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ПОЗДРАВЛЕНИЕ СО СТОЛЕТИЕМ «ЖУРНАЛА МИКРОБИОЛОГИИ, ЭПИДЕМИОЛОГИИ И ИММУНОБИОЛОГИИ»

Глубокоуважаемые коллеги!

В 2024 году «Журнал микробиологии, эпидемиологии и иммунобиологии» отмечает столетие своей деятельности!

От лица учредителей и издателя журнала — Общероссийской общественной организации «Всероссийское научно-практическое общество эпидемиологов, микробиологов и паразитологов» и Центрального научно-исследовательского института эпидемиологии Роспотребнадзора — позвольте поздравить читателей, авторов, рецензентов и всех, кто прилагает огромные усилия к развитию журнала, с юбилеем!

Сегодня «Журнал микробиологии, эпидемиологии и иммунобиологии» — одно из наиболее уважаемых научных периодических медицинских изданий России, площадка междисциплинарной интеграции фундаментальных и прикладных исследований для разработки технологий борьбы за санитарно-эпидемиологическое благополучие населения.

С момента создания журнала в редакционную коллегию входили выдающиеся российские учёные А.И. Абрикосов, С.В. Коршун, И.Л. Кричевский, Ф.Я. Чистович, академики В.Д. Беляков, А.Ф. Билибин, И.Н. Блохина, С.В. Прозоровский, П.А. Вершилова, Г.В. Выгодчиков, В.М. Жданов, П.Ф. Здродовский и др. Именно они стали основателями российских научных школ в области эпидемиологии, гигиены, микробиологии, иммунологии, патологической анатомии, инфекционных болезней, организации здравоохранения.

За истекший век журналом выпущено в свет 1028 номеров, опубликовано 19 903 статьи. В мирное время и в периоды потрясений «Журнал микробиологии, эпидемиологии и иммунобиологии» служил профилактике инфекций, строящейся на изучении их сущности, а также глубоком постижении теоретических основ эпидемиологии и иммунологии.

Сквозь столетие во главу угла своей деятельности журнал ставит доверие и уважение врачей и учёных. Именно поэтому «Журнал микробиологии, эпидемиологии и иммунобиологии» стремится публиковать самые актуальные, востребованные практикой результаты научных исследований, рассматривает развитие института рецензирования как гарантию качества научных публикаций и важную задачу для передачи научного опыта.

Бессменным учредителем журнала является Всероссийское научно-практическое общество эпидемиологов, микробиологов и паразитологов. В 2019 году в состав учредителей журнала вошёл и взял на себя функции издателя Центральный научно-исследовательский институт эпидемиологии Роспотребнадзора. Издатель, являясь головным научно-исследовательским институтом России в области эпидемиологии, как никто другой осознаёт важность развития научной основы для создания технологий и средств борьбы с инфекциями, особенно актуального в период внешних угроз и наличия технологических возможностей для использования инфекционных агентов в качестве управляемых факторов политического влияния.

Совместные усилия главного редактора «Журнала микробиологии, эпидемиологии и иммунобиологии» академика Российской академии наук Виталия Васильевича Зверева, издателя журнала — Центрального научно-исследовательского института эпидемиологии Роспотребнадзора, редакционной коллегии способствовали стремительному росту международной значимости издания. Пройдя все сложные времена, журнал не ставит своей задачей коммерческую прибыль. Политика журнала строится на бесплатном доступе российских и зарубежных читателей к полным текстам, неприемлемости публикации явной и скрытой рекламы, а также взимания платы с авторов.

Издатель прилагает организационные и финансовые усилия для развития «Журнала микробиологии, эпидемиологии и иммунобиологии» — использования самых современных издательских технологий, обеспечения доступности статей для мировой читательской аудитории, индексации ведущими международными библиографическими базами, привлечения ведущих учёных в качестве авторов и рецензентов.

Хочу выразить свою признательность и уважение нашим авторам, главному редактору, членам редакционной коллегии и редакционного совета, рецензентам, сотрудникам редакции — всем, кто прилагал силы к становлению и развитию журнала на протяжении 100 лет!

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The pilot study of the features of HIV-1 resistant variants spread using molecular clusters

Alina A. Kirichenko[1]✉, Dmitry E. Kireev[1], Yulia N. Sidorina[2], Natalia D. Abashina[2], Elena E. Brusentseva[2], Vasily G. Akimkin[1]

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Abstract

Introduction. As a result of routine testing of HIV-1 drug resistance (DR), a significant amount of viral nucleotide sequences and epidemiological data of HIV-infected individuals have been collected. Combined with the increasing use of bioinformatics methods in practice, it has become possible to study the features of HIV-1 resistant variants spread using molecular clustering analysis.

The **aim** of the study was to validate the molecular clustering analysis in a pilot region of Russia using a significant number of nucleotide sequences to study the features of the spread of HIV-1 resistant variants.

Materials and methods. HIV-1 nucleotide sequences were obtained from 899 HIV-infected patients who were registered at the Oryol AIDS Center in 2016–2021. HIV-1 genetic variants were determined using the Stanford University database, REGA and HIV BLAST. Resistance mutations and prognostic HIV-1 DR were determined using the Stanford University database. Phylogenetic analysis was carried out using the MEGA program. HIV-1 molecular clusters were identified using Cluster Picker software.

Results. In the pilot region, sub-subtype A6 dominated (85.7%); an increase in the share of CRF63_02A6 was noted. HIV-1 resistance was found in 13.6% of patients without antiretroviral therapy (ART) experience and in 52.0% with ART experience. Molecular clusters were more often formed by HIV-1 nucleotide sequences from ART-naïve patients. HIV-1 DR variants were less likely to fall into molecular clusters. The sources of transmitted mutations were more often patients with ART experience. The most actively and efficiently transmitted mutations were *K103N*, *V179E/T*, *Y181C* and *G190S*, associated with virus resistance to efavirenz and nevirapine.

Keywords: *HIV-1, drug resistance, resistance mutations, antiretroviral therapy, molecular clusters, transmission clusters, surveillance, genomic surveillance*

Ethics approval. The study was conducted with the informed consent of the patients. The local Ethics Committee of the Central Research Institute of Epidemiology (Protocol No. 93, June 18, 2019) approved the study.

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Оригинальное исследование
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Пилотное исследование по изучению особенностей распространения резистентных вариантов ВИЧ-1 с помощью молекулярных кластеров

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Аннотация

Введение. Благодаря рутинному тестированию лекарственной устойчивости (ЛУ) ВИЧ-1 накапливается значимое количество нуклеотидных последовательностей (НП) вируса и эпидемиологических данных о ВИЧ-инфицированных лицах, что вместе с активным внедрением в практику биоинформатических методов позволяет использовать их для изучения особенностей распространения резистентных вариантов ВИЧ-1 с помощью анализа молекулярных кластеров.

Цель исследования — апробация анализа молекулярных кластеров на территории пилотного региона России с использованием значимого количества НП для изучения особенностей распространения резистентных вариантов ВИЧ-1.

Материалы и методы. Получены НП ВИЧ-1 от 899 ВИЧ-инфицированных пациентов, состоявших на диспансерном учёте в Орловском центре СПИД в 2016–2021 гг. Определены генетические варианты ВИЧ-1 с помощью базы данных Стенфордского университета, REGA и HIV BLAST. Выявлены мутации резистентности и определена прогностическая ЛУ ВИЧ-1 с использованием базы данных Стенфордского университета. Проведён филогенетический анализ в программе «MEGA». Выявлены молекулярные кластеры ВИЧ-1 с помощью программного обеспечения «Cluster Picker».

Результаты. На территории пилотного региона доминировал суб-субтип A6 (85,7%), отмечено увеличение доли CRF63_02A6. Резистентность ВИЧ-1 была обнаружена у 13,6% пациентов без опыта антиретровирусной терапии (АРТ) и у 52,0% с опытом АРТ. Молекулярные кластеры чаще образовывали НП ВИЧ-1 от пациентов без опыта АРТ. ЛУ-варианты ВИЧ-1 реже попадали в молекулярные кластеры. Источниками передаваемых мутаций чаще являлись пациенты с опытом АРТ. Наиболее активно и эффективно передавались мутации *K103N*, *V179E/T*, *Y181C*, *G190S*, ассоциированные с устойчивостью вируса к эфавирензу и невирапину.

Заключение. Применение анализа молекулярных кластеров, предоставляющего информацию об особенностях распространения резистентных вариантов ВИЧ-1, может быть рекомендовано к использованию в России с целью разработки стратегий профилактики предотвращения передачи ЛУ-вариантов вируса и повышения эффективности лечения.

Ключевые слова: *ВИЧ-1, лекарственная устойчивость, мутации резистентности, антиретровирусная терапия, молекулярные кластеры, кластеры передачи, эпидемиологический надзор, геномный эпидемиологический надзор*

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

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

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Introduction

Widespread use of antiretroviral therapy (ART) significantly reduces morbidity and mortality of people living with HIV (PLHIV) [1, 2], as well as the risk of HIV transmission [3, 4]. However, the expansion of ART coverage in HIV-infected patients inevitably leads to the emergence and spread of drug resistance (DR) of the virus, which jeopardizes the efficacy of ART [5, 6]. At least 20% of HIV-infected patients[1] in Russia annually experience virologic failure of ART, the main cause of which is HIV-1 DR.

Lack of measures to counteract the emergence and spread of HIV-1 DR variants will lead to reduced ART efficacy, increased morbidity and mortality, poorer health of PLHIV, reduced therapeutic options available to patients, which will result in increased economic costs of counteracting the HIV epidemic [7, 8]. Thus, HIV-1 DR poses clinical, epidemiological and economic threats to achieving control of the HIV epidemic.

In this regard, recommendations were developed in Russia to identify HIV-1 DR in clinical practice to improve the effectiveness of treatment at the individual level[2] and as one of the components of epidemiological surveillance of HIV infection to improve the effective-

[1] Federal Scientific and Methodological Center for AIDS Prevention and Control of the Central Research Institute of Epidemiology of Rospotrebnadzor. Reference. HIV infection in the Russian Federation as of December 31, 2022. URL: http://www.hivrussia.info/wp-content/uploads/2023/09/Spravka-VICH-v-Rossii-na-31.12.2022.pdf (date of access: July 8, 2024).

[2] Clinical Guidelines “HIV Drug Resistance Analysis”. Moscow; 2017. URL: https://fedlab.ru/upload/medialibrary/f38/_-_10_04_2017_.pdf (date of access: July 8,.2024).

ness of ART and reduce the spread of HIV-1 DR at the population level among HIV-infected individuals[3].

In Russia, HIV-1 DR surveillance studies are rarely and unsystematically conducted; however, HIV-1 DR testing at the individual level is one of the routine types of analysis of ART efficacy, is included in the standards of primary health care for HIV infection[4] and is performed annually on at least 7,000 HIV-infected patients[5]. Thanks to routine HIV-1 DR testing, a significant amount of HIV-1 nucleotide sequences and related epidemiological data on HIV-infected individuals has been accumulated, which, together with the active introduction of bioinformatic methods into practice, has allowed the development of a new area called genomic epidemiological surveillance [9]. In 2021, during a pandemic caused by a new coronavirus infection, the World Health Organization (WHO) called on countries to strengthen the role of genomic epidemiological surveillance to better understand the transmission of infectious agents with pandemic and epidemic potential in order to develop vaccines, drugs, diagnostic test systems, as well as to take measures aimed at preventing the spread of infections[6].

One of the most important tools for genomic epidemiological surveillance of HIV infection is the identification of molecular clusters, i.e., HIV-1 nucleotide sequences that have high genetic similarity[7] and suggest an epidemiological link between the HIV-infected individuals from whom they are derived [10]. Clustering of HIV-1 nucleotide sequences indicates a more active transmission of the virus [11] and makes it possible to identify foci of increased morbidity, as well as to characterize the cohort with the most active HIV-1 transmission for effective anti-epidemic measures. Currently, the US Centers for Disease Control and Prevention (CDC) emphasize the identification of molecular clusters and rapid response to them as one of the main principles necessary to end the HIV epidemic, along with early diagnosis, rapid and effective treatment and prevention in at-risk groups [12].

For such studies, the key factor is the "sampling density" of HIV-infected persons, i.e. the proportion of persons for whom the nucleotide sequence of HIV-1 is known among all identified PLHIV in the region under study. If the "sample density" is less than 10%, the reliability and accuracy of the results obtained are significantly reduced [13].

Currently in Russia, bioinformatic methods in epidemiological surveillance are used only for the purpose of investigating cases of HIV infection, presumably related to the provision of medical care[8]. In turn, there have been no studies devoted to the analysis of molecular clusters in epidemiological surveillance of DR variants of HIV in Russia.

Therefore, the **aim** of this study was to validate molecular cluster analysis in a pilot region of Russia using a significant number of nucleotide sequences to study the features of the spread of resistant HIV-1 variants.

Materials and methods

Study samples

The study included 899 HIV-infected patients who were registered at the Oryol Region AIDS Center from 2016 to 2021.

The criteria for inclusion in the study were the diagnosis of HIV infection confirmed in accordance with national clinical protocols, as well as the availability of patient data: gender, date of the first positive immune blot result, and experience of taking antiretroviral drugs.

Inclusion criteria were a viral load of less than 500 copies/mL of HIV-1 RNA.

Informed consent was obtained for all patients or their legal representatives (if the patient was less than 18 years of age at the time of the study) before performing procedures related to this study. The study was approved by the local ethics committee of the Central Research Institute of Epidemiology of Rospotrebnadzor (protocol No. 93 of 18.06.2019).

RNA extraction and HIV-1 sequencing

Extraction of HIV-1 RNA from blood plasma and sequencing of amplified fragments of the pol gene encoding protease and part of reverse transcriptase (2253-3368 bp. relative to the reference strain HXB2, GenBank #K03455) were performed using the AmpliSense HIV-Resist-Seq reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor) with an Applied Biosystems 3500 Genetic Analyzer (Life Technologies) or with the in-house method using the next-generation sequencer MiSeq (Illumina).

Sequencing data were processed and consensus sequences were obtained using DEONA software (ver-

[3] Methodological Guidelines MG 3.1.5.0075/1-13 "Surveillance of the spread of HIV strains resistant to antiretroviral drugs" Moscow; 2013.

[4] Order of the Ministry of Health of the Russian Federation № 438n from June 23, 2022 "On approval of the standard of primary medical and sanitary care for adults with HIV infection (diagnosis, treatment and dispensary monitoring)".

[5] ITPC, Eastern Europe and Central Asia. Analysis of procurement of diagnostics for HIV treatment in Russia in 2020-2021. (2022) URL: https://itpc-eeca.org/wp-content/uploads/2022/07/monitoring-testov-vich-2020-21-gg-1.pdf

[6] World Health Organization. Global genomic surveillance strategy for pathogens with pandemic and epidemic potential, 2022–2032. 2022. 32 p. URL: https://www.who.int/publications/i/item/9789240046979 (date of access: 08.07.2024).

[7] CDC. A guide for health departments: detecting and responding to HIV Transmission Clusters, 2018. 2019. 131 p. URL: https://www.cdc.gov/hiv/programresources/guidance/cluster-outbreak/index.html (date of access: 08.07.2024).

[8] Methodological guidelines of Rospotrebnadzor MG 3.1.3342-16 "Epidemiological surveillance of HIV infection". Moscow; 2016.

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sions 1.2.3, 1.7.0) (RMBit) for classical sequencing data and with the help of the Trimmomatic [14] and VirGenA programs [15] for next-generation sequencing data with a 20% sensitivity threshold for HIV-1 minor variants.

All nucleotide sequences were subjected to quality control using the WHO BCCfE HIVDR QC instrument[9].

The obtained HIV-1 nucleotide sequences as well as related epidemiological and laboratory data on patients were uploaded to the Russian Database of HIV resistance to antiretroviral drugs[10].

Identification of HIV-1 genetic variants

HIV-1 genetic variants were identified using the Stanford University HIVdb (v. 9.1)[11] and REGA HIV-1 Subtyping Tool (v. 3.0)[12].

In case of discordant results between the two tools, nucleotide sequences were analyzed using the HIV BLAST (Basic Local Alignment Search Tool) tool of the Los Alamos Institute international database[13].

Determination of HIV-1 drug resistance

Resistance mutations were identified using the Stanford University HIVdb database (v. 9.1), according to which each mutation and combination of mutations are assigned penalty scores characterizing the level of HIV-1 prognostic DR: potential-low (10–14 points), low (15–29 points), intermediate (30–59 points) and high (more than 60 points). The DR variants of the virus were considered to be those for which 15 or more penalty scores were obtained.

HIV-1 prognostic DR was assessed to:

- protease inhibitors (PIs): atazanavir (ATV), darunavir (DRV), fosamprenavir (FPV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), tipranavir (TPV);
- nucleoside reverse transcriptase inhibitors (NRTIs): abacavir (ABC), zidovudine (ZDV), stavudine (d4T), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), tenofovir (TDF);
- non-nucleoside reverse transcriptase inhibitors (NNRTIs): doravirine (DOR), efavirenz (EFV), etravirine (ETR), nevirapine (NVP), rilpivirine (RPV).

The 2009 Surveillance Drug Resistance Mutation (SDRM) list, significant for surveillance of transmitted DR HIV-1, was used to assess mutations in ART-naïve patients [16].

Phylogenetic analysis

HIV-1 nucleotide sequences were aligned using the online software of the Los Alamos Institute International Database using the HMMER method.

Editing and trimming of the aligned nucleotide sequences was performed using the BioEdit 7.0.9.0 program.

Phylogenetic analysis was performed by maximum likelihood method with bootstrap 100 and general reversion model with invariant sites and gamma distribution (G+I) in MEGA6 program.

Identification of HIV-1 molecular clusters

HIV-1 molecular clusters were identified using the Cluster Picker 1.2.3 software [17] with a bootstrap threshold of 0.9 and a genetic distance threshold of 0.045 nucleotide substitutions per position (4.5%).

Molecular clusters were visualized using the MicrobeTrace online software[14].

Clusters were classified as large if they consisted of 4 or more nucleotide sequences and active if they contained at least 1 nucleotide sequence from a patient diagnosed with HIV infection between 2019 and 2021.

Statistical analysis

The data obtained in the study were statistically processed using Microsoft Excel and GraphPad Prism online software. The statistical significance of differences between quantitative indicators was assessed using Fisher's two-sided exact test. Differences were considered significant at $p < 0.05$.

Results

Patient characteristics

The study included 899 HIV-infected patients, representing 34.1% of identified PLHIV in the study region as of the end of 2021[15].

At the time of blood collection, 354 (39.4%) patients were ART-naïve and 545 (60.6%) patients were ART-naïve.

The median age of patients at the time of blood collection for the study was 37 (32–42) years. Among ART-experienced patients, there were 6 (1.7%) HIV-infected patients less than 18 years of age.

The route of HIV-1 transmission was known for 874 (97.2%) study patients. The main routes of HIV-1 transmission were sexual (507; 65.0%) and parenteral through intravenous drug administration (283; 31.5%).

[9] URL: http://pssm.cfenet.ubc.ca/who_qc/
[10] URL: https://ruhiv.ru
[11] URL: https://hivdb.stanford.edu/
[12] URL: http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool
[13] URL: https://www.hiv.lanl.gov
[14] URL: https://microbetrace.cdc.gov
[15] Федеральный научно-методический центр по профилактике и борьбе со СПИДом ФБУН ЦНИИ Эпидемиологии Роспотребнадзора. Информационный бюллетень № 46 «ВИЧ-инфекция». 2021. URL: http://www.hivrussia.info/wp-content/uploads/2022/05/Byulleten-46-VICH-infektsiya-za-2020-g.-.pdf (дата обращения: 08.07.2024).

Male patients made up the majority (512; 57.0%).

Table 1 presents the clinical and epidemiological characteristics of all patients included in the study.

HIV-1 genetic variants

The dominant genetic variant of HIV-1 was sub-subtype A6, which was detected in 85.7% of HIV-infected patients. The circulating recombinant form (CRF) 63_02A6 was detected with high frequency (10.6%), the prevalence of which increased among the study patients diagnosed with HIV infection in 2015-2021 (**Fig. 1**). The other HIV-1 genetic variants were much less common: subtype B, 2.4%; CRF02_AG, 1.0%; CRF03_A6B, 0.2%; and subtype F, 0.1%.

Drug resistance and HIV-1 resistance mutations

Among 545 ART-naïve patients, HIV-1 DR to at least one antiretroviral drug was detected in 74 (13.6%) HIV-infected individuals: most often to NNRTIs (11.4%), significantly less often to PIs (2.8%) and NRTIs (0.7%).

Among the NNRTI class, HIV-1 DR was detected most frequently to RPV (7.3%), NVP (6.4%), and EFV (6.1%), with predominantly high-level DR to 1st generation drugs (**Figure 2**). Among PIs, HIV-1 DR was most frequently detected to NFV (2.6%). Among drugs of the NRTI class, HIV-1 resistance was identified most frequently to ABC (0.7%), FTC (0.7%) and 3TC (0.7%).

HIV-1 DR among ART-naïve patients was predominantly detected to only one class of drugs — NNRTIs (10.1%). HIV-1 resistance only to PIs was detected in 12 (2.2%) patients, HIV-1 DR only to NNRTIs was not detected. Multidrug resistance was detected rarely and only simultaneously to two classes of antiretroviral drugs: PIs + NRTIs (0.6%) and NRTIs + NNRTIs (0.7%).

Analysis of HIV-1 DR patterns revealed that at least one resistance mutation, including polymorphic mutations for sub-subtype A6 — A62V and E138A, was detected in 273 (50.1%) HIV-1-infected individuals. The most common mutations were *K103N* (4.6%), *E138A* (4.2%), *G190S* (1.5%), *V179E* (1.3%), and *K101E* (1.1%), and the most common mutation for the NNRTI class was A62V (39.4%). The remaining mutations, including those to the PI class, occurred at a frequency of less than 1%.

Table 1. Clinical and epidemiological characteristics of patients

Characteristic	ART-experienced patients	ART-naïve patients	Total
Number of patients	354	545	899
Age, years, median (IQR)	37 (33–42)	36 (31–42)	37 (32–42)
Sex, *n* (%)			
male	196 (55.4)	316 (58.0)	512 (57.0)
female	158 (44.6)	229 (42.0)	387 (43.0)
Route of transmission, *n* (%)			
sexual (heterosexual)	177 (50.0)	330 (60.6)	507 (56.4)
sexual (homosexual)	1 (0.3)	6 (1.1)	7 (0.8)
sexual (unspecified)	46 (13.0)	24 (4.4)	70 (7.8)
parenteral (narcotic)	116 (32.8)	167 (30.6)	283 (31.5)
mother-to-child	7 (2.0)	0	7 (0.8)
unknown	7 (2.0)	18 (3.3)	25 (2.8)
Viral load, $\log_{10}$ copies/mL, median (IQR)	4.2 (3.7–4.9)	4.6 (4.0–5.2)	4.5 (3.9–5.1)
Sampling year, *n* (%)			
2016	22 (6.2)	0	22 (2.4)
2017	35 (9.9)	0	35 (3.9)
2018	139 (39.3)	341 (62.6)	480 (53.4)
2019	110 (31.1)	113 (20.7)	223 (24.8)
2020	6 (1.7)	0	6 (0.7)
2021	42 (11.9)	91 (16.7)	133 (14.8)

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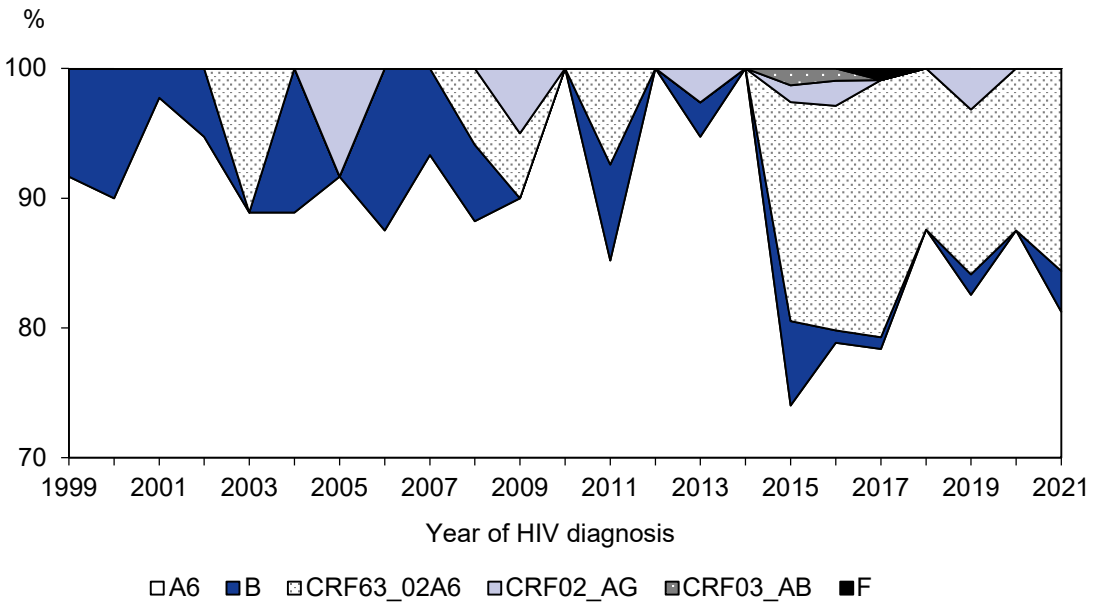

Fig. 1. Distribution of HIV-1 genetic variants by year of diagnosis of HIV infection.

SDRMs were detected in 6.8% of ART-naïve patients, and a tendency to increase their prevalence was noted. For example, in patients with a blood collection date in 2018, 2019, and 2021, at least one surveillance mutation was detected in 5.9% (95% CI 3.8–8.9%), 6.2% (95% CI 2.8–12.4%), and 8.8% (95% CI 4.3–16.6%) of cases, respectively.

The complete list of detected mutations among ART-naïve patients is presented in **Table 2**.

Among 354 ART-experienced patients, HIV-1 DR to at least one antiretroviral drug was detected in 52.0% of cases, most frequently to NNRTIs (44.6%) and NRTIs (36.2%). HIV-1 DR to PIs was detected rarely, in 5.4% of patients.

Among the NNRTI class, HIV-1 DR was detected most frequently to NVP (40.4%), EFV (40.4%), and RPV (32.8%) (**Figure 3**). Meanwhile, resistance was predominantly high to 1st generation NNRTIs (EFV

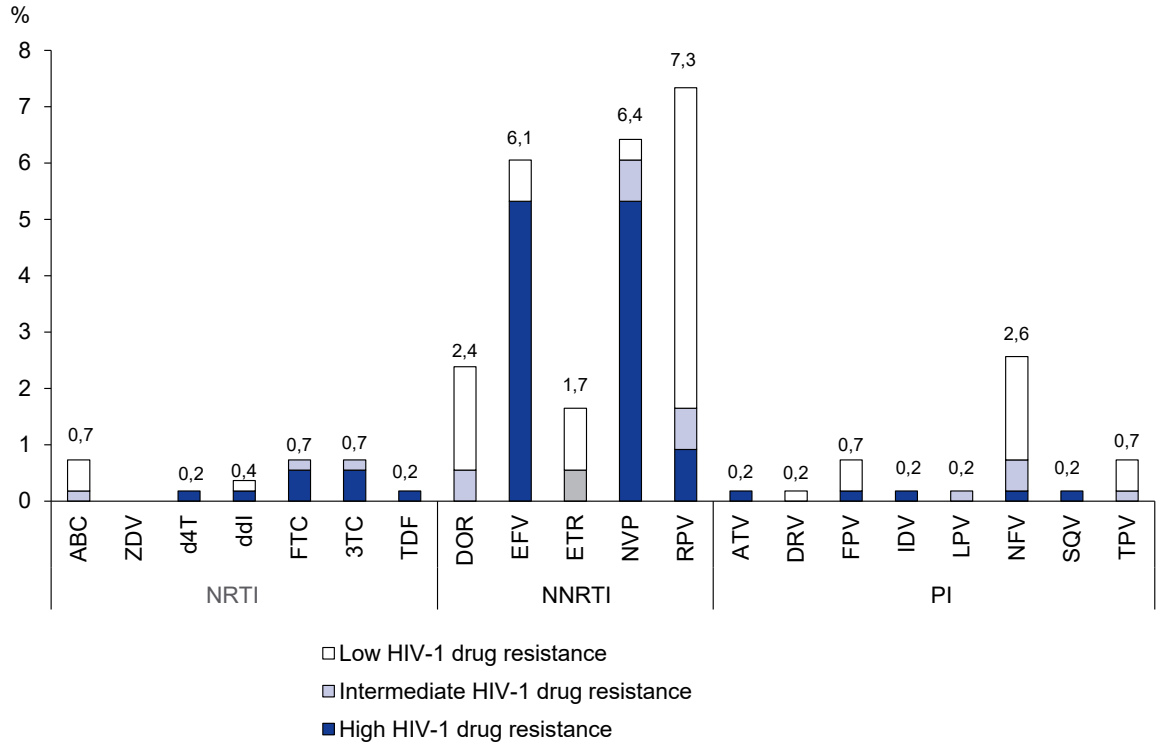

Fig. 2. Prevalence and level of HIV-1 drug resistance among ART-naïve patients.

and NVP). Among the NRTIs, HIV-1 DR was most frequently detected to ABC (35.3%), FTC (34.7%), and 3TC (34.7%), with predominantly high levels to the first 2 drugs. PIs to all drugs of the PI class did not exceed 5% and were most frequently detected to NFV (4.0%).

HIV-1 DR in ART-experienced patients was detected most often, in 27.1% of cases, to two drug classes (NRTI + NNRTI), and to NNRTI class drugs alone in 14.1% of cases. Multidrug resistance to 3 classes of drugs (PI + NRTI + NNRTI) was detected rarely, in 3.1% of patients.

In an analysis of HIV-1 DR patterns in ART-experienced patients, resistance mutations were found in 255 (72.0%) HIV-1-infected patients, including polymorphic mutations. The most frequent mutations to NNRTIs were *G190S* (20.1%), *K103N* (12.7%), *K101E* (10.2%), *Y181C* (6.8%), *E138A* (6.2%); to NRTIs, *A62V* (38.4%), *M184V/I* (26.8%), *K65R* (10.5%); and to PIs, *M46I* (2.5%). **Table 3** presents the list of identified resistance mutations among ART-experienced patients.

HIV-1 molecular clusters

Molecular cluster analysis revealed that 243 out of 899 HIV-1 nucleotide sequences (27.1%) formed 91 clusters. HIV-1 nucleotide sequences from ART-naïve patients were more frequently detected within clusters (69.5% vs 57.3%; $p = 0.0009$).

The prevalence of HIV-1 DR within clusters was 20.2% (49/243) vs 42.7% (209/656) outside clusters

Table 2. Prevalence of resistance mutations among ART-naïve patients

ARV class	Mutations	Mutation detection rate, *n* (%)
NRTI	*E44D*	3 (0.6)
	A62V	215 (39.4)
	*K65R**	1 (0.2)
	*M184I**	1 (0.2)
	*M184V**	2 (0.4)
NNRTI	*A98G*	1 (0.2)
	*K101E**	6 (1.1)
	*K103N**	25 (4.6)
	V106I	1 (0.2)
	V108I	3 (0.6)
	E138A	23 (4.2)
	E138G	5 (0.9)
	E138K	1 (0.2)
	V179D	3 (0.6)
	V179E	7 (1.3)
	V179T	3 (0.6)
	*G190S**	8 (1.5)
PI	*M46I**	3 (0.6)
	M46V	2 (0.4)
	*M46L**	1 (0.2)
	*I84V**	1 (0.2)

Note. *Mutations from the SDRM list.

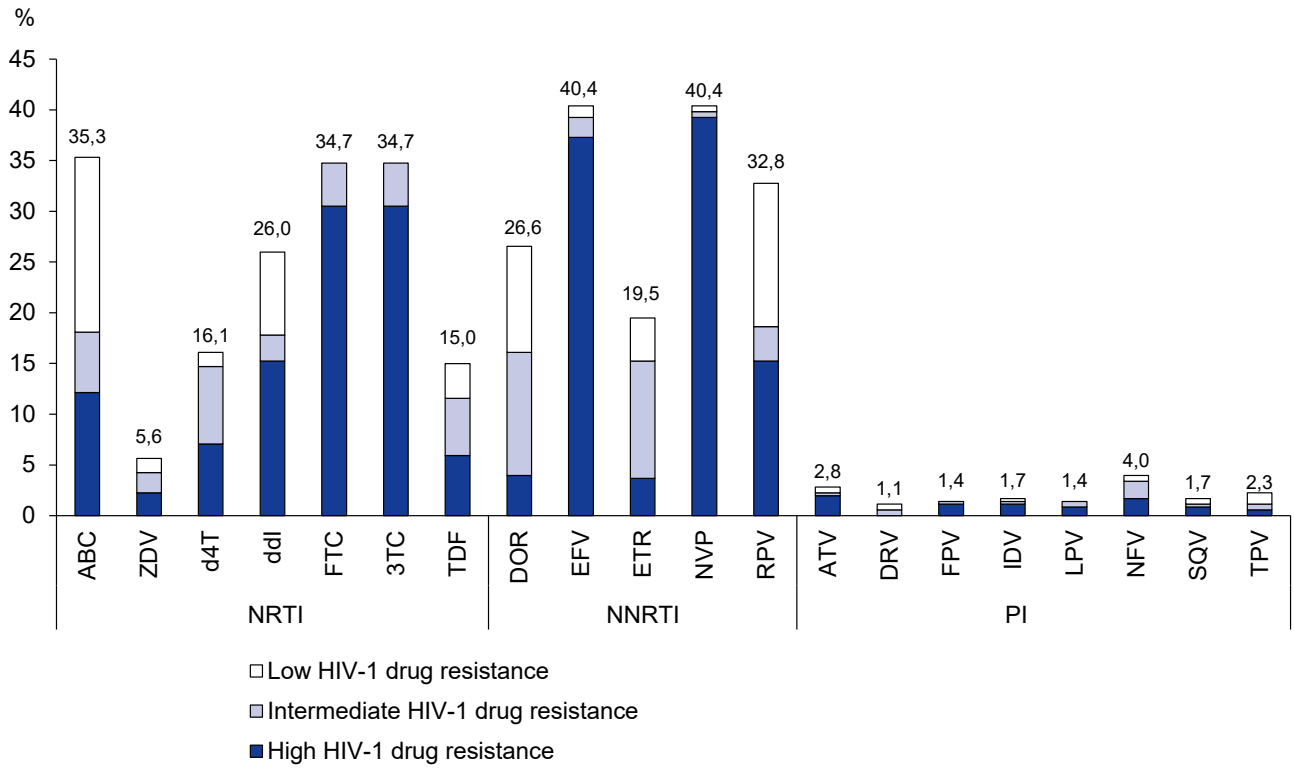

Fig. 3. Prevalence and level of HIV-1 drug resistance among ART-experienced patients.

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Table 3. Prevalence of resistance mutations* among ART-experienced patients

ARV class	Mutations*	Mutation detection rate, *n* (%)
NRTI	*A62V*	136 (38.4)
	K65R	37 (10.5)
	D67N	13 (3.7)
	K70R	10 (2.8)
	K70E	4 (1.1)
	L74I	6 (1.7)
	L74V	13 (3.7)
	M184I	19 (5.4)
	M184V	76 (21.5)
	T215Y	4 (1.1)
	K219E	4 (1.1)
	K219Q	7 (2.0)
NNRTI	*L100I*	4 (1.1)
	K101E	36 (10.2)
	K103N	45 (12.7)
	V106I	8 (2.3)
	V108I	7 (2.0)
	E138A	22 (6.2)
	E138G	8 (2.3)
	V179D	6 (1.7)
	V179E	7 (2.0)
	V179T	4 (1.1)
	Y181C	24 (6.8)
	G190S	71 (20.1)
	H221Y	5 (1.4)
	P225H	9 (2.5)
PI	*M46I*	9 (2.5)
	I50L	4 (1.1)

Note. *Mutations with a prevalence of at least 1% are represented.

($p = 0.0005$). However, there was no correlation of DR HIV-1 nucleotide sequence clustering with ART experience or any other clinical and epidemiological characteristics of patients.

Resistance mutations (excluding polymorphic mutations for sub-subtype A6 – *A62V*, *E138A*) were found in 54 HIV-1 nucleotide sequences in 33 clusters.

The clustering features of HIV-1 nucleotide sequences with mutations occurring with a frequency of more than 1.0% in the study sample were analyzed (**Table 4**). The most common mutations in the detected clusters were *K103N* (6.2%), *V179E* (4.1%), *G190S* (4.9%), and *M184V* (4.9%). At the same time, the *V179E* mutation was significantly more frequent in the clusters, and in contrast, the *M184V*, *K101E*, and *G190S* mutations were less frequent than among all the patients studied.

Transmitted mutations, i.e., those that occurred in at least 2 HIV-1 nucleotide sequences in a cluster, were found in 9 clusters in 27 nucleotide sequences (**Figure 4**). The profile of transmitted mutations was limited and included mutations associated with DR to NNRTIs (*K103N*, *V179E/T*, *G190S*, *Y181C*), NRTIs (*M184I/V*, *K65R*), and PIs (*L33F*).

Within clusters, the transmission efficiency of resistance mutations was determined as the ratio of the number of transmitted mutations in clusters to all mutations in clusters. The highest transmission efficiency (50% or higher) was found for *K103N* (10/15; 66.7%), *V179E/T* (11/12; 91.7%), *Y181C* (2/4; 50.0%) and *G190S* (6/12; 50.0%) mutations.

Based on the date of HIV diagnosis, putative sources of transmitted HIV-1 mutations were identified, which in 6/9 clusters (66.7%) were patients with a history of taking antiretroviral drugs.

In all clusters, patients with an earlier date of diagnosis transmitted the full mutation profile, except for one cluster in which only *K103N* was transmitted from the *M184V* + *K103N* profile.

Most clusters (7/9) with transmitted HIV-1 mutations were small and inactive. Only *K103N* (1 cluster) and *V179E* (1 cluster) mutations were identified in large active clusters.

Discussion

Against the background of a long history of taking antiretroviral drugs and an increasing number of patients on ART, the prevalence of DR HIV variants in Russia is increasing every year [18], which is the reason for increasing the coverage of HIV-1 DR testing among HIV-infected individuals. Thanks to the improvement of sequencing technologies and the strengthening of the national database of HIV resistance to antiretroviral drugs [19], a significant amount of genetic and epidemiological data is being accumulated, which, together with the development of bioinformatic research methods, makes it possible to use them for a more in-depth study of the features of HIV-1 spread.

In this study, a pilot region of Russia was used as an example to demonstrate for the first time in the country the potential of a new direction, genomic epidemiological surveillance, in terms of the spread of resistant HIV-1 variants. For the first time, a high coverage of HIV-1 sequencing of PLHIV was obtained for one region of Russia, which amounted to 34.1% at the end of

Table 4. Prevalence of HIV-1 DR mutations within clusters

ARV class	Mutations	Prevalence among all study patients (*n* = 899), %	Prevalence within clusters (*n* = 243), %	*p**
NRTI	*K65R*	4.2	2.1	0.0604
	D67N	1.4	0.4	0.2039
	K70R	1.1	0	0.0701
	M184I	2.2	2.1	1
	M184V	8.7	4.9	**0.0159**
NNRTI	*K101E*	4.7	2.1	**0.0211**
	K103N	7.8	6.2	0.3270
	V106I	1.0	1.2	0.7091
	V108I	1.1	1.2	0.7349
	E138G	1.4	1.6	0.7568
	V179D	1.0	1.6	0.2625
	V179E	1.6	4.1	**0.0006**
	Y181C	2.7	1.6	0.3515
	G190S	8.8	4.9	**0.0117**
	P225H	1.0	0	0.1233
PI	*M46I*	1.3	0.4	0.1975

Note. *Statistically significant differences are highlighted in bold ($p < 0.05$).

2021[16]. This makes it possible to obtain reliable results of the study.

The assessment of HIV-1 genetic diversity in the study region revealed 5 genetic variants (sub-subtype A6, subtype B, CRF63_02A6, CRF02_AG, CRF03_A6B) characteristic of the Russian genetic landscape [20], as well as subtype F atypical for the Russian epidemic, probably resulting from an imported case of HIV infection. The observed increase in the proportion of CRF63_02A6 among patients diagnosed between 2015 and 2021 reflects the general trend in the country [21, 22].

The analysis of HIV-1 DR revealed that HIV-1 resistance to at least one antiretroviral drug was detected in 13.6% of ART-naïve patients, most often to NNRTIs (11.4%): RPV (7.3%), NVP (6.4%) and EFV (6.1%), which is in line with the data obtained for Russia as a whole [21].

The prevalence of HIV-1 DR in the studied patients with ART experience was 52.0%, most frequently to the same NNRTI class drugs (44.6%): NVP (40.4%), EFV (40.4%) and RPV (32.8%), as well as to NNRTIs (36.2%): ABC (35.3%), FTC (34.7%) and 3TC (34.7%).

The described prevalence of HIV-1 DR in ART-experienced patients in Russia varies considerably from 50% [23] to 82.4% [24], reflecting the correct assignment of genotyping test. In the present study, the relatively low

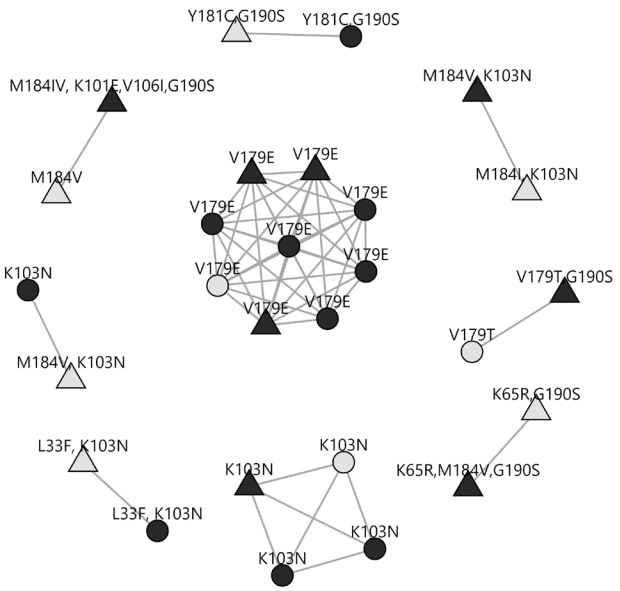

Fig. 4. Clusters with transmitted resistance mutations.

The triangle indicates HIV-1 nucleotide sequences from ART-experienced patients, and the circle indicates HIV-1 nucleotide sequences from ART-naïve patients. HIV-1 nucleotide sequences from patients with the earliest date of diagnosis of HIV infection in the cluster are marked in light grey.

[16] Federal Scientific and Methodological Center for AIDS Prevention and Control FBUN Central Research Institute of Epidemiology of Rospotrebnadzor. Information Bulletin No. 46 "HIV-infection". 2021. URL: http://www.hivrussia.info/wp-content/uploads/2022/05/Byulleten-46-VICH-infektsiya-za-2020-g.-.pdf. (date of access: July 8, 2024).

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HIV-1 DR is associated with the fact that virologic failure of ART wasn't observed in all patients.

It should be noted that high-level HIV-1 DR among all patients, regardless of ART experience, were most often established for NNRTIs (NVP, EFV) and NRTIs (FTC and 3TC), which can be explained by their widespread use and low genetic barrier to the development of HIV-1 DR [25].

During the study period, the most commonly used 1st-line ART regimen in the region included TDF, 3TC and EFV. According to the results of the present study, the primary resistance of HIV-1 to the used nucleoside base did not exceed 1%, and resistance to EFV amounted to 6.1%, which allows recommending replacement of the third component of the regimen in accordance with the national clinical guidelines for the treatment of HIV infection[17].

The most frequent mutations (excluding polymorphic mutations for sub-subtype A6 - *A62V* and *E138A*) among patients without ART experience were *K101E*, *K103N*, *V179E* and *G190S* to NNRTIs, among patients with ART experience — *K101E*, *K103N*, *V179E*, *Y181C*, *G190S* to NNRTIs and *M184V/I*, *K65R* to NRTIs. HIV-1 DR and PI class drug resistance mutations were rare among the study patients.

The prevalence of mutations significant for epidemiological surveillance of transmitted HIV-1 DR in the Orel region was 6.8%, which corresponds to the average level in Russia [18]. Multidrug resistance to three classes of ARVs (PIs + NRTIs + NNRTIs) was detected only in ART-experienced patients in 3.1% of cases. Thus, it can be concluded that the HIV-1 DR level in the study region is moderate, and the HIV-1 DR patterns identified correspond to the ART regimens used and are typical for Russia.

Assessment of HIV-1 DR levels and patterns provides important information about which drugs are effective at the time of the study, but does not allow us to determine the distribution of DR variants or predict which drugs will be effective in the future. For a more in-depth analysis, one of the tools of genomic epidemiological surveillance, the method of molecular cluster analysis, was applied to the spread of HIV-1 DR in this study.

The analysis found that molecular clusters were more likely to be formed by HIV-1 nucleotide sequences from ART-naïve patients, indicating that they were the main sources of HIV infection in the region and suggesting that there was no high risk of transmission of HIV-1 DR variants due to the relatively low prevalence of primary HIV-1 DR.

In addition, it was found that DR variants of the virus from both ART-naïve and ART-experienced patients were less likely to fall into molecular clusters, which is likely due to the fact that most of them have significantly reduced fitness [26].

Despite the fact that HIV transmission in the Orel region was mainly from patients without ART experience, the sources of resistant HIV variants were presumably patients with ART experience. It is interesting to note that foreign studies have described that the source of HIV-1 DR variants was, on the contrary, patients without ART experience [27-29], which is probably due to higher ART efficacy rates in the countries.

An evaluation of transmitted mutations of HIV-1 DR found that the profile is limited to 9 mutations: *K103N*, *V179E/T*, *Y181C*, *G190S* to NNRTIs; *K65R*, *M184I/V* to NRTIs; and *L33F* to PIs, thus predicting which drugs will be ineffective in the future. Thus, the most frequently transmitted mutation was *K103N*, which is associated with the emergence of HIV-1 resistance to NVP and EFV. The results of other studies have also shown that this mutation is transmitted more frequently than others [30, 31], which is due to the fact that viruses with this mutation and wild-type viruses have a similar fitness, as well as the fact that this mutation can persist in the patient's body for a long time [32, 33]. It is also important to note that the presence of this mutation in patients starting treatment with the TDF + 3TC + EFV regimen is associated with increased risks of virologic failure [34].

V179E/T mutations, which also occurred with high frequency in clusters, are associated with decreased response to NNRTI treatment (with the exception of DOR), but generally do not result in the occurrence of virologic failure [35]. However, in the presence of these mutations, it is not recommended to prescribe a regimen containing EFV if the patient has a high viral load at the start of treatment [36].

The next most common transmitted mutation in the clusters, *G190S*, is associated with a 200-fold and 130-fold reduction in HIV-1 susceptibility to NVP and EFV, respectively, and is often found in sub-subtype A6 viruses due to its predisposition to have this mutation [37]. It is noted that the fitness of virus containing this mutation is reduced [38].

The Y181C mutation reduces the susceptibility of the virus to all NNRTIs, especially NVP, and hardly impairs the fitness of the virus [39].

Transmitted mutations to the *M184I/V* NNRTI found in clusters are associated with high levels of HIV-1 resistance to 3TC, FTC and low levels to ABC, while increasing susceptibility to d4T, ZDV and TDF, allowing 3TC and FTC to be retained in regimens when they occur. Foreign studies have described that these mutations are often transmitted from patients with virologic failure of ART [40], and also persist for a long time in HIV reservoirs [41].

The *K65R* mutation is associated with decreased susceptibility to all NRTIs except ZDV, to which, on

[17] Clinical Recommendations "HIV infection in adults" (approved by the Ministry of Health of the Russian Federation). Moscow; 2020.

the contrary, it increases viral sensitivity. *M184V* and *K65R* mutations have been described to significantly reduce virus fitness [42, 43].

The only mutation to PIs, *L33F*, which was found in the clusters, is additional and only slightly affects the susceptibility of the virus to antiretroviral drugs.

The presence of the mutations described above in molecular clusters indicates their active transmission, but the greatest danger is posed by those mutations that have a high efficiency of transmission within these clusters. As a result of the efficiency assessment it was found that such mutations are *K103N*, *V179E/T*, *Y181C*, *G190S*, which cause resistance of the virus to the 1st generation NNRTIs – EFV and NVP.

Thus, based on the data obtained, we can conclude that there is no risk of increased transmission of HIV-1 DR in the Oryol region, as evidenced by the high degree of clustering of ART-naïve patients who have a relatively low level of primary resistance, the low degree of clustering of HIV DR variants, and the fact that transmission of mutations was found mainly in small and inactive clusters. The rapid and efficient transmission of mutations associated with virus resistance to 1st generation NNRTIs established in this study allows us to recommend limiting their use to prevent the spread of DR variants of HIV-1 in the region and to improve the efficacy of ART.

Conclusion

The results of molecular cluster analysis provide information on the peculiarities of HIV-1 DR variants distribution, in particular, on the dynamics of HIV-1 DR transmission, sources of resistant variants of the virus, efficiency of transmission of resistance mutations, which allows us to recommend this method for use within the framework of genomic epidemiological surveillance of HIV infection in Russia to develop prevention strategies to prevent transmission of DR variants of the virus and to improve the effectiveness of treatment.

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In vitro and *in vivo* tropism and biodistribution of recombinant simian adenovirus type 25

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Abstract

Introduction. Recombinant adenoviruses are widely used in the development of vaccines for a variety of infectious diseases. Despite numerous clinical trials, only a few types of human (types 5 and 26) and simian (isolate Y25) adenoviruses are currently used to produce vaccine formulations. Different types of adenoviruses vary in their cellular tropism, which plays a key role in their ability to elicit an immune response.

The **aim** of this study was to investigate the cellular tropism of the simian adenovirus type 25 *in vitro* and its biodistribution *in vivo* in comparison with human adenoviruses types 5 and 26.

Materials and methods. The efficiency of in vitro transduction was evaluated on 15 different cell lines using recombinant adenovirus vectors expressing the enhanced green fluorescent protein (EGFP) reporter gene. *In vivo* biodistribution and bioluminescence imaging were evaluated in BALB/c mice after administration of recombinant adenoviral vectors encoding the luciferase reporter gene. The acute toxicity of a recombinant simian adenovirus type 25 vector was assessed in mice and rats following intramuscular or intravenous administration.

Results. Recombinant simian adenovirus effectively transduces a wide range of cells. At the same time, a higher tropism to human glioblastoma cells (GL-6) was found compared to the other two studied adenoviruses. *In vivo* experiments have shown that recombinant adenoviruses are mainly localized at the injection site, and transgene expression persists for 21 days. Acute toxicity studies demonstrated that simian adenovirus type 25 vector was well-tolerated, with no animal deaths or detectable toxic effects.

Conclusion. The new platform based on the recombinant simian adenovirus type 25 is not inferior to the existing and well-established delivery systems based on human adenoviruses types 5 and 26. Due to ihigh transduction leveland favorable safety profile, the use of the simian adenovirus type 25 in medicine has the potential to offer many benefits for the development of vaccines against future infectious diseases.

Keywords: *adenovirus vector, human adenovirus, simian adenovirus, tropism, biodistribution, acute toxicity*

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 Committee on Biomedical Ethics of the Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, Russia (protocol No. 30, October 28, 2022).

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Оригинальное исследование
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Исследование тропизма и биораспределения рекомбинантного аденовируса обезьян 25-го серотипа *in vitro* и *in vivo*

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Аннотация

Введение. Рекомбинантные аденовирусы (rAd) широко используются для разработки вакцин против ряда инфекционных заболеваний. Несмотря на большое количество клинических исследований, на сегодняшний день только несколько серотипов аденовирусов человека (5-й и 26-й серотипы) и обезьян (изолят Y25) на постоянной основе применяются для создания вакцинных препаратов. Различные серотипы rAd отличаются тропностью к клеткам, что играет ключевую роль в их способности к индукции иммунного ответа.

Цель работы — изучить клеточный тропизм *in vitro* и биораспределение *in vivo* rAd обезьян 25-го серотипа (SAd25) в сравнении с аденовирусами человека 5-го и 26-го серотипов.

Материалы и методы. Эффективность трансдукции *in vitro* оценивали на 15 клеточных линиях с использованием rAd, экспрессирующих репортерный ген *EGFP*. Биораспределение и биолюминесцентную визуализацию *in vivo* оценивали на мышах BALB/c с использованием rAd, экспрессирующих репортерный ген люциферазы. Острую токсичность SAd25 оценивали на мышах и крысах при внутримышечном и внутривенном введении.

Результаты. SAd25 эффективно трансдуцирует всю панель клеточных линий, при этом обнаружена более высокая тропность к клеткам глиобластомы человека (GL-6) по сравнению с двумя другими исследованными rAd. В экспериментах *in vivo* показано, что rAd в основном локализуются в месте введения, экспрессия трансгена сохраняется в течение 21 дня. В экспериментах по оценке острой токсичности SAd25 животные хорошо переносили введение препарата, гибель животных не зафиксирована, токсические эффекты не обнаружены.

