержке проекта Российской Федерацией в лице Минобрнауки России.

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

Для цитирования: Грачева А.В., Дроков А.О., Смирнова Д.И., Хохлова Д.М., Корчевая Е.Р., Панкратов А.А., Трунова Г.В., Хохлова В.А., Воронцова М.С., Ленева И.А., Свитич О.А., Зверев В.В., Файзулоев Е.Б. Вирулентность и тканевая специфичность разных эпидемически значимых вариантов SARS-CoV-2 для золотистых сирийских хомячков. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):470–482.
DOI: https://doi.org/10.36233/0372-9311-528 EDN: https://www.elibrary.ru/jukmca

DOI: https://doi.org/10.36233/0372-9311-528

Introduction

Modeling viral diseases in laboratory animals is one of the most important problems of medical virology. The emergence in 2019 and global spread of SARS-CoV-2 coronavirus (*Severe acute respiratory syndrome-related coronavirus* species, *Betacoronavirus* genus, *Coronaviridae* family), accompanied by a high rate of hospitalization and mortality among those who became ill, necessitated the urgent development of treatments and specific prophylaxis for COVID-19, which is impossible without preclinical testing in adequate animal models of the disease. Since the beginning of the pandemic, considerable efforts have been made to develop effective and safe vaccines and therapeutic agents, and studies of the pathogenesis and features of the immune response to SARS-CoV-2 infection have been conducted [1]. The success of these studies largely depended on the availability of animal models for coronavirus infection developed in the first decade of the 2000s against the background of the threat of worldwide spread of SARS-CoV-1, the causative agent of severe acute respiratory syndrome [2], which belongs to the same species as SARS-CoV-2. Animal models for infection that reproduce clinical and pathological features of COVID-19 in humans are important tools for studying the pathogenesis of the disease, pathogen transmission and are indispensable for testing new antiviral drugs and vaccines [3–5].

To date, there are several animal models for COVID-19, primarily based on representatives of primate, carnivore and rodent groups. However, the problem of selecting the most adequate, informative and convenient model remains relevant. The value of primate-based animal models for coronavirus pneumonia lies in the fact that monkeys are similar to humans in their physiological characteristics and immune regulation. Rhesus macaques, African green monkeys, baboons, and common marmosets are most often used for COVID-19 modeling [1, 6, 7]. The main drawbacks of such models are the huge demand for animals, high cost, shortage of trained personnel and primate vivariums equipped according to biosafety level 3 requirements [1, 6].

Mink, ferrets and cats are also susceptible to SARS-CoV-like coronaviruses [7-11]. Notably, SARS-CoV-2 is found in the nasal cavity of ferrets and they can be infected by indirect contact, indicating the ability of ferrets and mink to transmit the virus by mimicking the SARS-CoV-2 transmission pathway in humans. A disadvantage of such models is that these animals are relatively large carnivores, so handling them is difficult. Therefore, there is a need for models based on small laboratory animals that are susceptible to the virus.

Mice and other rodents are most commonly used to model COVID-19. However, wild-type mice are not susceptible to infection with the ancestral Wuhan-like virus SARS-CoV-2 [5, 6, 12] because the virus is able to bind efficiently to the human ACE2 receptor (hACE2) but not to murine ACE2 (mACE2). Our previous findings indicate that Wuhan-like virus does not cause productive infection in BALB/c mice and, in contrast, when infected with Omicron-like virus multiplies in lungs, brain tissue, and other organs [5].

Several lines of genetically modified mice with hACE2 receptor are known, which have been adapted for studies on the pathogenesis of cardiovascular diseases and modeling of coronavirus infection [6, 12]. These transgenic mouse lines, with different origin, are capable of stable hACE2 expression in many organs. The mouse model also has some serious limitations, including differences in hACE2 expression patterns in different organs and tissues in humans and mice. Because hACE2 expression in transgenic mice is not physiologic, infection with SARS-CoV-2 can cause clinical manifestations and pathologic changes uncharacteristic of humans [1]. In addition, transgenic mice are not widely available in Russia and are characterized by high cost.

Among SARS-CoV-2 susceptible animals, the golden Syrian hamster (*Mesocricetus auratus*; hereafter hamsters) is of particular interest. Genetic comparison of hACE2 with analogous receptors of other mammals showed that the amino acid sequence of hamster ACE2 is very similar to that of the analogous human receptor, with which it has only 3-4 differences. In addition, hamster ACE2 has shown high affinity for the S-protein of SARS-CoV-2 and SARS-CoV in several studies [1, 3, 7].

The hamster model for coronavirus pneumonia is widely used in preclinical studies of vaccines and drugs [13]. Symptoms, disease pathogenesis and immune responses characteristic of COVID-19 in humans are well reproduced in hamsters [3, 14]. Hamsters are also in demand for modeling other human respiratory viral infections [14] caused by viruses such as SARS-CoV-1 [2], influenza viruses [15, 16] and adenoviruses [14, 17]. With advantages such as high reproduction rate, easy handling, affordable cost and availability in nurseries, hamsters are an optimal choice compared to other small laboratory animals.

Coronavirus disease caused in hamsters by Wuhan-like strains of SARS-CoV-2 has been well studied and characterized to date [13, 18-20]. Since at the current stage of the epidemic process, the ancestral SARS-CoV-2 virus has been replaced by new variants of concern (first Delta, then Omicron and its progeny), it is of interest to study their virulence and disease pathogenesis in infected hamsters. Previously, we conducted a study of the protective activity of a prototype live attenuated vaccine against SARS-CoV-2 in hamsters, which included their infection not only with the parental Wuhan-type virus, but also with strains belonging to Delta and Omicron variants [21]. In this article, we considered it appropriate to review and discuss the results obtained in more detail in the context of the pathogenicity of different virus variants for non-immunized hamsters.

The aim of the study was to assess the virulence and tissue specificity of SARS-CoV-2 strains belonging to different variants of concern in Syrian golden hamsters.

Materials and methods

Virus

Laboratory strains of SARS-CoV-2 isolated at the I.I. Mechnikov Research Institute of Virus Diseases from patients with confirmed diagnosis of COVID-19 during different periods of the pandemic were used in the study (**Table 1**). All works with SARS-CoV-2 virus were conducted in the conditions of the biosafety level 3 laboratory.

SARS-CoV-2 was cultured in Vero CCL81 (ATCC) kidney epithelial cell culture of African green monkey (hereinafter referred to as Vero) at 37ºC in DMEM medium based on Earle's buffer (PanEco) with 5% fetal bovine serum (Gibco), 300 μg/mL L-glutamine (PanEco), 40 μg/mL gentamicin (PanEco) in an atmosphere of 5% CO_2. A three-day-old monolayer of Vero cells was infected with SARS-CoV-2 virus at a multiplicity of infection MOI = 0.001. Virus adsorption was performed in a CO_2 incubator for 60 min, then maintenance medium (DMEM, 300 μg/mL L-glutamine, 40 μg/mL gentamicin) was added and incubated at 37ºC until the appearance of pronounced cytopathic action (CPA) in an atmosphere of 5% CO_2. After the appearance of pronounced CPA, the culture fluid was clarified by centrifugation at 4000 rpm for 10 min and stored at -80ºC until used in experiments.

The titer of SARS-CoV-2 was determined in Vero cell culture by CPA endpoint. Tenfold dilutions of virus in 4 repeats were added to the wells of a 96-well plate with a 3-day-old monolayer of Vero cells and incubated for 5 days at 37ºC in an atmosphere of 5% CO_2. Titration results were evaluated by microscopic examination of the cell monolayer for the presence of characteristic CPA (rounding of cells and detachment of cells from the monolayer). Virus titer was calculated as described by M.A. Ramakrishnan et al. [22], and expressed in log10 $TCID_{50}$/mL.

Animal models

4-week-old female SPF hamsters ($n = 30$) weighing 40-45 g (SPP Nursery for Laboratory Animals, Branch of the Institute of Bioorganic Chemistry of the RAS, Russia) were used in this work. Hamsters were randomly distributed into groups. The animals were kept in accordance with the rules for the arrangement, equipment and maintenance of experimental and biological clinics. The animals were fed with briquetted feed according to the approved norms. The authors complied with institutional and national standards for the use of laboratory animals in conducting the experimental animal study. The conduct of the study was approved by the Ethical Committee of the I.I. Mechnikov Research Institute of Veterinary Medicine (protocol No. 2 of 24.05.2021).

Study design

The study design is schematically presented in **Fig. 1**. Hamsters were divided into 5 groups of 6 animals each and intranasally infected with different virus strains (Table 1) at a dose of 10^4 $TCID_{50}$/head (100 μl each). For intranasal infection, animals were anesthetized and held in an upright position. The negative control group received an equivalent volume of phosphate-salt buffer pH 7.2. Weight control was performed daily. Four days after infection, the animals were humanely euthanized. The right hamster lung was fixed in 10% neutral buffered formalin for histologic examination. Tissues of lung, brain, nasal passages, heart, liver, spleen, kidney and blood were collected, homogenized in 1 ml DMEM medium with gentamicin (40 μg/ml, PanEco) using a Tissue Lyser LT homogenizer (Qiagen) and centrifuged at 10,000 rpm for 5 min at 4ºC. The supernatant was collected for measurement of virus titers and viral RNA concentration and stored at –80ºC until examination. Changes in body weight from day 1 to day 4 after infection, virus titer and viral RNA content in organs and tissues, and severity of inflammatory changes in the lungs of animals on day 4 after infection reflected the virulence of the strain, and the distribution of viral RNA and infectious virus in organs and tissues reflected its tissue specificity.

SARS-CoV-2 RNA quantification

The accumulation of viral RNA in organs and tissues was assessed by quantitative reverse transcription polymerase chain reaction as described previously [23]. Viral RNA was isolated from samples using the MagnoPrime UNI reagent kit (NextBio). To detect viral RNA, primers and probe designed for the SARS-CoV-2 nucleocapsid (N) gene were used, as proposed by J. Chan et al. [24].

Table 1. Characteristics of SARS-CoV-2 strains used in the study

Strain	Collection date	GenBank ID	Variant	Pangolin lineage	Passage level	Titer, log10 $TCID_{50}$/ml
Dubrovka	04.06.2020	MW514307.1	Wuhan-like	B.1.1.317	17	7,85
Podolsk	10.08.2021	ON032860.1	Delta	AY.122	16	7,0
Otradnoe	25.01.2022	ON032857.1	Omicron	BA.1.1	8	6,0
FEB2	11.10.2022	OP920753.1	Omicron	BA.5.2	4	6,5

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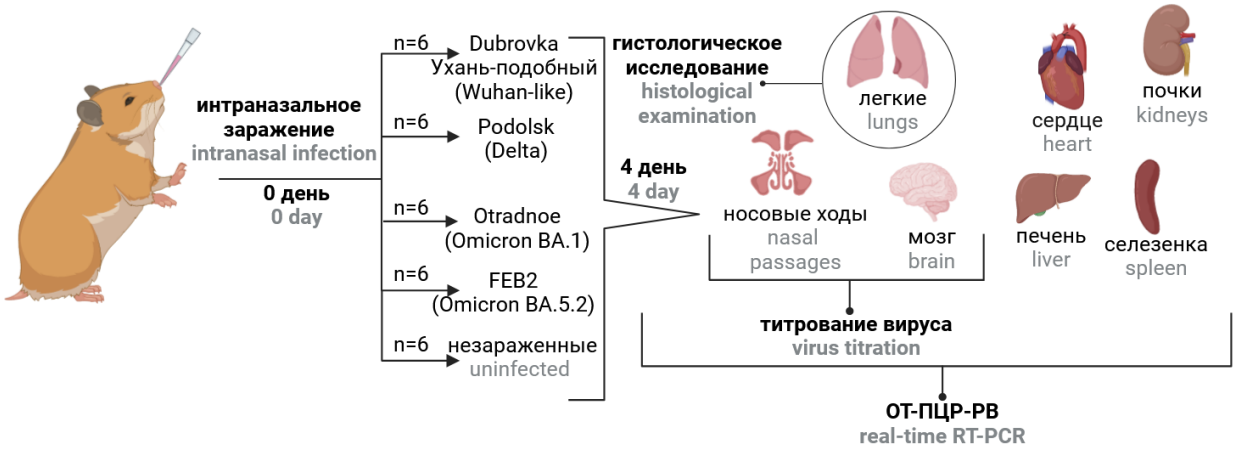

Fig. 1. Study design.
The infection dose of 4.0 log10 $TCID_{50}$ per animal.

Histologic examination of the lungs

The right hamster lung was fixed in 10% neutral buffered formalin (BioVitrum) for 24 h, dehydrated according to the standard histological technique and placed in Histomix paraffin medium (BioVitrum). On the Leica RM 2125 RTS rotary microtome we made stepwise longitudinal sections 3-5 microns thick, the preparations were stained with hematoxylin and eosin, enclosed in Canadian balsam (Sigma-Aldrich). Histological preparations were examined using a BX51 light microscope (Olympus). Photofixation of the obtained lung histologic preparations was performed with the help of an Olympus XC10 camera. Pathomorphologic changes in the lungs were evaluated by 2 specialists using a blind method, using a combined severity score from 0 to 3 for each of the morphologic criteria proposed by A.D. Gruber et al. [25]. The maximum possible score was 60.

Statistical processing of data

Statistical analysis was performed using the Graphpad Prism v. 8.0.01 software. Data are presented in graphs as mean, standard deviation (SD), standard error (SE), median, upper and lower quartiles. In box plots, the boundaries of the box are the upper and lower quartiles of the sample (25% and 75%), the ends of the whiskers are the boundaries of a statistically significant sample (without outliers), the line in the box itself is the median of the data. Statistical processing of the obtained results was carried out using the nonparametric method (Mann-Whitney U-test). Differences were considered statistically significant at $p < 0.05$.

Results

Morphologic changes were absent in histologic preparations of the right lung of uninfected animals (**Fig. 2**).

On the 4th day after infection, broncho-interstitial pneumonia was detected in histologic preparations of hamster lungs of all groups (**Fig. 2**). However, there were significant differences in the severity and prevalence of alterative-inflammatory changes between the groups.

On the 4th day after infection, the groups of animals infected with Wuhan-like Dubrovka strain and FEB2 strain (BA 5.2) showed similar in nature and severity inflammatory changes, the morphological picture of which corresponded to bronchointerstitial pneumonia in the viral stage. The lumen of bronchi and bronchioles in the foci of pneumonia often contained cellular debris, macrophages and neutrophils. The integrity of epithelial lining was focally disturbed due to migration of lymphoid cells, dystrophy, necrosis and desquamation of epitheliocytes. There were loci of epithelial hyperplasia. The wall of bronchi and bronchioles was moderately infiltrated with lymphocytes, histiocytes with a small admixture of polymorphonuclear lymphocytes. Dilated lymphatic vessels located along the course of the bronchial tree contained clusters of lymphocytes. Large lymphoid accumulations (hyperplasia of bronchoassociated lymphoid tissue) were found in the bronchial bifurcation zones. Inflammatory changes were also observed in the walls of medium and small branches of the pulmonary artery accompanying the airways. Perivascular lymphoid tissue was in a state of sharp hyperplasia.

Large confluent foci of pneumonia were observed in all lobes of the organ and were located along the course of the bronchial tree, spreading to the periphery. Their area, estimated at qualitative level, occupied 50-90% of the histologic section area of the organ. Respiratory department in the pneumonia foci represented airless fields, lumen of alveoli in which were not defined, interalveolar septa were destroyed due to expressed lymphoid-histiocytic infiltrate with insignificant admixture of neutrophils. Remains of dead cells nuclei, fibroblasts, erythrocytes were seen among the cells of inflammatory infiltrate. In fresher areas of pneumonia airiness of respiratory section was reduced due to sharp

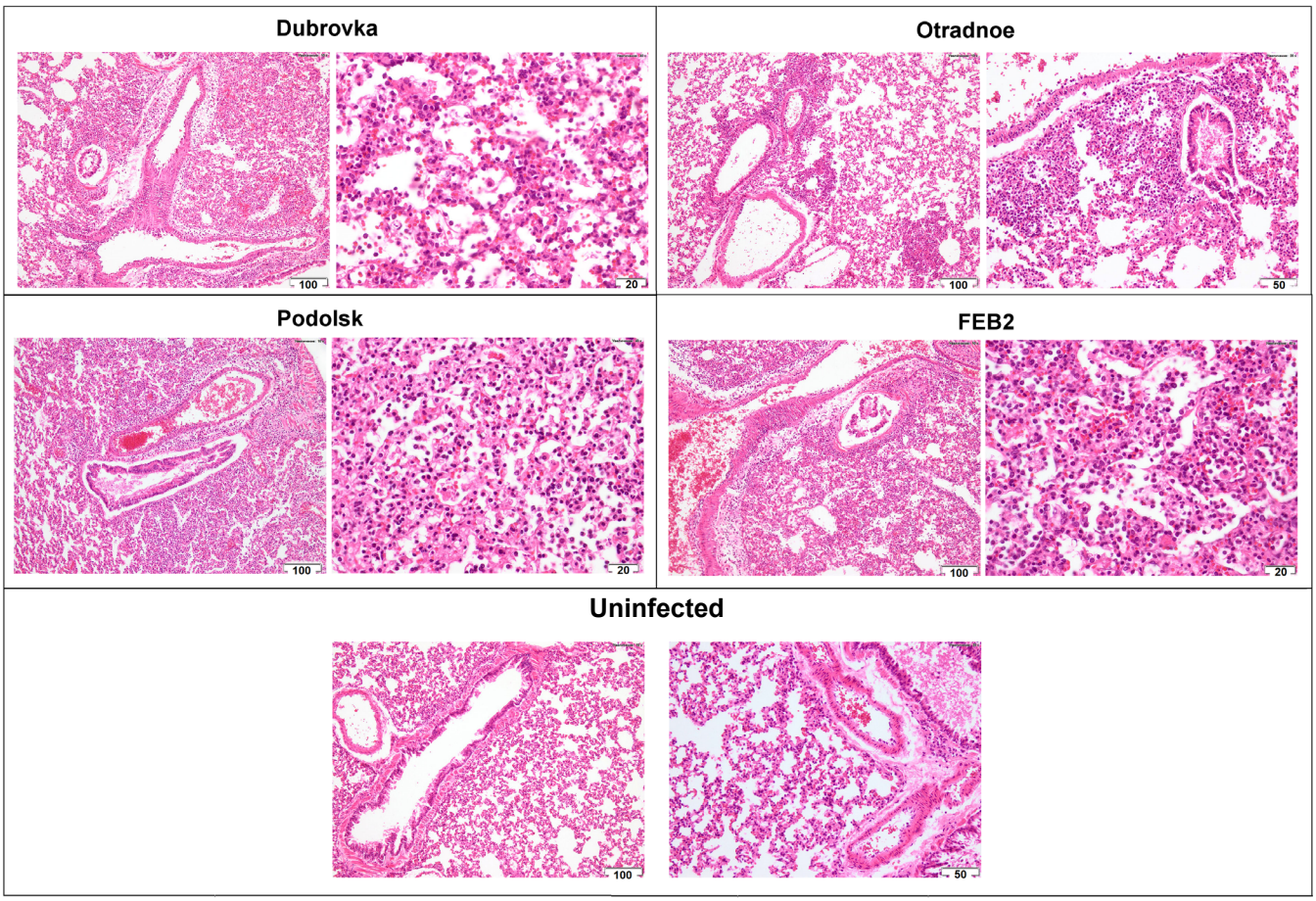

Fig. 2. Bronchointerstitial pneumonia in hamster on the 4th day post-challenge with different SARS-CoV-2 strains.

thickening of interalveolar septa and expressed exudation into the alveolar cavity of liquid blood and cells of inflammatory infiltrate: macrophages, lymphocytes, erythrocytes. Many alveoli contained eosinophilic filamentous material (presumably fibrin). In the interalveolar septa there was microvascular hypertension, interstitial edema and diffusely scattered lymphoid-histiocytic infiltrate.

On the 4th day after infection with Podolsk (Delta) strain, the severity and prevalence of inflammatory changes in hamster lungs were lower compared to those in the groups of animals infected with Wuhan-like virus and FEB2 strain (BA.5.2). Small foci of interstitial pneumonia were not located in all lobes, were located along the course of large lobular and segmental bronchi, and their area did not exceed 50% of the histologic section area of the organ. The lumen of bronchi and bronchioles in the foci of pneumonia were mostly free, contained single macrophages, lymphocytes, small groups of desquamated epitheliocytes. The epithelial lining looked preserved over a large length, with single lymphocytes in the field of view of the ×20 objective lens among the cells of the mesenteric epithelium. Airiness of pulmonary parenchyma in the foci of pneumonia was reduced due to thickening of interalveolar septa. Small groups of macrophages, lymphocytes, single neutrophils, erythrocytes and few dead cells (presumably, alveolocytes) were observed in the alveolar cavity. Proteinaceous exudate in the lumen of the alveoli was rare. At this period of the experiment airless and confluent foci of pneumonia were practically absent.

In histologic preparations of hamster lungs euthanized on the 4th day after infection with Otradnoe strain (BA.1.1), the least pronounced pathomorphological changes were observed compared to other groups. Small foci of interstitial pneumonia, which occupied no more than 5–7% of the total section area, were located in 2–3 lobes mainly in the root areas along the course of lobular bronchi. Inflammatory changes in the wall of bronchi and accompanying vessels were weakly expressed.

During histologic examination of the lungs of infected and uninfected hamsters, the morphologic manifestations of coronavirus pneumonia were graded using the recommendations of A.D. Gruber et al. [25]. In infected animals, the cumulative score reflecting the severity of the inflammatory process ranged from 20.8 to 49.8, while in uninfected animals it was close to zero (**Fig. 3**). In the group of animals infected with Wuhan-like virus, the mean value of the cumulative severity score was 50 ± 6, Delta — 30 ± 5, BA.1.1 — 21 ± 7, BA.5.2 — 39 ± 6.

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-528

In addition to the severity of pathologic changes in hamster lungs, weight dynamics was an important criterion in assessing the virulence of different SARS-CoV-2 strains. The greatest difference in weight of infected and uninfected animals was observed on the 3rd or 4th day after infection. In the group of animals infected with Wuhan-like virus, the delay in weight gain was 14.6% compared to uninfected animals. The similar figure in animals infected with Delta, BA.1.1 and BA.5.2 averaged 2–3% (**Fig. 4**).

Since the main target organs for SARS-CoV-2 are the lungs, nasal passages and brain, not only the viral RNA content but also the infectious activity of the virus was investigated in these organs. The mean values of virus titer in tissues and organs of animals differed significantly depending on the strain used for infection. Thus, on the 4th day after infection, the highest titer values were observed in lung homogenates in groups of animals infected with Delta and Wuhan-like viruses — on average 7.4 log10 and 6.2 log10 $TCID_{50}$/mL of homogenate, whereas in groups infected with BA.1.1 and BA.5.2, the virus titer was significantly lower — 4.6 and 5.0 log10 $TCID_{50}$/mL of homogenate, respectively (**Fig. 5**). In nasal passages homogenates, infectious virus was detected in animals of all groups at a titer of 4.9–6.8 log10 $TCID_{50}$/mL of homogenate. In brain tissue, infectious virus was detected only in animals infected with Wuhan-like virus (on average 3.7 log10 $TCID_{50}$/mL of homogenate). It should be noted that the tissue homogenates were toxic to the Vero cells in which the titration was performed; therefore, the limit of sensitivity was 2.0 log10 $TCID_{50}$/mL of homogenate.

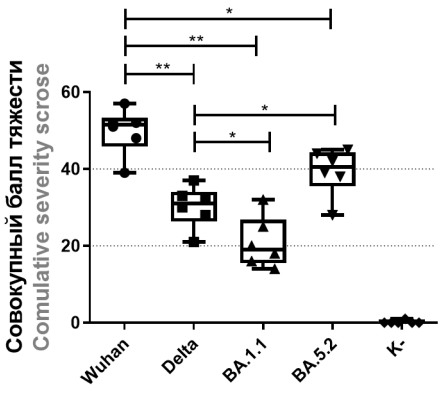

Fig. 3. Histopathology score of hamster lungs on day 4 post-infection with different SARS-CoV-2 strains.
*$p < 0.05$; **$p < 0.01$.

In the lungs of infected animals, the concentration of viral RNA varied depending on the strain from 7.6 to 9.3 on average, in the nasal passages from 8.3 to 9.3, and in the brain from 3.8 to 7.6 log10 RNA copies/mL of homogenate (**Fig. 6**). The highest level of viral RNA in the lungs, nasal passages, brain and other organs of hamsters was observed in the groups of animals infected with Wuhan-like virus and Delta. The concentration of viral RNA in brain homogenates of animals infected with Wuhan-like virus was 7.6, Delta — 5.6, BA.1.1 and BA.5.2 — 3.8 and 4.1 log10 RNA copies/mL, respectively.

Viral RNA was also detected in the heart, liver, kidney, spleen, and blood of most infected animals, but at much lower levels than in the lungs and nasal

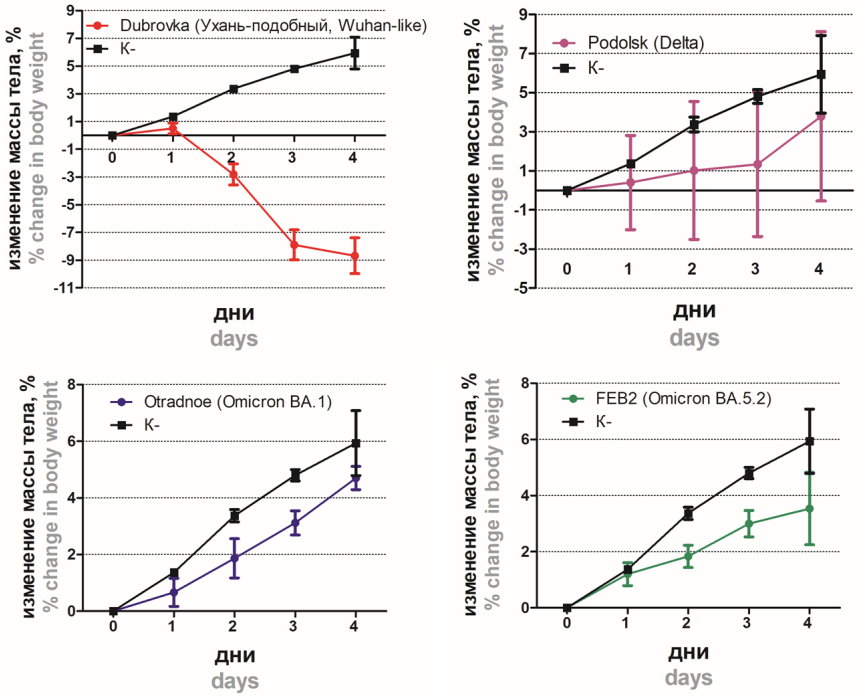

Fig. 4. Weight dynamics in hamsters infected intranasally with different SARS-CoV-2 strains.
K– — uninfected hamsters.

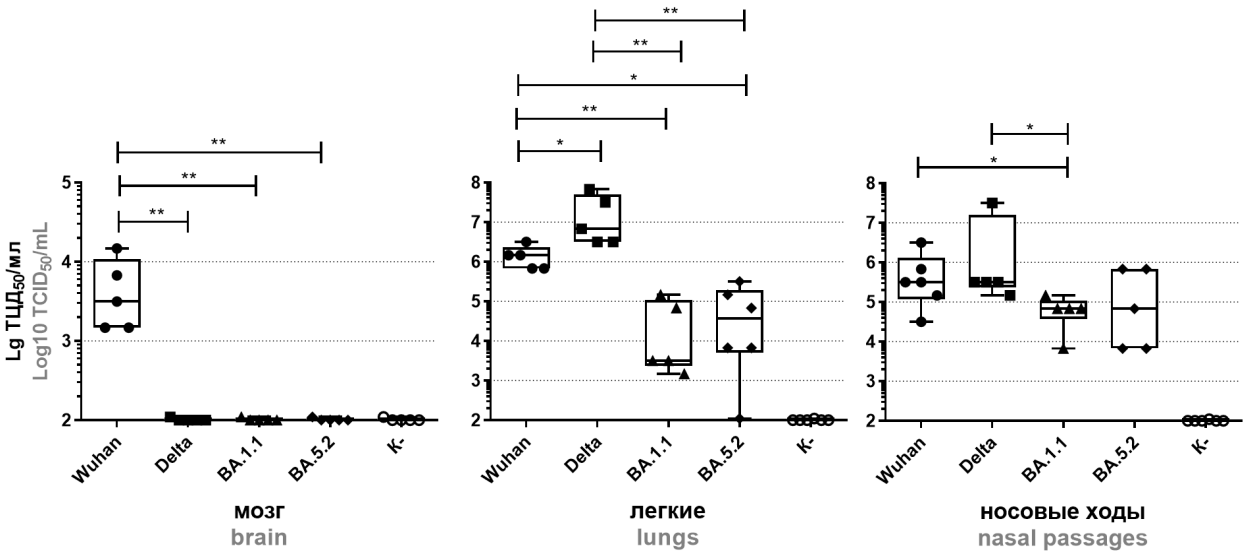

Fig. 5. Titer values of different SARS-CoV-2 strains in the organs of Syrian hamsters on the 4th day after infection.
*$p < 0.05$; **$p < 0.01$.

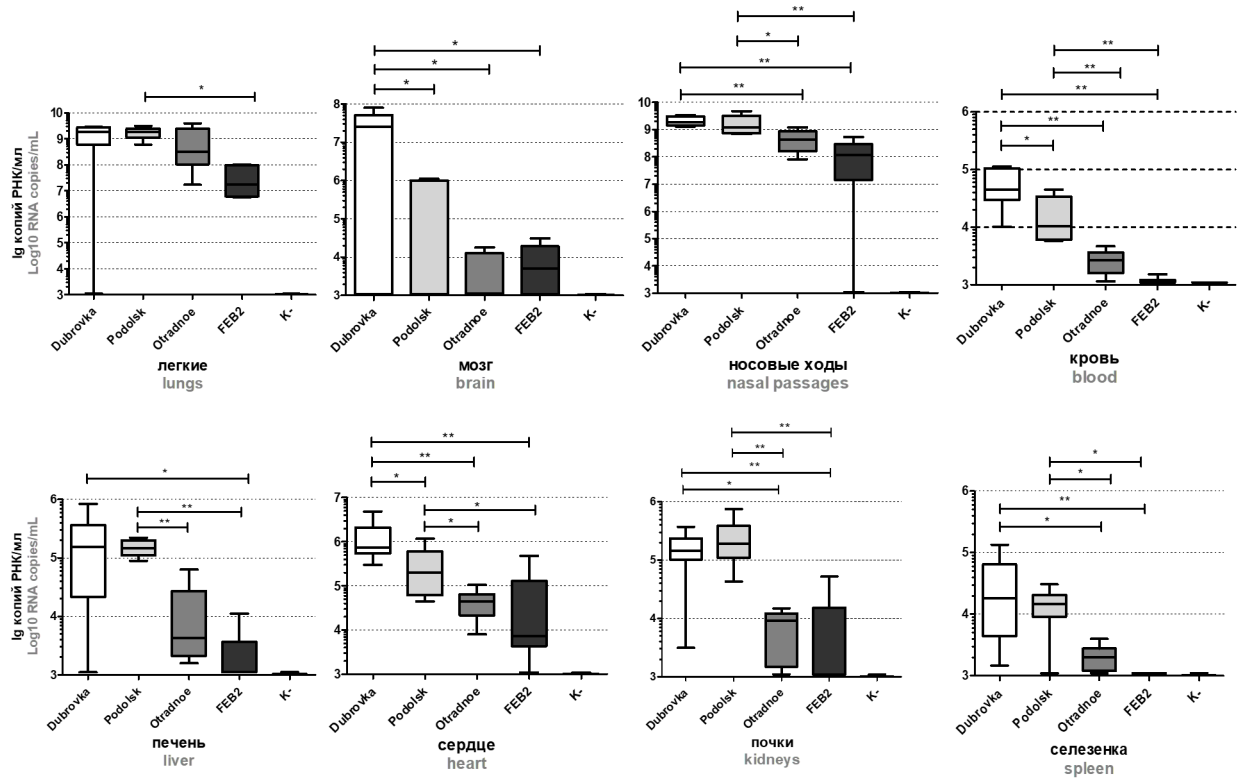

Fig. 6. Distribution of viral RNA in the organs of hamsters infected with different SARS-CoV-2 strains.
*$p < 0.05$; **$p < 0.01$.

passages (Fig. 6). The concentration of viral RNA in the above organs of animals infected with BA.1.1 and BA.5.2 was significantly ($p < 0.05$) lower than in Wuhan-like virus and Delta infection (Fig. 6). The lowest viral RNA content in organs was observed in BA.5.2 infection, while no viral RNA was detected in the blood, kidney and spleen of most animals. It is noteworthy that in all groups of infected animals a significant content of viral RNA was observed in the heart — from 4.7 to 6.1 log10 RNA copies/mL of homogenate.

Discussion

The design of the study implied equality of all conditions, including a single dose of 10^4 $TCID_{50}$/head, except that different virus strains were administered to animals of different groups. Since SARS-CoV-2 is a re-

DOI: https://doi.org/10.36233/0372-9311-528

spiratory virus and is transmitted by airborne droplets, the correctness of our choice of intranasal route of virus administration in COVID-19 modeling is undoubted. This method of administration mimics the natural route of infection and is the simplest, fastest and non-invasive way to infect small laboratory animals such as mice and hamsters [4].

Our study revealed differences in virulence and tissue specificity of SARS-CoV-2 strains belonging to different variants of concern. The greatest virulence was possessed by the Wuhan-like Dubrovka strain, which was manifested by the development of subtotal pneumonia and maximum weight gain delay by 14.6% on average. Hamsters infected with Podolsk (Delta), Otradnoe and FEB2 strains (Omicron BA.1 and BA.5.2) lost significantly less weight, 2-3% ($p > 0.05$). Greater weight loss and severe pneumonia in hamsters infected with Wuhan-like virus was associated with increased virus content in organs and viral damage to the brain. The neurovirulence of Wuhan-like virus was manifested by significantly higher viral RNA content in the brain and isolation of infectious virus from brain homogenates. A number of studies [26, 27] revealed different tropism of SARS-CoV-2 variants to brain cells and lower neurovirulence of the Omicron variant compared to Wuhan-like virus and Alpha and Delta variants, which were dominant earlier [26, 27]. Comparison of the literature data with our own data on the increased tropism of Wuhan-like virus to brain tissues suggests that it was the lesion of the central nervous system that could negatively affect the weight and general condition of the animals [28].

The results of histological examination of the lungs confirmed the data on the different virulence of the virus strains used to infect hamsters. Wuhan-like virus caused the most severe lesions in the lungs with extensive foci of bronchointerstitial pneumonia (cumulative severity score of 50) than Delta- and Omicron-like viruses (cumulative severity score of 21 to 39); $p < 0.05$. The results obtained, indicating lower virulence for hamsters of Omicron-like strains compared to the ancestral Wuhan virus, are consistent with the lower pathogenicity of the Omicron variant for humans [29], as confirmed by the lower reproductive activity of the virus in human Calu-3 lung cell culture [30]. On the other hand, early conclusions about the lower virulence of Omicron-like strains may have overestimated their attenuation for humans, since they did not separate the real decrease in virulence from the effect of prior immunity, since vaccinated and re-infected individuals naturally carry the disease more easily.

The severity of pathological changes in the lungs during infection with different strains of Omicron and Delta variants also differed significantly: the mean value of the cumulative severity score was 21 ± 7 for BA.1.1, 39 ± 6 for BA.5.2, and 30 ± 5 for Delta. The observed higher virulence of BA.5.2 compared to Delta ($p < 0.05$) does not agree with the data presented in the article by S. Mohandas et al. [31], who found greater virulence of the Delta-like strain compared to the Omicron variant BA.5.2 sublineage. In this connection, it is important to note that the virulence of different virus strains may be determined not only by their belonging to a particular genetic variant, but also by strain-specific differences and the number of passages the virus isolate underwent in cell culture. It is known that virus isolation and its passages in cell culture are accompanied by the accumulation of mutations that promote virus adaptation to a new host, while virulence decreases for model laboratory animals [32]. Thus, the greater virulence of strain FEB2 (BA.5.2) can be explained by the fact that in our study this strain had undergone 4 passages in Vero cell culture before infection of hamsters, whereas the Podolsk (Delta) strain underwent 16 passages and the Otradnoe (BA.1.1) strain underwent 8 passages.

It is noteworthy that a significant content of viral RNA (up to 6.1 log10 RNA copies/mL) was detected in the heart of animals infected with different strains of SARS-CoV-2. Heart damage by SARS-CoV-2 virus in hamsters has been observed in a number of studies [33, 34]. This observation is also interesting in the context of the high probability of myocarditis in humans after COVID-19. Since ACE2 receptor expression is upregulated in human myocytes [35], the probability of SARS-CoV-2 virus infection of cardiac tissues and the risk of myocarditis development are increased [36, 37].

Among small laboratory animals, COVID-19 modeling is possible in various mouse lines, with the K18-hACE2 transgenic mice being the most susceptible to SARS-CoV-2 [5]. However, at the moment this line of mice is difficult to access, and effective reproduction of the virus in organs other than lungs makes it difficult to use this animal model for modeling viral pneumonia, because these animals have a high percentage of mortality due to causes unrelated to pneumonia. Thus, the main cause of death in K18-hACE2 mice is central nervous system damage and development of viral encephalitis and other neurological diseases due to high expression of ACE2 receptor in brain cells [18, 38, 39]. The disease in K18-hACE2 mice is more severe and has differences in the character of clinical manifestations compared to those in humans.

In view of the above, the hamster model for coronavirus pneumonia is one of the most adequate, accessible and informative among small laboratory animals. Hamsters, when infected with SARS-CoV-2, show clinical signs of respiratory disease and develop mild to moderate pneumonia [18, 40]. Furthermore, they have the ability to spread the virus with infection of contact naive animals [3, 39]. The hamster-based animal model has been widely used in preclinical trials of antiviral drugs and vaccines because it reproduces the development of viral pneumonia without animal death [13, 18, 41, 42]. This study showed that the modeling of

COVID-19 caused by new virus variants (Delta, BA.1.1 and BA.5.2) in hamsters remains relevant. These virus variants retained the ability to cause pneumonia with extensive lesions in hamsters. While weight dynamics as an indicator of virulence has become less informative, such indicators as viral load (virus infectivity and viral RNA content in organs) and severity of inflammatory changes in the lungs have retained their informative value in assessing the severity of the disease. Scoring of the severity of pathomorphologic changes in the lungs is of particular value in comparing the severity of pneumonia, because it reduces subjectivity in the evaluation of the results of histologic examination and gives a semi-quantitative assessment of the pathologic process.

