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ORIGINAL RESEARCHES

Original Study Article
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POEM: POpulation Epidemiological Model for anti-epidemic measures efficiency prognosis in the Russian Federation

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Abstract

Introduction. The COVID-19 pandemic has revealed a whole complex of problems related to mathematical modeling of the epidemic process and assessing the effect of preventive and anti-epidemic measures in modern complex societies. Along with this, the accumulation of significant factual data has spurred the active development of agent-based models, in which each agent (a hypothetical person) has a unique set of characteristics and interaction methods determined based on real sociological and demographic data.

Aim and objectives. Development and demonstration of the capabilities of the epidemiological agent-based model POEM platform (POpulation Epidemiological Model).

Materials and methods. The POEM platform is developed based on the source code of one of the most widely used agent-based models worldwide, Covasim, taking into account the demographic and organizational-administrative conditions specific to the Russian Federation.

Results. Computational experiments have shown that due to individual variability in the dynamics of infection development and the specifics of disease registration, even mass events, while leading to an actual increase in the number of infected individuals, do not have a significant impact on the shape of the curve of registered disease incidence. It has been shown that intercity traffic flows at a level of 0.1% of the population per day have a minimal effect on the dynamics of the epidemic's development, while the effect of a 1% population outflow per day sharply reduces the effect of strict anti-epidemic measures implemented in only one particular city. Using the example of the Voronezh region, the transition from the Delta variant to Omicron in early 2022 was modeled, and a high degree of correlation was shown between the model dynamics and the actual ratio of virus variants observed.

Conclusion. The model is fully implemented within the Russian system on the server of the Research Institute for System Biology and Medicine of Rospotrebnadzor and can be used to conduct digital epidemiological experiments to predict the effectiveness of proposed anti-epidemic measures.

Keywords: *agent-based modeling, computational epidemiology, epidemic process, anti-epidemic measures*

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Оригинальное исследование
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ПОЭМА: ПОпуляционная Эпидемиологическая Модель Агентная для прогноза эффективности противоэпидемических мероприятий в Российской Федерации

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Аннотация

Введение. Пандемия COVID-19 выявила целый комплекс проблем, связанных с математическим моделированием эпидемического процесса и оценкой эффекта от проводимых профилактических и противоэпидемических мероприятий в современном сложноорганизованном обществе. Вместе с этим накопление значительных массивов фактических данных дало импульс активному развитию агентных моделей, в которых каждый агент (условный человек) имеет уникальный набор характеристик и способов взаимодействия, определяемых на основе реальных социологических и демографических данных.

Цели и задачи. Разработка и демонстрация возможностей эпидемиологической агентной модели ПОЭМА (ПОпуляционная Эпидемиологическая Модель Агентная).

Материалы и методы. Платформа ПОЭМА разработана на исходном коде одной из наиболее широко используемых во всем мире агентной модели Covasim с учетом демографических и организационно-административных условий, характерных для Российской Федерации.

Результаты. Вычислительные эксперименты показали, что из-за индивидуальной вариабельности в динамике развития инфекции и особенностей регистрации заболеваемости даже массовые мероприятия хотя и приводят к фактическому увеличению числа инфицированных, но не оказывают существенного влияния на форму кривой регистрируемой заболеваемости. Показано, что транспортные потоки между городами на уровне 0,1% населения в день оказывают минимальный эффект на динамику развития эпидемии, в то время как эффект от перетока 1% населения в день резко снижает эффект от введения строгих противоэпидемических мероприятий, проводимых только в одном отдельно взятом городе. На примере Воронежской области проведено моделирование смены циркулирующего варианта SARS-CoV-2 с Дельта на Omicron в начале 2022 г. и показана высокая степень корреляции между модельной динамикой и наблюдаемым в реальности соотношением вариантов вируса.

Заключение. Модель реализована полностью в Российском контуре на сервере НИИ системной биологии и медицины Роспотребнадзора и может быть использована для проведения цифровых эпидемиологических экспериментов с целью прогноза эффективности предполагаемых к проведению противоэпидемических мероприятий.

Ключевые слова: *агентное моделирование, вычислительная эпидемиология, эпидемический процесс, противоэпидемические меры*

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

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Introduction

The COVID-19 pandemic has shown that socio-economic factors are an objective constraint in implementing the necessary set of anti-epidemic measures. Different countries have chosen different approaches to preventing the spread of the new coronavirus infection, in all cases justifying the validity of the decisions being made. Against this backdrop, the need for computational epidemiological models became evident, which would allow for the most objective assessment of the potential effect of individual anti-epidemic measures and correlate it with the socio-economic losses from the epidemic. Thus, the COVID-19 pandemic served as a catalyst for the development of methods for computer modeling of epidemics.

Computational models for studying the spread of infectious diseases can be divided into two main types: compartmental and agent-based. Compartment models appeared almost 100 years ago and were actively used due to the speed and ease of calculations. In the basic compartmental model (SIR – Susceptible, Infected, Recovered), all individuals are identical and can only be in three states. Although modifications to the SIR model have been described for analyzing additional parameters, accounting for individual combinations of properties of the subjects within the population is practically non-existent. Agent-based models allow each virtual entity to be endowed with unique characteristics of any complexity. They are much more computationally expensive, but they allow for the creation of a digital environment that most accurately reproduces the conditions of the epidemic process's development [1].

The complexity of development has led to the widespread practice of using ready-made open-source agent models, which are then customized by the user to solve a range of specific tasks. Among the main computational approaches considered for creating artificial populations whose agents are humans, the FRED [2], AceMod and Covasim [3] platforms have gained the widest adoption in modeling respiratory infections.

The open-source platform Covasim stands out because it contains numerous built-in algorithms for modeling epidemic control measures and allows for modification of the software code to implement additional functionalities. It has been used to address a wide range of issues, such as determining the optimal testing strategy for asymptomatic carriers [4], modeling the circulation of different SARS-CoV-2 variants [5], the effect of lifting school closures [6] and the likelihood of a second wave [7] in the UK, developing pandemic control strategies [8, 9] and vaccination strategies [10] in Australia, and vaccination strategies in the USA [11] and Italy [12]. In most of these works, a model was used that was refined to take into account regional characteristics and specific tasks.

The aim of the study is to develop an agent-based epidemiological model POEM (Population Epidemiological Model) based on the Covasim platform for modeling epidemics, studying the influence of various factors on pathogen spread, as well as evaluating the effectiveness of anti-epidemic measures in an artificial population. The application of the developed computational tool was demonstrated in a series of digital experiments, comparing it with observed epidemiological patterns.

Materials and methods

The development was based on the open-source program Covasim, released under the Creative Commons Attribution-ShareAlike 4.0 license and available on the GitHub server (github.com/institutefordisease-modeling/covasim). APOEM, like Covasim, is implemented in the programming languages Python and JavaScript.

Modeling two or more settlements and their interactions

The simulation of 2 or more settlements was implemented using parallel computing as follows: during the initialization phase, additional cells are created in each city for agents who may arrive in that city. Then each city is modeled in a separate computational thread. The main simulation includes three stages: adding agents who have arrived in the city, performing the main simulation step (one simulated day), and selecting agents who will leave the city in the next simulation step. For each displaced agent, the duration of their stay in another city is chosen based on a Poisson distribution with a mean value of 14 days (this parameter can be changed by the user). An agent who has temporarily moved from one settlement to another only has random interactions, but their number increases by an average of 3 times (the coefficient can be changed by the user). The graphical interface has been enhanced with the ability to define one or more transportation flows (agent movement between settlements). The user specifies the origin and destination, as well as the proportion of agents moving daily. The graphical interface also allows you to view simulation results for individual locations in different tabs or compare them on a single graph.

Modeling the conduct of mass gatherings

The simulation of mass gatherings is implemented as follows: the user inputs a table (through a graphical interface or by calling functions in Python) containing the date of each event, the number of participants and the number of such gatherings. To implement this module, a new contacts layer was added. This layer is dynamic, with contacts updated daily to simulate new events. An agent can only participate in one event per day.

In the first step, a list of people who will participate in mass events is randomly determined. Next, participants break into groups for separate meetings.

For each group, contacts are randomly established so that each agent has an average of 9 connections, as literature suggests that even for large mass events, the average number of contacts lasting 15 minutes or more, during which the virus can be transmitted, is 9 [13]. The probability of infection transmission through contact at mass events is assumed to be the same as workplace contacts.

Functional extensions of the Covasim model implemented in the APOEM software platform

New variants of agent susceptibility distributions and their infectivity have been introduced. A variant has been implemented where agents have individual but identical values for susceptibility and infectivity for a given agent, with the aim of modeling the behavior of agents with high values for both indicators.

Accounting for the time delay between receiving a positive test result and the date the information is submitted to state control bodies has been added. Each agent is assigned a delay in receiving test results (a random number from a given distribution), which is added to the test date during the simulation.

To account for the socio-demographic characteristics of the modeled regions of the Russian Federation, an additional module for configuring the artificial population construction has been implemented. The module is implemented based on the open-source library SynthPops (URL: https://github.com/InstituteforDiseaseModeling/synthpops). The ability to upload the artificial population parameters in the Excel table format has been added to the interface.

A set of pathogen parameters corresponding to the Omicron variant has been added based on literature data. The set of factors for modeling the Omicron variant of the SARS-CoV-2 virus was selected based on a literature search in the PubMed and Google Scholar databases using the following keywords: Omicron transmissibility, transmissibility of Omicron, symptomatic factor, critical factor of Omicron, severe factor of Omicron, death of Omicron, and recovery of asymptomatic Omicron. The search was conducted for articles published since 2021, and 45 publications were selected that presented the parameters necessary for the stochastic agent-based model. To calculate the average median value of these parameters, only studies with a sample size of at least 100 people were used.

Computational experiments were conducted in two stages. The first stage involves using a web interface, the results were evaluated under various scenarios without repetitions. During the second stage, to assess the variability of the results due to the stochastic nature of the model, the most informative scenarios were executed 30 or 100 times (in different tasks) using a Python script.

Results

Covasim Source Code Modifications

The creation of the Population Epidemiological Model (POEM) is based on the open-source programming environment Covasim, with modifications or additions of code to specific modules to implement new features, increase the amount of information, and localization (**Table 1**).

1. Assigning susceptibility and infectivity to distribution agents

Table 1. Comparison of Covasim and APOEM

Covasim	POEM
The susceptibility of the agent to the pathogen is defined discretely based on age category	The susceptibility of the agent to the pathogen is defined according to the chosen distribution option
The infectivity of an agent is determined by a distribution that is independent of the susceptibility	The infectivity of an agent can be defined in conjunction with the susceptibility to that agent
Modeling the spread of SARS-CoV-2 variants – Wuhan variant, Alpha, Beta, Gamma, Delta	The ability to model infection caused by the Omicron variant of SARS-CoV-2 has been introduced; there is also the possibility of creating a custom virus variant
The epidemic spread is modeled within a single synthetic population (a hypothetical city) that is created based on average European demographic and statistical data	The epidemic spread is modeled in parallel for multiple synthetic populations (hypothetical cities) with different set parameters. Model cities are defined as independent of each other or connected by an interaction coefficient. It is possible to build model cities based on real demographic and statistical data
The ability to model anti-epidemic measures – quarantine, mask mandates, vaccination	The ability to model both one-time (single) and regular mass events has been added
Anti-epidemic measures are implemented simultaneously and with equal intensity in all institutions of the same type in the hypothetical city	There is the possibility of selectively closing individual schools and businesses when a certain percentage of diagnosed cases is reached (the epidemic threshold)
The results of the agent testing are reflected on the incidence graph on the day of testing	The test results are displayed on an incidence graph with a delay, calculated based on real data from Rospotrebnadzor (18 million records)
Basic programming skills are required for setup	Parameter settings and advanced features for visualization in the web version

The key parameters of the infectious process are the susceptibility of individuals to infection (innate and acquired immunity) and contagiousness – the ability of the pathogen to be transmitted from one individual to another. At the same time, contagiousness is considered a characteristic of the pathogen, as individual characteristics of subjects are averaged at the population level. In the agent-based model, it is possible to define an individual agent's ability to spread the virus (e.g., due to a higher viral replication rate, behavioral characteristics, severity of respiratory infection symptoms, or a reduced infectious dose). To describe this characteristic of the agent (subject), the term "infectiousness" will be used below.

The original Covasim code assumes that agents' susceptibility to the virus is rigidly tied to the agent's age (**Table 2**) [14]. However, the authors of Covasim note that the susceptibility characteristics they adopted contradict the results of another study, where susceptibility to infection did not change with age [15]. At the same time, it is evident that the susceptibility of agents should correspond to mathematical distributions commonly observed in biology, and other studies have investigated, for example, the spread of infection in a population using uniform, Gaussian, and bimodal Gaussian distributions [16].

Since the exact characteristics of the distribution of human susceptibility to SARS-CoV-2 and other respiratory viruses are unknown, the possibility of applying the following normalized distributions was included in the POEM model:

- age-stratified susceptibility distribution proposed in the base Covasim model;
- normal (independent of age);
- lognormal (independent of age);
- lognormal, sharply peaked with a thin tail (independent of age).

To ensure the comparability of the impact of the susceptibility distribution on infection spread parameters, the sum of the conditional susceptibility of agents in the population (the area under the susceptibility distribution curve) in the APOEM model is a constant.

In the original Covasim code, the agent's susceptibility characteristic is defined (Table 2), and infectiousness is determined by the probability of pathogen transmission. However, during the spread of COVID-19, significant heterogeneity in the number of infections from a single patient has been described, with 80% of secondary infections being caused by 15% of patients [17]. It is also known that the variability in the amount of SARS-CoV-2 virus detected by PCR testing among different patients (and consequently, the possibility of virus transmission) varied by several orders of magnitude [18]. In the POEM model, the ability to establish a direct relationship between a subject's susceptibility to the virus and its infectiousness was introduced, or to set these parameters as independent, as there is evidence in the literature supporting both options. For example, the ability to become infected and maintain a high level of replication could theoretically be linked to the level of viral receptor expression [19]. On the other hand, it has been shown that some people produce significantly more microdroplets than others and may be more infectious simply because of this [20], meaning that susceptibility and infectiousness may not be linked at the individual level.

2. Implementing a temporary delay in displaying test results

One of the drawbacks of the Covasim base code is the instantaneous availability of results from testing conducted on an artificial population of agents. However, the actual registration of testing data in the overall statistics may be significantly delayed under the increased strain on the healthcare system. The integration of realistic delay into the POEM model, as close as possible to the conditions for recording morbidity in Russia, was performed based on an analysis of records of over 18 million cases of COVID-19, for which the date of a positive sample collection was compared with the date the information was received by Rospotrebnadzor. The resulting distribution of the difference between these dates for each sample was used to determine the probability of receiving delayed data after testing (**Table 3**).

3. Implementation of a block forming two or more settlements, with or without their interaction.

The COVID-19 pandemic has demonstrated that imposing isolation on a specific territory or settlement cannot guaranty the prevention of the pathogen's spread. For national health policy formation, modeling an epidemic in a single region without considering its interconnectedness with other regions has limited value. In the original Covasim code, there is an option to model the importation of a fixed number of infected individuals into the simulated population (a specified number of agents become ill each day without a source of infection), while the outflow of infected agents is not provided for. This approach doesn't allow for the

Table 2. Age-specific coefficients of susceptibility to the SARS-CoV-2 virus, as defined in the original version of Covasim[14]

Parameter	Age, years									
	0–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	90+
Susceptibility*, %	0.34	0.67	1.00	1.00	1.00	1.00	1.00	1.24	1.47	1.47

Note. *The infection probability coefficient upon contact between agents, used as part of the formula that determines the probability of virus transmission with each contact.

Table 3. Distribution of the delay in reporting positive test results to Rospotrebnadzor, days after sample collection

Parameter	Delay, days														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Percentage, %	< 1	14	22	12	10	9	7	6	5	4	3	2	2	2	2

dynamics of the epidemic's development in related regions to be taken into account. To overcome this limitation, the ability to model multiple populations (cities) in parallel, with independent parameters for epidemic control measures and the possibility of regulating the movement of agents between them, was developed. In the absence of interaction between cities, each can be considered an independent epidemiological experiment, allowing for a comparison of the effect of population characteristics or the nature of anti-epidemic measures. The interaction coefficient between cities is determined empirically as the percentage of the population travelling between cities each day, reflecting hypothetical transportation flows.

4. Building a model artificial population based on real demographic and sociological data

Modeling the epidemic process under conditions close to real societies is implemented in the APOEM model thru the ability to upload demographic and sociological data for a specific locality or region. The data is provided by the user in the form of an electronic spreadsheet created according to a template and includes population size and its age distribution, household size distribution, educational institution sizes and the number of teachers and other staff working in them, the distribution of workforce sizes, and the employment rate.

As an example of implementing real data loading into the POEM software model, profiles for the following Russian regions have been created: Blagoveshchensk and the Amur region, Nizhny Novgorod and the Nizhny Novgorod region, Voronezh and the Voronezh region.

5. Modeling the spread of the Omicron variant

In late November 2021, the WHO designated a new genetic variant of SARS-CoV-2 as a variant of concern (B.1.1.529), naming it Omicron [21]. At the time this SARS-CoV-2 variant emerged, it had several times more mutations in the spike protein than any other variant [22]. Thanks to these mutations, the Omicron variant of the virus binds more effectively to the host cell's ACE2 receptors compared to previous variants. Most neutralizing antibodies produced by vaccinated individuals or those who have recovered from other SARS-CoV-2 variants are ineffective against Omicron, but it is associated with milder symptoms and a higher proportion of asymptomatic carriers compared to other variants [23].

The degree of danger of a coronavirus variant depends on factors such as contagiousness, immune evasion, and the severity of the clinical course of the infection [24]. The basic reproduction number (R_0) of the Omicron variant is 8.2 [25], which corresponds to a much more effective spread compared to earlier virus variants. In the original Covasim environment, the transmissibility of virus variants is implemented based on coefficients relative to the original Wuhan variant. In the POEM model, the probability of infection transmission from agent to agent was set to 3.34 (relative to the Wuhan variant), calculated as the average obtained from the analysis of several publications. The probability of symptomatic infection with the Omicron variant was established at 0.1 based on a multicenter retrospective observational study conducted in Shanghai [26]. The risk of hospitalization with the Omicron variant infection was 56% lower compared to the Delta variant [27], and the risk of severe illness was 2–3 times lower [28]. Based on a systematic review and meta-analysis of severity and clinical outcomes in people infected with the Omicron variant, an odds ratio of 0.46 for critical illness compared to the Delta variant and an odds ratio of 0.39 for mortality were used. According to the literature, the incubation period (in the terminology of the Covasim platform, actually the latent period, the time from infection to the onset of viral shedding) for the Omicron variant is about 2–3 days, and the average duration of the asymptomatic period is 2.2 days [29].

The median duration of virus detection was 9 days (ranging from 7 days in asymptomatic cases to 10 days in severe cases) [30]. Acute symptoms in those infected with the Omicron variant were observed for a shorter period – an average of 6.9 days, compared to patients infected with Delta, who experienced symptoms for an average of 8.9 days [24]. The length of stay in intensive care units for Omicron variant infections was 5 days shorter than for Delta variant infections, with a median value of 5.3 days [31]. The overall mortality rate was higher for patients with the Delta variant, at 0.5%, compared to patients with the Omicron variant (0.1%). The average time from diagnosis to death when infected with the Delta variant was 13 days, compared to 10 days with the Omicron variant [31].

Computational experiments

1. Mass gathering modeling

Mass gatherings have a significant impact on the dynamics of epidemics [32–34] and are one of the priority targets for epidemic control measures.

Two cities with a population of 100,000 agents have been given, with anti-epidemic measures in place in the form of social distancing (reducing the frequency of random contacts by 50%, and contacts at school and work by 90%). On the first day in both cities, 30 people were infected with the Delta variant of SARS-CoV-2. The probabilities of testing asymptomatic and symptomatic agents were 1% and 10%, respectively. Every 10 days, mass events are held in one of the cities, with a total number of participants equal to 20,000. The results of 10 simulations for each city are presented in **Fig. 1**. The occurrence of mass events leads to a sharp increase in the number of infected individuals in the model. However, even a significant number of simultaneous infections (500–1000 compared to the usual 1000–2000 infected per day) cannot be detected through daily testing due to the smoothing of the incidence rate caused by the varying lengths of the incubation period and the uneven delay in reporting positive results, which negates sharp increases in infections.