Заключение. Новая платформа на основе SAd25 не уступает уже существующим и хорошо зарекомендовавшим себя системам доставки на основе аденовирусов человека 5-го и 26-го серотипов. Благодаря высокому уровню трансдукции и благоприятному профилю безопасности SAd25 может предложить ряд преимуществ для разработки вакцин против новых инфекционных заболеваний.

Ключевые слова: *аденовирусный вектор, аденовирус человека, аденовирус обезьян, тропизм, биораспределение, острая токсичность*

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

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Introduction

Adenoviruses (Ad) are non-enveloped DNA-containing icosahedral-shaped viruses. The *Adenoviridae* family consists of 6 genera, including viruses of the *Mastadenovirus* genus that infect mammals, including humans [1]. Human Ads are divided into 7 species (*Mastadenovirus adami*, *Mastadenovirus blackbeardi*, *Mastadenovirus caesari*, *Mastadenovirus dominans*, *Mastadenovirus exoticum*, *Mastadenovirus faecale*, *Mastadenovirus russelli*) depending on their morphological, virological, serological and genetic characteristics. The fundamental biological properties of Ad species *Mastadenovirus caesari* (formerly human adenoviruses of subgroup C) have long been widely studied, which has made them popular targets for the development of vector systems for the delivery of foreign genetic information *in vivo* and *in vitro* [2, 3].

To date, significant progress has been made in the use of Ad as vector vaccines [3-6]. Over the past 10 years, vaccines for the prevention of Ebola virus disease and COVID-19 coronavirus infection have been approved and registered in Russia [7, 8]. The experience of vaccination during the COVID-19 pandemic proved the safety and efficacy of Ad-based vector vaccines. Three Ad platforms were used for large-scale vaccination: based on human Ad of type 5 (Ad5), type 26 (Ad26) and chimpanzee adenovirus (isolate Y25) [5, 7, 9]. Despite a large number of clinical studies, only a few human and chimpanzee Ad types (Ad6, Ad35, ChAd63, ChAd3) have been studied as the basis for vector vaccines [10]. Different types have distinct characteristics in cell tropism, which may play a key role in the induction of immune response by affecting the expression and distribution of the target antigen [11]. The key characteristics in selecting alternative types of Ad are low seroprevalence in the human population and the ability to induce high levels of specific immune responses to the target antigen.

Previously, a technology platform based on simian Ad type 25 (SAd25) was developed [12]. **The aim** of this study was to compare the tropism of Ad5, Ad26 and SAd25 *in vitro* and *in vivo*.

Materials and methods

Cell lines

15 cell lines of different origins were used in the experiments:

1) human cells: HEK 293 (embryo kidney cells transformed by E1 region of Ad5), H292 (lung mucoepidermoid carcinoma cells), H460 (large cell lung carcinoma cells), H1299 (non-small cell lung cancer cells), A549 (lung adenocarcinoma cells), A431 (epidermoid carcinoma cells), GL-6 (glioblastoma cells), LHR-T (embryonic lung cells), HeLa (cervical carcinoma cells);

2) monkey cells: two green monkey kidney cell lines (CV-1 and Vero E6);

3) mouse cells: L929 (fibroblast cells) and EPNT-5 (glioblastoma cells);

4) hamster cells: CHO (Chinese hamster ovary cells) and BHK-21 (newborn Syrian hamster kidney cells).

Recombinant adenoviruses

Recombinant Ad5- and Ad26-vectors carrying the reporter gene of the enhanced green fluorescent protein EGFP (rAd5-EGFP and rAd26-EGFP), the luciferase gene (rAd5-Luc, rAd26-Luc) were obtained earlier [7, 12, 13]. To clone the luciferase gene or the SARS-CoV-2 virus glycoprotein S gene into the rSAd25 genome, we used the technique described previously [12]. The pArms-SAd25-Luc or pArms-SAd25-S-CoV2 carrying expression cassettes with the reporter gene or antigen were linearized and added to pSAd25-EGFP containing the ΔE1/ΔE3 gene. After electroporation in *E. coli* BJ5183 cells, homologous recombination resulted in pSAd25-Luc or pSAd25-S-CoV2 encoding the ΔE1/ΔE3 genome of SAd25 with an expression cassette. rAd was rescued and grown in HEK 293 cells.

Laboratory animals

All animal experiments were performed in strict compliance with the recommendations of the National Standard of the Russian Federation (GOST R 53434-2009 Principles of Good Laboratory Practice), and the methods used were approved by the Biomedical Ethics Committee of N.F. Gamaleya NRCEM (Protocol No. 30 of 28.10.2022). Six-week-old female BALB/c mice (18-20 g) were obtained from the Pushchino Nursery (Russia; accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care). Outbred (mongrel) mice and rats were obtained from the Andreevka branch of the Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency of Russia. The animals had free access to water and standard rodent food.

Determination of the infectious titer of adenoviruses

The quantity of infectious Ad particles was determined by an endpoint dilution assay TCID50 (50% tissue culture infectious dose) in HEK 293 cell culture. Cells were plated into 96-well plates at 3×10^4 cells per well. Then, serial 10-fold dilutions of virus were added in 8 repeats. The plates were incubated for 12–14 days, and the result was recorded visually by the presence of cytopathic effect (CPE). The virus titer was calculated according to the Reed-Mench formula and expressed in $TCID_{50}$/mL [14].

Determination of adenovirus replication

To study the kinetics of Ad replication, HEK 293 cell culture was plated into 96-well plates at the rate of 3×10^4 cells per well. Cells were then infect-

ed with rAd5-EGFP, rAd26-EGFP, and rSAd25-EGFP at a dose of 0.01 $TCID_{50}$/cell. Culture plates were incubated for 6–7 days at 37ºC and 5% CO_2. The results were recorded using a Lionheart FX Automated Microscope (BioTek Instruments Inc.).

Transduction of cell lines by recombinant adenoviruses

Two to three hours before transduction, cells were plated into 48-well plates at the rate of 10^5 cells per well. Cells were then transduced with rAd5-EGFP, rAd26-EGFP and rSAd25-EGFP at a dose of 1 $TCID_{50}$/cell. Culture plates were incubated at 37ºC and 5% CO_2. Fluorescence intensity was measured on a Synergy H1 Hybrid Multifunctional Reader (BioTek Instruments Inc.) using Gen5 Microplate Reader and Imager Software.

Administration of recombinant adenoviruses to animals

For biodistribution studies, rAd was injected once intramuscularly at a dose of 10^{10} or 10^{11} viral particles (v.p.) per animal ($n = 3$). Animals in control group were injected with sterile phosphate-buffered saline (PBS) (Paneco). The research protocol was approved by the Committee on Biomedical Ethics of the Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, Russia (protocol No. 30, October 28, 2022).

Determination of adenovirus DNA in animal organs and tissues

Animals were euthanized 24 h after rAd administration. Organs were harvested, weighed and homogenized in PBS. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). Viral DNA was determined by real-time polymerase chain reaction on a CFX 96 thermocycler (Bio-Rad): primers (5'-GGCGGCGGCTGGCGGTAGA-3' and 5'-GCAACATCTGGAACCGCGCG-3'), qPCRmix-HS SYBR mix (Eurogen). The initial denaturation step (5 min at 95ºC) was followed by 40 cycles of 15 s at 95ºC, 30 s at 61ºC and 30 s at 72ºC. The data were processed using Bio-Rad CFX Manager software.

Bioluminescence imaging in vivo

Luciferin (Promega; 2.5 mg/animal) was diluted in PBS and injected intraperitoneally into mice on days 1, 3, 7, 14 and 21 after rAd administration. Animals were anesthetized with isoflurane (Piramal Critical Care) for 5-10 min, followed by bioluminescence imaging on an IVIS Lumina Series II instrument (Caliper). Exposure times were adjusted to avoid pixel oversaturation, and flux measurements were converted to photons per second for comparative evaluation of luminescence at different time points. Luminescence image data were analyzed using the Living Image v. 4.2 software.

Methods of acute toxicity assessment

In the toxicity experiment, the effect of rSAd25-S-CoV2 on experimental animals (mice and rats) was evaluated by intramuscular (for mice and rats) and intravenous (for mice) administration. For each route of administration, 4 groups of outbred mice of both sexes (10 females and 10 males per group) were formed and injected with rAd at different doses (10^9, 10^{10} and 10^{11} v.p. per animal). The control group was injected with PBS. Three groups of rats (10 females and 10 males per group) were formed and injected with rAd at different doses (10^{10} and 10^11 v.p. per animal). The control group was injected with PBS. After a single injection of the drug, the animals were monitored for 14 days with daily clinical examination. Parameters of functional state, appearance, physiologic functions were recorded. On days 0, 7 and 14, the animals were weighed. On the 14th day animals were euthanized and complete necropsy was performed. At necropsy, the external state of the body, internal surfaces and passages, cranial cavity, thoracic, abdominal and pelvic cavities with organs and tissues in them, neck with organs and tissues, and skeletal-muscular system were examined.

Statistical analysis

Data were processed using the following computer programs: GraphPad 8.0 and Microsoft Excel. For quantitative results, arithmetic mean, geometric mean, standard error of the mean and standard deviation were calculated. When analyzing data from unrelated samples, Student's test, Mann–Whitney test or Kraskell–Wallis test were used. The a-priori level of significance was taken as $\alpha = 0.05$. Differences were considered statistically significant at the achieved significance level $p < \alpha$.

Results

Construction of recombinant vectors based on simian adenovirus serotype 25

A recombinant replication-defective vector based on SAd25 with an *EGFP* reporter gene (rSAd25-EGFP) was obtained earlier [12]. Recombinant vectors carrying the luciferase gene (rSAd25-Luc) and the S-protein gene of SARS-CoV-2 coronavirus (rSAd25-S-CoV2) were obtained in a similar manner (**Fig. 1**, *a*).

To evaluate the reproductive efficiency of the recombinant vector based on the simian adenovirus in permissive culture, rSAd25-EGFP carrying the gene of a fluorescent protein reporter was used. rAd5-EGFP and rAd26-EGFP carrying a similar transgene served as comparison vectors. HEK 293 cells were infected with the studied Ads at a rate of approximately 1 infectious particle per 100 cells. Visual evaluation of the results was performed starting 96 h after transduction every 16 h (**Fig. 1**, *b*).

After 96 h, intense fluorescence induced by recombinant viral vectors was observed. In contrast to the

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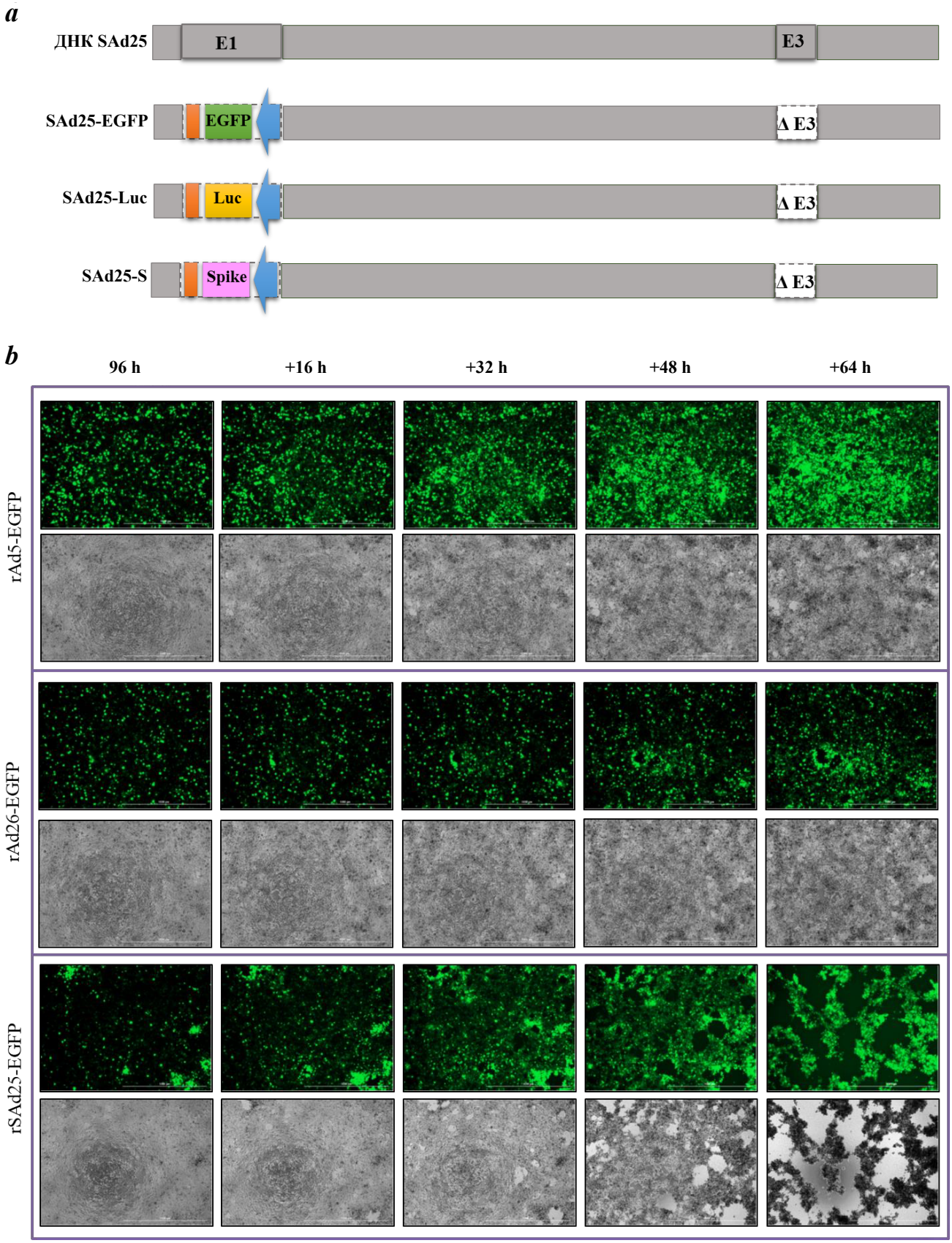

Fig. 1. Schematic representation of the recombinant SAd25 genomes (*a*) and comparison of the reproduction efficiency of rSAd25-EGFP, rAd5-EGFP and rAd26-EGFP in HEK 293 (*b*).

comparison vectors, the formation of fluorescent focuses was detected only in wells with rSAd25-EGFP, indicating a higher rate of accumulation of viral progeny in the infected cells. Over time, the focuses increased in size, leading to cell monolayer lysis and a pronounced cytopathic effect. Thus, the rSAd25 vector has lytic potential; replication leads to significantly greater cell damage compared to rAd5 and rAd26.

An in vitro study of rSAd25-EGFP tropism

To determine the tropism of rSAd25, the transduction efficiency of different cell types (human, hamster, monkey, mouse origin) was examined in comparison with the commonly used vectors rAd5 and rAd26. The investigated replication-defective vectors contained the EGFP gene under the control of the cytomegalovirus promoter, which provides similar levels of expression after transduction. All cell lines were transfected with rSAd25-EGFP, rAd5-EGFP, and rAd26-EGFP at a dose of 1 $TCID_{50}$/cell. The indicated dose of virus, on the one hand, has no cytotoxicity and, on the other hand, allows to expect that each cell will contain approximately only 1 v.p. Transduction with a higher dose leads to infection of one cell with several viral particles and, consequently, transgene expression is not linear. Transduction efficiency was assessed by the level of EGFP fluorescence in cells and expressed as relative units. The total value was determined by subtracting the fluorescence level of intact cells from the fluorescence level of transduced cells (**Fig. 2**).

EGFP expression was detected in all studied cell lines. The transduction of Vero E6 monkey cells differed when different adenoviral vectors were used. The highest level of EGFP expression in Vero E6 cells was observed for rSAd25-EGFP.

The investigated adenoviruses penetrated into hamster (BHK-21, CHO) and mouse (L929, EPNT-6) cells with comparable efficiency.

The tropism of rSAd25-EGFP, rAd5-EGFP and rAd26-EGFP differed in human cells. SAd25-EGFP penetrated human glioblastoma GL-6 cells with higher efficiency. The fluorescence level of rSAd25-EGFP in human lung carcinoma H292 and H460 cells was significantly higher than that for rAd5-EGFP. Meanwhile, in another lung carcinoma cell line (H1299), rAd26-EGFP showed an advantage.

In vivo biodistribution assessment of recombinant adenoviruses

The biodistribution features of rAd5-EGFP, rAd26-EGFP and rSAd25-EGFP were studied in experiments on BALB/c mice using two methods: viral genome detection and transgene expression. 24 h after intramuscular injection of rAd at a dose of 10^{10} v.p., the number of Ad genome copies in tissues and organs was analyzed by real-time polymerase chain reaction (**Fig. 3**).

Among the 14 selected organs and tissues, viral DNA was mainly detected in muscle samples from the injection site. Moreover, the copy number of rSAd25-EGFP DNA in muscle was significantly higher than that of rAd26-EGFP and rAd5-EGFP DNA, respectively. In addition to the injection site, small amounts of rAd5 genomes were detected in the lower lymph nodes as well as in the blood (rSAd25 and rAd5).

For bioluminescent imaging, we used rAds expressing the luciferase gene, which were injected into animals intramuscularly at a dose of 10^{11} v.p. For all Ad types, the bioluminescent signal was detected only at the injection site (**Fig. 4**). After a single injection of rAd5-Luc, rAd26-Luc, or rSAd25-Luc, the highest lu-

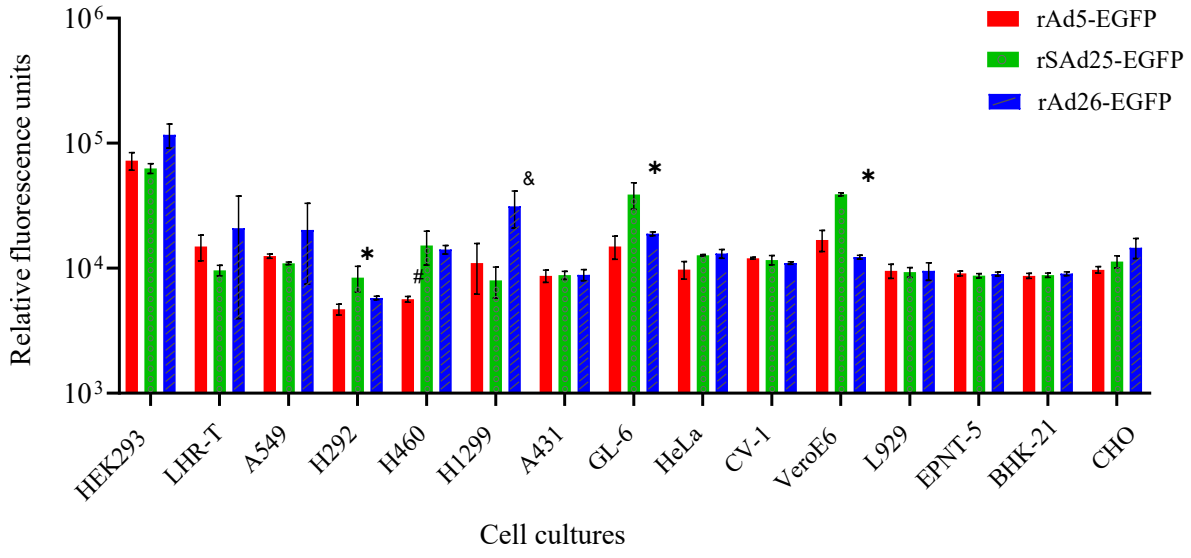

Fig. 2. Tropism of rAd5-EGFP, rAd26-EGFP and rSAd25-EGFP to different mammalian cell cultures.
* — statistically significant difference with rSAd25-EGFP; & — statistically significant difference with rAd26-EGFP; # — statistically significant difference with rAd5-EGFP.

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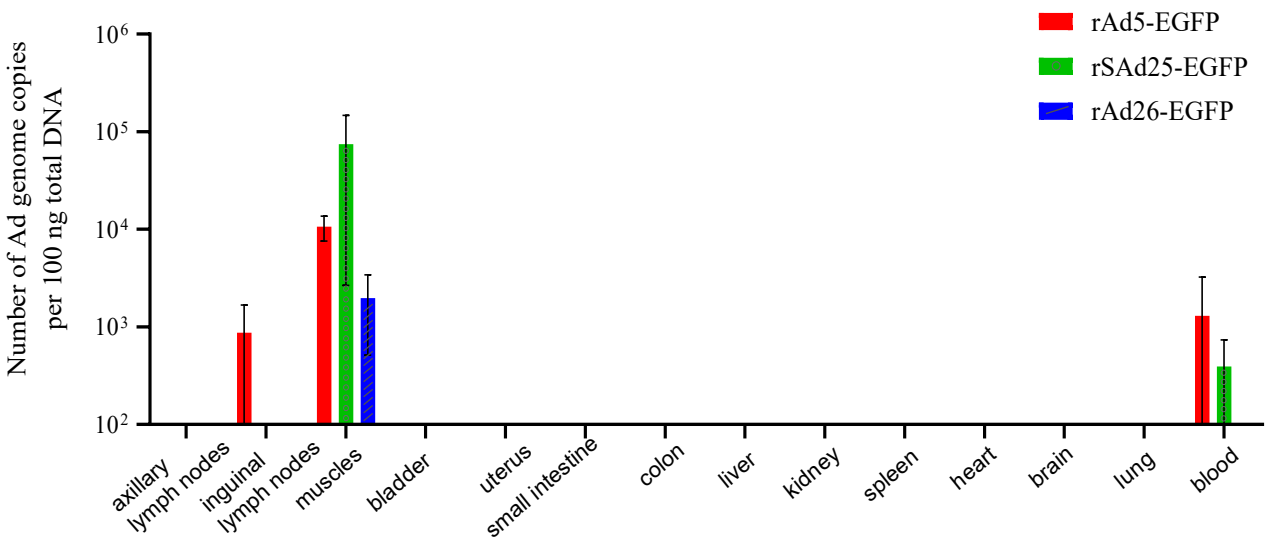

Fig. 3. Comparative analysis of adenovirus biodistribution in mice.
Each column represents the average number of genomes with a standard deviation.

ciferase expression was detected 1 day after injection (**Fig. 4**, *a*). The highest luciferase activity was observed in mice injected with rAd5-Luc, whereas the lowest activity was observed in the group of mice with rAd26-Luc. Luciferase activity gradually decreased by day 21 for all adenoviruses (**Fig. 4**, *b*). However, the smallest drop in transgene expression level (approximately 100-fold) was observed in mice injected with rSAd25-Luc. For rAd5-Luc and rAd26-Luc, luciferase activity decreased more than 250 and 400 times, respectively (**Fig. 4**, *c*).

Acute toxicity study of rSAd25

The acute toxicity of rSAd25 expressing the target gene of protein S of SARS-CoV-2 virus (rSAd25-S-CoV2) was studied in mice and rats of both sexes a single intramuscular or intravenous injection. The use of rAd with the target antigen allows the most adequate assessment of toxicity of both the vector itself and the insertion.

The tested animals (mice and rats) tolerated well both intramuscular and intravenous administration of adenovirus preparation in all doses. During the observation period, no animal died and no clinical signs of intoxication were observed. Integral indices of animal condition did not differ between experimental and control groups. Positive dynamics of body weight change was observed in all groups (**Fig. 5**).

Macroscopic examination of mice revealed no effect of rSAd25 on the state of internal organs; no differences were found between control and experimental groups. Macroscopic examination of male rats revealed differences between experimental and control groups. In 4 out of 10 male rats injected with 10^{11} v.p. and in 2 out of 10 male rats injected with 10^{10} v.p., areas of decreased airiness of lung tissues (atelectasis) and foci of coagulation necrosis with signs of organization were observed. It is important to note that no pathomorphologic changes of lungs were detected in the studied females. The results of macroscopic examination of other internal organs of rats of experimental groups did not differ from those of the control group.

Discussion

Ad-based vectors are excellent tools for the delivery of foreign genetic information into mammalian cells due to their large packaging capacity and high functional titers. Ad vectors are widely used for the development of vector vaccines and gene therapy drugs. However, their use may be limited when target cells lack receptors involved in attachment and internalization. In this case, the use of vectors based on alternative types of Ads will be significant only if the reproductive potential of the selected vector is high.

The standard strategy for producing recombinant replication-defective Ad vectors is to delete the E1 region of the genome. One important aspect of successful complementation of E1-deleted Ad is the functional interaction of the E1B 55K protein (produced by a trans-complementary cell line) with the E4 34K protein in the virus genome. At the same time, the development of complementary cell lines for different types of replication-defective vectors is a labor-intensive process. Therefore, the availability of non-human Ad types capable of replicating in cells such as HEK 293 (human cells transformed with E1 region of Ad5 genome) is a great advantage. Although the sequence similarity between the E1B 55K proteins of Ad5 and SAd25 is about 56%, rSAd25-EGFP was produced and accumulated in high titers in HEK 293 cell culture without additional viral genome modifications. The results obtained differ significantly from the data of other studies, which show

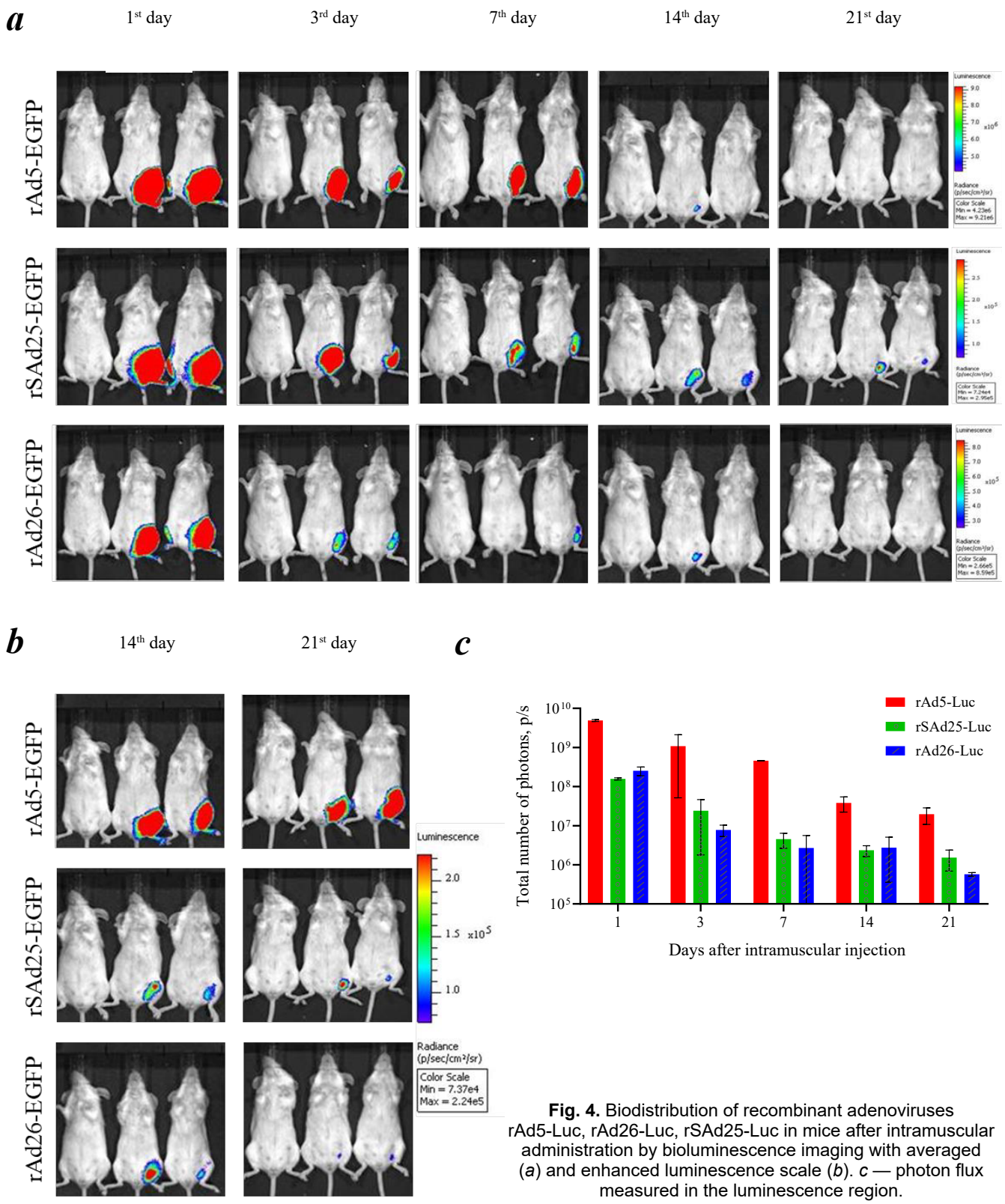

Fig. 4. Biodistribution of recombinant adenoviruses rAd5-Luc, rAd26-Luc, rSAd25-Luc in mice after intramuscular administration by bioluminescence imaging with averaged (*a*) and enhanced luminescence scale (*b*). *c* — photon flux measured in the luminescence region.

that the replacement of open reading frame 6 in the E4 region is necessary for successful replication of the recombinant vector [15].

The nature of Ad receptor expression on the surface of cells (both primary and secondary) determines the tropism of the virus, which determines the range of vector applications. SAd25 uses CAR (coxsackie and adenovirus receptor) as a primary cellular receptor, like most other Ad types [16, 17]. The amino acids involved in the interaction with CAR are located in the loop of AB knob-domain of the fiber. The key amino acids in Ad5 are Ser408, Pro409, Lys417 and the corresponding

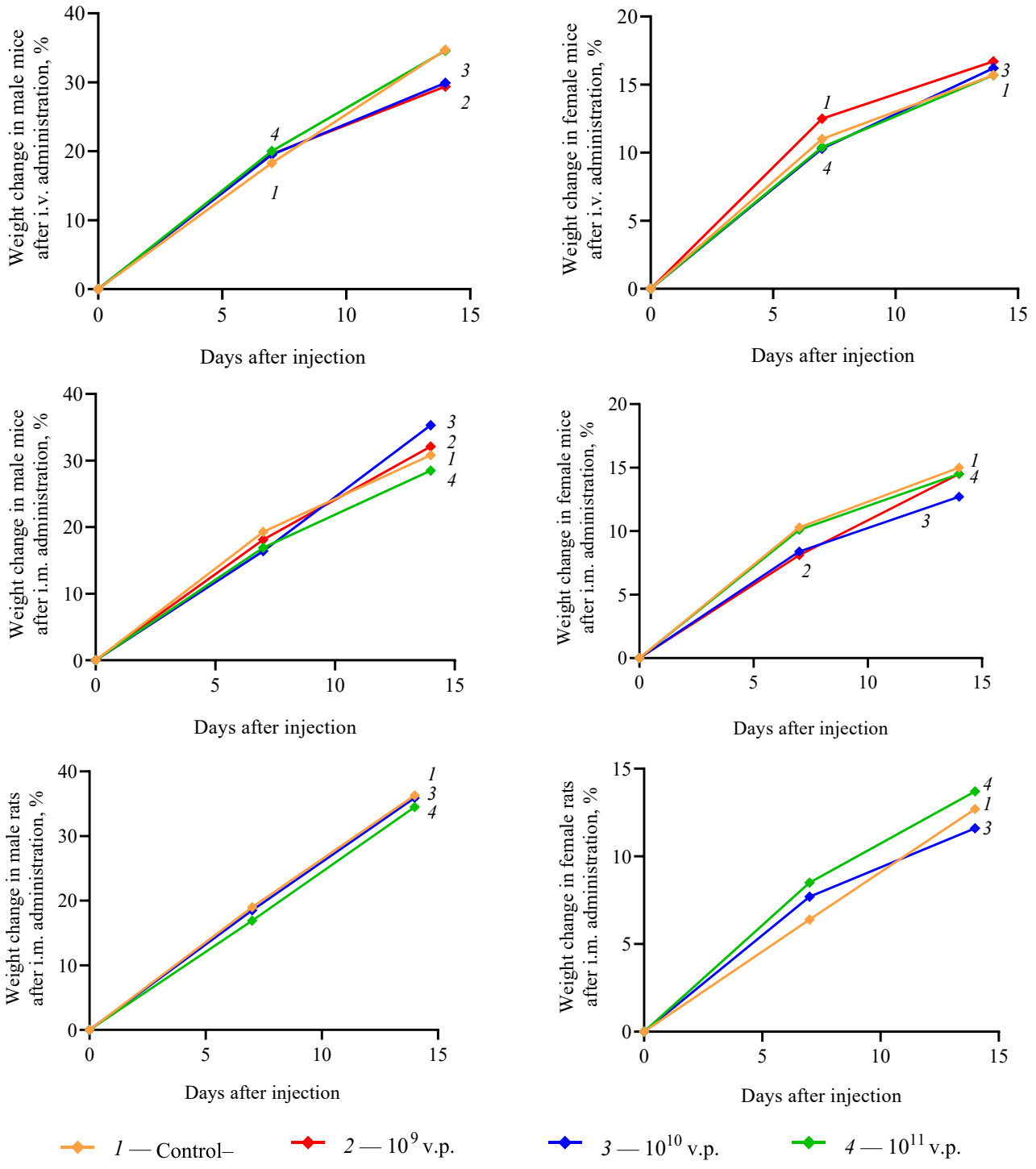

Fig. 5. Study of acute toxicity of rSAd25-S-CoV2 in mice and rats.
i.v. — intravenous; i.m. — intramuscular.

amino acids in other types: Ser196, Pro197, Lys205 in Ad26 and Ser255, Pro256, Lys267 in SAd25 [17]. The Ad5 and SAd25 fiber sequences are 63.9% similar, and it is not surprising that both recognize the same receptor. Like Ad5 and Ad26, SAd25 uses αv-integrins as a secondary receptor for internalization due to the presence of an RGD (Arg-Glu-Asp) sequence at the base of the penton [18].

To better understand the tropism of SAd25, we analyzed the transduction efficiency of SAd25 versus Ad5 and Ad26 using replication-defective vectors expressing EGFP (SAd25-EGFP, Ad5-EGFP, Ad26-EGFP). Experimental results showed that SAd25 efficiently transduced all cell lines tested. Its broad transduction profile was attributed to its interaction with CAR and αv-integrins. However, interaction with these receptors

is not the only factor determining the cellular tropism of the virus. The observed differences in Ad tropism of subgroups C, D, and E can be significantly influenced by the length of the fiber, as it is the main factor that determines the strategy of Ad attachment to the cell. Therefore, an interesting aspect of our analysis was the increased tropism of SAd25 to human neuroblastoma cells. Further studies are needed to specifically define the mechanism of virus entry into these cells.

In the present study, we showed the biodistribution of SAd25 by viral DNA detection in organs or tissues and transgene expression. It should be noted that these two indicators do not necessarily coincide with each other for several reasons: the same promoter may have variable activity in different cell types; different virus types may have different fates after entering the same cell type; degraded virus in the lysosome can no longer express the transgene, but viral DNA is still detectable.

Considering that the intramuscular route is the most commonly used vaccination strategy, a comparison of vectors based on different Ad types after a single injection was performed. Local expression of the luciferase gene continued for 3 weeks and gradually decreased. The expression level after rSAd25-Luc injection was lower than after rAd5-Luc but higher than after rAd26-Luc injection. Thus, the data showed that SAd25 could be an excellent vector for vaccine development, along with Ad5 and Ad26.

The results of acute toxicity determination demonstrated that a single intramuscular or intravenous administration of SAd25 to mice at doses exceeding the equitherapeutic dose by 100 and 1000 times was well tolerated by the animals. Death and clinical manifestations of toxic reactions were not observed. A single intramuscular administration of SAd25 to rats in the range of the studied doses did not result in significant toxic effects. Certain male rats showed pathomorphologic changes in the lungs, which were not accompanied by clinical manifestations. No other toxic reactions were detected. It is not clear whether these changes were related to Ad-vector administration or caused by other factors, and whether the changes were temporary or permanent. These factors require further investigation, including a full cycle of preclinical studies. In summary, the data obtained are consistent with the results obtained for other vectors based on different Ad vectors [19–21].

Conclusion

The new SAd25-based platform is not inferior to the existing and well-established Ad5 and Ad26-based platforms. Given the current challenges, such as the emergence of new viral infections, such as the COVID-19 pandemic, and the return of known pathogens to the population, the use of simian Ad can significantly accelerate the process of developing and introducing new vaccines. Ultimately, this will contribute to the improvement of public health, both in Russia and abroad.

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Current data on the circulation of the Q fever pathogen in the Republic of Guinea

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Abstract

Background. Q fever is the one of the best-studied zoonoses, which is widespread throughout almost the entire territory of Africa, excluding the territory of the Sahara. However, the current data on the incidence of coxiellosis and the circulation of *Coxiella burnetii* on this continent are limited and vary according different sources.
In 1980–1990, the Soviet-Guinean Research Virology and Microbiology Laboratory conducted studies to estimate the distribution of the Q fever pathogen, assess the herd immunity in humans and identify specific antibodies in the sera of livestock. However, in subsequent years, the research was suspended.
The aim of this study is to obtain up-to-date data on the distribution of *C. burnetii* in all landscape and geographical zones of the Republic of Guinea.
Materials and methods. The study was carried out in the laboratory of the Russian-Guinean Center for Epidemiology and Prevention of Infectious Diseases (Kindia, Republic of Guinea). The study involved 332 sera of febrile patients and 3156 sera from practically healthy volunteers, 1074 blood samples of livestock, 1648 suspensions of ticks, 319 specimens of small mammals and 298 of bats. The study was carried out using ELISA and PCR methods, selected samples were subjected to in-depth genetic analysis.
Results and discussion. The study of the distribution of *C. burnetii* on the territory of all landscape-geographical zones of the Republic of Guinea was carried out. For the first time, an officially registered case of human Q fever case has been identified. The role of livestock, small mammals and bats in the circulation of the pathogen has been established. It has been shown that the main vectors in Guinea are ixodid ticks of the *Amblyomma variegatum, Hyalomma truncatum and Rhipicephalus decoloratus* species. Employing molecular methods, *C. burnetii* strains carrying the QpH1 plasmid, capable of causing diseases in humans and animals were identified. For the first time, the complete nucleotide sequence of 16S rRNA of the Q fever pathogen (OQ152497–OQ152500) identified on the territory of Guinea was determined and registered in the GenBank database.
Conclusion. Taking into account the high epidemiological significance of Q fever, the study of the specifics of *C. burnetii* circulation in Guinea remains an urgent task. Regular monitoring and assessment of risk factors for diseases caused by coxiella are necessary for the development of an algorithm for laboratory diagnosis and recommendations for clinicians.

Keywords: *Q fever, Coxiella burnetii, cases of the disease, immune stratum, carriers and vectors, Republic of Guinea*

Ethics approval. The study was conducted with informed consent of patients or their official representatives. The study protocol was approved by the decision of the National Ethical Committee of the Ministry of Health of the Republic of Guinea (protocol No. 129/CNERS/16 of August 31, 2015). The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the "Consensus Author Guidelines for Animal Use" (IAVES, July 23, 2010). The study was approved by the Bioethics Commission of the Russian Anti-Plague Institute "Microbe" (Protocol No. 8 of November 11, 2023).

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Современные данные о циркуляции возбудителя лихорадки Ку на территории Гвинейской Республики

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Аннотация

Введение. Лихорадка Ку является наиболее изученным зоонозом, широко распространённым практически на всей территории Африки, исключая территорию Сахары. Однако сведения о заболеваемости коксиеллезом и циркуляции *Coxiella burnetii* на этом континенте являются ограниченными и неоднородными. В Гвинейской Республике в 1980–1990 гг. на базе Советско-Гвинейской научно-исследовательской вирусологической и микробиологической лаборатории проводились исследования по изучению распространения возбудителя лихорадки Ку, получены данные об иммунной прослойке населения и выявлены специфические антитела в сыворотках крови сельскохозяйственных животных. В последующие годы исследования были приостановлены.

Цель работы — получение современных данных о распространении *C. burnetii* на территории всех ландшафтно-географических зон Гвинейской Республики.

Материалы и методы. Исследования проводили в лаборатории Российско-Гвинейского центра эпидемиологии и профилактики инфекционных болезней (Киндия, Гвинейская Республика), для чего были получены 332 сыворотки крови лихорадящих больных и 3156 сывороток крови практически здоровых людей, 1074 образца крови сельскохозяйственных животных, 1648 суспензий клещей, 319 экземпляров мелких млекопитающих и 298 — рукокрылых. Исследование проводили методами иммуноферментного анализа и полимеразной цепной реакции, отдельные образцы подвергали углублённому генетическому анализу.

Результаты и обсуждение. Проведено изучение распространения *C. burnetii* на территории всех ландшафтно-географических зон Гвинейской Республики. Впервые выявлен и подтверждён лабораторными методами случай заболевания человека лихорадкой Ку. Установлена роль сельскохозяйственных животных, мелких млекопитающих и рукокрылых в циркуляции возбудителя. Показано, что основными переносчиками на территории Гвинеи являются иксодовые клещи видов *Amblyomma variegatum, Hyalomma truncatum* и *Rhipicephalus decoloratus*. При проведении молекулярно-генетических исследований выявлены штаммы *C. burnetii*, несущие плазмиду QpH1, способные вызывать заболевания людей и животных. Определены полные нуклеотидные последовательности 16S рРНК возбудителя лихорадки Ку, обнаруженного на территории Гвинеи, которые в последующем зарегистрированы в базе данных GenBank (OQ152497–OQ152500).

Заключение. С учётом полученных сведений о распространении возбудителя лихорадки Ку актуальной задачей остается продолжение изучения особенностей циркуляции *C. burnetii* на территории Гвинеи. Регулярный мониторинг и оценка факторов риска возникновения заболеваний, вызываемых коксиеллами, позволят разработать алгоритм лабораторной диагностики и рекомендации для врачей.

Ключевые слова: *лихорадка Ку, Coxiella burnetii, случаи заболевания, иммунная прослойка, носители и переносчики, Гвинейская Республика*

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Этическое утверждение. Исследование проводили при информированном согласии пациентов или их официальных представителей. Протокол исследования одобрен решением Национального этического комитета Министерства здравоохранения Гвинейской Республики (протокол № 129/CNERS/16 от 31.08.2015). Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с «Consensus Author Guidelines for Animal Use» (IAVES, 23.07.2010). Исследование одобрено комиссией по биоэтике Российского противочумного института «Микроб» (протокол № 8 от 21.11.2023).

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

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Introduction

Q fever or coxiellosis is a common natural focal disease for humans and animals, the etiological agent of which is the bacteria *Coxiella burnetii* (family *Legionellaceae*, class *Gammaproteobacteria*), characterized by polymorphism of the clinical picture in humans and various mechanisms of transmission of the pathogen. In natural foci, the main vector of *C. burnetii* is considered to be ixodes, less frequently argas ticks, and the reservoir — wild mammals, among farm animals — small and large cattle [1, 2].

The disease in humans runs in the form of fever with general toxic symptoms with possible transition to a chronic form. Due to the widespread infection, a variety of transmission routes (contact, food, airborne and dust), Q fever is an important medical and social problem worldwide.

Furthermore, this infectious disease has an important veterinary significance, as it causes reproductive disorders (abortions and stillbirths) in small and large cattle, which causes significant economic losses, especially in those regions where livestock breeding is the main branch of agricultural production [2].

Coxiellosis is one of the best-studied zoonoses within Africa. There is emerging evidence that *C. burnetii* infection is responsible for non-malarial febrile illnesses and community-acquired pneumonia in many African countries. However, current information on the incidence of Q fever and circulation of *C. burnetii* in this continent is limited and heterogeneous [3].

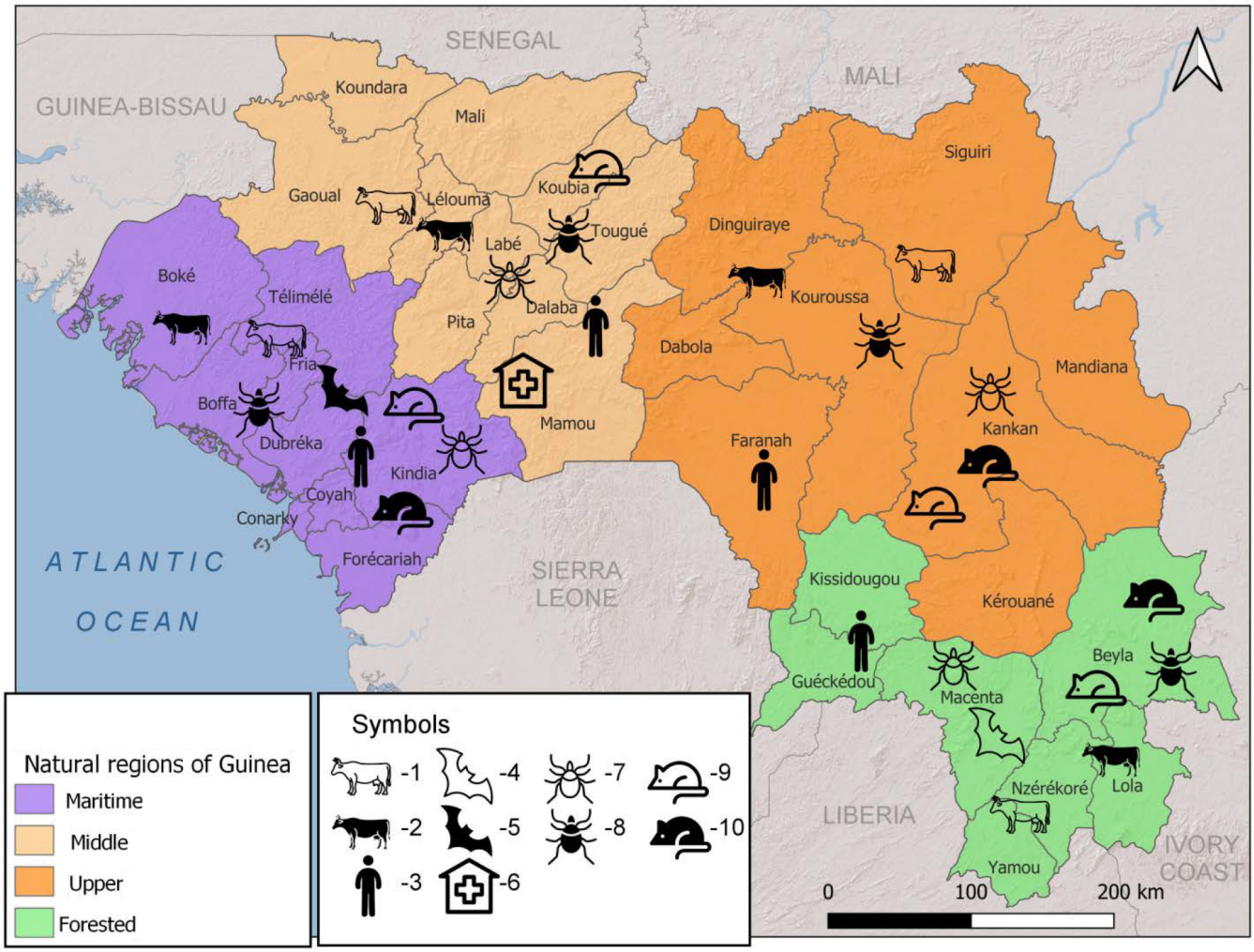
Available publications on previous studies indicate a fairly high level of detection of specific antibodies to the pathogen in the population of some African countries [4–9]. For example, the study of blood serum samples from residents of some settlements of the Sin-Saloum region (Republic of Senegal) showed that specific antibodies to *C. burnetii* were detected in 3.7–24.8% of samples (depending on the place of residence of the subjects) [5]. Similar data were obtained during serosurvey in pastoralist communities in Marsabit County, northern Kena, when positive results were obtained in 13.2% of cases [6]. Males in Kenya were significantly more likely than females to have immunologic markers identified [7].

It has been shown that pastoralist tribes are at the highest risk of contracting coxiellosis because of their nomadic lifestyle and well-preserved traditions, which increase the likelihood of eating unboiled dairy products and raw meat from infected animals. It is also possible that infection occurs through contact with urine, feces, and blood of infected animals, as well as amniotic fluid after abortion or premature birth [6–9].

It is also known that Q fever causes significant losses among not only farm animals but also wild animals such as antelopes, giraffes, lions and cheetahs, causing irreparable damage to the numbers of these rare mammals [10].

This infectious disease is also becoming relevant as a “travelers' disease” due to the popularization of the African continent and rapidly developing tourism in this territory. An outbreak of Q fever was described in the literature, when during a safari in a natural park in Kenya, 4 (8%) out of 50 participants of the tourist route were infected, which caused importation of cases to Europe [11].

Most of the information published in the open press on the circulation of the Q fever pathogen relates to East Africa. There is much less information on the situation in the western part of the continent. There are data on studies conducted in natural foci of coxiellosis in Ghana, Nigeria, Mali [12–14], as well as in Senegal, where in 2023 two new genotypes of *C. burnetii* were



Landscape and geographical zones of the Republic of Guinea, where markers of the Q fever pathogen were identified.

1 — DNA in blood sera of cattle; 2 — IgG antibodies in blood sera of cattle; 3 — IgG antibodies in blood sera of residents; 4 — DNA in suspensions of bat organs; 5 — antigens in suspensions of bat organs; 6 — cases of human disease; 7 — DNA in suspensions of ixodes ticks; 8 — antigens in suspensions of ixodes ticks; 9 — DNA in suspensions of organs of small mammals; 10 — antigens in suspensions of organs of small mammals.

identified in samples from small mammals, the pathogenicity of which remains to be studied [15].

The Republic of Guinea is located in West Africa, on the coast of the Atlantic Ocean. The population of the country, as of the end of March 2024, is about 14.5 million people [16]. Based on geographical and natural-climatic features, the territory of the state is conditionally divided into 4 landscape-geographical zones: Lower (Maritime), Middle, Upper and Forested (**Figure**) [17].

In 1980–1990, the Soviet-Guinean Research Virology and Microbiology Laboratory conducted a large number of studies to investigate the spread of the Q fever pathogen in the Republic of Guinea, including data on the immunity of the country's population and the detection of specific antibodies in the blood sera of farm animals [18]. In the following decades, due to the prevailing economic and political conditions, research was suspended, and the significance of Q fever in the overall morbidity has not been determined. In 2017, as part of the research work of the Russian-Guinean Center for Epidemiology and Prevention of Infectious Diseases (hereinafter referred to as the Center), established on the basis of orders of the Government of the Russian Federation on the premises of the Institute of Applied Biology of Guinea (Kindia), research was continued [19–21].

The aim of the study was to obtain up-to-date data on the distribution of *C. burnetii* and the features of natural foci of Q fever in different landscape and geographical zones of Guinea.

Materials and methods

Collection of samples of clinical and biological material and subsequent diagnostic work was carried out on the basis of the Center's laboratory by Russian and Guinean specialists, guided by the requirements of sanitary rules and regulations 3.3686-21 “Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases”.

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Human blood serum

Blood serum of practically healthy people was collected in regional hospitals in Guinea and by local specialists. Fasting blood sampling was performed in the morning hours from the ulnar vein in the amount of 5–10 ml into a disposable sterile vacuum tube with a clot activator in compliance with the rules of asepsis.

Then the obtained samples were delivered to the laboratory of the Center in compliance with the rules of biological safety and temperature regime. All serum samples were preliminarily analyzed by immunochromatographic analysis for detection of malaria plasmodium antigens with the SDBIOLINE Malaria Ag P.f./Pan reagent kit (Standart Diagnostics, Inc.). To exclude the possibility of nonspecific reactions, samples containing antigens of malaria pathogens were not included in subsequent studies.

A panel of 3156 sera from virtually healthy individuals living in all 4 landscape-geographic zones of Guinea was compiled to study the herd immunity to the Q fever pathogen in the Guinean population. Different age groups were included: 1519 (48.2%) women and 1637 (51.8%) men. The work was carried out using the enzyme-linked immunosorbent assay (ELISA) reagent kit "Enzyme-linked immunosorbent assay for detection of class G antibodies to *C. burnetii* antigens" (Pasteur Research Institute of Epidemiology and Microbiology, Russia). The sera were tested at a dilution of 1 : 100.

To clarify the possibility of human disease with Q fever, 332 sera from febrile patients who sought medical help in regional hospitals in Guinea with complaints of prolonged fever and other symptoms not excluding coxiellosis were collected and examined. The material was also collected by Guinean specialists using the methodology described above. For molecular genetic studies, blood was also collected in an amount of 5–10 ml in a disposable sterile vacuum tube with 3.8% sodium citrate.

Samples were examined by polymerase chain reaction (PCR) to detect *C. burnetii* DNA with the AmpliSens *Coxiella burnetii*-FL reagent kit (Central Research Institute of Epidemiology, Russia) and ELISA using the diagnostic kit *Coxiella burnetii* ELISA IgM (Vircell) to detect IgM antibodies to the pathogen.

The study of samples of clinical and biological material from humans was conducted with informed consent of patients, for minors — with the permission of parents (official representatives). The study protocol was approved by the decision of the National Ethical Committee of the Ministry of Health of the Republic of Guinea (protocol No. 129/CNERS/16 of August 31, 2015).

Blood serum of farm animals

Blood samples were obtained at slaughterhouses, using generally accepted methods, from adult animals (more than 1.5 years old) without signs of infectious diseases after their examination by a veterinarian. In total, a panel of 1074 cattle blood samples was formed for the work. The obtained sera were tested by ELISA and PCR methods using "ID Screen Q Fever Indirect Multi-species" reagent kits for detection of IgG antibodies specific to *C. burnetii* (ID Screen), which is recommended as a veterinary assay, and *C. burnetii* DNA was detected by the AmpliSens *Coxiella burnetii*-FL reagent kit (Central Research Institute of Epidemiology, Russia).