Conclusion

The results of this study showed that infection in Syrian golden hamsters infected with SARS-CoV-2 strains belonging to different evolutionary lineages proceeds differently. The Wuhan-like virus was found to be more virulent and neurotropic than the Delta and Omicron variants, which became widespread later. Modeling on hamsters of COVID-19 caused by sublineages of the Omicron variant remains relevant, despite insignificant weight loss of animals, in contrast to infection with Wuhan-like virus. Histologic examination and such indicators as viral load in the lungs, nasal passages, brain, heart and a number of other organs continue to play a decisive role in assessing the pathogenicity of Omicron-like strains for hamsters.

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DOI: https://doi.org/10.36233/0372-9311-528

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The article was submitted 01.05.2024;
accepted for publication 02.07.2024;
published 29.08.2024

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Статья поступила в редакцию 01.05.2024;
принята к публикации 02.07.2024;
опубликована 29.08.2024

Original Study Article
https://doi.org/10.36233/0372-9311-498

Molecular genetic characteristics of *Streptococcus pneumoniae* serogroups 15 and 11 representatives circulating in Russia and their relationship with global genetic lineages

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Abstract

Aim of the study. Genetic analysis of *Streptococcus pneumoniae* serogroups 15 and 11 circulating in Russia according to the following parameters: serotype affiliation; clonal complex (CC); presence of resistance and virulence determinants; relatedness to genetic lineages circulating in the world, and justification of inclusion of the actual serotypes of serogroups 15 and 11 in the future conjugate vaccine composition.

Materials and methods. The study included whole genome data of *S. pneumoniae* serogroups 11 and 15.

Results. Genomes of serogroup 15 strains from Russia are represented mainly by serotypes 15B and 15C, the majority of which belong to CC-1025 and CC-1262. CC-1025 is characterized by a more frequent association with invasive diseases. Representatives of CC-1025 and CC-1262 contain virulence determinants unique to these genetic lineages within the studied population of serogroup 15: oligopeptide transporters, fructose-specific PTS system, unique hydrolase variants, additional iron ion transporters, the gene of zinc metalloprotease ZmpC (activating human MMP9). The genomes of serogroup 11 are represented mainly by serotype 11A, the majority belong to CC-62 and CC-1012. The virulence determinants unique to CC-62 (within the studied serogroup 11) include bacteriocins, components of oligopeptide transport, flavin reductase-like protein (adhesin, also protects bacteria from oxidative stress), fucose processing operon, PsaA (adhesin, also a component of the ATP-binding cassette transporter that imports manganese ions).

Conclusion. In the Russian Federation, serogroups 15 and 11 are the most common non-vaccine serogroups. No antimicrobial resistance determinants have been identified in the genomes of representatives of these serogroups. For each of the genetic lineages prevalent in Russia and associated with serogroups 15 and 11, unique virulence determinants within the studied serogroup have been identified, which may contribute to the success of these lineages. It is advisable to include serotypes 15B and 11A in vaccines promising for the Russian Federation.

Keywords: *Streptococcus pneumoniae, serogroups 11 and 15, sequence types, genetic lineages, vaccination, virulence.*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. The research protocol was approved by the Ethics Committee of the SAPIENS (protocol 3.1, January 27, 2020).

Funding source. The study was conducted under the SAPIENS (Scientific Assessment of Pneumococcal Infection Epidemiology Networks) grant. Sponsor of the study: Rostropovich-Vishnevskaya Charitable Foundation “For the sake of children's health and future” with the assistance of the All-Russian Public Organization “Pediatric Respiratory Society”.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Isaeva G.Sh., Tsvetkova I.A., Nikitina E.V., Zaripova A.Z., Bayazitova L.T., Isaeva R.A., Polev D.E., Saitova A.T., Kraeva L.A., Goncharov N.E., Kalinogorskaya O.S., Gordeeva S.A., Sidorenko S.V. Molecular genetic characteristics of *Streptococcus pneumoniae* serogroups 15 and 11 representatives circulating in Russia and their relationship with global genetic lineages. *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):483–501.
DOI: https://doi.org/10.36233/0372-9311-498
EDN: https://www.elibrary.ru/gciets

Оригинальное исследование
https://doi.org/10.36233/0372-9311-498

Молекулярно-генетическая характеристика *Streptococcus pneumoniae* серогрупп 15 и 11, циркулирующих в России, и их связь с глобальными генетическими линиями

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Аннотация

Цели исследования — генетический анализ *Streptococcus pneumoniae* серогрупп 15 и 11, циркулирующих в России, по параметрам: серотиповая принадлежность; клональный комплекс (CC); наличие детерминант резистентности и вирулентности; взаимосвязь с циркулирующими в мире генетическими линиями; наличие уникальных генов, значимых для проявления вирулентности; обоснование актуальных серотипов серогрупп 15 и 11 для включения в состав будущей конъюгированной вакцины.

Материалы и методы. В исследование включены полногеномные данные *S. pneumoniae* серогрупп 11 и 15.

Результаты. Российские геномы серогруппы 15 представлены в основном серотипами 15B и 15C, большинство относится к CC-1025, CC-1262. Для CC-1025 характерна более частая ассоциация с инвазивными заболеваниями. Представители CC-1025 и CC-1262 содержат уникальные для данных генетических линий, в пределах изучаемой популяции серогруппы 15, детерминанты вирулентности: транспортеры олигопептидов, фруктозоспецифичную фосфотрансферазную транспортную систему, уникальные варианты гидролаз, дополнительные транспортеры ионов железа, ген цинковой металлопротеазы ZmpC (активирующей матриксную металлопротеиназу 9 человека). Геномы серогруппы 11 представлены в основном серотипом 11A, большинство относится к CC-62 и CC-1012. К уникальным для CC-62 детерминантам вирулентности (в пределах изучаемой серогруппы 11) относятся бактериоцины, компоненты транспорта олигопептидов, флавинредуктазаподобный белок (адгезин, также защищает бактерии от окислительного стресса), оперон процессинга фукозы, PsaA (адгезин, также является компонентом АТФ-связывающего кассетного транспортера, импортирующего ионы марганца).

Выводы. В России среди невакцинных серогрупп распространены серогруппы 15 и 11. В геномах представителей этих серогрупп детерминант антимикробной резистентности не выявлено. Для каждой из распространённых в России генетических линий, ассоциированных с серогруппами 15 и 11, идентифицированы уникальные в пределах изучаемой серогруппы детерминанты вирулентности, которые могут способствовать успешности данных линий. В перспективные для России вакцины целесообразно включение серотипов 15B и 11A.

Ключевые слова: *Streptococcus pneumoniae, серогруппы 11 и 15, сиквенс-типы, генетические линии, вакцинация, вирулентность*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов или их законных представителей. Протокол исследования одобрен Этическим комитетом SAPIENS (протокол № 3.1 от 27.01.2020).

Источник финансирования. Исследование проведено в рамках гранта SAPIENS (Scientific Assessment of Pneumococcal Infection Epidemiology Networks). Спонсор исследования: Благотворительный Фонд Ростроповича–Вишневской «Во имя здоровья и будущего детей» при содействии Общероссийской общественной организации «Педиатрическое респираторное общество».

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

Для цитирования: Исаева Г.Ш., Цветкова И.А., Никитина Е.В., Зарипова А.З., Баязитова Л.Т., Исаева Р.А., Полев Д.Е., Саитова А.Т., Краева Л.А., Гончаров Н.Е., Калиногорская О.С., Гордеева С.А., Сидоренко С.В. Молекулярно-генетическая характеристика *Streptococcus pneumoniae* серогрупп 15 и 11, циркулирующих в России, и их связь с глобальными генетическими линиями. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):483–501.
DOI: https://doi.org/10.36233/0372-9311-498
EDN: https://www.elibrary.ru/gciets

Introduction

Invasive pneumococcal diseases (pneumonia, meningitis and sepsis) are the most common cause of mortality among children under 5 years of age and adults against the background of reduced immune defense [1, 2].

More than 100 serotypes of *Streptococcus pneumoniae* are known, some of which are highly virulent and capable of causing invasive pneumococcal infection. After the introduction of pneumococcal vaccination with conjugated polysaccharide vaccines into national childhood immunization programs, the previously widespread serotypes have been replaced by non-vaccine serotypes [3]. Two conjugated polysaccharide vaccines are approved for use in Russia: 10-valent (Synflorix, GlaxoSmithKline) and 13-valent (Prevenar 13, PCV13, Pfizer), as well as 23-valent polysaccharide vaccine (Pneumomax 23, Merk Sharp & Dohme). PCV13 is included in the national immunization schedule for vaccination of children.

Already early after the start of the national PCV13 vaccination program, a change in the serotype composition of the *S. pneumoniae* population among healthy children was observed, with the coverage of circulating serotypes by the PCV13 vaccine being about 50% [4]. Among the serotypes not covered by PCV13 vaccine, pneumococci of serogroups 15 and 11 predominate in vaccinated healthy children both in the early (2016-2018) [4] and late (2020–2022) periods after the start of vaccination [5–7]. It should be noted that serotypes 15BC and 11AD, which were not widespread in the pre-vaccination period, were found in children [8], as well as in adults [9, 10] with pneumococcal meningitis in the corresponding period [8].

In a pneumococcal population, there is often an association of a serotype with a particular genetic lineage - a group of closely related isolates belonging to one or more closely related clonal complexes (CC) or dominant sequencing types (ST). Populations of pneumococci of serogroups 15 and 11 have regional peculiarities. Thus, representatives of serogroup 15 are associated with genetic lineages CC-199 and CC-63 in the USA and Iceland, with CC-1025 and CC-1262 - in Russia (data from PubMLST database). Representatives of serogroup 11 are mainly associated with the ubiquitous genetic lineage CC-62, but the genetic lineage CC-1012 is also common in Russia. In some regions (Japan), an increase in the prevalence of multidrug-resistant strains of serotype 15A has been noted [11]. Thus, monitoring the antibiotic sensitivity of emerging epidemiologically significant genetic lineages is also important.

Due to the significant increase in the prevalence of serotypes of serogroups 15 and 11 among various population groups against the background of the widespread vaccination with PCV13, as well as due to their association with invasive diseases, the analysis of these strains is of fundamental and practical importance. In particular, identification of individual serotypes within these serogroups (since routine molecular typing methods do not allow differentiation of close serotypes), analysis of accumulated data on cross-immunogenicity of close serotypes, study of the invasive potential of genetic lineages associated with these serotypes — all this is important for determining the serotype composition of the future conjugated polysaccharide vaccine promising for Russia.

Objectives of the study — genetic analysis of *S. pneumoniae* serogroups 15 and 11 circulating in Russia according to the following parameters: serotype affiliation; clonal complex; presence of resistance and virulence determinants; relatedness to genetic lineages circulating in the world; presence of unique genes significant for virulence; justification of inclusion of the actual serotypes of serogroups 15 and 11 in the future conjugate vaccine composition.

Materials and methods

Sampling

The study included strains of serogroups 11 and 15 of *S. pneumoniae* from Russia for which full genomic data were available: isolates isolated at the Children's Research and Clinical Center for Infectious Diseases and the Botkin Clinical Infectious Diseases Hospital (St. Petersburg), Kazan Research Institute of Epidemiology and Microbiology (as part of the SAPIENS project). S.P. Botkin (St. Petersburg), Kazan Research Institute of Epidemiology and Microbiology (within the SAPIENS project), as well as full genomic data of isolates from different Russian cities obtained during the PEGAS study [10, 12].

The study was conducted with the voluntary informed consent of patients or their legal representatives. The study protocol was approved by the SAPIENS Ethical Committee (version 3.1 of 27.01.2020).

The choice of serotypes is explained by the significant spread of pneumococci belonging to these serotypes against the background of PCV13 vaccination, with only serotypes 11A and 15B included in the new PCV20 (Pfizer, currently not registered in Russia) and in Pneumomax 23. The selected isolates were isolated in different time periods (from 2001 to 2022) from carriers and patients with invasive diseases, from patients of different age groups. Two datasets were supplemented with full genomic data of *S. pneumoniae* strains isolated in different regions of the world — 23 strains for serogroup 11 dataset and 13 strains for serogroup 15 dataset. When selecting full-genomic data of *S. pneumoniae* from other regions of the world, the datasets included representatives of all available in the PubMLST database STs associated with the analyzed pneumococcal serotypes from different regions of the world with an interval of 1–4 years (depending on the prevalence).

DOI: https://doi.org/10.36233/0372-9311-498

Serogroup 15 dataset included genomes of 45 isolates: 32 from Russia and 13 from other regions of the world. The analysis included whole genome data from isolates obtained from various clinical samples: patients with meningitis (n = 11; source of isolation — liquor), pneumonia (n = 11; source of isolation: 10 — sputum, 1 — not specified), acute otitis media (n = 3; source of isolation: middle ear fluid), carriers (n = 20; source of isolation — nasopharynx).

Serogroup 11 dataset included genomes of 38 isolates: 15 from Russia and 23 from other regions of the world. The analysis included whole genome data from isolates obtained from various clinical samples: patients with meningitis (n = 3; source of isolation — liquor), pneumonia (n = 8; source of isolation — sputum), acute otitis media (n = 3; source of isolation — middle ear fluid), carriers (n = 20; source of isolation — nasopharynx), in 1 case there was no information about the diagnosis (source of isolation – blood). For 3 isolates there was no information about the diagnosis and source of isolation.

Whole genome sequencing

Whole genome sequencing (WGS) of pneumococcal isolates isolated in St. Petersburg or within the SAPIENS project was performed at the Pasteur Research Institute of Epidemiology and Microbiology. DNA was isolated from pure cultures of *S. pneumoniae* using the QIAamp DNA Mini Kit (Qiagen). WGS was performed on the DNBSEQ-G50 platform (MGI). Libraries for WGS were prepared using the MGIEasy Fast FS DNA Library Prep Set (MGI) according to the manufacturer's standard protocols. The median length of library fragments was 430 bp (identified using the QIAxcel Advanced system capillary gel electrophoresis system). Sequencing to obtain paired-end reads was performed on the DNBSEQ-G50 platform (MGI) using DNBSEQ-G50RS kits (FCL PE150/FCS PE150). Whole genome data of 11 *S. pneumoniae* isolates uploaded to GenBank (BioProject PRJNA971376, BioProject PRJNA1009429, BioProject PRJNA1076328, BioProject PRJNA1154393).

Bioinformatics analysis

For isolates sequenced at the Pasteur Research Institute of Epidemiology and Microbiology, the quality of the obtained nucleotide sequences was assessed using the program FastQC v. 0.11.8 (Babraham Bioinformatics). Quality filtering of reads and removal of PCR adapters and primers used in library preparation were performed using the program Cutadapt v. 1.15. For *de novo* genome assembly, we used the algorithm SPAdes v. 3.15.4. Final quality assessment was performed using the Quast v. 5.0.2 program. ST determination by MLST typing (Multilocus sequence typing) was performed using the MLST v. 2.0 program[1]. Genomes were annotated using RAST server (Rapid Annotations using Subsystems Technology). The serogroup and serotype affiliation of the strains were determined using the blastall program with an E-value threshold < 0.01. The obtained matches were filtered by bit-score and identity values. Searches were performed against a locally customized *cps*-locus sequence database of 90 serotypes. Genes and mutations associated with antibiotic resistance were identified against the CARD database [13]. Methods for nuclear genome and pan-genome analysis (R package micropan: Microbial Pan-Genome Analysis v. 2.1) were used to compare genomes [14]. Clusters of orthologs were identified based on distances calculated by pairwise comparison of amino acid sequences. The clustering was based on the complete-linkage clustering method, in which the distance between clusters is equal to the maximum distance between points from different clusters, with threshold distance criterion being 0.75. To identify associations of unique clusters of orthologs with genetic lineages, the presence/absence/variability statistics of genes in the genomes of the analyzed isolates were estimated using the Scoary v. 1.6.16 package[2] [15].

Statistical analysis

For statistical processing we used the Scoary program, which allows us to obtain a list of genes significant for the corresponding trait, associated with the trait positively or negatively, sorted by p-values.

Results

To analyze the populations of *S. pneumoniae* serogroups 15 and 11 circulating in Russia and to characterize the genetic relationships between the genetic lines of serogroups 15 and 11 circulating in Russia and worldwide, pan-genome analysis was performed. For this purpose, two samples were formed, which included full genomic data of *S. pneumoniae* belonging to serogroups 15 and 11 from Russia and other regions of the world.

Analysis of S. pneumoniae serogroup 15

The study included full genomic data of 45 isolates of pneumococcus serogroup 15, including 32 isolates from different cities of Russia, as well as 13 isolates from other regions of the world (**Table 1**). Among the isolates of serogroup 15 isolated in Russia, 15 (46.9%) isolates belonged to serotype 15B, 12 (37.5%) to 15C, 3 (9.4%) to 15F, and 6 (6.3%) to 15A. Representatives of serotypes 15B/C were associated with 3 common STs (ST-1025, ST-199, ST-1262, of which only ST-199 is not found in Russia), as well as with rare STs. Serotypes 15A/F were associated predominantly with ST-63. ST-1025 isolates were isolated predominantly from sterile loci (isolation biomaterial — blood, liquor)

[1] Center for Genomic Epidemiology. URL: https://cge.food.dtu.dk/services/MLST/

[2] URL: https://github.com/AdmiralenOla/Scoary

Table 1. Characteristics of serogroup 15 strains

Sample	PubMLST ID / ENA_accession	Country	Region	Year of isolation	Serotype	ST	Patient's age, years	Diagnosis	Source of isolation	Penicillin	Erythromycin	Tetracycline	Chloramphenicol	Co-trimoxazole
PEGAS-5-1079	51104 [10, 12]	R	Yaroslavl	2016	15B	1025	11	MNG	CSF	S	S	S	S	R
PEGAS-5-1659	51117 [10, 12]	R	Yaroslavl	2017	15B	1262	2	MNG	CSF	S	S	S	S	S
PEGAS-2019-106	73021 [10, 12]	R	Yaroslavl	2019	15B	1262	1	MNG	CSF	S	S	S	S	S
PEGAS-2019-269	73025 [10, 12]	R	Yaroslavl	2019	15B	1025	0,2	MNG	CSF	S	S	S	S	R
PEGAS-2019-73	142552 [10, 12]	R	Yaroslavl	2019	15B	1025	78	PN	CSF	S	S	S	S	R
PEGAS-5-638	51109 [10, 12]	R	Smolensk	2016	15B	1025	50	MNG	CSF	S	S	S	S	R
PEGAS-2019-184	73023 [10, 12]	R	Smolensk	2019	15F	6202	52	MNG	CSF	S	S	S	S	S
PEGAS-2019-237	142578 [10, 12]	R	Smolensk	2019	15C	1025	63	PN	SP	S	S	S	S	R
PEGAS-2020-201	142624 [10, 12]	R	Yuzhno-Sakhalinsk	2020	15C	1025	23	PN	SP	S	S	S	S	R
PEGAS-2019-213	142574 [10, 12]	R	Yuzhno-Sakhalinsk	2019	15C	16380	2	PN	SP	R	S	S	S	R
PEGAS-2020-146	142613 [10, 12]	R	Kirov	2020	15C	1262	1	PN	SP	S	S	S	S	S
PEGAS-2019-343	142585 [10, 12]	R	Seversk	2019	15A	12518	55	PN	SP	S	S	S	S	S
PEGAS-2019-347	142587 [10, 12]	R	Seversk	2019	15C	16349	70	PN	SP	S	S	S	S	R
PEGAS-2019-373	142591 [10, 12]	R	Tomsk	2019	15C	1262	3	PN	SP	S	S	S	S	S
PEGAS-2019-375	142593 [10, 12]	R	Tomsk	2019	15B	1262	86	PN	SP	S	S	S	S	S
PEGAS-2019-390	142595 [10, 12]	R	Tomsk	2019	15C	1262	61	PN	SP	S	S	S	S	S
PEGAS-2020-229	142634 [10, 12]	R	Tolyatti	2020	15F	16421	45	PN	SP	S	S	S	S	S
ST_12518_2	ERR1788193	R	Moscow	2014	15A	12518	5	PHR	NPS	S	S	S	S	S
ST_3201_3	ERR1788219	R	Moscow	2015	15B	3201	2	–	NPS.	R	S	S	S	R
ST_1262_2	ERR1788207	R	Moscow	2013	15B	1262	5	–	NPS	S	S	S	S	R
ST_1262_3	ERR1788225	R	Moscow	2015	15B	1262	5	PHR	NPS	S	S	S	S	R
ST_1025_5	ERR1788208	R	Moscow	2014	15C	1025	5	PHR	NPS	S	S	S	S	R
ST_3557_1	ERR1788206	R	Moscow	2013	15B	3557	2	PHR	NPS	R	S	R	S	R

End of the Table 1

Sample	PubMLST ID / ENA_accession	Country	Region	Year of isolation	Serotype	ST	Patient's age, years	Diagnosis	Source of isolation	Penicillin	Erythromycin	Tetracycline	Chloramphenicol	Co-trimoxazole
6_2F1	PRJNA1154393	R	Moscow	2011	15F	6202		–	NPS	S	S	S	S	S
27_Kz	PRJNA971376	R	Kazan	2020	15C	1025	3	–	NPS	S	S	S	S	R
12001	PRJNA1076328	R	Saint-Petersburg	2016	15B	1262	3	–	NPS	S	S	S	S	S
12456	PRJNA1076328	R	Saint-Petersburg	2016	15B	1025	5	–	NPS	S	S	S	S	R
108	PRJNA1154393	R	Saint-Petersburg	2021	15C	1349		MNG	CSF	R	S	S	S	R
76_B	PRJNA1076328	R	Saint-Petersburg	2021	15B	1025	44	MNG	CSF	S	S	S	S	R
137_B	PRJNA1076328	R	Saint-Petersburg	2022	15C	1025	38	MNG	CSF	S	S	S	S	R
138_B	PRJNA1076328	R	Saint-Petersburg	2022	15C	1025	38	MNG	CSF	S	S	S	S	R
336_B	PRJNA1076328	R	Saint-Petersburg	2022	15B	Unkn_21	64	MNG	CSF	S	S	S	S	S
ST_63_3	ERR065297	U	Massachusetts	2004	15A	63	6	–	NPS	R	R	S	R	S
ST_63_4	ERR068032	U	Massachusetts	2004	15A	63	6	–	NPS	R	R	S	R	R
ST_63_5	ERR069724	U	Massachusetts	2004	15A	63	6	–	NPS	R	R	S	R	S
ST_199_1	ERR069751	U	Massachusetts	2001	15C	199	2	–	NPS	S	S	S	S	S
ST_199_2	ERR069691	U	Massachusetts	2004	15B	199	2	–	NPS	S	S	S	S	S
ST_199_3	ERR069774	U	Massachusetts	2001	15C	199	2	–	NPS	S	S	S	S	S
ST_199_4	ERR065975	U	Massachusetts	2001	15B	199	2	–	NPS	S	S	S	S	S
ST_199_11	ERR540653	I	Reykjavik	2010	15B	199	2	–	NPS	S	S	S	S	S
ST_199_16	ERR755466	I	Reykjavik	2013	15C	199	2	OM	MEF	S	S	S	S	S
ST_199_17	ERR755326	I	Reykjavik	2013	15B	199	3	OM	MEF	S	S	S	S	S
ST_199_13	ERR470151	I	Koupavogur	2009	15C	199	4	–	NPS	S	S	S	S	S
ST_199_18	ERR755336	I	Habnarfjordur	2013	15B	199	2	OM	MEF	S	S	S	S	S
ST_199_21	ERR755384	I	Habnarfjordur	2014	15C	199	4	–	NPS	S	S	S	S	S

Note. MNG — meningitis; PN — pneumonia; Phr — pharyngitis; OM — otitis media; CSF — cerebrospinal fluid; SP — sputum; NPS — nasopharyngeal smear; MEF — middle ear fluid; R/S — presence/absence of determinants of resistance (source: Prediction of antimicrobial resistance in PATRIC and RAST, URL: https://www.bv-brc.org/job).

and more frequently were associated with invasive diseases. Most isolates of this serogroup 15 were sensitive to antibiotics of different classes. Detailed characteristics of the analyzed isolates (ST, source of isolation, year of isolation, presence of antibiotic resistance determinants in the genomes, etc.) are presented in **Table 1**.

The pan-genome of *S. pneumoniae* isolates of serogroup 15 was characterized by comparing all proteins (blast-all-all). In representatives of serogroup 15 the share of the main (conserved) part of the genome was 59.8% — 1286 genes were present in all genomes of the analyzed sample (**Fig. 1**). In the population of serogroup 15, 2097 clusters of orthologs were identified, the most numerous cluster was represented by 296 proteins. The pan-genome of pneumococcus serogroup 15 isolates belongs to the closed pan-genome (alpha index value > 1), and its size approaches a constant as more genomes are used (Hipps' law) [14]. This may indicate that the genome diversity of serogroup 15 representatives has reached saturation, regardless of the time period and geographic region of isolates isolation, as well as their belonging to the genetic lineage.

All representatives of the genetic lineage ST-1025 are associated with a homogeneous dendrogram cluster describing the relationship between strains based on pan-genome analysis and taking into account both the presence or absence and homology of available amino acid sequences (**Fig. 2**). All ST-1025 representatives contain in their genomes a unique operon encoding oligopeptide transporter components. Furthermore, ST-1025 representatives contain in their genomes a unique operon encoding components of the fructose-specific phosphotransferase transport system (PTS). ST-1025

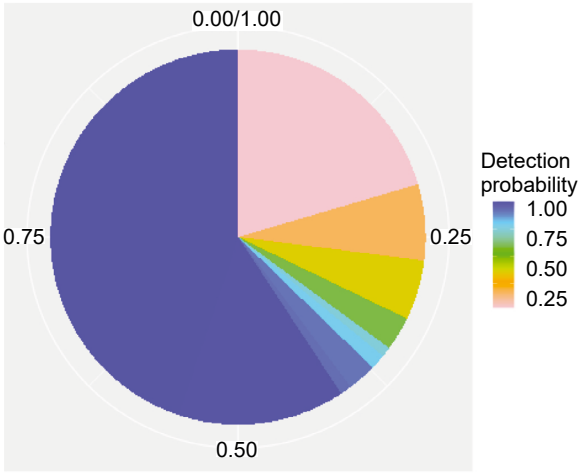

Fig. 1. Distribution of gene families of the pan-genome of *S. pneumoniae* serogroup 15 strains.
The color of the sector reflects the probability of identification of the gene family in the genomes of isolates. The blue color shows highly conservative («core genome») gene families.

isolates also contain unique variants of hydrolases, iron ion transporters, and the zinc metalloprotease gene *ZmpC* (**Table 2**).

Along with ST-1025, the prevalence of ST-1262 may be associated with the presence in the genomes of its representatives of factors that provide higher adaptability to stress conditions (**Table 3**).

Analysis of S. pneumoniae serogroup 11

The sample of serogroup 11 representatives included full genomic data of 15 isolates from different cities of Russia, as well as 23 isolates from other re-

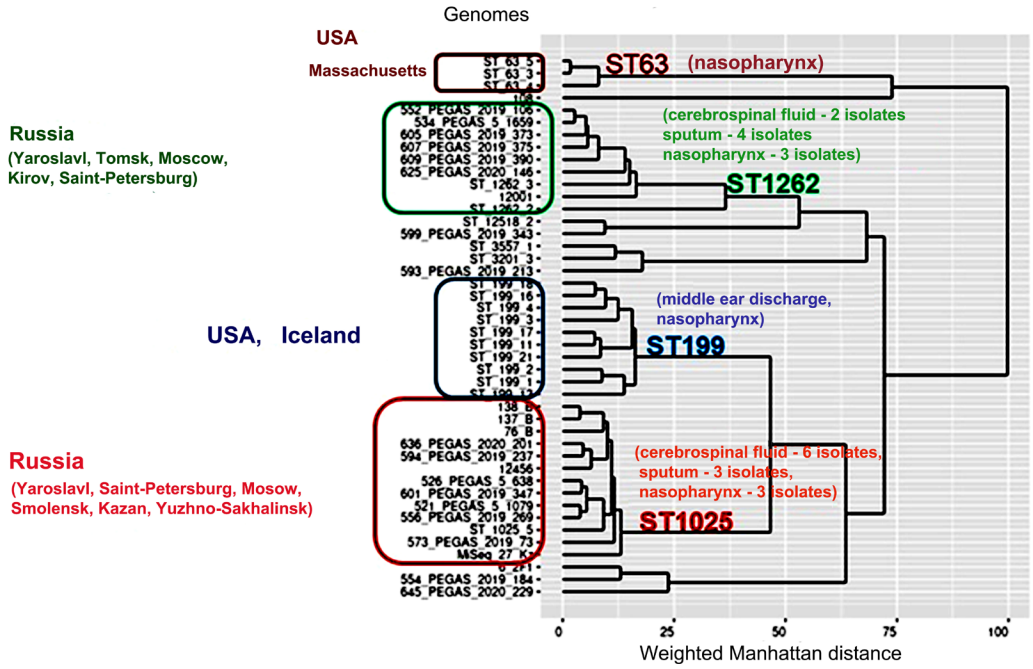

Fig. 2. A dendrogram describing the clustering of *S. pneumoniae* isolates of serogroup 15 by pan-genome R micropan analysis (presence/absence and gene homology).

DOI: https://doi.org/10.36233/0372-9311-498

Table 2. Unique proteins of the CC-1025 genetic lineage representatives*

Sequence ID	Homology with known proteins, %	Protein name	Proposed function
27_Kz_seq27	100	ABC iron (III) transporter, permease	Transport of iron III+ ions
27_Kz_seq161	96	ABC transporter, permease	Transport of iron III+ ions
27_Kz_seq266	97,9	Membrane succinate permease DctA, sodium symporter	Transport of dicarboxylic acids
27_Kz_seq792	100	Component IIC of the phosphotransferase system (PTS)	Protein-N(PI)-phosphohistidine-fructose-PTS
27_Kz_seq793	99	Component IIB of the PTS	
27_Kz_seq794	100	Component IIA of the PTS	
27_Kz_seq795	100	Hypothetical nitrogen regulatory protein IIA of the PTS system	
27_Kz_seq796	99,9	A hypothetical transcription antiterminator of the BglG family	
27_Kz_seq1007	100	High affinity permease Fe^{2+}/Pb^{2+}	Ferrum ions transport
27_Kz_seq1008	99,7	DyP-type peroxidase (IPR006314)	DyP proteins have characteristics that distinguish them from other peroxidases: broad substrate specificity, lack of homology with most other peroxidases, and the ability to function well under conditions of lower pH values
27_Kz_seq1359	99,9	Zinc-dependent metalloproteinase ZmpC	Cleaves and activates human matrix metalloproteinase-9. The role in the virulence and pathogenicity of pneumococcus in the lungs
27_Kz_seq1361	100	Hypothetical acetyltransferase	Unknown
27_Kz_seq1489	100	N-acetylneuramic acid epimerase	Mutarotation of sialic acids. The presence of sialic acids in the elements of the bacterial cell surface helps them evade the innate immune response of the host
27_Kz_seq1490	100	Substrate-binding subunit AppA, ABC component of the oligopeptide transporter	Transport of oligopeptides
27_Kz_seq1494	99,8	Hypothetical glycosylhydrolase family 32	Unknown

Note. *These proteins are encoded in the genomes of 13 isolates: 556_PEGAS_2019_269, 573_PEGAS_2019_73, 594_PEGAS_2019_237, 601_PEGAS_2019_347, 636_PEGAS_2020_201, 76_B, MiSeq_27_Kz, ST_1025_5, 12456, 137_B, 138_B, 521_PEGAS_5_1079, 526_PEGAS_5_638)

gions of the world. Among the isolates of serogroup 11 isolated in Russia, 13 (86.7%) isolates belonged to serotype 11A and 2 (13.3%) to serotype 11D. Representatives of serogroup 11 were associated with two common genetic lineages: CC-62 (circulating ubiquitously) and CC-1012, as well as with rare STs. Isolates belonging to CC-62 were isolated predominantly from the nasopharynx. Isolates belonging to CC-1012 were frequently associated with invasive diseases (biomaterial of isolation was liquor). Most isolates of serogroup 11 were sensitive to antibiotics of different classes (**Table 4**).

Pan-genome analysis of *S. pneumoniae* isolates of serogroup 11 showed a higher degree of genome heterogeneity in this group (**Fig. 3**). The share of the main (conserved) part of the genome was 36% — 820 genes were present in all genomes of the analyzed sample (**Fig. 3**). In the population of serogroup 11, 1864 clusters of orthologs were identified, the most numerous cluster was represented by 191 proteins. The pan-ge-

Table 3. Unique proteins of the CC-1262 genetic lineage representatives*

Sequence ID	Homology with known proteins, %	Protein name	Proposed function
552_PEGAS_2019_106_seq440	100	Phage shock protein PspC	The integrity of the inner membrane in response to extracytoplasmic stress conditions
552_PEGAS_2019_106_seq590	100	Satellite phage hypothetical protein (*Streptococcus satellite phage Javan725*)	Prophage component
552_PEGAS_2019_106_seq591	100	Satellite phage hypothetical protein (*Streptococcus satellite phage Javan296*)	Prophage component
552_PEGAS_2019_106_seq592	100	Primase C-terminal 1 domain-containing protein	Prophage component
552_PEGAS_2019_106_seq624	100	Methionine tRNA ligase	The initiation of protein synthesis
552_PEGAS_2019_106_seq686	98,6	ABC transporter, ATP-binding subunit, GlnQ	Transport of glutamine
552_PEGAS_2019_106_seq915	99	Superfamily 2 helicase	Unknown
552_PEGAS_2019_106_seq1038	99,4	O-acetylhomoserine aminocarboxypropyltransferase	Synthesis of methionine
552_PEGAS_2019_106_seq1080	91	AAA ATPase	ATP hydrolysis
552_PEGAS_2019_106_seq1081	85	Serine protease	Possible signaling function
552_PEGAS_2019_106_seq1112	100	Hypothetical macrolide efflux transporter	Possible macrolide efflux
552_PEGAS_2019_106_seq1113	100	Hypothetical protein	Unknown
552_PEGAS_2019_106_seq1114	100	Group I pyridoxal-dependent decarboxylase (cleaves Orn/Lys/Arg and glycine)	Amino acid metabolism

Note. *These proteins are encoded in the genomes of 10 isolates: PEGAS_2019_106, 605_PEGAS_2019_373, 607_PEGAS_2019_375, 609_PEGAS_2019_390, 12001, 625_PEGAS_2020_146, ST_1262_2, ST_1262_3, 534_PEGAS_5_1659, 552_PEGAS_2019_106

nome of the pneumococcal isolates of serogroup 11 serogroup 11 belonged to the open pan-genome — the alpha index value < 1 (0.82), i.e. the pan-genome size of this group should increase, as more genomes are included in analysis. This may indicate greater variability of genomes of this group and greater diversity of the additional part of the genome of representatives of serogroup 11 (**Fig. 4**), their potentially greater adaptability. This fact is consistent with the high prevalence of CC-62 in different regions of the world in different periods of time.

SS-62 representatives contain in their genomes a unique operon encoding the synthesis of bacteriocin involved in interspecific competition, oligopeptide transporter components, and flavin reductase-like protein that promotes adhesion and protects the bacterium from oxidative stress, which increases the virulence of the microorganism (**Table 5**). Also, all representatives of SS-62 contain a fucose processing operon and PsaA

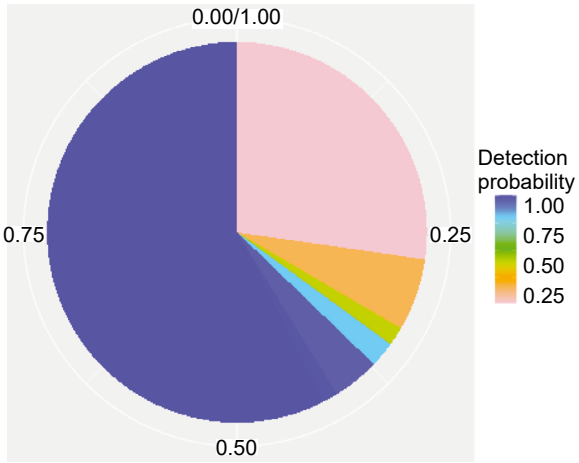

Fig. 3. Distribution of gene families of the pan-genome of *S. pneumoniae* serogroup 11 strains.

The color of the sector reflects the probability of identification of the gene family in the genomes of isolates. The blue color shows highly conservative («core genome») gene families.