2. Modeling of anti-epidemic measures

The most severe clinical course of COVID-19 was observed in older individuals. The most intensive anti-epidemic measures were aimed at protecting the at-risk age group. One of the common measures to reduce morbidity was the closure of schools to decrease the spread of the virus in society and, consequently, the risk of household transmission to the elderly. Individual examples of the impact of school closures on reducing infections in the elderly have been described [35], but overall, epidemiological data do not confirm the effectiveness of such measures with sufficient certainty, and the results of mathematical modeling described earlier have been contradictory [36, 37].

The following hypothetical scenarios were tested on the POEM model. The most effective measures (100% isolation of all contacts) were applied only to young people (< 20 years old) or only to the elderly (> 60 years old) on days 30–90 or 60–120 after the start of the epidemic (at the time the measures were implemented, approximately 1% or 20% of the population was infected, respectively), with the assumption that administrative resources allowed for restrictions to be in place for only 60 days.

When measures were implemented early, during low COVID-19 prevalence, there was a pronounced effect on the number of infected individuals. However, due to the lifting of restrictions on day 90, by day 140, the number of infected individuals in all groups was practically indistinguishable (**Fig. 2**). Meanwhile, isolation of both young people and, especially, the elderly led to a 17% and 35% reduction in deaths, respectively, although the confidence intervals for these results partially overlapped.

The late implementation of measures to isolate young people led to a 25% decrease in the number of cases, but the reduction in fatalities was less pronounced, at 16%. Late isolation of the elderly led to a smaller decrease in overall morbidity, but a significant reduction in mortality (66%). Thus, isolating young people may have an effect on the incidence of illness in the elderly, but less noticeable than the direct isolation of the elderly.

The modeling results obtained are consistent with the understanding that the later separation of organized children's and adolescent groups, implemented during the rise in COVID-19 incidence, is accompanied by an

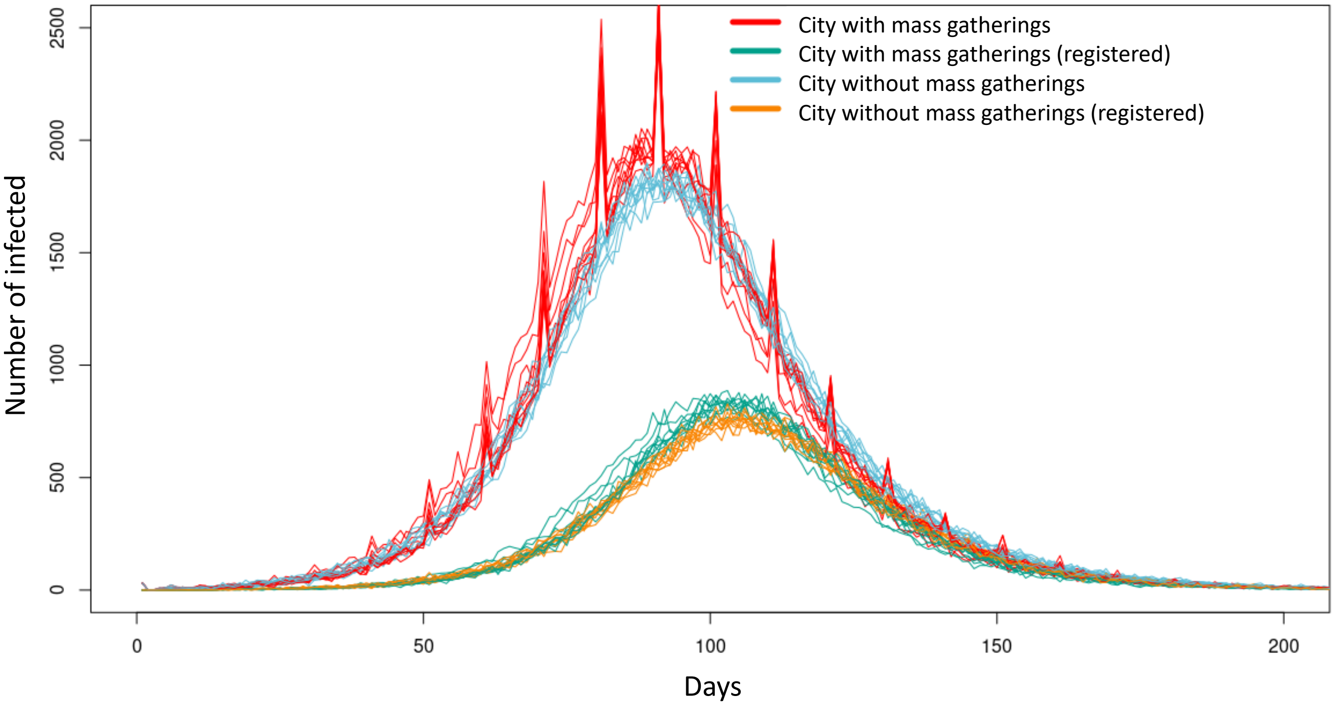

Fig. 1. The impact of holding mass gatherings on the number of SARS-CoV-2 infections in a city with a population of 100,000 agents.

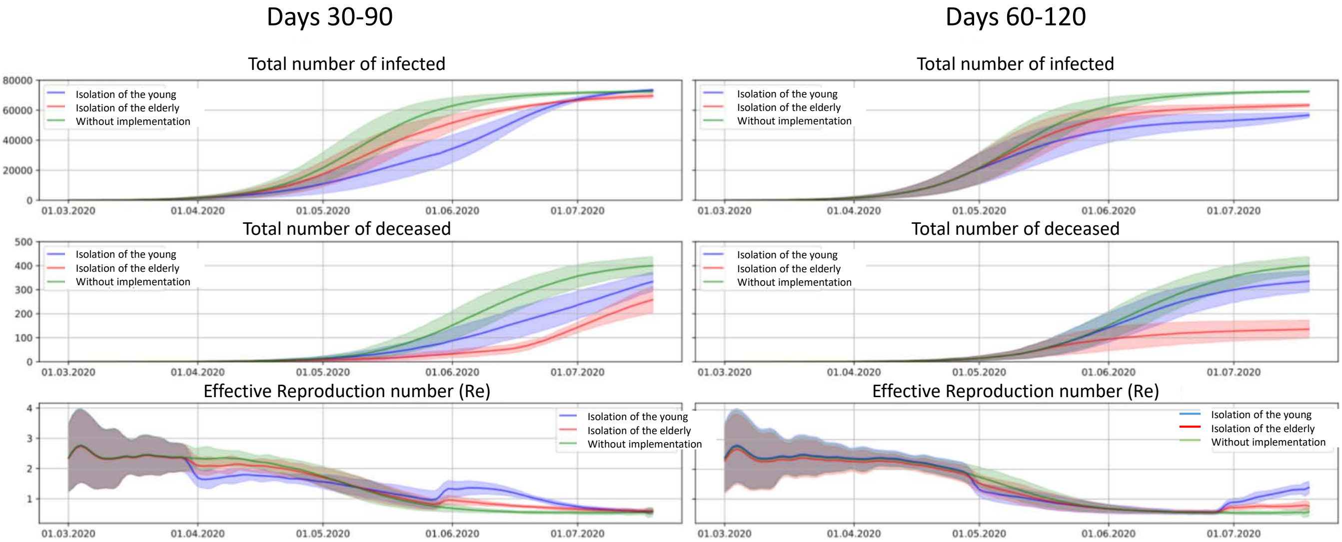

Fig. 2. The impact of implementing anti-epidemic measures on morbidity and mortality across different age groups.

The blue line on the graph represents the results when young individuals were isolated, and the red line represents the results when elderly individuals were isolated. The green line represents epidemic modeling in the city without the implementation of measures.

intensification of infection transmission within households due to longer and closer domestic contact (students transferred to distance learning infect elderly individuals who are temporarily involved in supervising minors).

3. Passenger flow modeling

During the COVID-19 pandemic, restrictions on movement between cities and countries were almost universal and aimed to prevent the virus from being brought into the region from areas with an unfavorable epidemiological situation. To model the effectiveness of traffic flow restrictions, a simulation of the epidemic's spread was conducted in two cities. In both cities, anti-epidemic measures were implemented on the 50th day of the simulation (soft measures in the first city, strict ones in the second). On the 90th day of the simulation, only mild epidemic control measures were implemented in both cities, which continued until the end of the simulation. Soft epidemic control measures included only mandatory mask-wearing at work and in public places with a conditional effectiveness of 25%, hard measures included mandatory mask-wearing at work and in public places with a conditional effectiveness of 75%, and school closures. The spread of the epidemic was modeled under conditions of minimal transportation flows (0.01% of the population moving between cities daily), average flows (0.1% of the population daily, roughly corresponding to transportation flows between Moscow and St. Petersburg), and intensive flows (1% of the population daily).

In the city with mild measures, the number of cases reached 60,000 by day 200 (compared to 72,000 without measures), and the number of deaths was 210 compared to 275 (**Fig. 3**). With minimal traffic flow, the number of cases in the city with strict measures reached 50,000 by the 200th day, and the number of deaths was 130. With moderate traffic flow, the number of cases in the city with strict measures has barely changed, while the number of deaths has increased to 150. With heavy traffic flow, the effect of strict epidemic control measures in the second city was practically nullified.

4. Modeling the introduction of measures when a threshold number of cases is reached

A classic tool in epidemiology is the implementation of anti-epidemic measures upon reaching a set level (epidemic threshold). This method is well-established for influenza[1]. Since epidemic threshold values have not been developed for COVID-19, three fairly strict values were tested in the modeling: 20, 70 and 150 cases per 10,000 population. At the same time, it was agreed that administrative resources are limited, and strict measures (mask mandate with 70% effectiveness in public places and at work, school closures) will only be implemented for 30 days, followed by another 30 days of less stringent measures (mask mandate with 30% effectiveness in public places, at work, and in schools). Epidemiological measures had an immediate effect on the pathogen transmission efficiency in the model population (reducing the effective reproductive number below 1). The total number of infected individuals was 30–35 thousand in all scenarios, but the number of deaths in the case of the latest implementation of measures (when the total number of diagnosed cases reached 150 per 10 thousand population) reached 145,

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[1] State Sanitary and Epidemiological Rationing of the Russian Federation. 3.1.2. Respiratory tract infections. Methodology for calculating epidemic thresholds for influenza and acute respiratory viral infections in the subjects of the Russian Federation. Methodological recommendations. MP 3.1.2.0118-17. Moscow; 2017.

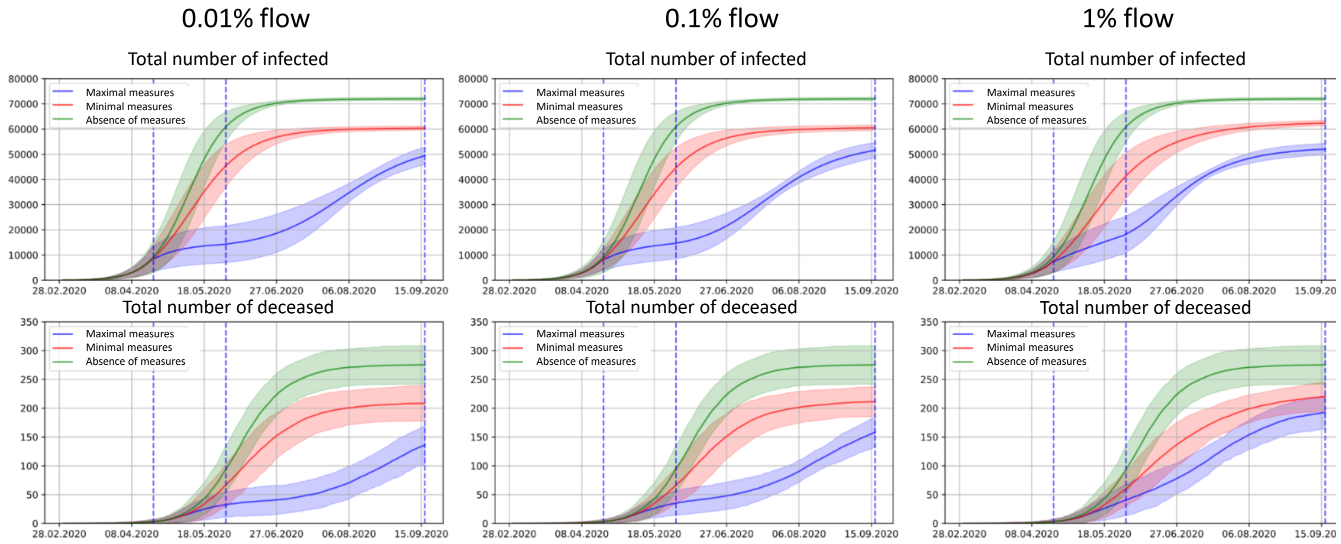

Fig. 3. The impact of intercity traffic flows on morbidity and mortality at different levels of implementation of anti-epidemic measures.

The blue line on the graph shows the simulation results for the city with the most effective measures, and the red line shows the results for the city with less strict measures. The green line shows modeling an epidemic in the city without the implementation of measures and with no transportation. Dotted lines show the dates of the implementation and cancelation of measures and the end of the simulation.

while in all other scenarios the values were in the range of 91–111 people (**Fig. 4**). Thus, the POEM model allows for simulating the development of an epidemic under the conditions of the response standards adopted in Russia for disease spread, and accounting for registration delays based on real data allows for planning the fight against the spread of infection, taking this factor into account.

5. Modeling the circulation of two virus variants

The Voronezh region was chosen as a model, because a sufficiently large number of SARS-CoV-2 virus whole-genome sequences were available on the VGARus platform in December 2021 – January 2022, allowing for a comparison of the calculated ratio of virus variants in the simulation and the actually observed one. Demographic data was loaded into the model, and based on this, a digital replica of the Voronezh region was created. On April 1, 2021, 30 agents infected with the Delta variant appeared in it, and on December 15, 2021, 30 agents infected with the Omicron variant appeared. This simulation was repeated 100 times, and the results are presented in **Fig. 5**. The Pearson correlation coefficient between the average result from 100 simulations and the real data for the proportion of the Omicron variant among circulating SARS-CoV-2 in the population was 0.9985.

Discussion

Computational epidemiology is a new field of knowledge that combines the principles of classical epidemiology, statistics, mathematics and computer science to analyze large datasets, model the spread of diseases in the human population, and inform public health policy.

A successful example of implementing this approach is the Baroyan–Rvačev model for predicting influenza epidemics in the USSR [38]. The model used detailed data on intercity migration flows, which allowed for highly accurate predictions of the start of the epidemic process and its peak in the capitals of the union republics.

The rapid development of computational technologies in the late 20th and early 21st centuries allowed for the transition to large-scale agent-based modeling with explicit interactions between individuals to simulate the spread of infectious diseases. The development of such approaches allows for a number of fundamental features to be taken into account: the diversity of agents (*e.g.*, age, gender, social status, *etc.*), the heterogeneity of social contacts, migration flows, the implementation of disease control measures for specific population groups, etc. The COVID-19 pandemic fully demonstrated the need to utilize the predictive power of stochastic discrete models for conducting computational epidemiological experiments to select the optimal set of response measures.

The Covasim agent-based modeling platform, combined with the SynthPops synthetic population creation module, is currently one of the most advanced in epidemiology. Over the past 3 years, a significant amount of research has been conducted using Covasim to develop measures to prevent the spread of epidemics, such as:

- studying test strategies and impact of test characteristics [4, 9, 39];
- studying the effectiveness of isolation and quarantine [6, 39];
- modeling the achievement of herd immunity [10];

- analysis of vaccination strategies [6, 11, 12];
- assessing the risks of the consequences of rescinding management decisions [8, 40].

In certain cases, the objectives were achieved by expanding, calibrating, or modifying the Covasim model. The most extensive additions were proposed in the new ProMES model based on Covasim [41].

This study presents the POEM model, which overcomes the limitations associated with using the Covasim platform for the population of various regions of the Russian Federation and adds a number of new features that allow for computational experiments with greater detail and reliability than provided by the original code.

Agent susceptibility profiles to SARS-CoV-2 have been developed, reflecting the latest accumulated data, which differ from the results of early COVID-19 studies. A population structure based on real demographic data and a correct registration delay based on an analysis of 18 million COVID-19 cases have been implemented. The ability to assess the role of mass gatherings and traffic flows has been added, and based on a meta-analysis of published data, the ability to simulate Omicron variant morbidity has been added. The web interface for running simulations and visualizing results makes it possible for users without programming skills to work with the POEM software platform. For scientific research, it is possible to run multiple simulations with statistical significance assessment and output of results not only in graphical format but also as the raw values of all parameters.

Digital epidemiological experiments performed to demonstrate the model capabilities show that the model corresponds to the actually observed development of epidemics and the effect of anti-epidemic measures. At the same time, the model allows for practicing not only standard but also extreme scenarios, such as the emergence of highly contagious pathogens. Since the key constraint during the COVID-19 pandemic was public acceptance of restrictions, using the APOEM

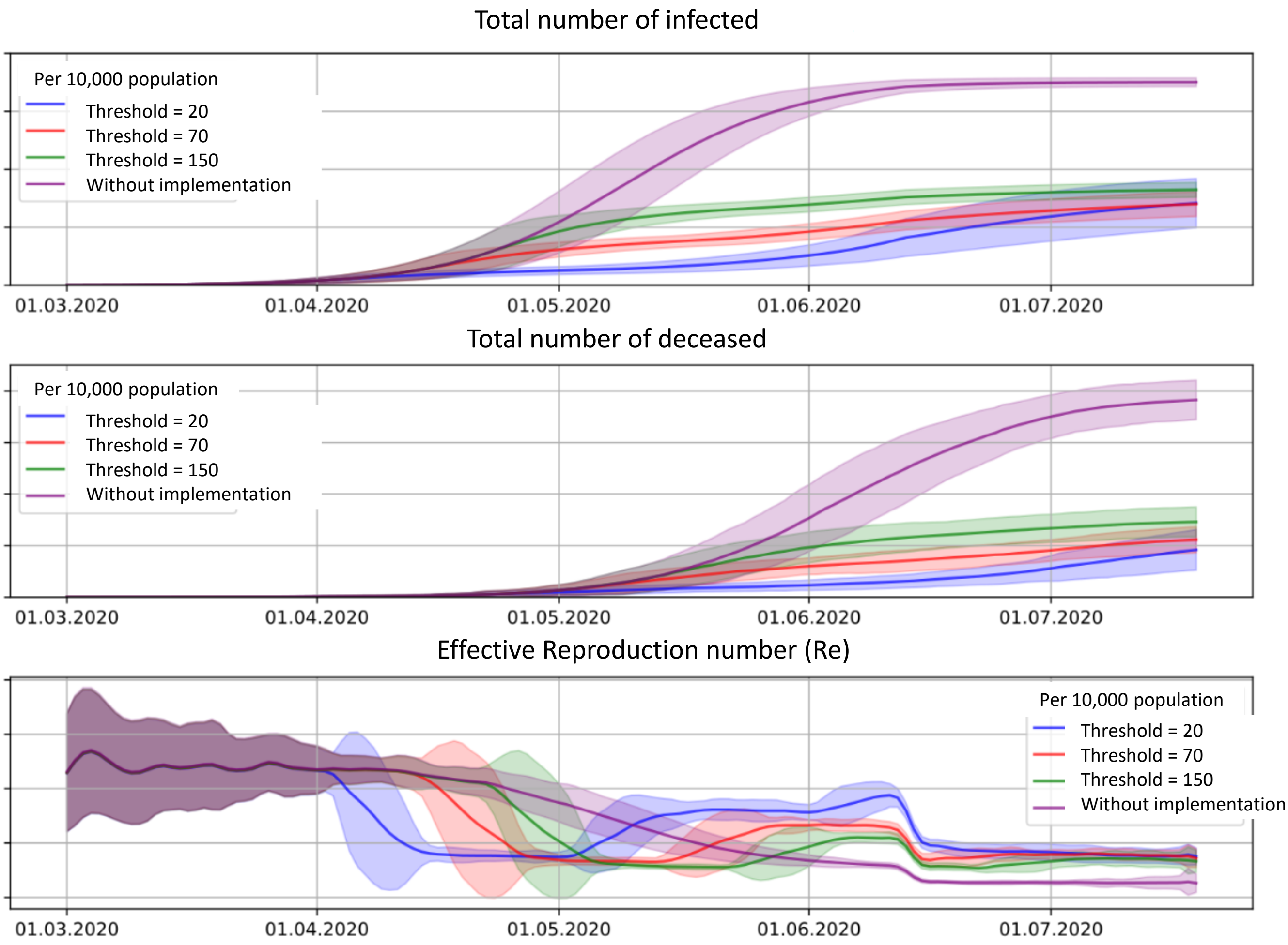

Fig. 4. The impact of implementing epidemic control measures upon reaching the threshold of registered cases on incidence and mortality.