Tick suspensions

Ixodes ticks were collected in all 4 landscape-geographical zones of Guinea. Ectoparasites were removed manually, using personal protective equipment, from humans, farm animals, domestic and stray dogs and cats, small mammals, and reptiles. A total of 4709 specimens of ticks were collected during the studies, which were classified into 11 species based on morphological features: *Amblyomma variegatum* Fabricius, 1794; *Haemaphysalis leachi* Audouin, 1826; *Hyalomma rufipes* Koch, 1844; *Hyalomma truncatum* Koch, 1844; *Rhipicephalus (Boophilus) decoloratus* Koch, 1844; *Rhipicephalus (Boophilus) geigyi* Aeschliman & Morel, 1965; *Rhipicephalus (Boophilus) annulatus* Say, 1821; *Rhipicephalus (Boophilus) microplus* Canestrini, 1888; *Rhipicephalus lunulatus* Neumann, 1907; *Rhipicephalus sanguineus* Latreille, 1806; *Rhipicephalus senegalensis* Koch, 1844 according to the identification guide [22]. Further, 1648 pools were formed taking into account species, sex, developmental phase and fatness of individual mites, as well as places of ectoparasite collection. Ectoparasites were washed twice with 70% ethanol to remove external contaminants and external microflora. Samples were prepared using a TissueLyser II laboratory homogenizer (Qiagen) in 500 µl of sterile phosphate-salt buffer solution.

The obtained material was tested by PCR and ELISA methods using the AmpliSens *Coxiella burnetii*-FL reagent kit (Central Research Institute of Epidemiology) and ELISA-Qu-antigen (kit N1) test system for detection of *Coxiella burnetii* antigens (Pasteur Research Institute of Epidemiology and Microbiology), respectively. Some samples containing both DNA and antigens of *C. burnetii* were analyzed using high-throughput sequencing on the Ion S5 platform (Thermo Scientific); further reads were mapped to the 16S rRNA sequence from the NCBI GenBank database using the BWA algorithm [23]. The plasmid profile with specific primers to the QpH1, QpRS and QpDV plasmid loci was also studied.

Small mammal organ suspensions

During epizootological monitoring on the territory of Guinea, 319 specimens of small mammals (Rodentia, Eulipotyphla) were captured. The species spectrum of rodents was represented by 13 species: *Arvicanthis ansorgei* Thomas, 1910 (Sudan grass mouse); *Heliosciurus gambianus* Ogibly, 1835 (Gambian squirrel);

Cricetomys gambianus Waterhouse, 1840 (Gambian hamster rat); *Lemniscomys striatus* Linnaeus, 1758 (striped mouse); *Lophuromys sikapusi* Temminck, 1853 (rusty-bellied stiff-necked mouse); *Mastomys erythroleucus* Temminck, 1853 (Guinean multispotted mouse); *Mastomys natalensis* A. Smith, 1834 (Natal mouse); *Mus minutoides* A. Smith, 1834 (dwarf mouse); *Mus musculoides* Temminck, 1853 (Temminck's mouse); *Praomys daltoni* Thomas, 1892 (Dalton's mouse); *Rattus rattus* Linnaeus, 1758 (black rat); *Crocidura olivieri* Lesson, 1827 (African giant whitetooth); *Crocidura* sp. Wagler, 1832 (whitetooths) [24–28].

We obtained 298 specimens of bats (Chiroptera) of 14 species: *Eidolon helvum* Kerr, 1792 (palmate wing); *Epomophorus gambianus* Ogilby, 1835 (great epaulet wing); *Lissonycteris angolensis* Bocage, 1898 (Angolan flying dog); *Rousettus aegyptiacus* E. Geoffroy, 1810 (Egyptian flying dog); *Chaerephon pumillus* Cretzschmar, 1830 (dwarf folding spoonbill); *Mops condylurus* A. Smith, 1833 (Angolan folding spoonbill); Hipposideros caffer Sundevall, 1846 (South African leafhopper); *Hipposideros jonesi* Hayman, 1947 (Jones' leafhopper); *Hipposideros ruber* Noack, 1893 (red leafhopper); *Nycteris hispida* Schreber, 1775 (shaggy slitmouth); *Neoromicia guineensis* Bocage, 1889 (Guinean leatherback); *Scotophilus dinganii* A. Smith, 1833 (African smooth-nosed platypus); *Scotophilus leucogaster* Cretzschmar, 1830 (white-bellied house smooth-nosed platypus); *Rhinolophus alcyone* Temminck, 1853 (Ghanaian horseshoe) [24-27].

Mammals were dissected in compliance with the rules of biological safety no later than 3 h after capture. In the absence of such an opportunity, animal carcasses were frozen at –20ºC and delivered to the laboratory, where they were subsequently dissected and organ samples were taken. Combined suspensions of lungs and kidneys were used as material for work, in which markers of the coxiellosis pathogen (DNA and antigens) were searched with the reagent kits mentioned above.

The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the "Consensus author guidelines for animal use" (IAVES July 23, 2010). The study was approved by the Bioethics Commission of the Russian Anti-Plague Institute "Microbe" (protocol No. 8 of November 21, 2023).

During statistical processing of the material we calculated the proportion of detected pathogen markers in each sample, 95% confidence intervals (CI) for the proportions using Wilson's method.

Results and discussion

Identification of human cases of Q fever in Guinea

To date, there has been no detection of suspected cases of Q fever in Guinea. This can be explained by the lack of alertness of health workers in local hospitals to this infectious disease. In this regard, a case of the disease reported for the first time in the country is of great interest.

Patient D., 28 years old, living in Mamou (Middle Guinea), was admitted to the infectious disease department of a regional hospital. At hospitalization, the patient had prolonged (more than 1 month), subfebrile fever, muscle and joint pains and breathing difficulties. The results of immunochemistry and microscopy tests for detection of malaria pathogens were negative.

For further differential diagnosis, the patient's blood serum was sent to the Center's laboratory. The obtained material was analyzed by reverse transcription PCR to detect RNA of Ebola, yellow fever, West Nile, dengue, Zika, Crimean-Congo hemorrhagic fever, hepatitis C, 16S RNA of leptospirosis pathogens, DNA of hepatitis B virus, pathogens of Q fever and rickettsioses. Based on the results, *C. burnetii* DNA was detected in the patient's serum. The material was also tested using ELISA and specific IgM antibodies to coxiellae were detected in a titer of 1 : 400. Subsequently, the obtained blood sample was used to determine the nucleotide sequence of the DNA of the Q fever pathogen. Partial sequencing with specific primers revealed 99% identity of the tested sample with the genome of *C. burnetii*. Phylogenetic analysis using the BLAST algorithm[1] showed 96% homology with strains isolated in Namibia.

When interviewed, patient D. mentioned close contact with farm animals, specifically owned cattle, and also indicated that there had been cases of spontaneous abortions of cattle in the patient's village.

Neither genetic markers of *C. burnetii* nor specific IgM antibodies to coxiellae were detected in the remaining serum samples from febrile patients transported from Guinea hospitals.

Determination of the herd immunity of Guineans to the Q fever pathogen

In the present study, specific IgG antibodies to the Q fever pathogen were detected in sera of participants of all age groups living in different zones of Guinea, and there was no dependence of the antibody detection rates on the sex of the examined persons (**Table 1**). In the whole country, specific immunoglobulins to *C. burnetii* were registered in 366 out of 3156 serum samples, which amounted to 11.6% (95% CI 10.5-12.8).

Determination of the herd immunity of farm animals to the Q fever pathogen

One of the indicators of circulation of pathogens of naturally occurring infectious diseases in a certain area is the detection of specific IgG immunoglobulins in blood sera of farm animals living in this region.

As a result of this work, antibodies to the Q fever pathogen were detected in 172 samples, which amount-

[1] URL: https://blast.ncbi.nlm.nih.gov/Blast.cgi

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Table 1. Identification of specific IgG antibodies to the Q fever pathogen in the blood sera of residents of the Republic of Guinea

Age, years	Number of samples						
	total	men			women		
		total	positive	% (95% CI)	total	positive	% (95% CI)
< 10	197	108	8	7.4 (3.8–13.9)	89	6	6.7 (3.1–13.9)
10–20	498	275	26	9.4 (6.5–13.4)	223	27	12.1 (8.4–17.0)
20–30	699	340	32	9.4 (6.7–12.9)	359	29	8.1 (5.7–11.4)
30–40	595	300	39	13.0 (9.7–17.3)	295	33	11.2 (8.1–15.3)
40–50	559	285	35	12.3 (8.9–16.6)	277	29	10.5 (7.4–14.6)
50–60	339	230	37	16.1 (11.9–21.4)	109	26	23.8 (16.8–32.6)
> 70	269	102	20	19.6 (13.1–28.3)	167	19	11.4 (7.4–17.1)
Total	3156	1637	197	12.1 (10.5–13.7)	1519	169	11.3 (9.6–12.8)

ed to 16.0% (95% CI 13.9-18.3). Positive results were recorded in all landscape-geographical zones).

Detection of specific markers (DNA and antigens) of the Q fever pathogen in tick suspensions

PCR and ELISA methods were used to examine suspensions of ixodid ticks of various species, which are the main vectors of *C. burnetii*, collected in all landscape and geographical zones of Guinea. The pathogen DNA was detected in 294 (17.9%) samples and antigen in 307 (18.7%). Positive findings were noted among all ixodid tick species represented in the work, but the majority were attributed to *Am. variegatum*, *Hy. truncatum*, *Rh. decoloratus* (**Table 2**).

Analysis of plasmid profiles is an important tool for studying the spread of Q fever and determining the type of the pathogen. For genetic typing of *C. burnetii*, a panel of 20 samples from different tick species was created, in which both DNA (Ct level not more than 15) and antigen of the pathogen were detected simultaneously. PCR with specific primers to the QpH1, QpRS and QpDV plasmid loci revealed the presence of QpH1 alone in 5 samples. Sequences of QpRS and QpDV plasmid fragments were not detected in any of the samples. In the course of analyzing the results obtained and literature data, it was found that strains carrying the QpH1 plasmid are widespread in Equatorial Africa and are capable of causing diseases in humans and animals

Table 2. Identification of *C. burnetii* markers in suspensions of ixodid ticks of different species collected in the territory of the Republic of Guinea

Tick species	Number of samples (copies)	The number of positive samples; % (95% CI)	
		PCR	ELISA
Am. variegatum	872 (2493)	159; 18.2 (15.8–20.9)	193; 22.1 (19.5–25.0)
Ha. leachi	16 (56)	0	0
Hy. truncatum	52 (95)	14; 26.9 (16.8–40.3)	16; 30.8 (19.9–44.3)
Rh. annulatus	58 (161)	36; 62.1 (49.2–73.4)	29; 50 (37.5–62.5)
Rh. decoloratus	391 (1104)	47; 12 (9.2–15.6)	37; 9.5 (6.9–12.8)
Rh. geigyi	210 (668)	36; 17.1 (12.7–22.8)	29; 13.8 (9.8–19.1)
Rh. microplus	18 (47)	0	1; 5.6 (1.0–25.8)
Rh. sanguineus	10 (42)	0	1; 10 (1.8–40.4)
Rh. senegalensis	18 (29)	2; 11.1 (3.1–32.8)	1; 5.6 (1.0–25.8)
Rh. lunulatus	2 (11)	0	0
Hy. rufipes	1 (3)	0	0
Total	1648 (4709)	294; 17.8 (16.1–19.8)	307; 18.6 (16.8–20.6)

[29, 30], which does not exclude the possibility of their circulation in Guinea.

The generated panel of samples was analyzed using high-throughput sequencing methods on the Ion S5 platform (Thermo Scientific). As a result, the nucleotide sequence of 16S rRNA of the Q fever pathogen was determined in 8 samples, which coincides 99.9% with the reference strain presented in the NCBI GenBank database. When comparing the obtained consensus sequence with the NCBI BLAST database, *C. burnetii* strains isolated in Namibia were found to show homology (96%) with the studied sample. Furthermore, the obtained reads were classified by the kraken2 algorithm using the Greengenes 16S RNA base, which also showed that the investigated sample belonged to the *C. burnetii* species. Some of the obtained 16S rRNA nucleotide sequences with the highest quality reads were deposited in the international GenBank database under the numbers OQ152497-OQ152500.

Detection of specific markers (DNA and antigens) of the Q fever pathogen in organ suspensions of small mammals

As a result of PCR and ELISA tests, markers of the Q fever pathogen were detected in the material collected in all zones of Guinea. Antigen was detected in 0.9% of samples and DNA in 5.1%. The maximum number of positive findings was obtained when samples from *Mastomys erythroleucus* rodents was examined (**Table 3**). These data may indicate the participation of animals of this systematic group in the spread of *C. burnetii* in Guinea.

Detection of specific markers (DNA and antigens) of the Q fever pathogen in organ suspensions of bats

Examination of pooled samples of lungs and kidneys obtained from bats revealed both *C. burnetii* antigens (1% of cases) and DNA (2%). The majority of positive samples were formed from organs of *Scotophilus leucogaster* (**Table 4**). Representatives of this species are widely distributed throughout sub-Saharan Africa. The data obtained confirm the role of bats in the circulation of the Q fever pathogen, which indicates the need for additional studies to determine the role of these mammals in the ecology of the pathogen.

Conclusion

As a result of this work, the distribution of *C. burnetii* in all landscape-geographical zones of Guinea was studied, including the first case of human case of Q fever identified and laboratory confirmed (**Table 5**). The role of farm animals, small mammals and bats in the circulation of *C. burnetii* has been established. It was shown that the main vectors of the pathogen on the territory of Guinea are ixodes ticks of *Am. variegatum*, *Hy. truncatum* and *Rh. decoloratus* species. Molecular genetic studies of the material collected in Guinea revealed *C. burnetii* strains carrying the QpH1 plasmid, which are capable of causing disease in humans and animals, and for the first time for this region the com-

Table 3. Identification of *C. burnetii* markers in suspensions of organs of small mammals of different species collected on the territory of the Republic of Guinea

Small mammal species	Number of samples (copies)	The number of positive samples; % (95% CI)	
		PCR	ELISA
Arvicanthis ansorgei	3	0	0
Heliosciurus gambianus	4	0	0
Cricetomys gambianus	7	1; 14.3 (2.6–51.3)	0
Lemniscomys striatus	3	0	0
Lophuromys sikapusi	1	0	0
Mastomys erythroleucus	124	8; 6.5 (3.3–12.2)	2; 1.6 (0.4–5.7)
Mastomys natalensis	32	2; 6.3 (1.7–20.1)	0
Mus minutoides	5	0	0
Mus musculoides	16	1; 6.3 (1.1–28.3)	0
Praomys daltoni	6	0	0
Rattus rattus	96	4; 4.2 (1.6–10.2)	1; 1.0 (0.2–5.7)
Crocidura olivieri	7	0	0
Crocidura sp.	15	0	0
Total	319	16; 5.0 (3.1–8.0)	3; 0.9 (0.3–2.7)

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Table 4. Identification of *C. burnetii* markers in organ suspensions of bats of various species collected on the territory of the Republic of Guinea

Bat species	Number of samples (copies)	The number of positive samples; % (95% CI)	
		PCR	ELISA
Eidolon helvum	1	0	0
Epomophorus gambianus	4	0	0
Lissonycteris angolensis	4	0	0
Rousettus aegyptiacus	15	0	0
Chaerephon pumillus	3	0	0
Mops condylurus	26	1; 3.8 (0.7–18.9)	0
Hipposideros caffer	32	0	0
Hipposideros jonesi	25	0	0
Hipposideros ruber	41	1; 2.4 (0.4–12.6)	1; 2.4 (0.4–12.6)
Nycteris hispida	5	0	0
Neoromicia guineensis	23	1; 4.3 (0.8–21.0)	0
Scotophilus dinganii	1	0	0
Scotophilus leucogaster	117	3; 2.6 (0.9–7.3)	2; 1.7 (0.5–6.0)
Rhinolophus alcyone	1	0	0
Total	298	6; 2.0 (0.9–4.3)	3; 1.0 (0.3–2.9)

plete nucleotide sequence of the 16S rRNA gene of the Q fever pathogen was determined and registered in the international GenBank database.

Our study allowed us to expand the data on the circulation and distribution of *C. burnetii* in West Africa. The study of the specifics of *C. burnetii* circulation in Guinea remains an urgent task. Systematically obtained data on the detection of the pathogen and assessment of risk factors for outbreaks of diseases caused by coxiellae are necessary for the development of an algorithm for laboratory diagnosis and recommendations for clinicians. Regular monitoring of the spread of Q fever, carried out with the participation of both medical and veterinary services in Guinea, will make it possible to forecast the epidemiological situation and coordinate preventive (anti-epidemic) measures within the framework of the One Health concept[2].

[2] WHO. One health. URL: https://www.who.int/health-topics/one-health#tab=tab_1 (дата обращения: 26.05.2024).

Table 5. Identification of markers of the Q fever pathogen in various materials collected on the territory of the Republic of Guinea

Type of the studied samples	Number of samples	The number of positive; % (95% CI)		
		PCR	ELISA	
		DNA	antigen	IgG
Lower (Maritime) Guinea				
Blood serum of practically healthy people	943	N. i.	N. i.	106; 11.2 (9.4–13.4)
Blood serum of cattle	371	6; 1.6 (0.7–3.5)	N. i.	64; 17.2 (13.7–21.4)
Suspensions of ticks	624	111; 17.8 (15.0–21.0)	131; 21.0 (18.0–24.4)	N. i.
Suspensions of organs of small mammals	149	8; 5.4 (2.7–10.2)	0; 0 (0–2.5)	N. i.
Suspensions of bat organs	107	0; 0 (0–3.4)	3; 2.8 (0.9–7.9)	N. i.
Middle Guinea				
Blood serum of practically healthy people	778	N. i.	N. i.	82; 10.5 (8.6–12.8)
Blood serum of cattle	257	3; 1.2 (0.4–3.4)	N. i.	49; 19.2 (14.7–24.3)
Suspensions of ticks	402	71; 17.7 (14.2–21.7)	77; 19.1 (15.6–23.3)	N. i.
Suspensions of organs of small mammals	55	3; 5.4 (1.9–14.8)	0; 0 (0–6.5)	N. i.
Suspensions of bat organs	61	0; 0 (0–5.8)	0; 0 (0–5.8)	N. i.
Upper Guinea				
Blood serum of practically healthy people	655	N. i.	N. i.	77; 11.8 (9.5–14.4)
Blood serum of cattle	182	2; 1.1 (0.3–3.9)	N. i.	35; 13.2 (9.6–17.8)
Suspensions of ticks	245	53; 21.6 (16.9–27.2)	28; 0.8 (0.3–2.9)	N. i.
Suspensions of organs of small mammals	43	2; 4.6 (1.3–15.4)	1; 2.3 (0.4–12.1)	N. i.
Suspensions of bat organs	54	0; 0 (0–6.6)	0; 0 (0–6.6)	N. i.
Forest Guinea				
Blood serum of practically healthy people	780	N. i.	N. i.	101; 12.9 (10.7–15.5)
Blood serum of cattle	264	3; 1.1 (0.4–3.3)	N. i.	35; 13.2 (9.6–17.8)
Suspensions of ticks	377	59; 15.6 (12.3–19.7)	61; 16.2 (12.8–20.2)	N. i.
Suspensions of organs of small mammals	72	3; 4.2 (1.4–11.5)	2; 2.8 (0.8–9.5)	N. i.
Suspensions of bat organs	76	6; 7.9 (3.7–16.1)	0; 0 (0–4.8)	N. i.
General by country				
Blood serum of practically healthy people	3156	N. i.	N. i.	366; 11.6 (10.5–12.7)
Blood serum of cattle	1074	14; 1.3 (0.8–2.1)	N. i.	172; 16.0 (13.9–18.3)
Suspensions of ticks	1648	294; 17.8 (16.1–19.8)	307; 18.6 (16.8–20.6)	N. i.
Suspensions of organs of small mammals	319	16; 5.0 (3.1–8.0)	3; 0.9 (0.3–2.7)	N. i.
Suspensions of bat organs	298	6; 2.0 (0.9–4.3)	3; 1.0 (0.3–2.9)	N. i.

Note. N. i. — not investigated.

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Enhancement of systemic and lung-localized CD4$^+$ T-cell immune responses by truncation of NS1 protein of a seasonal live influenza vaccine strain

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Abstract

Introduction. There is a large variety of licensed influenza vaccines worldwide, but their common limitation is rather narrow specificity and inability to protect against antigenic-drift variants of influenza virus. Therefore, optimization of immunogenic and cross-protective properties of licensed influenza vaccines is an urgent priority of public health agenda. One such approach is to modulate the immunogenic properties of live attenuated influenza vaccine (LAIV) by truncating the open reading frame of influenza virus non-structural protein 1 (NS1). The **main objective** of this study is to evaluate the immunogenic properties of the H1N1 seasonal LAIV strain by truncation of the NS1 protein to 126 amino acides.

Materials and methods. Using reverse genetics technique, two H1N1 LAIV strains with full-length and truncated NS1 protein with three consecutive stop codons added after the 126th amino acid residue were obtained.C57BL/6J mice were immunized intranasally with the vaccine candidates, twice at a three-week interval. Seven days after the second immunization, cells were isolated from spleen and lung tissues and stimulated with whole wild-type H1N1 influenza virus. Levels of systemic and tissue-resident cytokine-producing CD4$^+$ and CD8$^+$ memory T cells were assessed by intracellular cytokine staining assay with flow cytometry. Replication of engineered vaccine strains in *in vitro* and *in vivo* systems was also evaluated.

Results. Truncation of NS1 protein of the LAIV strain significantly increased the levels of virus-specific CD4$^+$ effector memory T cells in spleens and the levels of CD4$^+$ tissue-resident memory T cells in lungs of mice after two-dose immunization, indicating a higher potential for protection against influenza infection of the LAIV NS_{126} vaccine strain compared to the classical variant of LAIV. Importantly, the LAIV NS_{126} strain also had a more pronounced attenuated phenotype in mice than its classical counterpart.

Keywords: *influenza virus, live attenuated influenza vaccine, NS1 protein, memory T cells, tissue-resident memory T cells, tissue-resident memory T cells, CD4$^+$ T cells, CD8$^+$ T cells*

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 Institute of Experimental Medicine (protocol No. 1/20, February 27, 2020).

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Оригинальное исследование
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Усиление системного и локализованного в лёгких CD4⁺-Т-клеточного иммунного ответа при укорочении белка NS1 штамма сезонной живой гриппозной вакцины

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Аннотация

Введение. В мире существует большое разнообразие лицензированных вакцин против гриппа, но их общими недостатками являются достаточно узкая специфичность и неспособность защищать от дрейфовых вариантов вируса. Соответственно, оптимизация иммуногенных и кросс-протективных свойств лицензированных гриппозных вакцин — актуальная задача практического здравоохранения. Одним из таких подходов является модуляция иммуногенных свойств живой гриппозной вакцины (ЖГВ) за счёт усечения рамки считывания неструктурного белка 1 вируса гриппа (NS1). Основной **целью** данной работы является оценка иммуногенных свойств сезонной ЖГВ подтипа H1N1 при усечении рамки считывания белка NS1 до 126 аминокислот.

Материалы и методы. Методами обратной генетики сконструированы 2 штамма ЖГВ подтипа H1N1 с полноразмерным и с усечённым белком NS1, где после 126 аминокислот добавлены 3 последовательных стоп-кодона. Мышей линии C57Bl/6J иммунизировали интраназально двукратно с 3-недельным интервалом. Через 7 дней после повторной иммунизации у мышей выделяли клетки из тканей селезёнки и лёгких, стимулировали цельным диким вирусом H1N1 и оценивали уровни системных и тканерезидентных цитокинпродуцирующих CD4⁺- и CD8⁺-Т-клеток памяти методом внутриклеточного окрашивания цитокинов. Также была проведена оценка репродукции штаммов в системах *in vitro* и *in vivo*.

Результаты. Укорочение белка NS1 в ЖГВ значительно повышало уровни вирусспецифических CD4⁺-Т-клеток эффекторной памяти в селезёнке и уровни тканерезидентных CD4⁺-Т-клеток в лёгких мышей после двукратной иммунизации, что указывает на более высокий потенциал защиты от гриппозной инфекции у ЖГВ с усечённым белком NS1 по сравнению с классическим вариантом ЖГВ. Важно отметить, что ЖГВ с усечённым белком NS1 также имела более выраженный аттенуированный фенотип в эксперименте на мышах, чем её классический аналог.

Ключевые слова: *вирус гриппа, живая гриппозная вакцина, белок NS1, Т-клетки памяти, Т-клетки эффекторной памяти, тканерезидентные Т-клетки, CD4⁺-Т-клетки, CD8⁺-Т-клетки*

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Introduction

Influenza viruses pose a constant threat to the global population due to their high contagiousness and ability to cause severe epidemics, which kill up to 650,000 people annually [1]. The most effective means of fighting influenza infection remains vaccination, which is mainly aimed at preventing the development of severe cases of the disease, as well as its complications. There is a sufficiently large variety of licensed influenza vaccines, but their effectiveness in different epidemic seasons varies greatly due to the narrow specificity of the induced immune response to vaccination [2]. In this regard, the search for new approaches to improve the immunogenicity and broaden the spectrum of action of seasonal influenza vaccines is of paramount importance for global health care.

The non-structural NS1 protein of influenza A virus is multifunctional protein and participates in various stages of virus-cell interaction: it is an antagonist of the antiviral cellular response and a regulator of viral and cellular gene expression [3, 4]. In particular, the NS1 protein of influenza virus acts as an interferon (IFN) antagonist and thereby promotes productive infection by disrupting one of the most important links of antiviral immunity [5]. Furthermore, the C-terminus of NS1 protein is attributed to the function of decreasing dendritic cell activation and, consequently, impairing the stimulation of naive T cells [6]. Accordingly, the immunogenicity of live attenuated influenza vaccine (LAIV) can be enhanced by truncating the NS1 protein from the C-terminus to attenuate its anti-IFN activity. Previously, we constructed a vaccine strain of H7N9 subtype LAIV that encoded an NS1 protein shortened to 126 amino acids. Experiments in mice showed that this modification resulted in a significant enhancement of the T-cell response to the immunodominant epitope NP_{366} compared to immunization with full-length NS1 [7].

The aim of the present study was to evaluate the modulation of immunogenic properties of the seasonal H1N1 subtype influenza vaccine strain by truncation of the NS1 protein reading frame to 126 amino acids. The systemic and local T-cell response to all influenza virus antigens was studied by stimulating immune cells with whole live epidemic influenza H1N1 virus.

Materials and methods

Viruses

Experimental reassortant strains of the H1N1 subtype were obtained by standard reverse genetics methods based on the attenuation donor of the domestic LAIV A/Leningrad/134/17/57 (H2N2) (Len/17) [8]. The parental epidemic influenza virus was the A/Guangdong-Maonan/SWL1536/2019 (H1N1) [H1N1/wt] strain obtained from the NIBSC collection (UK). The H1N1 LAIV vaccine strain with full-length NS1 protein contained PB2, PB1, PA, NP, M and NS genes from the attenuation donor Len/17, and hemagglutinin and neuraminidase genes from the epidemic H1N1/wt virus. To obtain a recombinant influenza virus expressing a truncated NS1 protein, 3 consecutive stop codons were added after 126 amino acids of the open reading frame of the NS1 protein of Len/17 virus by site-directed mutagenesis using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and specific primers (Evrogen Ltd.). Viruses were cultured in 10-11-day-old embryonated chicken eggs (ECE) (Siniavinskaya poultry farm) at 33ºC (for vaccine strains of LAIV) or at 37ºC (for epidemic strain H1N1/wt) and stored at –70ºC in aliquots.

Cell lines

MDCK dog kidney cells (ATCC CCL-34) and Vero green monkey kidney cells (ATCC CCL-81) were cultured in DMEM growth medium containing 10% fetal bovine serum and supplemented with an antimycotic antibiotic (Capricorn reagents listed).

Determination of the infectious activity of influenza viruses

The infectious titers of viruses were determined by the limit dilution method. To infect ECE, viruses were diluted in phosphate-salt buffer (PBS) and each dilution infected 4-6 embryos in a volume of 200 µl. The ECEs were incubated at 33ºC and 38ºC for 48 h or at 26ºC for 6 days, after which the embryos were cooled; the presence of virus in allantois fluid was determined by hemagglutination assay with chicken erythrocytes. Determination of infectious titers of viruses on cell cultures was performed by infecting daily monolayer cultures in 96-well plates with serial 10-fold dilutions of viruses. After adsorption, the inoculum was removed, cells were washed, incubated in DMEM medium containing 1 µg/mL TRSC trypsin and antibiotic-antimycotic at 33ºC for 4 days. The presence of viruses in the wells was determined by staining the cells fixed in acetone with monoclonal anti-NP-antibody conjugated with horseradish peroxidase (PPDP LLC). Illumination was performed using TMB substrate (Thermo Fisher Scientific), and optical density was measured on an xMark flatbed spectrophotometer (BioRad). Wells were considered positive at optical density values (λ = 450 nm) exceeding the values of negative control wells at least 2-fold. Virus titers in ECE and Vero and MDCK cells were calculated according to the method proposed by L.J. Reed et al. [9] and were expressed in 50% infectious doses (log10 EID_{50}/mL and log10 $TCID_{50}$/mL).

Accumulation and purification of influenza virus on a sucrose gradient

For immunologic tests, H1N1/wt influenza virus was purified on a sucrose gradient to remove nonspecific proteins from chicken embryos and to concentrate the virus. Viruses were purified on a 30%/60% density

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gradient using an ultracentrifuge (BeckmanCoulter) in several steps:

1) clarification of the collected allantois fluid by centrifugation for 15 min at 4ºC at 3500*g*;

2) sedimentation at 16,000*g* for 2 h at 4ºC and resuspension of the formed precipitate in 2 ml of FSB;

3) layering of the resuspended precipitate on a 30%/60% sucrose step gradient followed by ultracentrifugation for 2 h and at 4ºC at 23,000*g*;

4) collection of concentrated virus at the boundaries of the gradient and its washing in 10 ml of FSB by centrifugation for 1.5 h at 23,000*g*. In the last step, the viral precipitate was resuspended in 1 ml of PBS and stored at –70ºC in aliquots.

Mice immunization and organ harvesting

Female mice of the C57Bl/6J line, supplied from the Stolbovaya Branch Nursery of the National Center for Biomedical Research of the Federal Medical and Biological Agency of Russia, were used. Mice were immunized twice intranasally with an interval of 21 days with one of the recombinant strains of LAIV at a dose of 10^6 EID_{50} in a volume of 50 μl using light ether anesthesia. Control animals received an equal volume of PBS. On the 3rd day, lungs and nasal passages were collected from 4 vaccinated mice from each group and further homogenized using a TissueLyser LT automatic homogenizer (Qiagen). Lung and nasal tissue homogenates were used to determine the infectious titer of the virus in the ECE system. At 7 days after re-immunization, lung tissue and spleens were collected from 6 mice from each group for further evaluation of T-cell immunity. The research protocol was approved by the Ethics Committee of the Institute of Experimental Medicine (protocol No. 1/20, February 27, 2020).

Assessment of the T-cell immune response

Determination of systemic and memory T cells localized in the lungs was performed according to the previously described method [7] with minor modifications. Single splenocytes were isolated in CR-0 medium (RPMI-1640 supplemented with antibiotic-antimycotic, 25 mM HEPES (listed reagents from Capricorn) and 50 μM 2-mercaptoethanol (Sigma-Aldrich) using 70 μm pore size filters (BD Biosciences). Erythrocytes were then lysed using erythrocyte lysis buffer (BioLegend). For intracellular cytokine staining, 2×10^6 cells were added to 100 μL of CR-10 medium (CR-0 medium containing 10% fetal bovine serum) in sterile U-bottom microplates. Then, 100 μl of CR-10 medium containing purified whole H1N1/wt virus at a dose of 2 infectious units per cell was added to each well and incubated for 18 h at 37ºC, 5% CO_2. GolgiPlug solution (BD Biosciences) was then added to the samples at a final concentration of 1 : 1000 to stop protein transport. Stimulation with phorbolmyristyl acetate (Sigma-Aldrich) was used as a positive control; unstimulated control samples and isotypic control samples were also prepared. Cells were incubated for 5 h at 37 ºC, 5% CO_2, then stained for 20 min at 4ºC in the dark with ZombieAqua fluorescent live/dead cell detection dye and a mixture of the following fluorescently labeled surface antibodies: CD4-PerCP/Cy5.5, CD8-APC/Cy7, CD44-PE, and CD62L-BV421 (reagents listed are BioLegend). The Cytofix/Cytoperm kit (BD Biosciences) was used for fixation/permeabilization, after which cells were stained with antibodies to cytokines: IFN-γ — FITC, tumor necrosis factor-α (TNF-α) — APC, interleukin-2 (IL-2) — PE/Cy7 for 20 min at 4ºC in the dark. Samples were fixed with Cyto-Last buffer (antibodies and buffer – BioLegend) and analyzed using a Navios cytofluorimeter (BeckmanCoulter).

To detect tissue-resident memory T cells (T_{RM}), lungs perfused with PBS solution were cut into small pieces with sterile scissors and treated with a mixture of DNAase I and collagenase (Sigma-Aldrich) for 40 min at 37ºC. A suspension of individual cells was then prepared using 70 μm pore size filters. Erythrocytes were lysed as described above and stimulated with whole H1N1/wt virus followed by detection of virus-specific effector memory T cells (T_{EM}; $CD44^+CD62L^-$) expressing tissue-resident markers ($CD69^+CD103^+$). The staining kit for surface markers and intracellular cytokines included: CD4 — PerCP/Cy5.5, CD8 — APC/Cy7, CD44 — APC, CD62L — BV421, CD69-PE/Cy7, CD103 — FITC, while intracellular staining was performed for only one cytokine, IFN-γ — PE/Dazzle (listed reagents — BioLegend). The number of cytokine-positive cells in stimulated groups was counted and the level of spontaneous cytokine secretion in unstimulated control samples was subtracted.

Statistical processing of the results

Flow cytometry data were analyzed using Kaluza Analysis software (BeckmanCoulter). Statistical analysis and preparation of illustrations were performed using the GraphPad Prism v. 7.0 program. ANOVA with Tukey's correction or Mann–Whitney U-test was used to compare data; differences were considered significant at $p < 0.05$.

Results

In the present study, a strain of seasonal H1N1 subtype LAIV expressing a truncated nonstructural protein 1 truncated to 126 amino acids (LAIV H1N1 NS_{126}) was obtained by genetic engineering methods. In *in vitro* experiments, it was shown that the classical vaccine strain H1N1 and the modified variant H1N1 $NS1_{126}$ had similar growth characteristics in various culture systems, but the variant with $NS1_{126}$ was significantly more weakly propagated at a temperature reduced to 26ºC (**Table**). These results are consistent with the previously obtained data on the phenotypic characteristics of vaccine strains of LAIV with $NS1_{126}$ [7, 10]. Further-

Replicative properties of LAIV vaccine strains *in vitro* and *in vivo* systems

Vaccine strain	Virus titer in ECE, lg EID_{50}/ml			Virus titer in cells, lg $TCID_{50}$/ml		Virus titer in mouse organs, lg EID_{50}/ml	
	26ºC	33ºC	38ºC	MDCK	Vero	lungs	nose
LAIV H1N1	5,8 ± 0,4	8,7 ± 0,3	1,9 ± 0,4	7,2 ± 0,3	6,2 ± 0,2	< 1,2	2,3 ± 1,2
LAIV H1N1 $NS1_{126}$	4,4 ± 0,7	7,6 ± 0,5	< 1,2	5,8 ± 0,8	5,4 ± 0,4	< 1,2	< 1,2

more, the modified strain practically did not propagate in the upper respiratory tract of mice, in contrast to the classical strain of LAIV, which is also consistent with the previously obtained data on the enhancement of the attenuating properties of the vaccine when the reading frame of the NS1 protein is truncated.

Two-dose immunization of mice with LAIV and LAIV with truncated NS_{126} stimulated the induction of high levels of virus-specific T_{EM} cells with $CD44^+CD62L^-$ phenotype in the spleens of mice, and truncation of NS1 protein in the vaccine strain of LAIV significantly increased the levels of polyfunctional $CD4^+$-T_{EM} cells secreting two (IFN-γ, TNF-α) or three (IFN-γ, TNF-α and IL-2) cytokines simultaneously **(Fig. 1**, *a*). Furthermore, only the group of mice that received LAIV NS_{126} showed significantly higher levels of IFN-γ-producing cytotoxic memory T cells compared to controls (Fig. 1, *b*). Thus, the data obtained indicate that the modified strain of LAIV NS_{126} may have a higher potential for protection against influenza infection than the classical LAIV variant.

Further, we investigated subpopulations of virus-specific T_{EM} cells in the lungs with evaluation of the expression of surface markers of memory T_{RM} cells by these cells. Evaluation of the levels of IFN-γ-producing T_{EM} cells in the lungs of immunized mice revealed a significant enhancement of the $CD4^+$ T-cell response in the group of animals inoculated with the prototype LAIV $NS1_{126}$ compared with the classical version of the vaccine (**Fig. 2**, *a*). For cytotoxic $CD8^+$-T_{EM} cells in the lungs, a comparable level of immunogenicity of LAIV was shown, independent of NS1 protein modification (Fig. 2, *d*). At the same time, the level of expression of tissue residency markers was comparable in both vaccine groups (Fig. 2,

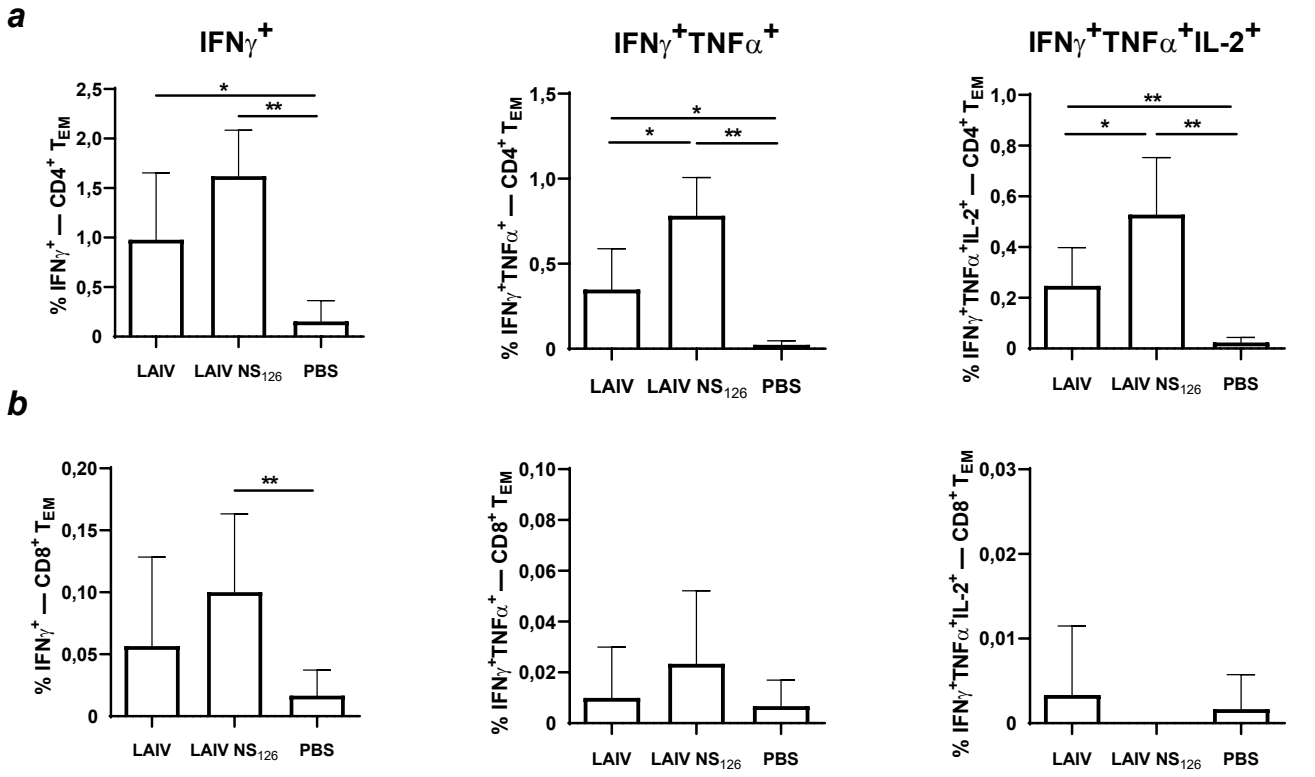

Fig. 1. Number of effector memory T cells ($CD44^+CD62L^-$) with $CD4^+$ (*a*) and $CD8^+$ (*b*) phenotype expressing IFNγ (left panel), IFNγ and TNFα (middle panel) and IFNγ, TNFα and IL-2 (right panel) in groups of mice immunized with LAIV or LAIV with truncated NS1 protein, as well as in the control group (PBS).

Significant differences between groups (Mann–Whitney test), $*p < 0.05$, $**p < 0.01$.

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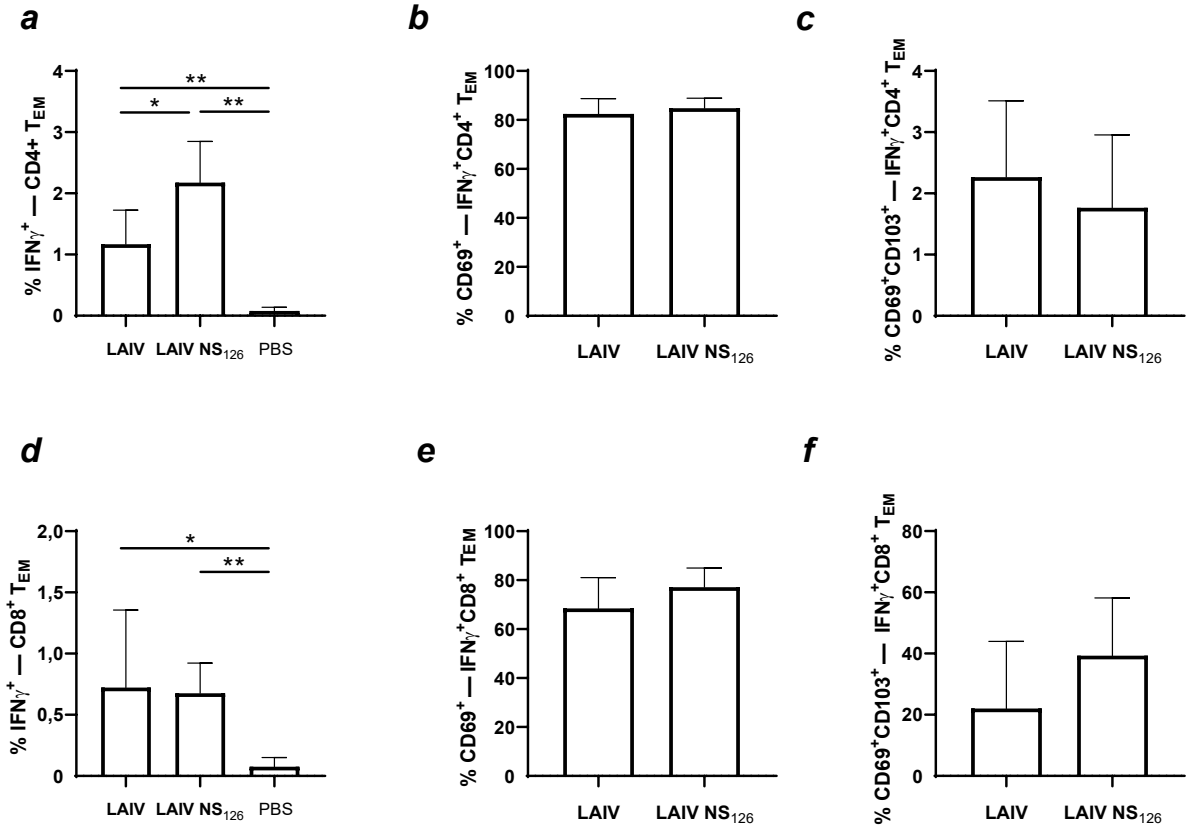

Fig. 2. Induction of virus-specific memory T cells in the lungs after immunization of mice with the study vaccine viruses.

(*a*, *d*) Number of IFNγ-producing effector memory T cells ($CD44^+CD62L^-$) with CD4+ (*a*) and $CD8^+$ (*d*) cell phenotype in groups of mice immunized with LAIV or LAIV with truncated NS_{126}, as well as in controls. Proportion of virus-specific tissue-resident memory cells with $CD69^+CD103^-$ phenotype among $IFN\gamma^+$ cells in $CD4^+$ and $CD8^+$ T cell populations (*b* and *f*, respectively). Proportion of virus-specific tissue-resident memory cells with $CD69^+CD103^+$ phenotype among $IFN\gamma^+$ cells in $CD4^+$ and $CD8^+$ T cell populations (*c* and *f*, respectively). * $p < 0.05$, ** $p < 0.01$ between groups (Mann–Whitney test).

b, c, d, e, f), indicating the localization of the identified virus-specific cells in the lung epithelium, in close proximity to the potential site of pathogen entry.

Discussion

Existing influenza vaccines induce predominantly neutralizing antibodies targeting hypervariable epitopes of the main influenza virus antigen, the hemagglutinin molecule, necessitating almost annual updates of the vaccine strain composition. Over the past decade, significant progress has been made in the development of influenza vaccines with a broader protective spectrum, targeting conserved viral antigens such as the hemagglutinin stem domain, neuraminidase, or M2e; and the development of T cell-based approaches that have the greatest potential to induce long-term cross-protective memory cell responses [11]. To date, a massive amount of evidence has accumulated on the ability of influenza viruses expressing the truncated NS1 protein to stimulate the formation of a more pronounced adaptive immune response while rendering the virus more attenuated [12-15]. However, the vast majority of studies have used a model laboratory strain A/Puerto Rico/8/34 (H1N1) or a strain based on wild-type influenza virus, which has a significant disadvantage – the probability of reverting to a virulent phenotype in case of possible reassortment with other circulating viruses. In this current study, the strain of the domestic licensed live influenza vaccine, widely used in public health practice in Russia and in a number of foreign countries, was used as the basis for our investigation [16].

We have previously shown that truncation to 126 amino acids of the NS1 protein of the H7N9 LAIV vaccine strain leads to an enhanced humoral and T-cell response in a mouse experiment [7]. In contrast to the above mentioned study, where the T-cell immune response was assessed by stimulation of cells with synthetic peptides corresponding to immunodominant $CD8^+$ T-cell epitopes NP_{366}, in the present study, a stimulation of immune cells of immunized mice with whole purified H1N1/wt influenza virus was performed. Such stimulation better reflects the actual clinical situation, since during influenza the organism is exposed to the circulating virus in its natural form, and infected cells present a large variety of T-cell viral epitopes on MHC-I and MHC-II complexes.

The present study demonstrated an enhanced $CD4^+$ T-cell response in mice when they were immu-

nized with a live influenza vaccine strain with modified NS1 protein, and this effect was expressed both at the systemic (splenocytes) and local levels (cells from lung tissues). At the same time, systemic virus-specific $CD4^+$ T cells were characterized by a polyfunctional phenotype, producing, in addition to IFN-γ, other key proinflammatory cytokines involved in the antiviral response, such as TNF-α and IL-2. T-lymphocytes capable of secreting several cytokines simultaneously in response to antigenic stimulation are known to be more accurate predictors of the organism's ability to resist reinfection than monofunctional cells secreting only IFN-γ [17]. For systemic $CD8^+$-T_{EM} cells, no significant increase in the proportion of cytokine-producing T cells was detected with NS1 protein shortening, likely due to the small number of animals in the group and high dispersion. These data are in general agreement with previous results obtained for the H7N9 vaccine strain expressing a shortened variant of NS1 protein, where $CD8^+$ T-cell response was assessed after stimulation of splenocytes with a peptide corresponding to the immunodominant epitope NP_{366} [7]. It is important to note that a more pronounced T-cell response formed directly in the lung tissues of mice immunized with the vaccine strain of LAIV with $NS1_{126}$ indicates the potential for the development of an accelerated immune response during subsequent contact with the pathogen, since T_{RM} cells in the lungs represent the first line of immune defense of the organism against respiratory pathogens [18, 19].

Conclusion

The present study provides evidence of enhanced systemic and lung-localized $CD4^+$ T-cell immune response upon truncating of NS1 protein of a seasonal H1N1/wt strain. Since virus-specific T cells were detected when lymphocytes were stimulated with whole live H1N1/wt virus, it can be assumed that upon re-infection with a modern circulating virulent virus of this subtype, mice immunized with the H1N1 $NS1_{126}$ variant of H1N1 LAIV will be better protected from clinical manifestations of the disease than animals that received the classical variant of LAIV.

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Circulation of *Mycobacterium tuberculosis* strains of the Beijing Central Asian Outbreak genotype in the Kemerovo region — Kuzbass in 2018–2022

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Abstract

Introduction. The Kemerovo region — Kuzbass is characterized by a high prevalence of multidrug-resistant (MDR) tuberculosis (TB), including coinfection with HIV (HIV/TB). A previously unknown in Russia relationship between MDR and the Beijing Central Asian Outbreak (CAO) subtype has been discovered, which updates studies of *Mycobacterium tuberculosis* taking into account this resistant variant.

Objective: to study the molecular genetic structure of the *M. tuberculosis* population, to assess the prevalence and possible routes of emergence of Beijing CAO strains in the Kemerovo region — Kuzbass.

Materials and methods. A total of 325 *M. tuberculosis* strains were studied in 2018–2022 using spoligotyping, MIRU-VNTR 24 and SNP typing. Whole genome sequencing and bioinformatics analysis were performed for seven Beijing CAO strains.

Results. Primary MDR and pre-extensive drug resistance (pre-XDR) were detected in 39.4% and 11.5% of strains, respectively. In the total sample, MDR was 43.4%, pre-XDR — 19.7%. In the structure of the *M. tuberculosis* population, the Beijing genotype prevailed (78.8%), with its subtypes Central Asian Russian (40.9%) and B0/W148 (32.6%). The Euro-American lineage (27.3%) was represented by the genotypes T (6.5%), LAM (5.8%), Ural (4.9%), H (0.9%); one strain CAS1-Delhi was detected, the genotype of 2.8% of strains was not identified. The proportion of Beijing CAO was 12.6% of the total sample; this subtype was significantly more often detected among HIV/TB (20.6%) than in HIV-negative TB patients (9.1%; $p = 0.005$). The results of the Beijing CAO genome analysis from the Kemerovo region indicate the absence of a direct chain of transmission between these TB cases. A hypothesis has been put forward about the introduction of Beijing CAO to the Kemerovo region from Central Asia and its endemic circulation in the region.

Conclusion. A high level of MDR and pre-XDR was detected in Beijing genotype strains in the *M. tuberculosis* population of the Kemerovo region — Kuzbass, especially the B0/W148 (97.2%) and CAO (87.5%) subtypes. Beijing CAO strains, detected mainly in newly diagnosed HIV/TB patients, require further monitoring and control of their spreading.

Keywords: *Mycobacterium tuberculosis, Beijing Central Asian Outbreak, Beijing B0/W148, multidrug resistance, Tuberculosis, HIV infection*

Ethics approval. The study was conducted with the informed consent of the patients.The research protocol was approved by the Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problem (protocol No. 4, April 12, 2023) and Ethics Committee of the Kemerovo State Medical University (protocol No. 255/к, November 11, 2020).

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Оригинальное исследование
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Циркуляция штаммов *Mycobacterium tuberculosis* Beijing Central Asian Outbreak в Кемеровской области — Кузбассе в 2018–2022 годах

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Аннотация

Введение. Кемеровская область — Кузбасс характеризуется распространённостью туберкулёза (ТБ) с множественной лекарственной устойчивостью (МЛУ), в том числе сочетанного с ВИЧ-инфекцией (ВИЧ/ТБ). Обнаружена высокая частота МЛУ среди штаммов Beijing, в том числе субтипа Central Asian Outbreak (CAO), что актуализирует исследования возбудителя с учётом этого резистентного варианта.
Цель исследования: изучить молекулярно-генетическую структуру популяции *Mycobacterium tuberculosis*, оценить распространённость и возможные пути появления штаммов Beijing CAO в Кемеровской области — Кузбассе.
Материалы и методы. Изучено 325 штаммов *M. tuberculosis*, выявленых за 2018–2022 гг., методами сполиготипирования, MIRU-VNTR 24 и SNP-типирования. Для 7 штаммов Beijing CAO проведены полногеномное секвенирование и биоинформатический анализ.
Результаты. Первичная МЛУ и преширокая лекарственная устойчивость (пре-ШЛУ) обнаружены у 39,4 и 11,5% штаммов соответственно. В общей выборке МЛУ составила 43,4%, пре-ШЛУ — 19,7%. В структуре популяции *M. tuberculosis* преобладал генотип Beijing (78,8%), его субтипы Central Asian Russian (40,9%) и B0/W148 (32,6%). Евро-американская линия (27,3%) представлена генотипами T (6,5%), LAM (5,8%), Ural (4,9%), H (0,9%); обнаружен 1 штамм CAS1-Delhi; 2,8% штаммов не идентифицированы. Доля Beijing CAO составляла 12,6% общей выборки, данный субтип значимо чаще обнаруживали среди ВИЧ/ТБ (20,6%), чем у ВИЧ-негативных больных ТБ (9,1%; p = 0,005). Результаты анализа геномов Beijing CAO из Кемеровской области свидетельствуют об отсутствии цепи передачи между этими случаями ТБ. Выдвинута гипотеза о заносе Beijing CAO из Центральной Азии и его эндемичной циркуляции в Кемеровской области.
Заключение. В популяции *M. tuberculosis* выявлен высокий уровень МЛУ и пре-ШЛУ у штаммов Beijing, в особенности субтипов B0/W148 (97,2%) и CAO (87,5%). Штаммы Beijing CAO, выявленные преимущественно у впервые выявленных больных ВИЧ/ТБ, требуют дальнейшего наблюдения и контроля их распространения.