Table 4. Characteristics of serogroup 11 strains

Sample	PubMLST / ENA_accession number	Country	Region	Isolation year	Serotype	ST	Patient's age, years	Diagnosis	Source of isolation	Penicillin	Erythromycin	Tetracycline	Chloramphenicol	Co-trimoxazole
PEGAS-2019-401	73030 [10, 12]	Russia	Krasnodar	2019	11A	1012	61	MNG	CSF	S	S	S	S	S
PEGAS-2019-64	142555 [10, 12]	Russia	Yaroslavl	2019	11A	156	66	PN	SP	S	S	R	R	R
PEGAS-2019-113	142568 [10, 12]	Russia	Smolensk	2019	11A	1012	57	PN	SP	S	S	S	S	S
PEGAS-2019-344	142586 [10, 12]	Russia	Seversk	2019	11D	62	67	PN	SP	S	S	S	S	S
PEGAS-2019-349	142588 [10, 12]	Russia	Seversk	2019	11A	1012	85	PN	SP	S	S	S	S	S
PEGAS-2020-149	142616 [10, 12]	Russia	Kirov	2020	11A	6191	62	PN	SP	S	S	S	S	R
PEGAS-2020-150	142617 [10, 12]	Russia	Kirov	2020	11A	62	1	PN	SP	S	S	S	S	S
PEGAS-2020-226	142631 [10, 12]	Russia	Tolyatti	2020	11A	62	34	PN	SP	S	S	S	S	S
PEGAS-2019-114	142560 [10, 12]	Russia	Moscow	2019	11A	1012	72	PN	SP	S	S	S	S	S
ST_62_27	ERR1788222	Russia	Moscow	2012	11A	62	5	–	NPS	S	S	S	S	S
ST_62_28	ERR1788215	Russia	Moscow	2014	11A	62	5	PhR	NPS	S	S	S	S	S
ST_1012_3	ERR1788171	Russia	Moscow	2013	11A	1012	3	MNG	CSF	S	S	S	S	S
ST_1012_4	ERR1788140	Russia	Moscow	2011	11A	1012	3	MNG	CSF	S	S	S	S	S
105_Kz	PRJNA1009429	Russia	Kazan	2020	11D	62	4	–	NPS	S	S	S	S	S
25_B	PRJNA1076328	Russia	Saint Petersburg	2021	11A	1050	60	–	BL	S	S	S	S	S
ST_62_3	ERR069801	USA	Massachusetts	2001	11A	62	2	–	NPS	S	S	S	S	S
ST_62_4	ERR069822	USA	Massachusetts	2001	11A	62	3	—	NPS	S	S	S	S	S
ST_62_5	ERR065964	USA	Massachusetts	2001	11A	62	3	—	NPS	S	S	S	S	S
ST_62_6	ERR069804	USA	Massachusetts	2001	11A	62	6	–	NPS	S	S	S	S	S
ST_62_7	ERR065326	USA	Massachusetts	2004	11A	62	2	—	NPS	S	S	S	S	S
ST_62_8	ERR069707	USA	Massachusetts	2004	11A	62	2	–	NPS	S	S	S	S	S
ST_62_9	ERR069727	USA	Massachusetts	2004	11A	62	2	–	NPS	S	S	S	S	S
ST_62_10	ERR065310	USA	Massachusetts	2004	11A	62		–	NPS	S	S	S	S	S

End of the Table 4

Sample	PubMLST / ENA_accession number	Country	Region	Isolation year	Serotype	ST	Patient's age, years	Diagnosis	Source of isolation	Penicillin	Erythromycin	Tetracycline	Chloramphenicol	Co-trimoxazole
ST_62_11	ERR124268	USA	Massachusetts	2007	11A	62	6	–	NPS	S	S	S	S	S
ST_62_12	ERR129079	USA	Massachusetts	2007	11A	62	6	–	NPS	S	S	S	S	S
ST_62_13	ERR129211	USA	Massachusetts	2007	11A	62	6	–	NPS	S	S	S	S	S
ST_62_14	ERR129131	USA	Massachusetts	2007	11A	62	6	–	NPS	S	S	S	S	S
ST_62_15	ERR470324	Iceland	Reykjavik	2009	11A	62	3	–	NPS	S	S	S	S	S
ST_62_16	ERR449847	Iceland	Reykjavik	2009	11A	62	65	PN	NA	S	S	NA	NA	NA
ST_62_20	ERR470201	Iceland	Reykjavik	2010	11A	62	11	OM	MEF	S	S	S	S	S
ST_62_21	ERR540645	Iceland	Reykjavik	2010	11A	62	5	–	NPS	S	S	S	S	S
ST_62_22	ERR540483	Iceland	Reykjavik	2010	11A	62	60	PN	NA	S	S	S	S	S
ST_62_17	ERR470261	Iceland	Mosfellsbaer	2009	11A	62	17	OM	MEF	S	S	S	S	S
ST_62_18	ERR449827	Iceland	Mosfellsbaer	2009	11A	62	42	PN	NA	S	S	S	S	S
ST_62_19	ERR470192	Iceland	Selfoss	2010	11A	62	1	OM	MEF	S	S	S	S	S
ST_62_23	ERR755493	Iceland	Hafnarfjörður	2014	11A	62	5	–	NPS	S	S	S	S	S
ST_62_24	ERR755501	Iceland	Hafnarfjörður	2014	11A	62	5	–	NPS	S	S	S	S	S
ST_62_26	ERR755548	Iceland	Kopavogur	2014	11A	62	6	–	NPS	S	S	S	S	S

Note. MNG — meningitis; PN — pneumonia; Phr — pharyngitis; OM — otitis media; CSF — cerebrospinal fluid; SP — sputum; NPS — nasopharyngeal smear; MEF — middle ear fluid; R/S — presence/absence of determinants of resistance (source: Prediction of antimicrobial resistance in PATRIC and RAST. URL: https://www.bv-brc.org/job).

(a component of the ATP-binding cassette transporter that imports manganese ions and is also an adhesin).

Representatives of the SS-1012 genetic lineage are less common, also mostly associated with serotype 11A, but isolated mainly from liquor and sputum. The unique features of this genetic lineage include the presence of the Streptococcus satellite phage Javan359. Representatives of SS-1012 have a bacteriocin unique to this genetic lineage. Also, SS-1012 isolates may have peculiarities of amino acid synthesis and riboflavin biosynthesis, which may be related to virulence, but this assumption needs to be verified in additional studies.

Discussion

Since the introduction of PCV-13 into national immunization schedules, reports of increased circulation of *S. pneumoniae* serogroup 15, which is not covered by PCV13, have begun to appear [16–18]. 15B is one of the serotypes currently associated with relatively high mortality rates [19–22], development of invasive forms, particularly meningitis [23, 24]. According to recent-

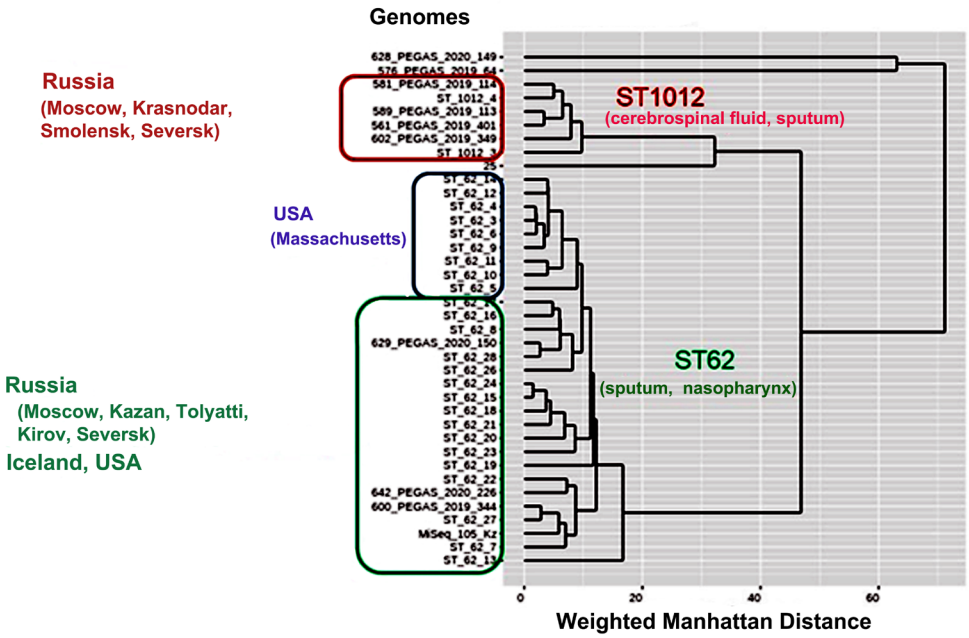

Fig. 4. Dendrogram describing the clustering of *S. pneumoniae* serogroup 11 isolates by pan-genome R micropan analysis (presence/absence and gene homology).

ly published results of Chinese researchers, the most common circulating among children in China is pneumococcal serogroup 15 [25]. In Russia there is also a tendency of expansion of this serogroup [5, 6]. According to the results of our analysis, the two most common genetic lineages of serogroup 15 circulating in Russia, CC-1025 and CC-1262, are often associated with invasive diseases. Isolates of CC-1025 and CC-1262 are represented by serotypes 15B/C and have genetic determinants that may contribute to better adaptation and success of these genetic lineages and may potentially be associated with virulence (**Tables 2, 3**). In particular, oligopeptide transporters, in addition to transporting bacteriocins and chemokines, may be associated with the regulation of the expression of choline-binding proteins [26, 27]. A unique variant of fructose-specific PTS may also contribute to the selection of ST-1025 representatives in carriers on the background of vaccination due to energetic advantages. The zinc metalloprotease ZmpC specifically cleaves and activates human matrix metalloproteinase-9, which in turn degrades components of the extracellular matrix [28]. All ST-1262 strains contain a gene encoding a peptide that accounts for resistance to abortive phage infection (**Table 3**). As part of the satellite prophage, all representatives of ST-1262 have a gene encoding a phage shock protein that ensures the integrity of the cell inner membrane in response to extracytoplasmic stress conditions. It is possible that ST-1262 representatives have peculiarities of amino acid metabolism (**Table 3**), but this assumption needs to be verified.

Thus, potentially virulent pneumococci of serotypes 15B and 15C are circulating in Russia. It was previously established that the structural difference between these serotypes is based on variations in the short tandem repeat of thymine-adenine nucleotides in the *wciZ* O-acetyltransferase gene, which ensure mutual switching of serotypes 15B and 15C [29, 30]. The cross-immunogenicity of serotypes 15B/C with the formation of stable antibody titers was confirmed in earlier studies [30, 31]. Thus, vaccines containing serotype 15B could potentially limit the spread of virulent genetic lineages associated with serotypes 15B/C in the pneumococcal population.

According to the results of various studies, serotype 11A is currently spreading worldwide [32], both in pneumococcal carriers [33] and in invasive diseases [34]. According to A.B. Brueggemann et al, serotype 11A is more associated with asymptomatic carriers than with invasive disease, indicating a relatively low virulence potential [35]. However, some ST-62 strains of serotype 11A are capable of causing invasive diseases with high lethality [36]. According to the results of our study, ST-62 representatives contain in their genomes loci potentially capable of increasing the adaptability and virulence of the microorganism: loci encoding the synthesis of bacteriocins, transporters, including oligopeptides, adhesion proteins, flavin reductase, oxidative stress defense factors, complement activation regulators, and transcription regulators (**Table 5**). Our results are confirmed by the data of previous studies [37]. Thus, the research group of M.A. Higgins et al. previously showed the inability of *S. pneumoniae* to grow on fucose, despite the presence of regulatory and biochemical mechanisms of fucose metabolism [38]. It is assumed that the fucose processing pathway of

Table 5. Unique proteins of the serogroup 11 genetic lineages representatives

ID последовательности Sequence ID	Homology with known proteins, %	Protein name	Proposed function
		CC-62* — 29 isolates	
GID11_seq178	100	Bacteriocin	Interspecific competition
GID11_seq180	87,5	Transposase ISSmu1	Prophage component
GID11_seq303	98,8	O6-methylguanine DNA methyltransferase	DNA repair. Maintaining the stability of the genome
GID11_seq357	100	L-fuculose phosphate aldolase	Metabolism of fucose
GID11_seq358	99,3	RbsD/FucU family transport protein	
GID11_seq359	98,6	Enzyme IIA component of the phosphotransferase system (PTS)	
GID11_seq363	99,6	Hypothetical protein	Unknown
GID11_seq364	99,8	F5/8 type C domain-containing protein	It can act as a protective agent. Possibly, regulation of complement activation (lectin pathway)
GID11_seq373	56	Pneumococcal surface protein A-like protein	An adhesive and a component of an ATP-binding cassette conveyor importing manganese ions. It is possible that PsaA, like many other virulence factors, performs two functions during infection: direct adhesion and participation in the absorption of manganese
GID11_seq740	97,7	Hypothetical helicase	Unknown
GID11_seq974	51,8	ABC transporter, permease	Transport
GID11_seq975	52,7	ABC transporter, ATP-binding subunit	
GID11_seq976	43,3	ArsR family transcriptional regulator	
GID11_seq1078	96,9	Superfamily II group DNA or RNA helicases	Possible regulation of expression
GID11_seq1083	100	Flavin reductase-like domain-containing protein	Flavin reductase is present on the surface of pneumococci. It promotes virulence by protecting against oxidative stress and mediating adhesion
GID11_seq1103	95,5	Transcription regulator BlpS	The domain binding to DNA
GID11_seq1185	28,8	Component of the antimicrobial peptides ABC transport system	Interspecific competition
GID11_seq1585	28	HECT domain containing protein	Ubiquitin-protein ligases — protein utilization
		CC-1012 — 6 isolates**	
GID12_seq99	100	Guanosine triphosphate cyclohydrolase	The opening of the imidazole ring of guanosine triphosphate is catalyzed. An obligatory stage of biosynthesis of a variety of coenzymes (riboflavin and folate), tRNA bases
GID12_seq198	100	Hypothetical macrolide efflux protein	Possible macrolide efflux

End of the Table 5

ID последовательности Sequence ID	Homology with known proteins, %	Protein name	Proposed function
GID12_seq199	99,8	Hypothetical protein	Unknown
GID12_seq200	100	Group I pyridoxal-dependent decarboxylase (cleaves Orn/Lys/Arg and glycine)	Amino acid metabolism
GID12_seq887	98,3	Competence system transport protein	Natural competence system
GID12_seq1238	87,9	DNA-binding protein of the satellite phage *Streptococcus satellite phage Javan359*	Prophage component
GID12_seq1240	100	Hypothetical satellite prophage protein *Streptococcus satellite phage Javan735*	Prophage component
GID12_seq1279	91,4	Argininosuccinate synthetase, rgG	Amino acid biosynthesis; L-arginine biosynthesis (L-arginine from L-ornithine and carbamoyl phosphate
GID12_seq1281	98,4	Bacteriocin-like peptide	

Note. *The ST62 group: 642_PEGAS_2020_226, MiSeq_105_Kz, ST_62_10, ST_62_11, ST_62_12, ST_62_13, ST_62_14, ST_62_15, ST_62_16, ST_62_17, ST_62_18, ST_62_19, ST_62_20, ST_62_21, ST_62_22, ST_62_23, ST_62_24, ST_62_26, ST_62_27, ST_62_28, ST_62_3, ST_62_4, ST_62_5, ST_62_6, ST_62_7, ST_62_8, ST_62_9, 600_PEGAS_2019_344, 629_PEGAS_2020_150.
**The ST1012 group: ST_1012_3, ST_1012_4, 561_PEGAS_2019_401, 581_PEGAS_2019_114, 589_PEGAS_2019_113, 602_PEGAS_2019_349.

S. pneumoniae plays a non-metabolic role in the interaction of this bacterium with the human host. Pneumococcal surface adhesin A (PspA) prevents activation of both classical and alternative complement pathways through its interaction with the C3b component [39]. PspA also interacts with human lactoferrin, inhibiting its bactericidal action [39]. Flavin reductase is present on the surface of pneumococci and promotes virulence by protecting against oxidative stress and mediating adhesion, and provides protection against pneumococcal infection [40]. The immune response to this protein increases with age [40]. SS-62 representatives contain other hypothetical regulators of complement activation, ABC-transporters and transcription regulators. Probably, the presence of a large number of adaptive factors allowed the genetic lineage ST-62, associated mainly with serotype 11A, to spread widely throughout the world.

Serogroup 11 includes 6 antigenically different serotypes (11A-11F) with highly homologous *cps* loci. The structural difference between the serotypes is due to either the mutations in the *wcjE* gene (manifested in serotypes 11A and 11E by differences in the degree of β-galactose-6-O-acylation) [41], or the N112S mutation in the *wcrL* glycosyltransferase gene (manifested by the addition of an additional carbohydrate residue to the repeating unit of the carbohydrate chain of the capsule in serotype 11D) [42]. Studies have shown that vaccines containing serotype 11A are very likely to limit the spread of serotype 11E, but not serotypes 11B, 11C, 11F, nor 11D (due to the presence of 2 types of carbohydrate chain structural units in its capsule) [43]. However, all serotypes except 11A are not widely distributed, and their inclusion in a future vaccine is not yet necessary.

There is no doubt that specific prophylaxis with pneumococcal vaccines plays a huge role in reducing invasive forms of pneumococcal infections both among children and adults, as evidenced by numerous publications from various countries that have introduced this vaccination into national calendars. However, the undeniable fact is the increased prevalence of non-vaccine serotypes of pneumococci, the invasive potential of which still requires clarification and additional research. One of the ways to further improve specific prophylaxis, some authors suggest the development of new vaccines with high valence. But it should also be taken into account that structural similarity between capsular polysaccharides of closely related serotypes of pneumococci may lead to induction of cross-reacting antibodies against serotype not covered by PCV, which may provide additional protective clinical effect.

Conclusion

Vaccination against invasive variants of pneumococci has played an important role in the spread of non-vaccine serotypes, and the epidemic processes associated with their expansion are a consequence and evidence of the effectiveness of vaccination. Serotype-specific vaccination leads to the spread of serotypes not covered by vaccines, some of which may exhibit increased virulence and/or antimicrobial resis-

tance. In Russia, serogroups 15 and 11 are common among non-vaccine serogroups. No antimicrobial resistance determinants have been identified in the genomes of representatives of these serogroups. For each of the genetic lineages associated with serogroups 15 and 11 common in Russia, virulence determinants unique within the serogroup under study have been identified, which may contribute to the success of these lineages. Given the high virulence potential and prevalence, we can predict an increase in the epidemiologic importance of these genetic lineages in Russia. Inclusion of serotypes 15B and 11A in vaccines for use in Russia is advisable.

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teria, made a significant contribution to the search and analytical work and preparation of the article, read and approved the final version before publication.

The article was submitted 17.04.2024;
accepted for publication 22.06.2024;
published 29.08.2024

Баязитова Л.Т. — организация сбора и обработки материала; *Исаева Р.А., Полев Д.Е., Саитова А.Т., Гончаров Н.Е., Калиногорская О.С., Гордеева С.А.* — сбор и обработка материала; *Краева Л.А.* — организация обработки материала; *Сидоренко С.В.* — концепция и дизайн исследования, редактирование. Все авторы подтверждают соответствие своего авторства критериям ICMJE, внесли существенный вклад в проведение поисково-аналитической работы и подготовку статьи, прочли и одобрили финальную версию до публикации.

Статья поступила в редакцию 17.04.2024;
принята к публикации 22.06.2024;
опубликована 29.08.2024

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-548
ORIGINAL RESEARCHES

Оригинальное исследование
https://doi.org/10.36233/0372-9311-548

Распространённость генов *qacEΔ1, qacE, oqxA, oqxB, acrA, cepA* и *zitB* среди мультирезистентных *Klebsiella pneumoniae*, выделенных в кардиохирургическом стационаре

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Аннотация

Актуальность. Инфекции, вызванные *Klebsiella pneumoniae* с множественной лекарственной устойчивостью (МЛУ), являются основной причиной смертности во всём мире. Широкое использование дезинфицирующих средств и антисептиков привело к появлению *K. pneumoniae* со сниженной чувствительностью к ним, что в сочетании с МЛУ может представлять существенную эпидемиологическую угрозу.

Целью исследования была оценка распространённости генов эффлюксных насосов и транспортёров, ассоциированных с устойчивостью к биоцидам, и их связи с резистентностью к антибиотикам среди изолятов *K. pneumoniae*, выделенных в кардиохирургическом стационаре.

Материалы и методы. Изоляты *K. pneumoniae* (*n* = 50), выделенные из клинического материала пациентов и смывов с медицинского оборудования, были проверены методом полимеразной цепной реакции на присутствие генов 4 типов эффлюксных насосов (*qacEΔ1*, *qacE*, *oqxA*, *oqxB*, *acrA*) и 2 транспортёров, участвующих в оттоке катионов (*cepA*) и ионов цинка (*zitB*). Для оценки силы ассоциации между генами устойчивости к биоцидам, генами бета-лактамаз и мобильных генетических элементов использовали тест ранговой корреляции Спирмена.

Результаты. Встречаемость *K. pneumoniae*, содержащих в геноме *qacEΔ1*, *qacE*, *oqxA*, *oqxB*, *acrA*, *cepA* и *zitB*, оказалась высокой: 54, 62, 100, 84, 100, 72 и 96% соответственно. Наиболее часто были обнаружены *K. pneumoniae* с комбинацией всех исследуемых насосов (32%), причём такие культуры были в 100% случаев мультирезистентными. Гены *qacE*, *qacEΔ1* были тесно связаны с устойчивостью к цефалоспоринам, карбапенемам, фторхинолонам, генами карбапенемаз и интегронами. Среди клинических изолятов *K. pneumoniae* с МЛУ были широко представлены гены различных эффлюксных насосов, ассоциированных с устойчивостью к биоцидам, и их комбинации.

Заключение. Высокая распространённость генов эффлюксных насосов, ассоциированных с устойчивостью к четвертичным соединениям аммония, хлоргексидину и солям цинка, и их значимая связь с антибиотикорезистентностью у нозокомиальных *K. pneumoniae* подчёркивают важность дальнейшего изучения механизмов кросс-резистентности к биоцидам для совершенствования методов борьбы с патогенами с МЛУ.

Ключевые слова: *Klebsiella pneumoniae, гены эффлюксных насосов и транспортеров, устойчивость к антибиотикам, гены бета-лактамаз, мобильные генетические элементы*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Институциональным наблюдательным советом Института экологии и генетики микроорганизмов (протокол № 26, дата утверждения 22.05.2024).

Благодарность. Авторы статьи выражают благодарность к.м.н. Ларисе Геннадьевне Кудрявцевой и д.м.н. Виктору Ивановичу Сергевнину за предоставленный биологический материал для молекулярно-генетического анализа.

Источник финансирования. Работа выполнена в рамках НИОКТР № 124020500028-4.

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

Для цитирования: Михайловская В.С., Селиванова П.А., Кузнецова М.В. Распространённость генов *qacEΔ1, qacE, oqxA, oqxB, acrA, cepA* и *zitB* среди мультирезистентных *Klebsiella pneumoniae*, выделенных в кардиохирургическом стационаре. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):502–511.
DOI: https://doi.org/10.36233/0372-9311-548
EDN: https://www.elibrary.ru/fcoyee

Original Study Article
https://doi.org/10.36233/0372-9311-548

Prevalence of *qacEΔ1, qacE, oqxA, oqxB, acrA, cepA* and *zitB* genes among multidrug-resistant *Klebsiella pneumoniae* isolated in a cardiac hospital

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Abstract

Background. Infections caused by multidrug-resistant (MDR) *Klebsiella pneumoniae* are the leading cause of mortality worldwide. The widespread use of disinfectants and antiseptics has caused the emergence of *K. pneumoniae* with reduced sensitivity to them, which, in combination with MDR, can pose a significant epidemiological threat.

The aim of the study was to assess the prevalence of efflux pump and transporter genes associated with biocide resistance and their association with antibiotic resistance among *K. pneumoniae* isolated in a cardiac surgical hospital.

Materials and methods. *K. pneumoniae* isolates (*n* = 50) from the patients and medical equipment were tested by polymerase chain reaction for the presence of genes of 4 types of efflux pumps (*qacEΔ1*, *qacE*, *oqxA*, *oqxB*, *acrA*) and 2 transporters involved in the outflow of cations (*cepA*) and zinc ions (*zitB*). Spearman's rank correlation test was used to assess the strength of the association between the efflux pumps, beta-lactamase genes and mobile genetic elements.

Results. The occurrence of *K. pneumoniae* containing *qacEΔ1*, *qacE*, *oqxA*, *oqxB*, *acrA*, *cepA* and *zitB* was high: 54, 62, 100, 84, 100, 72 и 96% respectively. *K. pneumoniae* with a combination of all the studied pumps was most often detected (32%), and these isolates were MDR in 100% of cases. The *qacE*, *qacEΔ1* genes were closely associated with resistance to cephalosporins, carbapenems, fluoroquinolones, carbapenemase genes, and integrons. The results of the study showed that the genes of various efflux pumps associated with biocide resistance and their combinations were widely represented among the clinical isolates of MDR *K. pneumoniae*.

Conclusion. The high prevalence of efflux pump genes associated with resistance to quaternary ammonium compounds, chlorhexidine and zinc salts and their significant association with antibiotic resistance in nosocomial *K. pneumoniae* underlines the importance of further studying the mechanisms of cross-resistance to biocides to improve methods of combating MDR nosocomial pathogens.

Keywords: *Klebsiella pneumoniae, genes of efflux pumps and transporters, resistance to antibiotics, beta-lactamase genes, mobile genetic elements*

Ethics approval. The study protocol was approved by the Institutional Review Board of the Institute of Ecology and Genetics of Microorganisms (protocol No. 26, May 22, 2024).

Acknowledgement. The authors of the article express their gratitude to Larisa G. Kudryavtseva, Cand. Sci. (Med.) and Viktor I. Sergeevnin D. Sci. (Med.) for providing the biological material for molecular genetic analysis.

Funding source. The work was carried out within the framework the state assignment No. 124020500028-4.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Mihailovskaya V.S., Selivanova P.A., Kuznetsova M.V. Prevalence of *qacEΔ1, qacE, oqxA, oqxB, acrA, cepA* and *zitB* genes among multidrug-resistant *Klebsiella pneumoniae* isolated in a cardiac hospital. *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):502–511.
DOI: https://doi.org/10.36233/0372-9311-548
EDN: https://www.elibrary.ru/fcoyee

Введение

Klebsiella pneumoniae является одним из ведущих возбудителей инфекций, связанных с оказанием медицинской помощи в кардиохирургических стационарах. Представители этого вида, согласно классификации Всемирной организации здравоохранения, относятся к группе приоритетных патогенов, поскольку инфекции, вызванные мульти- и панрезистентными *K. pneumoniae*, ассоциированы с высокой смертностью пациентов [1]. Необходимой частью программы инфекционного контроля и предотвращения внутрибольничных инфекций является обработка поверхностей дезинфектантами и антисептиками [2]. Часто использующиеся в качестве дезинфицирующих средств четвертичные аммониевые соли (ЧАС) обладают способностью прикрепляться к клеточной стенке бактерий благодаря положительному заряду, что вызывает её структур-

DOI: https://doi.org/10.36233/0372-9311-548

ную дезорганизацию и лизис клеток. Хлоргексидин может ковалентно связываться с мембраной, что в конечном итоге приводит к деполяризации и гибели клеток бактерий [3]. Широкое применение дезинфицирующих средств и антисептиков (в том числе в общественных и медицинских учреждениях во время пандемии COVID-19) вызвало возникновение резистентных к ним *K. pneumoniae*, что в сочетании с множественной лекарственной устойчивостью (МЛУ) может представлять существенную эпидемиологическую угрозу [4, 5].

Одним из механизмов резистентности *K. pneumoniae* к биоцидам является экспрессия эффлюксных насосов (ЭН). Существуют 6 семейств эффлюксных систем: основное суперсемейство мембранных транспортеров (MFS), суперсемейство оттока лекарственных и токсичных веществ (MATE), суперсемейство АТФ-связывающих бактериальных кассетных транспортеров (ABC), суперсемейство малых транспортеров множественной лекарственной устойчивости (SMR), суперсемейство связывающе-транспортирующих протеинов (RND) и недавно описанная протеобактериальная антимикробная эффлюксная структура (PACE) [6, 7]. AcrAB, OqxAB, EefAB и KexD относятся к белкам суперсемейства RND, которое является наиболее важным у грамотрицательных бактерий. Среди ЭН наибольшую значимость представляют AcrAB и OqxAB [8]. Гены *qacE* и *qacEΔ1* впервые были описаны у *K. pneumoniae* в 3'-консервативном сегменте интегрона плазмиды R751 [9]. Ген *qacEΔ1* представляет собой модифицированную форму *qacE*, которая возникла в результате вставки сегмента ДНК, содержащего ген устойчивости к сульфаниламиду вблизи 3'-конца гена *qacE*. упомянутые ЭН относятся к суперсемейству SMR и обусловливают устойчивость к органическим катионам. Известно, что *cepA*, кодирующий насос оттока катионов, связан с устойчивостью к хлоргексидину у *K. pneumoniae* [10]. ZitB участвует в конститутивном пути экспорта цинка, способствуя гомеостазу клетки во время воздействия низких и умеренных концентраций ионов металла [11].

Гены группы *qac* часто выявляются в ассоциации с генами, кодирующими устойчивость к антибиотикам разных групп, в том числе к бета-лактамам [12]. В нескольких работах отмечается, что присутствие насоса AcrAB-TolC способствовало снижению минимальной ингибирующей концентрации ципрофлоксацина [13] и бета-лактамов [4] и формированию МЛУ. Важно, что гены ЭН, связанных с устойчивостью к биоцидам, располагаются не только на хромосоме (*emrE, mdfA, sugE, ydgE, ydgF*), но и на мобильных генетических элементах (*oqxA, oqxB, qacEΔ1, qacE, qacF/H/I, qacG, sugE*), таких как плазмиды, интегроны и транспозоны [14]. Более того, гены ЭН могут быть локализованы на тех же мобильных элементах, что и гены устойчивости к антибиотикам, что приводит к перекрестной или ко-резистентности к дезинфектантам, антисептикам и антимикробным препаратам [14].

Ранее мы представили молекулярно-генетическую характеристику изолятов *K. pneumoniae*, выделенных от пациентов и проб окружающей среды в кардиохирургическом стационаре [15].

Цель исследования — оценка распространённости генов ЭН и транспортёров, ассоциированных с устойчивостью к биоцидам, и их связи с резистентностью к антибиотикам среди изолятов *K. pneumoniae*, выделенных в условиях кардиохирургического стационара.

Материалы и методы

Бактериальные изоляты

Исследовали 50 изолятов *K. pneumoniae*, выделенных в 2021–2022 гг. из клинического материала пациентов (мокрота, кровь, моча, содержимое ран) и смывов с медицинского оборудования кардиохирургического стационара. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Институциональным наблюдательным советом Института экологии и генетики микроорганизмов (протокол № 26, дата утверждения 22.05.2024).

Бактериологические исследования были выполнены на автоматическом анализаторе «WalkAway-96 Plus» («Beckman Coulter») с использованием панели NBC 41. Чувствительность к антибиотикам (ампициллин, цефотаксим, цефтазидим, цефтриаксон, цефепим, меропенем, имипенем, ампициллин/сульбактам, ампициллин/клавуланат, ципрофлоксацин, левофлоксацин, амикацин, гентамицин) и присутствие генов бета-лактамаз (bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{OXA}, bla_{KPC}, bla_{VIM-2}, bla_{IMP-1}, bla_{NDM-1}) оценивали ранее [15]. Фенотип МЛУ определяли как нечувствительность штаммов хотя бы к одному препарату 3 и более классов антибиотиков.

Детекция генов ЭН и мобильных генетических элементов

ДНК изолятов *K. pneumoniae* экстрагировали путём прогрева суспензии клеток бактерий в течение 15 мин при 97ºС в твёрдотельном термостате «Термит», пробы охлаждали, центрифугировали 5 мин при 13 000 об/мин, супернатанты переносили в чистые пробирки Эппендорф и хранили при –20ºС. Методом ПЦР детектировали гены ЭН, ассоциированных с устойчивостью к ЧАС и хлоргексидину (*qacEΔ1*, *qacE*, *oqxA, oqxB, acrA*), ген транспортёра катионов (*cepA*), ген *zitB*, кодирующий транспортёр цинка, ген интегронов класса 1 (*int1*), а также плазмиды IncQ, распространённой в клинических *K. pneumoniae*. Праймеры, синтезированные ООО «Синтол» согласно рекомендациям ав-

торов, условия проведения ПЦР и размеры ампликонов указаны в **табл. 1**. Амплификацию проводили в 25 мкл реакционной смеси с использованием реагентов производства ООО «Синтол» на термоциклере «DNA Engine Dyad Thermal Cycler» («Bio-Rad»). Электрофоретическое разделение продуктов реакции проводили в 1,2–2,0% агарозном геле в трисборатном буфере при напряжении электрического поля 6 В/см. Визуализацию полос и документирование данных осуществляли с помощью системы гель-документации «Gel-DocXR» («Bio-Rad»).

Статистический анализ

Для выявления значимых различий между качественными показателями выборок определяли точный критерий Фишера (двусторонний). Значения $p < 0,05$ считали достоверными. Тест ранговой корреляции Спирмена использовали для оценки силы ассоциации между генами ЭН и транспортёров, генами бета-лактамаз и мобильных генетических элементов. Сила связи была классифицирована по значению коэффициента r_S на очень слабую (0,00–0,19), слабую (0,20–0,39), среднюю (0,40–0,59), сильную (0,60–0,79) и очень сильную (0,80–1,0).

Результаты

Все протестированные гены ЭН встречались с высокой частотой среди изолятов *K. pneumoniae*, выделенных из биологического материала пациентов и медицинского оборудования кардиохирургического стационара (**рис. 1**, *а*). Распространённость генов *qacE, qacEΔ1, oqxB* и *cepA*, ассоциированных с устойчивостью к хлоргексидину и ЧАС, оказалась значительной и составила 62% (31/50), 54% (27/50), 84% (42/50) и 72% (36/50) соответственно. Гены *oqxA* и *acrA* были обнаружены у всех изолятов. Встречаемость *K. pneumoniae*, содержащих ген системы выброса ионов цинка (*zitB*), составила 96% (48/50). В целом в геномах *K. pneumoniae* было обнаружено 3–7 генов разных ЭН и транспортёров одновременно, причём в исследуемой выборке более половины культур имели 6 и более ЭН (58%, 29/50). Важно отметить, что изоляты с МЛУ содержали больше разных эффлюксных систем, чем чувствительные *K. pneumoniae* ($p < 0,05$, *t*-тест) (**рис. 1**, *б*).

Детектировано 14 индивидуальных паттернов, среди которых комбинация всех исследуемых генов (*qacE* + *qacEΔ1* + *cepA* + *zitB* + *oqxA* + *oqxB* + *acrA*) встречалась наиболее часто (32%, 16/50). Эти 16 культур были в 100% случаев с МЛУ, в частности устойчивы ко всем протестированным бета-лактамным антибиотикам и фторхинолонам. Каждая из последующих комбинаций встречалась с частотой менее 12% (**табл. 2**).

В **табл. 3** показана ассоциация между генами ЭН и транспортёров и фенотипом резистентности к антибиотикам *K. pneumoniae*. Гены *qacE*, *qacEΔ1* были тесно связаны с устойчивостью к цефалоспо-

Таблица 1. Использованные в исследовании последовательности праймеров, условия проведения ПЦР и ожидаемый размер ампликона
Table 1. Primer sequences used in the study, the conditions for PCR and the expected size of the amplicons

Ген Gene	Нуклеотидная последовательность (5'–3') Nucleotide sequence (5'–3')	Условия ПЦР PCR conditions	Размер, п.н. Size, bp	Ссылка Reference
ЭН и транспортёры | Efflux pumps and transporters				
qacEΔ1	F: TAGCGAGGGCTTTACTAAGC R: ATTCAGAATGCCGAACACCG	93ºC, 2 m; 35 [93ºC, 30 s; 55ºC, 30 s; 72ºC, 1 m]; 72ºC, 5 m	300	[16]
qacE	F: CCCGAATTCATGAAAGGCTGGCTT R: AAGCTTTCACCATGGCGTCGG		350	
cepA	F: CAACTCCTTCGCCTATCCCG R: TCAGGTCAGACCAAACGGCG	94ºC, 5 m; 30 [94ºC, 30 s; 53ºC, 60 s; 72ºC, 2 m]; 72ºC, 7 m	1051	
oqxA	F: CTCGGCGCGATGATGCT R: CCACTCTTCACGGGAGACGA	95ºC, 1 m; 35 [95ºC, 45 s; 60ºC, 45 s; 72ºC, 1 m]; 72ºC, 5 m	392	[17]
oqxB	F: TTCTCCCCCGGCGGGAAGTAC R: CTCGGCCATTTTGGCGCGTA		512	
acrA	F: GCTGTCGACGGTTAATGACTTTACAGAGG R: ACATCCGAGAATTCCAGCGT	94ºC, 3 m; 34 [94ºC, 45 s; 52ºC, 45 s; 68ºC, 1 m]; 72ºC, 5 m	107	[18]
zitB	F: TACGACGCTTCAGTTCAGC R: CACTTTCGGTTGGCTAAGAC	95ºC, 5 m; 30 [94ºC, 30 s; 53ºC, 60 s; 72ºC, 60 s]; 72ºC, 7 m	449	[19]
Мобильные генетические элементы | Mobile genetic elements				
intI	F: GGCATCCAAGCAGCAAG R: AAGCAGACTTGACCTGA	94ºC, 5 m; 35 [94ºC, 30 s; 55ºC, 30 s; 72ºC, 2 m]; 72ºC, 5 m	–*	[20]
IncQ	F: CTCCCGTACTAACTGTCACG R: ATCGACCGAGACAGGCCCTGC	94ºC, 5 m; 35 [94ºC, 30 s; 61ºC, 30 s; 72ºC, 2 m]; 72ºC, 5 m	436	[21]

Примечание. *Продукт амплификации может быть представлен несколькими последовательностями разного размера.
Note. *The amplification product may be represented by several sequences of different size.

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-548
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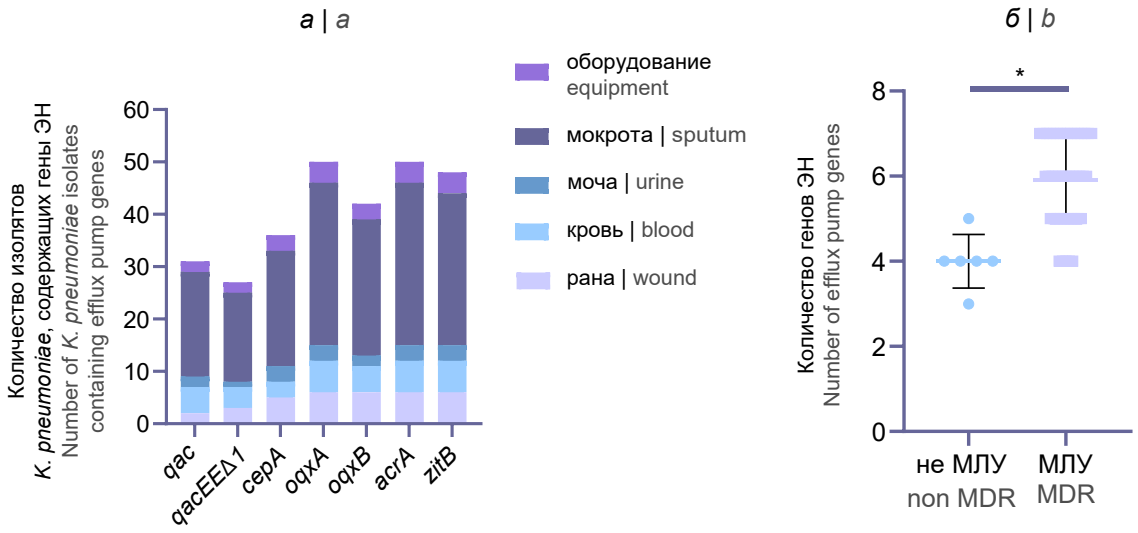

Рис. 1. Распространённость *K. pneumoniae*, содержащих гены ЭН, с учётом источника выделения (*а*); количество детектированных генов ЭН среди МЛУ и не-МЛУ *K. pneumoniae* (*б*).