The blue line on the graph shows the simulation results when measures are implemented upon reaching a threshold of 20 diagnosed cases, the red line shows the results when the threshold is 70, the green line shows the results when the threshold is 150 diagnosed cases, and the purple line represents the modeling of the epidemic in the city without the implementation of measures.

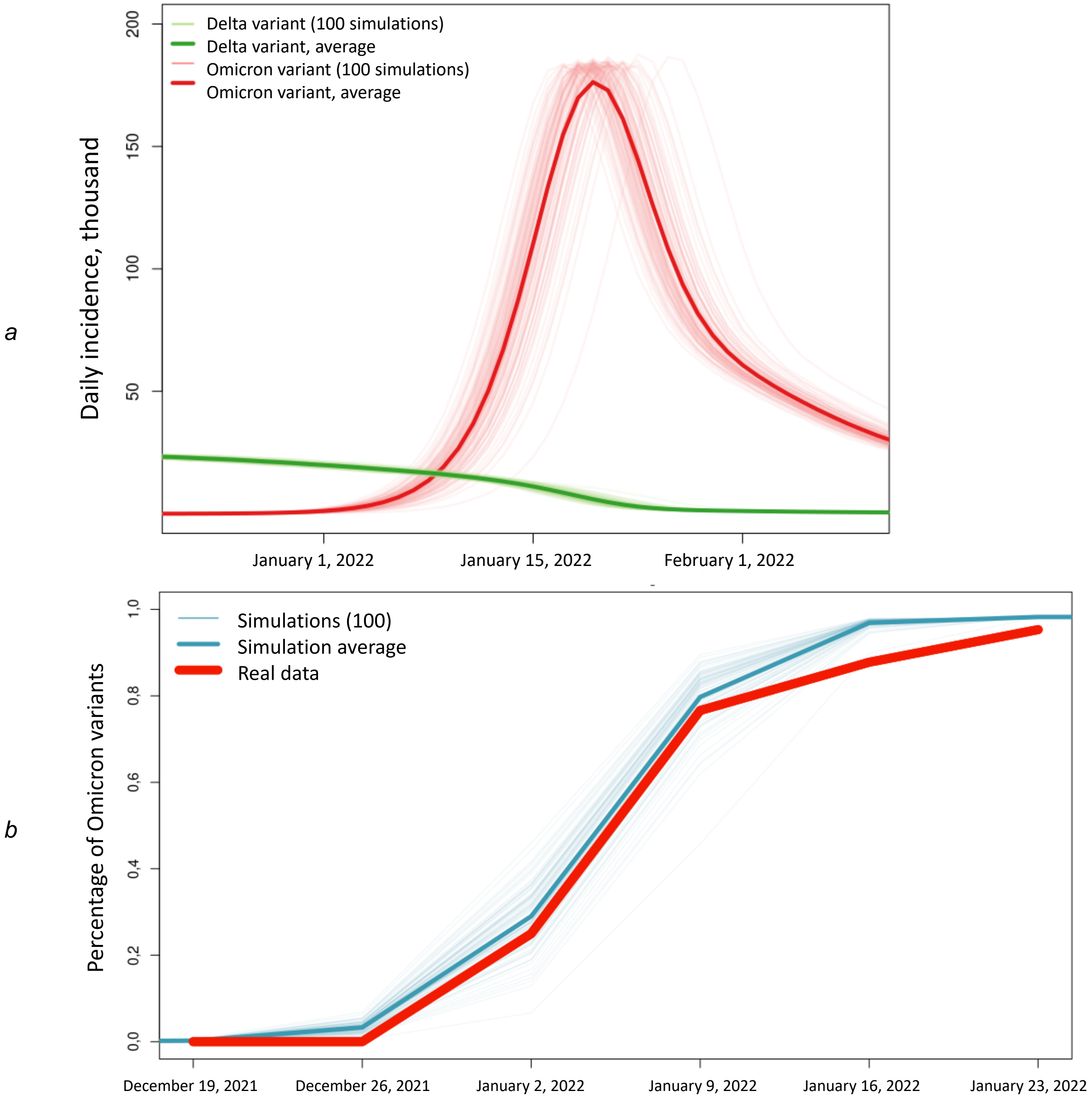

Fig. 5. Simulation of the dominant SARS-CoV-2 variant change in the Voronezh region in the winter of 2021–2022.

a — graph of the daily number of infected individuals, broken down by SARS-CoV-2 variant. The Delta variant is marked in green, and the Omicron variant is marked in red. The daily number of infections in 100 simulations is shown in pale colors, and the average values are shown in bright colors;

b — the proportion of the Omicron variant among the SARS-CoV-2 circulating in the Voronezh region. The values calculated from the results of 100 simulations are shown in light blue, the average value is in dark blue, and the real data from the whole-genome sequencing analysis results are in red.

platform makes it possible to select the most effective measures, taking into account limited administrative resources. The Covasim model was developed with a focus on performance, ease of use and flexibility: realistic scenarios can be run on a standard laptop in less than a minute [3]. In the POEM system, modeling an epidemic in a population of 100,000 people over 180 days and generating all the graphs takes 30 seconds on a single-core processor. Simulating a population of 1 million people over 180 days takes 6 minutes.

The limitation of the POEM platform is the lack of epidemic development prediction functions. There are studies where this possibility is implemented by dynamically adjusting the virus transmission efficiency based on current morbidity data. This approach can be quite effective for short-term (around 2 weeks) disease prediction, but the primary goal of this work was to reproduce the population and epidemic spread in as much detail as possible to develop epidemic control strategies. Dynamically adjusting the model is an easy way to compensate for model imperfections, but it can reduce its value as a planning tool.

Conclusion

POEM is the first platform in Russia for agent-based modeling of epidemics, which is expected to be accessible to a wide range of professional epidemiologists in the future. The model flexibility allows for virtually unlimited scaling of the platform by adding regions and traffic flows based on demographic and statistical data, defining new variants of the coronavirus and other pathogens. The model is fully implemented within the Russian framework on the server of the Research Institute of System Biology and Medicine of Rospotrebnadzor and can be used to conduct digital epidemiological experiments to predict the effectiveness of anti-epidemic measures in the Russian Federation.

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Association of *TRIM22* gene polymorphisms (*N155D* and *T242R*) with HIV infection in the Northwestern Federal District of Russia

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Abstract

Introduction. The human immunodeficiency virus (HIV) remains a global health challenge. The *TRIM22* gene, which encodes a protein with antiviral activity, is a promising candidate for research, but its role in the pathogenesis of HIV infection in population of the Russian Federation has not been previously studied.

The aim of the study was to analyze the polymorphic variants rs7935564 (*N155D*) and rs1063303 (*T242R*) of the *TRIM22* gene in HIV-infected individuals in the Northwestern Federal District.

Materials and methods. Polymorphic variants rs7935564 (*N155D*) and rs1063303 (*T242R*) of the *TRIM22* gene were analyzed in groups of HIV-infected individuals with virological failure of antiretroviral therapy (n = 378) and practically healthy individuals (n = 319). Genotyping was performed using the polymerase chain reaction method followed by sequencing. Statistical analysis included testing for Hardy-Weinberg equilibrium of genotype distributions, assessing associations under 3 inheritance models (dominant, recessive, additive) with odds ratio (OR) and 95% confidence interval (CI) calculation, linkage disequilibrium analysis, and haplotype frequency analysis.

Results. The distribution of genotypes for the analyzed polymorphic variants was conformed to Hardy-Weinberg equilibrium expectations ($p > 0.05$). A significant association was found between the G allele of the rs7935564 polymorphism and the presence of HIV infection in both recessive (OR = 1.76) and additive (OR = 1.37) inheritance models. For polymorphism rs1063303, a significant association was observed only in the dominant model (OR = 1.40). Moderate linkage disequilibrium was found between the loci (D' = 0.4478; $r^2 = 0.1572$; $p < 0.001$). The *G-G* haplotype (rs7935564_G — rs1063303_G) was associated with the presence of infection (OR = 1.57).

Conclusion. In the Russian population, the polymorphic variant rs7935564 (*N155D*) of the *TRIM22* gene is a significant genetic factor associated with HIV infection, while the results for rs1063303 (*T242R*) were statistically ambiguous. The association of the *G-G* haplotype with the presence of infection suggests a potential synergistic effect of these alleles. The data obtained highlight the importance of considering a population genetic background when evaluating the genetic determinants of the interaction between HIV and the host organism.

Keywords: *HIV infection, virus-host interaction, TRIM22, polymorphism, prognostic markers, laboratory diagnostics*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Saint-Petersburg Pasteur Institute (protocol No. 110/a, November 27, 2020).

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Оригинальное исследование
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Ассоциация полиморфных вариантов гена *TRIM22* (*N155D* и *T242R*) с ВИЧ-инфекцией в Северо-Западном федеральном округе России

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Аннотация

Введение. Вирус иммунодефицита человека (ВИЧ) остается глобальной проблемой здравоохранения. Ген *TRIM22*, кодирующий белок с противовирусной активностью, является перспективным кандидатом для исследований, однако его роль в патогенезе ВИЧ-инфекции в популяциях Российской Федерации ранее не изучалась.

Целью работы был анализ полиморфных вариантов rs7935564 (*N155D*) и rs1063303 (*T242R*) гена *TRIM22* у ВИЧ-инфицированных лиц в Северо-Западном федеральном округе.

Материалы и методы. Проведён анализ полиморфных вариантов rs7935564 (*N155D*) и rs1063303 (*T242R*) гена *TRIM22* в группах ВИЧ-инфицированных лиц с вирусологической неэффективностью антиретровирусной терапии (n = 378) и практически здоровых лиц (n = 319). Генотипирование выполнено методом полимеразной цепной реакции с последующим секвенированием. Статистический анализ включал проверку соответствия распределения генотипов равновесию Харди–Вайнберга, оценку ассоциаций по 3 моделям наследования (доминантной, рецессивной, аддитивной) с расчётом отношения шансов (ОШ) и 95% доверительного интервала (ДИ), анализ неравновесия сцепления и частот гаплотипов.

Результаты. Распределение генотипов для анализируемых полиморфных вариантов соответствовало равновесию Харди–Вайнберга (p > 0,05). Выявлена достоверная ассоциация аллеля *G* полиморфизма rs7935564 с наличием ВИЧ-инфекции в рецессивной (ОШ = 1,76) и аддитивной (ОШ = 1,37) моделях наследования. Для полиморфизма rs1063303 значимая ассоциация наблюдалась только в доминантной модели (ОШ = 1,40). Обнаружено умеренное неравновесие сцепления между локусами (D’ = 0,4478; r^2 = 0,1572; p < 0,001). Гаплотип *G-G* (rs7935564_G — rs1063303_G) был ассоциирован с наличием инфекции (ОШ = 1,57).

Заключение. В российской популяции полиморфный вариант rs7935564 (*N155D*) гена *TRIM22* является значимым генетическим фактором ассоциации с ВИЧ-инфекцией, в то время как для rs1063303 (*T242R*) получены статистически неоднозначные результаты. Ассоциация гаплотипа *G-G* с наличием инфекции свидетельствует о потенциальном синергетическом эффекте данных аллелей. Полученные данные подчёркивают важность учёта популяционной специфики при оценке генетических детерминант взаимодействия ВИЧ и организма-хозяина.

Ключевые слова: *ВИЧ-инфекция, взаимодействие вирус–хозяин, TRIM22, полиморфизм, прогностические маркеры, лабораторная диагностика*

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

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Introduction

The human immunodeficiency virus (HIV) continues to be one of the most significant global public health challenges. According to the latest epidemiological data[1], over 40 million people are living with this infection in 2024. Despite significant advancements in the development of antiretroviral therapy (ART), which has transformed the infection from a death sentence into a manageable chronic disease, complete eradication of the virus from the body remains an unattainable goal [1]. One of the key obstacles along this path is the virus's high genetic variability and the complex nature of its interaction with the host's immune system [2]. The most important component of innate immunity against viral infections is interferon-stimulated genes, whose products form a multi-component antiviral system [3]. In recent years, increasing attention has been given to the role of cellular restriction factors among these components, which represent an evolutionarily ancient first line of defense against pathogens [4]. These constitutively expressed proteins are capable of recognizing and directly inhibiting viral replication in the early stages of its life cycle, before the adaptive immune response is triggered.

Among the numerous factors of innate immunity, the TRIM (Tripartite Motif) family is of particular interest. It encompasses proteins with E3 ubiquitin ligase activity that are involved in various cellular processes, including proliferation, apoptosis, autophagy, and antiviral defense [5, 6]. The key mechanism of their action is the ubiquitin-mediated degradation of viral components or the activation of signaling pathways leading to the synthesis of type I interferons and pro-inflammatory cytokines [7, 8]. Members of this family, such as TRIM5α, are well-known for their ability to suppress retroviruses, including HIV, by specifically binding to the viral capsid and prematurely destabilizing it [9]. However, another member of this family, TRIM22, located on chromosome 11 in a cluster with other TRIM family genes, is attracting increasing attention as a potent regulator of the antiviral response, although its role in HIV infection has been much less studied and remains controversial [10]. It has been shown that TRIM22 is capable of suppressing the replication of a wide range of viruses, but data on the function of this protein in relation to HIV are complex and often contradictory. Several studies demonstrate its direct antiviral activity. Thus, it has been established that TRIM22 can inhibit HIV-1 transcription by suppressing the activity of its long terminal repeats (LTR), as well as disrupt virion processing and assembly thru interaction with the Gag precursor protein [12]. Furthermore, TRIM22 expression is negatively correlated with viral load in patients, and its overexpression *in vitro* leads to effective suppression of HIV-1 replication [11, 12]. These data suggest that TRIM22 is an important component of cellular defense against HIV. On the other hand, there is evidence of a potentially proviral role for TRIM22 in certain contexts. Some studies suggest that TRIM22 may enhance the pro-inflammatory response thru NF-κB activation, which could theoretically contribute to chronic immune activation and disease progression [13]. Furthermore, there are suggestions that depending on the cell type and phase of infection, TRIM22 can have opposing effects, acting either as a restriction factor or as a modulator of the immune response, indirectly influencing viral persistence [14]. This ambiguity highlights the complexity of the host immune system's functioning and the necessity for further research to elucidate the precise role of TRIM22 in HIV infection.

An important aspect determining the functional activity of TRIM family proteins is their genetic polymorphism. Single nucleotide polymorphisms (SNPs) in the coding and regulatory regions of the *TRIM22* gene can affect its expression level, protein stability, or functionality, thereby modulating individual susceptibility to infection and disease progression rate. However, the results of studies on the association between *TRIM22* polymorphism and HIV infection are often contradictory in populations with different genetic backgrounds. Ethnic differences in allele frequencies, haplotype structure and the presence of specific genetic modifiers can profoundly influence the phenotypic manifestations of polymorphic variants. Therefore, data obtained from Asian, African or Western European populations cannot be automatically extrapolated to the Russian population, which is characterized by a unique and complex genetic landscape shaped by centuries of mixing of Slavic, Finno-Ugric, Turkic and other groups [15]. Specifically, populations in the Northwestern Federal District (NWFD) of Russia exhibit a distinct genetic profile within the country, making them a particularly interesting subject for population genetic research [16]. Furthermore, the HIV strains circulating in the region are predominantly genotype A6 [17, 18], which has a limited global distribution and is characterized by certain molecular biological features. The reciprocal adaptation that occurs between the genetic background of a specific human population and the viral variants circulating within it shapes a unique evolutionary dynamic, the understanding of which requires local regional studies. Despite its obvious importance and potential clinical significance, the analysis of associations between TRIM22 gene polymorphisms and HIV infection in Russian populations, particularly in the North-Western Federal District, has been virtually non-existent until now. Filling this gap is a necessary step toward a deeper understanding of the pathogenesis of HIV infection, identifying genetic markers of individual risk, and, in the future, developing personalized approaches to the prevention and treatment of this disease. In

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[1] Global HIV & AIDS statistics — Fact sheet / UNAIDS 2024 epidemiological estimates. URL: https://www.unaids.org/en/resources/fact-sheet (data of access: 14.08.2025).

light of the above, the polymorphic variants rs7935564 (A>G N155D) and rs1063303 (C>G T242R) of the *TRIM22* gene, previously characterized in other geographical regions, are of particular interest.

The aim of the study was to analyze the polymorphic variants rs7935564 (*N155D*) and rs1063303 (*T242R*) of the *TRIM22* gene in HIV-infected individuals in the Northwest Federal District.

Materials and methods

Whole blood samples were used as material for the study, obtained from 378 HIV-infected patients experiencing virological failure of ART and 319 practically healthy individuals without acute or chronic infectious or somatic diseases at the time of examination, permanently residing in the Northwestern Federal District. In the control group, the age ranged from 18 to 60 years, with an average of 38.9 years; there were slightly more men (*n* = 166; 52.04%) than women (*n* = 153; 47.96%). In the group of HIV-infected individuals, the age ranged from 18 to 73 years, with an average of 38.3 years; the proportion of men (*n* = 240; 63.49%) was significantly higher than that of women (*n* = 138; 36.51%).

An important aspect of sample formation was the control of HIV infection risk factors. All participants in the study (both the target and control groups) are natives and residents of the Northwestern Federal District, deny involvement in any high-risk groups for HIV infection (injection drug users, commercial sex workers, men who have sex with men, individuals who have sexual contact with representatives of key risk groups), and also practice risky sexual behavior (inconsistent use of barrier contraception, multiple sexual partners), which suggests a common social and epidemiological environment. This approach was aimed at minimizing potential bias associated with unequal probability of encountering the virus in the compared groups, and allows for a more confident interpretation of the identified associations as being related specifically to genetic characteristics rather than behavioral factors.

The choice of patients with virological failure of ART as the target group was due to the necessity to ensure the inclusion of individuals with a confirmed diagnosis of HIV infection in the analysis, excluding possible errors or undetected seroreversion. This cohort of patients is under constant observation, which ensured the reliability of diagnosis verification and the availability of necessary biological samples.

All participants were informed about the purpose and methodology of the study and signed informed consent. A positive decision for conducting this study was obtained from the local Ethics Committee of the Pasteur St. Petersburg Research Institute of Epidemiology and Microbiology (protocol No. 110/a dated November 27, 2020).

Total DNA/RNA extraction was performed using the RIBO-prep reagent kit (Central Research Institute of Epidemiology) with the use of the Hemolytic reagent. Primers described previously [19, 20] were used to amplify gene fragments containing the target loci of *TRIM22* (rs7935564 A>G *N155D*; rs1063303 C>G *T242R*). The amplification mixture consisted of a buffer solution containing Tris-HCl pH 8.8 (at 25°C), KCl, 6–7 mM $MgCl_2$, deoxynucleoside triphosphates, glycerol, Tween-20 and Taq+Phusion polymerases. PCR was performed under the following conditions: after denaturation at 95°C for 15 minutes, 45 amplification cycles were set up in the following mode: 95°C for 30 seconds, 52–64°C for 30 seconds, 72°C for 1 minute 30 seconds — 3 minutes 30 seconds; then final elongation at 72°C for 10 minutes. The quality of amplification was determined visually in a 2% agarose gel (120 V, 40 min; 1xTBE) stained with ethidium bromide, using a gel documentation system and subsequent analysis for the presence of target fragments and their length.

Amplification products, as well as the subsequent sequencing reaction products, were purified by ethanol precipitation. The purified fragment with a concentration of 50–100 ng, depending on the nucleotide composition of the analyzed region, was used to set up sequencing reactions with forward and reverse amplification primers. The sequencing reaction was performed using the ABI PRISM BigDye Terminator v. 3.1 reagent kit (Applied Biosystems). The obtained fragments of the analyzed samples were sequenced using the ABI PRISM 3500 genetic analyzer (Applied Biosystems).