Ключевые слова: *Mycobacterium tuberculosis, Beijing Central Asian Outbreak, Beijing B0/W148, множественная лекарственная устойчивость, туберкулёз, ВИЧ-инфекция*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Этическим комитетом Научного центра здоровья семьи и репродукции человека (протокол № 4 от 12.04.2023) и Этическим комитетом Кемеровского государственного медицинского университета (протокол № 255/к от 11.11.2020).

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Introduction

Kemerovo region – Kuzbass, despite the decrease trends, maintains high levels of tuberculosis (TB) incidence and prevalence. The region is also characterized by high rates of HIV infection. In 2021, TB incidence was 63.07 per 100,000, which is more than 2 times the national level (31.1 per 100,000), prevalence — 138.0 per 100,000 [1]. During the same period, HIV incidence (1274.03 per 100,000) was 1.62 times higher than the national average (782.0)[1]

HIV infection increases not only the risk of active TB, but also gives low treatment efficacy.[2] The widespread of drug-resistant strains of *Mycobacterium tuberculosis* is also among the main causes of high TB incidence.

Therefore, the study of the features of modern strains of M. tuberculosis is of absolute relevant. Previously, a number of molecular genetic studies of *M. tuberculosis* were conducted in the Kemerovo region and the associations of multidrug resistance (MDR) with strains of the Beijing genotype were revealed [2–4]. However, the prevalence of Beijing strains of the Central Asian Outbreak (CAO) subtype frequently characterized by MDR [5] has not been evaluated, although they were present in collections from other West Siberian regions [3,6]. The **aim** of the study: to investigate the molecular genetic structure of *M. tuberculosis*, to assess the prevalence and possible pathways of emergence of Beijing SAO strains in the Kemerovo Region – Kuzbass.

Materials and methods

Clinical isolates and drug sensitivity testing

We examined 325 clinical isolates of *M. tuberculosis* from TB patients from the Kemerovo region – Kuzbass: from 2 screening studies in 2020–2021 (n = 86) and 2022 (n = 163); from the 2018–2019 cryo-collection (n = 76) of the Novosibirsk Research Institute of Tuberculosis, which is a reference center for TB control in Siberia and the Russian Far East (**Fig. 1**).

Bacterial isolates were characterized by standard bacteriological and biochemical methods. Drug susceptibility testing (DST) was performed by the method of proportions on the Loewenstein-Jensen medium (and/or modified proportion method on Middelbrook 7H9 liquid nutrient medium using a Bactec MGIT 960 bacteriological analyzer) [3, 4].

The terminology of drug resistance according to the Clinical Guidelines "Tuberculosis in adults, 2022" was used:

- mono-resistance is resistance of *M. tuberculosis* to only one antiturberculosis drug;
- poly-resistance is resistance of *M. tuberculosis* to 2 or more antiturberculosis drugs, except for simultaneous resistance to isoniazid and rifampicin;
- MDR is resistance of *M. tuberculosis* to isoniazid and rifampicin simultaneously, regardless of resistance to other antiturberculosis drugs;
- pre-extensive drug resistance (pre-XDR) is resistance of *M. tuberculosis* to rifampicin with or without resistance to isoniazid, combined with resistance to any fluoroquinolone.

Genotypic identification

DNA was extracted using previously described methods [3]. Isolates were first differentiated into Beijing and non-Beijing genotypes [3]. Beijing strains were divided into two major groups known to be prevalent in Russia (subtypes B0/W148 and Central Asian Russian) by testing specific SNP markers [3, 4]. Beijing Central Asian Russian strains were genotyped for Central Asian Outbreak (CAO) markers [6] by the authors' previously reported real time PCR test for a specific SNP in the pks *8 gene* (pos.1884305) [7]. All non-Beijing strains were subjected to spoligotyping [3] and MIRU-VNTR 24 loci typing [4].

Whole-genome sequencing, bioinformatics and statistical analysis

Genomic libraries were prepared using the DNA Flex kit (Illumina). Whole-genome sequencing (WGS) of samples was performed on a NextSeq 550 sequencer (Illumina) using the reagent kit v2.5 and a flow cell (High output) of 300 cycles. The 33O, 59Kem, 145O, 155c genomes have been deposited at NCBI (bioproject PRJNA1139960). A total of 229 complete *M. tuberculosis* Lineage 2 CAO genomes [5] were retrieved from the online WGS Short Read Archive (NCBI), being part of the following bioprojects: PRJEB2138, PRJEB21922, PRJEB6273, PRJEB7281, PRJEB9680, PRJNA980215. Primary data processing involved removing short reads of poor quality ($Q < 20$) and cutting out technical sequences using the CutAdapt program [8]. Next, the short reads were mapped to the NC_000962 reference genome with single nucleotide position determination [9]. As a result of data validation and normalization, the concatenated sequence of nucleotide sequences of the 3568 bp genome data array was used for phylogenetic analysis. Antiturberculosis drug resistance genes, their promoters, and genes with high variability (PP, PE, PPE) were excluded from the sequence. The genome of the Beijing family belonging to the ancient variant (Asian Ancestral 1), which was isolated in Omsk (bioproject PRJNA489691), as well as the closest ancestors of Beijing CAO – Beijing Central Asian genomes (bioproject PRJEB9680) were used as

[1] ВИЧ-инфекция за 2021 г. Бюллетень № 47. URL: http://www.hivrussia.info/wp-content/uploads/2023/05/Byulleten-47-VICH-infektsiya-za-2021-g.pdf (date of access: March 20, 2024).

[2] World Health Organization. Global Tuberculosis Report 2022. URL: https://iris.who.int/bitstream/handle/10665/363752/9789240061729-eng.pdf?sequence=1 (date of access: March 20, 2024).

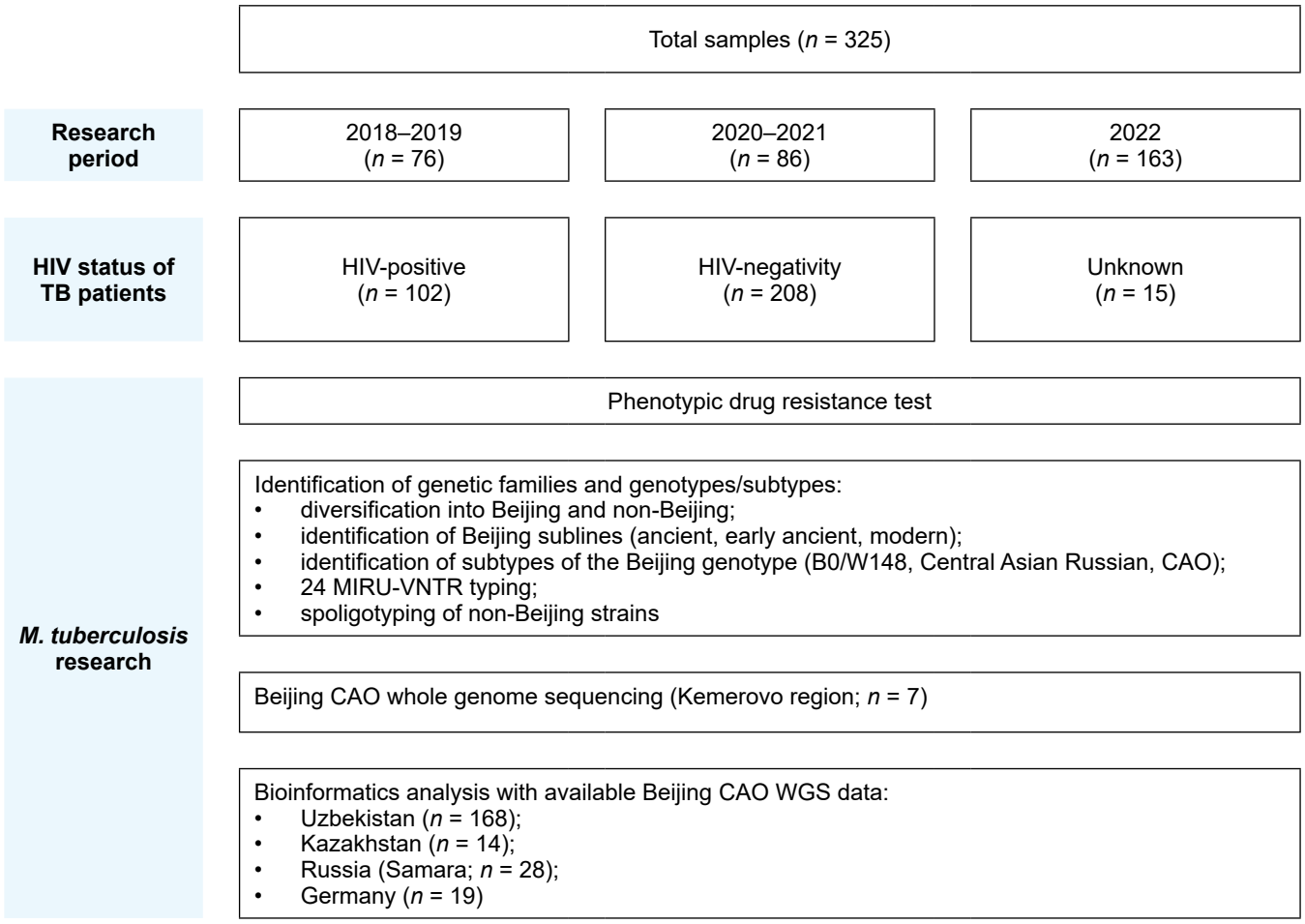

Fig. 1. Study design.

an outgroup. The phylogenetic tree was constructed using the IQ-TREE2 program by the maximum likelihood method; the phylogenetic tree was visualized using the FigTree program [9]. The reliability of the tree topology was evaluated based on bootstrap analysis with 1000 iterations. Transversion Substitution Model was chosen as the nucleotide substitution model because it had the best Akaike values calculated using IQ-TREE Model Finder. Evolutionary rate and tree topology were analyzed using the Total Recovery Time Substitution Model with gamma-distributed substitution rate at each site and 4 rate categories.

Statistical analysis was performed using the web resource http://www.medcalc.org/calc/odds_ratio.php, calculating Fisher's exact criterion values and odds ratios. Differences between groups were identified by the χ^2 test, and significance was confirmed at $p < 0.05$.

Results

General characterization and drug resistance of M. tuberculosis

In a sample of 325 clinical isolates, specimens from patients with infiltrative (30.8%), disseminated (31.1%), and fibrotic cavernous forms of pulmonary TB (20.6%) predominated, followed by tuberculomas (10.5%). Disseminated TB was significantly more common in HIV-infected patients (48.0%; 49/102) than in HIV-negative patients (22.6%; 47/208; $p < 0.0001$), while fibrotic cavernous TB and tuberculomas, on the contrary, had higher rates in HIV-negative patients, thus reflecting the clinical manifestations of TB characteristic of coinfection. A total of 226 69.5% isolates were obtained from patients with new TB cases (**Table 1**). New TB cases accounted for a significantly higher proportion of new TB cases in the HIV-infected group (84.4%; 84/102) than in the HIV-negative group (64.4%; 134/208; $p = 0.002$).

The results of the drug sensitivity test revealed a high prevalence of *M. tuberculosis* clinical isolates with drug resistance (75.7%; 246/325) to antituberculosis drugs (**Table 2**). 63.1% (205/325) of isolates were resistant to the main first-line antibiotics — rifampicin and isoniazid. Among them, 19.7% (64/325) cases were also accompanied by resistance to fluoroquinolones, which defined them as pre-XDR strains; their number was expectedly higher among previously treated TB patients than among new cases (44.6% vs. 11.5%; $p < 0.001$). The proportion of MDR (non-pre-XDR) strains among first-time cases was 39.4% and 43.4%

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Table 1. Demographic and clinical data of TB patients, *n* (%)

Characteristic	Total *n* = 325	New cases *n* = 226	Previously treated *n* = 99	χ^2; *p*
Gender				
female	102 (31,4)	69 (30,5)	33 (33,3)	0,251; *p* = 0,616
male	223 (68,6)	157 (69,5)	66 (66,7)	
Age, years				
19–45	241 (74,2)	159 (70,4)	82 (71,1)	5,589; *p* = 0,018
≥ 46	84 (25,8)	67 (29,6)	17 (28,9)	
HIV status*				
HIV-positive	102 (32,9)	84 (38,5)	18 (19,6)	10,54; *p* = 0,0012
HIV-negative	208 (67,1)	134 (61,5)	74 (80,4)	
TB clinical forms				
infiltrative	100 (30,8)	89(39,4)	11 (11,1)	25,827; *p* = 0,007
disseminated	101 (31,1)	82 (36,3)	19 (19,2)	9,389; *p* = 0,002
fibrous-cavernous	67 (20,6)	20 (8,8)	47 (47,5)	62,759; *p* < 0,001
focal	5 (1,6)	5 (2,2)	0	1,004; *p* = 0,316
tuberculomas	34 (10,5)	19 (8,4)	15 (15,2)	3,343; *p* = 0,068
caseous pneumonia	11 (3,4)	5 (2,7)	6 (5,1)	0,587; *p* = 0,444
intrathoracic	3 (1,0)	3 (1,3)	0	0,272; *p* = 0,602
TB meningitis	1 (0,3)	1 (0,4)	0	0,439; *p* = 0,602
TB pleurisy	1 (0,3)	1 (0,4)	0	0,439; *p* = 0,602

Note. *HIV status of 15 patients was not indicated; absolute data and percentage calculations are given for 310 patients (218 newly diagnosed and 92 previously treated).

in the total sample. MDR+pre-XDR levels were not significantly different between HIV-negative patients (62.0%; 129/208) and HIV/TB patients (57.8%; 59/102; $p = 0.480$).

Five isolates with drug resistance to bedaquiline were identified, 4 of which were characterized by mono- and poly-resistance and 1 by MDR. Drug sensitivity to linezolid was assessed for 163 isolates from a sample of 2022, when this test was included in routine bacteriologic studies in the Kemerovo region; all isolates remained sensitive to linezolid. The proportion of pre-XDR isolates was significantly lower in HIV/TB coinfected patients (12.7%; 13/102) than in HIV-negative patients (24.0%; 50/208; $p = 0.022$), as the latter group had significantly more previously treated TB cases.

Genotypic structure of M. tuberculosis

The Beijing genotype was detected in 256 (78.8%) of 325 *M. tuberculosis* isolates. Two isolates were attributed to the early ancient sublineage (intact NTF and RD 181 [10]), while the other 254 isolates belonged to the modern Beijing sublineage. The proportion of Beijing strains was significantly higher in the previously treated group (90.9%) than in the group of newly diagnosed TB patients (73.5%; $p < 0.001$; **Table 3**), with no significant difference in HIV-negative TB and HIV/TB patients (80.3%; vs. 75.5%; $p = 0.940$). The most abundant Beijing genetic groups were B0/W148 (32.9%) and Central Asian Russian, including the CAO subtype (41.5%; 134/325). The CAO subtype accounted for 12.6% of the total sample (15.6% of Beijing), and did not differ significantly between newly diagnosed (11.9%) and previously treated (13.1%) TB patients. However, among new cases, CAO strains were detected more frequently in the HIV/TB group (21.4%) than in HIV-negative cases (6.7%; $p = 0.005$). Overall, the proportion of Beijing CAO strains was higher in the HIV/TB group (20.6%; 21/102) than in HIV-negatives (9.1%; 19/208; $p = 0.005$).

Isolates of B0/W148 Beijing subtype was detected more frequently among previously treated TB patients than in new cases (45.5% vs. 27.0%, respectively; $p = 0.001$). No statistically significant differences between 'B0/W148 strains were found when the association by HIV status, treatment history of TB patients with other Beijing subtypes were analyzed.

All Beijing CAO strains from the Kemerovo region shared a common MIRU-VNTR 94-32 profile. Information to assess the association between cases was insufficient, but analysis of available data on 27 out of 40 Beijing CAO cases indicates that there was no association between these patients by place of residence. Only 8 cases of TB with Beijing CAO were among residents of different districts of Kemerovo, the rest of the

Table 2. Drug resistance of *M. tuberculosis* isolates, *n* (%)

Genotypes, subtypes	Total *n* = 325	New cases			Previously treated		
		all *n* = 226	TB *n* = 134	HIV/TB *n* = 84	all *n* = 99	TB *n* = 74	HIV/TB *n* = 18
Susceptible	79 (24,3)	74 (32,7)	45 (33,6)	27 (32,1)	5 (5,1)	3 (4,1)	1 (5,6)
Monoresistant	17 (5,2)	16 (7,1)	10 (7,5)	5 (6,0)	1 (1,0)	0	1 (5,6)
Polyresistant	24 (7,4)	21 (9,3)	12 (9,0)	9 (10,7)	3 (3,0)	3 (4,1)	0
MDR not XDR	141 (43,4)	89 (39,4)	50 (37,3)	35 (41,7)	52 (52,5)	35 (47,3)	11 (61,1)
pre-XDR	64 (19,7)	26 (11,5)	17 (12,7)	8 (9,5)	38 (38,4)	33 (44,6)	5 (27,8)

diseases were detected in 10 industrial cities of Kuzbass, distant from the regional center by 27–90 km.

Non-Beijing isolates belonged to Lineage 4 (Euro-American lineage) and Lineage 3 (CAS1-Delhi) spoligotypes. Spoligotyping of 69 non-Beijing isolates identified 31 spoligotypes. Apart from one Lineage 3 isolate, the rest represented 4 Lineage 4 genetic families: LAM, T, Ural and H (60 isolates) and 9 unclassified strains. Isolates belonging to the Euro-American lineage were identified in 23 spoligotypes; of these, 7 were represented by clusters of 2 to 10 isolates: SIT53/T, 10 isolates; SIT42 and SIT254/ LAM, 8 isolates each; SIT35/ Ural, 7 isolates; SIT262/Ural, 6 isolates; SIT1480/ Ural and SIT2128/ T, 2 isolates each.

Drug resistance and genotypes

The proportion of rifampicin- and isoniazid-resistant Beijing genotype strains was 74.6% (191/256), which was significantly higher than among non-Beijing strains, 17.4% (12/69; $p < 0.001$). The frequency of MDR+pre-XDR detection of Beijing genotype strains among previously treated cases (82.8%; 82/99) was more pronounced than among new TB cases (48.2%; 109/226; $p < 0.001$). Because of the small number of strains of other genetic families (non-Beijing), no significant differences in MDR frequency could be detected. Non-Beijing strains unclustered by spoligoprofile possessed MDR in 26.9% (7/26) of cases, while those combined by a single spoligotype possessed MDR in 11.6% (5/43; $p = 0.194$).

The highest MDR+pre-XDR frequencies were found in Beijing isolates of subtypes B0/W148 (97.2%; 103/106) and CAO (87.5%; 35/40). The proportions of MDR+pre-XDR isolates of Central Asian Russian and Beijing (other, including early ancient sublineage) were 46.8% (44/94) and 68.8% (11/16), respectively (**Fig. 2**).

Bioinformatic analysis showed that 7 Beijing CAO isolates whose full genomic data are presented in this study (kem 59, 33O, 155c, 145c, IM117-8c, IM115-6c, IM134-53c), isolated from 5 HIV-infected and 2 TB patients without HIV infection, belonged to 4 different clusters. It is noteworthy that one of them

Table 3. Genotypes and subtypes of *M. tuberculosis* isolates, *n* (%)

Genotypes, subtypes	Total *n* = 325	New cases			Previously treated		
		all *n* = 226	TB *n* = 134	HIV/TB *n* = 84	all *n* = 99	TB *n* = 74	HIV/TB *n* = 18
Beijing total	256 (78,8)	166 (73,5)	99 (73,9)	61 (72,6)	90 (90,9)	68 (91,9)	16 (88,9)
Beijing B0/W148	106 (32,9)	61 (27,0)	38 (28,4)	19 (22,6)	45 (45,5)	35 (47,3)	7 (38,9)
Beijing Central Asian Russian non-CAO	94 (28,9)	68 (30,1)	46 (34,3)	20 (23,8)	26 (26,3)	19 (25,7)	5 (27,8)
Beijing CAO	40 (12,6)	27 (11,9)	9 (6,7)	18 (21,4)	13 (13,1)	10 (13,5)	3 (16,7)
Beijing other	16 (4,9)	10 (4,4)	6 (4,5)	4 (4,8)	6 (6,1)	4 (5,4)	1 (5,6)
Non-Beijing total	69 (21,2)	60 (26,5)	35 (26,1)	23 (27,4)	9 (9,1)	6 (8,1)	2 (11,2)
T	21 (6,5)	19 (8,4)	14 (10,4)	4 (4,8)	2 (2,0)	1 (1,4)	0
LAM	19 (5,8)	15 (6,6)	6 (4,5)	9 (10,7)	4 (4,0)	3 (4,1)	1 (5,6)
Ural	16 (4,9)	15 (6,6)	9 (6,7)	6 (7,1)	1 (1,0)	0	1 (5,6)
H	3 (0,9)	3 (1,3)	0	2 (2,4)	0	0	0
Unknown	9 (2,8)	7 (3,1)	6 (4,5)	1 (1,2)	2 (2,0)	2 (2,7)	0
CAS1-Delhi	1 (0,3)	1 (0,4)	0	1 (1,2)	0	0	0

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was phylogenetically close to strains from Uzbekistan, suggesting the probability of origin of this strain outside the Kemerovo region (**Fig. 3**, highlight 1). Six others were included in 3 separate groups on the most recent highly resolved branches (bootstrap from 87% to 100%) among modern strains from Kazakhstan and Europe (Germany). This arrangement of the Kemerovo samples on the tree confirms the absence of a direct chain of transmission between these TB cases (**Fig. 3**, highlights 2–4).

Discussion

A study of a modern sample of *M. tuberculosis* strains from the Kemerovo region shows that the overall genotype structure is typical for the Asian part of Russia, where the dominant genotype Beijing (78.8%) is represented mainly by two subtypes: Central Asian Russian (41.2%) and B0/W148 (32.9%) [11–14]. However, a marked predominance of Beijing was found among previously treated TB cases (90.9%), and primarily due to a significant increase in the proportion of B0/W148 (from 27.0 to 45.5%). The increase in the proportion of Beijing B0/W148 was seen among both HIV-negative and HIV-infected TB patients. This unidirectional accumulation in both groups reflects the properties of this epidemic subtype, which in most cases carries primary MDR. Overall, its qualities reduce the efficacy of treatment of new TB cases and likely reduce adherence to MDR-TB therapy in repeated courses of treatment.

The Beijing genotype, which occurred equally frequently in TB patients with different HIV status, revealed higher MDR and pre-XDR levels in both groups: no significant association of MDR-TB with HIV infection was found. Thus, the previously found excess MDR-TB frequency among TB/HIV patients in Russian studies [13, 15–17] and their association with MDR strains was not confirmed in the present study.

The main distinctive feature of the *M. tuberculosis* population in Kemerovo region was the detection of about 30% (40/134) of CAO subtype strains in the structure of Beijing Central Asian Russian. According to our study, MDR frequency among Beijing Central Asian Russian strains was second only to Beijing B0/W148 (95.3%): about 60% of strains were MDR+pre-XDR (79 out of 134). This subtype, which unites a heterogeneous group of strains, in the Kemerovo region showed not only an exceptionally high presence of Beijing CAO (12.9% of the total collection) compared to other regions of Russia (**Table 4**), but also extremely high levels of MDR+pre-XDR (87.5%; 35/40). In turn, exclusion of CAO samples from the Central Asian Russian group leads to a decrease in the proportion of strains with MDR to 48.6% (44 out of 94), which corresponds to the average for the Beijing genotype (49.1% MDR; 53/108) without considering B0/W148 in this group. Importantly, Beijing CAO was significantly more frequently detected among HIV-infected people (20.6%). Thus, not only B0/W148 but also strains of the CAO subtype were the most successful among Beijing strains in the Kemerovo region — Kuzbass. The latter clearly benefited from the acquisition of MDR/pre-XDR, expressed in a wide spread among HIV-infected people in the region.

The cross-border route of transmission of TB caused by Beijing CAO from Central Asian countries to the population of the Kemerovo region can be considered as the most obvious one for the emerging of

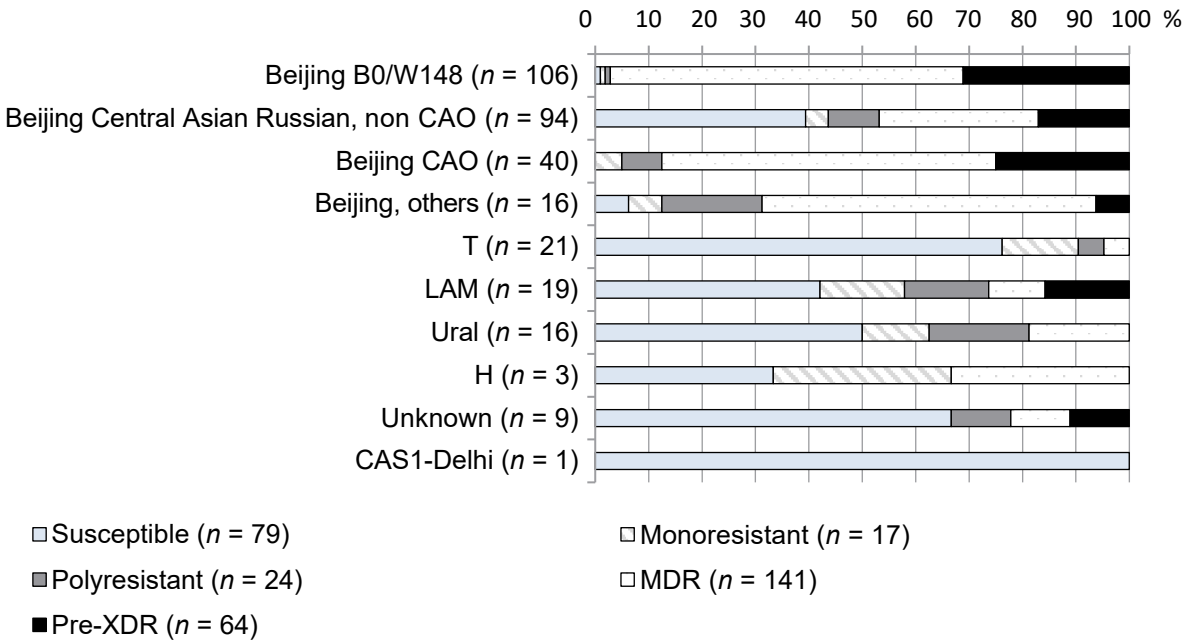

Fig. 2. Drug resistance of *M. tuberculosis* isolates in the Kemerovo region, %.

the first cases. An outbreak of CAO in Karakalpakstan (Uzbekistan) described by M. Merker et al., evaluated on strains from 2001–2005 [5], and then the detection of accumulation since 2015 and the significant presence of Beijing SAO in modern samples in Kazakhstan [21–23] indicate its successful spread outside Uzbekistan and the setting up of conditions for endemic circulation. All Beijing CAOs detected in the Kemerovo collection belonged to the MIRU-VNTR 94-32 supercluster [6], although other MIRU-VNTR profiles of CAOs in Russian regions have also been described [10, 22], emphasizing their current evolution due to the origin from 94-32 [6].

It is highly probable that transmission could have been carried out by labor migrants from Central Asia, whose regular influx to the Kemerovo region has been observed over the past 30 years [23]. Despite the fact that the registered incidence of TB in the Kemerovo region among migrants from Central Asia is estimated to be low (less than 1 case per 100,000 migrants) [24],

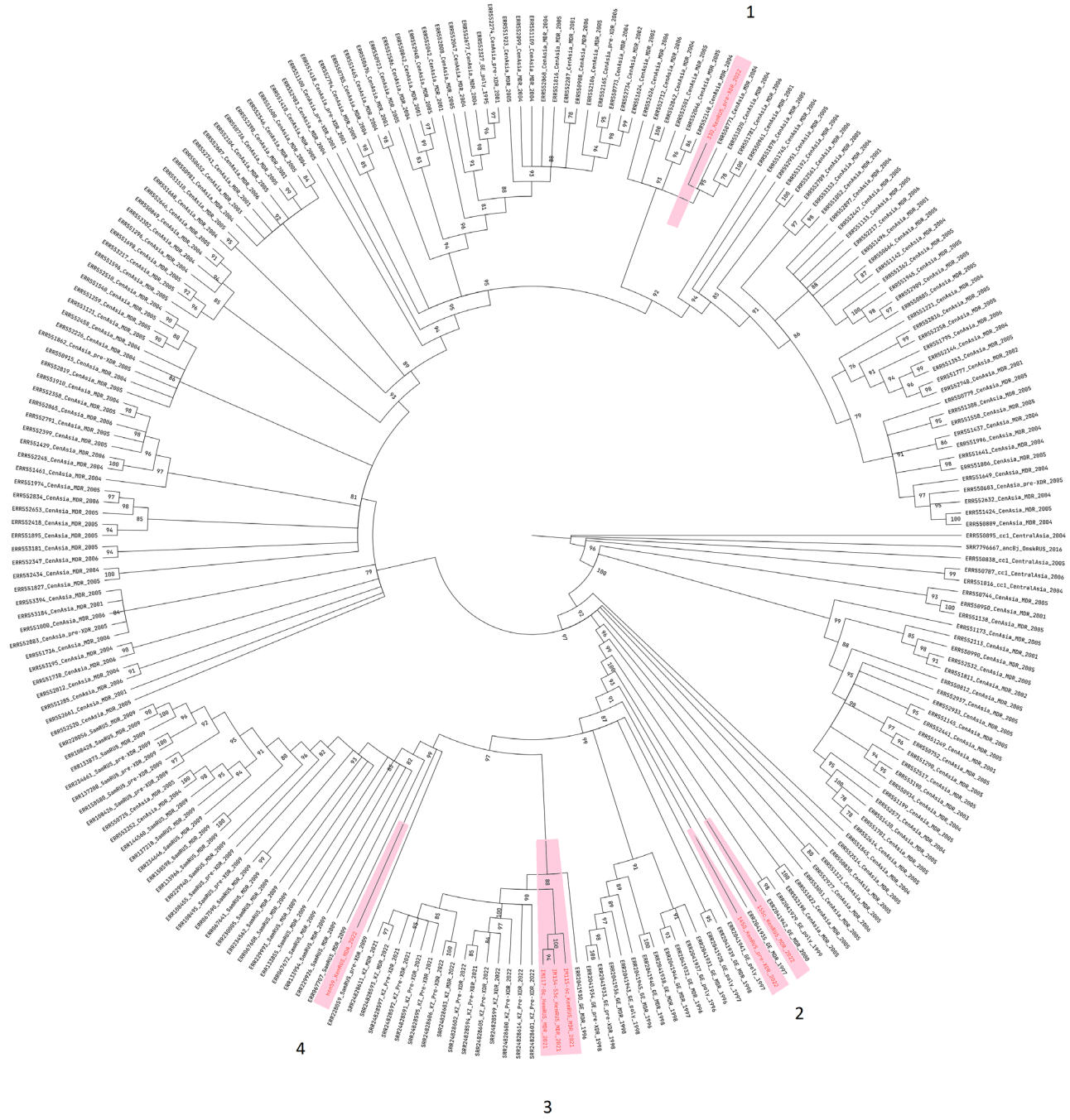

Fig. 3. Maximum-likelihood phylogenetic tree for sequences of 241 clinical isolates of Beijing CAO *M. tuberculosis*.

The genomes from the Kemerovo region are marked; numbers 1–4 mark their groups; sequences from other regions are marked in black with following designations: from Central Asia — Uzbekistan (CenAsia), Kazakhstan (KZ), Samara — Russia (SamRus), Germany (GE).

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Table 4. Beijing CAO of *M. tuberculosis* isolates from Central Asian countries and Russia, *n* (%)

Countries (total number of isolates)	*n*	Number of isolates, % of total			Source
		Beijing total	Beijing Central Asian Russian	Beijing CAO	
Central Asia					
Uzbekistan	235	136 (57,9)	58 (24,7)	–	[18]
Uzbekistan	277	237 (85,5)	174 (62,8)	173 (62,5)	[5]
Tajikistan	206	154 (78,4)	49 (23,8)	–	[18]
Kyrgyzstan	166	121 (72,9)	38 (22,9)	–	[18]
Kazakhstan	701	538 (76,7)	314 (51,7)	116 (16,5)	[19]
Kazakhstan	29	24 (82,8)	20 (69,0)	15 (51,7)	[20]
Russia					
Komi	130	73 (56,2)	45 (34,6)	2 (1,5)	[10]
Vologda	82	51 (62,2)	41 (50,0)	7 (8,5)	[16]
Kaliningrad	73	46 (63,0)	21 (28,8)	4 (5,5)	[10]
Karelia	67	36 (53,7)	18 (26,9)	3 (4,5)	[10]
Murmansk	67	35 (52,2)	23 (34,3)	1 (1,5)	[10]
Pskov	78	45 (57,7)	28 (35,9)	2 (2,6)	[10]
Samara	428	354 (82,7)	214 (50,9)	28 (6,5)	[5]
Omsk	131	93 (71,0)	51 (38,9)	5 (9,8)	[6]

Note. Dash indicated the absence of data.

the possibility of transmission of the *M. tuberculosis* to the local communities. This is consistent with the data from the neighboring Novosibirsk region, where TB cases among migrants were registered among citizens of Uzbekistan, Tajikistan, Kyrgyzstan and Kazakhstan, which prevail in seasonal labor migration in Siberia [25]. In the Kemerovo region, more than 25 thousand people who come to work every year are officially registered. Labor migrants are employed in construction, manufacturing industry, wholesale and retail trade, although they account for no more than 1% of the region's employed population [23].

In the present study, the fact that Beijing CAO strains were detected in 40 TB cases among the local population of the Kemerovo region, in particular, in 20.6% — HIV/TB, suggests the presence of conditions of endemic circulation of Beijing CAO in local setting.

Kuzbass is a highly urbanized industrial region [23] with an epidemic level of HIV infection. Despite the downward trends in the total number of registered HIV-infected persons since 2016, high levels of new cases remain [26]. In 2021, the all-Russian incidence rate was 41.7 per 100,000 population, and in the Kemerovo region — 83.23. The role of HIV-infected people in the setting up of condition for circulation of successful variants of *M. tuberculosis* in local area cannot be excluded. The risks of developing bacillary forms of TB in HIV-infected people against the background of insufficient efficacy of MDR-TB chemotherapy are in favor of some increase in the infectiousness of HIV-infected people [27]. Furthermore, a recent study shows that among coinfected people, the probability of recent TB transmission is more than 2 times higher than in the general population of TB patients. Moreover, endemic strains had a higher chance of being responsible for recent transmission among HIV-positive patients [28]. In the present study sample, Beijing CAO subtype was identified among new cases significantly more frequently in HIV/TB (17/84) than in HIV-negative TB patients, which may also be a consequence of their recent infection.

Phylogenetic reconstruction using WGS data confirms that the spread of CAO strains in the Kemerovo region is most likely the result of the expansion of Beijing isolates of this subtype into Russia from Uzbekistan. Previously, M. Merker et al. reconstructed the evolutionary history of CAO strains obtained in 2001-2006 as a result of an outbreak in Uzbekistan and showed the appearance of the first strains in the mid-1970s [5]. Phylogeography of CAO strains shows that the expansion from Uzbekistan is directed not only to different regions of Russia (Table 4), but also to European countries, as evidenced by the CAO strains from Germany and Kazakhstan presented in the tree (Fig. 3), among which 6 isolates from Kemerovo are clustered. The presence of one Kemerovo isolate in the cluster of CAOs from Uzbekistan indicates the possibility of recent transboundary transmission of this subtype from Central Asia to the Kemerovo region.

[3] ВИЧ-инфекция за 2021 г. Бюллетень № 47. URL: http://www.hivrussia.info/wp-content/uploads/2023/05/Byulleten-47-VICH-infektsiya-za-2021-g.pdf (date of access: March 20, 2024).

Thus, in the Kemerovo region, a significant distribution of strains of not only highly transmissible subtype B0/W148 (31.9%), but also of Beijing CAO subtype (12.6%), which is rarely found in Siberia, was revealed, carrying MDR and pre-XDR in most cases. The hypothesis of Beijing CAO emergence in Kuzbass as a result of repeated introductions from Uzbekistan directly or indirectly through neighboring countries is presented. The phenomenon of Beijing CAO anchoring in Kuzbass as well as the endemic circulation of these strains among HIV-infected people requires further study using WGS.

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Production of a new variant of soluble trimer Env of HIV-1 CRF63_02A6 SOSIP.664

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Abstract

Introduction. Obtaining stabilized recombinant HIV-1 Env trimers that have a close to the native conformation is one of the directions in the field of development of vaccines against HIV-1.

The aim of the study was to obtain and characterize the stabilized trimer Env of HIV-1 SOSIP.664 based on the circulating genetic variant of the recombinant form CRF63_02A6.

Materials and methods. Bioinformatics resources were used to design the trimer Env gene based on the HIV-1 recombinant genetic variant CRF63_02A6. The designed gene was synthesized and cloned as part of an integration plasmid vector. A stable producer of trimer Env was obtained by transfection of the CHO-K1 cell line using the developed plasmid vector. Purification of the protein complex was performed using affinity chromatography and gel filtration. Antigenic properties of trimer Env were studied using immunochemical analysis using broadly neutralizing HIV-1 monoclonal antibodies (bnAbs).

Results. A new variant of the stabilized trimer Env of the surface glycoprotein of the recombinant form CRF63_02A6 HIV-1 SOSIP.664 was designed, including additional stabilizing modifications. Based on the CHO-K1 cell line, a stable producer was obtained and a purification protocol for the designed trimer Env was developed. It was found that the trimer Env CRF63_02A6 SOSIP.664 is effectively recognized by bnAbs 2G12, VRC01 and PGT126.

Conclusion. The obtained results indicate the prospects for further study of the structural features of the trimer Env CRF63_02A6 SOSIP.664, as well as its immunogenicity and the possibility of using it as a vaccine antigen.

Keywords: *HIV-1, CRF63_02A6, Env trimers, SOSIP.664*

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Оригинальное исследование
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Получение нового варианта растворимого тримера Env ВИЧ-1 CRF63_02A6 SOSIP.664

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Аннотация

Введение. Получение стабилизированных рекомбинантных тримеров Env вируса иммунодефицита человека-1 (ВИЧ-1), близких к нативной конформации, является одним из направлений в разработке вакцин против ВИЧ-1.

Цель работы — получить и охарактеризовать стабилизированный тример Env ВИЧ-1 SOSIP.664 на основе циркулирующего генетического варианта рекомбинантной формы CRF63_02A6.

Материалы и методы. Для дизайна гена тримера Env на основе генетического варианта ВИЧ-1 рекомбинантной формы CRF63_02A6 использовали биоинформатические ресурсы. Спроектированный ген синтезирован и клонирован в составе интеграционного плазмидного вектора, с использованием которого получен стабильный продуцент тримера Env на основе клеточной линии CHO-K1. Очистку белкового комплекса проводили с помощью аффинной хроматографии и гель-фильтрации. Антигенные свойства тримера Env исследовали с помощью иммунохимического анализа с использованием широко нейтрализующих ВИЧ-1 моноклональных антител (bnAbs).

Результаты. Спроектирован новый вариант стабилизированного тримера Env поверхностного гликопротеина рекомбинантной формы CRF63_02A6 ВИЧ-1 SOSIP.664, включающего дополнительные стабилизирующие модификации. На основе клеточной линии CHO-K1 получен продуцент, стабильно продуцирующий спроектированный тример Env, и разработан протокол его очистки. Установлено, что тример Env CRF63_02A6 SOSIP.664 эффективно распознается bnAbs 2G12, VRC01 и PGT126.

Выводы. Полученные результаты свидетельствуют о перспективности дальнейшего изучения структурных особенностей тримера Env CRF63_02A6 SOSIP.664, а также его иммуногенности и возможности использования в качестве вакцинного антигена.

Ключевые слова: *ВИЧ-1, CRF63_02A6, тример Env, SOSIP.664*

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Introduction

According to the Joint United Nations Program on HIV/AIDS (UNAIDS), the total number of people worldwide living with HIV-1 in 2023 was 39.9 million, and the number of recent HIV infections was more than 1 million[1]. In Russia during the year 2023, there were about 1.2 million people with a confirmed diagnosis of HIV infection[2]. The development of an effective vaccine to prevent HIV infection continues to be one of the most pressing unresolved problems.

Induction of an immune response capable of preventing infection with different HIV-1 subtypes is a critical goal for vaccines with the purpose of protection against HIV-1 infection. It is known that broadly neutralizing HIV-1 antibodies (bnAbs) can prevent HIV-1 infection by binding to envelope glycoproteins (Env) on the virion surface [1–4]. Work is actively underway to obtain native Env complexes to study their immunogenicity and determine their suitability as a vaccine antigen [5–8]. Over the last decade, significant progress has been made in this field. For example, various modifications, including SOSIP.664, have been developed to obtain a stabilized structure of recombinant Env trimers close to the native conformation and thus increase their ability to induce desired NAb responses against heterologous viruses [6, 9, 10]. Variants of stabilized Env trimers of genetic subtypes of HIV-1 (A, B, and C) circulating predominantly in Europe, America, and Africa were obtained, which showed the ability to induce neutralizing antibodies [6, 8, 11].

The recombinant form CRF63_02A6 is currently dominant in Siberian regions [12, 13]. According to M.V. Sivay et al., the recombinant form CRF63_02A6 was first registered in the Novosibirsk region in 2006. [14]. After that, it actively spread to other territories of Siberia (Omsk, Altai, Tomsk, Krasnoyarsk, and Kemerovo), displacing the A6 subtype and becoming the dominant strain. By 2020, CRF63_02A6 was detected in 20 regions of Russia and 6 countries of Central Asia. A study by the authors showed that CRF63_02A6 is detected in more than 80% of new HIV-1 cases in Siberia [14].

The aim of this study was to obtain a stabilized trimer Env recombinant form CRF63_02A6 of HIV-1 SOSIP.664, including additional stabilizing modifications.

Materials and methods

Monoclonal antibodies, bacterial strains, cell lines

Monoclonal antibodies PGT126, 2G12, VRC01 were obtained from the NIH HIV Reagent Program. CHO-K1 cell line was taken from the Collection of Cell Cultures of State Research Center of Virology and Biotechnology "Vector".

Design of an Env stabilized trimer of the recombinant form CRF63_02A6

The nucleotide sequence encoding the HIV-1 env trimer was designed based on the env 22RUAR13 gene of the recombinant form CRF63_02A6 (Collection of bacteria, bacteriophages and fungi of FBUNC Vector, Rospotrebnadzor, registration number P-124) using the SnapGene v. 3.2.1 and BioEdit v. 7.2.5 programs. Simulations were performed using Alphafold2 neural network colab program[3]; visualized using RCSB PDB website online tool, Mol*Plagin 3.43.1 3D Viewer tool. After the design process, codon optimization of the gene for expression in mammalian cells was carried out using online Codon Adaptation Tool[4]. The synthesis of the gene was carried out by DNA Synthesis. The obtained gene was cloned as part of an integration plasmid vector[5]. The integrity of the genetic construct was confirmed by sequencing (SB RAS Genomics Core Facility of the Institute of Chemical Biology and Fundamental Medicine).

Obtaining a stable producer of the trimer Env recombinant form CRF63_02A6 SOSIP.664

A stable Env trimer producer was obtained from the CHO-K1 cell line as described in [15]. After obtaining a polyclonal cell pool, it was used to isolate monoclonal cell cultures. For this purpose, the polyclonal cell culture was seeded in a 96-well plate at 1 cell per well in 200 µl of complete DMEM/F12 growth medium (Servicebio) containing 10% fetal bovine serum (HiMedia) and the antibiotic puromycin (Invivogen) at a concentration of 10 µg/mL and grown until a monolayer was achieved within 7-14 days. After this time, the grown monoclonal cultures were crossed into a 24-well plate. After the monoclonal cell lines formed a monolayer, they were analyzed for transgene expression using dot-blot analysis.

The selected monoclonal culture was then used for protein production. The monoclonal cell culture CHO-K1-gp140.SOSIP.664.opt was cultured in roller vials for 7 days, then the culture medium was collected and centrifuged to remove cellular debris. A puri-

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[3] URL: https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb

[4] URL: https://www.jcat.de

[5] Patent No. 2800471 Russian Federation, MPK C12N15/00 (2006.01), C07K16/10 (2006.01), C12N1/00 (2006.01), C12N15/63 (2006.01). Plasmid genetic construct pVEAL3-10H10ch, recombinant cell line strain CHO-K1-10H10ch and chimeric antibody 10H10ch against tick-borne encephalitis virus produced by said cell line strain CHO-K1-10H10ch: 2022126714: applied. 13.10.2022: publ. 21.07.2023 / Shanshin D.V., Nesmeyanova V.S., Shcherbakov D.N. et al. URL: https://patents.google.com/patent/RU2800471C1/ru (date of reference: 18.09.2024).

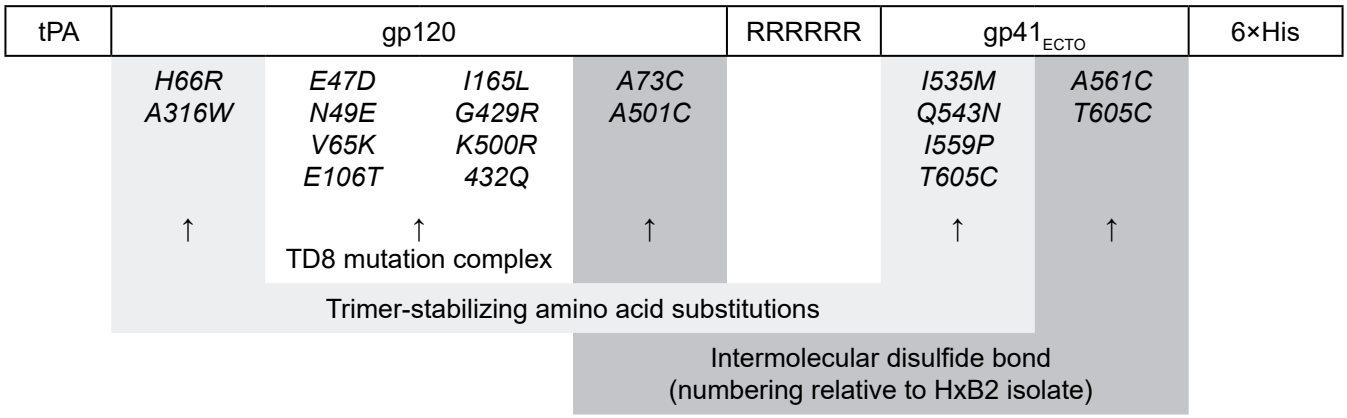

Fig. 1. Design of the Env trimer gp140.SOSIP.664.opt.

fication procedure was performed using IMAC Seplife FF (Sunresin). The degree of purification of the target protein was assessed by polyacrylamide gel electrophoresis (PAAG) under denaturing conditions in the presence/absence of reducing agents followed by Coomassie G250 staining. Fractions containing the target protein were pooled and dialyzed against buffer (75 mM NaCl, 10 mM Tris, pH 8.0); concentrated using a 100 kDa centrifuge concentrator (Jet Biofil). Affinity-purified proteins were then separated by exclusion chromatography using a Chrom-LinXTM 16/1000 Tiderose GF200 column (Taidu Biotech) at a flow rate of 1 min/mL.

Dot-blot and western blot analyses

Dot-blot and Western blot analyses were performed according to standard methods using the SNAP i.d. 2.0 system (Millipore) and bnAb PGT126. Goat antibodies against human IgG conjugated with alkaline phosphatase (Sigma) were used as secondary antibodies. The immune complex was visualized by adding NBT/BCIP solution (Sigma).

Enzyme immunoassay

Enzyme immunoassay was performed according to standard methods. The primary antibodies used were bnAbs PGT126, 2G12, VRC01, and secondary antibodies were horseradish peroxidase-labeled goat anti-human IgG antibodies (Sigma). Tetramethylbenzidine solution (Imtek) was used as a chromogenic substrate. The reaction was stopped with 1 N hydrochloric acid solution. The optical density was measured on a Varioskan LUX instrument (Thermo Scientific) at a wavelength of 450 nm.

Statistical processing of data was performed using the GraphPad Prism 9 program (GraphPad Software Inc.).

Results

In this study, the characterized *env* 22RUAR13 gene, which belongs to the current recombinant form CRF63_02A6 of HIV-1 currently circulating in the Siberian region, was used for the design. To improve the formation of soluble trimers, the construct contained the following modifications compared to the natural sequence: the natural signal peptide was replaced by the tPA (tissue plasminogen activator signal peptide) signal sequence; 2 cysteines were added to form the gp120-gp41 disulfide bond (501C and 605C); added stabilizing amino acid substitutions in gp41 (*I559P*, *H66R* and *A316W*); added amino acid substitutions in gp41 (*I535M* and *Q543N*) and TD8 mutation complex (*E47D*, *N49E*, *V65K*, *E106T*, *I165L*, *G429R* and *K500R*; *432Q*); to enhance cleavage, the REKR motif in gp120 was replaced with RRRRRR (R6); the MPER region, transmembrane and cytoplasmic domains were also deleted for better expression of the designed protein; after amino acid residue 664 at the C-terminus, 6×His was added for further purification [6] (**Fig. 1**). The resulting Env trimer variant of the recombinant form CRF63_02A6 was designated as gp140.SOSIP.664.opt.

Spatial structure modeling showed that the designed amino acid sequence was capable of forming specific monomers and trimeric complexes (**Fig. 2**).

Furthermore, a polyclonal cell culture CHO-K1-gp140.SOSIP.664.opt was obtained by transfection of

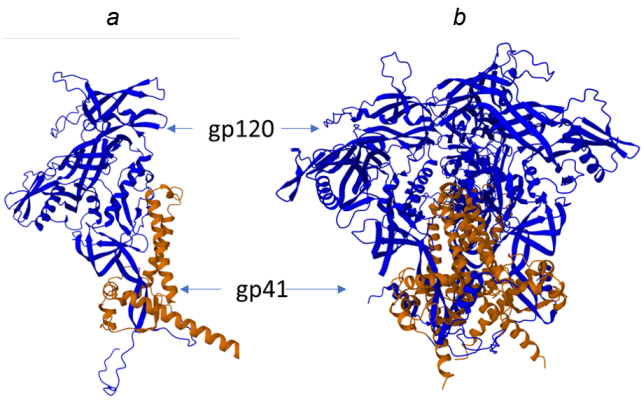

Fig. 2. Structure model of the monomer (*a*) and trimer (*b*) of gp140.SOSIP.664.opt.

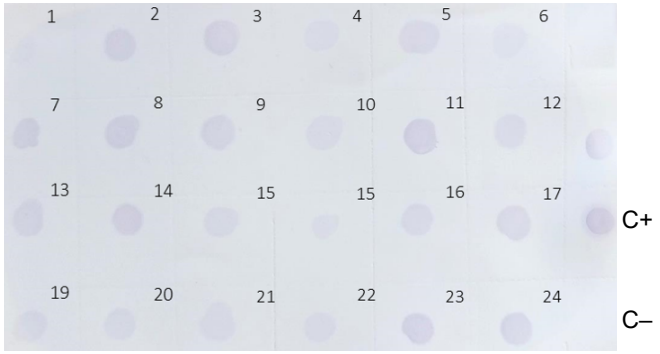

Fig. 3. Dot blot analysis of the culture medium collected from monoclonal cell cultures CHO-K1-gp140.SOSIP.664.opt.

Recombinant Env trimer CRF63_02A6 was used as C+, and culture medium collected from non-transformed CHO-K1 cells was used as C–.

the CHO-K1 cell line, which was used to isolate monoclonal cell lines by the limiting dilution method. As a result, 24 monoclonal cultures were obtained and examined for their ability to produce the target protein into the culture medium using dot-blot analysis. As a result, a monoclonal culture (No. 11) with high transgene expression was identified (**Fig. 3**), which was used for further research.

For preparative production of the target protein, monoclonal cell culture of CHO-K1-gp140.SOSIP.664.opt was cultured in roller bottles followed by purification of the recombinant Env trimer. At the end of cultivation, the culture medium was collected, and the target protein was purified by metal-chelate affinity chromatography, dialysis and subsequent gel filtration. The protein production was about 15 mg/L.