*$p < 0,05$ (*t*-тест).

Fig. 1. The prevalence of *K. pneumoniae* isolates containing efflux pump genes by the source of isolation (*a*); the number of detected efflux pump genes among multidrug-resistant (MDR) and non-multidrug-resistant (non-MDR) *K. pneumoniae* (*b*).

*$p < 0,05$ (*t*-test).

ринам, карбапенемам и фторхинолонам, а *oqxB* — к цефалоспоринам. Важно отметить, что только *K. pneumoniae* с МЛУ кодировали *qacE* и *qacEΔ1*. Не выявлено значимых ассоциаций генов *cepA* и *zitB* с фенотипом устойчивости к антибиотикам.

Таблица 2. Встречаемость индивидуальных комбинаций генов ЭН, ассоциированных с устойчивостью к биоцидам, среди изолятов *K. pneumoniae*

Table 2. The occurrence of individual combinations of efflux pump genes associated with biocide resistance among *K. pneumoniae*

Комбинации генов ЭН Efflux pump genes combinations	*n* (% от всех культур) (% of all isolates)
qacE + qacEΔ1 + cepA + zitB + oqxB + oqxA + acrA	16 (32)
cepA + zitB + oqxB + oqxA + acrA	6 (12)
cepA + zitB + oqxA + acrA	5 (10)
qacE + qacEΔ1 + zitB + oqxB + oqxA + acrA	5 (10)
qacE + cepA + zitB + oqxB + oqxA + acrA	4 (8)
zitB + oqxB + oqxA + acrA	3 (6)
qacE + zitB + oqxB + oqxA + acrA	3 (6)
qacEΔ1 + cepA + zitB + oqxB + oqxA + acrA	2 (4)
zitB + oqxA + acrA	1 (2)
qacE + qacEΔ1 + cepA + zitB + oqxA + acrA	1 (2)
qacE + qacEΔ1 + zitB + oqxA + acrA	1 (2)
qacE + qacEΔ1 + cepA + oqxB + oqxA + acrA	1 (2)
cepA + oqxB + oqxA + acrA	1 (2)
qacEΔ1 + zitB + oqxB + oqxA + acrA	1 (2)

С помощью корреляционного анализа выявлена позитивная связь между *qacEΔ1* и генами бета-лактамаз: bla_{OXA} (r_S = 0,31), bla_{VIM-2} (r_S = 0,68), bla_{NDM-1} (r_S = 0,64), bla_{CTX-M} (r_S = 0,51), bla_{SHV} (r_S = 0,51). Эти же гены показали значимую связь с *qacE* (**рис. 2**). В нашем исследовании гены *cepA* и *zitB* не показали позитивных корреляций с генами устойчивости к антибиотикам. Интегроны класса 1 длиной от 800 п.н. до 2500 п.н. были выявлены у 32 (64%) изолятов. Плазмида группы несовместимости Q была обнаружена у 90% (45/50) *K. pneumoniae*. Корреляционный анализ показал значимую позитивную связь между генами насосов *qacE, qacEΔ1* и интегронами (r_S = 0,70 и r_S = 0,65; рис. 2).

Обсуждение

K. pneumoniae — распространённый патоген, вызывающий инфекции, связанные с оказанием медицинской помощи. МЛУ этих бактерий к антибиотикам первой линии существенно затрудняет лечение. К тому же широкое использование биоцидов (особенно в сублетальных концентрациях) может вызвать резистентность к ним и, как следствие, способствовать сохранению *K. pneumoniae* в окружающей среде, в том числе на поверхностях медицинских приборов. Сниженная чувствительность бактерий к ЧАС, хлоргексидину и солям цинка в высокой степени связана с экспрессией ЭН. Во многих исследованиях показана важность ЭН для повышения устойчивости штаммов *K. pneumoniae* и к различным классам антибиотиков [22–24]. Важно отметить, что гены устойчивости к противомикробным препаратам часто находятся на

Таблица 3. Ассоциация между резистентностью к антибиотикам и генами ЭН изолятов *K. pneumoniae*
Table 3. Association between antibiotic resistance and efflux pump genes of *K. pneumoniae* isolates

Антибиотик/группа Antibiotic/group	Профиль Profile	Количество изолятов *K. pneumoniae*, позитивных на гены ЭН, *n* (%) The number of *K. pneumoniae* positive for the efflux pump genes, *n* (%)				
		qacEΔ1	*qacE*	*cepA*	*oqxB*	*zitB*
Ампициллин Ampicillin	R (*n* = 50)	27 (54)	31 (62)	36 (72)	42 (84)	48 (96)
	S (*n* = 0)	0	0	0	0	0
Цефалоспорины Cephalosporins	R (*n* = 45)	27 (60)*	31 (68,9)*	34 (75,6)	38 (84,4)	44 (97,8)
	S (*n* = 5)	0	0	2 (40)	4 (80)	4 (80)
Карбапенемы Carbapenems	R (*n* = 38)	27 (71,1)*	30 (78,9)*	29 (76,3)	35 (92,1)*	37 (97,4)
	S (*n* = 12)	0	1 (8,3)	7 (58,3)	7 (58,3)	11 (91,7)
Ампициллин/сульбактам Ampicillin/sulbactam	R (*n* = 45)	27 (60)*	31 (68,9)*	34 (75,6)	38 (84,4)	44 (97,8)
	S (*n* = 5)	0	0	2 (40)	4 (80)	4 (80)
Амоксициллин/клавуланат Amoxicillin/clavulanic acid	R (*n* = 42)	26 (61,9)*	29 (69,1)*	32 (76,2)	36 (85,7)	41 (97,6)
	S (*n* = 8)	1 (12,5)	2 (25)	4 (50)	6 (75)	7 (87,5)
Фторхинолоны Fluoroquinolones	R (*n* = 44)	27 (61,4)*	31 (70,5)*	33 (75)	38 (86,4)	43 (97,7)
	S (*n* = 6)	0	0	3 (50)	4 (66,7)	5 (83,3)
Гентамицин Gentamicin	R (*n* = 29)	20 (69)*	21 (72,4)	19 (65,5)	25 (86,2)	28 (96,6)
	S (*n* = 21)	7 (33,3)	10 (47,6)	17 (81)	17 (81)	20 (95,2)
Амикацин Amikacin	R (*n* = 23)	16 (69,6)	15 (65,2)	17 (73,9)	20 (87)	22 (95,7)
	S (*n* = 27)	11 (40,7)	16 (59,3)	19 (70,4)	22 (81,5)	26 (96,3)
Фенотип МЛУ MDR phenotype	МЛУ | MDR (*n* = 44)	27 (61,4)*	31 (70,5)*	33 (75)	38 (86,4)	43 (97,7)
	не-МЛУ non-MDR (*n* = 6)	0	0	3 (50)	4 (66,7)	5 (83,3)

Примечание. *$p < 0,05$ — разница между выборками статистически значима (*F*-тест).
Note. *$p < 0,05$ — difference between the samples is statistically significant (*F*-test).

мобильных генетических элементах, которые могут передаваться путём горизонтального переноса от одного штамма к другому. В данной работе у наиболее значимого нозокомиального патогена — *K. pneumoniae* мы изучили распространённость ЭН, связанных с устойчивостью к ЧАС и хлоргексидину, транспортёров, ассоциированных со сниженной чувствительностью к солям цинка, а также их связь с резистентностью к антибиотикам, наличием генов бета-лактамаз и мобильными генетическими элементами.

Одним из механизмов устойчивости к биоцидам грамотрицательных бактерий является экспрессия эффлюксных систем семейства SMR, которые кодируются генами *qacE* и *qacEΔ1* [25, 26]. С устойчивостью *K. pneumoniae* к хлоргексидину тесно связан *cepA*, кодирующий белки системы оттока катионов [27]. В выборочных исследованиях по изучению резистентности клинических штаммов *K. pneumoniae* к бензалкония хлориду ген *qacΔE1* обнаруживался в диапазоне от 53,1 до 68,0% [28, 29]. Согласно A. Abuzaid и соавт., среди *K. pneumoniae* ген *cepA* встречался в 87,5% случаев [28]. В исследовании К.Г. Косяковой и соавт. частота встречаемости *qacE*, *qacEΔ1* и *cepA* составила 33,3, 23,3 и 83,3% соответственно [25]. В нашем исследовании *qacEΔ1* и *qacE* были распространены в группе нозокомиальных *K. pneumoniae* с более высокой частотой: 62 и 54% соответственно. Встречаемость *cepA* оказалась несколько ниже — 72%. В последнее время возрастает роль OqxA- и OqxB-насосов, входящих в семейство связывающе-транспортирующих белков [22]. Ранее J. Yuan и соавт. обнаружили гены *oqxAB* во всех исследованных штаммах *K. pneumoniae*, что позволило сделать предположение о геноме *K. pneumoniae* как возможном резервуаре *oqxAB* [32]. У клебсиелл в основном эти гены расположены в хромосоме, но могут находиться на плазмидах и часто ассоциированы с устойчивостью к фторхинолонам, тигециклину, а также ЧАС и бигуанидным дезинфицирующим средствам [30, 31]. Меньший процент встречаемости данных детерминант показали M. Dehnamaki и соавт.: 57 и 56% изолятов несли гены *oqxA* и *oqxB* соответственно [17]. В исследовании L. Ni и соавт. частоты обнаружения *oqxA* и *oqxB* составили 60,9 и 17,2% [24]. В нашем исследовании ген *oqxA* детектирован у всех изолятов кардиохирургического стационара, тогда как *oqxB* содержали 84% культур. ЭН AcrAB-TolC обеспечивает толерантность бактерий к различным

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-548

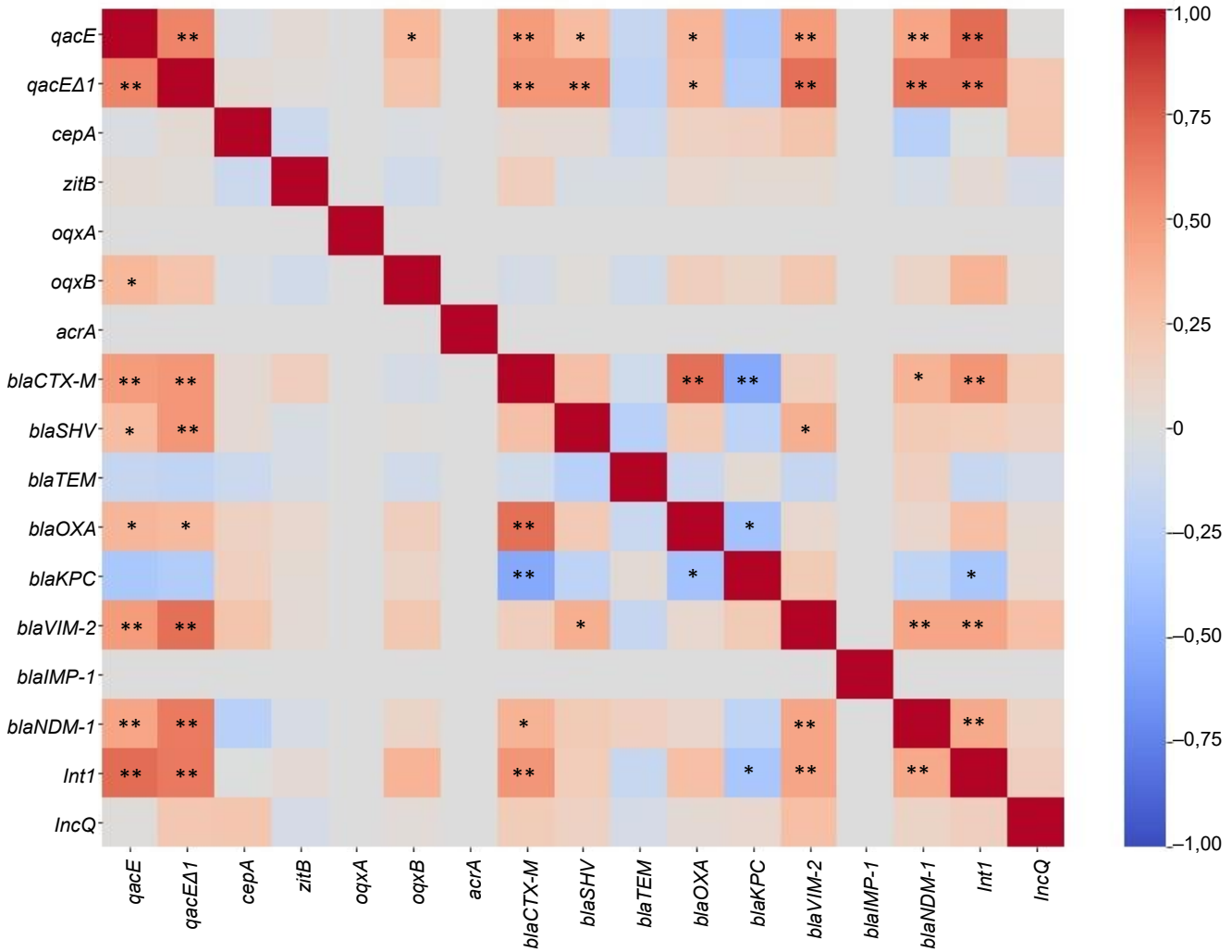

Рис. 2. Корреляционная матрица, отражающая силу связи между генами ЭН, генами бета-лактамаз и мобильными генетическими элементами.

Цветовое значение каждой ячейки соответствует коэффициенту корреляции Спирмена и пропорционально силе корреляции. *p < 0,05, **p < 0,01. Цветной вариант рисунка см. на сайте журнала.

Fig. 2. A correlation matrix reflecting the strength of the relationship between the efflux pumps genes, beta-lactamase genes and mobile genetic elements.

The color of each cell corresponds to the Spearman correlation coefficient and is proportional to the correlation strength. *p < 0.05, **p < 0.01. For a color version of the picture, see the journal's website.

антибиотикам, включая фторхинолоны, а также к биоцидам, включая этанол, хлоргексидина ацетат и бензалкония бромид [33]. Помимо вклада в фенотип МЛУ, AcrAB может представлять собой новый фактор вирулентности, необходимый *K. pneumoniae* для сопротивления иммунным защитным механизмам в лёгких, способствуя развитию пневмонии [34]. Ген *acrA*, кодирующий белок, соединяющий 2 интегральных мембранных белка, считается более консервативным, и его часто используют для обнаружения данного ЭН. В нашем исследовании все изоляты содержали *acrA*, тогда как, по данным W. Guo и соавт. [33], только 19% резистентных к карбапенемам *K. pneumoniae* включали данный ген. Важно отметить, что соли и наночастицы тяжёлых металлов активно используются в антисептических композициях. Сегодня ведётся активная разработка противомикробных поверхностных веществ на основе меди, серебра и цинка для создания бинарных или тройных контактно-нейтрализующих поверхностных покрытий [35]. Было доказано, что ZnO более токсичен для бактерий в форме наночастиц, что делает его более перспективным. В данной работе распространённость культур, кодирующих транспортёр, опосредующий отток ионов цинка [36], — ZitB, составила 96%.

В нескольких работах отмечается, что резистентность к антибиотикам и биоцидам может многократно увеличиваться за счёт одновременной экспрессии ЭН разных семейств [4, 19, 22]. Важно отметить, что в исследуемой выборке наиболее часто встречались *K. pneumoniae* с комбинацией всех исследуемых насосов (32%), причём такие культуры были в 100% случаев с МЛУ. Ранее мы показали

высокую распространённость в изолятах кардиохирургического стационара bla_{OXA}, bla_{VIM-2} и bla_{NDM-1}. В этом исследовании выявлено, что гены группы *qac* чаще встречались в ассоциации с генами, кодирующими устойчивость к карбапенемам, — bla_{VIM-2} (r_S = 0,68) и bla_{NDM-1} (r_S = 0,64), что может быть обусловлено их локализацией в плазмид-опосредованных интегронах класса 1 и требует дальнейшего изучения. В исследовании Y. Chen и соавт. также было показано, что гены *qacEΔ1* и *cepA* были достоверно чаще представлены среди карбапенемо-резистентных культур [16]. Глобальное распространение детерминант устойчивости к бета-лактамным антибиотикам, в том числе карбапенемам, является результатом их распространения посредством конъюгативных плазмид. Известны 5 групп несовместимости плазмид (Inc), которые описаны в литературе как потенциально ответственные за распространение генов bla_{KPC} и bla_{NDM} у изолятов *K. pneumoniae* [21]. Другие мобильные генетические элементы — интегроны — могут включать кассеты устойчивости к противомикробным препаратам и дезинфектантам [37]. По данным F. Firoozeh и соавт., 100% изолятов *K. pneumoniae* (n = 150) с МЛУ несли гены *intI* [38]. В исследовании É.M. Oliveira и соавт. у клинических изолятов *K. pneumoniae* в госпитале г. Ресифи (Бразилия) наиболее часто обнаруживались плазмиды групп несовместимости Q и FIB [21]. Данные, полученные W.M.B.S. Martins и соавт., свидетельствуют о том, что *K. pneumoniae* с высококопийной плазмидой IncQ, несущей bla_{KPC-2}, может эффективно передавать её путём конъюгативного переноса другим штаммам [39]. Плазмида IncQ, способная реплицироваться в широком круге хозяев, была обнаружена в клиническом изоляте *K. pneumoniae*, выделенном от пациентов с COVID-19 в России [40]. В нашем исследовании интегроны класса 1 были детектированы у 64% *K. pneumoniae*, а плазмиды IncQ — у 90%. При этом выявлена значимая позитивная связь между генами ЭН *qacE, qacEΔ1* и интегронами.

Заключение

Проведено исследование по распространённости генов ЭН, а также транспортёров, участвующих в оттоке ионов металлов, оценена их связь с антибиотикоустойчивостью и генами, ассоциированными с резистентностью к бета-лактамам, среди культур *K. pneumoniae* с МЛУ, выделенных от пациентов кардиохирургического стационара с нозокомиальной инфекцией. Показана значимая связь между устойчивостью к антибиотикам и присутствием генов ЭН, ассоциированных с устойчивостью к ЧАС, хлоргексидину и ионам цинка, что подчёркивает важность дальнейшего изучения механизмов кросс-резистентности к биоцидам для совершенствования методов борьбы с патогенами с МЛУ.

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Статья поступила в редакцию 01.06.2024;
принята к публикации 20.08.2024;
опубликована 29.08.2024

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The article was submitted 01.06.2024;
accepted for publication 20.08.2024;
published 29.08.2024

Original Study Article
https://doi.org/10.36233/0372-9311-524

Evaluation of symbiotic relationships of oral microorganisms and their effect on the development of inflammatory changes of the oral mucosa in the complete absence of teeth

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Abstract

Introduction. By fixing on the exposed surfaces of complete removable dentures and oral soft tissues, bacteria form a biofilm, thereby increasing their overall virulence and resistance. The microorganisms that make up the biofilm are often in a symbiotic relationship, which allows them to increase their pathogenic potential and cause the development of denture stomatitis. Accordingly, when a particular strain is present in the oral cavity, the risks of symbiosis are significantly increased.

The **aim** of the study was to evaluate the effect of symbiotic relationships of oral bacteria on the development of inflammatory changes in the oral cavity in the absence of teeth.

Materials and methods. Two groups of patients belonging to the elderly age according to WHO systematization (60–74 years) with complete absence of teeth (K08.1) were formed, differing in the presence of clinical manifestations of inflammation (82 men and 49 women). Biological material sampled from the oral cavity of patients was studied using the culture method and RT-PCR. To quantify the interaction between members of the microbiocenosis, we used the Jaccard similarity coefficient.

Results. Coagulase-negative and coagulase-positive staphylococci, *Neisseria*, *Candida* spp. fungi, *Enterobacterales* and *F. nucleatum* were more frequently found in patients with complete absence of teeth. Expressed symbiotic relations between microorganisms of the *"Enterobacterales* order, *Lactobacillus*, *Neisseria* and *Corynebacterium* genera, as well as *S. salivarius*, *C. albicans*, *F. nucleatum* were established. The nature of these relations depended on the presence of inflammatory changes in the oral mucosa and, in turn, influenced the development of the latter. Thus, in the absence of inflammation, *Corynebacterium*, *Lactobacillus* and *S. salivarius* showed stable synergism. In case of inflammation, the association between these bacteria was accompanied by the introduction of *F. nucleatum* and displacement of *S. salivarius*.

Conclusion. Thus, conditionally pathogenic microorganisms, forming microbial associations with multidirectional symbiotic relations increase their virulence, which allows them to occupy free niches in the oral cavity and subsequently trigger the development of pathological process of inflammatory character of prosthetic bed tissues.

Keywords: *symbiosis, microorganisms, inflammation, oral cavity, complete absence of teeth, denture stomatitis*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the E.A. Vagner Perm State Medical University (protocol No. 9, September 30, 2021).

Funding source. This study was not supported by any external sources of funding.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Shulyatnikova O.A., Yakovlev M.V., Godovalov A.P. Evaluation of symbiotic relationships of oral microorganisms and their effect on the development of inflammatory changes of the oral mucosa in the complete absence of teeth *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):512–519.
DOI: https://doi.org/10.36233/0372-9311-524
EDN: https://www.elibrary.ru/ioeegb

Оригинальное исследование
https://doi.org/10.36233/0372-9311-524

Оценка вклада симбиотических отношений микроорганизмов ротовой полости в развитие воспалительных изменений слизистой оболочки рта при полном отсутствии зубов

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Аннотация

Введение. Фиксация на открытых поверхностях съёмных пластиночных протезов и мягких тканей ротовой полости бактерий в виде биоплёнки обеспечивает повышение вирулентности и резистентности микробного сообщества. Микроорганизмы, входящие в состав биоплёнки, зачастую находятся в симбиотических отношениях, что позволяет им увеличивать свой патогенный потенциал и вызывать развитие протезных стоматитов.

Цель исследования — оценка вклада симбиотических отношений бактерий ротовой полости в развитие воспалительных изменений слизистой оболочки рта при полном отсутствии зубов.

Материалы и методы. Сформированы две группы пациентов в возрасте 60–74 лет (82 мужчины и 49 женщин) с полным отсутствием зубов (К08.1), различающиеся по наличию клинических проявлений воспаления. Биологический материал, отобранный из ротовой полости пациентов, изучали с использованием культурального метода и полимеразной цепной реакции для выявления микроорганизмов полости рта. Для количественного выражения взаимодействия между членами микробиоценоза использовали коэффициент сходства Жаккара.

Результаты. У пациентов с полным отсутствием зубов в микробиоте протезного ложа доминировали коагулазоотрицательные и коагулазоположительные *Staphylococcus* spp., *Neisseria* spp., *Candida* spp., *Fusobacterium* spp. и представители порядка *Enterobacterales.* Установлены выраженные симбиотические связи между микроорганизмами порядка *Enterobacterales*, родов *Lactobacillus*, *Neisseria* и *Corynebacterium*, а также *Streptococcus salivarius*, *C. albicans*, *F. nucleatum.* При этом характер этих отношений зависел от наличия воспалительных изменений слизистой оболочки рта и, в свою очередь, влиял на развитие последних. Так, в отсутствие воспаления устойчивый синергизм проявляют *Corynebacterium* spp., *Lactobacillus* spp. и *S. salivarius*. В случае присоединения воспаления в ассоциации этих бактерий наблюдается внедрение *F. nucleatum* и вытеснение *S. salivarius*.

Заключение. Условно-патогенные микроорганизмы, формируя микробные ассоциации с разнонаправленными симбиотическими отношениями, могут увеличивать свою вирулентность, что, вероятно, позволяет им занимать свободные ниши в ротовой полости, а в последующем обеспечивать развитие патологического процесса воспалительного характера тканей протезного ложа.

Ключевые слова: *симбиоз, микроорганизмы, воспаление, полость рта, полное отсутствие зубов, протезный стоматит*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Проведение клинико-экспериментальных исследований одобрено на заседании локального этического комитета ПГМУ им. акад. Е.А. Вагнера (протокол № 9 от 30.09.2021).

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.

Для цитирования: Шулятникова О.А., Яковлев М.В., Годовалов А.П. Оценка вклада симбиотических отношений микроорганизмов ротовой полости в развитие воспалительных изменений слизистой оболочки рта при полном отсутствии зубов. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):512–519.
DOI: https://doi.org/10.36233/0372-9311-524
EDN: https://www.elibrary.ru/ioeegb

DOI: https://doi.org/10.36233/0372-9311-524

Introduction

A comprehensive approach to dental treatment requires a detailed diagnosis of pathologies of the dento-mandibular system. Treatment of patients with complete absence of teeth in most clinical cases, in addition to the main dental disease, is accompanied by several concomitant pathologies that are associated with acute, subacute or chronic inflammatory process in the soft tissues of the denture bed [1]. The development of denture stomatitis is caused by both general and local etiological factors, often contributing to the rapid chronicization of the pathological process. Among such factors, the representatives of the oral microbiome play a decisive role.

Currently, various authors consider the oral cavity as a functionally and morphologically limited ecosystem, the main part of which are microorganisms (MO) [2, 3]. At the same time, direct contact with the external environment creates conditions for the establishment of an extensive range of transient microbes, many of which are fixed on open tissues and subsequently populate the oral cavity, becoming part of the permanent microbiota of the biotope [4, 5]. It should be noted that the differences in microanatomy, humidity, mobility and aeration of individual structures of the oral cavity, as well as the presence of dental structures and fillings in it contribute to the emergence of comfortable niches for the attachment and reproduction of opportunistic pathogenic microbes with both anaerobic and aerobic type of metabolism [6]. The latter, in turn, possess an extensive spectrum of pathogenicity factors, one of which is adhesive ability. Fixing on open surfaces of hard and soft tissues of the oral cavity, as well as artificial solid media, bacteria form a biofilm through cooperation and complex interaction, due to which their general virulence and resistance increase [7]. This spatial and structural association of individual strains of MO in the extracellular polysaccharide matrix is the main factor in the occurrence of the overwhelming spectrum of pathological processes of inflammatory character observed in the oral cavity [8, 9]. The latter, in turn, are often induced by exo- and endotoxins released by microbial cells and representing activators of mediated action on the macroorganism. The danger of such a bacterial ecosystem lies not only in resistance to most antibacterial drugs, but also in resistance to the factors of cellular and humoral immunity of the macroorganism, which is especially relevant for elderly people [10].

In the mid-1970s, the plaque-specific theory was formulated from the standpoint of clinical microbiology, which adheres to the concept of monoethiology of infectious and inflammatory diseases [11]. According to this theory, the development of the inflammatory process should be associated with the presence or relative predominance either in the biofilm composition or in the planktonic state of one etiologically significant MO species. However, due to the high contamination of the oral cavity and the presence of "comfortable" conditions for the formation of bacterial films, the doctrines described above somewhat lose their relevance.

It has been confirmed [12, 13] not only the importance of the bacterial composition of biofilms formed in the oral cavity on the surface of hard tissues of teeth and elements of dental structures in the development of inflammatory pathologies of the oral mucosa (OM), but also the amount of dental plaque and the time of its stay in direct contact with the soft tissues of the biotope in question.

Due to the formation of a close spatial-structural association, the bacteria included in the biofilm are often in symbiotic relations, which allows them to increase their pathogenic potential. At the same time, the introduction of a particular type of MO into such a symbiosis can both significantly change the orientation of symbiotic relations and influence the manifestations of the clinical picture. It is of interest to study the nature of interaction of oral cavity MOs in the presence and absence of inflammatory process.

The aim of the study was to evaluate the effect of symbiotic relations of oral cavity MOs on the development of inflammatory changes in the oral cavity in the absence of teeth.

Materials and methods

The basis for the formation of patient groups for the study was the assessment of the state of denture bed tissues in the complete absence of teeth. The study included individuals who, according to the World Health Organization systematization, belonged to the elderly age group (60-74 years old; **Table 1**).

In order to determine the changes in the interspecies relationships of the oral cavity MO in the presence/absence of inflammatory changes in the denture bed tissues, the patients were divided into two groups. The first group (n = 66) included patients who had been using previously fabricated complete removable plate pros-

Table 1. Study group composition

Participants	Group 1		Group 2	
	n	age, years	*n*	age, years
Males	40	64.3 ± 1.2	42	66.1 ± 1.1
Females	26	65.7 ± 1.4	23	64.8 ± 1.5

theses made of acrylic plastic Etacryl-02 for at least 6 months before the examination, who had passed the adaptation period and had no clinical signs of inflammatory phenomena in the oral cavity and periodontal tissues. The second group (n = 65) included persons who had been using complete removable plate prostheses made of acrylic plastic Etacryl-02 for at least 6 months before the study, who had passed the adaptation period and whose objective clinical examination revealed signs of inflammation of soft tissues of the prosthetic bed (chronic prosthetic stomatitis), the bacterial etiology of which was confirmed by microbiological analysis.

The clinical and experimental studies were approved at the meeting of the local ethical committee of the E.A. Vagner Perm State Medical University (protocol No. 9 from 30.09.2021).

The material for the study was obtained from the OM of the denture bed in the area of the apex of the alveolar process of the maxilla (projection of the 1st and 2nd premolars on the maxilla - taking into account the outlet of the duct of the parotid salivary gland) using a swab-probe and Amies transport medium. After preliminary dilution of the material, the contents were sown on blood agar, Endo and Sabouraud media, selective media for streptococci isolation. Incubation was carried out at 37°C in a humid atmosphere under microaerophilic conditions. The isolated strains were identified by culture, tinctorial and biochemical characteristics.

DNA of periodontal pathogens was detected and quantified in biological material using the Dentoscreen reagent kit (Litech Co. Ltd.) by real-time polymerase chain reaction with hybridization-fluorescence detection.

To quantify the interaction between members of the microbiocenosis, the Jaccard similarity coefficient (q) was used, calculated by the following formula:

$$q = c/(a + b - c) \times 100,$$

where a — number of observations with type a; b — number of observations with type b; c — number of observations containing both types of MO.

If $q \leq 30\%$ — conditions in the biotope are antagonistic, with q from 30 to 70% bacteria are capable of coexistence, and their ecological commonality is great (synergism), $q \geq 70\%$ — only joint existence of bacteria is possible (a state close to mutualism).

Statistical analysis of the data was performed using four-field conjugation tables and χ^2-criterion.

Table 2. Frequency of MO detection in the oral mucosa in the denture bed of patients (% of cases)

MO		Group 1 (n = 66)	Group 2 (n = 65)	p between groups
Staphylococcus spp.		95.5	98.5	0.32
Coagulase-positive staphylococci		33.3	76.9	0.19
including:	*S. aureus*	45.5	66.0	0.001
	S. intermedius	4.5	32.0	0.001
	S. hyicus	50.0	2.0	0.003
Coagulase-negative staphylococci		98.9	75.4	0.001
Streptococcus spp.		83.3	50.8	0.001
S. salivarius		33.3	6.2	0.001
S. pyogenes		15.2	52.3	0.001
Neisseria spp.		48.5	53.8	0.54
Candida spp.		46.9	78.5	0.001
including:	*C. albicans*	48.4	39.2	0.3
Enterobacterales		66.7	50.8	0.065
including:	*E. coli*	11.4	45.5	0.027
	Klebsiella spp.	25.0	51.5	0.19
	Enterobacter spp.	47.7	48.5	0.36
Lactobacillus spp.		33.3	55.4	0.012
Corynebacterium spp.		34.8	49.2	0.096
Enterococcus spp.		18.2	26.2	0.27
F. nucleatum		28.8	76.9	0.001
T. denticola		0	0	

DOI: https://doi.org/10.36233/0372-9311-524

Results

In patients with the complete absence of teeth, the microbial associations were characterized by a high diversity of MO species and complex relationships between them. Thus, in patients with complete absence of teeth, a significant proportion of coagulase-negative and coagulase-positive *Staphylococcus* spp. other than *S. aureus*, as well as *Neisseria* spp., yeast fungi of the *Candida* genus, *Enterobacterales*, and *Fusobacterium nucleatum* were found in the microbiota (**Table 2**). Among the representatives of the *Enterobacterales* order, representatives of the *Klebsiella* spp. and *Enterobacter* spp. genera were often found. Among the commensal species *Streptococcus* spp. strains with a wider set of pathogenicity factors, *S. pyogenes* were dominant.

According to the conducted research, the development of inflammatory complications of OM in the denture bed of patients with complete absence of teeth corresponded to colonization of the biotope by coagulase-positive species of *Staphylococcus* genus, increase in the share of *S. pyogenes* and decrease — of *S. salivarius*, increase in the occurrence of yeast fungi of *Candida* genus and *Escherichia coli*. Furthermore, it was found that more frequent detection of *F. nucleatum* markers was associated with the presence of denture stomatitis.

In the present study, no MO strains were isolated as a monovariant, and the minimum number of associates was at least 3. At the same time, no significant difference in the number of associates in microsymbioses among the compared groups was found. When evaluating pairwise relationships by Jaccard's coefficient, pronounced symbiotic relationships were found between the MOs of the *Enterobacterales* order, *Lactobacillus*, *Neisseria* and *Corynebacterium* genera, as well as *S. salivarius*, *C. albicans*, *F. nucleatum*. The nature of these relationships depended on the presence of inflammatory changes in the oral cavity. Among coagulase-negative staphylococci inhabiting the oral cavity of group 2 patients, it was noted that *S. epidermidis* showed the ability to coexist with *S. pyogenes* ($q = 50$), and *S. schleiferi* – with bacteria of the genus Lactobacillus ($q = 50$). In group 1, coagulase-negative staphylococci showed antagonistic properties against *E. coli*, *S. mitis* and *Neisseria* spp.

Corynebacterium spp. and *Lactobacillus* spp. with active participation of *S. salivarius* (Fig. 1) are characteristic symbionts for the oral cavity mucosa of group 1 patients, which are found in the vast majority of cases, and mutualistic relationships are formed between them and *S. salivarius* ($q > 70$). The formation of such an association allows participants to exert an antagonistic effect on representatives of the order *Enterobacterales*, the most common in dry mucosa of the oral cavity [14]. A negative point in group 1 patients should be recognized as a persistent ecological commonality between *Corynebacterium* spp. and *Fusobacterium* spp. ($q = 75$), which may be due to the syntrophy of these bacteria. Thus, *Corynebacterium* spp. synthesizes several free fatty acids essential for *Fusobacterium* spp. which, in turn, facilitates the availability of *Corynebacterium* amino acids [15]. The associations of these species, according to some authors, are most frequently registered in oral squamous cell cancer [16].

Yeast fungi of the *Candida* genus were found in 46.9% of cases in group 1 patients, which showed synergism with *Neisseria* spp. ($q = 42.4$) and *Enterobacterales* ($q = 63.6$). *Neisseria* spp. probably adapted to coexist with *Candida* spp. in this community because fungi are unique biochemical transformers, and the products of their metabolism are convenient for utilization by *Neisseria* spp. [17]. The synergism between fungi of the *Candida* and *Enterobacterales* is due more to increased antimicrobial resistance [18].

S. salivarius strains isolated from group 1 patients were found to exhibit pronounced (up to mutualistic) symbiotic relationships with lactic acid-producing *Lactobacillus*. However, as shown in the study [19], *Streptococcus* spp. bacteriocins can inhibit the production of this metabolite by *Lactobacillus* spp.

S. salivarius, which exhibited pronounced (up to mutualistic) symbiotic relationships with *Lactobacillus* spp. in group 1, do not participate in the formation of a persistent association of MO in the complete absence of teeth and accession of inflammation (Fig. 2). This situation leads to the formation of synergism not only between *Corynebacterium* spp. and *Fusobacterium* spp. ($q = 60.8$), but also between *Lactobacillus* spp. and *Fusobacterium* spp. ($q = 62.3$), indicating a closer incorporation of *Fusobacterium* spp. into the oral microbiocenosis and requiring a comprehensive targeting

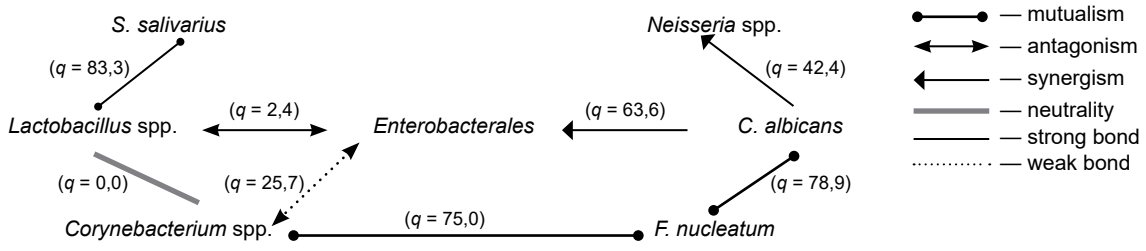

Fig. 1. The nature of the symbiotic relationship of the oral MOs of group 1 patients.

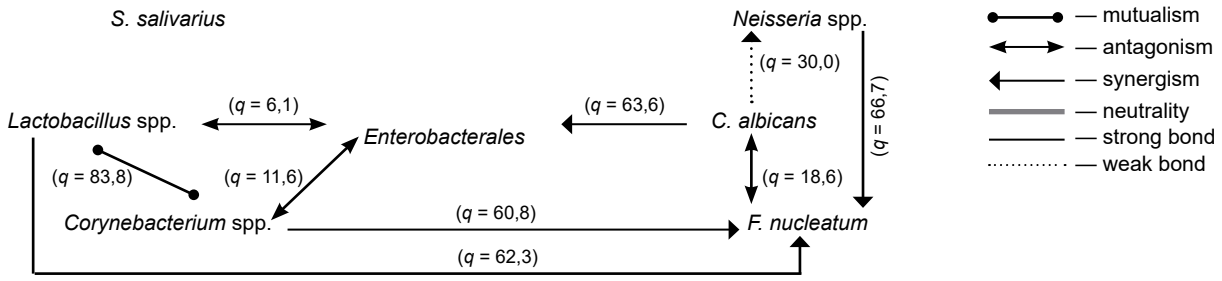

Fig. 2. Nature of symbiotic relationship of oral MOs of group 2 patients.

approach to oral antibacterial treatment before prosthodontics.