Statistical data processing was performed using licensed software: MS Excel (Microsoft) and Prizm 9.5.1 (GraphPad Software Inc.). We checked the genotype distribution for compliance with the Hardy–Weinberg principle. To assess the significance of the differences, depending on the characteristics of the samples, Fisher's exact test or the χ^2 test with Yates' correction were used, and the odds ratio (OR) with a 95% confidence interval (CI) was calculated. Additionally, the values of the non-equilibrium linkage coefficient were assessed, and haplotype frequencies were calculated. The significance level for differences was set at a probability value of $p < 0.05$.

Results

The distribution of genotypes and allele frequencies for the studied polymorphic variants rs7935564 (A>G *N155D*) and rs1063303 (C>G *T242R*) of the *TRIM22* gene, with an assessment of Hardy–Weinberg equilibrium compliance, is presented in **Table 1**.

In the analyzed groups, the distribution of genotypes for all polymorphic variants conformed to Hardy–Weinberg equilibrium expectations ($p > 0.05$), indicating the absence of significant deviations caused by selection, migration, or inbreeding, confirming the representativeness of the formed samples and the reliability of the obtained genetic data.

Based on the data obtained, the odds ratio (OR) was calculated within the framework of 3 alternative inheritance models: recessive, dominant and additive, to comprehensively assess the association between the studied polymorphic variants and the presence of HIV infection. The results are presented in **Table 2**. We assessed the equilibrium/disequilibrium of the analyzed loci: D = 0.0969; D' = 0.4478; $r^2 = 0.1572$; $p < 0.001$. Thus, moderate linkage disequilibrium is shown for the two analyzed SNPs in the study groups.

The frequency of haplotypes in the groups was estimated and their association with HIV infection was analyzed (**Table 3**).

Discussion

The TRIM22 protein exhibits a complex and multi-level mechanism of antiviral activity, particularly against HIV. Its expression in peripheral blood lymphocytes and its constitutive presence in a number of human tissues are induced by both type I and type II inter-

Table 1. Distribution of genotype and allele frequencies, and assessment of conformity to Hardy–Weinberg equilibrium

Polymorphism	Genotypes, alleles	Control group (*n* = 319)			HIV-infected individuals (*n* = 378)		
		Distribution of genotypes, alleles		p_{HWE}	Distribution of genotypes, alleles		p_{HWE}
		n	%		*n*	%	
rs7935564 (A>G *N155D*)	*A/A*	103	32.29	0.3	98	25.93	0.68
	A/G	165	51.72		195	51.59	
	G/G	51	15.99		95	25.13	
	A	0.58			0.5		
	G	0.42			0.5		
rs1063303 (C>G *T242R*)	*C/C*	127	39.81	0.24	121	32.01	0.2
	C/G	140	43.89		197	52.12	
	G/G	52	16.3		60	15.87	
	C	0.62			0.58		
	G	0.38			0.42		

Note. p_{HWE} — significance level under Hardy–Weinberg equilibrium.

Table 2. Assessment of the association of polymorphic variants with HIV infection

Polymorphism	Model	Genotype	OR	95% CI	*p*
rs7935564 (A>G *N155D*)	Dominant	*A/A*	1		0.065
		A/G-G/G	1.36	0.98–1.89	
	Recessive	*A/A-A/G*	1		0.0029
		G/G	1.76	1.21–2.58	
	Additive		1.37	1.11–1.71	0.0035
rs1063303 (C>G *T242R*)	Dominant	*C/C*	1		0.032
		C/G-G/G	1.4	1.03–1.92	
	Recessive	*C/C-C/G*	1		0.88
		G/G	0.97	0.65–1.45	
	Additive		1.17	0.94–1.45	0.16

Table 3. Association of haplotypes rs7935564/rs1063303 with HIV infection

rs7935564	rs1063303	Control group	HIV-infected individuals	OR, 95% CI	*p*
A	*C*	0.454	0.3912	1	–
G	*G*	0.2518	0.3065	1.57 (1.16–2.12)	0.0033
G	*C*	0.1667	0.1895	1.21 (0.91–1.60)	0.2
A	*G*	0.1275	0.1128	0.89 (0.61–1.30)	0.55

ferons, and it is also modulated in response to various viral pathogens and their antigens [21]. One of the key mechanisms of TRIM22 antiviral action is the suppression of HIV replication, which has been demonstrated in models of promonocytic cell lines and primary macrophages differentiated from human monocytes. The molecular mechanisms of this suppression are multifaceted: TRIM22 is capable of inhibiting the basal activity of the HIV promoter without affecting Tat-dependent or NF-κB-mediated transactivation, but effectively blocking LTR-mediated and phorbol ester and ionomycin-induced viral gene expression.

Although TRIM22, like other members of the TRIM family, does not have the ability to directly bind to DNA, it mediates its effect by interfering with cellular transcription factors. Specifically, it has been shown that TRIM22 disrupts the binding of the transcription factor Sp1 to its consensus sites in the HIV LTR, which is explained by the coiled-coil (CC) ability of the domain of these proteins to engage in heterotypic protein-protein interactions [22]. The two-domain organization of TRIM22 defines the duality of its functions: the N-terminal RING domain exhibits E3 ubiquitin ligase activity, leading to the polyubiquitination of viral target proteins and their subsequent degradation via the proteasomal pathway [23]. At the same time, the C-terminal CC domain is involved in the formation of high-molecular-weight protein complexes, mediating more complex and less studied restriction mechanisms that may include: disruption of virion assembly, interference with intracellular transport of viral components, or modulation of innate immunity signaling pathways [24]. It is important to note that this process is not limited to the direct degradation of targets, but also mediates the activation of key components of the innate immune system. Specifically, it has been shown that oligomerized TRIM22 is able to specifically activate NOD2 (nucleotide-binding oligomerization domain-containing protein 2), an intracellular pattern recognition receptor that plays a central role in initiating the pro-inflammatory response upon pathogen detection [25]. Activation of NOD2 triggers a signaling cascade leading to the activation of NF-κB and the production of pro-inflammatory cytokines, which enhances the cell's antimicrobial defense. Thus, amino acid substitutions in the coiled-coil domain caused by the polymorphic variants studied in this work could potentially disrupt the TRIM22 multimerization process, which in turn could alter its polyubiquitination ability and weaken the activation of the NOD2-mediated immune response, reducing the effectiveness of antiviral defense [26]. *In vitro* data indicate that these amino acid substitutions are associated with reduced protein suppressor activity against HIV transcription and correlate with a more severe disease course in infected individuals [10], suggesting their critical role in the protein's functional integrity and its ability to coordinate a multi-component antiviral response.

The conducted study represents the first analysis of the association between non-synonymous polymorphic variants of the *TRIM22* gene, rs7935564 (*N155D*) and rs1063303 (*T242R*), and HIV infection in the population of the Northwest Federal District. The data obtained revealed a complex and multifaceted nature of the association between the studied genetic variants and the presence of HIV infection.

The key finding of this study is the identification of a significant association between rs7935564 and the presence of HIV infection. Compelling evidence has been obtained for the association of the *G* allele with the presence of HIV infection. This is indicated by the significance in the most powerful additive model (OR = 1.37; 95% CI 1.11–1.71; $p = 0.0035$), as well as in the recessive model (OR = 1.76; 95% CI 1.21–2.58; $p = 0.0029$). This suggests that the effect of this variant is most pronounced in the homozygous state (*G/G*), which may be related to a more significant change in the charge and structure of the protein at position 155, which is critical for its function. Our results are consistent with the findings of a study in an Italian cohort, where the *G* allele of the TRIM22 gene rs7935564 was linked to HIV infection [10]. It is interesting to note that in the cited study, the most significant association with rapid HIV infection progression was identified within a dominant inheritance model.

The observed discrepancy with our data, in which the maximum association was found for the recessive model, may have several explanations. Firstly, fundamental differences in the genetic background between the Italian and Northwest Russian populations can significantly influence patterns of genetic associations, including the inheritance model, which is consistent with the well-known phenomenon of population specificity in the genomic architecture of complex traits. Secondly, the study design appears to be a critical factor: unlike our work, which analyzed the overall group of HIV-infected individuals with virological failure of ART, the study by S. Ghezzi et al. stratified patients into subgroups with different rates of disease progression. This approach allows for the identification of genetic determinants that specifically influence the rate of disease progression, which may remain unnoticed when analyzing an unstratified cohort. Additionally, it cannot be ruled out that different inheritance patterns reflect the pleiotropic effect of the studied polymorphism, which manifests differently at various stages of the infectious process — from initial infection to the late stages of immunopathology.

For the rs1063303 (*T242R*) polymorphism, our results are less conclusive. Despite the identification of a weak association in the dominant model (OR = 1.40; 95% CI 1.03–1.92; $p = 0.032$), the lack of significance in the additive and recessive models, as well as the wide confidence interval crossing unity, do not allow for a definitive conclusion about its independent role in the studied sample. Similar studies in other geographical regions have yielded conflicting results. Thus, in the aforementioned study of HIV progressors in Italy, no association was found between rs1063303 and the disease [10]. At the same time, in the aforementioned study, the haplotype rs7935564 G + rs1063303 G showed a significantly higher prevalence among HIV-infected individuals with rapid disease progression compared to those with slow progression. Additionally, it is known that the rs1063303 polymorphism has the opposite functional effect, increasing TRIM22 expression and decreasing its antiviral activity [27], and increased TRIM22 expression in peripheral blood mononuclear cells of HIV-infected patients is associated with a lower viral load [28].

An important aspect of our analysis was the assessment of linkage disequilibrium between the loci studied. The observed values (D' = 0.4478; $r^2 = 0.1572$) indicate the presence of moderate but statistically significant historical linkage, which, however, is not sufficient to consider these polymorphisms markers for each other. The low r^2 value, in particular, indicates that only about 15% of the variation in one locus can be explained by variations in another. This confirms that the identified association for rs1063303 is likely independent and not mediated thru linkage with rs7935564. It should be noted that according to open genetic databases, such as the 1000 Genomes Project [29], the analyzed SNP show linkage disequilibrium ranging from weak to moderate. Strong linkage disequilibrium (D' values can be high, ~0.8–1.0) can be expected in East Asian populations, but even here the r^2 value (which is most important for prediction) often remains low (< 0.3), meaning that despite a historical tendency for co-inheritance, one SNP cannot be reliably predicted from another. In European populations, linkage is weaker (r^2 values are often low, < 0.2) or absent [10]. Despite weak or moderate linkage, the "preferred" (co-occurring alleles) haplotype rs7935564 - rs1063303 is *A-C*, while the haplotype associated with the presence of HIV infection is G-G. In the current study, haplotype *A-C* was the most common in both the control and target groups. However, we did not find a logical protective effect of this haplotype against HIV infection; only a trend toward a protective effect was shown, which did not reach statistical significance. Conversely, for the "risk haplotype" *G-G*, an association with HIV infection was confirmed (OR = 1.57; 95% CI 1.16–2.12; $p = 0.0033$), indicating a possible synergistic effect of the two amino acid substitutions. This haplotype may determine a protein conformation that is less effective at suppressing HIV replication or modulates the immune response in a way that indirectly contributes to viral persistence.

A limitation of this study is its case-control design, which does not allow us to establish causality, as well as the relatively small sample size, which may have limited the power to detect weak associations, particularly for rs1063303. The results illustrate the phenomenon of population-specific genetic associations, which is widely recognized in modern complex disease genetics [30, 31]. This highlights the necessity for further longitudinal studies with increased sample sizes and thorough clinical-genetic comparisons to fully elucidate the role of *TRIM22* gene polymorphisms in the pathogenesis of HIV infection.

Conclusion

The study conducted demonstrates a significant association between the non-synonymous polymorphic variant rs7935564 (*N155D*) of the *TRIM22* gene and the presence of HIV infection in the population of the North-Western Federal District, while statistically ambiguous results were obtained for the variant rs1063303 (T242R). The identified association of haplotype *G-G* (rs7935564_G — rs1063303_G) with the presence of infection suggests a potential synergistic effect of these alleles. The obtained data contribute to understanding the molecular mechanisms of innate antiviral immunity, highlighting the role of the TRIM22 protein in modulating the virus-host interaction during HIV infection, and reflect the importance of considering population specificity of genetic factors when studying the interaction between HIV and the host organism.

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Genetic typing of DNA of isolates of *Coxiella burnetii* isolated from patients with Q fever in the Stavropol Territory

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Abstract

The aim of the study is to genetically type DNA of isolates of *Coxiella burnetii* obtained in the Stavropol Territory (ST) from 2009 to 2023, and to analyze the genetic structure of the Q fever pathogen population in the region.

Materials and methods. The study used blood sera from febrile patients obtained from the Center for Hygiene and Epidemiology in the Stavropol Territory between 2009 and 2023. MST typing of *C. burnetii* was performed, and the MST type was determined using an online resource (http://ifr48.timone.univ-mrs.fr). The phylogenetic tree was constructed using the MEGA software. Plasmid typing was performed using type-specific primers for the QpH1, QpRS and QpDV plasmid loci, and the resulting amplification products were visualized by electrophoresis in a 2% agarose gel. The territorial distribution of genetic variants was analyzed using ArcGiS 10.1 software.

Results. Molecular genetic typing of *C. burnetii* has established that strains of the pathogen of Q fever belonging to two genotypes, *MST7* and *MST28* of monophyletic group I, are circulating in the Stavropol Territory. The dominant genotype in the eastern regions of the Stavropol territory is *MST7*, and in the northern regions, it is *MST28*. The pathogen of Q fever circulating in the Stavropol Territory is of a single plasmid type, QpH1.

Conclusion. Determining the plasmid and MST type allows for the genotyping of *C. burnetii* DNA isolates without isolating a pure culture, which can be helpful in outbreak investigations and the creation of a regional DNA isolate database.

Keywords: *Q fever, Coxiella burnetii, multi-spacer typing, plasmid typing, Stavropol Krai*

Ethics approval. The study was conducted with the informed consent of the patients. The study protocol was approved by the Local Ethics Committee of the Stavropol State Medical University (protocol No. 112, May 05, 2023).

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Оригинальное исследование
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Генетическое типирование ДНК-изолятов *Coxiella burnetii*, выделенных от больных лихорадкой Ку в Ставропольском крае

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Аннотация

Цель работы — генетическое типирование ДНК-изолятов *Coxiella burnetii,* выделенных на территории Ставропольского края (СК) в 2009–2023 гг., анализ генетической структуры популяции возбудителя лихорадки Ку в регионе.

Материалы и методы. В работе использовали сыворотки крови от лихорадящих больных, полученные из Центра гигиены и эпидемиологии в Ставропольском крае в 2009–2023 гг. Проводили мультиспейсерное типирование (MST) *C. burnetii*, определяли принадлежность к MST-типу с помощью интернет-ресурса

(http://ifr48.timone.univ-mrs.fr). Филогенетическое дерево строили в программе «MEGA». Плазмидное типирование осуществляли с использованием типоспецифичных праймеров к локусам плазмид QpH1, QpRS, QpDV, визуализацию полученных продуктов амплификации — путём проведения электрофореза в 2% агарозном геле. Территориальное распространение генетических вариантов анализировали с использованием программного обеспечения ArcGiS 10.1.

Результаты. В ходе молекулярно-генетического типирования *C. burnetii* установлено, что на территории СК циркулируют штаммы возбудителя коксиеллёза, относящиеся к двум генотипам: *MST7* и *MST28* монофилетической группы I. Доминирующим генотипом на территории восточных районов СК является *MST7*, северных районов — *MST28*. На территории СК циркулирует возбудитель лихорадки Ку с единственным плазмидным типом — QpH1.

Заключение. Определение плазмидного и MST-типа позволяет генотипировать ДНК изоляты *C. burnetii* без выделения чистой культуры, что может помочь при расследовании вспышек и формировании базы данных региональных ДНК изолятов.

Ключевые слова: *лихорадка Ку, Coxiella burnetii, мультиспейсерное типирование, плазмидное типирование, Ставропольский край*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен локальным этическим комитетом Ставропольского государственного медицинского университета (заключение № 112 от 19.05.2023).

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

The Q fever is a naturally occurring zoonotic disease characterized by polymorphic clinical symptoms, capable of causing severe complications in humans and becoming chronic.

The source of human infection is usually small and large cattle. The main routes of infection are aspiration and contact, and to a lesser extent, alimentary. The transmission factors for the pathogen are raw animal products, meat and dairy products, animal care items, and other objects infected with *Coxiella* [1, 2].

Currently, sporadic and outbreak cases of Q fever are observed in almost all regions of the world [3–9]. In Russia, coxiellosis is registered in more than 50 administrative territories. In the Stavropol Territory, the incidence of Q fever has been recorded annually since 2016. The regional incidence rate is 27.5 times higher than the national average and 3.6 times higher than the average for the North Caucasus Federal District[1].

Thus, the current epizootiological and epidemiological situation regarding coxiellosis necessitates the implementation of systematic monitoring of *Coxiella burnetii* circulation within the territory of the North Caucasus.

In recent years, the role of molecular genetic research has increased in monitoring the state of populations of highly dangerous pathogens and in epidemiological investigations of disease cases [10]. The differentiation of *C. burnetii* strains and the study of population structure are associated with difficulties due to the complexity of cultivating *Coxiella* in laboratory conditions, their high pathogenicity, and the low degree of genetic heterogeneity within the species [4].

A combination of methods is used for genetic typing of *C. burnetii* strains and isolates: restriction fragment length polymorphism analysis [11], pulsed-field gel electrophoresis [12–14], selective amplification of restriction enzyme-digested fragments [15], copy number typing of the mobile element IS1111 [16], and single nucleotide polymorphism typing [17].

Methods based on the analysis of variable number tandem repeats (VNTRs) in the genome [18], as well as the multi-spacer typing (MST) method [19], have the greatest differentiating power and versatility.

MST typing is based on the analysis of the sequence of intergenic regions located between open reading frames. The main advantage of the method is the use of potentially highly variable targets that are not subject to evolutionary pressure, which allows for better differentiation of strains within conservative biological species [19, 20]. Currently, the MST method is used in most laboratories worldwide for identifying *C. burnetii* strains and allows for intraspecies subtyping of

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[1] The State report "On the state of sanitary and epidemiological welfare of the population in the Stavropol Territory in 2023". Stavropol; 2023. 129 p. URL: https://26.rospotrebnadzor.ru/doc/gd/doklad23.pdf (In Russ.)

genetic variants of different geographical origin without culturing [21, 22].

The MST method can be successfully applied to trace the origin of the pathogen isolates, which will be most relevant in investigation of epidemic outbreaks of this infection. Accumulating information about the genetic characteristics of *C. burnetii* strains circulating in individual regions will contribute to improving the effectiveness of epidemiological surveillance for Q fever. Using the MST method for genotyping the Q fever pathogen isolated in the territory of the North Caucasus will provide new information about the genetic structure of the *C. burnetii* population in the region and form a database for epidemiological investigations of coxiellosis cases.

The aim of the study is to perform DNA typing of *C. burnetii* isolates obtained in the Stavropol Territory in 2009–2023 and to analyze the molecular genetic structure of the pathogen population in the region.

Materials and methods

A total of 1472 blood serum samples from febrile patients, obtained from the Center for Hygiene and Epidemiology in SK between 2009 and 2023, were tested for the presence of *C. burnetii* DNA. Work with clinical material was carried out with the voluntary informed consent of the patients.

DNA extraction from blood serum samples was performed using the RIBO-prep reagent kit (Central Research Institute of Epidemiology). The identification of *C. burnetii* DNA in the tested material was performed using the AmpliSens *Coxiella burnetii*-FL reagent kit (Central Research Institute of Epidemiology) according to MR 3.1.0281-22[2]. Plasmid profiling and MST genotyping were performed for positive samples with sufficient pathogen DNA concentration (Ct ≤ 25).