The purified Env protein product was characterized by PAGE electrophoresis in the presence or absence of β-mercaptoethanol. Based on the amino acid sequence of recombinant Env CRF63_02A6 (683 a. a.), the theoretically calculated molecular mass of the monomer should be 76.8 kDa. However, it is known that in eukaryotic cells the Env HIV-1 protein is highly glycosylated and about 50% of its molecular mass can be made up of glycans [16, 17], resulting in an increase in the mass of the monomer to 140 kDa. In the eukaryotic cell culture, glycosylation of Env protein may be incomplete and there may be glycosylation intermediates. For this reason, in the case of electrophoresis under denaturing conditions (in the presence of β-mercaptoethanol), 2 bands in PAGE are observed (**Fig. 4**, *a*), differing by approximately 10-20 kDa, which represent monomers of the recombinant Env

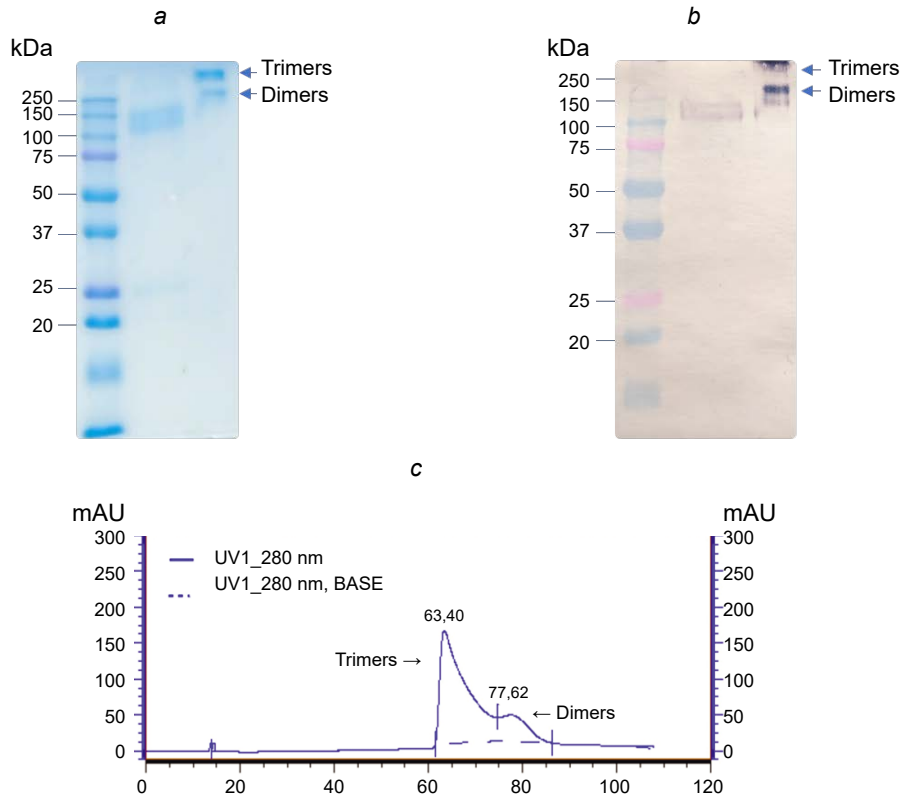

Fig. 4. Characterization of the Env protein.

a — electropherogram of separation of recombinant Env trimer gp140.SOSIP.664.opt in 12% PAGE: *1* — molecular weight marker of proteins Precision Plus Dual Color Standards (Bio-Rad, USA); *2* — in the presence of β-mercaptoethanol; *3* — in the absence of β-mercaptoethanol. *b* — immunoblotting of Env trimer gp140.SOSIP.664.opt with monoclonal antibody PGT126: *1* — molecular weight marker of proteins Precision Plus Dual Color Standards (Bio-Rad, USA); *2* — in the presence of β-mercaptoethanol; *3* — in the absence of β-mercaptoethanol; *c* — profile of size-exclusion chromatography of Env trimer gp140.SOSIP.664.opt on a Chrom-LinXTM 16/1000 Tiderose GF200 column.

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EC_{50} values calculated for gp140.SOSIP.664.opt compared with literature data

bnAbs	EC_{50} μg/mL			
	gp140.SOSIP.664.opt (CRF63_02A6)	BG505 (A)	JR-FL (B)	B41 (B)
PGT126	0,019	0,026–0,06 [18, 19]	N. d.	N. d.
2G12	0,313	0,027–0,07 [18, 19]	0,21 [19]	0,13 [19]
VRC01	0,098	0,163–1,35 [18, 19]	0,67 [19]	5,20 [19]

Note. N. d. — no data.

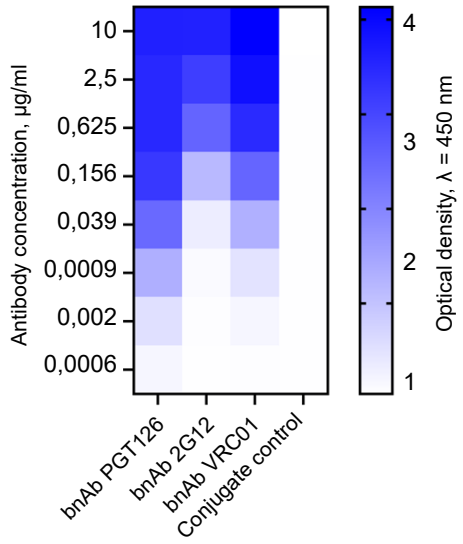

Fig. 5. Specific activity of monoclonal broadly neutralizing antibodies against Env trimer of the recombinant form CRF63_02A6 gp140.SOSIP.664.opt (presented as a heat map). The amount of absorbed antigen was 100 ng/well.

trimer with different degrees of glycosylation. Both are recognized by the monoclonal antibody PGT126 (Fig. 4, *b*).

In the absence of β-mercaptoethanol (Fig. 4, *a*, band 3), monomeric forms of protein are absent in PAGE, and higher order oligomers (more than 250 kDa) are detected.

Using gel filtration on a pre-calibrated ChromLinXTM 16/1000 Tiderose GF200 column (Taidu Biotech), it was found that the main peak of the target protein comes out in the region corresponding to the trimeric form (Fig. 4, *c*).

To study the antigenic properties of the Env trimer, an enzyme immunoassay was performed using bnAbs 2G12, VRC01 and PGT126 targeting an epitope including carbohydrate residues at positions 295, 332 and 392, a CD4-binding site and a V3-loop on the surface of gp120, respectively. The resulting Env gp140.SOSIP.664 trimer.opt was found to be efficiently recognized by monoclonal broadly neutralizing antibodies (**Fig. 5**), which may indicate that the Env trimer synthesized in the producer cells is properly stacked and maintains its conformation. The EC_{50} values for bnAbs 2G12, VRC01 and PGT126 were 0.313, 0.098 and 0.019 μg/mL, respectively (**Table**).

Discussion

Env trimers of various HIV-1 subtypes have been actively investigated worldwide in terms of both structural and antigenic features and immunogenic properties [6–8, 11]. Thus, native Env trimers of clades A, B, C, and G [20–26], as well as Env trimers based on the consensus sequence of clade C [27] and group M [6], respectively, have been characterized in detail using biochemical and structural approaches.

Predominantly, studies have focused on subtypes A, B, and C, with virtually no data on various CRFs [28], in particular the recombinant form CRF63_02A6, which is rapidly spreading in Russian regions and is currently dominant in the Siberian Federal District [12–14].

We previously obtained an Env trimer of the recombinant form CRF63_02A6 HIV-1, the nucleotide sequence of the *env* gene of the recombinant form CRF63_02A6 HIV-1 and known modifications of SOSIP.664 were also used in the design of this trimer [29]: Intermolecular disulfide bond (SOS, A501C-T605C) for binding gp120 and gp41; improved furin cleavage site (RRRRRR); point substitution of *I559P*, which helps stabilize gp41 subunits in a pre-fusion conformation; deletion of MPER region, transmembrane and cytoplasmic domains for better expression; addition of 6×His at the C-terminus for subsequent purification; the signal peptide was left natural. Using immunoassays, it is shown that CHO-K1 trimers synthesized in cells are recognized by both monoclonal broadly neutralizing antibodies and sera from HIV-positive patients [15].

Later, high-resolution crystal structures of BG505 SOSIP were obtained, which made it possible to identify additional positions of amino acid residues involved in trimer stabilization and contributing to the formation of well-ordered, homogeneous, and highly stable soluble trimers [6, 30]. The modified BG505 SOSIP.664-140 Env trimers obtained taking into account these data demonstrated the ability to induce a specific humoral immune response in laboratory animals. It was shown that such SOSIP.664-140 complex is actively recognized by neutralizing monoclonal antibodies, and, on the contrary, non-neutralizing antibodies do not bind to it [18].

In this study, bioinformatic resources were used to design an Env trimer gp140.SOSIP.664.opt of recombinant form CRF63_02A6, in the structure of which, along with the SOS disulfide bond (A501C-T605C) and

the amino acid substitution *I559P*, we introduced additional amino acid substitutions and the TD8 mutation complex in gp120, leading to additional stabilization of the protein complex [6]. As a result, an optimized stabilized trimer of Env CRF63_02A6 HIV-1 was obtained. Computer modeling using Alphafold2 showed that the designed amino acid sequence is able to fold into monomers and trimeric complexes (Fig. 1, *b*).

Using PAGE electrophoresis and Western blot analysis, we found that in the presence of β-mercaptoethanol, the purified HIV-1 Env trimer CRF63_02A6 denatures to a monomer (molecular mass ~140 kDa; Fig. 4, *a*) that is specifically recognized by bnAb PGT126 (Fig. 4, *b*). In the absence of β-mercaptoethanol, the monomer fraction is absent, indicating that the protein tends to aggregate into larger structures (Fig. 4, *a*). Gel filtration revealed that the protein in solution is predominantly in trimeric form (Fig. 4, *c*).

When CHO-K1-gp140.SOSIP.664.opt cells were cultured, the Env trimer was detected in the culture medium, indicating that it is soluble. The fact that the obtained Env trimer is efficiently recognized by monoclonal broadly neutralizing antibodies bnAbs 2G12, VRC01 and PGT126 (Fig. 5), which recognize conformational epitopes on the Env surface within the viral particle, testifies to its proper folding and preservation of conformation. It should be noted that the EC_{50} values obtained for the gp140.SOSIP.664.opt Env trimer differ from the EC_{50} values for Env trimers of other HIV-1 subtypes (Table). This may indicate the presence of antigenic differences between the above-mentioned HIV-1 strains.

Conclusion

An optimized stabilized trimer of Env HIV-1 gp140.SOSIP.664.opt based on the actual circulating genetic variant of the recombinant form CRF63_02A6 was created and characterized. The obtained data indicate the prospect of further study of its structural features, immunogenicity and the possibility of using it as a vaccine antigen.

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Self-replicating recombinant virus-like particles of lentivirus proliferating in glioblastoma cells and normal human macrophages

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Abstract

Introduction. Both attenuated and inactivated vaccines are used in disease control. Inactivated vaccines are very diverse and include whole cell and acellular vaccines containing protein target antigens or nucleic acids encoding target antigens. Immunity induced by inactivated vaccines is not believed to be long-lasting. It is very problematic to develop a vaccine against viruses that integrate into the genome of the host cell, as well as against persistent viruses that penetrate the central nervous system (CNS), which is typical for the human immunodeficiency virus type 1 (HIV-1).

Aim of the study: to evaluate the possibility of forming HIV-1 recombinant virus-like particles (recVLPs) and HIV-1B recombinant VLPs and simian immunodeficiency virus (SIV) — $SHIV_{89.6P}$ based on self-replicating RNAs (srRNAs) producing target lentivirus antigens on the alphavirus replicon platform (Sindbis virus or Venezuelan equine encephalomyelitis virus (VEEV)), and also to evaluate the ability of HIV-1B and $SHIV_{89.6P}$ VLPs to infect glioblastoma cells and normal human macrophages.

Materials and methods. BHK-21 cells were transfected with the srRNA mixture by electroporation. Recombinant virus-like particles (recVLP's) in recVLP's-infected cells were detected using the immunofluorescence assay (ELISA) and electron microscopy. recVLP's were used to infect glioblastoma cells and normal macrophages from a healthy donor.

Results. Based on the genomic RNA of the alphavirus, the plasmids were created, transcription from which makes it possible to obtain RNA that expresses lentiviral gene products in cells in quantities sufficient for the formation of mature VLPs. In BHK-21 cells infected with recVLP's, virus-specific antigens are detected only in the cytoplasm, but not in the nucleus. Both glioblastoma cells (U87) and normal human macrophages containing CD4 receptor and SSR5 and CXR4 co-receptors give infectious progeny of HIV-1B and $SHIV_{89.6P}$ recVLP's when infected with supernatant obtained after transfection of BHK-21 cells with srRNA.

Discussion. The results obtained show the possibility of expressing lentivirus structural proteins in glioblastoma cells (U87) and in normal human macrophages and can be used in the future to study the presentation of antigens in native and functional conformations in appropriate model systems to study the possibility of suppressing HIV infection in viral reservoirs in the CNS.

Keywords: *self-replicating VLPs, lentiviruses, CNS, VEEV, neuroinvasiveness*

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Оригинальное исследование
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Самореплицирующиеся рекомбинантные вирусоподобные частицы лентивирусов, размножающиеся в клетках глиобластомы и макрофагах человека

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Аннотация

Введение. В борьбе с заболеваниями применяются аттенуированные и инактивированные вакцины. Инактивированные вакцины весьма разнообразны и включают цельноклеточные и бесклеточные вакцины, содержащие белковые целевые антигены, или нуклеиновые кислоты, кодирующие целевые антигены. Считается, что иммунитет, индуцируемый инактивированными вакцинами, не долгосрочен. Весьма проблематична разработка вакцин против вирусов, интегрирующихся в геном клеток хозяина, а также против персистирующих вирусов, проникающих в центральную нервную систему (ЦНС), что характерно для вируса иммунодефицита человека типа 1 (ВИЧ-1).

Цель работы: оценить возможность образования на основе самореплицирующихся РНК (срРНК), продуцирующих целевые антигены лентивируса на платформе альфавирусного репликона (вирус Синдбис или вирус венесуэльского энцефаломиелита лошадей), рекомбинантных вирусоподобных частиц (рекВПЧ) ВИЧ-1В и рекВПЧ ВИЧ-1В и вируса иммунодефицита обезьян ($SHIV_{89.6P}$), а также их способность инфицировать клетки глиобластомы и макрофаги человека.

Материалы и методы. Клетки почки новорождённого хомяка (ВНК-21) трансфицировали срРНК с помощью электропорации; рекВПЧ в инфицированных клетках выявляли с помощью микроиммунофлуоресцентного анализа и электронной микроскопии и использовали для заражения клеток глиобластомы и макрофагов человека.

Результаты. На основе геномной РНК альфавируса созданы плазмиды, позволяющие получить срРНК, экспрессирующие в клетках продукты генов лентивирусов в количестве, достаточном для формирования зрелых рекВПЧ. В клетках ВНК-21, трансфицированных срРНК, вирусспецифичные антигены выявляются только в цитоплазме клеток. Клетки глиобластомы (U87), содержащие рецептор CD4 и корецепторы CCR5 и CXR4, а также макрофаги человека дают инфекционное потомство рекВПЧ ВИЧ-1В и $SHIV_{89.6P}$ при инфицировании супернатантом, полученным после трансфекции срРНК клеток ВНК-21.

Заключение. Полученные результаты показывают возможность экспрессии структурных белков лентивируса в клетках глиобластомы (U87) и в макрофагах человека и могут быть использованы в дальнейшем для изучения презентации антигенов в нативной и функциональной конформации в соответствующих модельных системах для исследования возможности подавления инфекции ВИЧ в резервуарах вируса в ЦНС.

Ключевые слова: *самореплицирующиеся ВПЧ, лентивирусы, центральная нервная система, вирус венесуэльского энцефаломиелита лошадей, нейроинвазивность*

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Introduction

Despite the introduction of antiretroviral therapy in 1996, patients with human immunodeficiency virus (HIV) infection exhibit the cognitive impairment known as HIV-associated neurocognitive disorders (HAND). Current estimates indicate that HAND is identified in 50% of people with long-term HIV infection[1,2]. The mechanisms of HAND associated with HIV-1 are not clear. The central nervous system (CNS) is highly compartmentalized and serves as a specific site of HIV-1 infection. HIV-1 replication in the CNS persists despite long-term combination antiretroviral therapy due to the inability of current antiretroviral drugs to penetrate and cross the blood–brain barrier (BBB) [1]. As a result of sustained HIV-1 replication in the CNS, even with combination antiretroviral therapy, a high incidence of HAND is observed [2, 3].

On the other hand, when analyzing the COVID-19 pandemic, there is increasing evidence that SARS-CoV-2 affects not only the respiratory tract but also the CNS, leading to neurological symptoms such as loss of smell and taste, headache, fatigue, nausea and vomiting, which are observed in more than one third of people with COVID-19 [4, 5]; acute cerebrovascular symptoms have been noted and disorders of consciousness have been reported [6]. Two strains of endemic SARS-CoV-2 infiltrate and persist in the CNS, and viral RNA has been detected in the brain and cerebrospinal fluid [7–10].

Thus, both HIV-1 and SARS-CoV-2 can be attributed to pantropic viruses, the neurotropicity of which may be due to both ophthalmic and cellular pathways of penetration into the CNS and other, as yet undetected mechanisms [11].

Back in the 1990s, we proposed to use vector viral systems that have lost their neurovirulence but retained their neuroinvasiveness to create vaccines against such pantropic viruses. Our analysis led us to the conclusion that vaccine strains TC-83 and 15 of Venezuelan equine encephalomyelitis virus (VEEV) are the most suitable for this purpose. Model experiments have confirmed that the use of a vaccine strain with these characteristics protects animals even against intracerebral infection with a virulent strain [12–14].

In 1998, we demonstrated the possibility of immunizing model animals by injecting self-replicating RNA (srRNA) of Sindbis virus carrying *env* and *gag* HIV-1B genes[3], which was confirmed by A.J. Geal et al. in 2012 when using VEEV srRNA [15].

In 2000–2003, we created VEEV replicon plasmids expressing Gag/Pol proteins and Env (gp160) $SHIV_{89.6P}$ protein as part of the International AIDS Control Organization, Vaccine Initiative, and National Institute of Allergy and Infectious Diseases National Institute of Health AIDS Vaccine Development Collaboration. The resulting plasmids were then used to study the effect of rapid degradation on the expression of the Gag portion of simian immunodeficiency virus (SIV) in a single-cycle vector. It was found that VEEV VLPs incapable of self-replication are more efficient in presenting the target antigen when the recombinant replicon is packaged into VLPs carrying spikes of wild-type VEEV glycoprotein [16].

Later, C.K. Jurgens et al. using the VEEV replicon plasmids created by D.A. Moshkoff and expressing $SHIV_{89.6P}$ Gag and $SHIV_{89.6P}$ Env (gp160) antigens, studied the expression of these proteins in monkey cells [17]. They found that expression of Gag and Env proteins on the VEEV RNA platform in primate cells resulted in the assembly of particles that morphologically and functionally resembled lentivirus virions and included an alphavirus replicon. Infection of $CD4^+$ cells with chimeric lentivirus-like particles was specific and productive, leading to RNA replication, expression of Gag and Env, and formation of daughter chimeric particles. Further genome modifications to enhance encapsidation of the chimeric virus genome and expression of attenuated SIV protease for particle maturation improved the ability of chimeric lentivirus-like particles to proliferate in cell culture. The ability to present lentivirus immunogens in native and functional conformation was demonstrated [17].

A review conducted by the 48th Central Research Institute of the Ministry of Defense examined the use of alphavirus vectors for the development of vaccines against a wide range of viruses [18]. It is emphasized that alphavirus RNA replicons combine the safety of inactivated and immunogenicity of live attenuated vaccines. Such constructs are suitable for the express development of vaccines for specific prevention of viral infectious diseases, and the presence of the TC-83 strain of VEEV suitable for human immunization determines the prospects for the creation of RNA replicons based on the genome of this pathogen [18]. Kazakh researchers integrated the gene of green fluorescent protein into the genome of VEEV under the control of a synthetic copy of the viral promoter of 26S subgenomic RNA. The RNA transcript of recombinant virus was transfected into BHK-21 cell culture. By 36 h after transfection,

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almost all cells in culture showed bright fluorescence of the marker protein [19]. Thus, the data obtained in the last 3 decades confirm the prospects of our chosen line of research.

The aim of this study is to evaluate the possibility of formation of HIV-1B and recombinant HIV-1B and $SHIV_{89.6P}$ VLPs based on srRNAs producing targeted lentivirus antigens on the alphavirus replicon platform, as well as the ability of HIV-1B and $SHIV_{89.6P}$ VLPs to infect human glioblastoma cells and macrophages.

Materials and methods

Plasmids expressing VEEV replicon RNA and chimeric viral genomes

The plasmids of recombinant VEEV replicon obtained by D.A. Moshkoff and used in the studies earlier are described in the corresponding papers [16, 17], the schematic representation of the plasmids is presented in **Fig. 1**.

Replicon particle production and titration

Linearized plasmids encoding target proteins served as a matrix for the synthesis of capped RNAs using the mMessage Machine T7 kit (Ambion). Subconfluent (80%) cells were harvested and prepared for electroporation. Cells were precipitated by centrifugation at 800g for 10 min, washed with phosphate buffer solution containing no ribonuclease, and resuspended to a concentration of 1.5×10^7 cells/mL in phosphate buffer solution containing no ribonuclease.

The *in vitro* synthesized srRNA (1 μg) was transfected into BHK-21 cells by electroporation using a BioRAD GenePulser (Bio-Rad). Cells were pulsed three times at a voltage of 850 V and a capacitance of 25 μF.

Recombinant VLPs (recVLPs) were collected and purified by precipitation through a sucrose solution pad.

The recVLPs were titrated by infecting U87.CD4-CCR5 and U87.CD4-CXCR4 glioblastoma cells with serial dilutions of purified recVLPs for 16 h at 37ºC followed by 10-min fixation with methanol at 4ºC. The fixed cells were rehydrated in phosphate-salt buffer pH 7.2 and incubated with the appropriate antiserum at a dilution of 1 : 100 for 1 h at room temperature. Antiserums to lentivirus proteins were obtained from Dr. D.C. Montefiore at the AIDS Reagent Receipt Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health). The evaluation of these neutralizing antibodies against HIV, SIV and SHIV is reported in [20]. Cells were washed and incubated with biotinylated anti-mouse IgG antibodies followed by streptavidin conjugated to Alexaflour Texas Red. RecVLP-infected cells were evaluated under the microscope by fluorescence under UV illumination.

Cells

All cells were obtained from the AIDS Reagent Receipt Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Newborn hamster kidney cells (BHK-21) were maintained in minimal essential alpha medium supplemented with 10% phosphate-salt buffer, 2 mM L-glutamine, and 100 units/mL penicillin and streptomycin at 37ºC in a CO_2 incubator [17].

U87 cells stably expressing CD4 and wild-type CCR5 co-receptor ($U87.CD4^+CCR5^+$ and $U87.CD4^+X4^+$) were cultured in Dulbecco's modified Iscov's medium (Termo Fischer Scientific) supplemented with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin (10 μg/mL) [21].

Commercial macrophage cultures were obtained from $CD14^+$ human peripheral blood monocytes (purity > 90%). Macrophages were cultured from monocytes in rhM-CSF-containing medium for several days and

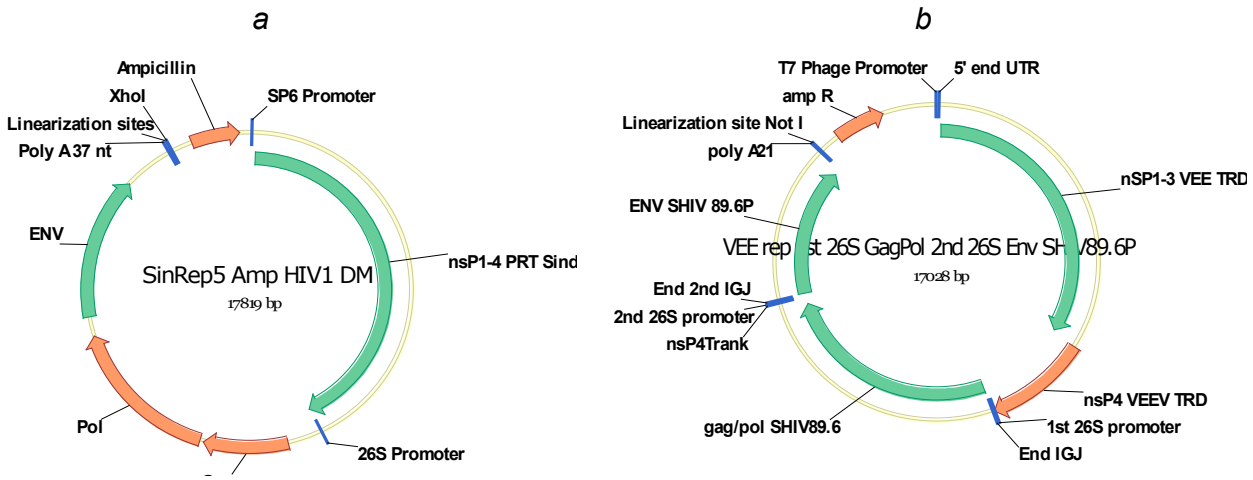

Fig. 1. Schematic representation of plasmids, transcribed RNA from which leads to translation of proteins capable of forming VLPs.

a — on the Sindbis virus replicon platform; *b* — on the VEEV replicon platform.

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ready for use. Human macrophages were isolated from a healthy adult donor. Macrophages were characterized using antibodies specific for CD14, CD11b. Commercial macrophages are negative for HIV-1, hepatitis B and C virus, mycoplasma, bacteria, yeast and fungi.

Microimmunofluorescence assay

To monitor transfection (electroporation) and determine the titer of recVLPs by microimmunofluorescence assay, BHK-21 cells were grown on 4- or 8-well LabTek slides (Nalge Nunc International) in a CO_2 incubator at 37ºC. 24 h after infection, slides were fixed in acetone-methanol (1 : 1) at 4ºC for at least 1 h. For the transfected cell assay, approximately 10^5 electroporated cells were seeded onto 4- or 8-well slides, incubated and fixed as described above. Fixed cells were rehydrated in phosphate-salt buffer solution pH 7.2 and incubated with a 1 : 100 dilution of appropriate antiserums to Gag and Env obtained from Dr. D.C. Montefiore for 1 h at room temperature, as well as with mouse anti-HIV p17 monoclonal antibodies and serum from an HIV^+ patient. After 3 washes in phosphate-salt buffer, goat anti-human or goat anti-mouse IgG-isothiocyanate fluorescein conjugate (Sigma) was added at a dilution of 1 : 100, and the slide was incubated for 30 min at room temperature followed by 3 additional washes. Slides were examined and photographed under a Zeiss LSM110 confocal fluorescence microscope (Carl Zeiss SMT, Inc.). Images were digitized and analyzed using Photoshop software (Adobe Systems Inc.).

Electron microscopy

Cell monolayers were washed briefly with serum-free medium and fixed with 4% glutaraldehyde in 0.15 M sodium phosphate buffer pH 7.4 overnight. The next 3 washes were made with phosphate buffer and monolayers were fixed for 1 h in a mixture of 1% OsO_4 and 1.25% potassium ferricyanide in 0.15 M sodium phosphate buffer. Cells were then washed with deionized water and dehydrated by increasing concentrations of ethanol (30, 50, 75 and 100%, 5 min each). Cells were infiltrated with two concentrations of Polybed 812 epoxy resin (Composition 1A : 2B, Polysciences, Inc.) for several hours at each change, polymerized for 24 h at 60ºC in pouring molds where they were separated from plates before sectioning. Ultrathin sections (70 nm) were cut with a Diatome diamond knife; sections were mounted on copper grids (200 cells) and stained with 4% aqueous uranyl acetate solution for 15 min, followed by Reynolds lead citrate for 7 min. Sections were photographed using a LEO EM910 transmission electron microscope (Carl Zeiss SMT, Inc.) at 80 kV.

Results

The prototype chimeric viral vaccine construct utilizes an incomplete alphavirus RNA genome (Sindbis or VEEV) containing *gag*/*pol* and *env* genes expressing the corresponding lentivirus proteins. Electroporation of cells with srRNAs transcribed from these plasmids results in the self-assembly of lentivirus-like particles.

DNA sequences encoding Gag/Pol and Env gp160 proteins of lentiviruses (HIV-1B and $SHIV_{89.6P}$) were obtained by standard genetic engineering methods. A plasmid containing DNA encoding HIV-1B proteins was constructed on the Sindbis virus replicon platform (Fig. 1, *a*). A plasmid containing DNA encoding $SHIV_{89.6P}$ proteins on the VEEV replicon platform was constructed from the $SHIV_{89.6P}$ KB9 molecular clone (GenBank # U89134) (Fig. 1, *b*).

In plasmid $SHIV_{89.6P}$, the 5'-primer contained the 26S subgenomic promoter sequences, and the 3'-primer contained the stop codon and AscI site for cloning into the VEEV replicon plasmid. The C-terminus of the NSP4 and 26S promoter sequences were amplified by polymerase chain reaction (PCR) and used in an overlapping elongation PCR with a fragment containing structural genes with the 26S replicon promoter for PCR. The fragment containing the C-terminus of NSP4, the complete VEEV 26S subgenomic promoter, the $SHIV_{89.6P}$ structural gene, and the AscI restriction site was introduced into the Zero Blunt PCR cloning vector (Invitrogen) and the nucleotide sequence was determined. The 26S-Gag fragment was ligated to pVR100 $SHIV_{89.6P}$ Env. The 26S-Env fragment was ligated to pVR21 $SHIV_{89.6P}$ Gag. Positive clones were identified by restriction analysis and confirmed by sequencing. The preparation of these plasmids and their further modification have been described in more detail previously [16, 17].

BHK-21 cells were transcribed from synthesized plasmids with recombinant srRNAs (1 μg each) expressing structural proteins of alphaviruses. The resulting VLPs were examined by electron microscopy. It was found that transfection of cells with both Sindbis virus recRNA (expressing HIV-1B structural proteins) and VEEV reVLPs (expressing $SHIV_{89.6P}$ structural proteins) resulted in VLP formation. The titers of VEEV recVLPs produced on the VEEV platform were 10^5–10^6 infectious units per 1 ml, which coincides with the data of other authors who used plasmids obtained by us [17].

Thus, we created constructs expressing the products of HIV-1B and $SHIV_{89.6P}$ genes in an amount sufficient for the formation of mature recVLPs (**Fig. 2**).

During the microimmunofluorescence assay, $SHIV_{89.6P}$ structural proteins were detected only in the cytoplasm of transfected cells (**Fig. 3**). The titers of recVLPs ranged from 10^4–10^6 infectious units per 1 ml.

CCR5 and CXCR4 are two major co-receptors required for HIV entry. The glioblastoma cell lines U87.CD4.CCR5 and U87.CD4.CXCR4 reliably support HIV-1 infection of various laboratory-adapted strains and primary isolates with different co-receptor utilization (R5, X4 and R5/X4), allowing us to investigate the antiviral efficacy of combined CCR5 and CXCR4 antagonist blockade [22].

Infection of these U87 cell lines with CD4 receptor and CCR5 and X4 co-receptors and human macrophages with chimeric lentivirus-like particles was specific and productive and resulted in srRNA replication, expression and processing of *gag*/*pol* and *env* HIV-1B, and $SHIV_{89.6P}$ gene products, and generation of daughter chimeric recVLPs capable of producing infectious progeny. Infection of human macrophages resulted in syncytium formation in both HIV-1B and $SHIV_{89.6P}$ recVLP infections (**Fig. 4**).

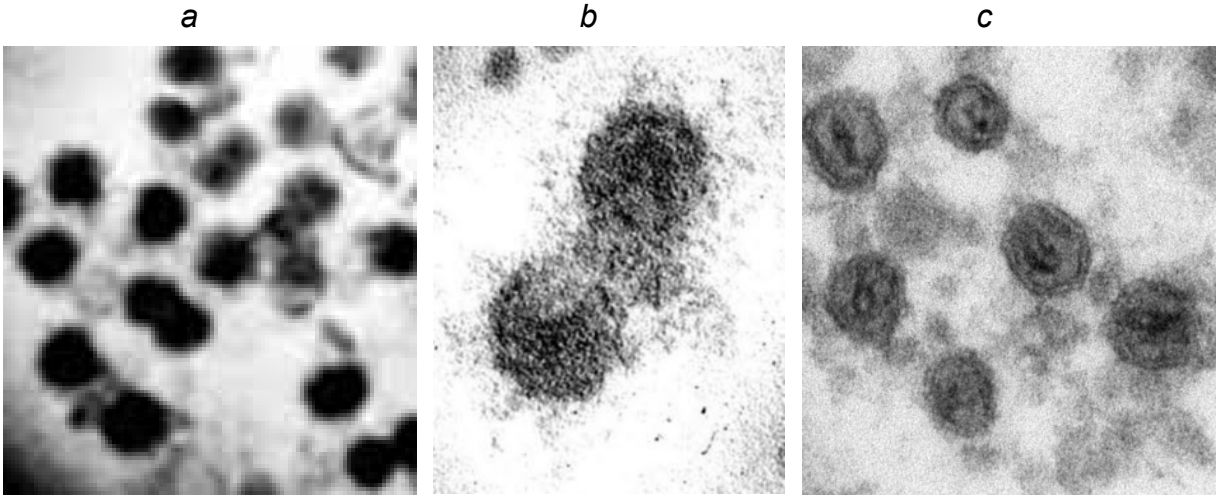

Fig. 2. Electron microscopic photographs of lentiviral VLPs.
a and *b* — HIV-1B VLPs; *c* — VLP $SHIV_{89.6P}$ VLPs.

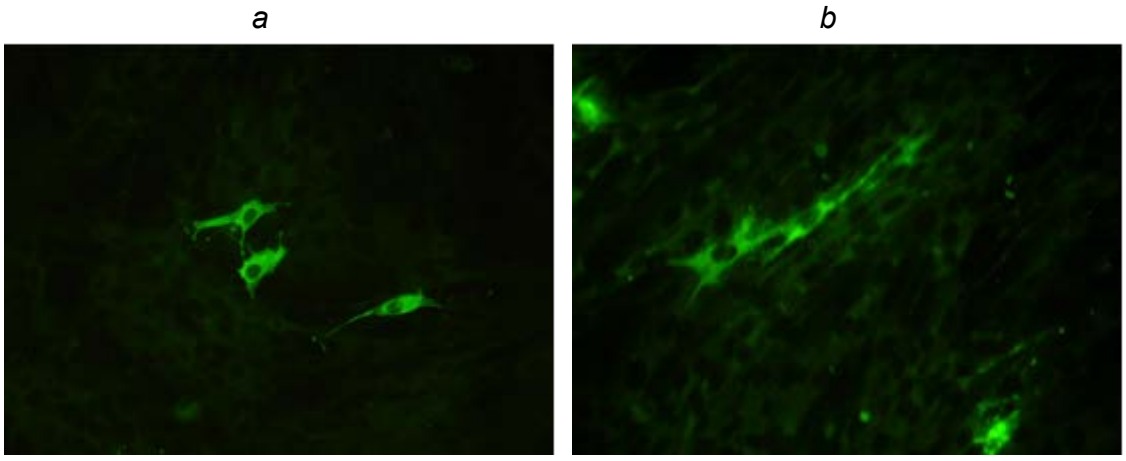

Fig. 3. ELISA, srRNA replication in the cytoplasm of BHK-21 cells and protein expression of the *gag* gene (*a*) and *env* gene (*b*) of $SHIV_{89.6P}$.

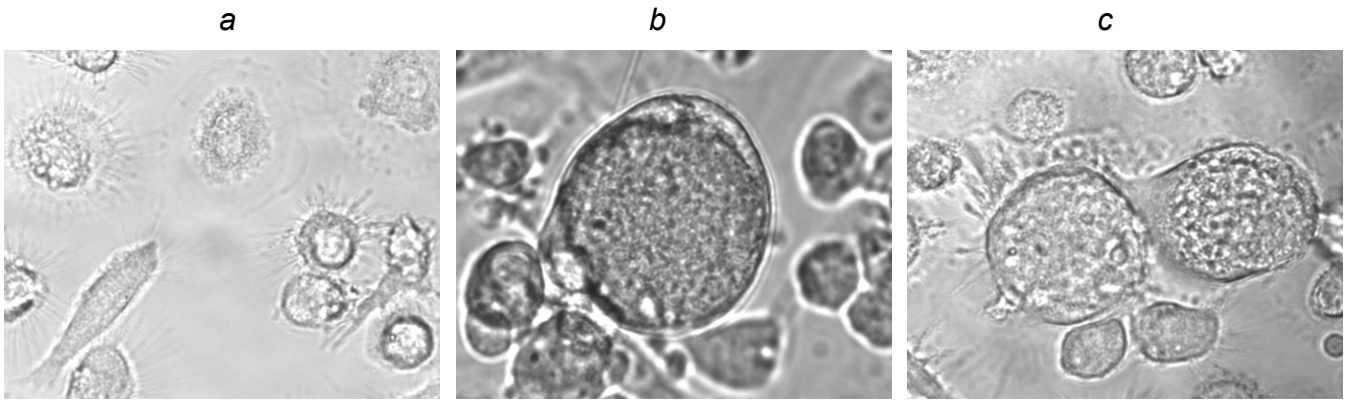

Fig. 4. Formation of syncytium on human macrophages infected with lentivirus recVLPs.
a — negative control, ×20; *b*, *c* — formed syncytium, ×40: HIV-1B recVLPs (*b*) and $SHIV_{89.6P}$ recVLPs (*c*).

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Thus, self-replicating lentivirus VLPs are able to replicate in glioblastoma cells and induce fusion of normal human macrophages.

Discussion

HIV vaccines has been in development for more than 30 years, but despite all the progress made, little success has been achieved. One of the reasons is the presence of a reservoir of HIV variants evading the immune system in the CNS. The solution to this problem could be the use of attenuated alphavirus vectors with lost neurovirulence but preserved neuroinvasiveness [12, 13, 23].

We have constructed plasmids that allow transcribing srRNAs, transfection of cells with these plasmids allows not only expression of target proteins, but also leads to the formation of infectious recVLPs. Apparently, these recVLPs can present antigen to cells of the immune system in conformations close to native ones [24–26]. When srRNA enters the cell, replication occurs and the srRNA is packaged into such chimeric particles. When the replicon genome is then released into the cytoplasm following entry of the chimeric particles into susceptible cells, the chimeric particles can potentially replicate and function as a live viral vaccine. Using engineered constructs, it was shown that expression of the *gag* and *env* genes of recombinant VEEV RNA in primate cells resulted in the assembly of particles that were morphologically and functionally VLP lentivirus and included recombinant alphavirus RNA that enables VLP self-assembly. Thus, HIV-1B and $SHIV_{89.6P}$ enveloped recVLPs are essentially recombinant viruses, which may help circumvent the complexities typically associated with noninfectious VLPs.

Infection of cells with CD4 receptor and CCR5 and X4 co-receptors with chimeric lentivirus-like particles was specific and productive and resulted in RNA replication, expression and processing of HIV *gag*/*pol* and *env* gene products, and generation of daughter chimeric particles. Further modifications of plasmids encoding $SHIV_{89.6P}$ proteins (Fig. 1, *c*), performed by other researchers and aimed at enhancing encapsulation of the chimeric virus genome and expression of the $SHIV_{89.6P}$ protease for particle maturation, improved the ability of chimeric lentivirus-like particles to proliferate in cell culture [17].

The studies performed here and previously led to RNAs capable of self-reproduction, and upon transfection with these RNAs, recVLPs are formed. Expression of lentivirus antigens by these recVLPs is observed only in the cytoplasm. Because there is no integration into the host genome, vaccination with these VLPs should not result in persistent or chronic infection. Alphaviruses are sensitive to interferon. Therefore, attenuating mutations can be incorporated into the genome to increase sensitivity to interferon [22, 23]. The VEEV vaccine strain approved for use in high-risk groups is able to penetrate the CNS of model animals through the BBB [12, 13, 27]. In our opinion, the data presented in the present study and those obtained earlier, as well as the analysis of published studies, allow us to believe that further research on the improvement of such vaccines is justified and should include, first of all, the use of adequate animal models, the study of the intensity of induced immunity and the evaluation of the safety of such vaccines.

In the case of srRNA production, the stage of obtaining viral progeny for subsequent immunization is not required. This seems to allow the use of mucosal or intradermal (ID) immunization methods, which are more effective, economical and immunogenic compared to intramuscular infection [28–30].

Our study and the data of the authors who used plasmids created by D.A. Moshkoff [17] provide evidence of the ability of our chimeric constructs to express structural proteins of lentivirus and to assemble into infectious particles to present lentivirus immunogens in their native and functional conformation.

HIV is capable of infiltrating the CNS, infecting immunocompetent cells that are able to cross the BBB. In this manner, HIV can infiltrate by circumventing the biological barrier that limits the entry of most other foreign molecules. Antiretroviral drugs are generally unable to effectively penetrate the BBB or are rapidly eliminated from the brain parenchyma, resulting in inefficient elimination of HIV from the brain and formation of viral reservoirs. Heterogeneous cellular reservoirs exist in the brain that can contain resting HIV. Such accumulation in the CNS can lead to virus rebound and recurrence of infection. There is a paradox in that foreign viral components cross the BBB and are transmitted to the CNS, while essential therapeutic drugs cannot penetrate it [3].

In both nonhuman primates and cats, neurovirulent variants have been isolated from CNS tissue or cerebrospinal fluid [31, 32], causing more rapid disruption of behavioral reactions and accelerated death. The isolation and cloning of lentivirus variants that cause death of animals in a short period of time opens the possibility of rapid testing of the effectiveness of protective drugs. SIVsmmPBj14 isolate (SIV-PBj14) is one of the most virulent known primate lentiviruses which causes acute disease and death within 6–10 days after intravenous inoculation into pig-tailed macaques [33, 34]. SIV-PBj14 replicated more efficiently than the initial virus pool in human peripheral blood mononuclear cells (PBMC) and also replicated in chimpanzee PBMC. Normal macaque PBMCs infected *in vitro* with SIV-PBj14 formed syncytia with human T-lymphoblasts from human lymphoma (Sup-T1), whereas the initial virus pool did not result in syncytia formation with these cells [36]. Infection of normal macrophages with $SHIV_{89.6P}$ VLPs also resulted in syncytia formation, which, in our opinion, is a positive property for

further development of a vaccine capable of suppressing HIV neuroinfection.

The increased reactogenicity of the TC-83 strain, usually regarded as an undesirable trait [35], in the case of controlling the inevitable entry of HIV into the CNS will apparently be a positive property. It is pertinent to recall the situation with fixed rabies virus, where high and rapid virus accumulation in the CNS and neuronal destruction, which is not observed in infection of rodents with the wild strain, involves inflammatory and immune responses, leading to the establishment of a distinct and robust defense [36]. The aim of the US studies was apparently to obtain attenuated variants of VEEV that had lost their ability to be transmitted by mosquitoes and were suitable for immunization of horses and were unable to revert to the wild type [37], as periodic outbreaks of VEEV resulted in the deaths of tens of thousands of horses [38]. Introduction of additional attenuating mutations into the TC-83 strain of VEEV resulted in variants with reduced cytopathic effects. Viremia was not detected in adult mice, and viremia in suckling mice, if detected, was low. Failure to induce viremia had a negative effect on the titers induced by neutralizing antibodies. They became 10 times lower than when immunized with the TC-83 strain. When the mutated nucleocapsid protein was placed under the control of the internal ribosome landing site of mouse encephalomyocarditis virus, although the variant became less virulent to suckling mice, it was less effective in inducing neutralizing antibodies. It did not induce viremia and did not penetrate the brains of adult mice [40]. The inactivated vaccine based on the highly attenuated TC-83 strain 230 did not protect animals from aerogenic infection, in which virulent strains easily penetrate into the brain. At the same time, a strain similar in characteristics to the TC-83 strain (with lost neurovirulence but preserved neuroinvasiveness) protected even against intracerebral infection with encephalitogenic strain of VEEV [12]. The conclusion that it is advisable to use vectors based on the VEEV platform for advanced CNS protection and that the TC-83 strain is the most suitable for the creation of a candidate vaccine [1] seems to be true at present. The feasibility of using the VEEV replicon to create effective vaccine candidates against a number of viruses is discussed in a review by A.A. Petrov et al. [18].

The authors of this article are aware of the complexities of the problem at hand-like any new technology, the use of srRNA has its pros and cons. In a review published in 2021 [39], A.V. Blagov et al. correctly note that the main advantage of RNA vaccines is the speed of their development and production. Especially since new efficient methods of cell-free synthesis of circular DNA are emerging. Minimizing the use of live bacterial cultures and viruses in vaccine production reduces the risks of contamination and makes production even faster and safer. The authors of the review also point out that the instability of RNA can be both a disadvantage because of the risk of degradation of the molecule and the development of too strong an inflammatory response, and an advantage because the mRNA itself can act as an adjuvant. Instability also affects the storage conditions of mRNA vaccines (–80ºC to –20ºC), forcing the maintenance of a cold chain or the use of lipid nanoparticles to increase the thermostability of mRNA vaccines [39]. The method of producing the srRNAs described in this study seems to be scalable, and the recVLPs themselves are produced in rodent and primate cells and are characterized by high immunogenicity. Recombinant VLPs on the VEEV platform have been well characterized both qualitatively and quantitatively; moreover, their efficacy against the highly virulent SIV strain for monkeys has been demonstrated [16, 17, 40].

At present, conditions appear to have been created for research to explore the possibility of creating an effective vaccine against HIV eluding immunity in CNS reservoirs. At the intermediate stage of research, the constructs developed by Kazakh researchers based on the TC-83 vaccine strain of VEEV [19] can be applied for subcutaneous infection of model animals (mice or rabbits). If this recombinant virus retained the ability to penetrate the BBB, a green fluorescent protein would presumably be expressed in the brains of infected animals. Since self-replicating VLPs can infect both CNS cells (glioblastoma) and macrophages, it is reasonable to introduce a fluorescent marker protein into the construct and study its expression as part of $SHIV_{89.6P}$ recVLPs in the CNS of primates. If the marker protein is expressed, it will be possible to proceed to the final stage of testing the efficacy of recVLPs expressing Env and Gag proteins on the SIV model, primates, or on the cat model, a variant of feline immunodeficiency virus that causes rapid death of animals.

Thus, the results obtained by us and other researchers make it possible to obtain recVLPs. These VLPs induce antigen production in monkey cells [17], are able to replicate in human CNS cells, and induce syncytium formation in human macrophages. In this study, lentivirus proteins and recRNAs are synthesized with srRNAs to form complete viral particles capable of infecting a susceptible cell line and producing virion progeny. Therefore, the VLPs obtained in this way are more correctly referred to as recVLPs. Once again, we draw attention to the fact that recVLPs infect human macrophages, which means that there is a system capable of both delivering target antigens to the CNS and presenting them to immunocompetent cells.

All of this allows us to consider that it is possible to present antigens in native and functional conformation to the CNS. Therefore, we can speak about the feasibility of using such constructs in further studies aimed at obtaining a candidate vaccine for HIV suppression in CNS reservoirs.

This study is part of the research on the development of an HIV vaccine capable of elimination of the virus from the CNS. Naturally, further work should be carried out under conditions of increased biological protection.

Conclusion

1. Plasmids containing genes encoding HIV Gag and Env proteins (Sindbis virus replicon) and Gag and Env proteins of SIV and HIV-1B recombinant $SHIV_{89.6P}$ (VEEV replicon) were constructed using the alphavirus replicon platform.

2. RNA transcribed from these plasmids results in VLPs when cells are transfected.

3. $SHIV_{89.6P}$ recVLPs are capable of infecting human CNS cells (U87 $CD4^+CCR5^+$ and U87 $CD4^+X4^+$ glioblastoma cell lines) and macrophages of a healthy donor.

4. Syncytium formation has been observed during infection of human macrophages with $SHIV_{89.6P}$ recVLPs.

5. The data obtained during the study of $SHIV_{89.6P}$ recVLPs by the authors and other researchers indicate that presentation of lentivirus immunogens is possible in native and functional conformation (induction of specific antibodies, binding to appropriate receptors on CNS cells, syncytium formation in macrophages), and support the feasibility of using such constructs in further development of a vaccine to suppress HIV in CNS reservoirs.

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Original Study Article
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Production of recombinant norovirus VP1 protein and its antigenic and immunogenic properties

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Abstract

Introduction. The importance of noroviruses in human infectious pathology and the danger of large epidemic outbreaks in organized groups determine the need to develop means of specific prevention of infection.

The aim of the study was to obtain recombinant norovirus VP1 protein and analyze its immunogenic and antigenic properties .

Materials and methods. Computer analysis of nucleotide and amino acid sequences, molecular cloning, polymerase chain reaction, electrophoresis of nucleic acids in agarose gel and proteins in polacrylamide gel, affinity chromatography, enzyme immunoassay.

Results and discussion. A genetic construct encoding recombinant VP1 of the GII genotype norovirus with codons optimized for highly effective expression in *Escherichia coli* has been created. The strain of *E. coli* Rosetta 2 (DE3) has been transformed by genetic construct. VP1 expression was carried out in *E. coli* cells, conditions for its production, purification and renaturation were optimized. A purified soluble recombinant VP1 protein forms virus-like particles with a diameter of 30–50 nm. Immunization of BALB/c mice by protein lead to antibodies production with a titer greater than 1 : 1000. When evaluating antigenic properties, it was shown that human IgG, IgM, and IgA antibodies interact with recombinant VP1. The total antibody detection rate was 47,4%. The results indicate the possibility of using recombinant VP1 for development of domestic vaccine for the prevention of norovirus infection.

Keywords: *norovirus, VP1, virus-like particles, vaccine*

Ethics approval. The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the "Consensus Author Guidelines for Animal Use" (IAVES, 07/23/2010). The research protocol was approved by the decision of the Local Ethics Committee of the Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology (protocol No. 4, November 25, 2021).

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Оригинальное исследование
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Получение рекомбинантного белка VP1 норовируса и его антигенные и иммуногенные свойства

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Аннотация

Введение. Значимость норовирусов в инфекционной патологии человека и опасность возникновения крупных эпидемических вспышек в организованных коллективах обосновывают необходимость разработки средств специфической профилактики инфекции.

Цель работы — получение рекомбинантного белка VP1 норовируса и анализ его иммуногенных и антигенных свойств.

Материалы и методы. Проведены компьютерный анализ нуклеотидных и аминокислотных последовательностей, молекулярное клонирование, полимеразная цепная реакция, электрофорез нуклеиновых кислот в агарозном геле и белков в полиакриламидном геле, аффинная хроматография, иммуноферментный анализ.

Результаты. Создана генетическая конструкция, кодирующая рекомбинантный VP1 норовируса генотипа GII с кодонами, оптимизированными для высокоэффективной экспрессии в *Escherichia coli*. Генетической конструкцией трансформирован штамм *E. coli* Rosetta 2 (DE3). Осуществлена экспрессия VP1 в клетках *E. coli*, оптимизированы условия для его продукции, очистки и ренатурации. Получен очищенный растворимый рекомбинантный белок VP1, формирующий вирусоподобные частицы диаметром 30–50 нм. Иммунизация белком мышей BALB/c вызывала образование антител с титром более 1 : 1000. При оценке антигенных свойств показано, что в крови волонтёров присутствуют антитела классов IgG, IgM, IgA, взаимодействующие с рекомбинантным VP1. Суммарная частота обнаружения антител составила 47,4%.

Заключение. Результаты обосновывают возможность использования рекомбинантного VP1 для создания отечественной вакцины для профилактики норовирусной инфекции.

Ключевые слова: *норовирус, VP1, вирусоподобные частицы, вакцина*

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 решением Локального этического комитета ННИИЭМ им. акад. И.Н. Блохиной (№ 4 от 25.11.2021).

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

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

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Introduction

In the etiologic structure of viral acute intestinal infections, noroviruses (NV; *Caliciviridae* family, *Norovirus* genus) rank second after rotaviruses. In countries that vaccinate against rotaviruses, NVs have taken the 1st place [1, 2]. The risk groups for NV infection include children, young and elderly people. Outbreaks of NV infection are reported throughout the year with an increase in incidence in the spring and summer months.

Human NV is an non-enveloped icosahedral virus, and has a genome in the form of a single-stranded positive-sense RNA approximately 7.5-7.7 kb long, encoding 3 open reading frames. ORF1 encodes a large polyprotein, the precursor of 6 non-structural proteins (NS1/2-NS7), ORF2 encodes the major structural capsid protein, and ORF3 encodes the minor structural capsid protein VP2, which is located within the viral particle. The virus capsid is constructed of an outer (VP1) and an inner (VP2) protein. VP1 is capable of self-assembling into virus-like particles that are virtually indistinguishable from native virions and have pronounced immunogenic properties.

Ten genogroups are known for NV, and 48 genotypes have been identified based on amino acid sequence analysis of the external capsid protein VP1. The most common NV genogroup is GII, which accounts for the majority of cases of NV-gastroenteritis of children in the first years of life in Russia. Thus, in the Sverdlovsk region in 2022, the most common occurring genotypic structure of circulating NVs was a norovirus belonging to the GII genogroup (58%). Similar data were obtained during molecular epidemiologic analysis of genetic variants of NVs in a number of other European countries, Japan and China. In certain years, GII genogroup accounted for up to 80-90% of cases of pediatric NV-gastroenteritis. The dominant virus variants of this genogroup include NV GII.4 [3, 4]. The importance of NV in human infectious pathology and the danger of large epidemic outbreaks in organized groups determine the need to develop means of specific prophylaxis of infection. The example of successful introduction of rotavirus vaccine shows that vaccination programs can significantly reduce the number of cases of gastroenteritis [5]. Based on NV proteins, several candidate NV vaccines are also being developed worldwide, two of which are currently in phase II/III clinical trials for the prevention of NV infection in children and adults [6, 7].