Synergism between *Enterobacterales*, fungi of the *Candida* genus and *Neisseria* spp. (q = 30–70) in group 2 patients persists and leads to the displacement of *S salivarius*. Furthermore, *F. nucleatum* forms more divergent relationships than strains of the same species isolated from group 1 patients. Thus, if in group 1 patients the neutrality between *Fusobacterium* spp. and *Neisseria* spp. was revealed, in group 2 patients these species show synergism (q = 66.7). The nature of relationships in the pair of *Fusobacterium* spp. and *C. albicans* changes in the case of inflammatory changes in the mucosa to antagonistic (q = 18.6), which, at first glance, does not seem logical, but is quite explainable by the fact that in antagonistic relationships between bacteria the spectrum of their metabolite changes, which is reflected in the clinical picture. Antagonistic relations between bacteria of the *Enterobacterales* order and the *Lactobacillus* genus are preserved in patients with and without clinical markers of inflammatory process.

Discussion

The oral microbiome is populated by representatives of more than 300 species of bacterial taxa alone. Within such a system, complex relationships are formed between individual members of associations, often not always mutualistic or synergistic. Different techniques have been proposed to study bacterial relationships, which, however, are characterized by complexity of replication and difficulty of interpretation. In the present study, we propose to use an index approach based on the Jaccard coefficient, which allows to reveal not only the directionality of the relationship, but also partly its expression.

Among all oral cavity MOs, *S. salivarius*, which belongs to the autochthonous symbionts of this biotope, should be emphasized. It has been shown that this species has a pronounced antimicrobial and antibiofilm activity [20], which was confirmed in the present study, when in patients without inflammatory changes in OM this species enhances antagonistic properties at the expense of *Lactobacillus* spp. and *Corynebacterium* spp. However, in the case of inflammatory changes, which are probably due to changes in the oral microbiome, an almost complete displacement of this species by *Streptococcus* spp. was observed.

According to the results of the study, *Enterobacterales* are allochthonous microbes that retain antagonistic relationships with autochthonous MOs (*Lactobacillus* spp., *Corynebacterium* spp.) and synergism with yeast fungi of the *Candida* genus both in the absence of clinical manifestations of the inflammatory process (prosthetic stomatitis) and in their presence. Such a picture indicates that bacteria with a wide range of pathogenicity factors and, accordingly, more pronounced virulence (e.g., *Enterobacterales*), which are not characteristic for the oral cavity, by fixation on the structural material of removable dental prostheses occupy free niches, and later, with a decrease in the activity of the immune system, poor hygiene or other provoking factors, it is these taxa that ensure the development of inflammatory processes in the soft tissues of the denture bed, together with autochthonous conditionally pathogenic MOs (*Candida* spp., etc.). At the same time, a change in the orientation of the relationship between *Enterobacterales* and *F. nucleatum* from mutualistic to antagonistic in the presence of inflammatory phenomena of denture bed tissues in patients with complete absence of teeth is likely to correlate with the worsening of the clinical picture when the association of these MOs is detected.

Conclusion

The obtained results allow us to consider *S. pyogenes*, *E. coli, F. nucleatum* and *Candida* spp. as initiators of pathological changes of inflammatory character in soft tissue periodontal tissues of persons using complete removable plate prostheses with a base made of acrylic polymer Etacryl-02. Conditionally pathogenic MOs, forming microbial associations with multidirectional symbiotic relations can increase their virulence, which allows them to occupy free niches in the oral cavity, and subsequently trigger the development of pathological process of inflammatory character of denture bed tissues.

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The article was submitted 15.04.2024;
accepted for publication 21.06.2024;
published 29.08.2024

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Статья поступила в редакцию 15.04.2024;
принята к публикации 21.06.2024;
опубликована 29.08.2024

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-527
ORIGINAL RESEARCHES

Оригинальное исследование
https://doi.org/10.36233/0372-9311-527

Сравнительный анализ структуры регуляторных генов штаммов *Vibrio cholerae* O1 биовара El Tor

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Аннотация

Актуальность. Экспрессия генов *ctxAB* и *tcpA-F*, кодирующих основные факторы патогенности возбудителя холеры, контролируется регуляторными генами, структура которых в штаммах возбудителя, выделенных в разные годы текущей пандемии, изучена не в полной мере.

Цель работы — сравнительный анализ структуры регуляторных генов в штаммах *Vibrio cholerae* O1 биовара El Tor, изолированных на территории России и сопредельных стран на протяжении 7-й пандемии холеры.

Материалы и методы. Использовали нуклеотидные последовательности полных геномов 29 токсигенных штаммов, выделенных с 1970 по 2023 г. Анализ проводили с помощью программ «BioEdit v7.2.6.1» и «Blast».

Результаты. Проведён анализ 10 регуляторных генов (*toxT, aphA, aphB, hns, hapR, vieA, luxO, luxT, carS, carR*). Установлено, что практически у всех штаммов в гене *hapR* имеется вставка тимина в позиции 219. Исключение составил *V. cholerae* M3208 (Тамбов, 2023), у которого обнаружена вставка 5 нуклеотидов в данном гене. У 44,8% изученных штаммов выявлены мутации в гене *luxO*, функциональное значение которых не установлено. У 46,7 и 33,3% изученных геновариантов с аллелем *ctxB1* обнаружены несинонимичные замены в генах *hns* (*G319A*) и *vieA* (*C235T*) соответственно. Все геновариенты с аллелем *ctxB7* имеют гены *hns* и *vieA* с мутациями. Три геновариента с аллелем *ctxB7*, завезённые в Россию в последние годы, содержат изменённую структуру гена *carR* (*G265A*).

Заключение. Структура генов (*toxT, aphA, aphB, carS, luxT, hapR*) является интактной у большинства изученных штаммов *V. cholerae* O1 El Tor. В то же время выявлена вариабельность генов *hns* (G319A), *vieA* (C235T) и *carR* (G256A). Мутации в данных генах могут быть использованы в качестве генетических меток современных геновариантов *V. cholerae* O1 El Tor.

Ключевые слова: *Vibrio cholerae, геновариенты, структура регуляторных генов, мутации*

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.

Для цитирования: Плеханов Н.А., Федоров А.В., Челдышова Н.Б., Кураташвили А.Ю., Заднова С.П. Сравнительный анализ структуры регуляторных генов штаммов *Vibrio cholerae* O1 биовара El Tor. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):520–529.
DOI: https://doi.org/10.36233/0372-9311-527
EDN: https://www.elibrary.ru/fjzedx

Original Study Article
https://doi.org/10.36233/0372-9311-527

Comparative analysis of the structure of regulatory genes of *Vibrio cholerae* serotype O1 biotype El Tor strains

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Abstract

Introduction. The expression of the *ctxAB* and *tcpA-F* genes encoding the main pathogenicity factors of the *Vibrio cholerae* is controlled by regulatory genes. The structure of these genes has not been fully studied in the pathogen strains isolated during different periods of the current pandemic.

The aim of the study was a comparative analysis of the structure of regulatory genes of *V. cholerae* O1 biovar El Tor strains isolated on the territory of the Russian Federation and neighboring countries during the seventh cholera pandemic.
Materials and methods. The nucleotide sequences of the complete genomes of 29 toxigenic strains isolated from 1970 to 2023 were analyzed. The analysis was carried out using BioEdit v7.2.6.1 software and Blast tool.
Results. The analysis of ten regulatory genes (*toxT, aphA, aphB, hns, hapR, vieA, luxO, luxT, carS, carR*) was carried out. Almost all strains were found to have a thymine insertion in the *hapR* gene at position 219. The exception was *V. cholerae* strain M3208 (Tambov, 2023), which had an insertion of five nucleotides in this gene. Mutations of the *luxO* gene with an unknown effect were detected in 44.8% of the studied strains. In 46.7% and 33.3% of the studied genetic variants carrying the *ctxB1* allele, non-synonymous substitutions were detected in the *hns* (G319A) and *vieA* (C235T) genes, respectively. All genetic variants with the *ctxB7* allele have mutations in both the *hns* and *vieA* genes. Three genetic variants with the *ctxB7* allele, imported to the Russian Federation in recent years, contain an altered structure of the *carR* gene (*G265A*).
Conclusion. The structure of genes (*toxT, aphA, aphB, carS, luxT, hapR*) of *V. cholerae* O1 El Tor strains remains unchanged for the majority of the studied isolates. At the same time, variability in the *hns* (*G319A*), *vieA* (*C235T*) and *carR* (*G256A*) genes was detected. Mutations in these genes can be used as genetic markers of modern *V. cholerae* O1 El Tor genetic variants.

Keywords*: Vibrio cholerae, genetic variants, regulatory gene structure, mutations*

Funding source. The authors declare no external funding for the study.

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this manuscript.

For citation: Plekhanov N.A., Fedorov A.V., Cheldyshova N.B., Kuratashvili A.Yu., Zadnova S.P. Comparative analysis of the structure of regulatory genes of *Vibrio cholerae* serotype O1 biotype El Tor strains. *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):520–529.
DOI: https://doi.org/10.36233/0372-9311-527
EDN: https://www.elibrary.ru/fjzedx

Введение

Холера — особо опасная инфекционная болезнь, вызываемая токсигенными штаммами *Vibrio cholerae*, остается серьёзной проблемой здравоохранения во многих странах Азии, Африки, Америки (регион Карибского бассейна). Ежегодно регистрируется около 2,9 млн случаев холеры, из которых более 95 000 заканчиваются летально. Согласно данным Всемирной организации здравоохранения, с середины 2021 г. отмечается рост заболеваемости и смертности от данной болезни[1]. Так, средний показатель летальности в 2021 г. составил 1,9% (в странах Африки — 2,9%), что является самым высоким за последнее десятилетие, и данная тенденция сохранилась в 2022–2023 гг. Остаётся высоким риск завоза возбудителя в любую страну мира [1].

Текущая, 7-я, пандемия холеры является самой длительной (продолжается уже более 60 лет) и включает несколько линий штаммов *V. cholerae* O1 биовара El Tor с определённой структурой генов *ctxAB* и *tcpA-F*, кодирующих основные факторы патогенности возбудителя холеры — холерный токсин (ХТ) и токсин-корегулируемые пили адгезии (ТКПА) [3, 4]. Начало пандемии было вызвано типичными штаммами *V. cholerae* O1 биовара El Tor, содержащими аллели *ctxB3* и $tcpA^{eltor}$. В 1990-х гг. появились генетически изменённые варианты *V. cholerae* O1 биовара El Tor (геновариантов), которые отличались от типичных штаммов повышенной продукцией ХТ в результате замены аллеля *ctxB3* на *ctxB1*, характерного для возбудителя 6-й пандемии — *V. cholerae* O1 классического биовара. Дальнейшие эволюционные преобразования геновариантов способствовали появлению «гипервирулентных» штаммов, которые не только имеют новые аллели *ctxB — ctxB7* и *tcpA* — $tcpA^{cirs101}$, но и включают около 70 генов с единичными нуклеотидными заменами, а также делеции в мобильных генетических элементах [2–6]. При этом рядом авторов показано, что повышение вирулентности геновариантов *V. cholerae* O1 El Tor связано с изменением структуры основных генов патогенности (*ctxB* и *tcpA*), другими — с появлением мутаций в структуре ряда регуляторных генов [6–8]. Как известно, экспрессия генов *ctxAB* и *tcpA-F* контролируется сложным регуляторным каскадом с участием различных положительных и отрицательных факторов транскрипции, а также зависит от плотности бактериальной популяции, сигналов внешней среды (температура, соли желчных кислот, pH среды, осмолярность и т.д.) и продуцируемых сигнальных молекул (в том числе 3′,5′-циклического дигуанинмонофосфата, c-di-GMP). Непосредственным транскрипционным активатором генов *ctxAB* и *tcpA-F* является белок ToxT, продукция которого контролируется белком ToxR, играющим важную роль в вирулентности холерного вибриона. Для активации транскрипции *toxT* белок ToxR взаимодействует с другими белками — ToxS и TcpPH. В свою очередь транскрипция

[1] Cholera – Global situation. WHO Report; 2023. URL: https://who.int/emergencies/disease-outbreak-news/item/2023-DON437

DOI: https://doi.org/10.36233/0372-9311-527

генов *tcpPH* зависит от белков AphAB. Показано, что активная экспрессия AphAB происходит при низкой плотности бактериальной популяции. В данных условиях фосфорилированный белок системы «quorum sensing» LuxO ингибирует экспрессию quorum-чувствительного регуляторного белка HapR (оказывающего негативное влияние на каскад вирулентности) — происходит продукция ХТ и ТКПА [9, 10]. Кроме AphA с промотером *tcpP* при нахождении патогена *in vivo* связывается и белок CarR, действующий совместно с CarS, что способствует увеличению колонизирующей способности штаммов *V. cholerae* O1 биовара El Tor. Кроме того, в присутствии катионных антимикробных пептидов (HD-5, α-дефензин, β-дефензин), продуцируемых эпителиальными клетками, CarR непосредственно регулирует экспрессию генов, кодирующих систему модификации липида А липополисахарида клеточной стенки (оперон *almEFG*), что придаёт устойчивость бактерий к катионным пептидам и обеспечивает нормальный рост вибрионов в кишечнике. Таким образом, белок CarR регулирует вирулентность возбудителя и способствует устойчивости патогена в макроорганизме [11].

При увеличении количества бактерий фосфолирирования LuxO не происходит, и он не блокирует *hapR*. Продуцируемый белок HapR подавляет транскрипцию *aphAB* и *tcpPH.* В итоге прекращается биосинтез факторов вирулентности [9, 10]. Кроме LuxO, транскрипцию *hapR* репрессирует и недавно обнаруженный у патогена белок LuxT, который также непосредственно связывается с промоторной областью *hapR* [11]. Негативным регулятором транскрипции генов *ctxAB* и *tcpA-F* является и ДНК-связывающий гистоноподобный белок H-NS, который репрессирует транскрипцию *toxT*, а также блокирует транскрипцию генов *ctxAB* и *tcpA*, связываясь с той же областью ДНК, что и белок ToxT [12]. Ещё одним белком, участвующим в регуляции продукции факторов вирулентности, посредством контроля содержания вторичного мессенджера c-di-GMP (cyclic diguanosine monophosphate), является VieA, кодируемый геном *vieA* из оперона *vieSAB*. Транскрипция гена *vieA* в клетках подавляется как белком H-NS, так и HapR. Данный белок содержит ДНК-связывающий участок, а также домен (EAL) с дигуанилат-фосфодиэстеразной активностью, гидролизующий c-di-GMP. Накапливаясь в клетках в высокой концентрации, c-di-GMP ингибирует транскрипцию генов *ctxAB* и *toxT*, и деградация этой молекулы способствует увеличению биосинтеза ХТ и ToxT [13].

Также стоит отметить, что регуляторные гены, контролирующие не только вирулентные, но и другие свойства бактерий (в том числе формирование биоплёнки), выступают в качестве перспективных мишеней для создания антимикробных препаратов нового поколения. В настоящее время проводятся исследования по поиску и синтезу антимикробных пептидов, способных снижать вирулентность возбудителя холеры и разрушать образование биоплёнок без токсического воздействия на макроорганизм. При этом перспективной мишенью выбран белок LuxO [14].

Таким образом, структура генов *ctxB* и *tcpA* у геновариантов *V. cholerae* O1 El Tor, завезённых в разные годы на территорию России и сопредельных стран, достаточно подробно изучена [15–18]. В то же время распространённость мутаций в регуляторных генах в данных штаммах исследована фрагментарно. **Цель** работы — сравнительный анализ структуры регуляторных генов в штаммах *V. cholerae* O1 биовара El Tor, изолированных на территории России и сопредельных стран на протяжении 7-й пандемии холеры.

Материалы и методы

В работе использовали полногеномные последовательности 29 токсигенных штаммов *V. cholerae* O1 биовара El Tor, завезённых с 1970 по 2023 г. на территорию России и сопредельных стран, депонированные в NCBI GenBank и в VGARus (штаммы M3208 и M3210). Характеристика использованных штаммов приведена в **таблице**.

Штаммы хранились в лиофильно высушенном состоянии в Государственной коллекции патогенных бактерий Российского противочумного института «Микроб». Для культивирования бактерий использовали бульон и агар LB (pH 7,2). Подготовку проб осуществляли согласно МУ 1.3.2569-09[2]. Подготовку образцов ДНК исследуемых штаммов проводили в боксе биологической безопасности II класса в противочумном костюме IV типа с использованием набора «TransGen EasyPure Genomic DNA Kit» в соответствии с протоколом производителя. Секвенирование ДНК осуществляли на платформе «DNBSEQ-G50» («MGI Tech») с использованием стандартного протокола подготовки ДНК-библиотек, соответствующего платформе.

Анализ структуры регуляторных генов *toxT, aphA/aphB, hns, hapR, vieA, luxO/luxT, carR/carS* проводили с помощью программы «BioEdit v7.2.6.1» и алгоритма «BLAST v2.15.0 NCBI GenBank».

Результаты

На первом этапе работы была изучена структура гена *toxT*, кодирующего регуляторный белок ToxT. Согласно данным литературы, указанный бе-

[2] Организация работы лабораторий, использующих методы амплификации нуклеиновых кислот при работе с материалом, содержащим микроорганизмы I–IV групп патогенности: Методические указания МУ 1.3.2569-09. Федеральный центр гигиены и эпидемиологии Роспотребнадзора. 2009.

Характеристика и результаты анализа структуры регуляторных генов штаммов *V. cholerae* O1 серогруппы биовара El Tor, использованных в работе
Characteristics and results of analysis of the structure of regulatory genes of *V. cholerae* O1 strains of the El Tor biovar serogroup used in the study

Штамм Strains	Год, место и источник выделения The year, site and source of isolation	Структура гена (замена аминокислот) | Gene structure (amino acid substitutions)						
		toxT VC0838	*aphA/aphB* VC2647/VC1049	*hns* VC1130	*hapR* VC0583	*vieA* VC1652	*luxO/luxT* VC1021/VCA0917	carR/carS VC1320/ VC1319
Типичные штаммы *V. cholerae* O1 El Tor с аллелем *ctxB3* | Typical *V. cholerae* O1 El Tor strains with the *ctxB3* allele								
M1062 (SSAB01)	1970, РФ, Астрахань, больной RF, Astrakhan, patient	int	int/int	int	insT219, C118G (*H40D*)	int	int/int	int/int
M893 (SSAA01)	1970, РФ, Астрахань, больной RF, Astrakhan, patient	int	int/int	int	insT219	int	int/int	int/int
M818 (LAHM01)	1970, РФ, Балаково, больной RF, Balakovo, patient	int	int/int	int	insT219	int	int/int	int/int
M1030 (NEDX01)	1972, Туркменистан, Йолотен, больной Turkmenistan, Ioloten, patient	int	int/int	int	insT219	int	*A521G* (*H174R*)/int	int/int
C-191 (WNZT01)	1973, РФ, Ставрополь, больной RF, Stavropol', patient	int	int/int	int	insT219, *C109T* (*R37C*)	insTTC p:365	int/int	int/int
123AZ (SMZB01)	1977, Азербайджан, больной Azerbaijan, patient	int	int/int	int	Δ	int	int/int	int/int
2278 (WNZM01)	1987, РФ, Краснодар, больной RF, Krasnodar, patient	int	int/int	int	insT219, *G76A* (*A26T*)	int	*A340G* (*K114E*)/int	int/int
Геноварианты *V. cholerae* O1 El Tor с аллелем *ctxB1* | Genetic variants of *V. cholerae* O1 El Tor with the *ctxB1* allele								
P13762 (LQYD01)	1988, Узбекистан, Ташкент, больной Uzbekistan, Tashkent, patient	int	int/int	int	insT219	int	int/int	int/int
M1270 (VXCC01)	1993, РФ, Татарстан, Набережные Челны, больной RF, Tatarstan, Naberezhnye Chelny, patient	int	int/int	int	insT219	int	*T98C* (*I33T*)/int	int/int
M1275 (LRAF01)	1993, РФ, Дагестан, Каспийск, больной RF, Dagestan, Kaspiysk, patient	int	int/int	int	int	int	int/int	int/int
M1293 (JFFW01)	1994, РФ, Дагестан, больной RF, Dagestan, patient	int	int/int	int	insT219	int	*T176A* (*L59H*)/int	int/int
20-A-11 (PYAR01)	1995, Украина, больной Ukraine, patient	int	int/int	int	insT219	int	int/int	int/int
R17644 (JRTW01)	1997, РФ, Ачинск, больной RF, Achinsk, patient	int	int/int	*G319A* (*G107S*)	insT219	int	*T62A* (*I21N*)/int	int/int
M1327 (LRFE01)	1998, РФ, Дагестан, больной RF, Dagestan, patient	int	int/int	int	insT219	int	int/int	int/int

Окончание таблицы | End of the Table

Штамм Strains	Год, место и источник выделения The year, site and source of isolation	Структура гена (замена аминокислот) | Gene structure (amino acid substitutions)						
		toxT VC0838	*aphA/aphB* VC2647/VC1049	*hns* VC1130	*hapR* VC0583	*vieA* VC1652	*luxO/luxT* VC1021/VCA0917	carR/carS VC1320/ VC1319
M1344 (NEDY01)	2001, РФ, Татарстан, Казань, больной RF, Tatarstan, Kazan, patient	int	int/int	*G319A* (*G107S*)	insT219	int	int/int	int/int
M1429 (LAEM01)	2004, РФ, Башкортостан, Белорецк, больной RF, Bashkortostan, Beloretsk patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	int/int	int/int
RND18826 (AYOM01)	2005, РФ, Тверь, больной RF, Tver, patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	*G424T* (*V142L*)/int	int/int
P-18899 (LAKM01)	2006, РФ, Мурманск, больной RF, Murmansk, patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	*C527T* (*A176V*)/int	int/int
89 (NDXR01)	2010, Украина, Ялта, вн. ср. Ukraine, Yalta, env.	int	int/int	int	insT219	int	*T287G* (I96S)/Δ	int/int
2011EL-301 (AJFN01)	2011, РФ, Таганрог, вн. ср. RF, Taganrog, env.	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	*G424T* (*V142L*)//int	int/int
81 (JRQM01)	2014, РФ, Ростов-на-Дону, вн. ср. RF, Rostov-on-Don, env.	int	int/int	*G319A* (*G107S*)	insT219	C235T (L79F)	G424T (V142L)/int	int/int
M3210 (*micr026579*)	2023, РФ, Ростов-на-Дону, вн. ср. RF, Rostov-on-Don, env.	*G436A* (*V146I*)	int/int	int	insT219	int	*G331A* (*A111T*)/int	int/int
Геновариванты *V. cholerae* O1 El Tor с аллелем *ctxB7* | Genetic variants of *V. cholerae O1* El Tor with the *ctxB7* allele								
L3226 (JDVX01)	2010, РФ, Москва, больной RF, Moscow, patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	*T287G* (*I96S*)/int	int/int
RND19191 (JNGT01)	2010, РФ, Москва, больной RF, Moscow, patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	int/int	int/int
76 (MPVL01)	2011, Украина, Мариуполь, больной Ukraine, Mariupol, patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	int/int	int/int
153 (MWRE01)	2011, Украина, Мариуполь, больной Ukraine, Mariupol, patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	*G268T* (*G90C*)/int	int/int
M1509 (NEDZ01)	2012, РФ, Москва, больной RF, Moscow, patient	int	int/int	G319A (G107S)	insT219	C235T (L79F)	int/int	*G256A* (*D89N*)/int
3265/80 (JRQL01)	2014, РФ, Москва, больной RF, Moscow, patient	int	int/int	*G319A* (*G107S*)	insT219	*C235T* (*L79F*)	int/int	*G256A* (*D89N*)/int
M3208 (*micr026578*)	2023, РФ, Тамбов, больной RF, Tambov, patient	int	int/int	*G319A* (*G107S*)	insCTAAA97 (33fs)	*C235T* (*L79F*)	int/int	*G256A* (*D89N*)/int

Примечание. В столбце «Штамм» в скобках указан сокращённый код доступа в GenBank, курсивом выделен код доступа в VGARus; вн. ср. — внешняя среда; ins — вставка нуклеотида(ов); int — структура гена идентична референс-штамму *V. cholerae* N16961 O1 биовара El Tor; fs — сдвиг рамки считывания; Δ — делеция гена.
Note. The GenBank accession number is indicated in parentheses in the Strains column, the VGARus accession number is indicated in italics; env. — environmental; ins − nucleotide insertion; int — the structure is identical to the reference strain *V. cholerae* N16961 O1 El Tor; fs − frameshift mutation; Δ — gene deletion.

лок включает 276 аминокислот. Наиболее важным участком является N-терминальный домен (1–164 аминокислоты). Показано, что стабильное сохранение структуры N-терминального участка необходимо для транскрипционной активности данного регулятора [19]. В результате проведённого нами анализа установлено, что структура данного гена у большинства взятых в исследование штаммов соответствует референс-штамму *V. cholerae* N16961 O1 биовара El Tor. Исключение составил штамм M3210 (Ростов-на-Дону, 2023) у которого обнаружена несинонимичная однонуклеотидная замена *G436A*, которая привела к смене аминокислоты валина на изолейцин в позиции 146 в N-терминальном домене белка ToxT.

Изменений в нуклеотидной последовательности генов *aphA* и *aphB* у взятых в исследование штаммов *V. cholerae* O1 биовара El Tor не выявлено.

При изучении структуры гена *luxO* показано, что у большинства типичных штаммов структура данного гена соответствовала референсному штамму. Исключение составили штаммы *V. cholerae* M1030 и 2278, имеющие несинонимичные замены, что привело к смене аминокислот в центральном и аминотерминальном участках белка LuxO. Среди геновариантов 11 штаммов имели несинонимичные SNP (таблица). Сведения о влиянии указанных аминокислотных замен на функциональную активность белка LuxO в литературе отсутствуют. Однако практически все изученные штаммы *V. cholerae* O1 биовара El Tor имели интактную последовательность гена *luxT.* Белок LuxT, как и LuxO, ингибирует транскрипцию *hapR*. Исключение составил штамм *V. cholerae* 89 (Ялта, 2010), у которого ген *luxT* не обнаружен.

Далее нами была изучена структура гена *hapR*, кодирующего белок HapR. Согласно данным литературы, в штамме *V. cholerae* N16961 O1 El Tor, который используется в качестве референсного, в последовательности гена *hapR* имеется делеция тимина в позиции 219, что приводит к сдвигу рамки считывания и синтезу функционально неактивного белка HapR [20]. У большинства изученных нами штаммов присутствует ген *hapR* со вставкой тимина, что указывает на продукцию ими полноценного белка HapR. Исключение составили типичные штаммы *V. cholerae* 123AZ, у которого данный ген отсутствует, а также M1062, C-191 и 2278 с заменами аминокислот в начале N-концевого участка, не влияющими на формирование зрелого белка HapR и его функцию [21]. У геновариант *V. cholerae* M3208 (Тамбов, 2023) в результате инсерции 5 нуклеотидов в начале гена происходит сдвиг рамки считывания. Данный штамм синтезирует белок HapR с изменённым аминокислотным составом и, вполне вероятно, с изменённой функциональной активностью.

При изучении нуклеотидной последовательности гена *hns,* кодирующего белок-репрессор H-NS, выявлено, что у типичных штаммов *V. cholerae* O1 биовара El Tor, а также у геновариантов, завезённых в 1988–1995 гг., нуклеотидная последовательность данного гена соответствует последовательности аналогичного гена референс-штамма *V. cholerae* N16961 O1 El Tor. Изменённая структура гена *hns* (замена G на A в позиции 319) выявлена у геновариант R17644 (Ачинск, 1997), имеющего аллель *ctxB1*. Мутация *G319A* привела к смене аминокислоты (*G107S*) в ДНК-связывающем участке белка H-NS (таблица). В последующие годы геновариант с аллелем *ctxB1* включали как интактный, так мутантный ген *hns*. В то же время мутация *G319A* в гене *hns* присутствует у всех геновариантов *V. cholerae* O1 биовара El Tor, имеющих аллель *ctxB7*.

Анализ гена *vieA* из оперона *vieSAB* показал наличие интактной его последовательности у 5 типичных штаммов. Исключение составил штамм *V. cholerae* C191, у которого обнаружена вставка 3 нуклеотидов, кодирующих лизин, в результате чего синтезируется белок VieA с изменённой аминокислотной последовательностью. Геновариант *V. cholerae* O1 El Tor с *ctxB1*, завезённые с 1988 по 2001 гг., имеют интактный ген *vieA*. В то же время у *V. cholerae* M1429 (Белорецк, 2004) уже присутствует SNP (*C235T*), приводящая к замене аминокислоты (*L79F*). В последующие годы белок VieA с данной аминокислотной последовательностью выявлен у большинства изученных геновариантов с аллелем *ctxB1*, а также у всех штаммов с аллелем *ctxB7.* Ранее подобные изменения в структуре белка VieA были выявлены K.J.F. Satchell с соавт. у геновариантов *V. cholerae* O1 биовара El Tor, выделенных в Зимбабве (2009), Бангладеш (2010), на острове Гаити (2010) [6].

Нуклеотидная последовательность гена *carS* является интактной у всех изученных штаммов, в то же время в гене *carR* выявлены изменения (таблица). В ранее проведённой работе нами обнаружены 2 геновариант (M1509, 3265/80), в гене *carR* которых присутствует замена *G256A*, приводящая к смене аминокислот D89N [22]. В результате данной мутации синтезируется нефункциональный белок CarR и изменяется диагностически значимый признак El Tor вибрионов — устойчивость к полимиксину B, штаммы становятся чувствительными к указанному катионному антибиотику [23]. В данной работе выявлен ещё один штамм — *V. cholerae* M3208 (Тамбов, 2023), который также содержит мутацию *G256A* в гене *carR* (таблица).

Обсуждение

Продукция основных факторов патогенности — ХТ и ТКПА в штаммах *V. cholerae* O1 серо-

DOI: https://doi.org/10.36233/0372-9311-527

группы биовара El Tor контролируется значительным количеством регуляторных белков, образующих регуляторную сеть. При этом некоторые из них являются полифункциональными и участвуют в других процессах бактериальной клетки. В данной работе нами был проведён анализ 10 регуляторных генов и выявлены важные изменения, характерные для современных штаммов возбудителя.

При анализе нуклеотидной последовательности гена *toxT* установлена её идентичность данному гену референс-штамма *V. cholerae* N16961 практически у всех изученных как типичных штаммов, так и геновариантов с разными аллелями *ctxB*. Исключение составил геновариант *V. cholerae* M3210 (Ростов-на-Дону, 2023), у которого присутствие несинонимичной SNP в гене *toxT* привело к продукции мутантного белка ToxT с заменой валина на изолейцин в позиции 146. В ранее проведённой работе показано, что при замене валина на аргинин (*V146A*) мутантный штамм сохранял высокий уровень продукции ХТ и ТКПА, сопоставимый с исходным, при выращивании его в разных средах (LB, AKI) при температуре 30ºC. В то же время культивирование мутантного штамма при температуре 37ºC приводило к значительному снижению биосинтеза ХТ (9% от исходного) и полному отсутствию продукции ТКПА [19]. Можно высказать предположение, что у штамма *V. cholerae* M3210 биосинтез ХТ и ТКПА также будет зависеть от температуры культивирования. Однако для проверки данного предположения необходимы дополнительные исследования.

Стабильное сохранение структуры генов *aphA* и *aphB* у штаммов *V. cholerae* O1 биовара El Tor, завезённых и выделенных на территории России и сопредельных стран в разные периоды текущей пандемии холеры, может указывать на их важную роль в биологии возбудителя холеры. Так, белок AphA участвует не только в контроле продукции ХТ и ТКПА, но и в биосинтезе ацетоина, который противодействует закислению среды при росте холерного вибриона в присутствии глюкозы, а также контролирует процесс формирования биоплёнки [24, 25]. Достаточно стабильной является и структура гена *hapR* — выявлен только 1 штамм (*V. cholerae* M3208), завезённый из Индии в 2023 г., который содержит вставку 5 нуклеотидов в данном гене, что приводит к сдвигу рамки считывания и, возможно, биосинтезу нефункционального белка HapR. В ранее проведённой работе показано, что наличие функционального белка HapR не является существенным для проявления патогенных свойств *V. cholerae*. Штаммы с делетированным геном *hapR* были вирулентными [26].

При изучении другого негативного регулятора — белка H-NS установлено, что 46,7% изученных геновариантов с аллелем *ctxB1*, а также все штаммы с аллелем *ctxB7* имеют несинонимичную SNP (*G319A*) в гене *hns*, что привело к замене аминокислот в позиции 107 (*G107S*). B.M. Carignan и соавт. показали, что в штаммах с данной мутаций белок H-NS теряет способность связываться с ДНК и репрессировать транскрипцию гена *toxT*, что приводит к увеличению продукции белка ToxT, и вследствие этого возрастает биосинтез ХТ и ТКПА и повышаются вирулентные свойства штаммов [8]. Стоит отметить, что мутация в гене *hns* возникла уже вскоре после появления первых геновариантов *V. cholerae* O1 биовара El Tor, т. к. уже в 1997 г. на территорию России были завезены штаммы с мутацией *G319A*. Для геновариантов с аллелем *ctxB7* указанная структура гена *hns* является уже характерным признаком (генетической меткой).

При анализе структуры гена *vieA* установлено, что штаммы с мутированным *vieA* (*C235T*) впервые были завезены в Россию в 2004 г. (таблица). Белок VieA играет важную роль в биологии штаммов классического биовара, т. к. регулирует транскрипцию 401 гена (10,3% генома). В то же время у El Tor вибрионов под контролем VieA находятся всего несколько генов, в том числе *vps* и *rbm*, кодирующие, соответственно, продукцию экзополисахарида и белковый матрикс биоплёнки [27]. Однако изменения в структуре гена *vieA* важны для El Tor вибрионов в совокупности с вариабельностью гена *hns.* Мутация *G319A* в гене *hns* приводит к повышению экспрессии штаммами ХТ, ТКПА, а также гемолизина HlyA и MARTX токсина, но при нахождении в кишечнике данные гипервирулентные штаммы становятся чувствительными к действию антимикробных пептидов и желчи хозяина. В то же время при изменении структуры гена *vieA* и продукции мутантного белка VieA данные штаммы *in vivo* приобретают устойчивость к действию указанных стрессоров [28]. В экспериментально полученных штаммах, содержащих нуклеотидную последовательность *hapR* с инсерцией тимина в позиции 219, наряду с наличием изменённой структуры *hns* (*G319A*) и/или *vieA* (*C235T*), биосинтез ХТ значительно возрастал [8]. Среди изученных нами ряд геновариантов с аллелем *ctxB1*, а также все штаммы с аллелем *ctxB7* включают указанные гены (*hns* и *vieA*) с мутациями, что указывает на участие данных изменённых регуляторов в повышении продукции ХТ в данных штаммах.

Появление мутации *G265A* в гене *carR* у геновариантов *V. cholerae* O1 El Tor, выделенных в последние годы, согласно данным литературы, приводит к снижению транскрипционной активности белка CarR, что выражается в уменьшении экспрессии *almEFG* оперона и снижению процесса модификации липополисахарида клеточной стенки. В результате данного процесса клетки становятся чувствительными к действию катионного антими-

кробного препарата — антибиотику полимиксину B, что фенотипически *in vitro* проявляется изменением диагностически значимого признака и отсутствию роста штаммов *V. cholerae* O1 биовара El Tor в его присутствии [23]. Однако, возможно, при нахождении *in vivo,* в условиях воздействия катионных антимикробных пептидов хозяина, геновариантты, синтезирующие мутантный белок CarR, будут устойчивыми к их действию, т. к. они продуцируют мутантный белок VieA, который восстанавливает устойчивость патогена к действию данных стрессоров [28].

При анализе структуры гена *luxO* установлена его вариабельность как у типичных штаммов, так и у геновариантов. В изученной нами литературе отсутствуют сведения о влиянии выявленных мутаций на функциональную активность LuxO. Можно предположить, что наличие функционального LuxT практически у всех штаммов, как и LuxO, выполняющего ингибирующую функцию в отношении *hapR*, может компенсировать снижение или отсутствие активности LuxO. В ранее проведённой работе установлено, что у «гипервирулентного» геновариантa *V. cholerae* MQ1795 O1 биовара El Tor (Бангладеш, 1994), наряду с мутациями в *hapR* (вставка T в позиции 219), *hns* (*G319A*), *vieA* (*C235T*), изменена и структура гена *luxO* (*C656T*) [8]. Однако у изученных нами штаммов данная мутация не выявлена.

Заключение

Проведённое исследование показало, что нуклеотидная последовательность ряда изученных регуляторных генов (*toxT, aphA, aphB, carS, luxT, hapR*) у штаммов *V. cholerae* O1 El Tor, завезённых и выделенных на территории России и сопредельных стран, остаётся неизменной. В то же время структура других генов изменяется. Наиболее значимыми являются мутации в гене *carR*, что привело к изменению диагностически значимого признака и появлению чувствительных к антибиотику полимиксину B геновариантов *V. cholerae* O1 El Tor, а также в генах *hns* и *vieA,* кодирующих негативные регуляторы продукции факторов патогенности. Можно высказать предположение, что постепенное повышение вирулентности геновариантов *V. cholerae* O1 El Tor явилось результатом изменения регуляторных механизмов продукции основных факторов патогенности — ХТ и ТКПА вследствие появления мутаций как в структурных генах *ctxB* и *tcpA,* так и в регуляторных *hns* и *vieA*, кодирующих белки-репрессоры. При этом мутации, выявленные в генах *hns* (G319A), *vieA* (C235T) и *carR* (G256A) у всех изученных штаммов с аллелем *ctxB7*, могут быть использованы в качестве генетических меток современных геновариантов *V. cholerae* O1 El Tor.

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Статья поступила в редакцию 12.04.2024;
принята к публикации 13.06.2024;
опубликована 29.08.2024

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The article was submitted 12.04.2024;
accepted for publication 13.06.2024;
published 29.08.2024

Systematic Review
https://doi.org/10.36233/0372-9311-467

Limitations in creating artificial populations in agent-based epidemic modeling: a systematic review

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Abstract

Introduction. The key step in agent-based modeling of epidemics, which allows researchers to take into account individual characteristics of people, is the creation of an artificial population. The main difficulty of this procedure is finding a balance between the detail of the population description and the computational efficiency of the calculations.

The aim and objectives of the review: Critically analyze and summarize the current evidence on how to create artificial populations; evaluate the limitations and advantages of available approaches in solving various problems in epidemiology.

Materials and methods. An analysis of literature sources devoted to agent-based modeling has been performed. The analysis is focused on algorithms for creating an artificial population with a given level of detail for modeling human respiratory infections.