MST typing was performed according to the method proposed by O.O. Glazunova et al. [19]. For the amplification of the nucleotide sequences of each of the 10 non-coding intergenic spacers, a separate reaction mixture with a primer pair to a conserved region was used (**Table 1**). Amplification was performed using the BioMaster HS-Taq PCR-Color (2×) ready-to-use reaction mixture (Biolabmix). Reaction mixture composition: primer F (C = 10 pmol/µL) — 1.25 µL, primer R (C = 10 pmol/µL) — 1.25 µL, BioMaster HS-Taq PCR-Color (2×) mix — 12.5 µL, sample volume — 10 µL. PCR was performed on a T100 DNA thermocycler (Bio-Rad), with the following reaction profile: initial denaturation (95°C for 5 min); second stage for 40 cycles (95°C for 20 s, 57°C for 30 s, 72°C for 1 min); final elongation (72°C for 5 min). Sanger sequencing was performed on an Applied Biosystems 3500 capillary sequencer. Sequence assembly was performed using the VectorNTI program[3]. When determining the MST group, a comparison was made with the reference genomic sequences of 85 *C. burnetii* strains isolated between 1945 and 2025 in different regions from the online MST type database[4] (**Fig. 2**). The dendrogram based on the MST typing results was constructed using the MEGA 10 software[5].

Table 1. Primer sequences for MST typing

Locus/length of fragment, bp	Primer direction	Sequence (5'–3')
Cox2/397	forward	GAAGCTTCTGATAGGCGGGA
	reverse	CAACCCTGAATACCCAAGGA
Cox5/395	forward	TGGTATGACAACCCGTCATG
	reverse	CAGGAGCAAGCTTGAATGCG
Cox18/557	forward	TTCGATGATCCGATGGCCTT
	reverse	CGCAGACGAATTAGCCAATC
Cox20/631	forward	TCTATTATTGCAATGCAAGTGG
	reverse	GATATTTATCAGCGTCAAAGCAA
Cox22/383	forward	CGCAAATTTCGGCACAGACC
	reverse	GGGAATAAGAGAGTTAGCTCA
Cox37/463	forward	ATTCCGGGACCTTCGTTAAC
	reverse	GGCTTGTCTGGTGTAACTGT
Cox51/674	forward	GCGAGAACCGAATTGCTATC
	reverse	TAACGCCCGAGAGCTCAGAA
Cox56/479	forward	ATGCGCCAGAAACGCATAGG
	reverse	CAAGCTCTCTGTGCCCAAT
Cox57/617	forward	GGTGGAAGGCGTAAGCCTTT
	reverse	TGGAAATGGAAGGCGGATTC
Cox61/611	forward	GGGATTTCAACTTCCGATAGA
	reverse	GAAGATAGAGCGGCAAGGAT

The analysis of the territorial distribution of genetic variants and the creation of the map were performed using ArcGIS 10.1 software.

Plasmid typing was performed using the PCR method with type-specific primers for the QpH1, QpRS and QpDV plasmid loci (**Table 2**) using the BioMaster HS-Taq PCR-Color (2×) kit (Biolabmix). Reaction mixture composition: primer F (C = 10 pmol/µL) — 1.25 µL, primer R (C = 10 pmol/µL) — 1.25 µL, BioMaster HS-Taq PCR-Color (2×) mix — 12.5 µL, sample volume — 10 µL. PCR was performed on a T100 DNA thermocycler (Bio-Rad) using the following program: initial denaturation (95°C — 5 min), 40 cycles (95°C — 20 s; 56°C — 30 s; 72°C — 45 s); final elongation (72°C — 5 min). Visualization of the amplifi-

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[2] Methodological recommendations MR 3.1.0281-22 "Epidemiological surveillance, laboratory diagnostics and prevention of Ku fever" (approved by the Head of Rospotrebnadzor on 04.08.2022).

[3] URL: https://vector-nti.software.informer.com/

[4] URL: http://ifr48.timone.univ-mrs.fr

[5] URL: https://www.megasoftware.net/

Table 2. Primer sequences for plasmid type determination

Plasmid type	Primer direction	Sequence (5'–3')
QpH1	forward	CTCCAGTAGGGTAATGGTGTCA
	reverse	GCCTTGGCTGGCACCTG
QpRS	forward	ATGTCAACAGATGACTCATC
	reverse	CTAGGATAATGAGAGTCTATC
QpDV	forward	GAGTCTACTCAGTGATAG
	reverse	TTACCGGTATTTTCTCGA

cation products was performed by electrophoresis in a 2% agarose gel with an intercalating dye (ethidium bromide) [4].

Results

When examining 1472 blood serum samples from febrile patients in Stavropol Territory, *Coxiella burnetii* DNA was detected in 192 samples. Molecular genetic typing (belonging to MST genotypes and plasmid profile types) was performed for 60 DNA isolates of *C. burnetii* with a sufficient amount of the pathogen's target DNA for analysis (Ct ≤ 25). The obtained data were used to study the distribution of *Coxiella burnetii* gene variants in the Stavropol Territory from 2009 to 2023.

Genetic typing of C. burnetii isolates by MST method

As a result of the MST typing performed, the *C. burnetii* DNA isolates obtained from the territory of the North Caucasus were found to belong to two genotypes: MST7 (55 samples, 2009–2023) and MST28 (5 samples, 2010–2011, 2020). MST7 genotype DNA isolates were detected in 55 samples from Q fever patients in 14 districts of the Stavropol Territory: Budennovsk (15), Kursk (14), Ipatovo (4), Sovetsk (4), Shpakovskoe (4), Neftekumsk (3), Blagodarnoe (3), Apanasenkovskoe (1), Georgievsk (2), Krasnogvardeyskoe (1), Levokumskoe (1), Turkmenskoe (1), Kirovskoe (1), and Grachevskoe (1); **Fig. 1**. Five DNA isolates of *C. burnetii* from the Apanasenkovsky (2), Ipatovsky (2) and Neftekumsky (1) districts were assigned to the MST28 genotype.

The *C. burnetii* DNA isolates studied in this research belonged to monophyletic group I and were located on the dendrogram within two separate clades (**Fig. 2**).

Plasmid DNA typing of C. burnetii isolates

In addition to MST typing for all extracted DNA isolates, the plasmid profile was determined. It was established that the *Coxiella burnetii* pathogen, belonging to the QpH1 plasmid type, is circulating in the Stavropol Territory [23]. DNA isolates containing plasmids QpRS and QpDV were not detected in the samples studied.

Discussion

During the interpretation of the obtained data, it was found that the genetic structures of *C. burnetii* populations in the North Caucasus and other regions of the Russian Federation differ. Previous genetic studies of the Q fever pathogen strain collection isolated from environmental samples in the Leningrad and Pskov regions revealed that the dominant genotype of *C. burnetii* in the studied regions is MST23 (monophyletic group II) [4].

Analysis of information contained in the MST7 genotype database of *C. burnetii* showed that MST7 genotype strains (3 strains) were previously isolated from clinical material in Russia (Leningrad, 1955, 1957) and France (1993). *C. burnetii* strains of genotype MST28 (4 strains) were isolated from clinical material in Kazakhstan (1962, 1965).

MST7 genotype strains (monophyletic group I), which are widespread in the North Caucasus region, were first isolated in Russia during investigations into Q fever outbreaks in Leningrad in 1955 and 1957 and were previously considered unique to Russia, having been imported from Central Asian countries, as all those who contracted Q fever were workers involved in processing cotton brought from Central Asia, where Q fever was enzootic at the time [4, 24].

C. burnetii DNA isolates belonging to genotype MST28 were identified in Russia for the first time; strains of this genotype were also previously isolated in Central Asia (Kazakhstan).

All of the above allows us to hypothesize that both genotypes (MST7 and MST28) circulating in the Stavropol Territory were likely introduced to the region from Central Asia. However, due to the limited data on the genetic characteristics of *C. burnetii* strains prevalent in Russia and other regions of the world, further study is needed on the territorial distribution of *C. burnetii* genotypes in various regions of Russia, including the Astrakhan and Rostov regions, where a high incidence of Q fever is registered annually, as well as in Central Asian countries, to identify dominant genetic variants and establish probable routes of Q fever pathogen introduction into individual regions of the Russian Federation.

The new information obtained in this study on the distribution of *C. burnetii* strains belonging to genotypes MST7 and MST28 in the North Caucasus region can be used in the epidemiological investigation of Q fever outbreaks and sporadic cases in Russian regions.

Based on the data from the conducted study, it is suggested that the *C. burnetii* population in the North Caucasus is homogeneous in terms of plasmid type. It is known that belonging to the QpH1 plasmid type is characteristic of strains from different geographical origins. Strains and isolates with the QpH1 plasmid genotype have been previously isolated in Russia and in European countries (France, Switzerland, Germany, Romania, Slovakia, Greece, Czechoslovakia, Ukraine,

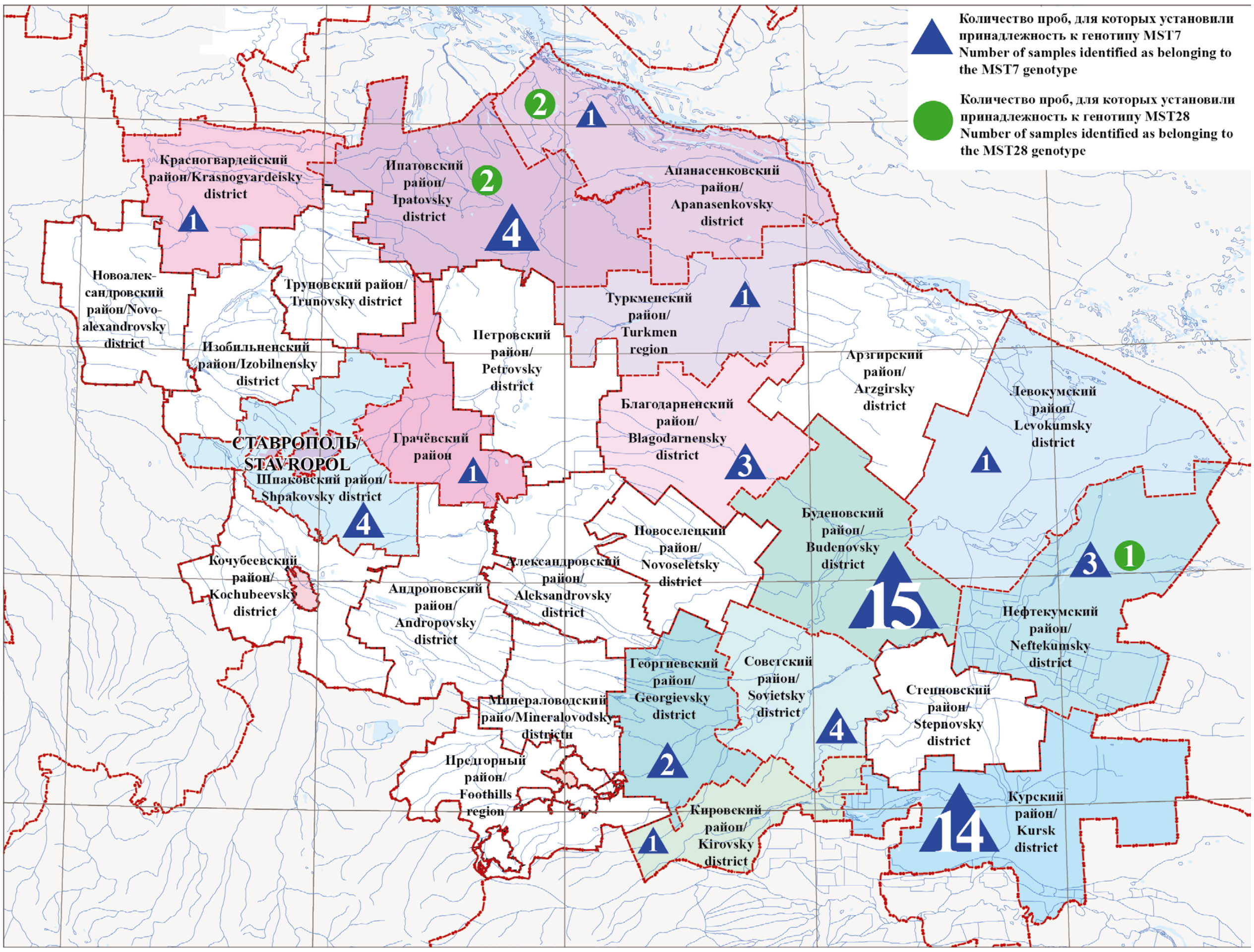

Fig. 1. Geographical distribution of C. burnetii MST genotypes in Stavropol Territory (2009–2023).

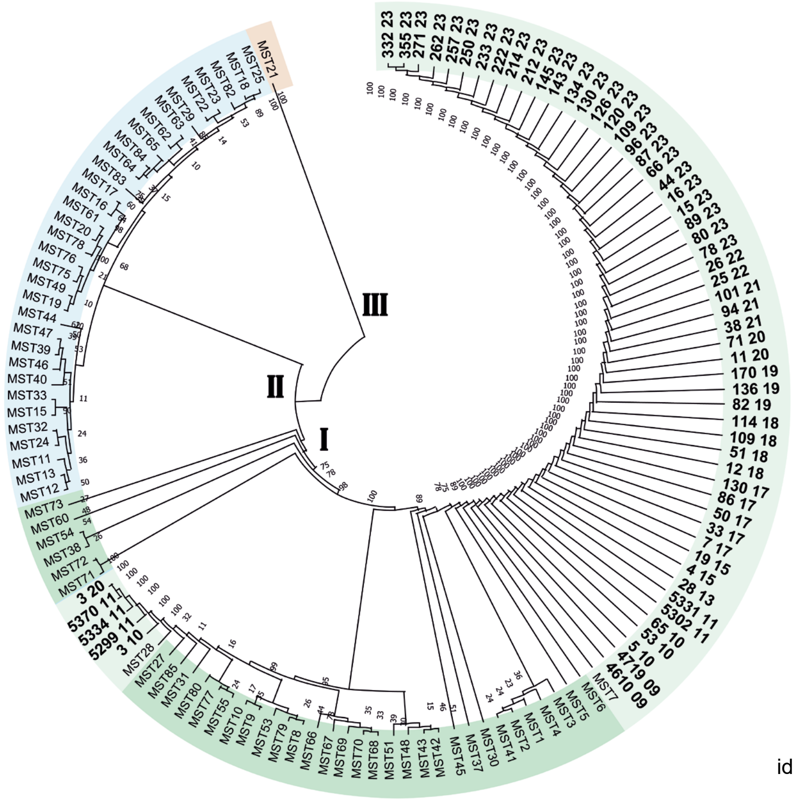

Fig. 2. Cluster analysis of 60 *C. burnetii* DNA isolates based on MST typing data.

The dendrogram was constructed using the UPGMA algorithm. Three monophyletic lineages (I, II, III) were identified. Isolates sequenced at the Stavropol Anti-Plague Institute of Rospotrebnadzor are marked in bold.

Austria), Central Asia (Uzbekistan, Kazakhstan, Mongolia), America (USA, Canada), and West Africa (Senegal and the Republic of Guinea).

When comparing the plasmid profiles and MST types of DNA isolates and strains from the online database identified in the Stavropol Territory, it was established that previously isolated strains of Q fever with MST7 (Russia, France) and MST28 (Kazakhstan) genotypes belonged to the QpRS plasmid type [19]. Conversely, the DNA isolates identified in this study with similar MST types belonged to the QpH1 plasmid type.

To date, plasmid genes definitively associated with pathogen virulence have not been identified. There is a proposal that strains with the QpH1 plasmid are more evolutionarily ancient representatives of the pathogen population circulating in natural foci, characterized by low epidemic potential [25]. Thus, the previously isolated strains of *C. burnetii* (Leningrad-2, Leningrad-4) that caused outbreaks in Leningrad (1955 and 1957) belonged to genotype MST7, plasmid type QpRS [24, 26]. At the same time, the *C. burnetii* strain NL3262, which caused a mass outbreak of Q fever in the Netherlands in 2007–2010 [1], belonged to the QpH1 plasmid type. Therefore, the assumption about the insufficient pathogenic potential of *C. burnetii* strains with the QpH1 plasmid type, which is necessary for the epidemic process to occur, requires further experimental and theoretical justification.

Conclusion

Molecular genetic typing of *C. burnetii* has established that the pathogen of Q fever circulating in the Stavropol Territory belongs to two genotypes: MST7 and MST28 of monophyletic group I. The dominant genotype in the eastern regions of the Stavropol Territory is MST7, while strains of genotype MST28 are prevalent in the northern regions of the territory. It was also established that Q fever pathogens with a single plasmid type, QpH1, circulated in the Stavropol Territory from 2009 to 2023.

The information obtained during this study on the *C. burnetii* genotypes found in the Stavropol Territory (MST and plasmid typing) will allow for an analysis of the genetic structure of the Q fever pathogen population and can be used in epidemiological investigations of potential outbreaks (imported cases) to determine the source and routes of infection spread.

Determining the plasmid and MST type allows for the genotyping of *C. burnetii* DNA isolates without isolating a pure culture, which can be helpful in outbreak investigations and the creation of a regional DNA isolate database.

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Suppression of biofilm formation and survival of clinical isolates of uropathogens within epithelial cells under the effect of Fluorothiazinone *in vitro*

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Abstract

The aim of this study was to investigate the effect of the innovative Russian antibacterial drug Fluorothiazinone, an inhibitor of the type III secretion system and flagellar function, on the virulence properties of urinary tract infection (UTI) pathogens.

Materials and methods. Clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* species were obtained from 4 patients with recurrent UTI. Cytotoxicity analysis was performed on HeLa cells using the CytoTox 96 kit. Bacterial flagellar motility was assessed in 0.25% agar by measuring the zone of motility. Biofilms were cultivated in 96-well plates and stained with crystal violet and Congo red. Biofilm structure was assessed by microscopy, and the bacterial biofilm and exopolysaccharide matrix were quantified by measuring the optical density of the dye bound to the biofilm. Intracellular development was studied in PC-3 cells by determining the number of intracellular bacteria using a cell lysate plating method and an immunochemical method involving staining the bacteria with specific antibodies.

Results. The Fluorothiazinone antibacterial specifically inhibited the cytotoxicity and motility of *E. coli* and *P. aeruginosa*. For *K. pneumoniae*, susceptibility to Fluorothiazinone was observed, associated with the suppression of cytotoxicity. For all the isolates studied, inhibition of biofilm formation on an abiotic surface was demonstrated. For *E. coli* and *K. pneumoniae*, a significant decrease in intracellular replication within human prostate adenocarcinoma cells and suppression of intracellular bacterial community formation were observed.

Conclusion. Fluorothiazinone disrupts mechanisms that contribute to pathogen persistence in the formation of chronic UTI, including biofilm formation on abiotic surfaces such as catheters, stents, and drains, and the formation of intracellular bacterial communities and dormant intracellular reservoirs.

Keywords: *Fluorothiazinone, Escherichia coli, Pseudomonas aeruginosa, Klebsiella, antibiotics, infectious diseases, intracellular bacterial communities, urinary tract infections, biofilms, flagellum, cytotoxicity*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol of a clinical trial No. 07-FT-2024, RCT No. 513 dated 10/30/2024.

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Оригинальное исследование
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Подавление образования биоплёнок и выживания в эпителиальных клетках клинических изолятов уропатогенов при действии препарата Fluorothiazinone *in vitro*

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Аннотация

Целью данного исследования было изучение влияния инновационного отечественного антибактериального препарата Fluorothiazinone, ингибитора системы секреции III типа и функционирования жгутика, на вирулентные свойства возбудителей инфекций мочевыводящих путей (ИМП).

Материалы и методы. Клинические изоляты видов *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* были получены от 4 пациентов с рецидивирующими инфекциями ИМП. Анализ цитотоксичности проводили на клетках HeLa с помощью набора «CytoTox 96». Жгутиковую подвижность бактерий оценивали в 0,25% агаре с измерением зоны подвижности. Биоплёнки культивировали в 96-луночных планшетах и окрашивали кристаллическим фиолетовым и Конго красным, оценивали структуру биоплёнок при микроскопировании и проводили количественную оценку бактериальной биоплёнки и экзополисахаридного матрикса при измерении оптической плотности связавшегося с биоплёнкой красителя. Внутриклеточное развитие изучали на клетках PC-3 при определении количества внутриклеточных бактерий методом высева лизатов клеток и иммунохимическим методом при окраске бактерий специфическими антителами.