The aim of the study was to obtain recombinant NV VP1 protein and analyze its immunogenic and antigenic properties.

Materials and methods

Nucleotide sequence analysis, oligonucleotide design, gene construction, calculation of protein molecular weight, isoelectric point and extinction coefficient were performed using the Lasergene 7.1.0 software package (Dnastar, Inc.). Codon optimization was performed using the Codonusage database[1]. The nucleotide sequence of VP1 of the epidemic variant of NV with genotype GII.4, prevalent in the territory of Nizhny Novgorod region, was used. Nucleotide sequences were sequenced using the ABI Prism 310 genetic analyzer (Thermo Fisher Scientific).

Escherichia coli cells, strain Rosetta 2 (DE3), transformed with the obtained genetic construct based on plasmid pET22b and encoding VP1 NV, were grown in LB-Miller medium, pH 7.0. Induction of protein synthesis was performed by adding isopropyl-β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM to each culture. Cell biomasses were obtained by centrifugation, lysed in a solution containing 25 mM HEPES (pH 7.5), 1 M NaCl, 10% glycerol, 1% Triton X-100, DNase I (10 μg/mL), RNaseA (10 μg/mL), lysozyme (50 μg/mL), and 0.2 mM phenylmethylsulfonyl fluoride, disintegrated by ultrasound using a QSonica Q55 (QSonica sonicators), a centrifuged and washed buffer containing 25 mM HEPES pH 7.5, 1 M NaCl, 10% glycerol, 1 M urea was added, followed by centrifugation.

The recombinant norovirus VP1 protein was purified by metal chelate chromatography under denaturing conditions using Ni-NTA Superflow sorbent (GEHealthcare). Renaturation of VP1 was performed by dialysis. Protein electrophoresis in 12% polyacrylamide gel in the presence of sodium dodecyl sulfate was carried out by the conventional method, immunoblotting was performed using human serum antibodies against NV VP1 and horseradish peroxidase-conjugated monoclonal antibodies to human IgG Hytest. After transfer, proteins on the membrane were stained in Super Signal West Dura Extended Duration Substrate solution (Thermo Scientific) and chemical luminescence was measured using a C-DiGit Blot Scanner (LiCog). Microphotographs of virus-like particles formed by NV VP1 were obtained using an NT7700 electron microscope (Hitachi). Female BALB/c mice 8 weeks old and weighing 16-18 g were used for immunization. Animals were kept in vivarium conditions in accordance with interstate standards GOST 33216-2014 and GOST 33215-2014. Biomaterial for the study was taken from mice in compliance with the principles of humanity set forth in the European Community directives (86/609/EC).

The studies were conducted according to the bioethical and ethical principles established by the Declaration of Helsinki (adopted in June 1964 and revised in October 2013). To evaluate the antigenic properties of recombinant proteins, we used 637 blood plasma samples obtained from the Hemohelp diagnostic center (TIAS LOTUS LLC) from individuals aged 19–44 years who applied for diagnostic studies and gave written consent for the use of their biomaterial in the study.

[1] URL: http://www.kazusa.or.jp/codon/

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ORIGINAL RESEARCHES

Antibodies to NV VP1 was determined by solid-phase enzyme-linked immunosorbent assay. VP1 was sorbed into wells of plates at a concentration of 1 μg/mL for 18 h at 20°C. Mouse serum to be tested was diluted in increments of 2, and volunteer plasma was diluted before testing in increments of 10. Serum from unimmunized mice was used as a negative control. When antibodies were determined in the blood of laboratory animals, rabbit antibodies against mouse immunoglobulins conjugated with horseradish peroxidase were used. When determining human antibodies, horseradish peroxidase-conjugated rabbit antibodies against immunoglobulins of classes G, M and A were used. The value of optical density greater than the average value of the negative control multiplied by three was taken as a cut-off for reactivity.

The obtained data were analyzed using Microsoft Excel software (Microsoft). Statistical processing of data was performed using the Graph Pad Prism 8 program (Graph Pad Software). Differences in data were considered statistically significant at $p \leq 0.05$.

Results

A site for the NdeI restriction endonuclease was added to the beginning of the nucleotide sequence encoding the VP1 protein of the epidemically significant strain of NV genotype GII.4. To the sequence encoding the C-terminal part of the protein, a nucleotide sequence encoding 6 histidines, a stop codon (TAA), and a site for the XhoI restriction endonuclease was added and used for subsequent molecular cloning. The schematic structure of the gene is shown in **Figure 1**. The resulting sequence was synthesized at Eurogen. The sequence encoding VP1 was transferred to the plasmid pET22b (Thermo Fisher Scientific), which allows high efficiency expression of recombinant proteins in *E. coli* strains containing DE3 lysogen in the genome [8].

The obtained genetic construct encoding NV VP1 was transformed into *E. coli* cells, Rosetta 2 strain (DE3). The efficiency of protein production was evaluated, which amounted to 20–40 mg of protein per 1 liter of cell culture. The protein formed inclusion bodies. The optimal medium composition and culture conditions for transformed *E. coli* cells were determined. The maximum cell culture density (OD_{600} = 2.8) corresponded to 5 g of biomass per 1 L of culture in LB medium containing 0.5% glycerol and 25 mM phosphate buffer pH 7.4. The optimal concentration of isopropyl-β-D-1-thiogalactopyranoside was 0.5 mM, the optimal temperature for protein expression was 30ºC, and the induction time was 4–8 h.

Subsequent purification by metal-chelate chromatography in the presence of 8 M urea and refolding by dialysis against a solution containing 25 mM HEPES pH 7.5, 150 mM NaCl, and 5% glucose resulted in a soluble protein consisting of 560 amino acids, having a calculated molecular mass of 60.6 kDa, an isoelectric

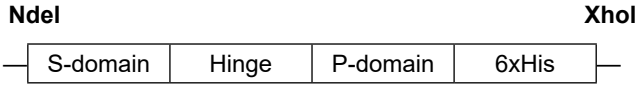

Fig. 1. Schematic representation of the genetic construct encoding VP1 of norovirus in pET22b.

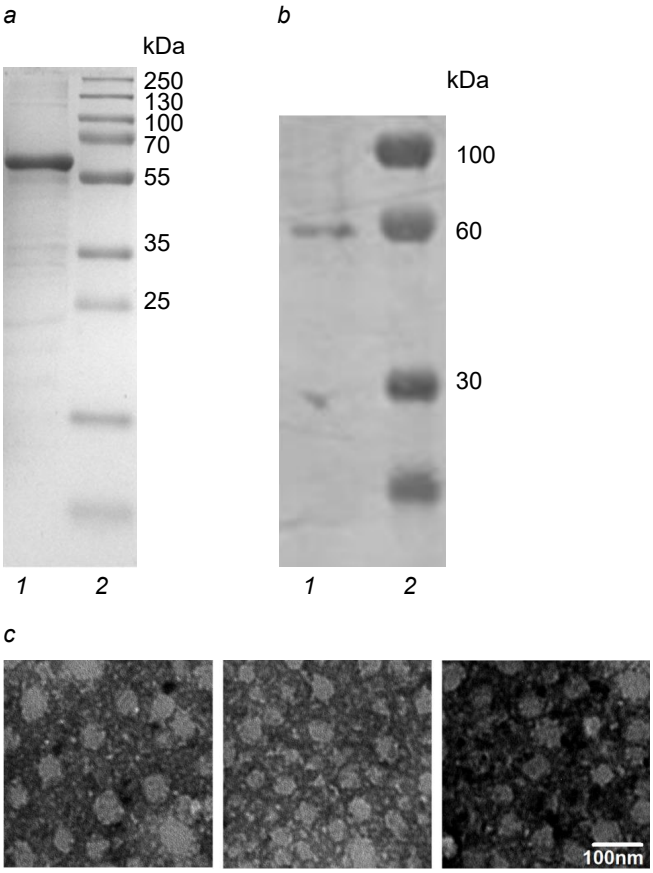

Fig. 2. Characteristics of the recombinant VP1 protein.
a — electrophoregram of purified norovirus VP1 protein; *b* — Western-blot of purified norovirus VP1 protein; *1* — VP1; *2* — molecular weight marker; *c* — electron microscopic photographs of virus-like particles formed by recombinant norovirus VP1. Contrast with 3% uranyl acetate, pH 4.6.

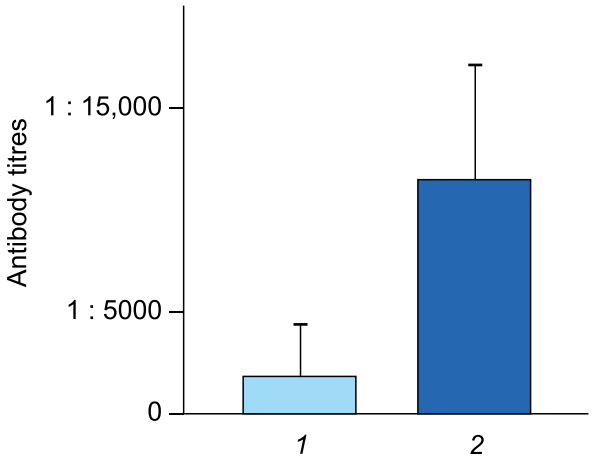

Fig. 3. Antibody titers in mice immunized with recombinant norovirus VP1.
1 — without aluminum hydroxide; *2* — with aluminum hydroxide.

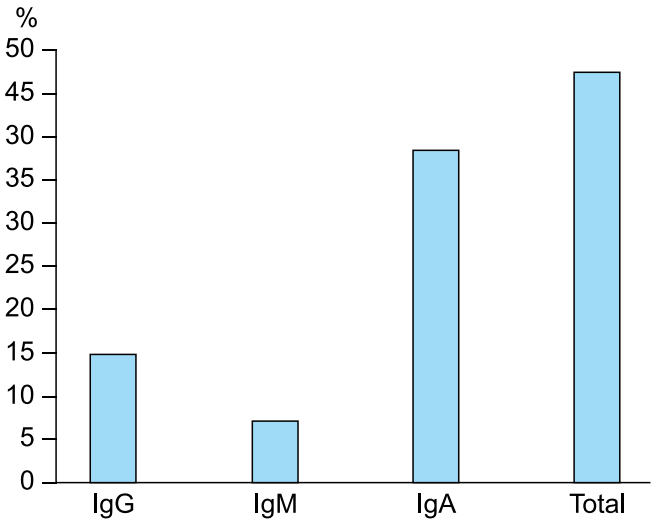

Fig. 4. Detection rate of antibodies of different classes to recombinant norovirus VP1 in the blood of healthy volunteers.

point equal to 6.15 and an extinction coefficient of 1.04 (**Fig. 2**, *a*).

The protein was produced in preparative quantities and used to assess the ability to form virus-like particles and for immunization of laboratory mice. Fig. 2, *b* shows an electron microscopic photograph indicating the ability of recombinant VP1 protein to form virus-like particles with a diameter of 30-50 nm, which is consistent with the data of other authors [9]. At the same time, western blot (Fig. 2, *c*) showed the ability of recombinant VP1 to interact with serum antibodies of seropositive individuals.

Two intraperitoneal immunizations of 10 laboratory mice with an interval of 2 weeks and subsequent obtaining of blood serum 3 weeks after the second immunization at a dose of 10 μg (0.5 ml) resulted in the formation of antibodies against NV VP1 in the blood of animals. Antibodies in the blood of animals were detected in titers from 1 : 1024 to 1 : 4096, the average titer was 1 : 1536. Immunization of animals with the same dose of protein according to the same scheme, but mixed with 100 mg of aluminum hydroxide caused the appearance of antibodies against NV VP1 in titers up to 1 : 32,768. On average, the antibodies titer was 1 : 13,720, which was almost an order of magnitude higher than the antibodies titers in animals immunized without aluminum hydroxide (**Fig. 3**). Thus, it was shown that the obtained recombinant protein is able to induce a pronounced antisense response, which significantly increases in the presence of the used adjuvant.

Since NV is widely circulating among the population, it is natural to expect the presence of antibodies to its proteins in human blood. We evaluated the presence of antibodies of different classes to the obtained recombinant protein in the blood of individuals living in the middle belt of Russia. The frequency of detection of antibodies to VP1 in blood plasma samples of 637 volunteers was determined. As shown in **Fig. 4**, IgG antibodies were detected in 14.8% of volunteers, IgM antibodies — in 7.1%, IgA antibodies — in 38.5%. The cumulative occurrence of antibodies was 47.4%.

The results indicate that the epitopes of the recombinant VP1 protein of NV GII.4 obtained by us are recognized by antibodies present in human blood. However, IgG antibodiy titers were mostly low and exceeded a value of 1 : 1000 or more in seropositive volunteers only in 4.3% of cases (4 of 94), indicating that these individuals had a long history of NV exposure. In volunteers who had IgA antibodies to NV VP1, high titers (equal to 1 : 1000 or more) were detected with similar frequency (4.9% of cases). In contrast, of the 25 volunteers who had IgM antibodies to NV VP1, titers equal to 1 : 1000 or more were detected in 16% of cases. It is likely that these individuals had recent exposure to the virus.

Discussion

The data obtained on the frequency of antibodies against the recombinant VP1 protein of NV GII.4 and their titers are consistent with the results found by other authors. A wide variation in the frequency of antibodies detection and their titers in individuals of different ages living in different countries has been reported. The cumulative detection rate ranges from 25 to 95%. Cross-reactivity with NV of other genogroups is observed [10, 11].

The NV VP1 protein consists of 2 domains involved in the self-assembly of virus-like particles [12]. The ability of NV VP1 to self-assemble can be used to obtain chimeric proteins forming virus-like particles, consisting of the S-domain of NV VP1 and fragments of other proteins acting as antigen. That is, there is a possibility of decorating virus-like particles of NV with different antigens, which has been demonstrated in the works of a number of authors [13–15]. The genetic construct encoding the recombinant protein we obtained can be used as a molecular platform for the creation of chimeric virus-like particles based on NV VP1.

Conclusion

The recombinant NV VP1 protein expressed in *E. coli* obtained by us is capable of forming virus-like particles, shows immunogenicity in mice in the absence and presence of adjuvant, and is recognized by human antibodies of IgG, IgM and IgA classes. The results of this work indicate the possibility of using recombinant VP1 as an antigen in the design of a vaccine for the prevention of NV infection based on virus-like particles.

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Original Study Article
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Detection of genetic determinants of potentially oncogenic representatives of the intestinal microbiota as biomarkers of colorectal cancer

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Abstract

Relevance. Colorectal cancer (CRC) is the second leading cause of cancer mortality worldwide. Non-invasive diagnostic methods based on the determination of hidden blood in the stool (fecal immunochemical test, guaiac test), which have been proven to be effective in clinical studies, are used for CRC screening. However, a significant disadvantage of the available non-invasive diagnostic methods is the low sensitivity in detecting the oncological process at the early stages. A number of recent studies discuss the relationship between the disease and various potentially oncogenic microorganisms in the human intestinal tract, which can be used to expand the arsenal of non-invasive methods for diagnosing CRC based on molecular genetic examination of a stool sample to identify oncogenic microorganisms.

The aim of this study was to evaluate the possibility of using genetic determinants of potentially oncogenic microorganisms as markers for colorectal cancer, based on a comparison of their prevalence in groups of patients with colorectal cancer, facultative precancerous diseases and patients without intestinal pathology.

Materials and methods. 215 participants were included in the "case–control" study: 70 patients with newly diagnosed colorectal cancer, 70 patients with inflammatory bowel disease, 75 participants without diagnosed intestinal pathology. Polymerase chain reaction (PCR) was used to identify and detect genes of potentially oncogenic microorganisms.

Results and discussion. An association was found between CRC and the presence of the *Bacteroides fragilis* fragilisin gene (OR 7.00; 95% CI: 2.55–22.50; $p < 0.001$), species-specific genes of the periodontal pathogenic microorganisms *Fusobacterium nucleatum* (OR 5.61; 95% CI: 2.87–11.30; $p < 0.001$) and *Porphyromonas gingivalis* (OR 16.3; 95% CI: 4.33–106.00; $p < 0.001$), the *clbB* gene of *pks* pathogenicity island of the enterobacteria (OR 3.44; 95% CI: 1.39–8.51; $p = 0.010$).

Conclusion. The presence of genetic markers of potentially oncogenic bacterial species and genotypes in the gut microbiome is associated with colorectal cancer. The results obtained support the possibility of using molecular genetic detection of the studied potentially oncogenic microorganisms as a method for non-invasive diagnosis of CRC.

Keywords: *colorectal cancer, microbiota, microbial markers, potentially oncogenic microorganisms, non-invasive diagnosis, screening*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the North-Western State Medical University named after I.I. Mechnikov (protocol No. 10, November 3, 2021).

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

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Оригинальное исследование
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Детекция генетических детерминант потенциально онкогенных представителей микробиоты кишечника в качестве биомаркеров колоректального рака

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Аннотация

Введение. Колоректальный рак (КРР) является второй по значимости причиной смертности от рака в мире. Для скрининга КРР применяются неинвазивные методы диагностики, основанные на определении скрытой крови в стуле (фекальный иммунохимический тест, гваяковый тест), хорошо зарекомендовавшие себя в клинических исследованиях. Однако существенным недостатком неинвазивных методов диагностики является невысокая чувствительность при выявлении онкологического процесса на ранних стадиях. В ряде современных работ обсуждается связь заболевания с различными потенциально онкогенными микроорганизмами (МО) в кишечном тракте человека, которые могут быть использованы для расширения арсенала неинвазивных методов диагностики КРР на основе молекулярно-генетического исследования образца кала для идентификации онкогенных МО.

Цель исследования — оценка возможности использования генетических детерминант потенциально онкогенных МО в качестве маркеров КРР, основанная на сопоставлении их распространённости в группах пациентов с КРР, факультативными предраковыми состояниями и пациентов без патологии кишечника.

Материалы и методы. В исследование, организованное по дизайну «случай–контроль», было включено 215 участников: 70 пациентов с впервые выявленным КРР, 70 пациентов с воспалительными заболеваниями кишечника, 75 участников без диагностированной патологии кишечника. Детекцию генов потенциально онкогенных МО осуществляли с помощью метода полимеразной цепной реакции.

Результаты и обсуждение. Установлена связь между КРР и наличием гена фрагилизина *Bacteroides fragilis* (ОШ = 7,00; 95% ДИ 2,55–22,50; $p < 0,001$), видоспецифических генов пародонтопатогенных МО *Fusobacterium nucleatum* (ОШ = 5,61; 95% ДИ 2,87–11,30; $p < 0,001$) и *Porphyromonas gingivalis* (ОШ = 16,3; 95% ДИ 4,33–106,00; $p < 0,001$), гена *clbB* острова патогенности *pks* энтеробактерий (ОШ = 3,44; 95% ДИ 1,39–8,51; $p = 0,010$).

Заключение. Наличие в составе кишечного микробиома генетических маркеров потенциально онкогенных видов бактерий ассоциировано с КРР. Полученные результаты обосновывают возможность применения молекулярно-генетической детекции изученных потенциально онкогенных МО в качестве метода неинвазивной диагностики КРР.

Ключевые слова: *колоректальный рак, микробиота, микробные маркеры, потенциально онкогенные микроорганизмы, неинвазивная диагностика, скрининг*

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

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

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Introduction

Colorectal cancer (CRC) is the 3rd most common type of cancer and the 2nd leading cause of cancer mortality in the world [1]. It is noteworthy that the incidence of colorectal cancer is increasing among people under 50 years of age [2]. Noninvasive (stool blood test, guaiac test and fecal immunochemical test (FIT)) and invasive (flexible rectoromanoscopy, colonoscopy) diagnostic methods are used to detect colorectal cancer. The obvious advantages of non-invasive diagnostic methods are their simplicity and availability for screening examinations of persons at risk.

The use of noninvasive diagnostic methods, such as FIT, in screening examinations can achieve a 27% reduction in mortality from CRC [3]. A meta-analysis of the results of 4 randomized controlled trials demonstrated that the use of guaiac test and flexible rectoromanoscopy contributes to the reduction of CRC mortality by 18% and 26%, respectively [4].

However, a significant disadvantage of noninvasive methods is their low diagnostic sensitivity at early stages of the disease. Thus, the sensitivity of FIT at stage I of CRC is 68% (95% CI 57–78%), at stage II — 92% (95% CI 87–96%), at stage III — 82% (95% CI 73–89%) [5]. Thus, there is a need to expand the arsenal of available non-invasive methods for CRC screening.

Specific features of the quantitative and qualitative composition of the microbiota can act as diagnostic markers of CRC, which is discussed in detail in reviews devoted to the role of the intestinal microbiota in the development and diagnosis of intestinal cancer [6, 7]. Numerous studies have revealed changes in the composition of the intestinal microbiome associated with the development of CRC, which suggests the possibility of using the identification of individual representatives of the intestinal microbiome as an independent method of noninvasive diagnosis of CRC or as an addition to existing noninvasive methods.

The first classification model based on intestinal microbial markers and allowing to distinguish patients with CRC from controls was proposed by G. Zeller et al. [8]. The classification algorithm included data on the relative abundance of 22 microorganism species (MOs), but at least half of the predictive power of the model was determined by only 4 species: two *Fusobacterium* species, *Porphyromonas asaccharolytica* and *Peptostreptococcus stomatis*, the presence of each of which was elevated in CRC.

Authors from the Chinese University of Hong Kong developed a diagnostic model demonstrating specificity of 81.2% and sensitivity of 93.8% when combining FIT and 4 bacterial markers (the *"m3"* marker gene of *"Lachnoclostridium"* sp., *Fusobacterium nucleatum*, *Hungatella hathewayi* (baseonym: *Clostridium hathewayi*) and *Bacteroides clarus*) [9]. In an earlier study, an experimental model based on 23 MOs from the *Oscillospiraceae* (heterotypic synonym: *Ruminococcaceae*) and *Lachnospiraceae* families, the *Bacteroides*, *Porphyromonas*, *Parabacteroides*, *Collinsella* genera and the *Enterobacteriaceae* family detected 91.7% of CRC cases in a sample of 490 patients, compared to 75.0% of cases detected by FIT [10].

The MOs most closely associated with CRC include both some oral pathobionts and intestinal bacteria. The former, in addition to the already mentioned representatives of the genera *Fusobacterium*, *Porphyromonas* and *Peptostreptococcus*, include *Parvimonas micra*, *Gemella morbillorum*, *Tannerella forsythia* and some other species [11]. Intestinal bacteria are most often represented by enterotoxigenic strains of *Bacteroides fragilis*, pathogenic and opportunistic bacteria of the *Enterobacteriaceae* family (*Escherichia coli*, *Salmonella spp.*, *Klebsiella pneumoniae*, *Citrobacter rodentium*), as well as *Campylobacter jejuni*, *Morganella morganii*, *Enterococcus faecalis*, *Clostridioides difficile* and others [12].

Based on the results of the literature review, we selected the MOs most frequently associated with CRC according to research data. We included colibactin-producing *Enterobacteriaceae* carrying the *clbA* and *clbB* genes as part of the *pks* pathogenicity island, fragilysin-producing *Bacteroides fragilis* carrying the *bft* gene, periodontal pathogenic bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis* among the potentially oncogenic MOs [6]. An additional factor in favor of including these MOs in the list of potential oncogenes was their association with the stage (progression) of CRC, prognosis/survival and resistance to therapy in patients with CRC [13, 14].

Taking into account probable regional, ethnic and other peculiarities of quantitative and qualitative composition of intestinal microbiota, it is urgent to assess the prevalence of the above mentioned potentially oncogenic MOs among the Russian population and their role in the development of CRC.

The aim of this study is to evaluate the possibility of using genetic determinants of potentially oncogenic MIs as markers of CRC, based on the comparison of their prevalence in groups of patients with CRC, facultative precancerous conditions and patients without intestinal pathology.

Materials and methods

On the basis of clinical departments of the Peter the Great Clinic of the I.I. Mechnikov NWSMU and the City Oncologic Dispensary of St. Petersburg in 2022–2024 a case-control study was conducted. A case-control study was conducted in 2022–2024 at the Peter the Great Clinic of the I.I. Mechnikov NWSMU and the City Oncologic Dispensary of St. Petersburg. The study included 215 participants: a group of 70 patients with diagnosed CRC (CRC group); a group of 70 patients with inflammatory bowel diseases (ulcerative colitis, Crohn's disease — IBD group); 75 participants without

Table 1. Nucleotide sequences of primers for identification of potentially oncogenic microorganisms

Target gene	GenBank Accession number	Forward primer/reverse primer	Source
The *clbA pks+* (polyketide synthase) gene of *E. coli*	CP155641.1	5'-CTCCACAGGAAGCTACTAAC-3' 5'-CGTGGTGATAAAGTTGGGAC-3'	[15]
The *clbB pks+ (*polyketide synthase) gene of *E. coli*	CP155641.1	5'-GCAACATACTCGCCCAGACT-3' 5'-TCTCAAGGCGTTGTTGTTTG-3' probe FAM (5'-CAAGGTGCGCGCTAGGCTGT-3');	[16]
The fragilisin (*bft*) gene synthesized by enterotoxigenic *B. fragilis*	AF103902.1	5'-GAACCTAAAACGGTATATGT-3' 5'-GTTGTAGACATCCCACTGGC-3'	[15]
fadA adhesion protein gene of *F. nucleatum*	DQ012973.1	5'-GCAGTTTCTGCTTCAGCATTT-3' 5'-TGCTTGAAGTCTTTGAGCTCTTT-3'	[17]
fimA gene for biofilm formation of *P. gingivalis*	AB195793.1	5'-TGCGACGCTATATGCAAGAC-3' 5'-TCTTCAAAACCACGCTGATG-3'	[17]

diagnosed bowel pathology (control group). The study was conducted with voluntary informed consent of the patients and was approved at the meeting of the Local Ethical Committee of the I.I. Mechnikov NWSMU (protocol No. 10 of 03.11.2021).

In the group of patients with CRC the distribution by stages of oncologic process taking into account TNM classification was as follows: stage 0 — 1 patient; stage 1 — 17 patients; stage 2 — 13 patients; stage 3 — 37 patients; stage 4 — 12 patients.

Inclusion criteria for patients from the CRC group: age over 18 years; diagnosis of CRC established for the first time on the basis of anamnesis, physical examination, morphological examination of tumor material, data of instrumental and laboratory methods of examination; receipt of clinical material (feces) from the patient.

Inclusion criteria for patients from the IBD group: age over 18 years; diagnosed IBD.

Inclusion criteria for patients from the control group: age over 18 years; no diagnosed IBD and CRC.

Exclusion criteria for all study groups: taking an antibacterial drug for the last 30 days and/or undergoing endoscopic examination (colonoscopy, rectomanoscopy) for the last 14 days before the submission of clinical material (feces).

Samples of clinical material obtained from study participants were stored at –20ºC until molecular genetic studies were performed.

DNA from fecal samples for polymerase chain reaction (PCR) was isolated by magnetic particle sorption using the MetaFec reagent kit (Raissol). The primer sequences used earlier for identification of potentially oncogenic MOs were applied in PCR (**Table 1**).

To identify potentially oncogenic MOs, quantitative PCR with hybridization-fluorescence detection (Bio-Rad CFX96 Thermal Cycler) was performed for *clbB pks*$^+$ and qualitative PCR (Bio-Rad T100 Thermal Cycler) with electrophoretic detection for other markers.

No cases of nonspecific amplification were detected.

The results of marker genes amplification were validated by capillary sequencing of amplicons on the GenomeLab GeXP device (Beckman Coulter, Inc.), and in all cases the sequences corresponding to the sequences of target fragments of marker genes presented in GenBank were obtained (**Table 1**). Aliquots of total DNA from the first 5 samples of biological material, in which positive PCR results were obtained, were used as positive controls after confirmation of amplification specificity by sequencing, and sterile deionized water served as a negative control.

R program (RStudio) was used for statistical data processing. To quantify the association of potentially oncogenic MOs with CRC, odds ratios (ORs) and 95% confidence intervals (CI) to them were determined. Sensitivity, specificity and CI were calculated using the Epitools[1] program. The results were considered statistically significant at $p < 0.05$.

Results

The study of clinical material samples by PCR allowed us to determine the detection rates of genetic markers of potentially oncogenic MOs in patients with CRC at different stages of the disease and in the comparison groups (**Fig. 1**). A higher prevalence of potentially oncogenic markers was detected in patients with CRC compared to patients with CRC and control group participants. There was a direct correlation between the stage of cancer and the prevalence of potentially oncogenic markers, in particular, *F. nucleatum* DNA, and a higher frequency of detection of periodontal pathogenic MOs at late stages of the cancer process. Colibactin-producing bacteria carrying the *clbB* gene of the *pks* pathogenicity island prevail at early tumor stages.

The association of some potentially oncogenic representatives of the intestinal microbiome with CRC was observed in the course of the case-control study (**Table 2**). Thus, the presence of the *bft* gene of entero-

[1] Epitools — Epidemiological Calculators. URL: http://epitools.ausvet.com.au

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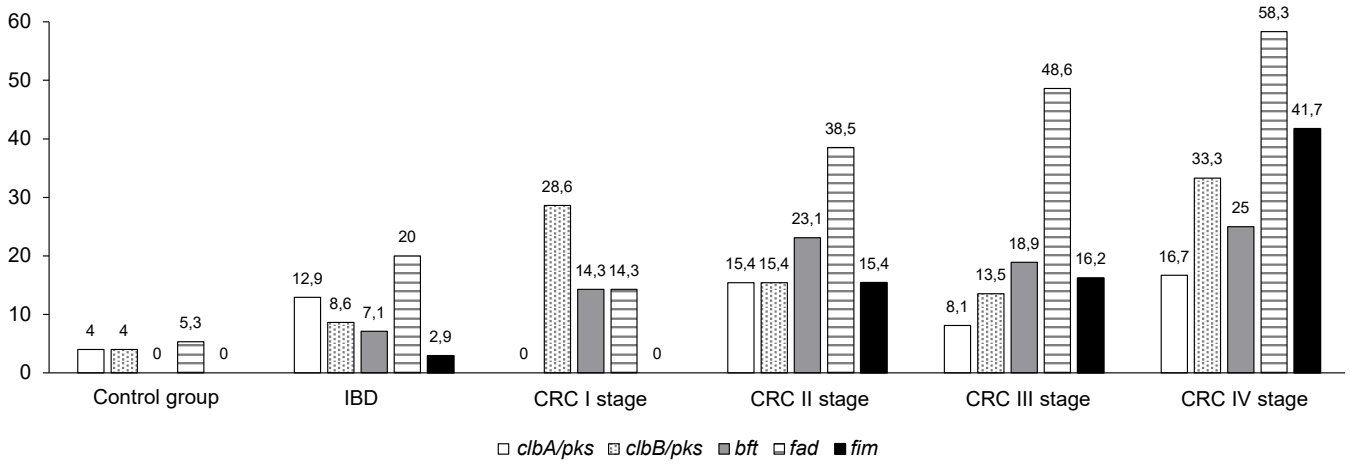

The prevalence of potentially oncogenic microorganisms in patients with CRC at various stages of the disease and in comparison groups.

toxigenic *B. fragilis* was significantly different between the groups of patients with IBD and patients with CRC and associated with the microbiome of patients with CRC (OR = 3.25; 95% CI 1.16–10.6; $p = 0.033$). Furthermore, a higher prevalence of this gene was found in patients with CRC compared to patients with IBD, while the enterotoxigenic *B. fragilis* gene was not detected in samples from participants in control group.

According to the data of this study, the presence of DNA of the periodontal pathogenic MO *F. nucleatum* was found to be associated with CRC, and its detection rate differed between the case and control groups. In patients with IBD, CRC was associated with the presence of DNA from another periodontal pathogenic MO, *P. gingivalis* (OR = 7.75; 95% CI 2.03–50.9; $p = 0.009$). The frequency of *F. nucleatum* DNA identification was higher in patients with CRC (44%) compared to patients with IBD (22%) and control group participants (5.3%). A high prevalence of *P. gingivalis* DNA was found in CRC patients (in 19% of samples) compared to IBD patients (in 2.9% of samples), *P. gingivalis* DNA was not detected in control group participants.

This study found that the presence of the *clbB* gene of the pathogenicity island of *Enterobacteriaceae pks* (OR = 5.47; 95% CI 1.49–20.14; $p = 0.015$) was associated with CRC and allowed us to separate the microbiome of patients with this pathology from that of control participants. The detectable *Enterobacteriaceae pks* pathogenicity island *clbB* gene was found in 19% of samples from patients with CRC, in 8.6% from patients with IBD, and in 4% from control participants. In contrast, the frequency of *clbA* gene identification did not differ between the comparison groups. The *clbA* gene was identified in 10% of samples from patients with CRC, in 13% of samples from patients with IBD, and in 4% of samples from control group participants.

In patients with CRC, the frequency of detection of markers of potentially oncogenic MOs did not depend on tumor localization: detection of *F. nucleatum* in tumors of distal location in 14 cases, proximal location — in 15 cases; *P. gingivalis* — in 5 and 7 cases; *clbB* gene of the *Enterobacteriaceae pks* pathogenicity island — in 6 and 7 cases; *clbA* gene — in 2 and 5 cases; fragilisin *bft* gene of enterotoxigenic *B. fragilis* – in 6 and 7 cases, respectively. No significant differences related to the morphological type of the tumor were revealed (detection of *F. nucleatum* in 18 cases of highly differentiated tumor, in 13 cases of low-differentiated tumor; *P. gingivalis* — in 6 and 7 cases; *clbB* gene of the *Enterobacteriaceae pks* pathogenicity island — in 7 and 6 cases; *clbA* gene — in 4 and 3 cases; fragilisin *bft* gene of enterotoxigenic *B. fragilis* — in 6 and 8 cases, respectively). Thus, the detection of markers of potentially oncogenic MOs allows to distinguish CRC regardless of the localization and morphological type of tumor.

Taking into account the obtained data, we calculated the sensitivity and specificity of the diagnostic test, which allows us to distinguish the microbiomes of patients with CRC from the microbiomes of patients with IBD and control group participants in case of separate and joint detection of genetic determinants of potentially oncogenic MOs (**Table 3**). The optimal (in terms of combination of sensitivity and specificity) variant of testing seems to be the one that takes into account the fact of DNA identification of at least 1 out of 5 potential microbial onco-markers.

Discussion

In the present study, we evaluated the prevalence of potentially oncogenic CRC-associated MOs among residents of a Russian megacity with diagnosed CRC, IBD and individuals without intestinal tract pathology.

It is known that *B. fragilis* is a commensal representative of the intestinal microbiota. Both non-toxigenic strains of *B. fragilis* (not associated with CRC) [18] and enterotoxigenic *B. fragilis* synthesizing fragilisin,

Table 2. Representatives of the intestinal microbiome associated with CRC

Target gene	CRC, *n* (%)	IBD, *n* (%)	Control, *n* (%)	OR (95% CI)			
				CRC/IBD	CRC/Control	CRC + IBD/Control	CRC/IBD + Control
Fragilisin *bft* gene of enterotoxigenic *B. fragilis*	14 (20%)	5 (7,1%)	0 (0%)	3,25 (1,16–10,6)	∞	∞	7 (2,55–22,5)
fadA adhesion protein gene of *F. nucleatum*	31 (44%)	14 (20%)	4 (5,3%)	3,18 (1,52–6,9)	14,1 (5,13–50,1)	8,41 (2,89–24,46)	5,61 (2,87–11,3)
fimA gene for biofilm formation of *P. gingivalis*	13 (19%)	2 (2,9%)	0 (0%)	7,75 (2,03–50,9)	∞	∞	16,3 (4,33–106)
The gene *clbA* of the pks pathogenicity island of *E. coli*	7 (10%)	9 (13%)	3 (4,0%)	0,75 (0,25–2,15)	2,67 (0,71–12,8)	3,09 (0,87–10,99)	1,23 (0,44–3,21)
The gene *clbB* of the pks pathogenicity island of *E. coli*	13 (19%)	6 (8,6%)	3 (4,0%)	2,43 (0,9–7,31)	5,47 (1,49–20,14)	3,77 (1,08–13,18)	3,44 (1,39–8,51)

Note. ∞ — due to the zero value of the denominator, it is not possible to calculate the OR indicator.

a toxin that cleaves the cell adhesion protein E-cadherin, disrupting the intestinal barrier and contributing to the development of diarrhea, are present in the gastrointestinal tract [19]. In addition, this toxin can activate the implementation of the Wnt/β-catenin signaling pathway, promoting cell proliferation, induction of inflammatory mediator production, and carcinogenesis [20]. The toxin of *B. fragilis* is encoded by the *bft* gene with 3 isotypes (*bft 1*, *bft 2*, *bft 3*), which is located in the pathogenicity island (PAI) and flanked by genes encoding mobilization proteins and representing CTn86 and CTn9343 conjugative transposon sequences. Non-toxigenic strains of *B. fragilis* do not possess a pathogenicity island, but in the presence of conjugative transposons in some strains PAI can transfer from enterotoxigenic *B. fragilis* to non-toxigenic strains of *B. fragilis* [21]. The role of enterotoxigenic *B. fragilis* as a "driver" in the "driver-passenger" model has been established, which consists in the damage of epithelial DNA by "driver" bacteria, which leads to the development of carcinogenesis and changes in the microbial community; further in the process of oncogenesis, "drivers" are displaced by commensal bacteria - "passengers" with tumor-promoting properties [22]. Enterotoxigenic strains of *B. fragilis* can inhibit the exosomal microRNA miR-149-3p, which mediates intercellular interactions by modulating the differentiation of Th17 cells, contributing to inflammation and carcinogenesis in the intestine [23].

The association of enterotoxigenic *B. fragilis* with CRC identified in our study was confirmed in different ethnic cohorts. For example, in the Iranian population, the frequency of *bft* gene detection in colorectal biopsy specimens of patients with CRC ranged from 30.5 to 47% compared with control group participants — up to 6.25% of biopsy specimens [24, 25]. Moreover, a higher prevalence of enterotoxigenic *B. fragilis* in biopsy specimens was found among patients from Tehran with ulcerative colitis compared to individuals without intestinal pathology [26]. In Canadian and French cohorts of patients with CRC, a high prevalence of enterotoxigenic *B. fragilis* was found (up to 32% of samples) compared to control subjects [15, 19]. The results of the European Prospective Investigation into Nutrition and Cancer (EPIC) showed that in the European cohort IgA- and IgG-seropositivity to enterotoxigenic *B. fragilis* and genotoxic *E. coli* was significantly associated with the development of CRC [27].

F. nucleatum is a Gram-negative non-spore-forming obligate anaerobic MO of the family Fusobacteriaceae and is the dominant MO in dental plaque biofilms [28]. *F. nucleatum* promotes carcinogenesis and metastasis through multiple mechanisms: promotes proliferation of myeloid suppressor cells; accelerates T cell apoptosis, suppresses T cell proliferation, thereby orchestrating a tumor microenvironment that promotes oncogenesis and metastasis; induces expression of the

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Table 3. Characterization of sensitivity and specificity of identification of potentially oncogenic microorganisms for the diagnosis of CRC

Parameter of the diagnostic method	CRC, *n*	IBD + control, *n*	Sensitivity, % (95% CI)	Specificity, % (95% CI)
Detection of the *clbA* gene	7	11	10 (5–19)	92 (86–95)
Detection of the fragilisin *bft* gene of enterotoxigenic *B. fragilis*	14	5	20 (12–31)	97 (92–99)
Detection the *fadA* adhesion protein gene of *F. nucleatum*	31	18	44 (33–56)	88 (81–92)
Detection the *fimA* gene for biofilm formation of *P. gingivalis*	13	2	19 (11–29)	99 (95–99)
Detection of the *clbB* gene	13	9	19 (11–29)	94 (89–97)
Detection of 1 or more microorganisms	36	27	51 (39–64)	81 (74–87)
Detection of 2 or more microorganisms	22	10	31 (21–44)	93 (88–97)
Detection of 3 or more microorganisms	12	4	17 (9–28)	97 (93–99)
Detection of 4 or more microorganisms	6	3	9 (3–18)	98 (94–100)
Detection of 5 or more microorganisms	2	2	3 (0,35–10,00)	99 (95–100)

molecular structure protein S100A9 and triggers activation of M2 macrophages via nuclear factor-κB, thereby activating tumor cell proliferation and migration; stimulates the proliferation of Foxp3+ regulatory T cells and inhibits the proliferation and function of effector T cells, impeding the antitumor immune response; induces the secretion of circulating exosomes, enhancing tumor invasion; furthermore, a possible role of *F. nucleatum* in resistance to tumor immunotherapy and chemotherapy [29]. A key virulence/oncogenicity factor of *F. nucleatum* is the adhesin FadA, which regulates annexin A1 expression via E-cadherin. Induction of annexin A1, which is a modulator of Wnt/β-catenin, specifically stimulates colorectal carcinoma cells, contributing to the progression of CRC [30]. Furthermore, *F. nucleatum* stimulates inflammatory and antiapoptotic responses in CRC cells through the release of ADP-heptose and activation of the ALPK1/TIFA axis [31]. Recently, a distinct Fna C2 clade of *F. nucleatum* associated with CRC has been characterized, which exhibits increased virulence [32].

According to a study conducted by J. Jones et al., it is *F. nucleatum* and enterotoxigenic *B. fragilis* represent two key pathobionts that promote oncogenic reprogramming of intestinal epithelial cells [11].

P. gingivalis is an anaerobic oral bacterium that causes chronic periodontitis. Over the past decade, the mechanisms by which *P. gingivalis* promotes tumor progression and stimulates cell invasion and metastasis of tumor cells have been identified. These mechanisms include increased expression of proinflammatory factors and matrix metalloproteinases that degrade the basal membranes and extracellular matrix of the intestinal epithelium [33].

According to these studies, an association was found between the presence of periodontopathogenic bacteria in fecal samples and biopsy material from CRC. A case-control study using metagenomic sequencing revealed that the detection rate of *Fusobacterium* was higher in CRC patients (31.9% vs. 11.7% in controls) and the development of CRC was associated with the presence of *F. nucleatum* (OR = 4.11; 95% CI 1.62–10.47; $p = 0.004$) and *P. gingivalis* (OR = 5.17; 95% CI 1.75–15.25; $p = 0.001$) [34]. A study conducted in France revealed that the prevalence of *F. nucleatum* was higher among patients with CRC (70.4%) compared to individuals without intestinal pathology [19].

P. gingivalis and *F. nucleatum*, oral bacteria belonging to the so-called red and orange complexes, can not only induce chronic inflammation but also promote oncogenesis in both the oral cavity and intestine, possibly having a synergistic effect [35].

It should also be noted that all three oncogenic bacteria (*F. nucleatum*, *B. fragilis* and *P. gingivalis*) are potentially associated not only with the development of CRC, but also with a worse prognosis for patients (lower survival rate) [13, 14]. Furthermore, the results of our study indicate a direct relationship between the stage of the cancer process and the prevalence of periodontal pathogenic MOs *F. nucleatum* and *P. gingivalis*.

Various authors have also found colibactin-producing genotypes of *Enterobacteriaceae* to be associated with CRC. Colibactin is a genotoxin that causes double-stranded DNA breaks, cell cycle arrest, and chromosomal instability in eukaryotic cells. It is synthesized by an assembly line of non-ribosomal polyketide synthase (*pks*) consisting of 19 genes (*clbA* to *clbS*) located on a 54 bp genomic island [36]. The *pks* island is present in the genomes of *K. pneumoniae*, *K. aerogenes* (basonym: *Enterobacter aerogenes*), *Citrobacter koseri*, and in the phylogenetic groups of *E. coli* [19]. Infection may occur at early stages of ontogenesis: it is known that transmission of colibactin-producing genotypes

of *Enterobacteriaceae* occurs in the perinatal period during passage through the birth canal (the *clbB* gene was detected in 87.5% of children born naturally, in 12.5% — by cesarean section) and as a result of breastfeeding [37]. The frequency of *clbN* gene detection was higher in CRC patients (49.4%) compared to control participants (24%; $p < 0.005$), with a high prevalence (72.2%) found in the last stage IV compared to stage I/II CRC (42.3%; $p < 0.05$) and stage III CRC (43.2%; $p < 0.05$) [19]. In a Japanese cohort study (543 colorectal neoplasia cases (22 CRC and 521 adenomas), 425 control participants), *pks*$^+$-*E. coli* was found in 32.6% of fecal samples from patients with colorectal neoplasia and in 30.8% from control participants [38]. Also among the Canadian cohort, the frequency of colonization with *pks*$^+$ bacteria was found not to differ between control group participants (42%) and CRC patients (46%), notably, *pks*$^+$ bacteria were common in advanced stages of CRC (40/79; 52%) compared to early tumor stages (3/15; 20%; $p < 0.05$) [15]. In a cohort study, *pks*$^+$-*E. coli* was detected in 9.44% (111/1175) of biopsy specimens from patients with CRC, with DNA *pks*$^+$-*E. coli* was inversely associated with the stage of tumor process ($p = 0.008$) [16]. Our study revealed a high prevalence of the *clbB* gene of the pathogenicity island of *pks*$^+$-*E. coli* at the I stage of the cancer process.

Conclusion

The results of this study allow us to conclude that CRC in patients of a large metropolitan area is associated with the presence of genes of potentially oncogenic bacteria in the intestinal microbiome, in particular, species-specific genes of periodontal pathogens *F. nucleatum*, *P. gingivalis*, toxin-fragilisin *bft* gene of *B. fragilis*, polyketide synthase *clbB* gene of *Enterobacteriaceae pks* pathogenicity island. The results obtained are consistent with the current ideas about the pathogenetic role of these bacteria and/or their toxin-producing strains in CRC.

Molecular genetic detection of the above genetic determinants of potentially oncogenic MOs can be applied as a method of non-invasive diagnosis of CRC regardless of the localization and morphological type of tumor, both separately and together with other recommended tests.

Given the available data on the association of these bacteria with the stage/progression of CRC, prognosis/survival and resistance to immunotherapy and chemotherapy, we can assume the possibility of their use as non-invasive biomarkers for predicting the course and outcomes of CRC, response to antitumor therapy, and in the future for the development of appropriate cancer prevention measures, including personalized correction of oral and intestinal dysbiosis and/or their sanitation.

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Analysis of influenza epidemics during the COVID-19 pandemic using an improved surveillance system (from 2021 to 2024)

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Abstract

Aim. Assessing the effectiveness of new baselines and intensity thresholds of epidemics based on rates of incidence and hospitalization with a diagnosis of "influenza" to clarify the timing of epidemics and their spread throughout the Russian Federation against the backdrop of the COVID-19 pandemic (from 2021 to 2024).

Materials and methods. At the A.A. Smorodintsev Influenza Research Institute, the software was reformed taking into account the need to solve the tasks set during the COVID-19 pandemic. Due to changes in influenza surveillance in relation to the diagnosis of "influenza", and hence the increase in the registration of influenza cases, the baseline and threshold of epidemics were adjusted for the influenza incidence and hospitalization rates in the surveyed cities in total (61) and for each Federal districts (over the entire population and by age groups) for 3 epidemics against the background of COVID-19 pandemic (2021–2022, 2022–2023, and 2023–2024).

Results. A comparison of baselines calculated over the previous 6 seasons based on the incidence and hospitalization rates of mostly clinically diagnosed influenza and new baseline levels of incidence and hospitalization rates of mostly laboratory-confirmed influenza during the pandemic showed minor changes in the indicators of baseline incidence and hospitalization rates while epidemic thresholds for these indicators increased.

Conclusion. Against the backdrop of the COVID-19 pandemic during the 2020–2021 season, there was no influenza epidemic. In 2021–2022, the A(H3N2) epidemic was of low intensity in terms of incidence, hospitalization rates and low mortality (2 cases). In 2022–2023, the influenza A(H1N1)pdm09 and B epidemic was of moderate intensity in terms of incidence, with a high frequency of hospitalizations and mortality (120 cases). Influenza A(H3N2) and B epidemic in 2023–2024 was of a "very high" level in terms of the influenza incidence, but average in terms of the frequency of hospitalizations and mortality (41 cases), with higher incidence rate compared to the previous epidemic (0.28% and 0.19% of the total population), including persons over 15 years of age (0.19% and 0.12%).

Keywords: *influenza, COVID-19, baselines, intensity thresholds, morbidity, hospitalization, mortality*

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Оригинальное исследование
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Анализ эпидемий гриппа на фоне пандемии COVID-19 с использованием усовершенствованной системы надзора (с 2021 по 2024 год)

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Аннотация

Цель работы — оценка эффективности новых базовых линий (БЛ) и порогов интенсивности (ПИ) эпидемий по заболеваемости и госпитализации с диагнозом «грипп» для уточнения сроков эпидемий и их распространения по территории России на фоне пандемии COVID-19 (с 2021 по 2024 г.).

Материалы и методы. В НИИ гриппа им. А.А. Смородинцева проведено реформирование программного обеспечения с учётом необходимости решения поставленных задач в период пандемии COVID-19. В связи с изменениями в надзоре за гриппом в отношении постановки диагноза «грипп» и увеличением регистрации случаев заболеваний, были откорректированы БЛ и ПИ эпидемий по заболеваемости гриппом и частоте госпитализаций в наблюдаемом 61 городе и для каждого федерального округа (по всему населению и по возрастным группам) за 3 эпидемии на фоне пандемии COVID-19 (2021–2022, 2022–2023 и 2023–2024 гг.).

Результаты. Сравнение БЛ, рассчитанных за предыдущие 6 сезонов по заболеваемости и частоте госпитализаций, в основном клинически диагностированным гриппом, и новых БЛ заболеваемости и госпитализации, в основном лабораторно подтверждённым гриппом в период пандемии, показало незначительные изменения в показателях БЛ заболеваемости и госпитализаций, а ПИ эпидемий по этим показателям увеличились.

Заключение. На фоне пандемии COVID-19 в сезон 2020–2021 гг. эпидемии гриппа не было. В 2021–2022 гг. эпидемия гриппа A(H3N2) была низкой интенсивности по заболеваемости, частоте госпитализации и низкой летальности (2 случая). В 2022–2023 гг. эпидемия гриппа A(H1N1)pdm09 и B была средней интенсивности по заболеваемости с высокой частотой госпитализаций и летальностью (120 случаев). Эпидемия гриппа A(H3N2) и B в 2023–2024 гг. по интенсивности заболеваемости была очень высокого уровня, но средней по частоте госпитализаций и летальности (41 случай), а заболеваемость, по сравнению с предыдущей эпидемией, была больше (0,28 и 0,19% всего населения), в том числе лиц старше 15 лет (0,19 и 0,12%).

Ключевые слова: *грипп, COVID-19, базовые линии, пороги интенсивности, заболеваемость, госпитализация, летальность*

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

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Introduction

A new coronavirus infection has been circulating in Russia for the last 4 seasons. Against this background, to accurately determine the start of the epidemic in the country, it is necessary to use incidence data only for influenza, without taking into account acute respiratory viral infection (ARVI), because incidence data for ARVI and COVID-19, which significantly outnumber influenza during the pandemic, mask the place, time of the start and nature of the development of the influenza epidemic. Due to the similarity of the clinical course of influenza, COVID-19 and other acute respiratory viruses, according to the Government Decree of December 14, 2022 № 2297, testing for influenza and other acute respiratory viruses in hospitals and polyclinics is carried out within the framework of the program of state guarantees of free medical care for citizens and simultaneously with the polymerase chain reaction (PCR) test for coronavirus[1].

The aim of the study is to assess the effectiveness of new base lines and intensity thresholds of epidemics in terms of morbidity and hospitalization with the diagnosis of "influenza" to clarify the timing of epidemics and their spread across Russia against the background of the COVID-19 pandemic (from 2021 to 2024).

Materials and methods

At the A.A. Smorodintsev Influenza Research Institute, the software was reformed taking into account the need to solve the tasks set during the SARS-CoV-2 pandemic. The introduction of a new electronic system, based on the creation of a flexible platform, allowed us to respond quickly by changing or adding electronic forms to the requirements for data collection and analysis and uploading the results to the WHO global database [1].

Previously, we calculated baselines and thresholds of influenza incidence for 6 seasons (from 2016-2017 to 2022-2023) using the moving epidemic method adopted in the European influenza surveillance system [2, 3]. Due to changes in influenza surveillance regarding the diagnosis of influenza and other acute respiratory infections, and hence the increase in influenza case registration, we adjusted the baselines and thresholds of epidemics for influenza incidence and frequency of hospitalizations diagnosed with influenza in the observed cities (61 in total) and for each federal district (FD) (for the whole population and age groups) for 3 epidemics occurring against the background of the COVID-19 pandemic (2021–2022, 2022–2023 and 2023–2024).

Statistical processing of the obtained results was carried out in the MS Excel program using Student's t-criterion, with significance P = 95%.

Results and discussion

Comparison of baselines calculated over the previous 6 seasons for incidence and hospitalization rates of mostly clinically diagnosed influenza and new baseline incidence and hospitalization rates of mostly laboratory-confirmed influenza during the pandemic showed little change in baseline incidence and hospitalization rates, while epidemic thresholds for these indicators increased (**Tables 1, 2**).