Results. The approaches to the creation of artificial populations are generalized. The main principles of realization of interaction between agents are revealed: by means of networks of contacts between agents and on the basis of taking into account the movement of agents between locations. The first approach is the most computationally efficient and simple; the second approach allows to better take into account the change in the behavior of agents during the development of the epidemic process.

Conclusion. Agent-based modeling is an optimal tool for selecting the best scenario for epidemic control and investigating the role of individual characteristics of people in the development of epidemics. When creating an artificial population, it is important to include in the model factors that can be targeted for control. A significant limitation is the lack of factual data on population structure, but this can be overcome by using indirect data.

Keywords: *Agent-based modeling, artificial population, epidemic process, computational epidemiology, systematic review*

Funding source. The work was supported by the Russian Science Foundation grant No. 21-15-00431 and Rospotrebnadzor subsidy No. 141-02-2023-208.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Maslova I.I., Manolov A.I., Glushchenko O.E., Kozlov I.E., Tsurkis V.I., Popov N.S., Samoilov A.E., Lukashev A.N., Ilina E.N. Limitations in creating artificial populations in agent-based epidemic modeling: a systematic review *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):530–545.
DOI: https://doi.org/10.36233/0372-9311-467
EDN: https://www.elibrary.ru/goxztx

Систематический обзор
https://doi.org/10.36233/0372-9311-467

Ограничения в создании искусственных популяций в агентном моделировании эпидемий: систематический обзор

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Аннотация

Введение. Ключевым этапом агентного моделирования эпидемий, позволяющим исследователям учитывать индивидуальные особенности людей, является создание искусственной популяции. Основная сложность этой процедуры — поиск баланса между подробностью описания популяции и вычислительной эффективностью расчётов.

Цели и задачи обзора: критически проанализировать и обобщить актуальные данные о способах создания искусственных популяций; оценить ограничения и преимущества имеющихся подходов при решении различных задач в эпидемиологии.

Материалы и методы. Проведён анализ источников литературы, посвящённых агентному моделированию. Анализ сфокусирован на алгоритмах создания искусственной популяции с заданным уровнем детализации для моделирования респираторных инфекций человека.

Результаты. Обобщены подходы к созданию искусственных популяций. Выявлены основные принципы реализации взаимодействия между агентами: с помощью сетей контактов между агентами и на основе учёта перемещения агентов между локациями. Первый подход является наиболее эффективным для вычислений и простым; второй подход позволяет лучше учитывать изменение поведения агентов в ходе развития эпидемического процесса.

Заключение. Агентное моделирование — оптимальный инструмент при выборе наилучшего сценария проведения противоэпидемических мероприятий и исследовании роли индивидуальных особенностей людей в развитии эпидемий. При создании искусственной популяции важно включать в модель факторы, на которые может быть направлен контроль. Существенным ограничением является отсутствие фактологических данных о структуре популяции, однако его можно преодолеть за счёт привлечения косвенных данных.

Ключевые слова: *агентное моделирование, искусственная популяция, эпидемический процесс, вычислительная эпидемиология, систематический обзор*

Источник финансирования. Работа поддержана грантом РНФ №21-15-00431 и субсидией Роспотребнадзора №141-02-2023-208.

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

Для цитирования: Маслова И.И., Манолов А.И., Глущенко О.Е., Козлов И.Е., Цуркис В.И., Попов Н.С., Самойлов А.Е., Лукашев А.Н., Ильина Е.Н. Ограничения в создании искусственных популяций в агентном моделировании эпидемий: систематический обзор. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2024;101(4):530–545.
DOI: https://doi.org/10.36233/0372-9311-467
EDN: https://www.elibrary.ru/goxztx

DOI: https://doi.org/10.36233/0372-9311-467

Introduction

Since the early 2000s, humanity has faced a number of viral epidemics, including Severe Acute Respiratory Syndrome (SARS, 2002-2003), Influenza A(H1N1)-California (swine flu) (2009), Middle East Respiratory Syndrome (MERS, 2012), Ebola outbreaks (2014-2016), Zika fever (2015-2016) and finally the COVID-19 pandemic caused by the novel SARS-CoV-2 coronavirus (2019-present). The COVID-19 pandemic has sparked the interest of epidemiology and public health professionals in using computational tools to predict epidemics and select optimal anti-epidemic measures. These tools include machine learning methods and computational epidemiologic models.

Computer simulations in epidemiology are designed to reproduce the dynamics of infectious disease spread, taking into account population demographics [1–3], contact network structure [4] and information on intervention strategies [5, 6]. These models provide a virtual laboratory to study hypothetical scenarios, evaluate the effectiveness of different interventions, and anticipate outbreak trajectories.

Numerical solution of ordinary differential equations and agent-based modeling (ABM) are the two most common modeling approaches in epidemiology [7, 8]. The first approach includes various compartmental models, such as the susceptible-infected-uninfected model [9] and its modifications; the second approach includes agent-based models, which take into account the heterogeneity of a population by modeling the actions and interactions of individual agents (people) within it [3, 4, 10].

Agent-based models consider each person as an autonomous agent with characteristics that determine his/her behavior and social interactions. The semantic blocks into which any synthetic population can be divided are presented in **Fig. 1**.

The agent-based approach is applicable for studying epidemic control measures [11–13], assessing the effectiveness of interventions on different populations [14], and conducting sensitivity analysis of modeling results to changes in parameter values [15]. The main goals of public health applications of ABM are to analyze and predict the public health consequences of proposed interventions, taking into account aspects of complex social structure. ABM-based models help to understand the underlying mechanisms that determine the dynamics and outcomes of epidemics. ABMs can be used for virtual experiments exploring different intervention strategies and other interventions to reduce morbidity in the population [16]. All this makes ABMs an important research and training tool for public health professionals.

The main difficulty in using ABM as a tool for social, political, and economic research lies in the proper matching of the purpose of modeling and the level of detail of the model [17]. The disadvantage of ABM can be excessive detail, which complicates the overall mod-

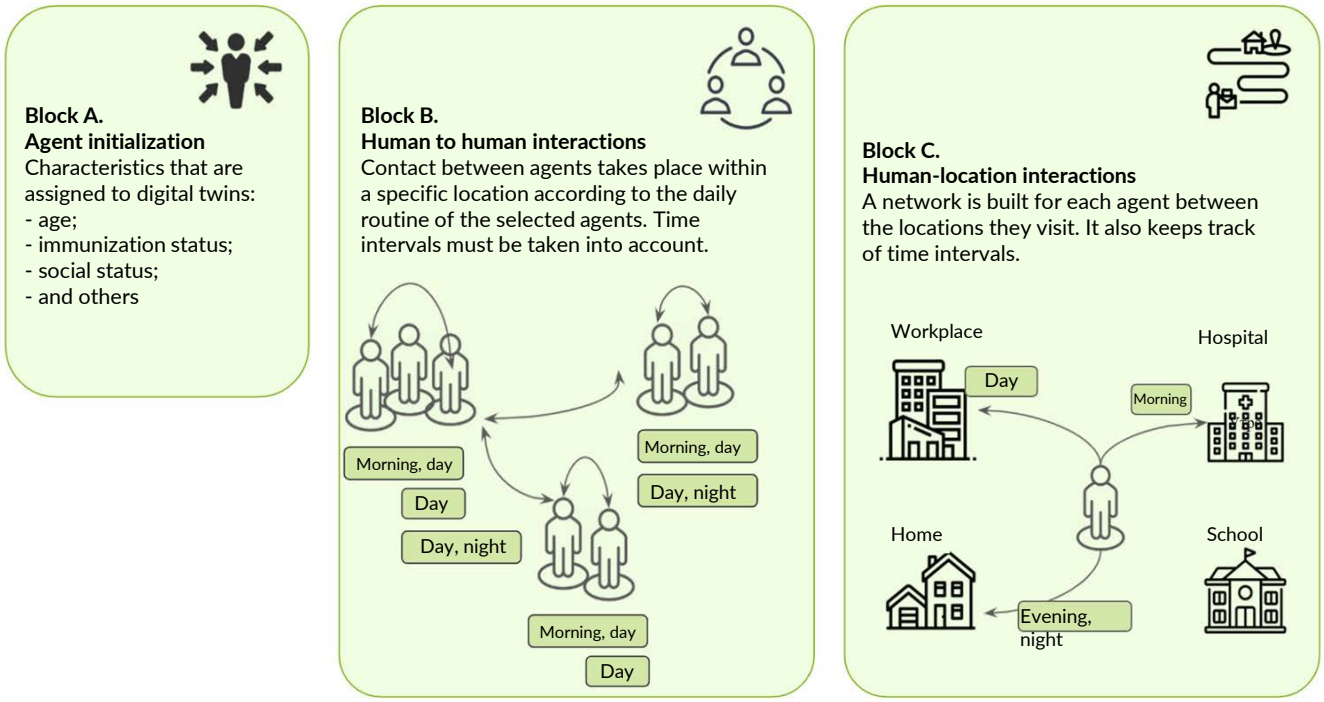

Fig. 1. The artificial population consists of agents with different demographic characteristics (block A). These agents are assigned specific tasks to perform at specific locations and times. This determines a network that connects agents to locations throughout the day, creating a person-location network (Block C). The person-to-person contact network (Block B) is developed based on the interactions obtained from the person-to-location graph.

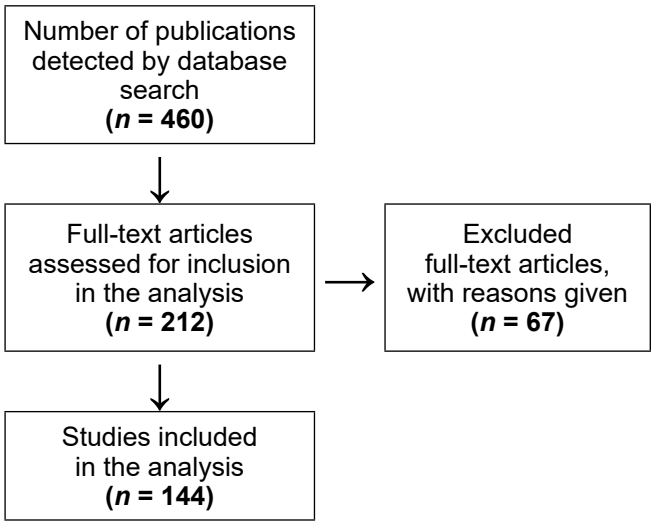

Fig. 2. Publication selection scheme for the systematic review.

eling task and leads to the creation of overly complex models with redundant parameters that do not contribute significantly to the modeling results [18].

Finding a balance in the choice of considered parameters and complexity when creating an artificial population (AP) for ABM is an open question facing researchers. This systematic review aims to identify the most common approaches to creating AP in agent-based modeling and to specify their limitations.

Materials and methods

This systematic review is based on the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. A systematic literature search was conducted using the PubMed database. The search was performed using the keywords: "agent-based" AND "epidemiology". Full-text articles published between 2020 and 2024 were considered. The initial assessment selected studies that used agent-based modeling, studied respiratory viral infections, and had a sufficiently detailed description of the model.

Papers studying the behavior of the virus in a single organism, as well as studies on modeling animal infections, were excluded from the study.

According to the search methodology, 144 studies published in international journals in English were selected and used for further analysis. No Russian-language publications meeting the selection criteria were found. The selected publications were systematized according to the ways of setting the AP by the criteria "location" (space consideration) and "agent properties". The agent's properties included such characteristics as gender, age, field of activity, ethnicity, income, and the like — i.e., characteristics determined on the basis of demographic and statistical data. We considered that the model accounted for locations if the probability of transmission depended on the agent's spatial location. This property of the model can be realized both by tracking the coordinates of each agent in the modeled space and by modeling individual spatial entities (e.g., store, school) that may house agents.

Results

In 2020-2024, the greatest interest of researchers was focused on modeling the spread of SARS-CoV-2 virus, the causative agent of COVID-19, in the population: 129 (89%) papers out of 144 selected modeled the spread of this virus, 10 (11%) papers modeled the spread of influenza virus. In several studies, researchers presented their models as suitable for studying several respiratory diseases (**Table 1**).

To systematize the types of APs used in the models, we analyzed the presence of agent properties and the consideration of their location. **Fig. 3** shows the distribution of publications considered in the review according to the type of APs described in them.

We can distinguish 4 variants of AP construction, based on combinations of presence and absence of consideration of agents' properties and consideration of locations.

Approaches to AP creation without consideration of location and agent properties (12 articles)

An artificial population without taking into account spatial localization and demographic properties of agents represents a graph — a network of contacting agents (**Fig. 4**). The stochasticity of such models is created by generating individual sets of connections at each node (agent) based on given probability distributions of the number of contacts.

At the same time, contacts or social ties can be the same or differ in the strength and frequency of interaction. In 6 (50%) out of 12 reviewed works all contacts are the same. In another 5 (41.7%) papers contacts are divided into 3 categories: close, permanent (family, friends) and casual, not close (contacts on the street, work, school). In 1 (8.3%) article, the division of interactions by types is more complex.

For example, in a study conducted by J. Whitman et al., the interactions are divided into two levels: intra-cohort (strong ties, high probability of transmission)

Table 1. Distribution of the articles according to the pathogen

Pathogen	Publication amount	
	n	%
SARS-CoV-2	129	89
Influenza	10	6,9
Measles	1	0,7
MERS-CoV	1	0,7
Unspecified respiratory diseases	4	2,7

DOI: https://doi.org/10.36233/0372-9311-467

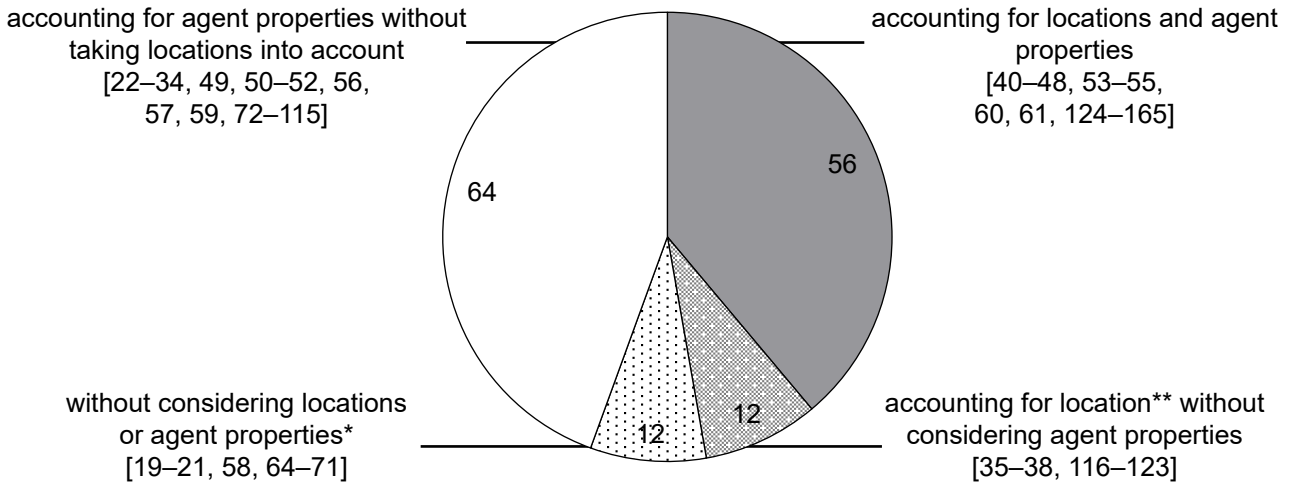

Fig. 3. Distribution of publications by artificial population type.
*At the same time, agents can be endowed with an individual level of protection against the pathogen (immunity) and the level of viral load.
**This group also includes papers that consider the spatial location of buildings and/or agents.

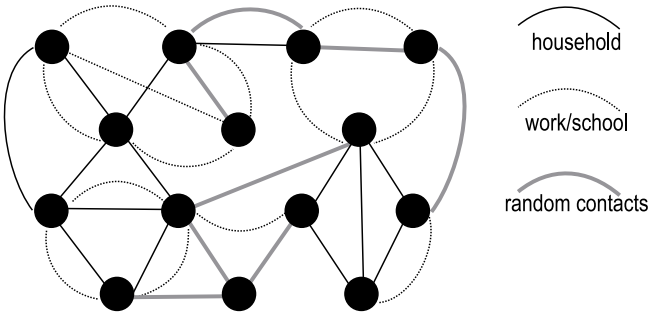

Fig. 4. A network of contacts without considering the properties of agents and spatial characteristics is illustrated.
Each node represents an agent, and the edges between nodes indicate a contact on one of the layers.

and inter-cohort (weak ties, rare cases of virus transmission, number of ties is smaller) [19]. This allowed us to account for the presence of clusters in the distribution of contacts and to reproduce the repetitive behavior of peaks in disease spread with significant stochasticity. Using this model, the researchers studied the behavior of the reproductive number at different values of the initial immune profile of the population, as well as the dynamics of the infection time series when the population size and contact matrix change.

A study by X. Guo et al. presented a multilevel model of the relationship between disease transmission and emotional stress in society [20]. In this paper, two independent networks of contacts are superimposed. Each node represents some group of people, infection and information exchange occurs through the edges of these nodes. Each node, in turn, models a set of individuals in each node, which increases the accuracy of the results.

In the study conducted by N.N. Chung et al. presents a contact network consisting of a set of overlapping networks (households, dormitories, workplaces, dynamic crowd network, dynamic social gathering network) [21].

Agent-based modeling based on the construction of AP without taking into account the spatial localization and demographic properties of agents makes it possible to solve a fairly wide range of problems without additional complication of the model. This approach was used to study the influence of such factors as population size, immunity parameters, the number and nature of agent relationships, and population density on the modeling results. This approach also allows us to analyze quarantine and testing strategies, the nature of repeated peaks of incidence, the dynamics of mutant infections, and the role of super-spreaders (agents with a large number of linkages).

The lack of detailing the properties of agents when creating the AP allows us to simplify the computational model and increase its interpretability. At the same time, the main limitations of the AP considered in this section are the lack of the possibility of introducing adjustable clustering (for example, separating pensioners into a separate group) and taking into account the behavior of the population, as well as the inappropriateness of such models to study the physical impact of social interactions.

Approaches to creating an AP that takes into account properties of agents without considering locations (64 studies)

APs in which agents with demographic, biological, and social properties interact with each other in an unstructured space are the most common in agent-based modeling. Many authors consider this type of AP to be optimal from the point of view of accuracy/performance balance. This approach is also popular due to the fact that high computational efficiency allows the agents to be endowed with an extensive set of parameters.

The construction of a network of contacts in the considered type of AP is often based on the creation of 4 main layers: households, work, schools and kindergartens, and society. In more complex models, up to 30 layers can be overlaid.

The considered agent-based models based on the formation of AP, taking into account the properties of agents without taking into account locations, according to the nature of the realization of social ties were distributed as follows:

- Uniform contact— 11 (17.2%) publications;
- Close/long distance contact — 1 (1.5%);
- Three or more types of contact — 52 (81.3%).

The most common agent characteristics include age (64/64) and sex (9/64). Age groups may differ in the likelihood of infection and the development of more severe cases of disease. The age structure of the population also affects the properties of contact networks between agents. For example, in models with homogeneous contacts, the network of interactions is built based on age-specific contact matrices [22, 23]. Work contacts may be excluded for the older generation, and some models construct additional blocks of contact networks for elderly care facilities [24-30].

The number and nature of contacts between agents may depend on the agent's occupation/profession. In the simplest case, professions such as teacher and hospital employee are modeled. Such an approach allows modeling elements of temporal dynamics of agents' interaction, e.g., five-day working day, possibility of vacation and skipping school/work, division of contact networks into daytime (school, work) and evening/nighttime (home, community) ones.

About 20% of the publications reviewed in this section use the Covasim environment for AP construction and modeling [10]. In its basic version, Covasim is an open-source modeling environment adapted to study the dynamics of the COVID-19 pandemic. The AP embedded in Covasim represents a set of people, each with attributes such as age, gender, and social status (**Figure 5**). In modeling the spread of infection, the model takes into account the frequency of contacts, the infectiousness of the virus, and the susceptibility of agents.

Using the open source agent-based modeling environment Covasim, researchers can explore different epidemic scenarios by changing infection parameters and modeling various interventions such as social distancing, isolation, testing, contact tracing, and vaccination campaigns. In a study conducted by A. Cattaneo et al., the Covasim environment was used to evaluate the effectiveness and optimization of a COVID-19 vaccination campaign in the Italian region of Lombardy [31]. The age structure of the population and the household characteristics were matched with data from the Italian National Institute of Statistics, while the rest of the contact network variables were constructed based on the default parameters embedded in Covasim. Different levels of constraints were modeled by reducing the number of contacts in the school, work and social interaction strata, and by varying the probability of transmission between household members. The Covasim environment also allows for the specification and tracking of dynamic characteristics of agent immunity. For example, vaccination, as well as disease, affects the dynamics of neutralizing antibodies and the level of protection of agents; cross-immunity with a given degree of effectiveness is realized when different strains of the virus are present in the population. In the study of A. Cattaneo et al., the Covasim model showed results consistent with the registered cases of COVID-19 infection, detection and mortality, the most effective vaccination strategy was determined and age priorities for vaccination were proposed [31].

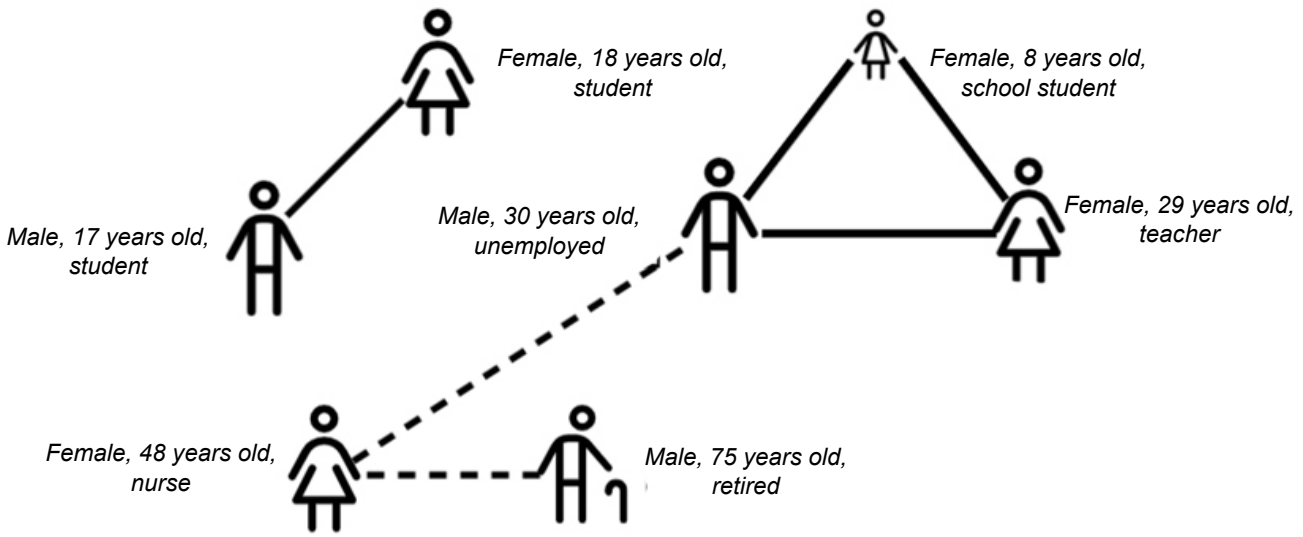

Fig. 5. Inter-agent interactions under the assumption that agents do have properties. Constant (solid lines) and dynamic (dashed lines) contact networks are modelled.

DOI: https://doi.org/10.36233/0372-9311-467

In general, agent-based modeling on AP, which takes into account the properties of agents without taking into account their locations, is used to study the development of an epidemic taking into account various demographic data, as well as to assess the effects of diseases on public health and the economy. In particular, such modeling makes it possible to assess the effectiveness of quarantine measures, analyze vaccination scenarios (including those targeting different age groups of the population), calculate the economic cost of introducing restrictive measures, and build population immunity.

One of the main limitations of this type of AP is the simplified representation of the network of contacts [32], as well as the idealization of individuals' activities during the day [33]. The authors also emphasize the potential importance of additional properties of agents, which are not taken into account in this approach to modeling [24, 34].

Approaches to agent location-aware and agent property-aware AP (12 studies)

The main purpose of AP modeling with and without taking into account the spatial movements of agents is to reflect both the mobility of agents and the spatial dynamics of their movements during the spread of an epidemic.

The most common tool for this approach is the NetLogo software. In this environment, a map of a closed space is represented either by a coordinate grid or by a set of cells, and agents move randomly across the map or according to specified movement patterns (**Fig. 6**) [35-37]. Infection in this type of representation is possible if the agents (infected and susceptible) collide, converge to some threshold distance, or fall into one cell.

In the agent-based models we have considered, based on the formation of AP with and without taking into account the location of agents, social ties were analyzed as follows:

- Uniform contact — 6 (50%) publications;
- Close/long distance contact — 4 (34%);
- Three or more types of contact — 2 (16%).

A good example of this approach is shown in the study by T. Daghriri et al., in which several ways of distancing were modeled and the movements of agents resulting from different scenarios were visually represented [35]. The model took into account the possibility of a part of the agents not respecting the distancing. The authors showed the importance of compliance with restrictive measures and depicted the correlation between the strictness of the social distancing policy and the spread of the disease.

The two main models describing the movement of agents in the environment are random walks and the gravity model, according to which the strength of interaction (intensity of flows) depends on the importance

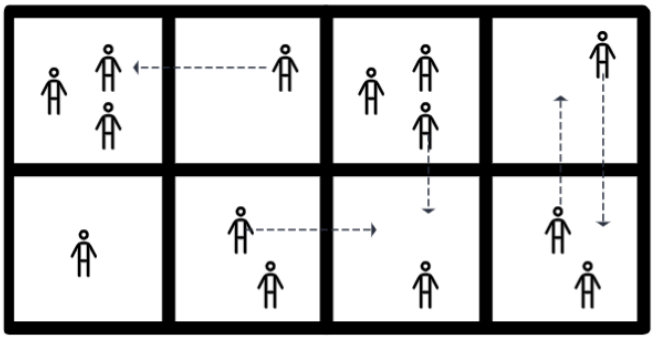

Fig. 6. Representation of an artificial population accounting for the movement of identical agents.
A contact is defined as a collision, approaching a critical distance, and/or agents entering the same cell.

(magnitude) of objects and the distance between them. For example, the study conducted by N. Kishore et al. showed that a densely populated center has a higher probability of being visited by agents [38].

The main goals of research in this approach are to study distancing strategies, the effectiveness of restrictive quarantine measures, the role of geographical factors in the spread of disease, and the role of super-spreaders. Such modeling also allows direct tracking of contacts of individuals in a population. However, it is not possible to model the implementation of anti-epidemic measures in different age and social groups of the population.

Approaches to creating an AP that takes into account the location and properties of the agents (56 studies)

When modeling with both geographic and demographic data, researchers try to achieve the closest approximation to the real population, with the goal of creating a digital twin. Typically, contact networks are divided into households, schools, workspace, and community, and geographic features are taken into account in two ways: modeling agents' movements on a map or capturing the location of buildings and determining the probability of agents visiting them. However, if in the group of APs that take into account the location of agents without taking into account their properties, the more common was the mapping of terrain, then in the works that take into account both the properties of agents and the properties of places, the division of the model space into conditional locations in which an agent can be located was more often used in the creation of APs (**Fig. 7**).

The most common framework for this type of model is FRED (a Framework for Reconstructing Epidemic Dynamics) [39]. FRED uses synthetic populations based on census data that reflect the demographic and geographic heterogeneity of the population. Each agent has associated demographic and socioeconomic information (e.g., age, gender, race, family income). Race, along with sex and age, can be used to account

for known disease prevalence. Households, educational and health institutions, places of work, and some other locations are georeferenced to a spatial grid of coordinates (at 1 km resolution). When calculating the probability of visiting different geographic locations, the agent's household income is taken into account. One of the features of this model is the ability to take into account the dynamic demography of agents, including aging, fertility and mortality. Works [40-43] have been performed on the basis of this model. Currently, FRED continues to be actively used to study seasonal influenza.

M.G. Krauland et al. studied the effect of a decrease in population immunity caused by the restriction of virus activity on its dynamics in subsequent years [43]. Modeling was conducted for a population representing Allegheny County (Pennsylvania, USA) with a population of about 1.2 million. This county includes both urban and suburban areas and is large enough to investigate influenza patterns. According to the results, a decrease in the incidence rate in the first season will lead to an increase in the incidence rate in the second season. Compensating for the decline in population immunity may help to increase vaccination. Depending on cross-immunity from previous infection and the transmissibility of the strain, the incidence rate could increase by up to 50%.

Many of the publications reviewed in this section describe complicated models where additional parameters have been added to the basic version of the AP. In particular, A. Truszkowska et al. modified the basic version of the model by adding the division of the able-bodied population into spheres of activity [44]. This allowed the model to reflect the complex structure of employment. And in the study conducted by C. Fosco et al., the labor force was divided into 4 groups according to different mobility in case of quarantine measures [45].

A number of works paid more attention to the division of the day into time segments. In 24 models, the temporal characteristics of agents' mobility were taken into account (taking into account the schedule, division of the day).

The goals of approaches that take into account both agent and location properties include:

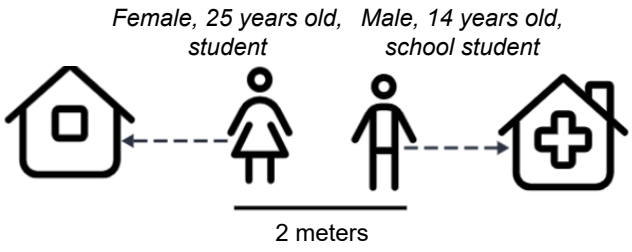

Fig. 7. Artificial population taking into account the location and characteristics of agents.

It is possible to overlay a network of contacts on a map or to simulate the movements and contacts of agents.

- management decision analysis;
- finding the optimal approach to implementing non-pharmaceutical interventions;
- study of infection spread using GPS;
- studying the spread of the pathogen in its early stages;
- studying the distribution of different strains;
- modeling contact tracing and virus transmission;
- studying the spread of the virus in different countries/cities;
- exploring vaccination strategies;
- studying the protection of the population depending on the past season.

In generating this type of AP, model developers often resort to various simplifications to allow for the inclusion of additional characteristics that they consider crucial [46]. Some assumptions exceed the current understanding of the mechanisms of epidemic development, allowing them to be included in the study only in an approximate form [47]. It is common practice to use updated real-world data as the basis for the creation of an AP-digital twin, which is then projected onto a sample of smaller size than the general population. Even if the sample replicates the structure of the real population, the results obtained for it may not fully reflect the situation in the real population [48].

Increasing complexity of AP formation

When creating a realistic population for epidemiologic studies, an extensive set of parameters is required, each of which cannot be taken into account at the moment. Basic versions of models allow describing the epidemiologic process in a general way and investigating regularities and trends in the dynamics of epidemics.

In order to make it more plausible, some authors have resorted to complicating the AP by introducing the following parameters:

- seasonality;
- comorbidities;
- dynamic immunity;
- ethnicity;
- profits;
- transport flows.

The heterogeneity of the population in terms of the susceptibility of individuals to the virus and the severity of the disease can be accounted for by a co-morbidity factor. In the simplest version, co-morbidities can be taken into account thanks to the binary parameter (present or absent) [28]. In a more complex version, by introducing an additional module for calculating the risk factor, it is possible to take into account both specific diseases (type 1 and type 2 diabetes, hypertension, cardiovascular diseases, etc.) and risk factors related to lifestyle (smoking, physical activity, increased body mass index, etc.) [49].

In certain studies, dynamic immunity modeling has been performed. A popular framework for account-

DOI: https://doi.org/10.36233/0372-9311-467

ing for immunity has been the Covasim model [50–52], which provides the possibility of dynamically changing the values of the level of specific immune defense for each agent and the population as a whole.

Seasonality can affect both the properties of the pathogen (mainly used in modeling seasonal influenza) and other parameters (effect of average daily temperature on susceptibility, effect of season on the contact network with sex distribution, etc.). [43, 53–58].

If appropriate data are available, it is possible to add sociological parameters of agents — income level and ethnicity, and these characteristics can be reflected in the model in different ways. In the study conducted by M.D. Patel et al., people of different nationalities had different susceptibility to the virus and tolerated the disease differently [59]. In the study conducted by C. Fosco et al., income level influenced the ability of workers to stay at home during the epidemic [45]. In the study conducted by M. Thakur et al. income was directly correlated with decreased vaccination rates [60].

Modeling of transport flows within the AP was used in 15 (10%) papers, 8 of which considered geographic and demographic population data, 7 — only demographic data.

Representation of transport was possible in the form of:

- an additional random network of contacts;
- more transportation stops/blocks;
- addition of common agent routing.
- Some researchers have resorted to dividing transportation into modes:
- automobiles, hitchhiking, public transport, walking, etc. (with the possibility of getting infected only in automobiles and public transportation) [25];
- metro, bus, shuttle bus [61].

Conclusion

AP formation is a key point in the construction of predictive agent-based models. The use of ABM allows us to consider the population at the level of individual representatives, which opens new opportunities for studying the development of epidemics and analyzing measures to prevent the spread of infection.

In our review, based on the analysis of 144 original studies, we consider 4 variants of AP construction with different degrees of detail. We intentionally used the PubMed database exclusively for the literature search because it is focused on biomedical research, including epidemiology. This choice allowed us to analyze the main publications published in ranked peer-reviewed journals in the field of interest, but it is possible that some part of the available publications was not considered. The review also considered articles published since the beginning of the COVID-19 pandemic. This allowed us to analyze the most relevant cross-section of papers, focusing on the demanded solutions in AP formation, while the review did not include the previously published EpiSimS [62] and TRANSIMS models [63].

It should be noted that all the considered variants of AP construction turned out to be suitable for solving the list of tasks in the field of infectious disease epidemiology stated by the developers. The limitations of the present study are dictated by the impossibility of experimental confirmation of the success of the implementation of the presented ABM to achieve the goals and objectives in the reviewed studies. In most cases, there is no possibility to critically conceptualize the model due to the availability of a general, often superficial description of its device given in the publication and the lack of access to the source code of the model. The selected literature was analyzed largely on the basis of the authors' evaluation of the results of the papers. In most cases, the authors do not provide an analysis of the sensitivity of the result to the parameters of the modeled pathogen and AP. Such analysis is an important feature of complex models and can show the real importance of parameters, and this review revealed a systematic shortcoming of a large part of the analyzed papers.

Among the identified limitations in AP creation, the most significant are the insufficiency and anachronism of real demographic and statistical data required for further accounting in the model. Works that take into account the properties of agents in the AP, as a rule, rely on census data or sociological surveys, which do not always have the required detail. Models that incorporate the movement of agents on a city map use information from specialized applications, databases and mapping services such as Google Maps and OpenStreetMap. Obtaining this data and incorporating it into the model can be challenging, so simplified models based on assumptions about agent behavior and interactions were used in some cases.

The use of complex and diverse real demographic and statistical data is possible when studying small groups (at the level of a room, building), but for larger studies, the computational complexity in case of increasing the number of parameters or population size may exceed the technical capabilities of the calculation and lead to unreliable or uninterpretable results.

Further research on the creation and use of AP in agent-based modeling can be focused on optimizing methods of model parameterization and finding a balance between model detail and interpretability to achieve maximum accuracy and precision of results. When creating a AP, it is important to consider the factors that can be targeted for control. This will improve the quality of public health decision-making and increase the effectiveness of epidemic response.

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The article was submitted 23.04.2024;
accepted for publication 10.07.2024;
published 29.08.2024

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Статья поступила в редакцию 23.04.2024;
принята к публикации 10.07.2024;
опубликована 29.08.2024

Review
https://doi.org/10.36233/0372-9311-553

The importance of the role of T-cell immunity in the development of modern tick-borne encephalitis vaccines

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Abstract

The tick-borne encephalitis (TBE) virus is highly pathogenic and can affect the central nervous system, leading to severe chronic effects or death. The only effective measure to combat TBE is vaccine prophylaxis. Vaccines that are currently used for mass immunization are based on inactivated TBE virus, they provide a protective immune response, but such vaccines require multiple administrations. A possible reason for short-term immunity is an incomplete functional T-cell response to these types of vaccines.

The **aim** of this review is to analyze the literature on the role of the T-cell immune response in protecting the body from TBE, its importance for vaccine development, and to consider approaches to the development of new TBE vaccines based on different platforms.

When preparing the review, we analyzed the literature presented in scientific databases — PubMed, Scopus, Elsevier, Google Scholar as of April 2024. The following keywords were used for the search: vaccine, tick-borne encephalitis virus, T-cell immune response, flaviviruses.

A several publications have demonstrated that T-cell responses following natural infection with TBE virus and after vaccination with inactivated virus are different. During viral infection, both Th1- and Th2-type $CD4^+$ T cells and $CD8^+$ T cells are activated and play an important role in the elimination of viral infection. After vaccination, the only Th2-type $CD4^+$ T-cell response predominates, which may be the reason for the short-lived immune response. To date, a number of different types of experimental TBE vaccines are being studied, such as live-attenuated vaccines, viral vector vaccines, subunit vaccines, virus-like particles, DNA and mRNA vaccines, and polyepitope immunogens. In our opinion, the most promising in terms of T-cell response activation are vaccines based on T-cell polyepitope immunogens delivered in the form of DNA or mRNA.

Keywords*: TBE vaccines, tick-borne encephalitis virus, T-cell immune response*

Acknowledgments. The authors express their gratitude to V.A. Yakovlev for assistance in preparing illustrative material and technical editing of the manuscript.

Funding source. The study was carried out within the framework of the state assignment of the State Research Center of Virology and Biotechnology "Vector".

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Tigeeva E.V., Nizolenko L.F., Karpenko L.I. The importance of the role of T-cell immunity in the development of modern tick-borne encephalitis vaccines *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):546–559.
DOI: https://doi.org/10.36233/0372-9311-553
EDN: https://www.elibrary.ru/icslhy

Научный обзор
https://doi.org/10.36233/0372-9311-553

Роль Т-клеточного иммунитета важно учитывать при создании современных вакцин против клещевого энцефалита

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Аннотация

Вирус клещевого энцефалита (КЭ) обладает высокой патогенностью, способен поражать центральную нервную систему, приводя к тяжелейшим хроническим последствиям либо летальному исходу. Единствен-

ной эффективной мерой борьбы с КЭ является профилактическая вакцинация. Используемые в настоящее время вакцины, полученные на основе инактивированного вируса КЭ, обеспечивают формирование протективного иммунного ответа, однако такие вакцины требуют многократного введения. Возможной причиной недолгосрочного иммунитета является формирование недостаточно напряжённого Т-клеточного ответа при использовании таких вакцин.