Результаты. Препарат Fluorothiazinone специфически подавлял цитотоксичность и подвижность *E. coli* и *P. aeruginosa*. Для *K. pneumoniae* наблюдали чувствительность к препарату Fluorothiazinone, связанную с подавлением цитотоксичности. Для всех изученных изолятов было показано ингибирование образования биоплёнок на абиотической поверхности. Для *E. coli* и *K. pneumoniae* выявили достоверное снижение внутриклеточного размножения в клетках аденокарциномы простаты человека и подавление формирования внутриклеточных бактериальных сообществ.

Заключение. Препарат Fluorothiazinone нарушает механизмы, способствующие установлению персистенции патогенов при формировании хронических ИМП, среди которых образование биоплёнок на абиотических поверхностях, таких как катетеры, стенты, дренажи, и формирование внутриклеточных бактериальных сообществ и покоящихся внутриклеточных резервуаров.

Ключевые слова: *Fluorothiazinone, кишечная палочка, синегнойная палочка, клебсиелла, антибиотики, инфекционные заболевания, внутриклеточные бактериальные сообщества, инфекции мочевыводящих путей, биоплёнки, жгутик, цитотоксичность*

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

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Introduction

Recurrent urinary tract infections (UTIs) are characterized by the reappearance of an acute bacterial infection caused by the same pathogen. Recurrent UTIs (rUTIs) are typically defined as 2 or more episodes of uncomplicated UTIs in the past 6 months or 3 or more episodes in the past 12 months [1–4]. Every year, 5–10% of adult women suffer from UTIs [5], which affects treatment costs and reduces quality of life, especially considering that more than 60% of women are diagnosed with a UTI at least once in their lifetime [6, 7]. The pathogen of most UTIs is uropathogenic *Escherichia coli* (UPEC) [8–10]. However, *Klebsiella pneumoniae* is increasingly becoming the second most common etiological agent of rUTIs [11, 12]. This gram-negative bacterium has become a common pathogen responsible for both community-acquired and nosocomial UTIs, particularly in individuals with risk factors for infection, weakened immune systems, and chronic comorbidities [13–15].

The pathogens of UTIs are characterized by a rather complex mechanism of infection development in the bladder, where bacteria, after adhering to the bladder mucosa receptors, cause an inflammatory response and penetrate the urothelium, forming intracellular bacterial communities (IBC) [16, 17]. Intracellular UPEC undergo several stages in their development: invasion, IBC formation, IBC maturation and the formation of dormant intracellular reservoirs. IBC is a transient bacterial state that persists until the bacteria multiply and exit the host cells. Quiescent intracellular reservoirs are formed in the deeper layers of the urothelium. Bacteria, capable of forming biofilms and dormant intracellular reservoirs can evade phagocytosis, survive within host cells while protected from antibiotics and immune factors. They form reservoirs of persistent pathogens in the urinary tract and periodically cause UTIs.

One significant risk factor in the development of rUTIs is bladder catheterization. Catheter-associated infections account for up to 40% of all nosocomial infections [18]. The risk of infection with short-term catheterization (up to 7 days) is up to 10% and increases by 3–7% each day the catheterization continues [19]. With prolonged catheterization (over 30 days), bacteriuria develops in almost 100% of patients [18]. In patients with catheter-associated UTIs, the formation of biofilms on the surfaces of catheters, stents, drains, etc. is a factor in the development of urosepsis, leading to significant complications in the course of the infection and treatment inefficacy [20].

UTIs are also largely associated with the rise of antibiotic resistance in uropathogens. Antibiotics remain the cornerstone of treatment and prevention of UTIs— approximately 25% of antibiotic prescriptions are issued for UTIs [21]. Worldwide spread of antibiotics, prolonged antibiotics prophylaxis or frequent use of different antibiotic combinations in patients with rUTIs can contribute to the development of further resistance. The accumulation of resistant forms of bacteria can develop within a few weeks. For example, *E. coli* resistant to at least 4 antibiotics was identified in fecal isolates from over 90% of patients who received prophylactic treatment with low-dose antibiotics for 2 weeks [22].

Identifying the characteristics of the complex interaction between uropathogens and the host organism is necessary for both a deeper understanding of the pathogenesis of UTIs and for selecting new treatment approaches for these widespread bacterial infections.

The aim of the study was to investigate the effect of the innovative Russian antibacterial drug Fluorothiazinone (FT; N.F. Gamaleya Research Institute of Epidemiology and Microbiology, RU LP-No. (005077)-(RG-RU)) on the virulence properties of isolates obtained from the urine of patients with chronic UTI.

Materials and methods

Bacterial isolates

Clinical isolates of *K. pneumoniae 5811*, *5672*, *P. aeruginosa 5595*, and *E. coli 5424/1* were isolated from the urine of 4 adult patients of both sexes from different age groups with clinical signs of UTI who were hospitalized at the N.A. Lopatkin Research Institute of Urology and Interventional Radiology, a branch of the National Medical Research Center of Radiology of the Ministry of Health of Russia. The study was conducted with the informed consent of the patients. Urine isolates were collected as part of a clinical trial according to Protocol 07-FT-2024, RCT No. 513 dated 10/30/2024.

Bacteria culturing

The bacterial species were confirmed by streak plate method on solid media: LB agar (BD Difco), cetrimide agar (“Himedia”), followed by analysis of the protein profile of single colonies using the Bruker MALDI Biotyper instrument (Beckman Colter Diagnostics).

Clinical uropathogenic isolates were grown for 18 hours at 37°C in Luria–Bertani broth (LB broth) with continuous aeration on a shaker (240 rpm) to a concentration of 10^9 microbial cells/mL, which was assessed using a McFarland standard on a Densi-La-Meter II densitometer (ERBA Lachema). The resulting bacterial suspension was diluted to the desired concentrations in fresh LB broth.

Study drug

The active pharmaceutical substance FT was used in the study.

To study the effect of FT on motility and biofilm formation by clinical isolates, a stock solution with a concentration of 2 mg/mL in 0.3 M CH_3COONa buffer, pH 7.0 ± 0.2, was used. The solution was prepared as follows: 0.021 g of the substance was added to a solution of 0.120 g of sodium hydroxide in 10 mL of distilled water. The mixture was then stirred on a magnetic stirrer until the FT crystals were completely dissolved, and subsequently acidified with glacial acetic acid to a pH of 7–8. The resulting solution was filtered through a 0.22 μm syringe filter into a sterile tube. In the tests, the antibacterial drug was used at a final concentration of 100 μg/mL.

To study the cytotoxicity and intracellular survival of the bacteria, a solution of FT in dimethyl sulfoxide at a concentration of 10 mg/mL was used, with a final concentration of FT in the medium of 20 μg/mL.

Cytoxicity analysis

Cytotoxicity analysis was performed according to the method [23] with some modifications. HeLa cells (cervical carcinoma cells, ATCC CCL-2™) grown in IMDM culture medium (BioloT) with 10% fetal bovine serum (HyClone) without the addition of antibiotics were infected with a bacterial culture at a multiplicity of infection (MOI) of 10 and 50, with 1 × 10^5 cells per well. FT at a concentration of 20 μg/mL was added immediately after the bacterial culture was introduced and incubated at 37°C and 5% CO_2 for 20 hours. The plates were centrifuged for 20 minutes at 1500 rpm.

The activity of released lactate dehydrogenase (LDH) was determined in the supernatants using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega). LDH is a cytosolic enzyme present in nucleated cells that is released into the extracellular environment after the cell membrane is damaged. The percentage of LDH release was calculated relative to an uninfected control (0% LDH release) and HeLa cells lysed with Triton X-100 (100% LDH release). LDH in bacterial cultures in the medium without a cell monolayer was not detected.

Swimming motility

To assess swimming motility, cultures were grown for 18 hours at 37°C with aeration on a shaker (240 rpm), diluted 100-fold in LB broth, and cultured for 3 hours under the conditions described above. 2 μL were inoculated into the depth of 0.25% agar containing 1% tryptone and 0.5% NaCl in 90 mm diameter Petri dishes. They were observed for 48 hours. Bacterial motility was assessed by measuring the diameter of the bacterial migration zone from the center of the plate. These values served as positive controls when assessing bacterial motility.

To assess the effect of FT on the motility of clinical isolates, an FT solution in acetate buffer at a concentration of 100 μg/mL was used. For this purpose, the solution of FT was added to the prepared and cooled to 45–50°C 0.25% agar, mixed thoroughly, and poured into Petri dishes. After the agar solidified, the bacterial culture was introduced into the depth of the agar in a volume of 2 μL. The samples were cultured at 30°C in a humid chamber for 48 hours. The effect of FT was evaluated by measuring the diameter of the bacterial movement zone. Isolates with a zone of motility less than 10 mm after 48 hours were classified as non-motile, while those with a zone greater than 10 mm were classified as motile. Acetate buffer without FT was added to the control cups.

Biofilm formation

The ability of the isolates to form biofilms was studied using a modified method by G.A. O'Toole *et al.* [24]. Biofilm cultivation was performed in TC-treated 96-well polystyrene microplates (Corning 3599). Bacterial cultures were grown in LB broth at 37°C overnight on a shaker at 240 rpm. Overnight cultures were grown to ≈ 10^7 CFU/mL in sterile LB broth supplemented with an FT solution in acetate buffer at a final concentration of 100 μg/mL, and 200 μL were added to each well of the plate. The drug was not added to the wells selected as a positive control. Sterile LB broth was added to the wells as a negative control.

The plates were incubated statically at 37°C for 48 hours. After incubation, the contents of the wells were aspirated and washed three times with 200 μL of sterile phosphate-buffered saline, air-dried, and fixed with 96% ethanol for 15 minutes. The ethanol was then removed and the wells were dried.

Staining was performed using two methods: for 15 minutes with 0.1% crystal violet (Merck) or for 30 minutes with 0.1% Congo red (Merck). After the specified time, the dye was aspirated, the wells were washed with distilled water and air-dried. For quantitative analysis, the dyes bound to the biofilm were extracted with 200 μL of 96% ethanol, and the extract was transferred to the wells of a clean plate.

Optical density was measured using a Multiskan EX spectrophotometer (Thermo Scientific) at a wavelength of 540 nm for crystal violet dye and 492 nm for Congo red. To study the biofilm structure, staining was performed similarly; the wells were washed and thoroughly air-dried, then the plates were examined under a light microscope (Nikon Eclipse 50i, Nikon) at 200× magnification. For each isolate, 3 parallel experiments were performed and each were repeated thrice.

Internalization and survival of bacteria in PC-3 cells

The study used the PC-3 cell line (human prostate adenocarcinoma cells, ATCC CRL-1435). The cells were incubated in RPMI-1640 cell culture medium (BioLot) supplemented with 10% fetal bovine serum (HyClone) without the addition of antibiotics, at 37°C with 5% CO_2 for 24 hours. Cell counts were determined using a Scepter 2.0 cell counter (Millipore).

Clinical isolates were grown for 18 hours at 37°C in LB broth with continuous aeration on a shaker (240 rpm) to a concentration of 10^9 microbial cells/mL.

A monolayer of PC-3 cells, grown in a 24-well culture plate with sterile 12 mm diameter round coverslips, was infected with a bacterial culture. Infection doses for the intracellular model were selected based on the cytotoxicity results obtained for the clinical isolates.

To assess cell invasion, cells were incubated for 1 hour, then washed three times with medium to remove unattached bacteria, and incubated for 1 hour at 37°C and 0.5% CO_2 in medium containing gentamicin (Gibco) to kill extracellular bacteria. The concentration of gentamicin was determined by the minimum inhibitory concentration (MIC) for each isolate. The cells were washed three times to remove gentamicin. To determine the number of bacteria, PC-3 cells infected with bacterial cultures were lysed with 0.25% Triton X-100 (Sigma) for 10 minutes at room temperature. Uninfected PC-3 cells were used as a negative control. Next, the lysates were plated on cetrimide or LB agar (depending on the species of the isolate being studied) using the serial dilution method. The number of intracellular bacteria was expressed in CFU/mL. The average value was calculated from the bacterial population of 2 wells in 3 experiments.

To assess the onset of intracellular reproduction, the cell monolayer was cultured for 4 hours, and all procedures for removing extracellular bacteria and reseeding were performed as described above.

Intracellular survival of bacteria in PC-3 cells was assessed 24 hours after infection with the bacterial culture. After this, 4 hours after infection, the cells were incubated with gentamicin for 1 hour to eliminate extracellular bacteria. The plate was then washed three times to remove the gentamicin, and fresh nutrient medium with a maintenance concentration of gentamicin was added. All procedures for removing extracellular bacteria and culturing were performed similarly to those described above.

To study the effect of FT, a solution of FT in dimethyl sulfoxide was added to the culture medium at a concentration of 20 μg/mL at different stages of the experiment: 1 hour before infection, at the moment of cell infection, 2 and 5 hours after infection and washing off gentamicin, followed by incubation in the presence of FT for 24 hours from the moment of infection.

For microscopic analysis, cells were fixed with acetone and stained with mouse polyclonal antibodies (antibodies were obtained for each isolate) for 30 minutes at 37°C and 5% CO_2 and with anti-mouse IgG-FITC conjugate (“Merck”) for 30 minutes at 37°C and 5% CO_2. Samples were analyzed using a Nikon Eclipse 50i fluorescence microscope (Nikon) at 1000× magnification.

Statistical analysis

Statistical processing and visualization of the analysis results for *in vitro* bacterial studies were performed using GraphPad Prism v. 8.4.3 (GraphPad Software). The data from the experimental and control groups were compared using the Student's t-test. The criterion for statistical significance of the difference in the data obtained was considered to be $p<0.05$. All experiments were conducted in triplicate.

Results

Characteristics of clinical isolates

Antibiotic susceptibility analysis of the studied clinical isolates was performed in the clinical diagnostic laboratory of the N.A. Lopatkin Research Institute of Urology and Interventional Radiology, a branch of the National Medical Research Center of Radiology of the Ministry of Health of Russia, and the results are presented in the **Table**.

FT does not affect the viability of clinical isolates

The effect of FT on bacterial viability was assessed by culturing in nutrient LB broth in the presence of FT at concentrations of 60, 80 and 100 μg/mL for 24 hours with continuous aeration on a shaker (240 rpm) and determining CFU by the streak/spread plate method. According to the mechanism of action, and as previously shown for bacteria of different species, FT did not affect the viability of the studied clinical isolates.

FT suppresses the cytotoxicity of clinical isolates

Both *K. pneumoniae* isolates were characterized by significant cytotoxicity. For *K. pneumoniae 5811* isolate, cytotoxicity at MOI_{10} was 65%, and at MOI_{50}, it was 78%. For *K. pneumoniae 5672* isolate, cytotoxicity at an MOI of 10 was 70%, and at an MOI of 50, it was 76%.

The cytotoxicity of *P. aeruginosa 5595* and *E. coli 5424/1* on the HeLa cell line was less pronounced. At MOI_{10}, the cytotoxicity of the *P. aeruginosa 5595* isolate was 50%, and at MOI_{50}, it was 61%. For *E. coli* isolate 5424/1, 38 and 60% at MOI_{10} and MOI_{50}, respectively.

In the presence of FT at a concentration of 20 μg/mL, cytotoxicity after 20 hours of culturing was significantly reduced, by 1.5–2.0 times at MOI_{10} and MOI_{50} for all 4 clinical isolates.

FT suppresses swimming motility

In a semi-liquid medium, bacterial cells are capable of swimming motility, moving by means of flagella. *K. pneumoniae* belongs to bacteria that do not have swimming motility via to flagella. In light of this, the effect of FT on swimming activity was evaluated for 2 uropathogenic isolates: *P. aeruginosa 5595* and *E. coli 5424/1*.

It has been established that both clinical isolates exhibit swimming motility. In the presence of FT at a concentration of 100 μg/mL in the culture medium, a pronounced suppression of motility associated with flagellar activity was observed (**Fig. 1**).

MIC values of antibiotics in relation to the tested clinical isolates

Antibiotic susceptibility testing for isolated microorganisms	*E. coli* 5424/1		*P. aeruginosa 5595*		*K. pneumoniae 5811*		*K. pneumoniae 5672*	
	quality result	MIC, mg/L	quality result	MIC, mg/L	quality result	MIC, mg/L	quality result	MIC, mg/L
Amikacin	S	≤ 8	S		S	≤ 8	S	≤ 8
Amoxicillin/clavulanic acid	R		R		R			
Ampicillin	R	> 16	R		R	> 16	R	> 16
Ampicillin/sulbactam					R			
Gentamicin	R	128	R	16	R	512	S	≤ 2
Imipenem	S	≤ 0.25	S		R	> 8	S	≤ 0.5
Colistin					R			
Levofloxacin			S		R	> 2	R	> 4
Meropenem	S	≤ 0.125	S		R	> 8	I	8
Nitrofurantoin			R					
Piperacillin/tazobactam	S	≤ 4/4	S		R		S	≤ 4/4
Tigecycline	S	≤ 1	R					
Tobramycin	S	≤ 2	S				S	≤ 2
Trimethoprim/sulfamethoxazole	S	≤ 2/38			I		S	≤ 2/38
Fosfomycin with glucose-6-phosphate	S	≤ 16					S	≤ 16
Cefazolin	R	> 32	R		R	> 32	R	> 32
Cefepime			S		R	> 8		
Ceftazidime			R		R	> 8	R	> 16
Ceftriaxone	S	≤ 1	R		R	> 4	R	> 4
Cefuroxime					R	> 16		
Ciprofloxacin	S	≤ 0.25			R	> 1	R	> 1
Ertapenem	S	≤ 0.25	R		R	> 1	R	> 2

Note: S — susceptible; R — resistant; I — intermediate.

The diameter of the bacterial growth zone in semi-solid agar for *P. aeruginosa 5595* after 48 hours was 65–67 mm, and in the presence of FT, it was 25–27 mm. For *E. coli 5424/1* isolate, the diameter of the zone of motility after 48 hours was 35–37 mm; in the presence of FT, the zone decreased to 12–14 mm. In control dishes with acetate buffer without the addition of FT, motility was not inhibited. This demonstrated the suppression of flagellar motility by FT.

FT suppresses biofilm formation

The ability to form biofilms and the effect of FT on this process were evaluated for 4 isolates. The biofilm density was quantified by staining the bacteria with crystal violet. After 48 hours, *P. aeruginosa 5595* formed an intense multi-layered mature biofilm, *K. pneumoniae 5672* and *E. coli 5424/1* isolates formed a dense monolayer with areas of multi-layered structure, and *K. pneumoniae 5811* isolate showed the slowest biofilm formation rate: by 48 hours, a monolayer with the formation of microcolonies was observed.

Adding FT simultaneously with the introduction of the bacterial culture led to a significant reduction in biofilm density and disruption of its structure. All isolates formed a sparse monolayer with a cellular structure on an abiotic surface under the influence of FT, but microcolony formation and biofilm maturation did not occur (**Fig. 2**).

When quantifying bacterial density in biofilms under the influence of phototherapy, a significant reduction in the number of bacteria within the biofilm was observed: the maximum suppression (by 85%) was for *K. pneumoniae 5672*, 79% for *E. coli 5424/1*, 75% for *P. aeruginosa 5595*, and 71% for *K. pneumoniae 5811* (Fig. 2).

The formation of the biofilm exopolysaccharide matrix (EPM) was assessed by Congo Red staining. It was found that all the isolates studied are capable of forming EPM, but the *K. pneumoniae 5811* biofilm was characterized by the lowest EPM intensity. Under the effect of FT, all isolates lost their ability to synthesize EPM (**Fig. 3**).