Monitoring for influenza incidence in the country during the 2023–2024 season showed its early increase: as early as week 45 of 2023 (2nd week of November), it exceeded the baseline (0.04 per 10,000 population) by 87.5% nationwide. Further, there was a steady increase in influenza incidence for 6 weeks with a peak (4.52 per 10,000 population) in week 51 of 2023. From the following week, the incidence began to decline but remained at a low level until week 18. The incidence rate at the peak of the epidemic (2023–2024) was 2.5 times higher than the 2022–2023 epidemic. The intensity of the last season's influenza epidemic was very high, while in the previous epidemic it was intermediate (**Fig. 1**).

Monitoring the incidence in different age groups of the population

In the 2023–2024 influenza season, influenza incidence exceeded the baseline in week 45 simultaneously in all age groups. Peak incidence was recorded in week 51 simultaneously in pediatric age groups and among persons over 15 years of age. The intensity of influenza incidence at the peak of the epidemic exceeded the very high intensity threshold in all age groups except children 0–2 years of age, in whom it was high. From week 52 onwards, incidence began to decline in all age groups, but remained above post-epidemic baseline in children 0–2 and 3–6 years of age until week 15 (inclusive), and among children 7–14 years of age and persons 15+ years of age until week 18.

During the 2023–2024 epidemic period, 0.27% of the total population of the observed cities (150,439 persons) became ill with influenza, including 0.88% of children aged 0–2 years, 0.8% of children aged 3–6 years, 0.57% of children aged 7–14 years, and 0.19% of persons aged 15 years and older. During the 2022–2023 epidemic period, 0.19% of the total population of the monitored cities (105,881 persons) became ill with influenza, including 0.81% of children aged 0–2 years, 0.66% of children aged 3–6 years, 0.46% of children aged 7–14 years, and 0.12% of persons aged 15 years and older (**Fig. 2**).

[1] Resolution of the Government of the Russian Federation of December 14, 2022 No. 2297 "On Amendments to Section IV of the Program of State Guarantees of Free Medical Assistance to Citizens for 2022 and for the Planning Period of 2023 and 2024" (publication number: 0001202212150010).

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Table 1. Baselines and thresholds of influenza incidence rate, per 10,000

Parameter		2016–2017 – 2022–2023					2021–2022 – 2023–2024				
		total	age, years				total	age, years			
			0–2	3–6	7–14	15+		0–2	3–6	7–14	15+
Russia											
BL	pre-epidemic	0,05	0,23	0,13	0,08	0,04	0,04	0,23	0,13	0,05	0,03
	post-epidemic	0,04	0,21	0,17	0,09	0,04	0,05	0,28	0,19	0,10	0,04
IT	medium	0,36	1,32	1,40	0,72	0,21	0,92	3,87	3,84	2,60	0,40
	high	1,12	5,39	4,38	2,35	0,65	2,95	9,83	9,28	6,91	1,92
	very high	1,46	7,20	5,69	3,07	0,84	3,85	12,47	11,69	8,81	2,59
Central Federal District											
L	pre-epidemic	0,06	0,24	0,24	0,13	0,03	0,05	0,20	0,13	0,07	0,05
	post-epidemic	0,03	0,12	0,12	0,05	0,02	0,05	0,11	0,16	0,08	0,02
T	medium	0,15	0,57	1,01	0,68	0,17	1,42	5,37	6,94	5,26	0,59
	high	1,82	9,56	9,25	5,32	1,21	3,97	16,44	18,35	13,70	1,94
	very high	2,56	13,54	12,89	7,38	1,67	5,10	21,33	23,39	17,42	2,54
Southern Federal District											
BL	pre-epidemic	0,06	0,12	0,16	0,15	0,07	0,07	0,11	0,11	0,26	0,06
	post-epidemic	0,06	0,25	0,17	0,08	0,06	0,06	0,34	0,14	0,16	0,06
IT	medium	0,47	1,89	1,74	0,73	0,27	0,57	3,43	2,20	0,81	0,28
	high	1,02	5,15	3,73	1,56	0,68	2,20	7,70	6,07	4,43	1,53
	very high	1,27	6,60	4,61	1,93	0,86	2,92	9,59	7,78	6,04	2,08
Northwest Federal District											
BL	pre-epidemic	0,03	0,16	0,17	0,07	0,02	0,03	0,16	0,15	0,03	0,02
	post-epidemic	0,05	0,26	0,26	0,20	0,04	0,07	0,31	0,31	0,24	0,04
IT	medium	0,36	1,31	1,38	0,93	0,21	0,54	3,56	2,90	2,24	2,42
	high	1,16	5,06	4,02	2,49	0,77	6,31	10,40	10,96	9,86	8,31
	very high	1,51	6,72	5,18	3,17	1,02	8,85	13,43	14,53	13,22	10,91
Far Eastern Federal District											
BL	pre-epidemic	0,17	0,31	0,30	0,30	0,10	0,16	0,34	0,16	0,21	0,12
	post-epidemic	0,11	0,66	0,60	0,24	0,08	0,11	0,83	0,70	0,30	0,10
IT	medium	0,91	3,07	2,69	1,45	0,58	1,56	6,27	5,33	3,74	0,80
	high	2,04	8,45	6,62	4,15	1,27	3,63	14,15	11,68	7,71	2,27
	very high	2,54	10,83	8,36	5,34	1,58	4,54	17,64	14,49	9,47	2,91
Siberian Federal District											
BL	pre-epidemic	0,02	0,09	0,09	0,03	0,01	0,02	0,09	0,08	0,03	0,01
	post-epidemic	0,04	0,17	0,14	0,08	0,02	0,05	0,13	0,14	0,08	0,03
IT	medium	0,40	1,77	1,49	0,80	0,21	0,53	3,14	2,03	1,53	0,20
	high	0,75	4,43	2,95	1,91	0,41	1,24	6,83	3,81	2,85	0,75
	very high	0,90	5,60	3,59	2,40	0,50	1,55	8,47	4,60	3,43	0,99
North Caucasian Federal District											
BL	pre-epidemic	0,03	0,14	0,29	0,22	0,03	0,19	0,27	0,29	0,20	0,53
	post-epidemic	0,04	0,14	0,39	0,11	0,04	0,06	0,49	0,23	0,27	0,04
IT	medium	0,17	2,83	2,38	0,77	0,13	0,41	6,24	4,68	3,28	1,02
	high	2,23	7,55	7,70	6,27	1,68	3,33	10,66	10,84	10,40	3,30
	very high	3,15	9,63	10,04	8,69	2,37	4,63	12,61	13,56	13,55	4,30

End of the Table 1

Parameter		2016–2017 – 2022–2023					2021–2022 – 2023–2024				
		total	age, years				total	age, years			
			0–2	3–6	7–14	15+		0–2	3–6	7–14	15+
Ural Federal District											
BL	pre-epidemic	0,03	0,07	0,06	0,03	0,03	0,03	0,12	0,08	0,05	0,02
	post-epidemic	0,04	0,09	0,10	0,04	0,04	0,04	0,09	0,06	0,02	0,05
IT	medium	0,23	0,24	0,33	0,28	0,24	0,26	1,02	0,65	0,51	0,35
	high	0,59	1,26	1,10	0,71	0,60	1,23	3,13	2,70	1,74	1,17
	very high	0,76	1,71	1,44	0,90	0,76	1,65	4,07	3,61	2,27	1,54
Volga Federal District											
BL	pre-epidemic	0,02	0,12	0,10	0,03	0,02	0,01	0,14	0,08	0,02	0,01
	post-epidemic	0,05	0,30	0,18	0,09	0,04	0,06	0,32	0,16	0,07	0,04
IT	medium	0,37	1,40	1,32	0,56	0,23	0,74	2,57	2,57	2,06	0,42
	high	0,95	3,53	2,76	2,08	0,67	2,68	10,73	7,75	5,24	1,87
	very high	1,21	4,48	3,40	2,75	0,86	3,54	14,34	10,03	6,65	2,51

Table 2. Baselines and thresholds for rates of hospitalization with “influenza”, per 10,000

Parameter		2016–2017 – 2022–2023					2021–2022 – 2023–2024				
		total	age, years				total	age, years			
			0–2	3–6	7–14	15+		0–2	3–6	7–14	15+
Russia											
BL	pre-epidemic	0,03	0,15	0,09	0,05	0,03	0,01	0,11	0,04	0,02	0,01
	post-epidemic	0,03	0,14	0,11	0,06	0,03	0,02	0,25	0,07	0,03	0,01
IT	medium	0,24	1,13	0,91	0,42	0,14	0,27	1,92	1,18	0,55	0,13
	high	0,49	3,04	1,86	0,80	0,29	0,62	4,24	2,35	1,01	0,35
	very high	0,60	3,89	2,28	0,97	0,35	0,77	5,26	2,87	1,22	0,45
Central Federal District											
BL	pre-epidemic	0,04	0,19	0,19	0,08	0,02	0,01	0,13	0,07	0,03	0,01
	post-epidemic	0,02	0,09	0,07	0,03	0,01	0,02	0,10	0,08	0,05	0,01
IT	medium	0,15	0,78	0,81	0,40	0,10	0,30	2,12	1,69	0,74	0,11
	high	0,45	3,82	2,67	1,03	0,23	0,69	5,59	3,79	1,41	0,30
	very high	0,58	5,16	3,49	1,31	0,29	0,86	7,12	4,71	1,71	0,39
Southern Federal District											
BL	pre-epidemic	0,06	0,11	0,15	0,14	0,06	0,06	0,10	0,11	0,08	0,03
	post-epidemic	0,05	0,21	0,72	0,07	0,04	0,05	0,25	0,11	0,13	0,05
IT	medium	0,45	1,93	1,71	0,68	0,24	0,47	3,32	1,95	0,83	0,20
	high	0,96	5,09	3,69	1,52	0,60	1,00	6,22	3,01	1,52	0,65
	very high	1,18	6,49	4,57	1,89	0,76	1,24	7,51	3,47	1,82	0,84
Northwest Federal District											
BL	pre-epidemic	0,02	0,20	0,15	0,06	0,01	0,03	0,23	0,16	0,07	0,02
	post-epidemic	0,03	0,19	0,18	0,17	0,03	0,04	0,17	0,16	0,09	0,02
IT	medium	0,24	1,21	0,94	0,56	0,13	0,33	1,91	1,21	0,78	0,37
	high	0,71	3,82	2,32	1,29	0,46	0,92	5,08	2,93	1,67	0,77
	very high	0,91	4,97	2,93	1,62	0,60	1,18	6,48	3,70	2,06	0,94

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End of the Table 2

Parameter		2016–2017 – 2022–2023					2021–2022 – 2023–2024				
		total	age, years				total	age, years			
			0–2	3–6	7–14	15+		0–2	3–6	7–14	15+
Far Eastern Federal District											
BL	pre-epidemic	0,13	0,23	0,23	0,24	0,09	0,00	0,08	0,14	0,12	0,07
	post-epidemic	0,08	0,41	0,36	0,12	0,06	0,07	0,74	0,19	0,22	0,08
IT	medium	0,62	2,40	1,62	0,87	0,42	0,62	3,50	1,70	0,92	0,38
	high	1,28	6,20	3,70	1,80	0,86	1,43	7,75	4,10	2,02	0,95
	very high	1,57	7,88	4,61	2,21	1,05	1,79	9,63	5,15	2,51	1,20
Siberian Federal District											
BL	pre-epidemic	0,02	0,08	0,07	0,03	0,01	0,01	0,13	0,03	0,03	0,00
	post-epidemic	0,02	0,14	0,11	0,06	0,01	0,02	0,08	0,07	0,03	0,01
IT	medium	0,21	1,33	0,81	0,36	0,09	0,22	2,26	0,79	0,38	0,07
	high	0,43	3,31	1,65	0,73	0,22	0,46	4,50	1,68	0,58	0,23
	very high	0,53	4,19	2,03	0,89	0,28	0,57	5,48	2,08	0,66	0,30
North Caucasian Federal District											
BL	pre-epidemic	0,01	0,00	0,18	0,17	0,02	0,00	0,00	0,00	0,14	0,00
	post-epidemic	0,02	0,14	0,25	0,06	0,03	0,01	0,35	0,00	0,00	0,00
IT	medium	0,15	2,00	1,42	0,74	0,10	0,08	3,29	1,48	1,44	0,15
	high	0,67	4,46	3,39	1,67	0,45	0,70	5,12	3,93	2,18	0,56
	very high	0,91	5,54	4,26	2,08	0,60	0,98	5,94	5,01	2,51	0,75
Ural Federal District											
BL	pre-epidemic	0,01	0,06	0,05	0,02	0,02	0,00	0,05	0,04	0,02	0,00
	post-epidemic	0,02	0,08	0,03	0,00	0,03	0,03	0,00	0,00	0,00	0,01
IT	medium	0,11	0,21	0,16	0,06	0,14	0,07	0,49	0,29	0,04	0,09
	high	0,27	0,82	0,46	0,22	0,30	0,26	3,32	0,91	0,45	0,20
	very high	0,34	1,09	0,59	0,29	0,37	0,34	4,57	1,18	0,62	0,25
Volga Federal District											
BL	pre-epidemic	0,02	0,12	0,10	0,03	0,02	0,01	0,08	0,06	0,01	0,01
	post-epidemic	0,03	0,23	0,14	0,08	0,02	0,02	0,15	0,04	0,02	0,01
IT	medium	0,24	1,05	0,76	0,32	0,16	0,22	1,38	0,70	0,31	0,15
	high	0,44	2,50	1,58	0,67	0,28	0,57	4,26	1,89	0,86	0,35
	very high	0,53	3,15	1,94	0,83	0,34	0,72	5,53	2,41	1,11	0,45

Geographical spread of the epidemic, its intensity in different Federal Districts

The epidemic started first of all (week 43) in the cities of the Urals Federal District; in week 44 it was registered in the other 3 Federal Districts (North-Western, Volga and Central), in week 46 in the Siberian Federal District, in week 47 in the Far Eastern Federal District, in week 49 in the Southern Federal District, and last of all in the North Caucasus Federal District (week 51).

The peak incidence was reached earliest (week 50) in the Far Eastern Federal District (4.6 per 10,000 population), in week 51 in the Northwestern (11.56), Central (5.41) and Ural Federal Districts (1.91), in week 52 in the Siberian Federal District (1.59), in week 2 in the Volga Federal District (3.57), and in week 5 in the North Caucasus (4.94) and Southern Federal Districts (3.86). The influenza epidemic ended earliest (at week 13) in the Siberian and Far Eastern FDs, at week 14 in the Volga and North Caucasus FDs, at week 17 in the Southern FD, and at week 19 in the Central FD; however, in the Northwestern and Ural FDs, influenza incidence remained above their post-epidemic baselines at week 19. Thus, the duration of the epidemic in the districts varied from 16 weeks in the North Caucasus FD to 29 weeks in the Urals FD.

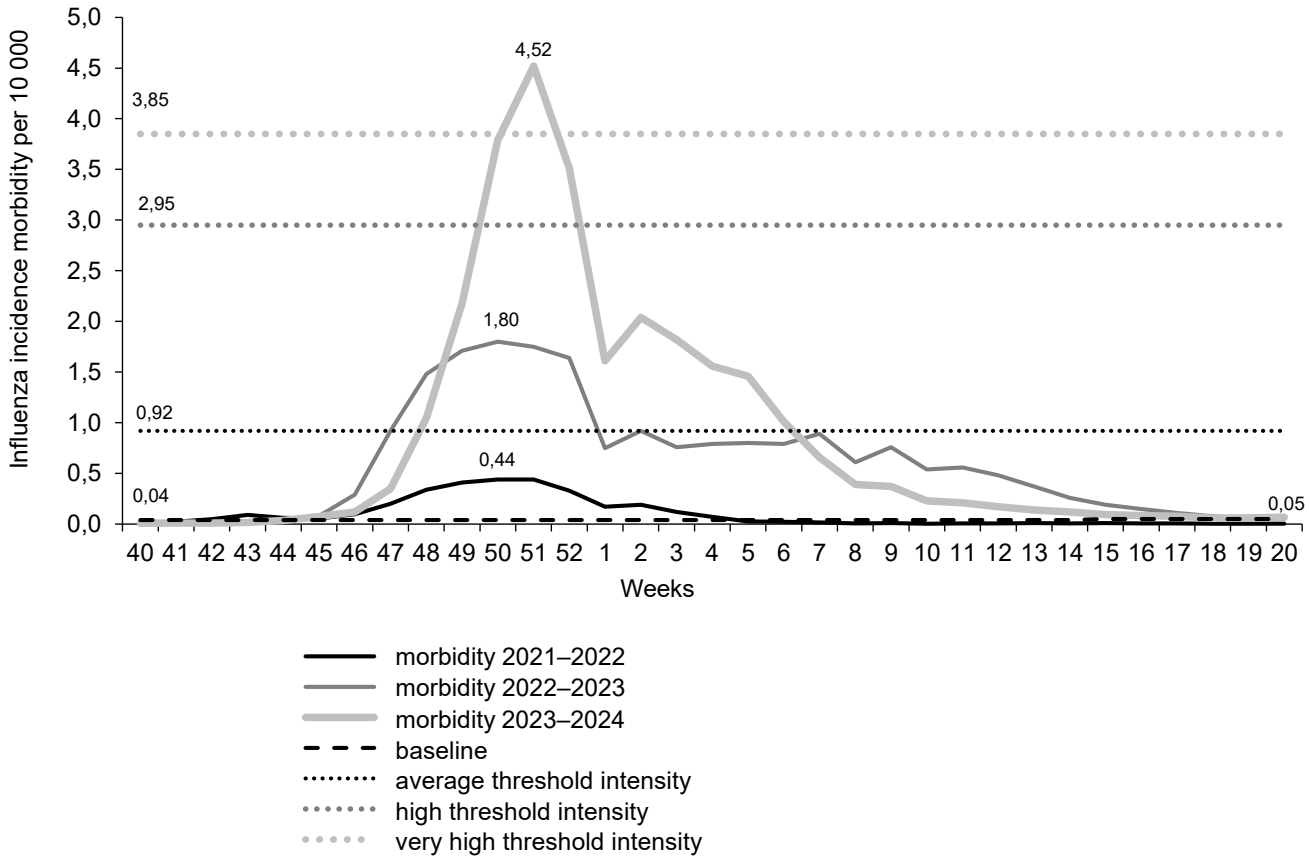

Fig. 1. Dynamics of influenza incidence in the population of the surveyed cities (61) in the seasons 2021–2022, 2022–2023, and 2023–2024.

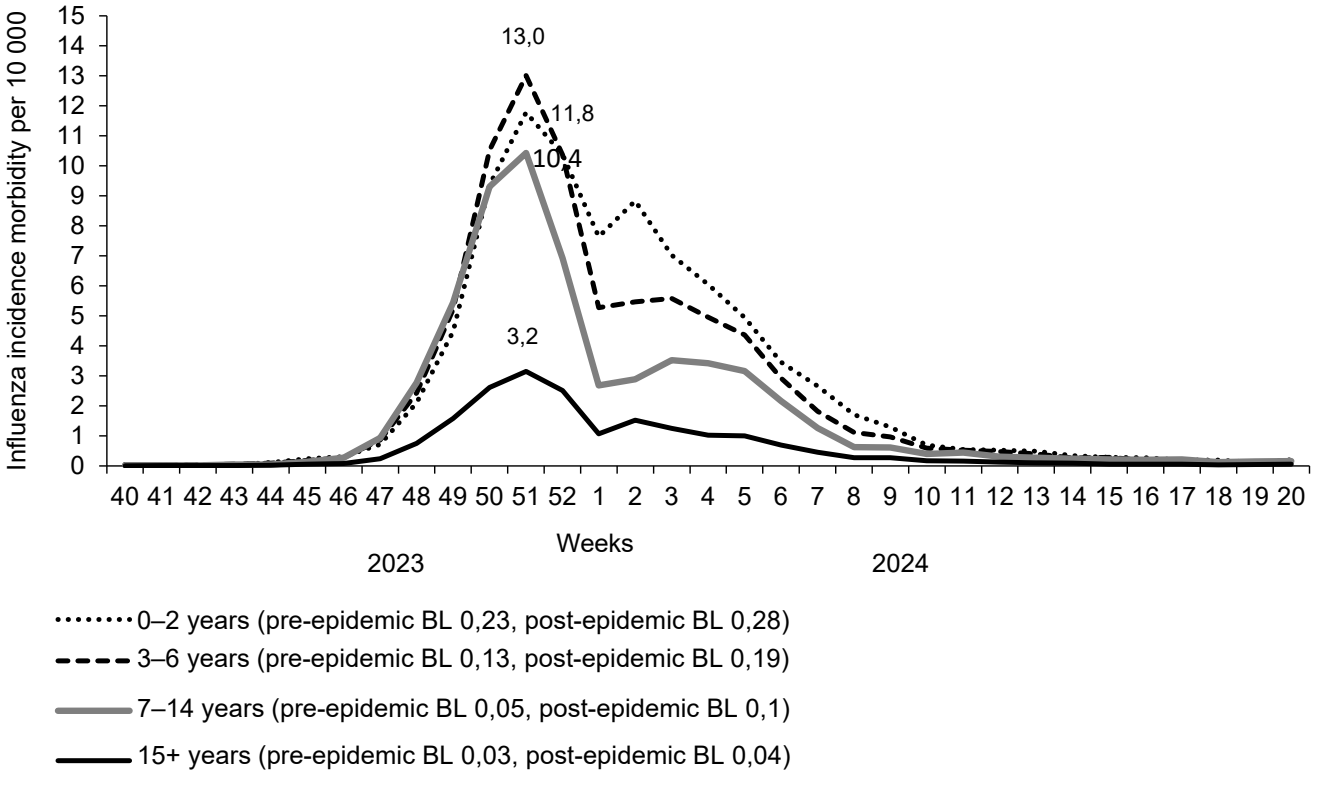

Fig. 2. Dynamics of influenza incidence in different age groups of the population of the surveyed cities (61) in the 2023–2024 season.

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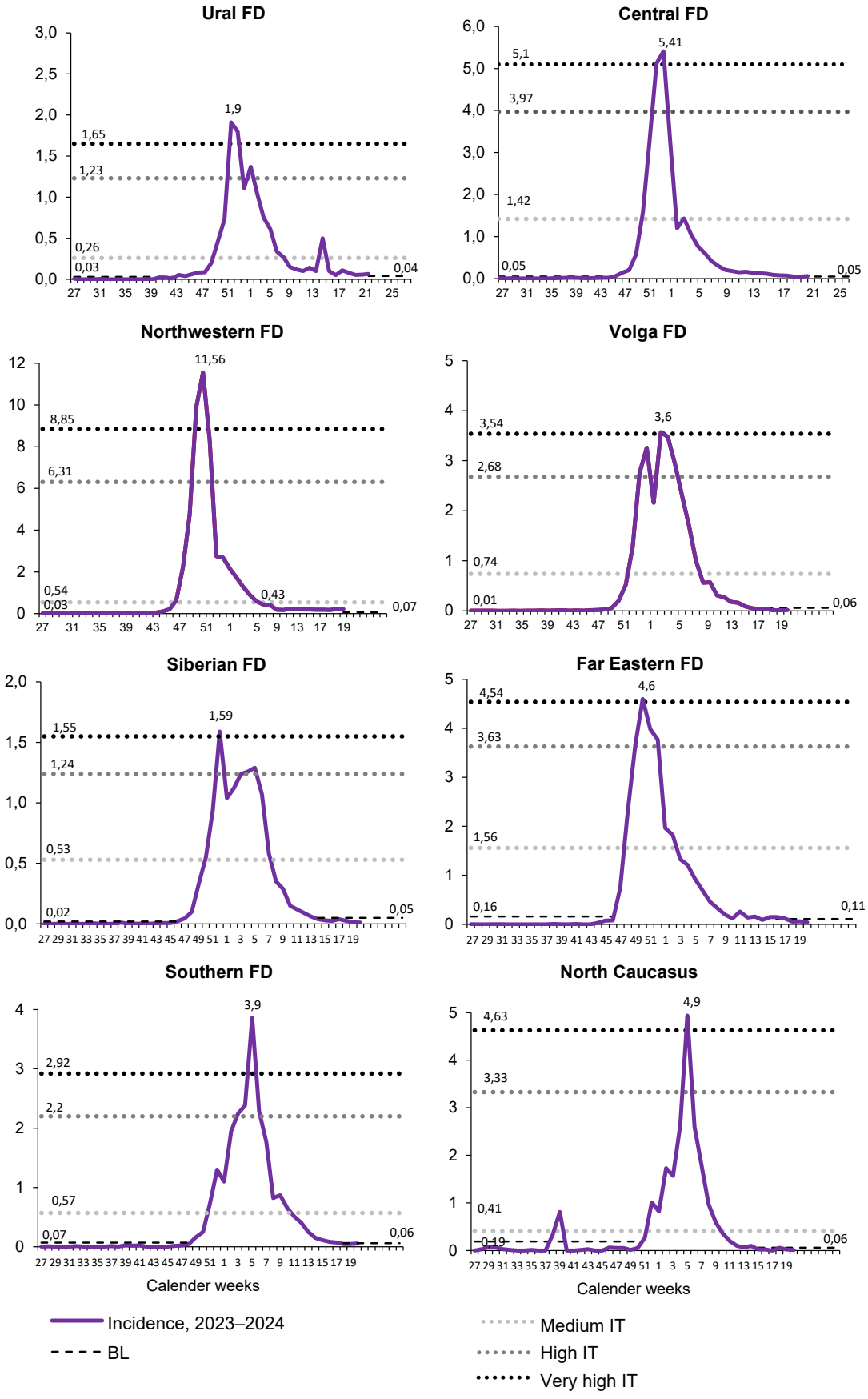

Fig. 3. Comparison of the dynamics and intensity level of influenza incidence in different Federal Districts (FD) in the 2023–2024 season.

Incidence intensity at the peak of the epidemic

The intensity of influenza incidence at the peak of the epidemic was very high, far exceeding the threshold of this level, in the Northwestern FD (11.6 vs. 8.85 per 10,000 of the population) and Southern FD (3.9 vs. 2.92 per 10,000 of the population); in the other FDs it also reached a fairly high level but only slightly exceeded this threshold (**Fig. 3**).

Hospitalization rates

An increase in the frequency of influenza hospitalizations led to the exceeding of the BL at week 45, at the same time as the onset of the upsurge. Hospitalization peaked (0.55 per 10,000 of the population) at week 51, when it exceeded the medium intensity threshold. Thereafter, hospitalization rates declined and were low intensity from week 6 and below base-

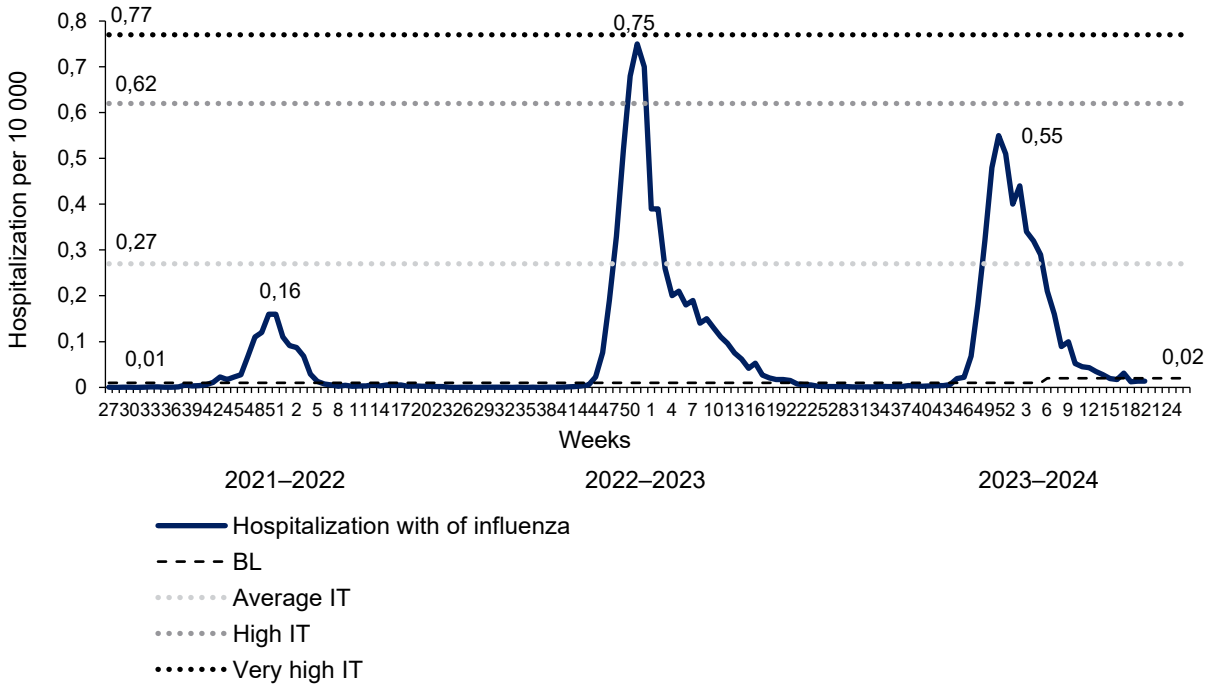

Fig. 4. Dynamics of the frequency of hospitalization of patients diagnosed with influenza in seasons 2021–2022, 2022–2023, and 2023–2024.

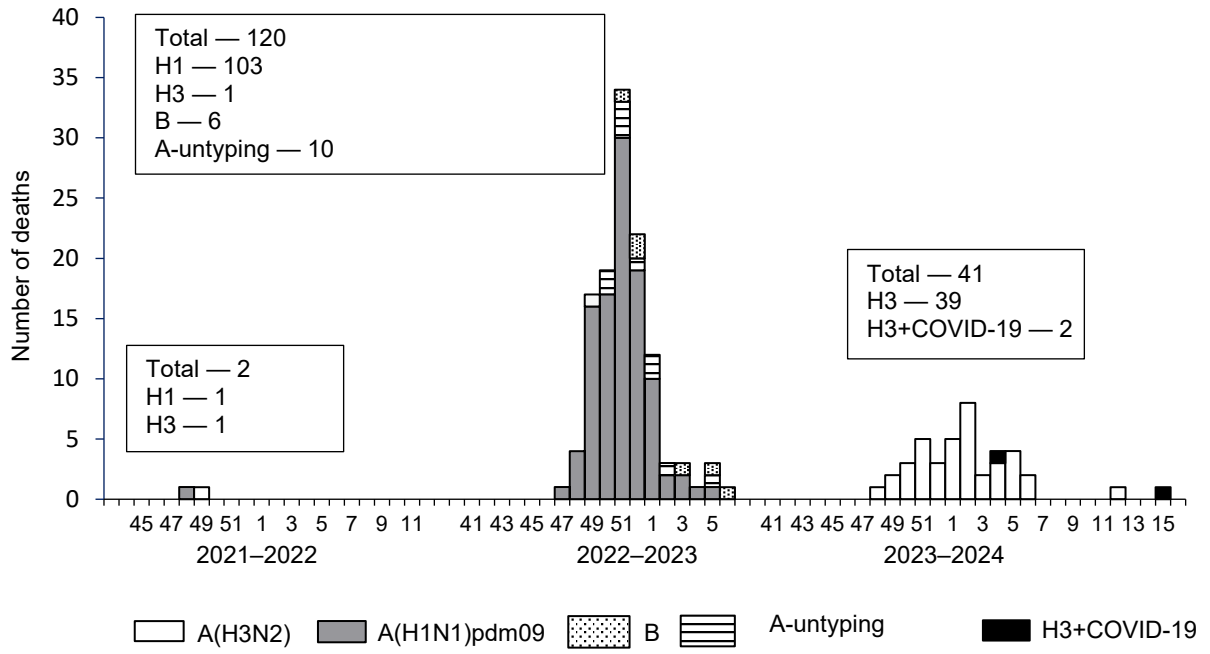

Fig. 5. The number and etiology of deaths from laboratory-confirmed influenza in the epidemic from 2021 to 2024.

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line from week 15 (**Fig. 4**). The decrease in hospitalization rates from March to May is apparently explained by the predominant circulation of influenza B virus at this time and a milder course of the disease than in influenza A(H3N2).

During the epidemic period, 0.05% of the total population was hospitalized, including 0.32% of children aged 0-2 years, 0.16% of children aged 3–6 years, 0.07% of children aged 7–14 years, and 0.02% of persons aged 15–64 years and 0.05% of persons over 65 years. The proportion of persons hospitalized during the epidemic period from the number of influenza cases averaged about 17.3%, including 35.9% among children aged 0–2 years, 19.6% among 3–6 years, 12.6% among 7–14 years, 12.7% among persons aged 15–64 years, and 26.7% among persons over 65 years old.

Influenza martality rates

In the 2023-2024 season, 41 deaths from laboratory-confirmed influenza were reported: 39 cases of influenza A(H3N2) and 2 cases of influenza A(H3N2)+-COVID-19. This season, deaths were recorded from week 47 of 2023 through week 15 of 2024 (November through April) and only from influenza A(H3N2), i.e., during its circulation period. Deaths from mixed A(H3N2) and COVID-19 infection were reported in January and April. There were no deaths from influenza B, despite its predominant circulation from March to May.

During the previous epidemic in the 2022–2023 season, when influenza A(H1N1)pdm09 was the main causative agent, 120 influenza-related deaths were laboratory-confirmed: 103 from influenza A(H1N1)pdm09, 6 from influenza B, 1 from influenza A(H3N2) and 10 from influenza A (not subtyped).

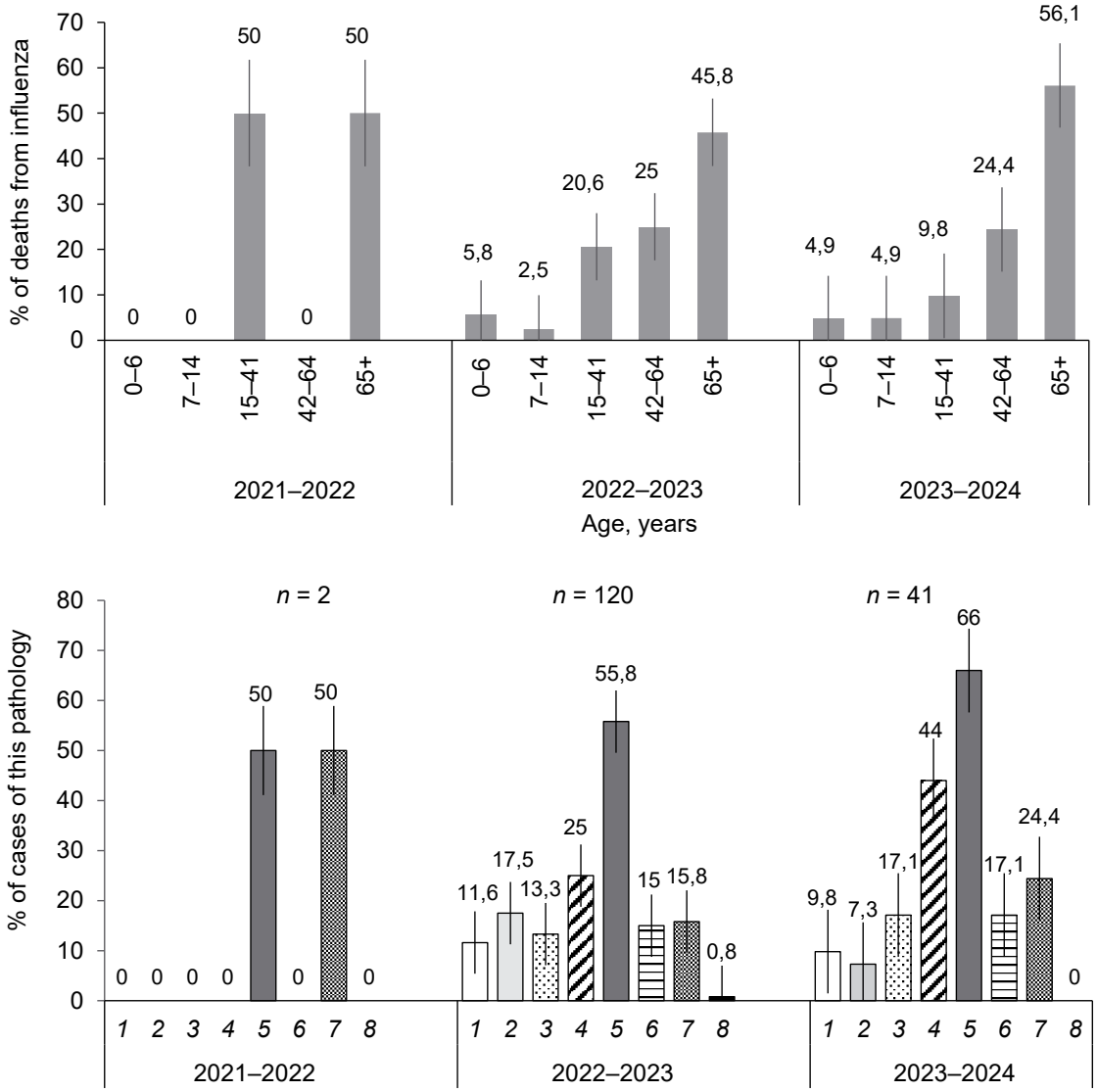

Fig. 6. Age structure and structure of background pathology of those who died from laboratory confirmed influenza during the epidemic in 2021–2024.

1 — obesity; *2* — diabetes; *3* — immunodeficiencies; *4* — diseases of internal organs; *5* — cardiovascular pathology; *6* — chronic lung diseases; *7* — diseases of the central nervous system; *8* — pregnancy.

It should be noted that in the 2021–2022 epidemic, where only influenza A(H3N2) virus was the pathogen, only 2 deaths from laboratory-confirmed influenza were reported (**Fig. 5**).

The age structure of those who died in the last season was dominated by elderly people aged 65+ years (56.1%; $p < 0.05$). The proportion of the deceased aged 42–64 years was 24.3%, the proportion of children under 6 years of age was 4.8%, and 7–14 years of age was 4.8% of the total number of the dead (**Fig. 6**). Patients with cardiovascular disease accounted for 66.0% ($p < 0.05$) and internal diseases for 44.0% ($p < 0.05$) of the deaths, which is associated with an increase in the elderly during the last epidemic. Other risk factors for fatal outcomes were such comorbidities as chronic lung diseases (17.1%) and immunodeficiency conditions (17.1%).

Comparison of influenza, ARVI and COVID-19 incidence dynamics showed alternation of influenza and COVID-19 pathogens (**Fig. 7**). In the 2021–2022 season, the Delta genetic variant wave preceded a low-intensity influenza A(H3N2) epidemic, which in turn was succeeded by a high-intensity Omicron genetic variant epidemic. In the 2022–2023 season, the epidemic caused by the daughter Omicron genetic variants was replaced by an influenza A(H1N1)pdm09 and B epidemic of medium intensity in terms of incidence. It was not until the 2023–2024 season that the COVID-19 wave and the A(H3N2) and B epidemics began simultaneously, with the COVID-19 wave being of low intensity and the influenza A(H3N2) and B epidemics being of very high intensity.

Other researchers provide data indicating possible interference of SARS-CoV-2, influenza and other ARVI pathogens with each other according to both sentinel and traditional surveillance data [4–6].

The peculiarity of the 2023–2024 influenza epidemic was an early onset (in week 45 of 2023), as in other influenza epidemics against the background of COVID-19 circulation (in 2021 — in week 45, in 2022 — in week 47). Early onset of influenza epidemics during COVID-19 circulation was also noted in Belarus; the influenza epidemic season of 2021–2022 and 2023–2024 started from week 47, and in Tashkent, the influenza epidemic season of 2023–2024 started from week 44 [7-9].

Such an early onset of influenza epidemics has been observed only in the history of influenza pandemics: influenza pandemic A/Singapore/57(H2N2) began in the 39th calendar week of 1957, pandemic

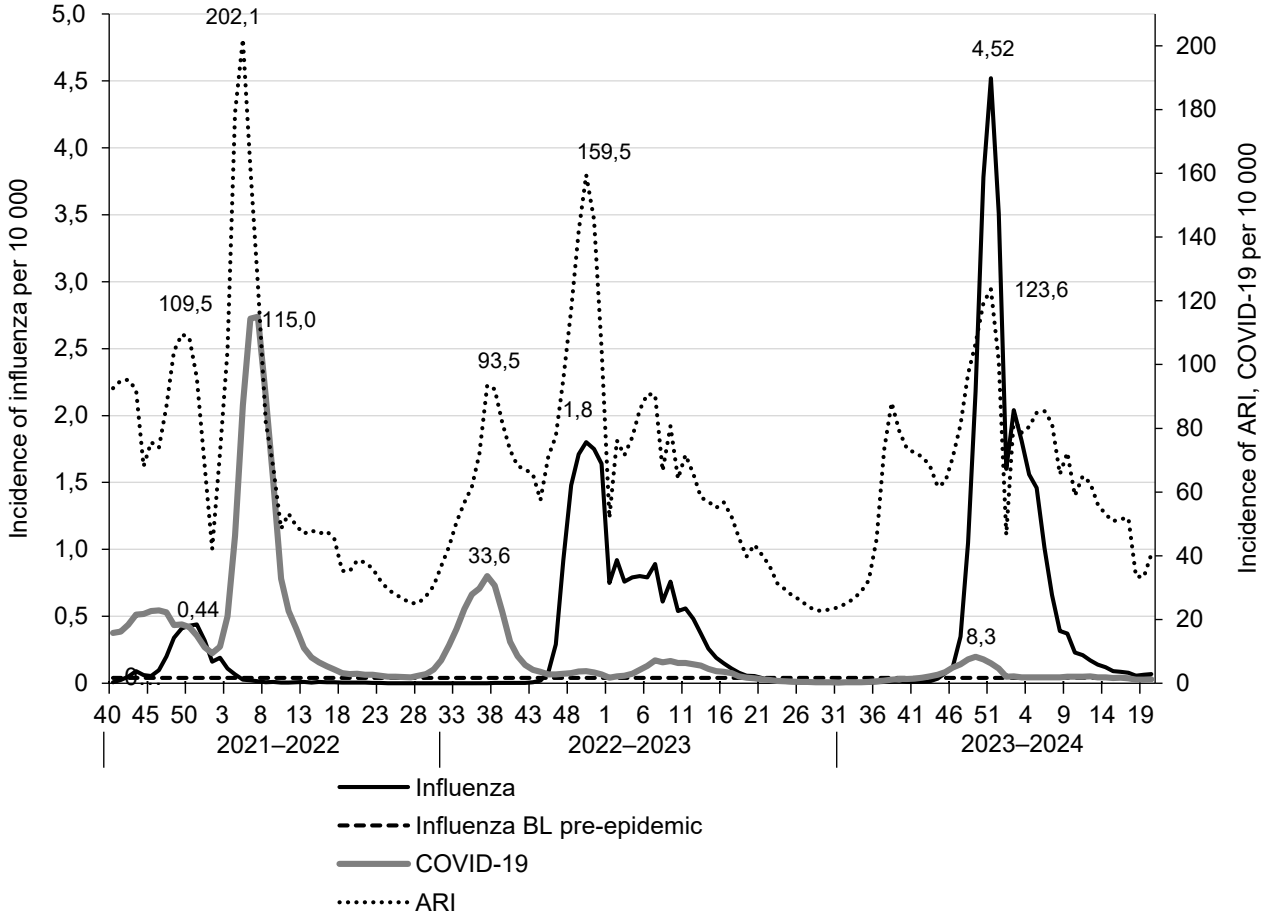

Fig. 7. Comparison of the incidence dynamics of influenza, ARVI and COVID-19 in the surveyed cities during the seasons from 2021 to 2024.

A/USSR/90/77(H1N1) began in November 1977, and pandemic A/California/09(H1N1) began in the 39th calendar week of 2009 [10–14].

It can be assumed that the early onset of influenza pandemics could be due to antigenic novelty of the pandemic influenza viruses, A/Singapore/57(H2N2), A/California/09(H1N1), to which the majority of the population was not immune, and, during the COVID-19 pandemic, due to decreased immunity to influenza in the population that had previously been infected with COVID-19.

Conclusion

Comparison of baselines calculated for 6 seasons before the COVID-19 pandemic and new BLs and PIs of epidemics in terms of incidence and PCR-confirmed influenza hospitalizations calculated for 3 epidemics against the background of the COVID-19 pandemic showed little change in baselines, while the intensity thresholds increased.

There was no influenza epidemic in the background of COVID-19 pandemic in the first season (2020–2021). In the 2021–2022 season, the A(H3N2) monoethiology epidemic was of low intensity in terms of morbidity and hospitalization rate, as well as low mortality (2 cases). In the 2022–2023 season, the influenza A(H1N1) pdm09 and B epidemic was of medium intensity in terms of incidence, with a high incidence of hospitalizations and high lethality (120 cases). The influenza A(H3N2) and B epidemic in the 2023–2024 season was of very high intensity in terms of influenza incidence, but medium in terms of hospitalization rate and mortality (41 cases). The incidence of the latter, compared to the previous epidemic, was higher (0.28 and 0.19% of the total population), including persons older than 15 years (0.19 and 0.12%). Thus, the influenza A(H1N1) pdm09 virus still remains the main cause of fatalities.

One of the reasons for the high incidence of influenza in the last epidemic may be the increase in influenza registration with increased PCR testing for influenza. The influence of interference between influenza and COVID-19 cannot be ruled out, with a decrease in COVID-19 incidence and an increase in influenza incidence in the last season.

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Original Study Article
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Multiplex real-time PCR for detection of *qac A/B* and *smr* genes in Gram-positive bacteria

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Abstract

Background. Disinfectants are effective means of non-specific prevention of infections associated with the provision of medical care. Violation of disinfectant use regimes leads to the formation of microorganism resistance to them. To monitor the spread of clinically significant microorganisms resistant to disinfectants, the development of methods for their detection, including molecular genetic methods, remains relevant.

The aim of the study was to develop a multiplex real-time PCR for the identification of *qacA/B* and *smr* genes, the determinants of resistance to cationic biocides, in Gram-positive bacteria.

Materials and methods. Conserved regions of *qacA, qacB* and *smr* genes were searched, and primers and probes were designed using BLASTN, GeneRunner, and Multiple Primer Analyzer programs. To evaluate the analytical sensitivity of the multiplex PCR, plasmids pTZ57-*qacA/B*, pTZ57-*smr*, and pTZ57-16S containing *qacA/B*, *smr* and 16S rRNA gene fragments of 197 bp, 127 bp, and 287 bp, respectively, were constructed. The method was tested on clinical isolates of Gram-positive bacteria (n = 30).

Results. A multiplex real-time PCR using TaqMan probes was developed for the detection of *qacA/B* and *smr* genes in Gram-positive bacteria. The 16S rRNA gene was used as an internal amplification control. The sensitivity of the multiplex PCR was 10^3 copies for all genes. Multiplex PCR validation showed that *qacA/B* genes were present in 30%, *smr* genes were present in 10% of the isolates tested. The reproducibility of the results was 100%.

Conclusion. The developed multiplex PCR differs from existing assays by high specificity and short turnaround time, as well as by the presence of an internal amplification control. It can be used for the detection of Gram-positive bacteria potentially resistant to cationic biocides.

Keywords: *disinfectants, resistance, qacA/B, smr, real-time PCR*

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Оригинальное исследование
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Мультиплексная ПЦР в режиме реального времени для выявления генов *qacA/B* и *smr* у грамположительных бактерий

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Аннотация

Актуальность. Дезинфицирующие вещества (ДВ) являются эффективными средствами неспецифической профилактики инфекций, связанных с оказанием медицинской помощи. Нарушение режимов применения ДВ приводит к формированию устойчивости микроорганизмов к ним. Для целей мониторинга распространения клинически значимых микроорганизмов, устойчивых к ДВ, остаётся актуальной разработка методов их выявления, в том числе молекулярно-генетических.

Целью исследования была разработка мультиплексной полимеразной цепной реакции в режиме реального времени (ПЦР-РВ) для выявления у грамположительных бактерий генов *qacA/B* и *smr* — детерминант устойчивости к ДВ из группы катионных поверхностно-активных веществ (КПАВ).

Материалы и методы. Поиск консервативных участков генов *qacA, qacB* и *smr* и разработку праймеров и зондов проводили с помощью программ BLASTN, GeneRunner и Multiple Primer Analyzer. Для оценки аналитической чувствительности мультиплексной ПЦР-РВ были сконструированы плазмиды pTZ57-*qacA/B,* pTZ57-*smr* и pTZ57-16S, содержащие фрагменты генов *qacA/B, smr* и 16S рРНК длиной 197, 127 и 287 п. н. соответственно. Апробацию метода проводили с использованием клинических изолятов грамположительных бактерий (n = 30).

Результаты. Разработана мультиплексная ПЦР-РВ с использованием зондов TaqMan для выявления генов *qacA/B* и *smr* у грамположительных бактерий. В качестве внутреннего контроля амплификации был использован ген 16S рРНК. Чувствительность мультиплексной ПЦР-РВ составила 10^3 копий для всех генов. Апробация мультиплексной ПЦР-РВ показала, что гены *qacA/B* присутствовали у 30% исследованных изолятов, *smr* — у 10%. Воспроизводимость результатов тестирования составила 100%. Специфичность разработанной мультиплексной ПЦР-РВ составила 100%.

Заключение. Разработанная мультиплексная ПЦР-РВ характеризуется высокой специфичностью и быстротой анализа, а также наличием внутреннего контроля амплификации и может быть использована для выявления грамположительных бактерий, потенциально устойчивых к ДВ из группы КПАВ, при проведении молекулярно-генетических исследований.

Ключевые слова: *дезинфектанты, устойчивость, qacA/B, smr, ПЦР в реальном времени*

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Introduction

The spread of pathogenic bacterial strains resistant to antimicrobials and disinfectants in recent decades is one of the most acute problems of modern public health care. Disinfectants are one of the most effective means of non-specific prevention of infectious diseases and play a leading role in the system of measures to prevent infections associated with the provision of medical care, as well as widely used in catering enterprises, food industry, municipal facilities, educational institutions and households. However, the phenomenon of microbial resistance to disinfectants observed since the 1950s leads to a sharp decrease in the effectiveness of disinfection measures [1], which is associated with the use of ineffective modes of their application, leading to the formation of microbial resistance to disinfectants, as well as cross-resistance to antibiotics due to the presence of common mechanisms of action [2, 3].

Cationic surfactants, which include quaternary ammonium compounds (QACs), guanidine derivatives, and tertiary amines, are the most commonly used surfactants [4, 5]. In 2023, QACs accounted for the largest

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share of disinfectants in the global market[1], and this trend is projected to continue for the next 10 years[2]. According to available data, their share in the Russian market is around 50–70% [6, 7].

The main mechanism of microbial resistance to QACs is their intracellular concentration decrease due to their elimination from the cell by efflux pumps [5, 8], which are united into 6 superfamilies based on structural similarity and peculiarities of functioning:

1) RND (Resistance-Nodulation Division);
2) SMR (Small Multidrug Resistance);
3) MATE (Multidrug And Toxic compound Extrusion);
4) MFS (Major Facilitator Superfamily);
5) ABC (ATP Binding Cassette);
6) PACE (Proteobacterial Antimicrobial Compound Efflux) [9, 10].

Resistance to QACs in Gram-positive bacteria is mainly associated with the efflux pumps QacA, QacB (MFS superfamily), as well as Smr (QacC), QacG, QacH, and QacJ belonging to the SMR superfamily [11, 12]. The prevalence of these efflux pumps among Gram-positive bacteria has been monitored in many countries using molecular genetic methods and has shown that *qacA*, *qacB* and *smr* genes are the most frequent [13–16]. At the same time, information on the distribution of these genes among Russian isolates of Gram-positive bacteria is practically absent. Only one publication presented data on the presence of *qacA* and *qacB* genes in *Staphylococcus aureus* isolates obtained from surface washes in public places in Novosibirsk [17]. Given that *qacA*, *qacB* and *smr* genes have predominantly plasmid localization, they can be widely disseminated by horizontal transfer, leading to the formation of acquired resistance to cationic surfactants in Gram-positive bacteria [12].

The aim of the study was to develop multiplex real-time PCR with fluorescently labeled probes to detect *qacA/B* and *smr* genes in Gram-positive bacteria.

Materials and methods

Bacterial isolates

The study used isolates of Gram-positive bacteria species *Staphylococcus aureus* (*n* = 12), *S. haemolyticus* (*n* = 6), *Enterococcus faecium* (*n* = 6) and *E. faecalis* (*n* = 4), *Streptococcus parasanguinis* (*n* = 1) and *S. epidermidis* (*n* = 1) obtained from medical institutions in Moscow. Isolates were obtained from washes taken from objects in the hospital environment, such as surfaces of ventilators and ultrasound machines, bedside tables, bed rails. Bacteria were cultured for 16 h at 37ºC in GRM-broth (State Research Center for Applied Biotechnology and Microbiology). The species affiliation of isolates was determined using a SMART MS 5020 mass spectrometer (Zhuhai DL Biotech Co., Ltd.).

DNA extraction

DNA was isolated from bacterial culture using ExtractDNA Blood & Cells DNA extraction kit (Evrogen) according to the manufacturer's recommendations and stored at –20ºC. The concentration of DNA samples was determined using a NanoDrop 2000C spectrophotometer (ThermoFS).