Цель обзора — анализ литературы, содержащей информацию о роли Т-клеточного иммунного ответа в защите организма от КЭ, о его значении для разработки вакцин, а также рассмотрение подходов к разработке новых вакцин против КЭ на основе различных платформ.

При подготовке обзора был проведён анализ литературы, представленной в базах PubMed, Scopus, Elsevier, Google Scholar по состоянию на апрель 2024 г. Для поиска использовали следующие ключевые слова: vaccine, tick-borne encephalitis virus, T-cell immune response, flaviviruses, вакцины, вирус клещевого энцефалита, Т-клеточный иммунный ответ, флавивирусы.

В ряде публикаций продемонстрировано, что структура Т-клеточного ответа при естественном заражении вирусом КЭ и после вакцинации инактивированным вирусом различна. В ходе вирусной инфекции активируются $CD4^+$-Т-клетки как Th1-, так и Th2-типа, а также $CD8^+$-Т-клетки, играющие важную роль в элиминации вирусной инфекции. После вакцинации преобладает ответ $CD4^+$-Т-клеток по Th2-типу, что может являться причиной недолговечного иммунного ответа.

На сегодняшний день исследуется ряд различных типов экспериментальных вакцин против КЭ, таких как вакцины на основе живых аттенуированных вирусов, вакцины на основе вирусных векторов, вирусоподобные частицы, субъединичные вакцины, ДНК- и мРНК-вакцины, полиэпитопные иммуногены. В плане активации Т-клеточного ответа наиболее перспективными выглядят вакцины на основе Т-клеточных полиэпитопных иммуногенов, доставляемых в форме ДНК или мРНК.

Ключевые слова: *вирус клещевого энцефалита, Т-клеточный ответ, вакцины против клещевого энцефалита*

Благодарность. Авторы выражают благодарность В.А. Яковлеву за помощь в оформлении иллюстративного материала и техническом редактировании рукописи.

Источник финансирования. Исследование было выполнено в рамках государственного задания ФБУН ГНЦ ВБ «Вектор» Роспотребнадзора.

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

Для цитирования: Тигеева Е.В., Низоленко Л.Ф., Карпенко Л.И. Роль Т-клеточного иммунитета важно учитывать при создании современных вакцин против клещевого энцефалита. *Журнал микробиологии, эпидемиологии и иммунобиологии*. 2024;101(4):546–559.
DOI: https://doi.org/10.36233/0372-9311-553
EDN: https://www.elibrary.ru/icslhy

The wide spread of the tick-borne encephalitis (TBE) virus is a serious concern for public health authorities in many countries. This is due to the fact that the virus, being highly pathogenic, can affect the central nervous system (CNS), leading to severe chronic consequences or death [1–3].

In 30% of cases, neurological complications develop in people who have contracted TBE. Mortality from infection varies depending on the strain of the virus. The highest percentage of fatal cases (up to 35%) is registered when infected with strains belonging to the Far Eastern subtype [4–6].

Vaccine prophylaxis is the most effective way to control the virus. All currently licensed vaccines are based on inactivated strains of TBE. It is considered that the average seroconversion rate for both Russian and European vaccines ranges between 86–100%, which ensures the formation of protective immunity in vaccinated individuals [2, 7]. At the same time, vaccines based on inactivated TBE virus have a number of disadvantages: complicated vaccination schedule, relatively high reactogenicity, complexity of production and storage; in addition, there are cases of breakthrough infections in vaccinated persons [2, 8, 9]. Among vaccinated persons, the incidence of TBE ranges from 3.7% [10] to 23.8% [11] of the total number of cases, depending on the endemic region. One possible reason for breakthrough infections is the lack of vaccines that take into account the genetic variability of the TBE virus. Another reason is due to insufficiently intense and short-lived specific immunity in a number of vaccinated individuals, especially the elderly [7–9, 12].

The T-cell immune response is an important part of protective immunity against viral infections such as TBE. There are an increasing number of publications on the role of the T-cell immune response in the defense against infection with TBE virus. Therefore, more and more researchers have begun to pay attention to this aspect of the adaptive immune response, especially in the context of studies devoted to the development of new vaccine preparations [7, 13]. The wide spread of the virus and the significant growth of the number of patients have stimulated interest in the development of new vaccines against TBE virus, taking into account the role of the T-cell immune response.

DOI: https://doi.org/10.36233/0372-9311-553

The **aim** of the review is to analyze the literature on the role of the T-cell immune response in protecting the body from tick-borne encephalitis, its importance for vaccine development, and to consider approaches to the development of new TBE vaccines based on different platforms.

In this review, we consider the main aspects of T-cell response formation in humans when infected with TBE virus and after vaccination with licensed vaccines, as well as the main directions of work on the search for safe and highly effective next-generation vaccines that can overcome the limitations of the existing ones.

Materials and methods

The following keywords were used for the search: vaccines, tick-borne encephalitis virus, T-cell immune response, flaviviruses.

In the first phase, a search using different combinations of keywords in the scientific electronic database PubMed retrieved 1754 sources. Restricting the search to the time of publication from 2019 to 2024 allowed us to narrow the search to 424 sources. A search without considering the year of publication in this research library found an additional 123 sources matching the subject matter. Similarly, the search was conducted using the scientific databases Scopus, Elsevier, Google Scholar.

During the literature search in these databases in Russian and English languages, which was carried out taking into account such selection criteria as year of publication and accessibility of publications to reading, about 2000 sources corresponding to the topic were analyzed. Due to article length limitations, 88 sources were selected.

Adaptive immune response during infection with TBE virus and after vaccination

The adaptive immune response consists of humoral (antibody-mediated) and cellular immune responses specific to the TBE virus. The figure schematically represents the immune reactions of the adaptive immune response occurring after vaccination or during infection with TBE virus

The efficacy of antibodies against the TBE virus has been demonstrated by protecting susceptible individuals exposed to the virus by administering them anti-TBEV-immunoglobulin. Humoral immunity is thought to play a crucial role in defense against the TBE virus by providing synthesis of antibodies specifically targeting the virus. These antibodies neutralize the virus and prevent its spread, helping to limit the severity of infection and providing long-term immunity against infection with the TBE virus (Figure, *A*). Antibodies are able to bind to viral particles, causing them to be engulfed and destroyed by phagocytic immune cells [7, 13].

Memory B cells and virus-neutralizing antibodies are formed in a person who has had TBE, which provide long-term protection against virus re-infection. The long-term maintenance of memory B cells allows the immune system to respond more quickly and effectively to re-infection. When the same virus is encountered again, these cells rapidly differentiate into plasma cells that produce antibodies that destroy the virus before it can cause widespread infection and disease [13–16].

When vaccinated with inactivated virus, the functionality of memory B-cell populations is relatively short-lived due to limited $CD4^+$ T-cell responses (Figure, *B*) [17].

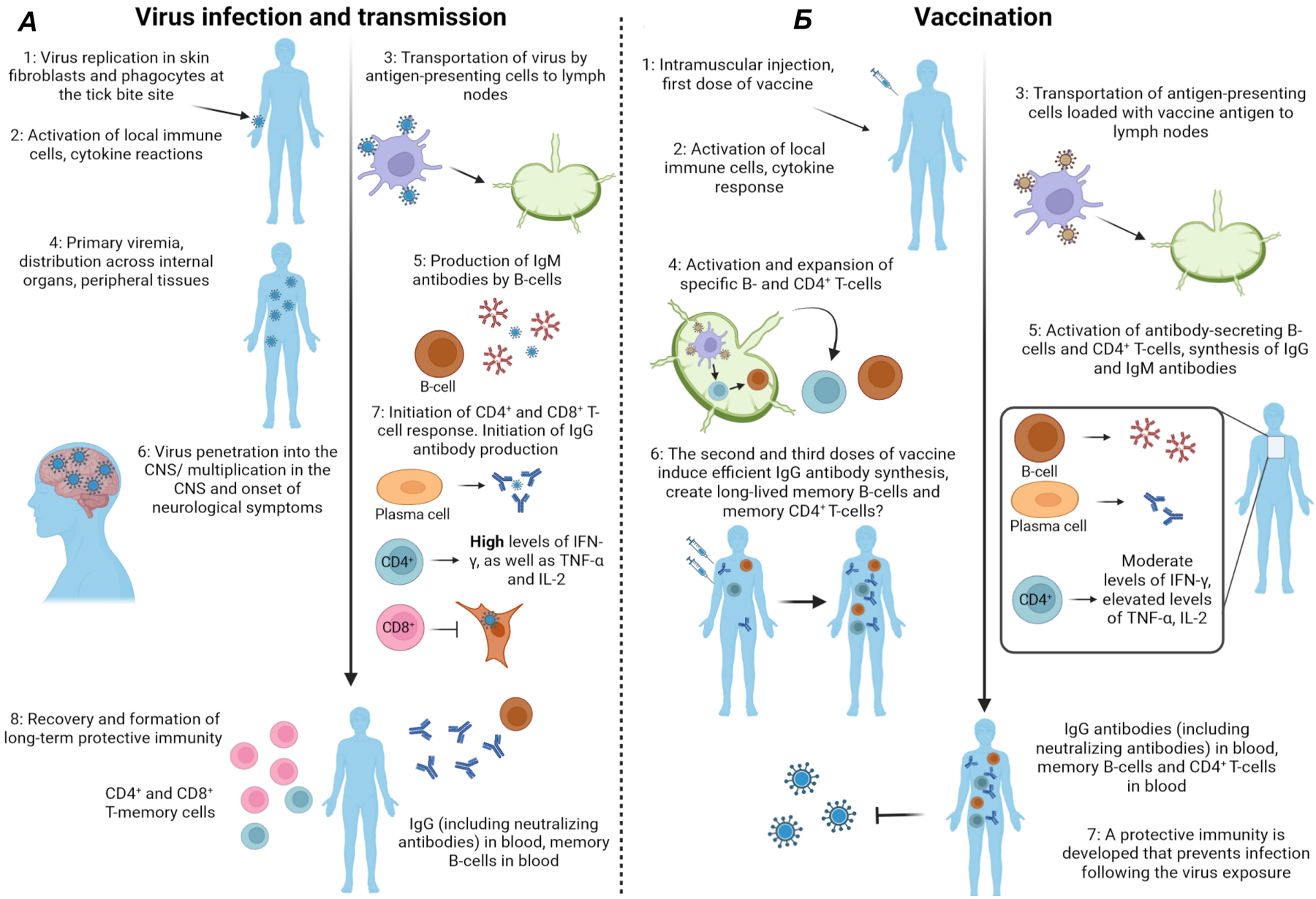
Immune response associated with $CD4^+$ T cells

$CD4^+$ T lymphocytes are important in the formation of both humoral and cellular immunity. $CD4^+$ T-cells are important producers of cytokines that help stimulate the antiviral immune response and provide B cells with the assistance needed to stimulate antibody synthesis (Figure, *A*). TBE virus encodes 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) and only 3 structural proteins: protein C (capsid) and the membrane-associated proteins prM/M (membrane/membrane precursor) and E (envelope) [18-20]. The structural proteins appear to contain the major epitopes that activate the $CD4^+$ T cell response during infection [8, 21], although there is evidence in the literature that several T helper epitopes are contained in the non-structural protein of the TBE virus, NS1 [22].

When analyzing peptide fragments of protein C predicted immunodominant epitopes, it was shown that mainly two peptide clusters are involved in $CD4^+$-cell activation both in natural disease and after vaccination. They are located in the α2- and α4-helixes of protein C [21]. The clusters of epitopes predicted for E protein were less effective with respect to $CD4^+$ activation, but this difference cannot be considered significant. It was found that in patients who underwent natural infection, specific stimulation of $CD4^+$ cells is provided by epitopes located in the 3rd domain, as well as in the stem region of the E-protein. In the group of vaccinated patients, stimulation was provided by peptide clusters from the 1st, 2nd and 3rd domains of the E-protein [21].

TBE virus-specific $CD4^+$ T-cells generated by vaccination appear to respond to a narrower range of viral targets than those generated by infection [8, 21], with vaccine-induced levels of interferon-γ (IFN-γ) reaching only about half the level of response induced by infection.

It is noteworthy that after natural infection with TBE virus, naive $CD4^+$ cells differentiate predominantly along the Th1 pathway, while during vaccination with inactivated virus — to a greater extent along the Th2 pathway [8, 9]. At the same time, against the background of natural infection, $CD4^+$ T cells acquire polyfunctionality, producing various cytokines, such as interleukin-2 (IL-2), IFN-γ and tumor necrosis factor-α (TNF-α; Figure, *A*) [4]. There is a correlation



Иммунные реакции, возникающие после заражения вирусом КЭ и после вакцинации против КЭ (по R. Ackermann-Gäumann и соавт. [13]).

Immune reactions occurring after infection with the TBE virus and after vaccination against TBE virus (adapted from R. Ackermann-Gäumann et al. [13]).

between the functionality of CD4+ T-cells and the level of virus-neutralizing antibodies, indicating that they are able to control the induction of neutralizing antibodies [8].

After vaccination, the number of CD4+ T-cells also positively correlates with the antibody response against TBE virus [17], and vaccine responders show increased proliferation of antigen-specific T-cells compared to non-responders (Figure, *A*) [23]. The response to vaccination tends to be skewed towards IL-2 and TNF-α production compared to infection (Figure, *B*) [9].

Immune response associated with CD8+ T cells

CD8+ T lymphocytes play an important role in viral infection by identifying and destroying infected cells, thereby limiting the spread of the virus in the body. To date, unlike CD4+ T cells, CD8+ T-lymphocyte-specific epitopes have only been found in non-structural proteins of the TBE virus, such as NS2A, NS3, NS4B and NS5 [24].

CD8+ T-cells are activated somewhat later than CD4+ T cells during natural infection, but nevertheless have a significantly higher level of activation, producing increased levels of granzyme B and perforin [4, 7]

K. Blom et al. showed that in patients with TBE at the peak of the T-cell response one week after hospitalization, CD8+ T-cell activation was significantly increased compared to CD4+ T-cells [25], indicating a tendency towards CD8+ dominance (Figure, *A*). These CD8+ T cells additionally exhibited an effector phenotype (CD45RA-CCR7) [24, 25] and had a highly activated Eomes+Ki67+T-bet+ transcriptional profile. However, these effectors tended to be monofunctional. After acute infection, when patients recovered, antigen-specific CD8+ T cells switched to the Eomes-Ki67-T-bet+ phenotype [25], which corresponds to a type 1 effector memory population.

Usually, CD8+ T-cell analysis is performed in patients with a severe course of the disease, in whom CD8+ T-cells are not only found in the blood, but sometimes also in brain tissue [13]. This fact limits the understanding of whether the CD8+ population is an important protective factor in mild or asymptomatic disease or an additional factor causing pathology [13]. In favor of the necessity of CD8+ T-cells for the body's defense against the TBE virus is the recent evidence that the severity of the disease, as well as its form, depends on the degree of T cell activation. Early activation of T-cell responses, including a subset of CD8+ T-lymphocytes, significantly correlated with a favorable outcome of the disease [26].

The results of animal studies are also mixed. The study by D. Růzek et al. showed that mice with severe immunodeficiency and mice with CD8 knockout had a higher survival rate after lethal infection caused by TBE virus compared to wild-type mice or mice with adoptively transferred CD8+ T-cells [27]. This may suggest a possible role of CD8+ T cells in the development of lethal infection. Thereafter D. Růzek et al. obtained data indicating that CD8+ T-cells are not responsible for the permeability of the blood-brain barrier during the disease, since its destruction during infection caused by this virus was observed both in wild-type and CD8 knockout animals [28].

The role of CD8+ T cells in virus clearance from neural tissues has been shown for other flavivirus infections using mice as laboratory animals [29]. Depletion of CD8+ T cells leads to enhanced infection by Zika and dengue viruses, but this effect is reversed after adoptive transfer of memory CD8+ T cells. Similar conclusions were obtained using mice deficient in various cytotoxic effector molecules in West Nile fever virus. During the initial stages of infections caused by yellow fever and Zika viruses, when mice has not yet formed a sufficient levels of virus-specific antibodies, effector CD8+ T cells are essential for infection control [29].

There are few data on the presence of specific CD8+ T cells in humans who have received the TBE vaccine (Figure, *B*). A. Sycheva et al. analyzed the formation of T-cell response in volunteers vaccinated with Tick-E-Vac and showed that in the peripheral blood of the vaccinated individuals a low level of CD8+ specific to the TBE virus was detected, and the overall response to the vaccine clearly depends on CD4+ [30, 31].

As mentioned above, the main epitopes of CD8+ T-cells are contained in nonstructural proteins of the virus [24]. Since non-structural proteins are synthesized only during active virus replication, such proteins are found in small amounts or are completely absent in currently used vaccines based on inactivated virus [32]. This fact may partially explain the low CD8+ T-cell response during vaccination. That said, TBE infection can induce a lifelong protective CD8+ response [14].

A number of vaccine platforms will be discussed below in terms of the possibility of inducing a T-cell immune response.

Vaccines against tick-borne encephalitis virus

Vaccines based on inactivated virus

There are currently a number of approved and licensed adult and pediatric TBE vaccines based on inactivated strains of the virus [33]. In Europe, two vaccines based on European strains of TBE virus are available: K23 and Neudorfl. In Russia, the Tick-E-Vac vaccine and its lyophilized analogue called TBE vaccine Moscow (FSBSI“Chumakov FSC R&D IBP RAS”) and EnceVir (Microgen), created on the basis of the Far Eastern strains of the TBE virus, Sofyin and 205, respectively, are licensed [12, 34–36]. The vaccine used in China is based on the Sen-Zhang strain (Far Eastern subtype of TBE virus) [37]. Vaccination against TBE virus has proven to be effective, as evidenced by the

results of mass vaccination campaigns in Austria [38] and Russia [39–42].

All licensed vaccines are capable of providing sufficiently effective prevention of TBE, especially when large-scale vaccination programs are implemented. However, the existing vaccines are not without disadvantages, including a complicated vaccination schedule due to the inability to maintain an adequate level of immune protection in the long term. Failure to adhere to a patient's vaccination schedule can lead to low levels of humoral immune response in the vaccinated, especially in the elderly [2, 7].

Vaccination with inactivated virus provides lower levels of antibodies than with natural infection [7]. It is suggested that this may be due to a change in the conformation of the E-protein as a result of the effect exposure of formaldehyde on the viral particle during the inactivation process. Thus, the availability of epitopes that bind to neutralizing antibodies is reduced [43]. As already mentioned, there is a difference in the immune response to infection and vaccination with inactivated virus, which is associated with the limited T-cell responses — a small number of specific $CD8^+$ T-lymphocytes, as well as reduced functionality of $CD4^+$ cells (Figure, *B*). In response to inactivated virus, mono- or bi-functional $CD4^+$ T cells are formed, capable of producing, for example, IL-2 alone or IL-2 and TNF-α alone, but the level of IFN-γ secretion is significantly reduced compared to natural infection [4, 8]. Vaccination leads to a shift of the response towards the Th2-pathway, whereas in natural disease, the cellular response is usually formed along the Th1-pathway, which may affect the efficacy of protection against the virus [7, 44]. Ideally, vaccines should elicit more robust responses of IFN-γ-producing $CD4^+$ T-cells.

The disadvantages of vaccines based on inactivated viruses include the fact that they are produced using only a specific strain of TBE virus and do not take into account its genetic variability. As a result, in endemic regions, the number of breakthrough infections among vaccinated individuals can reach 23.8% of the total number of cases [11]. Despite this, the direction of creating inactivated vaccines remains paramount. In 2017, phase I/II clinical trials of the Evervac vaccine were completed. The main difference of this vaccine from its analogs is the absence of adjuvants in its composition, as well as virus production in Vero cells, which allows for an improved safety profile. However, the problem of incomplete T-cell response has not been solved yet [45].

Approaches to TBE vaccine development and induction of cell-mediated immunity

To date, research on candidate vaccines against TBE virus and other flaviviruses has focused on several objectives at once:

- achieve high immunogenicity in all age and risk groups;
- ensure rapid and high levels of seroconversion;
- ensure the development of a long-term immune response by avoiding complex immunization regimens;
- reduce side effects;
- provide cross-protective immunity against several subtypes of TBE virus and induction of an effective $CD4^+$- and $CD8^+$-cell response [7].

In addition to the widely used approach of creating TBE vaccines based on inactivated virus, various experimental prophylactic vaccines based on different platforms are currently being developed [7].

Live attenuated vaccines

Live attenuated viruses that have lost their pathogenic properties at the genetic level, but contain the same antigens as the original pathogen and retain the ability to cause natural infection in the body in a weakened form, contribute to the formation of a pronounced and long-lasting B- and T-cell immunity, which is close to post-infection immunity in terms of intensity [46]. The first attempts to attenuate the TBE virus were not successful. Therefore, the Langat TR-21 virus, discovered in 1956 [7, 19], was considered as a more promising source of strains for live attenuated vaccines against TBE virus. However, a large study involving 650,000 volunteers showed that, in addition to induction of a high level of immune protection, the development of serious neurological consequences, including encephalitis, was often observed among those vaccinated with attenuated Langat virus [7]. Subsequent studies on the development of live-attenuated TBE vaccines have continued to improve their safety profile. Proteins such as C, E and NS5 were selected as the main targets for phenotype attenuation, which eventually led to several candidate vaccines with low reactogenicity and high levels of antibody production and T-cell response [47–49].

Work is actively underway to produce chimeric viruses combining fragments of the genomes of the TBE virus, most often the genes of the E and prM proteins, as well as West Nile, dengue, and Langat viruses [7, 50].

Although vaccines based on live attenuated viruses raise serious safety concerns, several vaccines have already been licensed worldwide against infections caused by other flaviviruses: yellow fever (YFV-17D), Japanese encephalitis (IMOJEV), and dengue fever (Dengvaxia). Two quadrivalent live attenuated dengue fever vaccines produced by Takeda Pharmaceutical and NIH/Butantan have successfully completed Phase III clinical trials [50].

Subunit vaccines

Compared to live attenuated vaccines, the production and use of subunit vaccines is characterized by safety due to the possibility of including individual antigenic components in the form of viral proteins or their

DOI: https://doi.org/10.36233/0372-9311-553

fragments in the vaccine. However, subunit vaccines induce mainly only a humoral immune response and a limited range of T-cell responses. They are unable to induce a prolonged immune response and therefore require the inclusion of adjuvants and booster immunizations.

Many potential vaccines being developed against TBE virus and infections caused by other flaviviruses have been based on structural protein E or its subunits containing epitopes recognized by neutralizing antibodies [7]. It was shown that immunization of mice with recombinant EDIII-domain of protein E in combination with various adjuvants allows to achieve not only induction of neutralizing antibodies, but also partial protection from virus infection [51]. Subunit vaccines against Dengue fever (V180) and West Nile fever (WN-80E), which contain truncated forms of protein E with adjuvants, have shown significant success in clinical trials [50].

Virus-like particles

Virus-like particles (VLPs) are formed as a result of simultaneous synthesis of structural proteins, most often prM/E, in different expression systems. The structure of VLPs is close to the native structure of the TBE virion, which allows the maximum number of T- and B-cell epitopes to be presented to immunocompetent cells. Such vaccines are characterized by the absence of potential pathogenic properties and a high level of safety [50]. VLPs administration is accompanied by the induction of a high titer of virus-neutralizing antibodies, activation of $CD4^+$ T-cells, and formation of central and effector memory T cells [7]. In one of the studies on the immunogenic properties of VLPs in a mouse model, it was shown that VLPs immunization promotes differentiation of $CD4^+$ T-cells along the Th2-pathway with a predominance of IL-4^+ phenotype [52]. A similar study indirectly confirmed this result. After VLPs administration to mice, a robust humoral immune response was observed; however, analysis of $CD4^+$ T-cells for IFN-γ, IL-2, and TNF-α showed no significant difference between experimental and control groups [53].

Vaccines based on viral vectors

Vaccines against TBE virus and diseases caused by related viruses are also being developed using an approach that has proven effective against other infections — viral vector-based vaccines. Such vaccines are recombinant or modified viruses encoding specific antigens and characterized by the ability or inability to replicate after introduction into the body. The main advantage of vaccines based on viral vectors is their high immunogenicity due to the intracellular expression of antigens and the presence of the viral vector itself, which can play the role of a natural adjuvant [50].

However, as with live attenuated vaccines, viral vector-based approaches, especially those capable of replication, raise questions about the safety of their use due to the increased risks of high viremia and the potential for acquisition of pathogenic properties. When viral vector-based vaccines are administered, an anti-vector immune response is formed, which reduces the efficacy of the vaccines in re-immunization. The disadvantages of such vaccines should also include complexity and costliness of their production [50].

Vectors based on such viruses as recombinant influenza A virus, recombinant adenovirus, modified Vaccinia virus, etc. are used in works on the development of experimental vaccines against flavivirus infections. [7]. Various combinations of TBE virus antigens, which are encoded in the genome of the viral carrier, make it possible to modulate the immune response if necessary. Thus, a number of studies have shown that various viral vectors encoding NS1 sequences induce the synthesis of virus-neutralizing antibodies and also provide partial protection against TBE virus [54]. At the same time, such vaccines can also activate the T-cell mediated immunity by inducing the formation of IFN-γ, IL-2 and TNF-α producing $CD4^+$- and $CD8^+$-T-lymphocytes.. A modified Vaccinia virus Ankara encoding the TBE virus prM and E protein sequence, when administered to mice, also induced high levels of virus-neutralizing antibodies and specific T-cell response, and provided complete protection against virus infection [55]. Similar results were obtained in a study investigating the properties of a candidate vaccine against Zika fever based on recombinant vesicular stomatitis virus encoding prM, E and NS1 proteins [56].

Despite the effectiveness of using viral vectors as a platform for vaccine development, only one vaccine has reached the clinical trial stage to date. MV-ZIKV against Zika fever has been developed on the platform of the Schwarz strain of measles virus and is in Phase I clinical trials [57].

mRNA and DNA vaccines

Recently, nucleic acid-based platforms, such as DNA- and mRNA-based vaccines, have been actively developed. The intracellular expression of antigens encoded by nucleic acid-based vaccines allows for the native structure of proteins due to posttranslational modifications [58]. This is important for further processing of the antigen, its presentation on the surface of immune cells, and activation of both $CD4^+$- and $CD8^+$ T cells.

The technology of production of such vaccines does not require complex manipulations or work with dangerous pathogens, which greatly facilitates the process of their creation and reduces its overall cost [58]. Furthermore, the use of such vaccines is believed to be safer compared to traditional approaches [59].

However, it should be noted that nucleic acid-based vaccines in their naked form have low immunogenicity; therefore, various delivery methods to immunocompetent cells, both chemical and physical, are used to improve their efficacy [50].

Several studies on obtaining experimental vaccines against TBE virus based on nucleic acids have been published. An experimental vaccine was obtained based on self-replicating non-infectious RNA of TBE virus containing several deletions in the C-protein gene region and point mutations in the prM gene region, but without loss of replicative function. The resulting mRNA vaccine effectively induced not only humoral but also cellular response, activating $CD8^+$ cells as well as the response of Th1-type $CD4^+$ T cells [60–62].

DNA vaccines have an advantage over mRNA vaccines due to their greater stability and less demanding storage conditions. The works of Y. Omori-Urabe et al. [63] and a group of researchers from University of Vienna (Austria) [64] described DNA constructs in the form of plasmid and viral vectors encoding E and prM proteins. Immunization with these constructs induced a strong immune response and a high level of virus-neutralizing antibodies. As a rule, the Th1 pathway of $CD4^+$-cell differentiation accompanied by the production of IFN-γ, TNF-α and IL-2 was observed during the administration of such vaccines; however, some variability in the shift of the Th1/Th2 ratio was demonstrated depending on the use of certain delivery methods [64].

Several experimental mRNA and DNA vaccines against other flavivirus infections (caused by dengue, Zika, and West Nile viruses) are also currently in clinical trials, and many others are being considered in preclinical studies [50].

Polyepitope vaccines

This vaccine platform specializes in the design of specifically T-cell immunogens and relies on two main strategies. The first polyepitope strategy is based on the design of artificial genes, delivered either by plasmid DNA or mRNA, or by a viral vector, encoding chains of $CD4^+$ and $CD8^+$ epitopes of various virus proteins, linked by linkers, lined up into a single artificial vaccine construct. This strategy gives the investigator the freedom to choose epitopes, which provides a narrower focus of responses on preferred epitopes [65]. Current knowledge of the mechanisms of $CD4^+$- and $CD8^+$-response formation to a productive viral infection allows us to develop algorithms for optimal selection of T-cell epitopes of the target pathogen, taking into account the peculiarities of the major histocompatibility complex (MHC) of a particular genotype. Currently, there are epitope databases such as the Immune Epitope Database [66], programs have been developed to predict T-cell epitopes in various viral proteins, and programs for rational vaccine design, such as PolyCTLDesigner [67].

The second strategy is to construct chimeric immunogens created from longer stretches of proteins covering the most conserved regions of viral proteins where T-cell epitopes are concentrated [65]. Bioinformatic approaches that are used in optimizing epitope compounds for polyepitope vaccines are also used in the design of conserved chimeric polyepitope proteins.

In the last three years, hundreds of papers have been published on the design of polyepitope immunogens for flaviviruses (Zika [68], dengue [69], Powassan [70, 71], and yellow fever [72] viruses), as well as SARS-CoV-2 [73–77], Ebola virus [78–80], Marburg virus [81], influenza [82–85], and others. The immunogenicity of polyepitope HIV-1 vaccines has been evaluated in clinical trials [86, 87].

D.N. Kisakov et al. described an experimental DNA TBE vaccine encoding an artificial polyepitope immunogen of the TBE virus [88]. The immunogen included predicted epitopes from the major proteins of the TBE virus (NS1, NS3, NS5, and E) restricted by the most common human allomorphs of HLA type I molecules and allelic variants of MHC type I molecules characteristic of BALB/c mice. Administration of this vaccine induces the formation of a protective virus-specific T-cell response in mice and provides 50% protection of immunised animals against infection with 100 LD50 of TBE virus (strain 205) [88].

Immunogens designed using computerized methods of T-cell epitope prediction and rational design of polyepitope antigens can become the basis for new effective methods of immunoprophylaxis of infectious diseases. They can be used to design both “universal” antigenic constructs covering a significant part of the target human population and personalized constructs tailored to the genetic features of a particular patient (taking into account his/her repertoire of allelic variants of class I and/or II MHC molecules).

With regard to vaccines against TBE virus the optimal way to improve vaccine efficacy may be an integrated strategy that combines the use of two immunogens in a prime-boost system, one of which induces virus-neutralizing antibodies (e.g., traditional inactivated vaccine) and the other induces T-cell responses (polyepitope immunogen).

Conclusion

With the accumulation of data on the peculiarities of the adaptive immune response in TBE, the role of the T-cell response in protective immunity during infection and vaccination, as well as its influence on the outcome of the disease, is becoming clearer. T-cell responses following natural infection with TBE virus and after vaccination with inactivated virus are different. During viral infection, both Th1- and Th2-type $CD4^+$ T cells and $CD8^+$ T cells are activated and play an important role in the elimination of viral infection. The absence of non-structural proteins of the TBE virus carrying the main epitopes of $CD8^+$ T-lymphocytes in the composition of inactivated vaccines leads to the activation of only a part of the T-cell immune response represented by $CD4^+$ T-cells of Th2-type, which mainly provide support for the B-cell response. Thus, the incomplete-

DOI: https://doi.org/10.36233/0372-9311-553

ness of the T-cell immune response occurring after vaccination with classical vaccines leads to reduced functionality of memory cells, which may underlie the short duration of the protective response to the vaccine.

Several questions regarding the T-cell response remain unclear, including the role of $CD8^+$ in the development of the pathologic process during infection. Nevertheless, many researchers conclude that a high level of virus-neutralizing antibodies combined with a T-cell response, including the response of specific $CD8^+$ T-cells, is a prerequisite for limiting the entry of TBE into CNS organs and mitigating immune pathology.

Attention to the T-cell response continues to grow also due to the need to improve classical inactivated vaccines against TBE virus. Research into next-generation vaccines is focused on finding a strategy that provides a balanced humoral and T-cell immune response. To date, a number of different types of experimental TBE vaccines are being studied, such as live-attenuated vaccines, viral vector vaccines, subunit vaccines, virus-like particles, DNA and mRNA vaccines, and polyepitope immunogens. In our opinion, the most promising in terms of T-cell response activation are vaccines based on T-cell polyepitope immunogens delivered in the form of DNA or mRNA. The optimal way to improve vaccine efficacy may be an integrated strategy that combines the use of two immunogens in a prime-boost system, one of which induces virus-neutralizing antibodies and the other induces T-cell responses.

The development of a safe, effective TBE vaccine that provides balanced T- and B-cell immunity will be a major advance in the fight against TBE virus.

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The article was submitted 14.06.2024;
accepted for publication 10.08.2024;
published 29.08.2024

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Статья поступила в редакцию 14.06.2024;
принята к публикации 10.08.2024;
опубликована 29.08.2024

Научный обзор
https://doi.org/10.36233/0372-9311-512

Оценка современного состояния фармацевтической разработки противостафилококковых профилактических препаратов

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Аннотация

Инфекция, вызванная *Staphylococcus aureus,* является самой распространённой, приводящей к развитию серьёзных осложнений у человека. *S. aureus* относится к высоколетальным патогенам при бактериемии со смертностью примерно 18% в благополучных странах и до 27% — в развивающихся.

Одним из самых поразительных и сложных аспектов клинических проявлений, вызванных *S. aureus*, считается способность бактерии вырабатывать устойчивость к антибиотикам. Своевременной необходимостью является разработка альтернативных способов лечения стафилококковой инфекции. Перспективным направлением следует рассматривать применение иммунотерапии и иммунопрофилактики для активации противоинфекционного иммунного ответа у пациентов.

Цель обзора — анализ основных тенденций в разработке вакцин, направленных на профилактику инфекций, вызываемых *S. aureus,* и факторов вирулентности *S. aureus*.

В обзоре рассмотрены проводимые в последние годы разработки лекарственных препаратов, направленные на профилактику и лечение инфекций, вызываемых *S. aureus*. Особое внимание уделяется факторам патогенности (капсула, поверхностные белки и ферменты), которые могут быть полезны для создания новых вакцин-кандидатов или иммунных терапевтических средств. За последние годы проведение многочисленных клинических исследований кандидатов-вакцин, созданных на основе различных антигенов, с учётом особо значимых факторов патогенности стафилококка, оказывающих влияние на заболеваемость, не увенчались успехом из-за их низкой эффективности или недостаточно обоснованной безопасности (развитие нежелательных явлений). Одним из важнейших факторов, сдерживающих разработку вакцины, является отсутствие успешной трансляции протективности вакцины, которая наблюдается в доклинических исследованиях на экспериментальных моделях, но не подтверждается в клинических исследованиях.

Таким образом, по мнению многочисленных исследователей, необходимо рассматривать использование в составе вакцин несколько антигенов, сосредоточив внимание на различных механизмах патогенности *S. aureus*, включая использование адъювантов.

Ключевые слова: *противостафилококковые вакцины, профилактика, стафилококковые инфекции*

Источник финансирования. Работа выполнена в рамках государственного задания ФГБУ «Научный центр экспертизы средств медицинского применения» Минздрава России № 056-00026-24-00 на проведение прикладных научных исследований (№ гос. учёта НИР 124022200103-5).

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

Для цитирования: Буйлова И.А., Савкина М.В., Саяпина Л.В., Кривых М.А., Обухов Ю.И. Оценка современного состояния фармацевтической разработки противостафилококковых иммунопрофилактических и иммунотерапевтических лекарственных препаратов и совершенствование методологических подходов к их экспертизе. *Журнал микробиологии, эпидемиологии и иммунобиологии*. 2024;101(4):560–572.
DOI: https://doi.org/10.36233/0372-9311-512
EDN: https://www.elibrary.ru/feqcvb

Review
https://doi.org/10.36233/0372-9311-512

Assessment of the current state of pharmaceutical development of anti-staphylococcal prophylactic drugs

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Abstract

Infection caused by *Staphylococcus aureus* is the most common infection leading to the development of serious complications in humans. *S. aureus* is among the highly lethal bacteremia-associated pathogens with a mortality rate of approximately 18% in industrial countries; in developing countries, the rate is even higher, up to 27%.

One of the most striking and challenging aspects of clinical manifestations caused by *S. aureus* is the ability of the bacterium to develop resistance to antibiotics. The development of alternative treatment options for staphylococcal infection is urgently needed. The use of immunotherapy and immunoprophylaxis to activate the anti-infection immune response in patients should be considered as a promising direction.

Objective: to analyze the main trends in the development of vaccines aimed at the prevention of *S. aureus* infection and its virulence factors.

The present review discusses vaccine development in recent years aimed at preventing infection caused by *S. aureus*. Particular attention is paid to pathogenicity factors (such as capsule, surface proteins and enzymes) that may be useful for the development of new candidate vaccines or immune therapeutics. In recent years, numerous clinical trials of candidate vaccines based on different antigens, taking into account particularly relevant *S. aureus* pathogenicity factors that influence morbidity, have not been successful due to their low efficacy or insufficiently substantiated safety (development of adverse events). One of the most important factors constraining vaccine development is the lack of successful translation of vaccine protective activity, which is observed in preclinical studies in experimental models but not confirmed in clinical trials.

Therefore, according to numerous researchers, the use of multiple antigens in vaccine formulations should be considered with the focus on different mechanisms of *S. aureus* pathogenicity and the use of adjuvants.

Keywords: *anti-staphylococcal vaccines, prophylaxis, staphylococcal infections*

Funding source. The work was carried out within the framework of the state assignment of the Scientific Center expertise of Medicinal Products No. 056-00026-24-00 for applied scientific research (state registration number 124022200103-5).

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Builova I.A., Savkina M.V., Sayapina L.V., Krivykh M.A., Obukhov Yu.I. Assessment of the current state of pharmaceutical development of antistaphylococcal immunoprophylactic and immunotherapeutic drugs and improvement of methodological approaches to their expertise. *Journal of microbiology, epidemiology and immunobiology.* 2024;101(4):560–572.
DOI: https://doi.org/10.36233/0372-9311-512
EDN: https://www.elibrary.ru/feqcvb

Цель данного обзора — анализ основных тенденций по разработке вакцин, направленных на профилактику инфекций, вызываемых *Staphylococcus aureus*.