Quantitative assessment showed a significant decrease in biofilm formation for all isolates: *K. pneumoniae 5811* — 76%, *K. pneumoniae 5672* — 74%, *P. aeruginosa 5595* — 70%, *E. coli 5424/1* — 67%

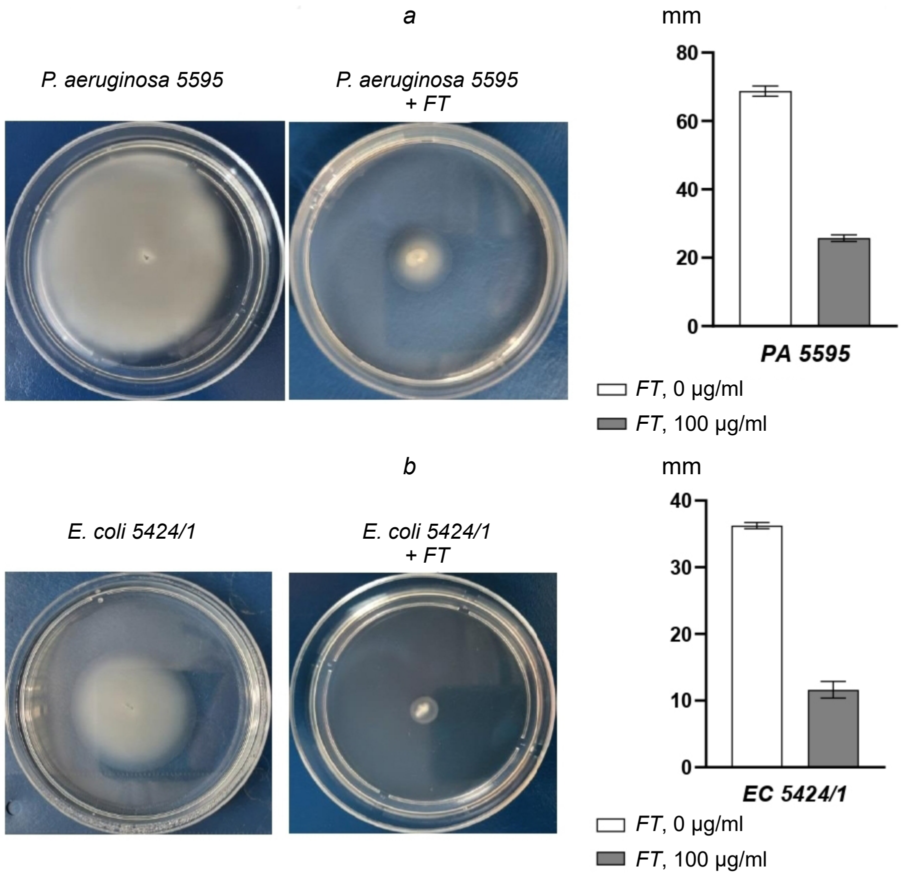

Fig. 1. Suppression of swimming motility of isolates after 48 hours of incubation in the presence of FT.

Representative images of swimming motility on Petri dishes and assessment of the change in the diameter of the zone of motility of *P. aeruginosa 5595* (*a*) and *E. coli 5424/1* (*b*) in the presence of FT at a concentration of 100 µg/mL. Error bars represent the SD of 2 replicates.

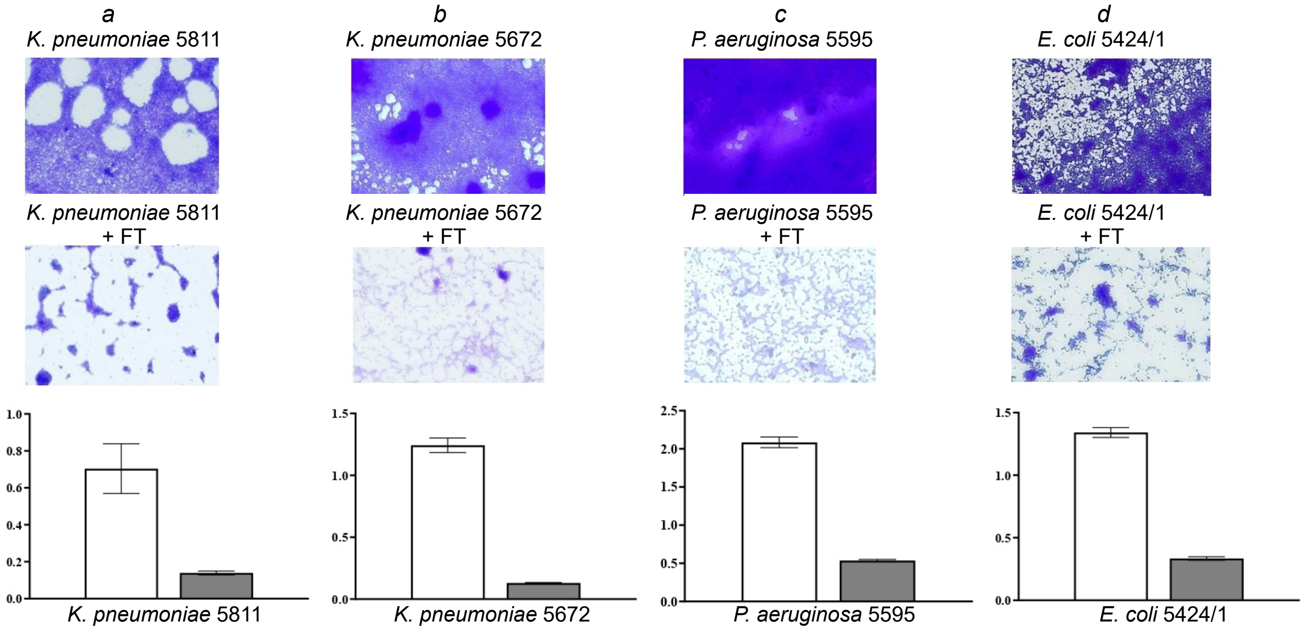

Fig. 2. FT alters the structure and biomass of biofilms formed by *isolates K. pneumoniae 5811* (*a*), *K. pneumoniae 5672* (*b*), *P. aeruginosa 5595* (*c*) and *E. coli 5424/1* (*d*).

Microphotographs of formed biofilm fragments from control and experimental samples with the addition of FT at a concentration of 100 µg/mL after 48 hours of incubation are presented. Biofilm biomass accumulation was assessed by staining with crystal violet at an optical density of 540 nm.

(Fig. 3). The solvent without FT had no effect on biofilm formation.

Thus, all the isolates studied are capable of formation of a biofilm on an abiotic surface, and FT inhibited the formation of a mature biofilm.

FT suppresses the invasion and intracellular replication of uropathogens

The ability of the studied uropathogenic isolates to form biofilms was assessed using the PC-3 cell line model (human prostate adenocarcinoma cells) by infecting the cells with the isolates and observing them during the invasion and intracellular replication stages at 4 and 24 hours. To assess the effect of FT on these processes, the drug was introduced 1 hour before infection, simultaneously with infection, and also after the medium was changed (at 2 and 5 hours).

The number of viable intracellular bacteria was assessed by culturing dilutions of the cell lysate after removing extracellular bacteria by gentamicin treatment for 1 hour. Simultaneously, the cell monolayer was analyzed by microscopy after staining with specific antibacterial antibodies.

For the *E. coli 5424/1* isolate, an inoculum of 1.5 × 10^6 CFU/mL was used for infection, which resulted in MOI_{55}. After 1 hour, the invasion rate was 0.47%, while under the effect of FT, it was 0.053%. After 4 hours, the number of intracellular bacteria in the control group was 2.1 × 10^5 CFU/mL, while in the presence of FT, it was an order of magnitude lower, at 1.1 × 10^4 CFU/mL. After 24 hours, an increase in the number of *E. coli 5424/1* was observed to 2.4 × 10^6 CFU/mL in the control samples, while bacterial growth was significantly reduced in the presence of FT to 7.5 × 10^2 CFU/mL.

In the micrographs of the infected cell monolayer in the control samples, intracellular cell conglomerates were detected as early as 4 hours, which formed typical IBC by 24 hours. In the presence of FT, IBC were practically not identified (**Fig. 4**).

For the *K. pneumoniae 5811* isolate, IBC formation was also observed, and it was suppressed in the presence of FT. This isolate showed lower rate of invasion, which was 0.005% for the control, while almost similar rate of invasion was observed under FT treatment. This isolate showed lower rate of invasion, which was 0.005% for the control and similar for the FT treatment group. (бы я исправила) This isolate showed to have effective intracellular replication: after 4 hours, the number of intracellular bacteria was 5 × 10^5 CFU/mL, and after 24 hours, it was 2.0 × 10^6 CFU/mL. Under the effect of FT, bacterial replication? was suppressed — 5.9 × 10^2 CFU/mL after 24 hours. The inhibition of IBC formation in the presence of FT is clearly demonstrated in the microphotographs (**Fig. 5**).

Discussion

The diagnosis of UTIs, which can mean both infectious disease and asymptomatic bacteriuria, is some-

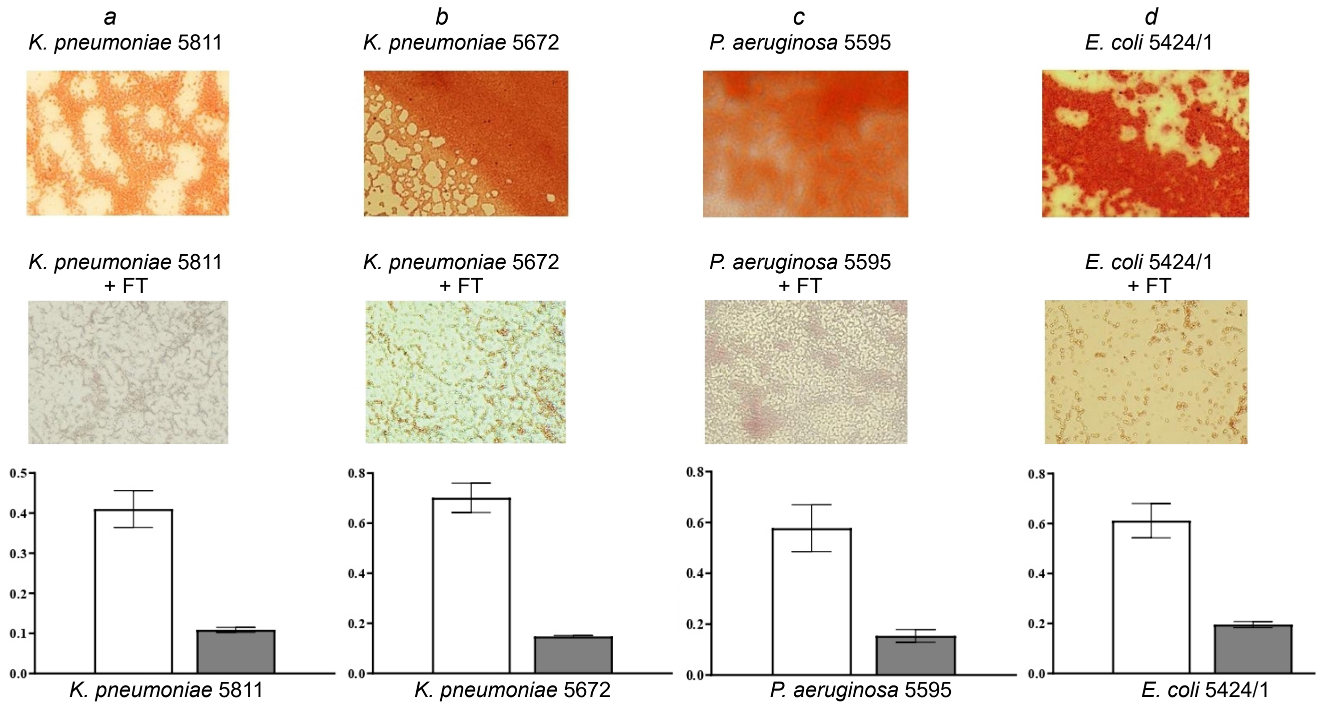

Fig. 3. FT inhibits the production of EPM biofilm by *K. pneumoniae 5811* (*a*), *K. pneumoniae 5672* (*b*), *P. aeruginosa 5595* (*c*), and *E. coli 5424/1* (*d*).

Microphotographs of biofilm fragments from control and experimental samples with the addition of FT at a concentration of 100 μg/mL are presented after 48 hours of incubation. EPM biofilm products were evaluated by the degree of Congo Red staining of the matrix at an optical density of 492 nm.

times difficult to establish, especially in recurrent cases, due to complex mechanism of interaction between uropathogens and tissues of the urinary tract and the body's immune system. International guidelines recommend relying solely on clinical symptoms of the disease [1]. Although UTIs are caused by pathogens from various taxonomic groups, and most of these bacteria are extracellular, they nevertheless utilize a common strategy for developing an infectious process: effective adhesion, invasion of epithelial cells, and the formation of pathogen reservoirs in the form of biofilms, dormant intracellular reservoirs, or surface biofilms (on catheters, foreign bodies). This strategy allows them to evade the action of antibiotics and host immunity factors, which determines clinical and epidemiological relevance of recurrent UTIs.

New approaches in treating such chronic infections should be based on understanding the pathogenesis mechanisms and on blocking of specific links of these mechanisms. In the current study, we evaluated the effect of the active pharmaceutical substance, antibacterial drug FT, on the expression of virulent properties of clinical isolates of 3 different species: *K. pneumoniae*, *P. aeruginosa* and *E. coli* under *in vitro* conditions.

FT is an original domestic antibacterial drug that differs from antibiotics in its mechanism of action, as it does not kill the bacteria but suppresses their virulence. The specific inhibition of the type III secretion system (T3SS), which is responsible for the production of toxins that destroy immune cells and promote intracellular bacterial survival, has been demonstrated for this antibacterial drug. FT also inhibits bacterial flagellar motility and biofilm formation. Suppression of the virulence factors leads to the halting of infectious process in the host organism, as demonstrated in animal experiments [25–27]. FT therapy is effective against bacteria resistant to different classes of antibiotics because the target of action is present regardless of the presence of resistance determinants [28].

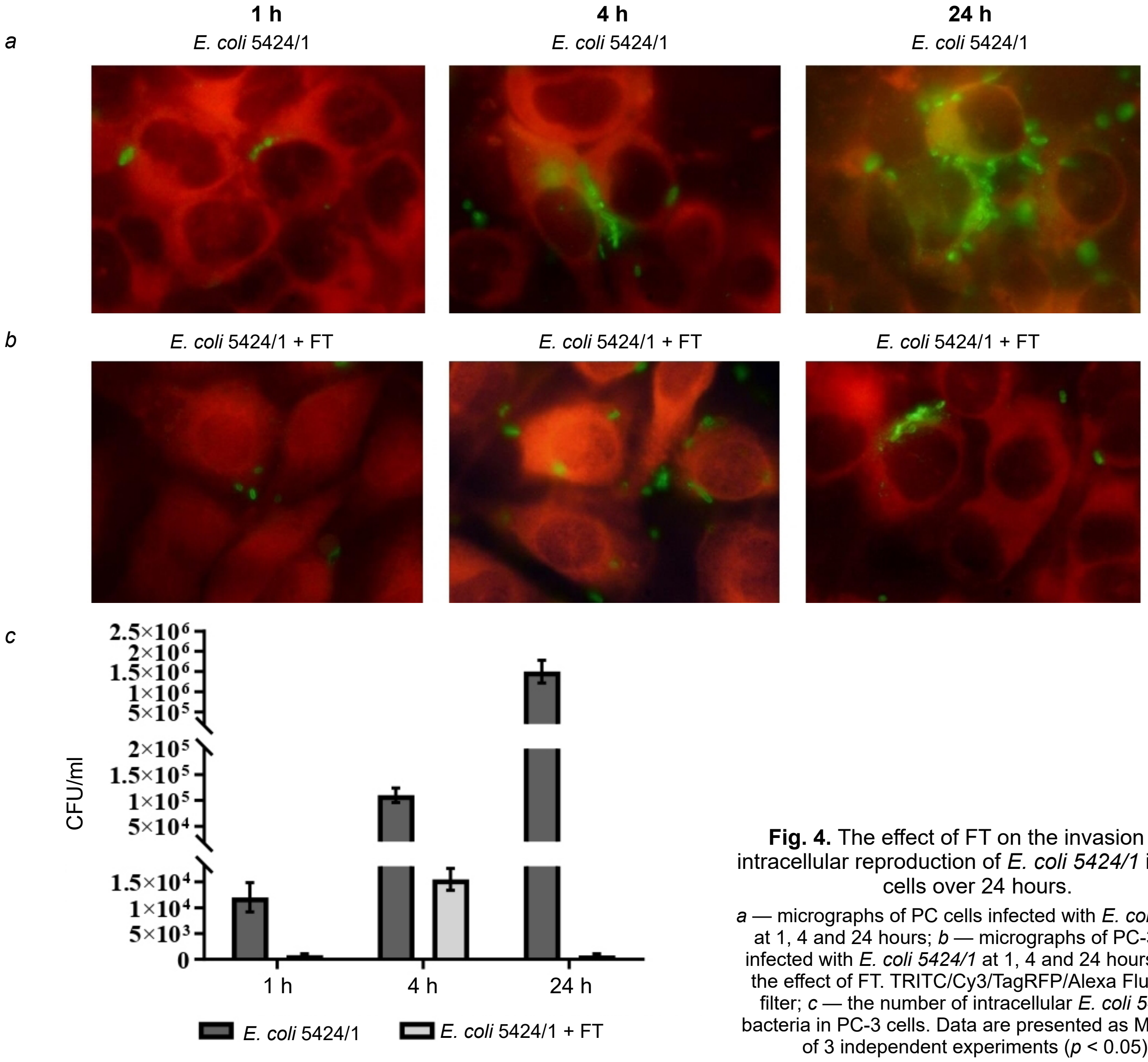

Fig. 4. The effect of FT on the invasion and intracellular reproduction of *E. coli 5424/1* in PC-3 cells over 24 hours.

a — micrographs of PC cells infected with *E. coli 5424/1* at 1, 4 and 24 hours; *b* — micrographs of PC-3 cells infected with *E. coli 5424/1* at 1, 4 and 24 hours under the effect of FT. TRITC/Cy3/TagRFP/Alexa Fluor 546 filter; *c* — the number of intracellular *E. coli 5424/1* bacteria in PC-3 cells. Data are presented as M ± SEM of 3 independent experiments ($p < 0.05$).

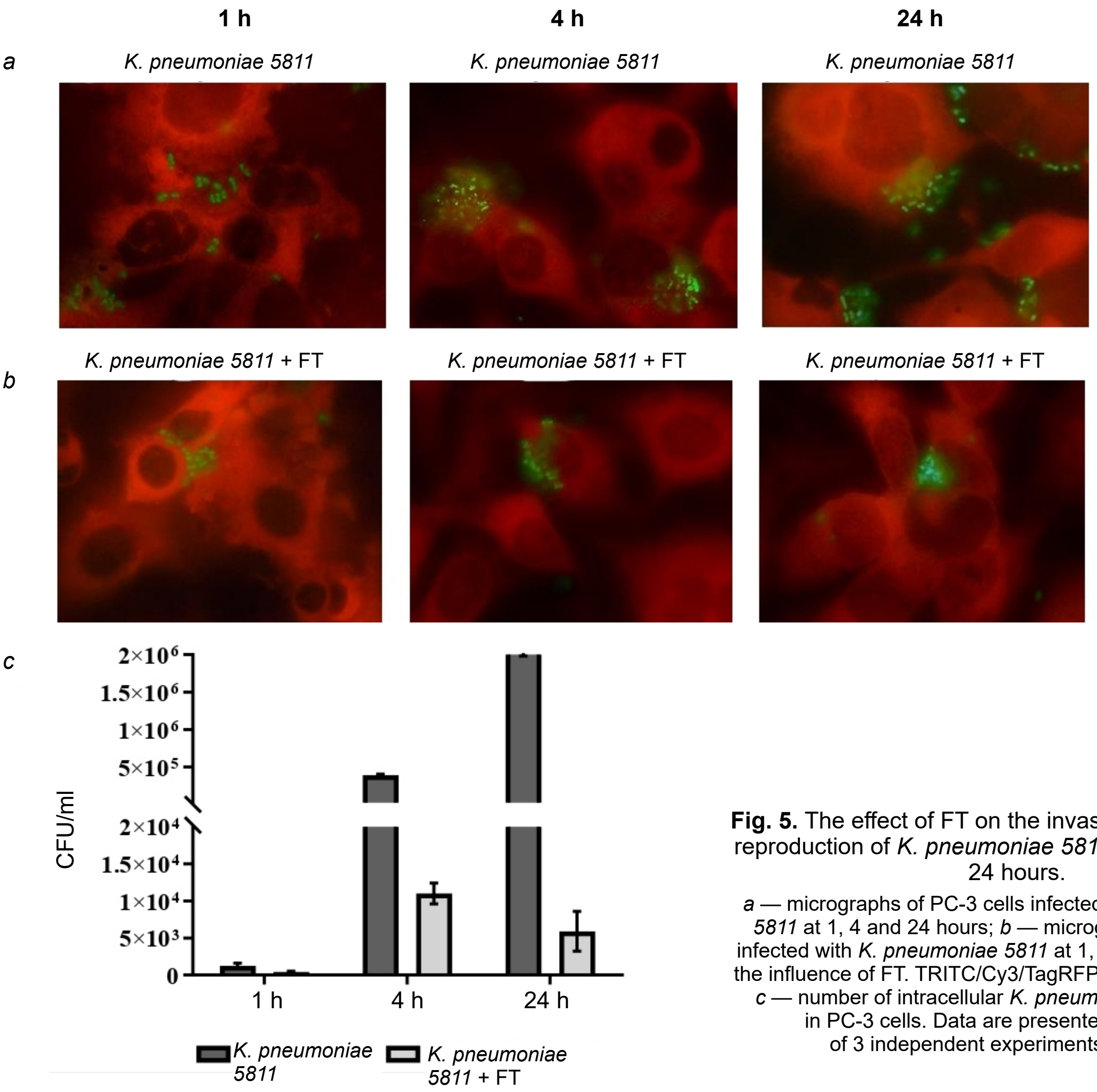

Fig. 5. The effect of FT on the invasion and intracellular reproduction of *K. pneumoniae 5811* in PC-3 cells over 24 hours.

a — micrographs of PC-3 cells infected with *K. pneumoniae 5811* at 1, 4 and 24 hours; *b* — micrographs of PC-3 cells infected with *K. pneumoniae 5811* at 1, 4 and 24 hours under the influence of FT. TRITC/Cy3/TagRFP/Alexa Fluor 546 filter. *c* — number of intracellular *K. pneumoniae 5811* bacteria in PC-3 cells. Data are presented as M ± SEM of 3 independent experiments (p < 0.05).