Development of primers and probes

The nucleotide sequences of the efflux pump genes *qacA*, *qacB*, *smr* and 16S rRNA of Gram-positive bacteria were taken from the GenBank database[3] and were analyzed using the BLASTN program[4]. Primers and probes were designed based on conserved gene regions using the GeneRunner v. 62.2.55 Beta[5] and Multiple Primer Analyzer[6] programs.

Multiplex real-time PCR testing

Multiplex real-time PCR was performed in a volume of 25 µl in 96-well PCR plates using the CFX96 Real-Time System thermocycler (Bio-Rad Laboratories, Inc.). The reaction mixture included 5× buffer and 2.5 U of Taq polymerase (Lytech LLC), primers (0.5 µM each), TaqMan probes (0.25 µM each; **Table**), and DNA sample (5 ng). TaqMan probes were synthesized by "DNA Synthesis LLC", primers were synthesized by Eurogen. Reaction profile: 95ºC — 2 min, 95ºC — 15 s, 56ºC — 20 s (36 cycles), 72ºC — 30 s.

Determination of analytical sensitivity of multiplex real-time PCR

To evaluate the analytical sensitivity of the method, plasmids pTZ57-*qacA/B*, pTZ57-*smr* and pTZ57-16S containing fragments of *qacA/B*, *smr* and 16S rRNA genes with lengths of 197, 127 and 287 bp, respectively, were constructed. Cloning was performed using the InsTAclone PCR Cloning Kit (ThermoFS) according to the manufacturer's recommendations. Plasmid concentrations were assessed using the Spectra Q BR kit (Sesana LLC) and the Qubit fluorimeter (ThermoFS). Sequencing of the obtained plasmids was per-

[1] Global antiseptics and disinfectants market size, share, trends & growth forecast report — segmented by type (alcohol and aldehyde, phenols and derivatives, biguanides and amides, quaternary ammonium compounds, iodine compounds and others), end user (domestic user and institutional user) and region — industry forecast from 2024 to 2029. URL: https://www.marketdataforecast.com/market-reports/antiseptics-and-disinfectants-market (date of access 23.10.2024).

[2] Global antiseptics and disinfectants market size to exceed USD 79.25 Billion by 2033 | CAGR of 10.67%. URL: https://finance.yahoo.com/news/global-antiseptics-disinfectants-market-size-160000859.html (date of access 23.10.2024).

[3] URL: https://www.ncbi.nlm.nih.gov/genbank

[4] URL: https://blast.ncbi.nlm.nih.gov/Blast.cgi

[5] URL: http://www.generunner.net

[6] URL: https://www.thermofisher.com

Nucleotide sequences of the primers and probes

Genes	Nucleotide sequence (5'–3')	Size, bp
qacA/B	qacA/B-D: 5'-CTGGCTTATACCTATTACCTA-3' qacA/B-R: 5'-TCCAACTAAAATTAATGCTAAAG -3' qacA/B-Pb: 5'-HEX- CGATTTGGACCGAAAATAGTGTTAC-BHQ1	197
smr	smr-F: AGTAAAACAATGCAACACCTAC-3' smr-R: ATACTATAGTTATTAGATTTATTTG-3' smr-Pb: 5'-FAM-TTAGTCTTAACAACCGTAGTCTCAAT-BHQ1	127
16S rRNA	16S-D: 5'-CAGCAGCCGCGGTAATAC-3' Bakt_805R: 5'-GACTACHVGGGTATCTAATCC-3' 16S-Pb: Cy5-5'-TGTAGCGGTGAAATGCG- BHQ2'	287

formed by the Sanger method. The samples of plasmids pTZ57-*qacA/B*, pTZ57-*smr* and pTZ57-16S with the concentration of 10^1-10^6 copies in 1 μl were obtained by tenfold dilutions and used as a template for real-time PCR. The results were analyzed using the CFX96 Real-Time System software (Bio-Rad Laboratories, Inc.).

Approbation and validation of the method

Multiplex real-time PCR validation was performed using DNA isolated from Gram-positive bacterial isolates (n = 30). To assess the variability of PCR threshold cycle (Cq) values, each sample was tested in 3-fold repetition and the mean Cq values, standard deviations (SD) and coefficient of variability (Cv, %) were calculated. The obtained amplicons of *qacA/B* and *smr* genes were analyzed by electrophoretic separation in 1.5% agarose gel and sequenced by the Sanger method. To validate multiplex real-time PCR, the previously described PCR methods with electrophoretic detection of *qacA/B* [18] and *smr* gene fragments [19] were used.

Results

For multiplex real-time PCR, primers and TaqMan probes complementary to highly conserved regions of *qacA*, *qacB* and *smr* genes were designed (Table), which were identified on the basis of multiple alignment of all full-length nucleotide sequences of these genes available in GenBank, EMBL[7] and DDBJ[8] databases. We analyzed 302 nucleotide sequences of the *qacA/B* genes and 220 sequences of the *smr* gene. Analysis of *qacA* and *qacA/B* genes showed that they differed by 8 nucleotides, so common primers and probe were designed for them.

In PCR testing, the use of an internal amplification control is important to exclude false negatives due to missing or an insufficient amount of DNA in the reaction mixture for detection. The 16S rRNA gene was used as an endogenous internal amplification control in the developed multiplex real-time PCR. The 16S-D primer and probe were designed based on the analysis of 5000 16S rRNA sequences (Table). The universal primer Bakt_805R, developed earlier, was used as a reverse primer [20].

Using the primers developed by us, we searched for isolates of Gram-positive bacteria (n = 30) containing *qacA/B* and *smr* genes. The amplicons obtained were analyzed by electrophoretic separation in 1.5% agarose gel. The amplicon lengths of the *qacA/B* and *smr* genes were as expected (Table; **Fig. 1**). The specificity of the primers was confirmed by sequencing the amplicons using the Sanger method.

To evaluate the analytical sensitivity of multiplex real-time PCR, plasmids pTZ57-*qacA/B*, pTZ57-*smr* and pTZ57-16S containing the obtained amplicons of *qacA/B*, *smr* and 16S rRNA genes were constructed. Using tenfold dilutions, their samples with concentrations ranging from 10^1 to 10^6 copies in 1 μL were obtained and used as a template for the developed multiplex real-time PCR. The sensitivity of multiplex real-time PCR amounted to 10^3 copies for all genes. The amplification efficiency of *qacA/B*, *smr*, and 16S rRNA genes was 95.1, 91.3 and 101.8%, respectively (**Fig. 2**).

The developed multiplex real-time PCR was tested using 30 clinical isolates of Gram-positive bacteria obtained from medical institutions of Moscow. Testing was performed in 3 repetitions. It was found that 30% of isolates (6 isolates of *S. haemolyticus* and 1 isolate each of *E. faecium*, *E. faecalis* and *S. parasanguinis*) had *qacA/B* genes, and 10% of isolates (2 isolates of *S. haemolyticus* and an isolate of *S. epidermidis*) contained the *smr* gene. Both *qacA/B* and *smr* were found in 2 *S. haemolyticus* isolates. Reproducibility of test results for all samples was 100%, and the coefficient of variability of Cq values ranged from 3.4 to 6.2%. The results of multiplex real-time PCR completely matched both the data of monoplex PCR assays with electrophoresis detection and the results of Sanger sequencing of amplicons. PCR methods with electrophoretic detection of *qacA/B* and *smr* genes, previously proposed by K.H. Lin et al. [18] and N. Noguchi et al. [19], respectively, were used for comparison with the developed multiplex real-time PCR. The results obtained using these methods were also in complete agreement. Thus, the specificity of our developed multiplex real-time PCR with probes labeled with fluorescent dyes was 100%.

[7] URL: https://www.embl.org
[8] URL: https://www.ddbj.nig.ac.jp

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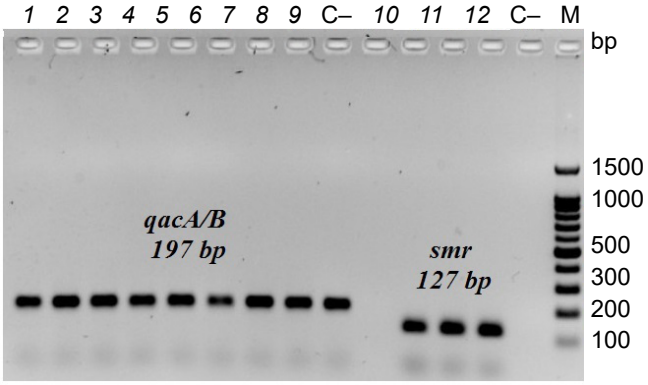

Fig. 1. Results of the amplification of *qacA/B* and *smr* genes.
1–9 — *qacA/B*-positive isolates; *10–12* — *smr*-positive isolates.
C– — negative control. M — DNA length marker.

Discussion

Detection of resistance genes by PCR methods is a widespread and available procedure for their monitoring. Analysis of literature sources has shown that several methods for detection of *qacA/B* and *smr* genes based on PCR with electrophoretic detection of results [9, 14, 21–23] and real-time PCR using intercalating DNA dyes [18, 24] have been proposed to date, but these methods have a number of disadvantages related to the duration of testing and reliability of results. The PCR method with electrophoretic detection requires gel electrophoresis, which increases the duration of the assay compared to real-time PCR and makes it laborious and inconvenient for testing a large number of clinical isolates. PCR using intercalating dyes allows real-time detection of amplicons, which reduces the duration of testing compared to the previous method, but may give false-positive results because intercalating dyes bind to all double-stranded DNA, including primer dimers and possible nonspecific PCR products, which requires careful selection of primers and observance of PCR conditions, including the reagents used [25].

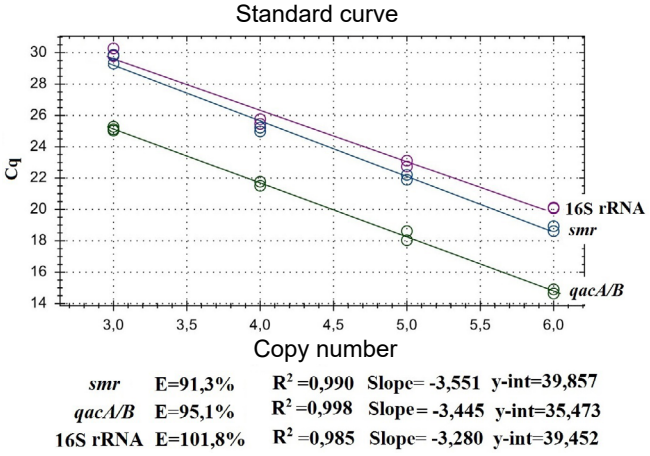

Fig. 2. Results of multiplex PCR sensitivity evaluation for detection of *qacA/B* and *smr* genes in gram-positive bacteria.

A multiplex real-time PCR method has been developed, which lacks the disadvantages of the above-mentioned methods due to the use of TaqMan probes labeled with fluorescent dyes, as well as the presence of internal amplification control.

Conclusion

A multiplex real-time PCR with probes labeled with fluorescent dyes for detection of *qacA/B* and *smr* genes of Gram-positive bacteria. It differs from the existing methods in its simplicity and rapidity of analysis, as well as by the presence of internal amplification control, which eliminates false-negative test results. The developed multiplex real-time PCR can be used to monitor the prevalence of *qacA/B* and *smr* genes to detect Gram-positive bacteria potentially resistant to cationic biocides. However, it should be taken into account that all molecular genetic assays are diagnostic tools and do not exclude the necessity to confirm the resistance phenotype by microbiological methods.

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Review
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Study of bacterial susceptibility to antibiotic and phage combinations: a literature review

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Abstract

The aim of the review is to describe existing laboratory methods for determining the sensitivity of bacteria to a combination of antibiotics and bacteriophages. However, more and more often there are scientific papers in which their combined action is described as synergism. The mechanisms of this phenomenon have not been fully studied, but it has been proven that not only virulent but also moderate phages can enter into synergy with antibiotics, allowing the minimum inhibitory concentration of the antibiotic to be reduced several times. Since synergy cannot yet be empirically predicted, microbiological laboratories use various *in vitro* methods, most of which are labor-intensive. The development of a new technique that can be introduced into the daily practice of microbiological laboratories is relevant.

Keywords: *resistance, susceptibility, antibiotic, bacteriophage, synergy*

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Определение чувствительности бактерий к комбинации антибиотиков и фагов: обзор литературы

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Аннотация

Цель обзора — дать описание существующих лабораторных методов для определения чувствительности бактерий к комбинации антибиотиков и бактериофагов.

Бактериофаги до сих пор рассматриваются некоторыми исследователями как альтернатива антибиотикам. Но всё чаще встречаются научные работы, в которых их совместное действие описывается в виде синергизма. Механизмы этого явления до конца не изучены, однако доказано, что в синергию с антибиотиками могут вступать не только вирулентные, но и умеренные фаги, позволяя снизить минимальную подавляющую концентрацию антибиотика в несколько раз. Поскольку синергию эмпирически пока предсказать невозможно, в микробиологических лабораториях используют различные методы *in vitro,* большинство из которых являются трудоёмкими. Актуальна разработка новой методики, которая может быть внедрена в ежедневную практику микробиологических лабораторий.

Ключевые слова: *резистентность, чувствительность, антибиотик, бактериофаг, синергия*

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

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

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Introduction

In recent years, the problem of resistance of microorganisms to antibiotics used in medicine has become increasingly urgent, and the widespread emergence of pathogens resistant to them is of concern to clinicians all over the world. Among etiologically significant bacteria, the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) is distinguished, which is characterized by a variety of antimicrobial resistance mechanisms. In May 2024, the World Health Organization published an updated list of antibiotic-resistant bacterial pathogens posing the greatest threat to human health. Depending on the necessity for the development of new antimicrobial drugs and new treatment options, microorganisms are categorized into priority groups. *A. baumannii* resistant to carbapenems and microorganisms belonging to the order *Enterobacterales*, including producers of extended-spectrum beta-lactamases, are classified as critically high-priority. High-priority pathogens include *Salmonella* spp. and *Shigella* spp. resistant to fluoroquinolones, *E. faecium* resistant to vancomycin, *P. aeruginosa* resistant to carbapenems, *Neisseria gonorrhoeae* resistant to third-generation cephalosporins and/or fluoroquinolones, and methicillin-resistant *S. aureus*. The medium priority level includes *Streptococcus* group A and *S. pneumoniae* resistant to macrolides, *Haemophilus influenzae* resistant to ampicillin, *Streptococcus* group B resistant to penicillin[1]. In Russia during the year 2017, the Strategy for the prevention and spread of resistance for the period up to 2030 was introduced, which provides for the introduction of modern methods to study the mechanisms of its formation, monitoring of its spread and ways of containment. Special importance and attention is given to ESKAPE pathogens in "Sanitary and Epidemiological Requirements 3.3686-21" as the main pathogens of infections associated with the provision of medical care[2].

Given the growing resistance of bacteria to chemical medicines, there is a necessity to introduce alternative approaches to the treatment of diseases caused by them. Instead of antibiotics, different authors suggest using probiotics, microbial enzymes, bacteriocins, bacteriophages and their lysins, synthetic phages, vaccines, serums and other biologics [1–6].

The most promising in this list are phages – bacterial viruses, because they do not have a toxic effect on the cells of the macroorganism and do not suppress immunity, so there are practically no contraindications for their prescription. At the same time, they have a narrowly targeted effect and do not cause negative changes in the composition of the human microbiota. Unlike other antimicrobial drugs, bacteriophages are able to overcome the bacterial immunity developed to them using several strategies. Compared to β-lactam antibiotics, which cause microbial cell death within 3 h, bacterial lysis by phages can occur in less than 10 min. However, unlike antibiotics, the action of bacteriophages does not lead to cumulative accumulation of endotoxin when destroying Gram-negative bacteria [7].

The only Russian manufacturer of medicinal bacteriophages is Microgen, which produces more than 14 unique drugs. Today, the market offers bacteriophages active against not only ESKAPE pathogens, but also against pathogens of diarrheal diseases — shigellosis, salmonellosis, escherichiosis. Medicines based on phages are produced either as combinations drugs — against several genera of bacteria, or as monotherapeutics specific against a particular type of pathogen. It should be noted that in Russia the use of bacteriophages is enshrined in regulatory documents, while most countries in Europe and Asia, Australia and the USA have only recently started to develop documents regulating the use of phages [8, 9].

Most studies have shown high efficacy and safety of tested phages, including those against priority bacterial pathogens [10]. Phage therapy without antibiotics has led to success against vancomycin-resistant enterococci, methicillin-resistant staphylococci (MRSA and MRSE) [11]. In rare cases, antagonism phenomena have been described when antibiotic and bacteriophage are administered together [10]. Therefore, before their administration, the sensitivity of a particular

[1] List of priority bacterial pathogens. URL: https://www.who.int/ru/news/item/17-05-2024-who-updates-list-of-drug-resistant-bacteria-most-threatening-to-human-health (date of access: 05.08.2024).

[2] Resolution of the Chief State Sanitary Doctor of the Russian Federation dated 28.01.2021 No. 4 "On Approval of Sanitary Rules and Norms 3.3686-21 'Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases'".

strain to antimicrobial agents should be determined. In Russia, the determination of bacterial sensitivity to bacteriophages is regulated by methodological recommendations for the rational use of bacteriophages[3], while sensitivity to antibiotics is regulated by clinical guidelines[4]. This raises the urgent question of determining the sensitivity of bacteria to the combination of antibiotics and phages in microbiological laboratories.

The aim of the review is to describe the existing laboratory methods for the combined determination of bacterial sensitivity to antibiotics and bacteriophages.

The combined effect of phage and antibiotics was first described by Neter and Clark in 1944 using *S. aureus* and penicillin as an example. In 2004, there were results of experiments on a chicken model devoted to studying the interaction of phage and enrofloxacin against *Escherichia coli* by Huff et al., and a few years later, A.M. Comeau and his research group conducted *in vitro* testing and noticed that subinhibitory concentrations of certain antibiotics can affect the production of virulent phages infecting *E. coli*. The authors named this phenomenon Phage-Antibiotic Synergy (PAS). For a long time, the mechanism of synergy remained unknown, until electron microscopy was used to study bacterial cultures treated with antibiotics and phages. It was discovered that chemical medicines which disrupt peptidoglycan synthesis lead to elongation of bacterial cells, which promotes phage replication and possibly its active attachment to the bacterium due to an increase in the cell wall surface area [12–14].

The PAS phenomenon has been extensively studied in many laboratories, resulting in evidence of synergism for various combinations of phages with antibiotics of different pharmacological groups. However, the methods used to evaluate these interactions are still not unified, so the approaches of various researchers have significant differences. The simplest way out of the situation is to borrow the method used to study the interaction of different classes of antibiotics, since combination antimicrobial therapy is administered to patients with bacteremia, pneumonia, surgical infection, and patients with septic shock in intensive care units. To date, 4 methods have been described by which synergy of chemical medicines can be assessed *in vitro*: the checkerboard method; combined testing of the bactericidal effect of several antimicrobial agents; E-test; analysis of the bacterial death graph depending on the time of antibiotic action, also known as time-kill assays [15]. Among the available methods of synergism determination, time-kill assays are the gold standard [16, 17], which was first used to confirm the synergism of phage and antibiotics[5]. Interactions detected *in vitro* are calculated and interpreted as synergistic, additive, indifferent or antagonistic depending on whether the antibacterial activity of the drugs in combination is greater, equivalent or less than the activity of the drugs used separately.

Broth microdilutions

In this method, 96-well plates are used in which wells are co-cultured with a broth suspension of bacteria, antibiotic and phage. The phage activity and the minimum inhibitory concentration (MIC) of the antibiotic are studied beforehand, since their sub-inhibitory concentrations are used for synergy studies. The result is evaluated by measuring growth kinetics by optical density (OD) using a spectrophotometer or by bacterial metabolism after staining with tetrazolium, which changes color in response to cellular respiration. Evaluation of the result with a real-time instrument allows to determine the time taken for partial inhibition, to detect late lysis and resumption of bacterial growth. However, it is impossible to infer bacterial viability from the OD alone and to distinguish dead (not yet destroyed bacteria) from live bacteria. Additional staining eliminates the error and allows detection of only metabolically active (live) bacteria. On the one hand, this method makes it possible not only to test any combinations of antibiotics and bacteriophages, but also to change their concentrations. On the other hand, it should be taken into account that the use of a single concentration of antibiotic (half of the previously known MIC) and phage (below the lysing concentration according to Appelman) does not always allow us to draw a conclusion about their interaction and reveal a pattern. At the same time, using a more labor-intensive method, combining several concentrations of antibiotic and phage, it is possible to find those combinations of two antimicrobial agents in which their synergy will be observed [18, 19]. Some researchers have achieved the PAS phenomenon even when the antibiotic was diluted 4, 10 and 100 times the MIC, and 100 and 1000 times the initial concentration of the phage [20].

In some cases, to study synergy, it is possible to use a bacteriophage lysing a bacterial strain of at least 3+, with the antibiotic taken in two concentrations: the MIC and half of the MIC. In case of resistance to the bacterial phage, the antibiotic is added in the maximum permissible concentration [19].

With the use of automated systems, this method allows the construction of sinograms in real time, studying the concentrations of antibiotics and bacteriophage titer. The instrument reads the absorbance value

[3] Rational use of bacteriophages in therapeutic and anti-epidemic practice: Methodological recommendations. Moscow;2022.

[4] Russian recommendations "Determination of sensitivity of microorganisms to antimicrobial agents". Smolensk;2024.

[5] International Organisation for Standardization. Susceptibility testing of infectious agents and evaluation of performance of antimicrobial testing devices. 2019;Part 1.
URL: https://iso.org/standard/70464.html

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from each well as a separate parameter and converts the data into a heat map representing the percentage of bacterial reduction. As a rule, sinograms can be divided into three parts: the area of antibiotic action, the area of bacteriophage action and the area of their joint action, by which it is possible to assess the effect of their interaction (PAS). The use of this method allows visualizing the effectiveness of the combination and selecting the optimal concentration of antibiotic and phage. An additional advantage of this method is the ability to simulate what is happening in the human body when adding biological fluids to the wells [21].

To simplify this technique, I. Nikolic et al. proposed the checkerboard method, which is used to study the interaction of 2 chemical medicines [22]. For more reliable results, the method is implemented in an automated version. The choice of dilution depends on the lytic activity of the phage and the MIC of the antibiotic, so these parameters should be determined in advance before the test. Antibiotic dilutions are added to the wells of a sterile flat-bottom plate from left to right to create a twofold serial decreasing concentration gradient in the range of 8-0.125 of the MIC. A two-fold serial decreasing gradient of phage concentration in the same range is created in the wells from top to bottom, after which a suspension of the test microorganism is added to the plate. The inhibitory concentrations of the antibiotic and phage allow the calculation of the fractional inhibitory concentration index (FIC) using the following formula:

$$\Sigma FIC = \frac{MICac}{MICa} + \frac{MICbc}{MICb},$$

where MICac — MIC of antibiotic combined with bacteriophage, μg/mL; MICa — MIC of antibiotic, μg/mL; MICbc — MIC of bacteriophage combined with antibiotic, MICb — MIC of bacteriophage, μg/mL.

The following results indicated that:

- FIC < 0.5 — synergy (combination of compounds increases the inhibitory activity of one or both compounds);
- FIC = 0.5–4.0 — no interaction (the combination has no increase in MIC due to the additive effect of both compounds);
- FIC > 4 — antagonism (combination of compounds increases MIC) [22, 23].

Broth microdilutions, although considered to be more reliable tests, are more complicated than the use of a solid medium. They require working with large volumes under aseptic conditions, preliminary determination of the MIC and lytic activity of the bacteriophage, and special equipment for continuous bacterial counts at short intervals throughout the day. In the absence of a spectrophotometer, OD measurement can be replaced by quantitative seeding from wells after a day of incubation, which makes this method less accurate and increases labor costs and the turnaround time by at least a day [20, 24]. The described approaches are not standardized in the Russian Federation, and they require a lot of time for staging, which has a limitation for determining the effects of PAS – joint administration of antibiotic and bacteriophage in practical laboratory conditions.

Use of nutrient dense media

Double-layer agar method

The effect of PAS against uropathogenic *E. coli* strain (UPEC) on a dense nutrient medium was first described by A.M. Comeau et al. [25]. They noticed that phage plaques were significantly larger around some antibiotic disks overlaid on the medium seeded in depth with the tested uropathogenic *E. coli* strain and bacteriophage. The authors hypothesized that a sublethal dose of β-lactam antibiotics stimulates phage activity. The results were further confirmed by adding antibiotics at different concentrations to a mixture of *E. coli* and phage, which were all poured together into semi-liquid agar: phage formed small plaques without cefotaxime and large plaques in the presence of the antibiotic at a concentration of 50 ng/mL. When the concentration of antibiotic was further increased, it completely inhibited the growth of the bacterium and the result of phage action could not be studied due to continuous lysis.

The simplicity of the described methodology allowed other researchers to conduct similar experiments using different bacterial strains, phage and antibiotic drugs, combining phages with bacteria or bacteria with an antibiotic in agar, and placing antibiotic disks or bacteriophage drops, respectively, on the surface of the solidified layer [26–28].

E-test

The gradient diffusion method can be used to determine synergy. There are two modifications of this technique. In the first variant, two strips impregnated with antibacterial drugs are placed perpendicularly to each other on a Petri dish seeded with the test culture, intersecting at the MIC level for each antibiotic. Much like with the checkerboard method, the interpretation of the synergy of the E-test is based on the calculation of the FIC index. In the second variant of the test, a strip with the antibiotic is placed on a lawn culture in a Petri dish, after one hour the strip is removed and replaced with a phage-impregnated strip. As a control, a second dish is used in which the antibiotic and bacteriophage strips are overlaid and not in contact with each other. Synergy is defined as a decrease in MIC by at least three 10-fold dilutions, indifference — as a decrease in MIC by at least two 10-fold dilutions, antagonism — as an increase in MIC by three or more 10-fold dilutions [15].

Disk-diffusion method

In this variant, a bacterial culture (0.5 McF) with bacteriophage (10^8 PFU/mL) is incubated for a day be-

fore the classical disk-diffusion method, after which a daily culture on a dense medium is obtained. The daily culture without pre-incubation with phage is used as control. Determination of antibiotic and phage synergy by this method is difficult because the diameter of growth retardation around the disk with antibiotic changes insignificantly [29]. The disadvantages of the method also include the double consumption of standard disks due to the use of controls.

Conclusion

The analysis of available sources shows that there are currently no available and reproducible methods to determine the interaction between bacteriophages and antibiotics in routine laboratory practice. When comparing known methods, it is not possible to obtain their 100% correlation; the coincidence varies from 44 to 88% when comparing time-kill assays with the checkerboard method, from 63 to 75% – when comparing time-kill assays with the E-test and about 90% – when comparing the E-test with the checkerboard method. Most of the studies propose an author's method without comparison with the existing ones, and use only one species and strain of microorganism as a test strain. At the same time, phage and antibiotic interactions depend not only on the selected drugs, but also on the test strain within the same species. Studies have shown that even predictions derived from artificial intelligence and machine learning require double-checking in the laboratory before treatment [15]. And although putative mechanisms of synergistic action of phages with antibiotics that either do or do not induce SOS repair have been described6, to answer the question whether phages can be combined with antibiotics to treat an infection caused by a particular strain, *in vitro* testing must be performed each time. To determine the sensitivity of bacteria to the combination of antibiotics and phages, all virulent bacteriophages should be included in the study, even if the bacteria are initially insensitive to them, since the restoration of strain sensitivity to phages in the presence of antibiotics and the manifestation of synergy of 2 drugs have been described. One of the new areas of research is the study of mechanisms of joint action of antibiotics and moderate phages, which have always been considered as an insurmountable obstacle to therapy. Synergy has already been described in 7 antibiotic groups with moderate bacteriophage [30].

One of the key objectives of the microbiology laboratory is to provide reliable information on the use of antimicrobial agents, including their combinations, for the treatment of infectious diseases. The methods by which a laboratory assesses sensitivity to antibiotics and bacteriophages individually are highly standardized and reproducible. It is this reproducibility that allows laboratories to obtain comparable results. Given that it is impossible to predict empirically the interaction between antibiotic and phage, and the combination of bacteriophages and antibiotics can cause both positive and negative shifts in chemopreventive MIC changes, it is necessary to develop the simplest possible methodology with a clear protocol and accessible equipment that can be implemented in any microbiology laboratory.

[6] A bacterial defense system that is activated in response to DNA damage or inhibition of replication and triggers a complex chain of defense reactions. SOS (save our souls) is an international distress signal in radiotelegraphic communication using Morse code.

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ЮБИЛЕИ

90 лет Ростовскому-на-Дону противочумному институту: история, достижения и перспективы

В 2024 г. Ростовский-на-Дону противочумный институт отмечает 90-летие. Основной задачей с момента основания института и до настоящего времени является борьба с особо опасными и природно-очаговыми инфекционными болезнями, разработка методов диагностики и профилактики, а также обеспечение биологической безопасности и противодействие биотерроризму. Претерпев ряд реорганизаций и сменив несколько названий, институт остается одним из ведущих многофункциональных научно-практических учреждений в стране, деятельность которого заключается не только в проведении научно-исследовательских работ, но и в организации эффективной работы референс-центров по мониторингу холеры и туляремии, центров индикации и секвенирования, ПЦР-центра и испытательного лабораторного центра, специализированных противоэпидемических бригад (СПЭБ).

История института начинается с 1934 г., когда в результате реорганизации Ростовского городского бактериологического института и Ростовской краевой противочумной станции был образован «Азово-Черноморский край. Институт противочумный, г. Ростов-на-Дону», переименованный в 1937 г. в «Ростовский-на-Дону государственный научно-исследовательский противочумный институт Юга РСФСР». Директором института был назначен Михаил Исаевич Любашевский, который организовал планомерную работу по формированию кадрового состава, обеспечению его научной и практической деятельности. В годы становления институт состоял из 8 структурных подразделений, в которых работали 24 научных сотрудника и 100 человек технического персонала, в непосредственном подчинении института было 9 противочумных пунктов.

С 1934 по 1940 г. специалистами института было проведено эпизоотологическое обследование природных очагов опасных инфекций Северо-Западного Прикаспия; разработаны и осуществлены широкомасштабные мероприятия по ликвидации эпизоотий чумы и туляремии на территории Азово-Черноморского края и Северного Кавказа; выполнены оригинальные исследования по эпизоотологии, эпидемиологии, патогенезу и иммуногенезу чумы и туляремии. В предвоенные годы в институте работали крупные учёные: Николай Николаевич Жуков-Вережников, Георгий Павлович Руднев, Петр Никитич Ступницкий, Иосиф Самсонович Тинкер. Их вклад в изучение чумы поистине неоценим.

С 1939 по 1964 г. институт возглавлял лауреат Сталинской премии, к. м. н. Александр Кондратьевич Шишкин, несомненной заслугой которого является организация работы института во время Великой Отечественной войны и в послевоенный период. В эти годы количество подразделений увеличилось до 13, в подчинении института находилось уже 30 противочумных лабораторий, отделений и станций. В 1941 г. А.К. Шишкин в труднейших условиях военного времени, сохранив все имущество и основные кадры, провёл эвакуацию института в с. За-

Ростовский-на-Дону государственный научно-исследовательский противочумный институт Юга РСФСР в 1930-е годы.

ветное, города Элисту, Астрахань и Гурьев (сначала в Калмыцкую АССР, а затем в Казахскую ССР). Лишь в апреле 1943 г. коллектив возвратился из эвакуации в освобождённый Ростов-на-Дону, немедленно приступив к восстановлению института и решению основной задачи — окончательной ликвидации эпизоотии чумы в очаге Северо-Западного Прикаспия. За успешное выполнение этой задачи 9 сотрудников института были удостоены Сталинской премии. Масштабную работу проводили по борьбе не только с чумой, но и с туляремией, бруцеллезом, брюшным тифом, дизентерией и холерой. Именно в военные годы (1943 г.) в институте был организован отдел холеры под руководством д. м. н., профессора Марии Семеновны Дрожевкиной, курировала работу отдела д. м. н., профессор, академик Зинаида Виссарионовна Ермольева. За образцовое выполнение заданий военного командования и Наркомздрава СССР сотрудники института были награждены орденами и медалями Советского Союза (в том числе боевыми). В трудные послевоенные годы на старых машинах, а зачастую даже пешком, специалисты института продолжали исследование северо-западного прикаспийского природного очага чумы. Нельзя не отметить вклад в эту работу д. б. н., профессора Николая Прокофьевича Миронова, который, несмотря на то что потерял левую руку в боях, не только принимал активное участие в этих мероприятиях, но и предложил схему конкретных действий по окончательной ликвидации энзоотии чумы в указанном очаге, создал лабораторию биологических методов борьбы с переносчиками и носителями возбудителей особо опасных инфекций.

Внимание исследователей в те годы было сосредоточено и на совершенствовании методов серологической диагностики инфекций. На базе института д. м. н., профессор Моисей Иосифович Леви разработал и внедрил в практику антигенные и иммуноглобулиновые эритроцитарные диагностикумы, которые используются и в настоящее время.

Мировым достижением является создание и внедрение в практику живой туляремийной вакцины под руководством Бориса Яковлевича Эльберта и Иосифа Самсоновича Тинкера на основе штамма Николая Акимовича Гайского с использованием жидкой желточной среды, разработанной Марией Семеновной Дрожевкиной. Проведённые исследования и введение массовой вакцинации населения существенно снизили заболеваемость туляремией в стране. Советское правительство высоко оценило эту работу и в 1946 г. удостоило звания лауреатов Сталинской премии Б.Я. Эльберта и Н.А. Гайского.

В 1963 г. в институте была создана лаборатория профилактики и лечения чумы, которую более 30 лет возглавляла д. м. н., профессор Любовь Николаевна Макаровская. Под её руководством Нина Кузьминична Завьялова защитила кандидатскую

Памятник З.В. Ермольевой в Ростове-на-Дону.

диссертацию «Опыт лечения чумы», пойдя осознанно на заражение этой болезнью с целью испытания на себе эффективности метода лечения.

Подполковник медицинской службы запаса д. м. н., профессор Григорий Моисеевич Мединский, который имел опыт организации противобактериологической защиты населения и разработки оперативных документов, предложил идею создания СПЭБ (Приказ Минздрава СССР от 30.09.1963 № 466 о формировании СПЭБ на базе противочумных учреждений), занимался проблемами санитарной охраны территорий от завоза и распространения карантинных инфекций. Сотрудники института в составе СПЭБ принимали участие в проведении комплекса противохолерных мероприятий в 1960-е гг. в республиках Средней Азии (Узбекистан, 1965), в локализации и ликвидации вспышек холеры в Астрахани (1970), Одессе (1970), Керчи (1970), Донецке (1971), Вилково Одесской области (1991), Республике Дагестан (1994); в работе по ликвидации последствий землетрясения в Армении (1988); в Чечне в условиях военного конфликта (1995) и др.

Под руководством д. м. н., д. б. н., академика РАМН и РАЕН Игоря Валериановича Домарадского, директора Ростовского-на-Дону противочумного института с 1964 г. по 1973 г., проводились фундаментальные исследования в области биохимии, генетики, молекулярной биологии возбудителей чумы и других особо опасных инфекций; пато- и иммуногенеза, лечения и профилактики инфекционных заболеваний, вызванных микроорганизмами I–II групп патогенности.

В 1970 г. на юге СССР возникла эпидемия холеры, в борьбе с которой сотрудники приняли активное участие. За практические успехи и научные достижения институт в 1971 г. получил статус головного по проблеме «Холера», сохраняющийся до

сих пор. Результаты научных исследований лаборатории микробиологии холеры под руководством эксперта Всемирной организации здравоохранения при ООН (1963–1973 гг.), к. м. н. Ранта Михайловича Саямова легли в основу признания роли вибрионов El Tor в этиологии холеры. Заведующий лабораторией патофизиологии к. м. н. Владимир Павлович Авроров с коллегами разработал, апробировал и внедрил в практику комплексный метод лечения диарейных заболеваний у людей, создав отечественный препарат для оральной регидратационной терапии холеры «Глюкосолан».

С 1973 г. под руководством заслуженного деятеля науки РФ, д. м. н., профессора Виктора Николаевича Милютина получило развитие новое направление по созданию сухих питательных сред для культивирования и диагностики возбудителей особо опасных инфекций и некоторых других патогенных микроорганизмов. Также в эти годы в Ростовском-на-Дону противочумном институте разработаны основы районирования территории страны по степени опасности заноса и распространения холеры. В 1970–1980 гг. д. м. н., профессор, заслуженный деятель науки РФ Борис Николаевич Мишанькин сформировал направление по изучению биохимии холерных вибрионов: были изучены и охарактеризованы многие ферменты, определены их роль и участие в патогенезе холеры; получены важные приоритетные как теоретические, так и практические данные о биохимическом потенциале холерных вибрионов. В 1982 г. создана лаборатория холерных диагностических фагов, которую более 30 лет возглавляла д. м. н. Татьяна Александровна Кудрякова. На основе совокупности биологических и морфологических признаков, качественной характеристики бактериофагов возбудителей особо опасных инфекций получены доказательства биологического разнообразия этой группы бактериальных вирусов, имеющих не только теоретическое, но и важное практическое значение. Наличие обширной коллекции фагов позволило разработать схему фаготипирования холерных вибрионов, признанную Всемирной организацией здравоохранения и используемую на практике.

За заслуги в организации борьбы с особо опасными инфекционными болезнями в 1984 г. Указом Президиума Верховного Совета СССР Ростовский-на-Дону противочумный институт был награждён орденом Трудового Красного Знамени.

С 1986 по 1988 г. институт возглавлял д. м. н., профессор Александр Сергеевич Новохатский, который способствовал внедрению в работу новых технологий и методов исследований: гибридомной технологии получения моноклональных антител, полимеразной цепной реакции, иммуноферментного анализ и др.

Успешно реализовал потенциал института как головного методического центра по холере заслуженный деятель науки РФ, д. м. н., профессор Юрий Михайлович Ломов (директор с 1988 по 2010 г.). В этот период интенсифицировались исследования, посвящённые экологии холерных вибрионов и их сохраняемости в окружающей среде. С 2008 г. институт является Референс-центром по мониторингу холеры на территории Российской Федерации, осуществляя мониторинг заболеваемости, формируя прогнозы развития эпидемиологической ситуации, изучая молекулярно-биологические свойства штаммов холерных и других патогенных для человека вибрионов, в том числе с атипичными свойствами.

В XXI в. институт возглавляли к. м. н Алексей Борисович Мазрухо (директор с 2011 по 2013 г.),

Ростовский-на-Дону противочумный институт в наши дни.

к. м. н. Светлана Викторовна Титова (директор с 2013 по 2019 г.) и к. м. н. Алексей Кимович Носков (директор с 2019 по 2024 г.), который для эффективного решения научных и практических задач, учитывая вызовы современности, оптимизировал структуру института. Алексей Кимович возглавлял исследования по совершенствованию тактики и разработки алгоритмов обеспечения санитарно-эпидемиологического благополучия на территории субъекта Российской Федерации, в том числе в условиях экстраординарного события на территории сопредельного государства и в условиях пандемического распространения болезней; активно внедрял в работу института высокопроизводительное секвенирование и переход к геномному эпидемиологическому надзору. Благодаря взаимодействию с учреждениями Роспотребнадзора в Ростовской области расширился спектр изучаемых традиционных инфекций за счёт болезней краевой инфекционной патологии — Крымской геморрагической лихорадки, клещевого боррелиоза, лихорадки Западного Нила и др. В рамках осуществления мероприятий по укреплению международной сети по противодействию чрезвычайным ситуациям санитарно-эпидемиологического характера 7 декабря 2021 г. прошла передача мобильного комплекса СПЭБ ФКУЗ «Ростовский-на-Дону противочумный институт» Роспотребнадзора Министерству здравоохранения и социального развития Киргизской Республики. Под руководством Алексея Кимовича специалисты СПЭБ участвовали в обеспечении санитарно-эпидемиологического благополучия в зонах чрезвычайных ситуаций, различных по характеру (г. Симферополь, 2021; г. Форос, 2021; г. Мариуполь, с 2022 по настоящее время; г. Геническ, 2023; г. Рассказово, 2023), в местах проведения массовых мероприятий (г. Сочи, 2024). Кроме того, была проведена работа в рамках международного сотрудничества (Республика Конго, 2023; Боливарианская Республика Венесуэла, 2023).

В 2024 г. на базе института создан Референс-центр по мониторингу туляремии, функции которого заключаются в оказании консультативно-методической и практической помощи органам и учреждениям Роспотребнадзора и медицинским организациям субъектов Российской Федерации по вопросам эпидемиологии, эпизоотологии, профилактики и диагностики туляремии. Референс-центр проводит анализ эпизоотологической и эпидемиологической ситуации по туляремии и углублённое изучение культур возбудителя туляремии с использованием традиционных и современных методов анализа. На основе результатов расширенной идентификации составляется геномный портрет штаммов туляремийного микроба. Проводится работа по созданию электронной базы данных и геоинформационной системы природных очагов туляремии на территории Российской Федерации. В институте существует пополняемая коллекция штаммов *Francisella tularensis*, паспортные данные которых содержат характеристики фенотипических и молекулярно-биологических свойств.

С момента основания института на его базе постоянно идёт подготовка кадров по различным аспектам особо опасных инфекций. Взаимодействие с учреждениями Роспотребнадзора и Минздрава происходит не только при обучении специалистов, но и в процессе выполнения общих научных и практических задач. Важным моментом является и сотрудничество с Ростовским государственным медицинским университетом, Южным федеральным университетом, Ростовским базовым медицинским колледжем и другими образовательными учреждениями Ростовской области, что обеспечивает взаимодействие с подрастающим поколением.

Весомые результаты 90-летней масштабной и разноплановой научной деятельности института отражены в тысячах опубликованных научных работ. В ходе выполнения научных тем сотрудниками института предложены новые и усовершенствованы методы лабораторной диагностики возбудителей опасных инфекционных болезней. Созданы диагностические препараты, базы данных и геоинформационные системы, разработаны методические документы федерального и регионального уровней, защищено более 50 докторских и 250 кандидатских диссертаций. На базе института подготовлены тысячи специалистов, составивших гордость отечественной науки и практического здравоохранения.

В настоящее время под руководством Натальи Евгеньевны Гаевской специалисты Ростовского-на-Дону противочумного института, основываясь на огромном опыте предшественников и современных знаниях, продолжают традиционные научные направления исследований.

Редакционная коллегия и редакция «Журнала эпидемиологии, микробиологии и иммунобиологии» поздравляет коллектив Ростовского-на-Дону противочумного института с юбилеем института и желает успехов в исследовательской и практической работе!

ХРОНИКА

РЕЗОЛЮЦИЯ V Всероссийской научно-практической конференции с международным участием «Современная иммунопрофилактика: вызовы, возможности, перспективы» (Москва, 10–11 октября 2024 года)

10–11 октября 2024 г. в Москве состоялась V Всероссийская научно-практическая конференция с международным участием «Современная иммунопрофилактика: вызовы, возможности, перспективы».

Мероприятие инициировано Федеральной службой по надзору в сфере защиты прав потребителей и благополучия человека и организовано Центральным научно-исследовательским институтом эпидемиологии Роспотребнадзора при содействии Российской академии наук, Всероссийского научно-практического общества эпидемиологов, микробиологов и паразитологов и Национальной ассоциации специалистов по инфекционным болезням имени академика В.И. Покровского.

Приветственное слово главы Роспотребнадзора А.Ю. Поповой участникам конференции представил директор Центрального научно-исследовательского института эпидемиологии Роспотребнадзора академик РАН В.Г. Акимкин. «Руководством страны поставлена стратегическая задача сохранения здоровья нации, снижения уровня смертности, преодоления демографического спада в стране, — говорится в приветствии. — Вакцинопрофилактика — наиболее эффективное и экономически рентабельное профилактическое мероприятие по сохранению здоровья населения и обеспечению активного долголетия, известное в современной медицине. Это один из главных инструментов по противодействию новым и уже известным биологическим угрозам».

Поздравления по поводу открытия конференции и пожелания плодотворной работы передал слушателям член президиума РАН, академик-секретарь отделения медицинских наук РАН В.И. Стародубов.

Приветственная часть завершилась награждением: золотая медаль имени В.И. Покровского за вклад в борьбу с инфекциями была вручена директору Центрального научно-исследовательского института эпидемиологии Роспотребнадзора академику РАН В.Г. Акимкину. Его программный доклад, посвящённый вопросам унификации и совершенствования системы эпидемиологического надзора за инфекционными болезнями, открыл пленарное заседание.

В своем выступлении В.Г. Акимкин отметил, что необходимость оптимизации продиктована рядом факторов: ростом числа нозологий, подлежащих эпиднадзору, внедрением информационных технологий эпидемиологической диагностики, правовыми и организационными аспектами. Сегодня появляются всё новые инфекционные болезни, и важнейшим научным направлением в эпиднадзоре становится молекулярно-генетический мониторинг патогенов. «Это достояние последнего десятилетия», — сказал В.Г. Акимкин, отметив, что Центральный НИИ Эпидемиологии Роспотребнадзора разработал и ввёл в действие платформу VGARus (Virus Genome Aggregator of Russia) — один из важнейших инструментов для обеспечения эпидблагополучия и биобезопасности в период пандемии. В национальную базу данных загружаются не только сведения о SARS-CoV-2, в настоящее время она содержит информацию уже о 47 патогенах, а к 2025–2026 гг. их число планируется расширить до 150. «Сегодня мы находимся на новом витке понимания эпиднадзора, — подчеркнул директор НИИ Эпидемиологии. — Молекулярно-генетические исследования позволят распространить опыт эпиднадзора и на другие инфекционные болезни, унифицировать его, в том числе для неинфекционных болезней, а также разработать универсальные средства и методы контроля за эпидемическим процессом».

За последние годы России удалось достичь существенного снижения заболеваемости инфекциями, управляемыми средствами вакцинопрофилактики, и обеспечить население защитой. Сегодня в мире разработано более 100 различных вакцинных препаратов, которыми контролируются около 46 заболеваний. Специфическая вакцинопрофилактика инфекционных болезней в ряде случаев при-

водит к снижению не только заболеваемости, но и смертности от заболеваний, имеющих неинфекционную природу.

Представляя «целую армию детских врачей», главный внештатный детский специалист по профилактической медицине Минздрава России академик РАН Л.С. Намазова-Баранова отметила важность ответственного отношения к своему здоровью, что невозможно без приверженности иммунизации.

Достижения отечественной науки в области разработки генно-инженерных вакцин против возбудителей особо опасных инфекций представил в своем докладе директор Государственного научного центр прикладной микробиологии и биотехнологии Роспотребнадзора академик РАН И.А. Дятлов.

На пленарном заседании также прозвучали доклады академика РАН В.В. Зверева, академика РАН Ю.В. Лобзина, академика РАН Н.И. Брико, профессора И.В. Фельдблюм.

В работе конференции приняли участие ведущие учёные научных учреждений, медицинские специалисты различного профиля, организаторы здравоохранения — всего в онлайн- и офлайн-формате более 2000 человек из 72 регионов Российской Федерации и 9 стран мира.

В рамках научной программы участники обсудили вопросы разработки эффективных средств и схем вакцинопрофилактики, совершенствования национального календаря профилактических прививок, подготовки высококвалифицированных кадров. Всего заслушано 104 доклада.

Сегодня иммунопрофилактика приобретает особое значение не только как единственная эффективная и перспективная технология борьбы с инфекциями, но и как неотъемлемый компонент системы здравоохранения, непрерывная работа которого обеспечивает эпидемиологическое благополучие по целому ряду инфекционных болезней. Выступления на конференции признанных экспертов в сфере иммунопрофилактики и эпидемиологии и научные дискуссии помогут в эффективной реализации конкретных направлений в этой отрасли здравоохранения, а также послужат обогащению участников современными знаниями, укрепят научные и деловые связи специалистов.

Участники V Всероссийской научно-практической конференции с международным участием «Современная иммунопрофилактика: вызовы, возможности, перспективы», обсудив широкий круг вопросов, связанных с текущим статусом системы иммунопрофилактики в Российской Федерации, решили:

1. Признать необходимость реализации в указанные сроки Плана мероприятий во исполнение «Стратегии развития иммунопрофилактики инфекционных болезней до 2035 года», утверждённой 18 сентября 2020 г. Распоряжением Правительства Российской Федерации № 2390, для совершенствования защиты от инфекционных заболеваний населения Российской Федерации, гарантированного обеспечения доступности для всех граждан страны качественной иммунизации самыми современными и эффективными вакцинами.

2. Ключевым направлением развития системы иммунопрофилактики считать совершенствование национального календаря профилактических прививок путём расширения перечня инфекций, против которых проводятся плановые прививки. Обеспечить внедрение прививок против менингококковой и ротавирусной инфекций в национальный календарь профилактических прививок в 2025 г.

3. Проанализировать и обобщить опыт субъектов Российской Федерации по созданию региональных программ, которые служат пилотными проектами для апробации подходов для расширения национального календаря профилактических прививок, на постоянной основе осуществлять оценку их эффективности, в том числе экономической.

4. В условиях санкционных мер в отношении поставок зарубежных иммунобиологических препаратов необходимо увеличить мощности отечественных предприятий, выпускающих вакцины, входящие в национальный календарь профилактических прививок, и обеспечивать современным оборудованием и кадрами научно-исследовательские центры, занимающиеся разработкой и испытаниями вакцин.

5. В соответствии со «Стратегией развития иммунопрофилактики инфекционных болезней до 2035 года» необходима государственная поддержка российских производственных предприятий по выпуску иммунобиологических препаратов, организации в Российской Федерации полного цикла производства поливалентных пневмококковой, менингококковой конъюгированных вакцин, ротавирусной и папилломавирусной вакцин, вакцины против вируса *Varicella zoster*, комбинированных пяти- и шестикомпонентных вакцин с бесклеточным коклюшным, Hib-компонентом и инактивированной вакциной против полиомиелита.

6. Применить отработанные на примере вакциноуправляемых инфекций и имеющие унифицированный характер методологические подходы к осуществлению эпидемиологического надзора и управления эпидемическими рисками на другие инфекционные и массовые неинфекционные болезни.

7. Повысить оперативность, чувствительность и специфичность эпидемиологического надзора за вакциноуправляемыми инфекциями, а также за побочными проявлениями после иммунизации путём применения современных молекулярно-биологических, в том числе молекулярно-генетических, технологий.

8. В рамках импортозамещения требуется разрабатывать и совершенствовать отечественные

иммуноферментные тест-системы для серодиагностики и серомониторинга вакциноуправляемых инфекций.

9. Необходимо развивать исследования проблемы восприимчивости и резистентности с применением метагеномной методологии, которые знаменуют переход к персонализированной иммунопрофилактике и иммунотерапии.

10. В условиях поэтапного перехода медицинских организаций к оказанию медицинской помощи на основе клинических рекомендаций следует развивать практику разработки и внедрения федеральных клинических рекомендаций по иммунопрофилактике отдельных инфекционных болезней, включая принципы «догоняющей» и сочетанной иммунизации, а также формирования индивидуального графика вакцинации. Организовать разработку клинических рекомендаций по вакцинопрофилактике опоясывающего лишая.

11. Учитывая высокую социально-экономическую значимость гриппа, необходима реализация современных коммуникационных стратегий для обеспечения 75% охвата иммунизацией.

12. Обострение эпидемической ситуации по кори в Российской Федерации требует активизации комплекса профилактических и противоэпидемических мероприятий в соответствии с утверждённой Роспотребнадзором и Минздравом России программой «Элиминация кори и краснухи, достижения спорадической заболеваемости эпидемическим паротитом в Российской Федерации (2021–2025 гг.)» и национальным планом по её реализации. Следует считать выполнение программы элиминации кори и краснухи на пространстве Содружества Независимых Государств одной из приоритетных задач сотрудничества государств-участников. Необходимо обеспечить бесперебойные поставки уже разработанных и сертифицированных отечественных вакцин в достаточном количестве в первичное звено, сохранить высокие темпы работы по плановой иммунизации населения против кори, активному выявлению и иммунизации лиц, не привитых против этой инфекции, в том числе среди иностранных граждан, а также усилению информационной работы с населением по пропаганде вакцинопрофилактики. Предлагается рассмотреть вопрос о формировании независимой экспертизы качества отечественных вакцин.

13. В условиях роста заболеваемости коклюшем оптимальной стратегией по предотвращению социальных и экономических потерь от этой инфекции является обеспечение своевременного охвата вакцинацией детей первых двух лет жизни в сроки, определённые национальным календарём профилактических прививок (приказ Минздрава России от 06.12.2021 № 1122), поэтапного внедрения возрастных ревакцинаций против коклюша детей, подростков и взрослых — каждые 10 лет с момента последней ревакцинации, а также догоняющей иммунизации лиц, не привитых своевременно. С целью профилактики летальных исходов коклюша у детей первых месяцев жизни следует использовать возможность создания у новорождённого пассивного иммунитета путём иммунизации беременных женщин.

14. Одним из приоритетных направлений в развитии иммунопрофилактики считать улучшение профессиональной подготовки медицинских работников всех специальностей по вопросам вакцинопрофилактики с использованием современных обучающих технологий, в том числе направленных на формирование навыков коммуникации для стимулирования спроса на иммунизацию.

15. В рамках эпидемиологического надзора за иммунопрофилактикой проводить на регулярной основе анализ приверженности вакцинопрофилактике медицинских работников и различных групп населения.