Задачи: оценка современных мировых исследований по разработке лекарственных препаратов, направленных на и лечение профилактику инфекций, вызываемых *S. aureus*, и перспективы их развития.

Инфекция, вызываемая *S. aureus,* является самой распространённой, приводит к развитию серьёзных осложнений у человека. При этом определённые группы людей, в том числе лица, получающие гемодиализ, пациенты с диабетом, имеющие сердечно-сосудистые или другие сопутствующие заболевания, подвержены более высокому риску развития осложнений при инфицировании бактериями или продукцией токсинов, например, при пищевом отравлении и синдроме токсического шока [1]. *S. aureus* принадлежит к семейству *Micrococcaceae* и представляет собой грамположительные кокки, расположенные в виноградоподобных кластерах. Методы дифференциации *S. aureus* от других видов стафилококков основаны на определении золотой пигментации колоний и положительных тестов на коагулазу, ферментацию маннита и дезоксирибонуклеазы [2].

S. aureus является комменсальной бактерией, при этом назальное носительство в популяции человека составляет более 30% [1]. *S. aureus* является вездесущим для человека патогеном, наиболее часто вызывающим инфекции кожи, мягких тканей, эндокардит, и становится основной причиной возникновения внутрибольничных инфекций, в том числе вентилятор-ассоциированной пневмонии, внутривенных катетер-ассоциированных инфекций, послеоперационных раневых инфекций, а также инвазивных инфекций у пациентов с иммуносупрессией [3–9].

S. aureus относится к высоколетальным патогенам при бактериемии со смертностью примерно 18% в благополучных странах и до 27% — в развивающихся [10, 11]. Инфекции, вызванные *S. aureus*, в настоящее время считаются наиболее частой причиной госпитализации для хирургического дренирования гноя у детей и бактериемии у лиц старше 65 лет, а также серьёзным осложнением инфицирования протезов и внутрисосудистых катетеров [1].

Одним из самых поразительных и сложных аспектов клинических проявлений, вызванных *S. aureus*, является способность бактерии вырабатывать устойчивость к антибиотикам. Этот эффект был продемонстрирован во время появления мети-

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-512

циллин-резистентного *S. aureus* (MRSA) в 1960-х гг. В последние годы выявляются штаммы, проявляющие умеренную, а в редких случаях полную устойчивость к ванкомицину (VRSA) — одному из основных препаратов, используемых для лечения инфекции, вызванной MRSA [10].

Средняя доля штаммов MRSA в Европейском союзе значительно различается между странами: от менее 1% в Дании, Исландии, Норвегии и Швеции до более 25% в других странах [1]. При этом MRSA часто вызывает развитие внутрибольничных инфекций во многих странах мира. Высокие показатели (> 50%) штаммов MRSA были зарегистрированы в Азии, на Мальте, в Северной и Южной Америке в начале 2010-х гг. [11]. В Азии наблюдается наибольшая распространённость внутрибольничного и внебольничного MRSA в мире.

Согласно докладу Всемирной организации здравоохранения по отчётам, представленным из 76 стран, медиана метициллин-резистентного *S. aureus*, вызывающего бактериемию и, как следствие, инфицирование различных органов человека, составляет 35% [13].

На основании вышеизложенного следует, что необходима разработка альтернативных способов лечения стафилококковой инфекции. Как перспективное следует рассматривать применение иммунотерапии и иммунопрофилактики для активации противоинфекционного иммунного ответа у пациентов. За последние два десятилетия научными сообществами была проделана значительная работа по созданию противостафилококковых вакцин, и тем не менее ни один кандидат на вакцину не доказал свою эффективность во время клинических испытаний [10].

В настоящее время в разработке вакцин против *S. aureus* широко используются современные инновационные подходы, направленные на совершенствование технологических процессов, улучшение параметров производимых продуктов, способных вызывать развитие гуморальных и клеточных реакций врождённого и приобретённого иммунитета [10].

S. aureus имеет несколько факторов патогенности, ориентированных на подавление ключевых компонентов иммунной системы. В многочисленных исследованиях показано, что *S. aureus* обладает способностью к колонизации, а именно: прикрепление к тканям хозяина и размножения, что, в свою очередь, приводит к включению неспецифических механизмов защиты [10].

В представленном обзоре рассматриваются проводимые в последние годы разработки вакцин, направленные на профилактику инфекций, вызываемых *S. aureus*. Особое внимание уделяется факторам патогенности, которые могут быть полезны для создания новых вакцин-кандидатов или иммунных терапевтических средств.

Факторы патогенности *S. aureus*, имеющие значение при разработке вакцин

Различные компоненты *S. aureus*, такие как капсула, поверхностные белки и ферменты, являются возможными мишенями для использования их в качестве основы для новых вакцин, способных обеспечить защиту людей от инфекций [14, 15]. Рассмотрим основные факторы патогенности *S. aureus* и приведём примеры их применения для разработки вакцин-кандидатов.

Капсулы

Обнаружение капсулы у *S. aureus* было впервые описано I. Gilbert в 1931 г. Роль капсулы заключается в защите бактерий от распознавания фагоцитирующими клетками, делая их устойчивыми к фагоцитозу [16, 17]. Примерно 90% выделенных клинических изолятов продуцируют капсульные полисахариды. Определены 11 серотипов инкапсулированных штаммов, из которых наиболее часто встречаются 5-й и 8-й серотипы. Капсульный полисахарид (CPS) представляет собой высокомолекулярные углеводные полимеры, состоящие из N-ацетил-D-фукозамина, N-ацетил-L-фукозамина и N-ацетил-D-манносаминуроновой кислоты. При этом 5-й и 8-й серотипы различаются между собой только связями между сахарами и сайтами О-ацетилирования остатков маннозаминуроновой кислоты [17, 18].

Капсульные антигены являются одними из первых целевых антигенов, использующихся в качестве основы в исследованиях при разработке вакцин, предназначенных для защиты от стафилококковой инфекции. Механизмы защиты капсульными прототипами вакцин обусловлены содействием патогену посредством опсонофагоцитоза. При разработке вакцин нового поколения учитывались ранее полученные результаты, которые показали, что очищенные полисахариды, ковалентно связанные с молекулами белка-носителя, приводят к увеличению уровня антител и активации Т-клеток, способных выполнять эффекторную роль, участвовать в распознавании антигенов и индуцировать иммунные реакции, при этом уровень антител сохраняется и остаётся стабильным [1, 19–21].

Белок А

Стафилококковый белок А (SpA), находящийся в оболочке клеточной стенки *S. aureus*, связывает Fcγ-домен иммуноглобулина (Ig) и сшивает Fab-домен В-клеточных рецепторов VH3-типа (IgM), также был привлекательным в качестве протективного антигена для нового прототипа вакцины [1]. Известно, что SpA, блокируя опсонофагоцитоз, предотвращает активацию компонентов системы комплемента, других медиаторов и клеток

иммунной системы хозяина, защищает *S. aureus* от разрушения/гибели.

F. Falugi и соавт. провели исследования на мышах с использованием в качестве вакцины-кандидата SpA против инфекции, вызванной *S. aureus*. Полученные результаты показали, что секретируемые продукты не способствуют связыванию SpA с Ig для противодействия фагоцитозу и перекрестному связыванию рецепторов B-клеток, опосредованных SpA для блокировки выработки антител у мышей [22], и эти субъединичные вакцины не были рекомендованы для дальнейших исследований. Предполагается, что экспрессия SpA у *S. aureus* и его связывание с Ig препятствуют ответу B-клеток при инфекции, тем самым подавляя развитие специфического иммунитета. Однако нарушение вирулентности стафилококка посредством мутаций в SpA, иммунизации нетоксигенным SpA или введения моноклональных антител, нейтрализующих SpA, может вызывать у мышей защитные антитела против высоковирулентных штаммов MRSA [22].

Адгезины

Значительная роль в ряду факторов патогенности во взаимодействии между *S. aureus* и клетками-хозяина отводится адгезинам, которые обеспечивают способность *S. aureus* прикрепляться к различным клеткам и веществам макроорганизма, таким как внеклеточный матрикс и белки плазмы. Наиболее охарактеризованными поверхностными адгезинами, ковалентно связанными с пептидогликановой клеточной стенкой из семейства белков MSCRAMM, являются белки ClfA и B, Cna, IsdA, B и H, FnBPA и B, SdrC, D и E [23–25].

Одним из вариантов создания прототипов вакцин в качестве антигенов исследователи рассматривают белок ClfA, являющийся антифагоцитарным, защищающим бактерии от опсонофагоцитоза, что подтверждает фактор патогенности в некоторых моделях развития инфекций, включая эндокардит, сепсис и септический артрит [26, 27].

Проводились работы по созданию прототипов вакцин против *S. aureus* с использованием различных кандидатов в антигены, в том числе продуцирующих *S. aureus* двух близкородственных фибронектин-связывающих белков (FnBP): FnBPA и FnBPB, которые участвуют в патогенезе инфекции *S. aureus*, способствуя прикреплению бактерий к клеткам-хозяевам. В экспериментальных работах C. Heilmann и соавт. установили, что значительную роль в индукции эндокардита, вызванного *S. aureus*, играет белок FnBPA, обладающий способностью прикрепляться к тромбоцитам и вызывать их агрегацию [28, 29]. Коллагеновый адгезин (Cna) представляет собой белок, отвечающий за связывание с несколькими типами коллагена. Cna является фактором вирулентности при септическом артрите и остеомиелите, который опосредует бактериальную колонизацию хрящей и костей. Проведёнными испытаниями установлено, что мыши, иммунизированные антигеном Cna-FnBP, выживали после заражения *S. aureus* значительно дольше, чем неиммунизированные мыши [30].

Как привлекательного кандидата в протективные антигены некоторые учёные рассматривают консервативный транспортный белок марганца C (MntC), являющийся высококонсервативным белком среди штаммов MRSA и VRSA, способный связывать каскад свертывания крови, в том числе плазминогена, посредством лизина, с различным внеклеточным матриксом. Полученные результаты при моделировании инфекции *S. aureus* у лабораторных животных показали, что введение белка MntC обеспечивает развитие иммунитета, защищающего мышей от инфекции *S. aureus*, за счёт значительного повышения уровня IgG в сыворотке крови при непосредственном участии T-иммунокомпетентных клеток [1, 31, 32]. В настоящее время MntC является компонентом вакцины SA4Ag, проходящей II фазу клинических испытаний [32].

Токсины

При изучении влияния различных факторов патогенности, используемых в качестве перспективных кандидатов при разработке вакцин, установлено, что значительная роль в данном направлении отводится токсинам *S. aureus,* обладающим гемолитическими, цитотоксическими и цитолитическими свойствами, а также способствующим успешной инвазии и размножению бактерий в организме хозяина [33]. Среди токсинов следует выделить 2 семейства: порообразующие токсины и суперантигены, из которых наиболее значимыми представителями, являются суперантигены [34–38].

Суперантигены представляют собой разнообразную группу белковых экзотоксинов, относящихся к наиболее мощным митогенам T-клеток. Они действуют путём перекрёстного связывания между MHC-II и β-цепью T-клеточного рецептора, индуцируют активацию антигенпрезентирующих клеток и T-лимфоцитов, что приводит к высвобождению большого количества провоспалительных цитокинов. К настоящему времени были идентифицированы различные типы стафилококковых энтеротоксинов, включая следующие варианты белков: A–E, G–J и R–T (SEA-SEE, SEG-SEJ, SER-SET), tSE-подобные токсины K–Q и U–X (SElK-SElQ, SElU-SElX) и TSST-1 [1, 39, 40].

Особого внимания заслуживают эксфолиативные токсины *S. aureus* ETA, ETB и ETD способные расщеплять десмосомальный кадгерин десмоглеин 1, опосредующий межклеточную адгезию в поверхностном слое кожи, приводя к стафилококковому синдрому обожжённой кожи. Данные суперанти-

DOI: https://doi.org/10.36233/0372-9311-512

гены обладают уникальными свойствами, которые активируют пролиферацию Т-клеток [1, 41].

Ферменты

Ферменты способны поставлять микробной клетке питательные субстраты и обеспечивать защиту от действия факторов иммунной системы. *S. aureus* может экспрессировать протеазы, липазу, дезоксирибонуклеазу и фермент, модифицирующий жирные кислоты. В нескольких исследованиях *in vitro* показано, что ферменты являются важными факторами патогенности *S. aureus*, который из-за диффузии различных факторов (фагоцитоз, цитокины, фактор некроза опухоли-α и др.) внутри организма хозяина изменяет свой фенотип с адгезивного на инвазивный [1].

Важным фактором патогенности при стафилококковых инфекциях является коагулаза (белок), наиболее известная своей способностью индуцировать свёртывание крови путём активации протромбина [42].

Разработка противостафилококковых вакцин-кандидатов

За последние годы проведение многочисленных клинических исследований кандидатов-вакцин, созданных на основе различных антигенов с учётом особо значимых факторов патогенности *S. aureus*, оказывающих влияние на заболеваемость, не увенчалось успехом из-за низкой их эффективности или недостаточно обоснованной безопасности (развитие нежелательных явлений). Как перспективные направления в разработке вакцин против *S. aureus*, по мнению исследователей, необходимо рассматривать использование нескольких антигенов, включая адъюванты, сосредоточив внимание на различных механизмах патогенности *S. aureus*.

В **таблице** приведены наименования созданных потенциальных кандидатов в вакцины, исследования которых к настоящему времени или проходят различные фазы клинических исследований или из-за недостаточности подтверждения эффективности и безопасности были прекращены.

Одним из наиболее перспективных кандидатов в вакцины является бивалентная полисахаридная вакцина **StaphVAX**, разработанная компанией «Nabi Biopharmaceuticals». Потенциальная вакцина включает два наиболее распространённых капсульных полисахарида — CP5 и CP8, способствующие развитию около 80% внутрибольничных инфекций, вызванных *S. aureus*, конъюгированных с детоксицированной формой экзотоксина А *Pseudomonas aeruginosa*. Клинические исследования фазы II вакцины-кандидата бивалентной полисахаридной StaphVAX проводили у пациентов с хронической почечной недостаточностью, получающих амбулаторный перитонеальный диализ. Полученные результаты свидетельствовали о её эффективности, т. к. введение StaphVAX обеспечивало развитие противостафилококкового иммунного ответа у пациентов после вакцинации и было безопасным [1]. Фазу III клинических исследований кандидата StaphVAX проводили у пациентов, которым предстояла операция через 3–54 нед после вакцинации. Однако во время фазы III (через 40 нед) клинических исследований наблюдали снижение уровня сывороточных антител у вакцинированных пациентов. В то же время в рамках исследования в определённые периоды были обнаружены и положительные явления, характеризующиеся частичным снижением бактериемии, вызванной *S. aureus*. Достоверных различий в количестве смертей в вакцинированных и контрольных группах не выявлено [17, 43, 44]. Несмотря на то, что при исследовании в фазе III были получены положительные результаты, для регистрации в США Управление по контролю качества пищевых продуктов и лекарственных средств рекомендовало проведение второго исследования фазы III. Результаты этого исследования показали, что StaphVAX снижает бактериемию, вызванную *S. aureus*, на 64% через 32 нед наблюдения, на 57% — через 40 нед, на 26% — через 54 нед. Таким образом, после анализа полученных результатов по эффективности и безопасности выявлено достаточно быстрое снижение титра антител, начиная с 32-й недели после вакцинации, что послужило основанием не рекомендовать кандидата в вакцину StaphVAX для регистрации и применения в медицинской практике [19, 45, 46].

Разработанная компаниями «Merck» и «Intercell» вакцина-кандидат **V710**, содержащая поверхностную детерминанту железа B (IsdB), представляет собой высококонсервативный поверхностный белок *S. aureus*. В доклинических исследованиях кандидата V710 показано развитие протективного иммунитета при моделировании заражения *S. aureus* у лабораторных животных. Положительные результаты доклинических исследований кандидата послужили основанием для проведения дальнейших исследований. Клинические исследования осуществлялись в 2007–2011 гг. В 2011 г. для оценки эффективности и безопасности препарата проводили клинические исследования фазы IIb/III у вакцинированных пациентов перед проведением кардиоторакальных операций. На основании полученных результатов установлено, что среди пациентов со срединной стернотомией введение препарата V710 не снижало частоту серьёзных послеоперационных инфекций, вызванных *S. aureus*, по сравнению с плацебо, что приводило к повышению риска смертности среди пациентов [47]. При этом установлено, что у вакцинированных пациентов, перенёсших хирургические операции, в сыворотке крови обнаруживали снижение уровня цитокинов —

Резюме клинических исследований различных антигенов вакцин-кандидатов против *S. aureus*
Summary of clinical studies of different candidate vaccine antigens against *S. aureus*

Вакцина кандидат Vaccine candidate	Антигены Antigens	Разработчик Developer	Клинические исследования Clinical trials	Адъювант Adjuvant	Ссылки References
StaphVAX	CP5 & CP8	«Nabi»	Провал фазы III Phase III failure	Отсутствует Absent	[19, 49, 50]
V710	IsdB	«Merck»	Провал фазы III Phase III Failure	Отсутствует Absent	[53]
SA75	Цельноклеточная вакцина Whole cell vaccine	«Vaccine Research International»	Фаза I Phase I	Отсутствует Absent	[20, 54]
SA4Ag	ClfA, MntC, CP5 & CP8	«Pfizer»	Фаза IIb Phase IIb	Отсутствует Absent	[19–21, 51, 52]
GSK2392103A	CP5, CP8, столбнячный анатоксин, мутантные формы α-гемолизина, ClfA CP5, CP8, tetanus anatoxin, mutant forms of alpha-hemolysin, ClfA	«GlaxoSmithKline»	Фаза I Phase I	AS03B	[53]
4C-Staph	Hla, FhuD2, Csa1A, EsxAB	«Novartis»	ДоКИ	TLR7-зависимый TLR7-dependent	[54–56]
1. STEBVAX 2. IBT-V02	1. SEB + Алюминий | Aluminium 2. SEB, SEA, TSST-1, LukS, LukF, LukAB, Hla + Алюминий | Aluminium	«Integrated BioTherapeutic»	Фаза I Phase I	Алгидрогель Alhydrogel	[1, 10, 57]
Pentastaph	StaphVax + тейхоевая кислота | teichoic acid, PVL (rLukS-PV/rAT), Hla	«GlaxoSmithKline»	Фаза I/II Phase I/II	Отсутствует Absent	[20, 59]
rFSAV	Hla, SpA, SEB, IsdB, MntC + Алюминий | Aluminium	«Olymvax»	Фаза II Phase II	Алюминий Aluminum	[60, 61]
S. aureus Toxoids	LukS-PV	Uniformed Services University of the Health Sciences	Фаза I Phase I	Алюминий Aluminum	[62, 63]

интерлейкина (ИЛ)-2 и ИЛ-17 и, как следствие, развитие осложнений, вызванных *S. aureus*, заканчивающиеся летальным исходом [19, 48]. Учитывая, что ИЛ-17 и ИЛ-2 играют решающую роль в задержке роста, размножении и гибели *S. aureus* в организме пациента, а введение препарата вызывает их снижение, приводящее к манифестации инфекционного заболевания, сделано заключение о прекращении проведения дальнейших клинических исследований препарата V710 из-за низкой эффективности и развития нежелательных реакций [49].

В 2006 г. компания «Vaccine Research International Plc» завершила I фазу клинических исследований вакцины-кандидата **SA75**, представляющей собой цельные клетки *S. aureus*, инактивированные хлороформом, предназначенной для профилактики внутрибольничных инфекций, вызванных *S. aureus*. По результатам исследований подтверждены эффективность и безопасность вакцины. Однако дальнейшие работы были приостановлены [1, 50].

Разработчиком кандидатной вакцины **SA4Ag**, состоящей из 4 антигенов: молекулы адгезии ClfA, переносчика марганца MntC и антифагоцитарных капсулярных полисахаридов CP5 и CP8, конъюгированных с белком CRM197, выступала компания «Pfizer». Вакцина SA4Ag показала хорошую эффективность против прогрессирующего развития инфекции *S. aureus* в исследованиях на животных. Иммунизация мышей комплексом антигенов SA4Ag резко снизила развитие у них бактериальной популяции при инфекции глубоких тканей, бактериемии и модели пиелонефрита. Однако благоприятные результаты доклинических исследований, полученные при введении препарата SA4Ag, в достаточной мере не смогли продемонстрировать его использование для предотвращения инвазивной инфекции *S. aureus*, связанной с хирургическим вмешательством [51]. Несмотря на то что вакцина-кандидат SA4Ag индуцировала сильные функциональные иммунные ответы на каждый антиген по сравнению с плацебо, она не показала эффективности в предотвращении послеоперационной инфекции, вызванной *S. aureus* (14 случаев в каждой группе до 90-го дня после операции) [52].

Вакцина-кандидат **GSK (GSK2392103A)** представляет собой четырёхкомпонентную стафилококковую вакцину, содержащую полисахариды 5 и 8,

DOI: https://doi.org/10.36233/0372-9311-512

конъюгированные со столбнячным анатоксином (TT) (CPS5-TT, CPS8-TT), мутантную форму гемолизина-1 (α-токсин; AT) и ClfA. Фаза I клинических исследований завершилась в 2012 г. Вакцина оказалась безопасной и вызывала гуморальные иммунные реакции после 1-й дозы вакцины[1]. При введении 88 здоровым добровольцам в возрасте 18–40 лет кандидата-вакцины CPS5-TT/CPS8-TT/AT/ClfA 5/5/10/10 мкг или 10/10/30/30 мкг дозы, через 0, 1, 6 мес концентрация антител у реципиентов резко возрастала к 14 сут после вакцинации [53].

Созданная компанией «Novartis» четырёхкомпонентная вакцина-кандидат **4C-Staph** включает 5 антигенов *S. aureus*: генетически детоксицированное производное секретируемого α-токсина или α-гемолизина (Hla), FhuD2 и Csa1A, а также EsxAB (слитый белок, содержащий EsxA и EsxB). Предложенный состав антигенов при введении мышам защищал их от инфекции *S. aureus* за счёт индукции специфических антител. Данный препарат 4C-Staph находится на стадии доклинических исследований. При проведении экспериментальных работ A. Torre и соавт. обнаружили, что введение 4C-Staph может компенсировать дефицит нейтрофилов у мышей с нейтропенией, активируя макрофаги и моноциты в очаге инфекции. Полученные результаты могут иметь важное значение в последующих исследованиях, направленных на разработку новых противостафилококковых вакцин [54–56].

Разработанный компанией «Integrated BioTherapeutic» прототип вакцины **STEBVAX** представляет собой рекомбинантную форму стафилококкового энтеротоксина B (SEB), содержащую 3 точечные мутации (*L45R*, *Y89A* и *Y94A*), которые блокируют взаимодействие токсина с человеческими рецепторами MHC класса II. В экспериментах на лабораторных животных иммунизация SEB защищала мышей не только от заражения энтеротоксином, но и от SEA, SEC1 (направленные стафиллококковые энтеротоксины типа A и C1) или TSST-1 (токсин синдрома токсического шока) [1]. В 2015 г. анализ результатов, полученных в фазе I клинических исследований[2], показал, что введение препарата индуцировало выработку специфических антител [57].

Компания «Integrated BioTherapeutics» в своё время также занималась разработкой 7-валентной вакцины-кандидата против *S. aureus*, состоящей из 7 анатоксинов *S. aureus*: Hla, F и S субъединицы лейкоцидина Пантона–Валентайна (PVL), лейкоцидина A/B, SEA, SEB и токсина синдрома токсического шока 1 [10]. Данные доклинических исследований показали, что препарат **IBT-V02**, созданный на основе анатоксинов, обеспечивает защиту мышей и кроликов от кожной инфекции, вызванной *S. aureus*. При этом защита полностью опосредована специфическими антителами, индуцированными кандидатом IBT-V02 [58]. Предварительные эксперименты на мышиной модели предоставляют обнадеживающую информацию для проведения последующих клинических исследований [10].

После провала StaphVAX компания «Nabi» в 2006 г. возобновила разработку модифицированной вакцины-кандидата **PentaStaph**, которая состояла из исходного состава StaphVax, тейхоевой кислоты, α-токсина и лейкоцидина PVL. После завершения фазы I клинических исследований вакцина PentaStaph была продана компании «GlaxoSmithKline Biologicals». В настоящее время препарат PentaStaph находится в фазе I/II клинических исследований [20, 59].

Безопасность, иммуногенность и эффективность вакцины-кандидата GSK против *S. aureus* (**GSK3878858A**) при введении здоровым взрослым и взрослым в возрасте от 18 до 64 лет с недавно перенесённой инфекцией кожи и мягких тканей, вызванной *S. aureus*, изучается в клиническом исследовании фазы I/II[3].

Компания «Olymvax» разработала противостафилококковый препарат **rFSAV**, в состав которого входят 5 рекомбинантных антигенов *S. aureus*: Hla, SEB, MntC, IsdB и SpA. При проведении клинических исследований в фазе II показана многообещающая эффективность, установленная в доклинических экспериментах на мышах [60]. В дополнение к стимулированию опсонофагоцитоза, сыворотки мышей, иммунизированных rFSAV, также нейтрализуют литическую активность Hla и предотвращают лёгкое истощение B-клеток селезёнки, наблюдаемое у мышей, опосредованных обработкой SpA [61]. Важно отметить, что вакцина представляет собой альтернативный аспект «иммуногенности» против *S. aureus*: стратегии ингибирования уклонения от иммунитета *S. aureus* (наличие Hla и SpA) [10].

Созданная Университетом здравоохранения унифицированных служб в сотрудничестве с «Nabi Biopharmaceutical» комбинация препарата ***S. aureus* Toxoids**, содержащая в составе α-гемолизин, LukS-PV и компонент лейкоцидина PVL, проходит клинические исследования фазы I. Антиген LukS-PV в сочетании с антигеном LukF-PV образуют порообразующий октамерный токсин, секретирующийся

[1] A Study to Evaluate the Safety, Reactogenicity and Immunogenicity of GSK Biologicals' Staphylococcal Investigational Vaccine in Healthy Adults. URL: https://clinicaltrials.gov/study/NCT01160172?term=GSK2392103A&rank=1

[2] Phase I STEBVax in Healthy Adults. URL: https://classic.clinicaltrials.gov/ct2/show/NCT00974935

[3] Safety, Immunogenicity and Efficacy of GSK S. Aureus Candidate Vaccine (GSK3878858A) When Administered to Healthy Adults (Dose-escalation) and to Adults 18 to 64 Years of Age With a Recent S. Aureus Skin and Soft Tissue Infection (SSTI). URL: https://classic.clinicaltrials.gov/ct2/show/NCT04420221

бактерией отдельно в виде мономеров. Молекула LukS-PV, связываясь со своим рецептором на клетке-мишени, способствует связыванию 4 молекул LukF-PV с эквивалентным количеством LukS-PV, что приводит к образованию октамерного комплекса. Токсин нацелен на полиморфноядерные фагоциты и моноциты [62, 63].

Минздрав России одобрил проведение клинических исследований препарата GSK3878858A (Sa-5Ag с адъювантом), защищающего от *S. aureus*, представленного компанией «GlaxoSmithKline». Вместе с тем, несмотря на полученное от Минздрава России разрешение на проведение клинических исследований, фаза II исследования препарата GSK не будет проводиться, т. к. компания сообщила, что отказывается от новых клинических исследований в России[4].

В России зарегистрирована **Вакцина стафилококковая лечебная** (Антифагин стафилококковый) для лечения стафилококковых инфекций (АО «Биомед»). Вакцина представляет собой комплекс пептидогликана и тейхоевых кислот, извлекаемый из микробных клеток водно-фенольной экстракцией.

В НИИ вакцин и сывороток им. И.И. Мечникова была разработана стафилококковая вакцина на основе комбинации протективных антигенов (пептидогликан, тейхоевые кислоты, белковые антигены клеточной стенки) из иммуногенных штаммов *S. aureus*, обладающих внутривидовой перекрёстной протективной активностью, а использование щадящего метода выделения антигенов (ацетон, водная экстракция) обеспечило сохранение иммуногенности. Предложенная вакцина, названная авторами **«Стафиловак»**, является активатором и стимулятором врождённого и адаптивного иммунитета. Показана защита от септической стафилококковой инфекции у мышей и кроликов. Вакцина в клинических исследованиях при включении в комплексную терапию хронических стафилококковых инфекций (пиодермия, фурункулёз и др.) оказывала длительный терапевтический эффект: снижала тяжесть обострений, значительно увеличивала период ремиссии, сокращала потребность в антибиотикотерапии, способствовала индукции интерферона-γ и антител [64–66].

Компания «Медгамал» (филиал НИЦЭМ им. Н.Ф. Гамалеи, Россия) зарегистрировала **Анатоксин стафилококковый очищенный** адсорбированный для профилактики инфекций *S. aureus* у лиц с повышенным риском заболевания, а именно: промышленные и сельскохозяйственные рабочие, подвергающиеся по роду своей деятельности частому травматизму, а также у больных, которым предстоят плановые операции[5].

S. aureus является одним из наиболее значимых патогенов для человека. Высокий профиль устойчивости *S. aureus* к антибиотикам вызывает необходимость поиска новых способов борьбы, в том числе разработки вакцин, в дополнение к исследованиям, направленным на разработку новых антибиотиков. Вместе с тем многолетние попытки зарубежных исследователей создать комбинированные вакцины против инфекции *S. aureus* не увенчались успехом. Некоторые кандидаты в вакцины, разработанные на основе различных антигенов *S. aureus*, были забракованы уже на стадии доклинических исследований. При этом многие прототипы вакцин успешно проходили дальнейшие исследования, часть из них успешно прошли фазу II, но из-за низкой эффективности или недоказанной безопасности на фазе III завершали свой путь из-за ряда обстоятельств и выявленных несоответствий заявленному применению.

В настоящее время исследования сосредоточены на выявлении новых составов вакцин, способных вызывать мощные гуморальные и клеточные иммунные реакции. Трансляционные научные исследования пытаются обнаружить корреляты защиты с использованием животных моделей, а также моделей *in vitro* и *ex vivo*, оценивающих эффективность вакцин-кандидатов. Многими учёными показано, что развитие исследований, направленных на поиски протективных компонентов, в частности среди поверхностных и секретируемых *S. aureus* белков, требует использования экспериментальных моделей, позволяющих определить белки, играющие большую роль в патогенезе инфекции [30].

Одним из важнейших факторов, сдерживающих разработку вакцины, является отсутствие успешной трансляции протективности вакцины, которая наблюдается в доклинических исследованиях на экспериментальных моделях, но не подтверждается в клинических исследованиях [10].

В случае вакцины-кандидата SA4Ag было показано, что индуцированный вакциной гуморальный иммунитет является антигенспецифическим по природе и способен индуцировать бактериальный опсонофагоцитоз. Опсонофагоцитарные гуморальные ответы были также продемонстрированы для вакцин-кандидатов V710 и StaphVAX, которые оказались неэффективными на поздних стадиях клинических исследований [10].

Таким образом, возрастает понимание того, что использование опсонофагоцитоза в качестве индикатора антистафилококковой иммуногенности

[4] GSK не будет проводить КИ вакцины от золотистого стафилококка // Фармацевтический вестник. 01.07.2022. URL: https://pharmvestnik.ru/content/news/GSK-otkazalas-provodit-KI-vakciny-ot-zolotistogo-stafilokokka.html

[5] Анатоксин стафилококковый очищенный. URL: https://medgamal.ru/products/anatoxin

недостаточно для одного определения эффективности противостафилококковой вакцины. В связи с этим следует заключить, что ни одна из вакцин-кандидатов, прошедшая поздние стадии клинических испытаний эффективности, не подтвердила индуцированные прототипами вакцин Т-клеточные реакции у людей, являющиеся потенциально решающим аспектом в формировании иммунитета против инфекций, вызванных *S. aureus*.

Ещё одним фактором, который должен учитываться при разработке вакцины против *S. aureus*, является врождённый (естественный) иммунитет у человека. Система врождённого иммунитета способствует ранней защите от *S. aureus* с помощью рецепторов распознавания структуры: Toll-подобного рецептора 2 и нуклеотид-связывающего домена олигомеризации 2, а также стимулирует выработку противомикробных пептидов и специфических путей передачи цитокинов ИЛ-1α и ИЛ-1β, привлекающих нейтрофилы в инфицированные ткани и предотвращающие развитие инфекции [1].

Адаптивный (приобретённый) иммунный ответ, включающий гуморальный и клеточный иммунитеты, также способствует защите хозяина. Развитие гуморального иммунитета является важным механизмом, участвующим в снижении инвазии *S. aureus*. Ранее сообщалось, что Т-клетки не являются необходимыми для защиты от *S. aureus* у мышей, но полученные в последнее время результаты показали, что Th1- и Th2-клетки могут играть как положительную, так и отрицательную роль при инфекции *S. aureus*. Активация Th1 приводит к секреции интерферона-γ, который может ускорить устранение системной инфекции, поражающей органы, с помощью усиления ответов макрофагов и повышения экспрессии молекул главного комплекса гистосовместимости. Кроме того, интерферон-γ считается стимулятором переключения изотипа иммуноглобулина на антитела классов IgG1 и IgG3 у человека или гомологичный IgG2A у мышей, который может действовать как опсонин, а Th2-клетки могут быть активированы компонентами клеточной стенки стафилококка, такими как пептидогликан и тейхоевая кислота. Цитокины Th2-клеток индуцируют и мобилизуют противомикробные пептиды, такие как человеческий β-дефенсин-3, являющийся заряженным катионным защитным пептидом, сохраняющим активность против *S. aureus* даже при повышенных концентрациях солей [1, 47, 67].

Учитывая, что цитокины ИЛ-17А и ИЛ-17F участвуют в продукции и привлечении нейтрофилов, Th17-клетки играют важную роль в первичной защите от инфекций, вызванных *S. aureus*. Ассоциированные Th17-лимфоцитами иммунные ответы могут быть направлены на стратегии смягчения развития удалённых инфекций, происходящих от персистирующего носительства *S. aureus* у человека [68]. Принимая во внимание данные обстоятельства, можно предположить, что непризнание кандидата V710 в вакцины против *S. aureus,* а также наличие низких концентраций ИЛ-2 и ИЛ-17А в сыворотке у привитых, приводящих к летальному исходу, говорит о том, что цитокин ИЛ-17А имеет решающее значение для устранения *S. aureus* у пациента [1].

В данном обзоре представлены последние разработки в мире и в России кандидатов вакцин, направленные на профилактику инфекций, вызываемых *S. aureus*. Вместе с тем создание многокомпонентной вакцины, которая может предотвратить заражение *S. aureus*, оказалось достаточно сложной задачей.

Для успешного прохождения клинических исследований необходимо продемонстрировать валидацию на доклиническом уровне с использованием моделей, которые могут коррелировать с состоянием человека при развитии инфекции [69]. Вакцины, проходящие клинические исследования, используют определённые группы населения с риском заражения, направлены на снижение патогенных свойств и факторов уклонения *S. aureus* от действия как гуморальных, так и клеточных звеньев иммунной системы. Для лицензирования вакцины необходимо доказать её эффективность на клиническом уровне, что не было продемонстрировано ни для одной вакцины-кандидата против *S. aureus*.

Необходимо отметить, что параллельно с созданием вакцин идёт разработка новых терапевтических препаратов против *S. aureus,* например, бактериофагов, моноклональных антител, центиринов и новых классов антибиотиков. При этом обсуждаемые способы лечения представляются как альтернатива вакцинации, вместо того чтобы в данных направлениях проводить параллельные исследования, обмениваться информацией и возможностями для сотрудничества. Например, при предоставлении доказательства эффективности при лечении инфекции *S. aureus* краткосрочная иммунотерапия может определить и предопределить антигенные мишени вакцин против *S. aureus*. Кроме того, учитывая, что в клинических исследованиях в качестве основы для терапевтической эффективности будут использоваться стандартные способы лечения, понимание того, как иммунотерапия, антибиотики и вакцины могут синергизировать друг друга, может быть очень важным при планировании будущих клинических исследований.

Выводы

Проведение за последние годы клинических испытаний кандидатов-вакцин, созданных на основе различных антигенов, оказывающих влияние на заболеваемость, вызванную несколькими фак-

торами патогенности стафилококка, не увенчались успехом из-за их низкой эффективности или недостаточно обоснованной безопасности (развитие нежелательных явлений).

Научное сообщество, несмотря на трудности, продолжает разрабатывать различные варианты с использованием современных технологий, включая сложные конструкции вакцин, способные активировать различные звенья иммунной системы, оказывающие влияние на основные механизмы патогенности *S. aureus*: например, использование цитокинов Th2-клеток, протективных компонентов, в частности, поверхностных и секретируемых *S. aureus* белков, октамерных токсинов, а также дальнейшее изучение опсонофагоцитарных гуморальных ответов.

Перспективными исследованиями для создания вакцин против инфекции *S. aureus* с направленностью на многочисленные факторы патогенности *S. aureus* следует рассматривать использование в составе вакцин комбинации нескольких антигенов, в том числе рекомбинантных белков, а также подбор адъювантов, способствующих усилению иммуногенности очищенных бактериальных антигенов.

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JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2024; 101(4)
DOI: https://doi.org/10.36233/0372-9311-512

Участие авторов: *Буйлова И.А.* — проведение поисково-аналитической работы и анализ научной литературы; *Савкина М.А.* — проведение анализа научной литературы; *Саяпина Л.В.* — анализ и редактирование статьи, подготовка материалов для публикаций; *Кривых М.А.* — анализ и редактирование статьи; *Обухов Ю.И.* — подготовка статьи для публикаций. Все авторы подтверждают соответствие своего авторства критериям Международного комитета редакторов медицинских журналов, внесли существенный вклад в проведение поисково-аналитической работы и подготовку статьи, прочли и одобрили финальную версию до публикации.

Статья поступила в редакцию 23.05.2024;
принята к публикации 28.07.2024;
опубликована 29.08.2024

Author contribution. *Builova I.A.* — conducting search and analytical work and analyzing scientific literature; *Savkina M.A.* — analyzing scientific literature; *Sayapina L.V.* — analyzing and editing the article, preparing materials for publication; *Krivykh M.A.* — analyzing and editing the article; *Obukhov Yu.I.* — preparing the article for publication. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published.

The article was submitted 23.05.2024;
accepted for publication 28.07.2024;
published 29.08.2024