In clinical trials on patients with acute UTI, it was found that FT reduced the development of recurrences by 10 times at 2 and 3 months after the end of therapy compared to cefepime, which can be explained by the effective eradication of uropathogens from their reservoirs, including the intracellular ones [29].

It is known that *P. aeruginosa* and *E. coli* secrete cytotoxins directly into eukaryotic cells via the T3SS. In this regard, we evaluated the effect of FT on the cytotoxicity of these isolates. The presence of the T3SS is not demonstrated for *K. pneumoniae*, but a cytotoxic effect associated with the presence of the capsular polysaccharide has been described [30].

For all analyzed isolates that were characterized by significant toxicity toward epithelial cells, a statistically significant suppression of cell death was demonstrated in the presence of FT.

Along with the flagellum, the target of FT is the cilium, which has a similar structure at the base of the organelle. For motile isolates of *P. aeruginosa* and *E. coli*, almost complete blockage of flagellar function was observed, which was expressed by the absence of a spreading zone in semi-solid agar. It should be noted that bacterial growth was not inhibited in the presence of FT, as shown in preliminary experiments.

The presence of surface structures in uropathogens, such as pili, fimbriae and flagella, facilitates adhesion to urothelial receptors as the first stage of bladder colonization, and thus they are an important target for suppression of infection development. One example of confirmation of the efficacy of this adhesion-blocking approach is the use of D-mannose in a clinical study, which showed a statistically significant reduction in the likelihood of recurrent UTIs [31].

Thus, the suppression of bacterial motility observed under the effect of FT, which is related to the function of flagella, is aimed at blocking of the first stage of infection development — the stage of urothelial colonization.

The key link in the development of a chronic infectious process with a recurrent course, caused by the long-term persistence of pathogens, is the bacteria's ability to actively invade urothelial cells and form intracellular biofilm-like communities.

The effect of FT on the 24-hour development cycle of *K. pneumoniae* and *E. coli* isolates in human prostate adenocarcinoma cells was evaluated. It was previously established that FT has high tissue availability due to its pharmacological properties, it penetrates cells, and retains activity in eukaryotic cells [32]. The introduction of FT into the culture medium significantly reduced the number of bacteria forming intracellular bacterial communities, as demonstrated by determining viable microorganisms and identifying intracellular structures under microscopy 24 hours after the pathogens came into contact with the cells.

Currently, the mechanisms by which FT suppresses intracellular bacterial replication are being studied. Given that FT does not have a direct antibacterial effect, the proposed mechanism involves blocking bacterial virulence factors responsible for interaction with the host cell during bacterial coexistence and persistence.

The ability of FT to suppress biofilms has been previously demonstrated for various pathogens, including carbapenem-resistant hospital-acquired infection agents. Studying the dynamics of biofilm formation *in vitro* and the effect of FT on this process showed that the FT blocks biofilm formation at the stage of microcolony aggregation and multilayer structure formation. Cultivation in the presence of FT has an inhibitory effect on the ability of isolates to form biofilms and, consequently, reduces the production of exopolysaccharides [26, 27]. The obtained results are of significant practical importance, as the EPM is a key factor in biofilm resistance to antibiotics and protection against the immune response.

Conclusion

In experiments conducted to study the effect of FT, the inhibitor of the type III secretion system and flagella, on the virulence properties of clinical isolates of three species of uropathogens under *in vitro* conditions, it was shown that FT suppresses key stages of bacterial persistence development in chronic infections, such as invasion of epithelial cells and the formation of pathogen reservoirs in the form of intracellular bacterial communities and surface biofilms. Properties of FT, such as biofilm suppression and intracellular replication inhibition, are a significant advantage of the innovative Russian antibacterial drug over currently used antibiotics.

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Characterization of the molecular genetic properties of epidemic strains of *Klebsiella pneumoniae* and *Staphylococcus aureus*, the pathogens of healthcare-associated infections circulating in the Nizhny Novgorod region

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Abstract

Introduction. Molecular epidemiological monitoring is aimed at obtaining up-to-date information on the genetic variants of healthcare-associated infections (HAIs) circulating in the region. Currently, special attention is being paid to monitoring representatives of the ESKAPE group, as they are a frequent cause of HAIs, complicate the course of the underlying disease, and are becoming an increasingly serious threat to the health and lives of patients due to their complex pathogenicity genes and diverse antibiotic resistance mechanisms.

The aim of the study is to analyze the results of whole-genome sequencing of epidemic strains of pathogens of HAIs — *Klebsiella pneumoniae* ssp. *pneumoniae* and *Staphylococcus aureus* — circulating in the Nizhny Novgorod region.

Materials and methods. Classical bacteriological methods, MALDI-TOF mass spectrometry, whole-genome sequencing, and bioinformatics methods were used.

Results. In-depth analysis revealed the circulation of a population of classical *K. pneumoniae* strains of sequence type (ST) 3-K type (K) 3 in the neonatal intensive care unit, containing a number of virulence genes and the $bla_{SHV\text{-}1}$ beta-lactamase. Circulation of a population of *K. pneumoniae* strains of the convergent pathotype ST395 and K39 was detected in a multidisciplinary hospital, and strains of the convergent pathotype *K. pneumoniae* ST395-K2, K47, as well as strains of the classical pathotype *K. pneumoniae* ST5209-K35, ST441-K62, ST147-K64, containing a spectrum of pathogenicity genes and beta-lactamases in their genome, including New Delhi metallo-beta-lactamase $bla_{NDM\text{-}1}$, were identified. *S. aureus* strains associated with catheter-associated bloodstream infections have significant pathogenic potential, belonging to 13 different STs and 19 spa types (t). Circulation of methicillin-resistant (SCC*mec* IV, ST8, t008) and methicillin-susceptible (ST1, t127) staphylococcal strains has been detected in hemodialysis centers and departments.

Conclusion. The data obtained indicate the circulation of convergent and classical strains of *K. pneumoniae* and virulent strains of *S. aureus* in medical and preventive organizations, which justifies the need for molecular epidemiological monitoring.

Keywords: *Klebsiella pneumoniae, Staphylococcus aureus, healthcare-associated infections, whole-genome sequencing, microbiological monitoring*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. The research protocol was approved by the Ethical Committee No. 1 on conducting scientific research with human subjects as a research object of Privolzhsky Research Medical University, Nizhny Novgorod (protocol No. 7, May 7, 2018.

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Оригинальное исследование
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Характеристика молекулярно-генетических свойств эпидемических штаммов *Klebsiella pneumoniae* и *Staphylococcus aureus* — возбудителей инфекций, связанных с оказанием медицинской помощи, циркулирующих на территории Нижегородской области

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Аннотация

Введение. Молекулярно-эпидемиологический мониторинг направлен на получение актуальной информации о генетических вариантах возбудителей инфекций, связанных с оказанием медицинской помощи (ИСМП), циркулирующих в регионе. В настоящее время особое внимание уделяется слежению за представителями группы ESKAPE, т. к. они являются частой причиной ИСМП, осложняют течение основного заболевания и становятся всё более серьёзной угрозой здоровью и жизни пациентов, поскольку обладают комплексом генов патогенности и разнообразными механизмами антибиотикорезистентности.

Цель работы — анализ результатов полногеномного секвенирования эпидемических штаммов возбудителей ИСМП — *Klebsiella pneumoniae* ssp. *pneumoniae* и *Staphylococcus aureus,* циркулирующих на территории Нижегородской области.

Материалы и методы. Использованы классические бактериологические методы, MALDI TOF масс-спектрометрия, полногеномное секвенирование, биоинформатические методы.

Результаты. Углублённый анализ показал циркуляцию в отделении новорождённых популяции классических штаммов *K. pneumoniae* сиквенс-типа (ST) 3-К-типа (К) 3, содержащих ряд генов патогенности и бета-лактамазу $bla_{SHV\text{-}1}$. В многопрофильном стационаре обнаружена циркуляция популяции штаммов конвергентного патотипа *K. pneumoniae* ST395-К39, а также выявлены штаммы конвергентного патотипа *K. pneumoniae* ST395-К2, К47 и штаммы классического патотипа *K. pneumoniae* ST5209-К35, ST441-К62, ST147-К64, содержащие в геноме спектр генов патогенности и бета-лактамаз, в том числе Нью-Дели металло-бета-лактамазу $bla_{NDM\text{-}1}$. Штаммы *S. aureus*, связанные с катетер-ассоциированными инфекциями кровотока, обладают выраженным патогенным потенциалом, относятся к 13 различным ST и 19 spa-типам (t). В гемодиализных центрах и отделениях гемодиализа выявлена циркуляция штаммов метициллин-резистентных (SCC*mec* IV, ST8, t008) и метициллин-чувствительных (ST1, t127) стафилококков.

Заключение. Полученные данные свидетельствуют о циркуляции в лечебно-профилактических медицинских организациях конвергентных и классических штаммов *K. pneumoniae* и вирулентных штаммов *S. aureus,* что обосновывает необходимость молекулярно-эпидемиологического мониторинга.

Ключевые слова: *Klebsiella pneumoniae, Staphylococcus aureus, инфекции, связанные с оказанием медицинской помощи, полногеномное секвенирование, микробиологический мониторинг*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов или их законных представителей. Протокол исследования одобрен Этическим комитетом № 1 по проведению научных исследований с участием человека в качестве объекта исследования Приволжского исследовательского медицинского университета (протокол № 7 от 05.07.2018).

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

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Introduction

Molecular epidemiological monitoring is becoming an integral task in organizing a system for epidemiological surveillance of infectious diseases, as it allows for tracking the circulation of opportunistic microorganisms and timely identification of signs of potential outbreaks, which include: isolation of a homogeneous spectrum of microorganisms from examined individuals; an increase in the incidence of infectious diseases caused by a single species or group of species of pathogens; and an increase in the detection rate of hospital strains. The objectives include monitoring the population structure of infectious agents, including healthcare-associated infections (HAIs), MLST typing of strains, analysis of pathogenicity genes and antibiotic resistance determinants, detection of new variants of hospital strains, and observation of their variability to assess epidemiological forecasting and justify timely intervention in the course of the epidemic process [1].

ESKAPE pathogens are a frequent cause of healthcare-associated infections, complicate the course of the underlying disease, and pose a serious threat to the health and life of patients, as they are able to quickly adapt and find new ways to resist the effect of drugs, disinfectants and antiseptics, and also transmit this ability to other pathogens at a genetic level [2, 3].

Among the bacteria that cause hospital infections, *Klebsiella pneumoniae* and *Staphylococcus aureus* are the leading causes, with an increasing proportion of carbapenem-resistant *K. pneumoniae* and the widespread prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) belonging to the MRSA group, which can cause outbreaks and lead to catheter-associated bloodstream infections (CABSI) [4,5].

The aim of the study is to analyze the results of whole-genome sequencing of epidemic strains of pathogens of healthcare-associated infections — *K. pneumoniae* ssp. *pneumoniae* and *S. aureus*, circulating in the Nizhny Novgorod region.

Materials and methods

Strains under study

55 epidemic strains of pathogens were studied: 17 strains of *K. pneumoniae* ssp. *pneumoniae* and 38 strains of *S. aureus*. Based on their place of isolation, the strains were divided into three groups:

The 1st group consisted of 7 *K. pneumoniae* strains isolated from sick children (gastric contents) (*n* = 4) in the neonatal intensive care unit of a pediatric hospital, and from equipment and care items (swabs from suction tubing, feeding syringe) (*n* = 3);

The 2nd group – 10 strains of *K. pneumoniae* isolated from patients in the multidisciplinary hospital departments (wound discharge) (*n* = 9) and from the department's external environment (swab from the intensive care unit sink faucet) (*n* = 1);

The 3rd group – 38 strains of *S. aureus*, including 31 strains from patients with CABSI who were receiving outpatient treatment at hemodialysis centers in the city and region and were hospitalized in medical organizations in the city (blood, wound at the catheter site, peritoneal fluid, nasal swab); 3 strains isolated from medical personnel (nasal swab), and 4 from the medical organization environment (equipment swabs).

Cultivation and identification of bacteria

Isolation of strains of conditionally pathogenic microorganisms was carried out using the classical bacteriological method, and identification was performed by MALDI-TOF mass spectrometry using an Autoflex mass spectrometer (Bruker Daltonics). The susceptibility of bacteria to antibiotics was studied using the disk diffusion method on "Nutrient Medium for Determining the Susceptibility of Microorganisms to Antibacterial Drugs — Mueller-Hinton II Agar (State Research Center for Applied Microbiology and Biotechnology of Rospotrebnadzor) using extended sets of disks for enterobacteria (set No. 7) and staphylococci (set No. 14) (Pasteur Research Institute of Epidemiology and Microbiology). The susceptibility of the strains to ceftazidime-avibactam was studied using ceftazidime + avibactam 10/4 mcg disks (Mast Group), and to tigecycline using tigecycline 15 mcg disks (Mast Group). The assessment was conducted in accordance with the clinical guidelines "Determination of the Susceptibility of Microorganisms to Antimicrobial Drugs"[1].

Whole-genome sequencing

Libraries were prepared using the TrueSeq kit (Illumina Inc.), and sequencing was performed on the MiSeq platform (Illumina Inc.). The raw reads were processed using the Trimmomatic utility, while the SPAdes v. 3.11.1 and Prokka v. 1.12 programs were used for *de novo* read assembly [6, 7]. All nucleotide sequences were deposited in the international GenBank database.

Whole-genome sequence analysis was performed using VFDB[2] [8], ResFinder[3] [9], the BIGSdb-Pasteur web platform[4] [10], the PubMLST resource[5] [11], and the Spa-typer and SCCmecFinder programs [12, 13]. Dendrograms were constructed using the maximum likelihood method to determine the genetic distance between microbial strains using the parsnp v. 1.7.4 program. The FastTree 2.1.1 algorithm and the Shimodaira–Hasegawa test [14] were used to assess the

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[1] MACMACH Recommendations version 2024. URL: https://www.antibiotic.ru/minzdrav/category/clinical-recommendations

[2] Virulence factor database. URL: http://www.mgc.ac.cn/VFs

[3] ResFinder. URL: http://genepi.food.dtu.dk/resfinder

[4] Institut Pasteur. *Klebsiella pneumoniae* species complex.
URL: https://bigsdb.pasteur.fr/klebsiella

[5] PubMLST. MLST Database. *Staphylococcus aureus.*
URL: https://pubmlst.org/organisms/staphylococcus-aureus

primary tree topology. Sequences from the GenBank database were used as references: GCF_000240185.1, GCA_000013425.1, and then the reference genome branch was removed. Phylogenetic trees were visualized using the iTol service [15].

Results

As a result of studying the molecular genetic properties of *K. pneumoniae* strains of group 1, it was found that all strains possess colibactin gene clusters, yersiniabactin genes, and genes responsible for the formation of type 3 fimbriae, and the *rmpA* gene, which regulates the hypermucoviscous phenotype, was detected in 3 strains (**Table**). A species-specific antibiotic resistance (ABR) determinant was identified in the genomes of all strains – the beta-lactamase bla_{SHV-1}, which confers natural resistance of microorganisms to aminopenicillins – ampicillin and amoxicillin. Phenotypically, the strains were susceptible to all antibiotics from other groups: 3rd–5th generation cephalosporins (cefazolin, cefotaxime, ceftriaxone, ceftazidime, ceftazidime/avibactam, cefepime, ceftaroline), aminoglycosides (gentamicin), fluoroquinolones (ciprofloxacin), carbapenems (ertapenem, imipenem, meropenem), monobactams (aztreonam), polymyxins (colistin), tetracyclines (tigecycline), and trimethoprim/sulfamethoxazole. As a result of the analysis of constitutional genes (housekeeping genes) and *wzi* gene alleles, the strains were identified as belonging to ST 3 and capsular type K 3 (Table).

K. pneumoniae strains of group 2 differed in their molecular genetic properties. Complete aerobactin siderophore gene clusters (*iucABCD* and *iutA*) were found in the genomes of *K. pneumoniae* strains 3254, 3260 and 3263, while *K. pneumoniae* strain 3263 was distinguished by the presence of the yersiniabactin gene cluster (*fyuA*, *irp1,2*, *ybtAEPQSTUX*) (**Fig. 1**). All strains were found to have a spectrum of ARGs – beta-lactamases and carbapenemases bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, bla_{OXA-1}, bla_{OXA-48}, and the fosfomycin resistance determinant – *fosA*. This explains the fact that all 3 strains had an MDR phenotype and were resistant to penicillins, cephalosporins, aminoglycosides, fluoroquinolones, monobactams and carbapenems (ertapenem). Based on the analysis of constitutional genes and *wzi* gene alleles, the strains were identified as belonging to ST395 and K39.

The virulence gene spectrum of *K. pneumoniae* strains 3245 and 3251, like that of *K. pneumoniae* strains 3254 and 3260, was represented by the aerobactin gene cluster (*iucABCD* and iutA) (Fig. 1). *K. pneumoniae* strain 3245 was found to have an identical spectrum of antibiotic resistance genes (bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, bla_{OXA-1}, bla_{OXA-48}) in its genome, with the exception of *fosA*. This strain had the same antimicrobial susceptibility and resistance phenotypes as the strains described above. *K. pneumoniae* 3251 was characterized by the presence of the $bla_{CTX-M-3}$ determinant, the absence of the bla_{OXA-48} and $bla_{CTX-M-15}$ genes, and was susceptible to carbapenems. *K. pneumoniae* 3245 and 3251 also belonged to ST395, but differed from *K. pneumoniae* strains 3254, 3260, and 3263 in their *wzi* gene alleles and belonged to K47.

K. pneumoniae strains 3255 and 3259 were characterized by the presence of complete aerobactin (*iucABCD* and *iutA*) and yersiniabactin (*fyuA*, *irp1,2*, *ybtAEPQSTUX*) gene clusters, with the salmochelin gene (*iro*) being detected in *K. pneumoniae* 3259. Their ABR gene spectrum was identical to the spectrum of determinants in *K. pneumoniae* 3254, 3260, 3263, 3245 (bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, bla_{OXA-1}, bla_{OXA-48}, *fosA*). *K. pneumoniae* 3255 and 3259 also had an MDR phenotype and were resistant to penicillins, cephalosporins, aminoglycosides, fluoroquinolones, carbapenems (ertapenem), and tigecycline. These strains also belonged to ST395, but to K2.

Only one aerobactin cluster determinant, *iutA*, was found in *K. pneumoniae* strains 3247, 3253, and 3256 (Fig. 1). *K. pneumoniae* isolate 3256 contained the beta-lactamases $bla_{CTX-M-14}$, $bla_{OXA-244}$ and bla_{SHV-11} in its genome, exhibited phenotypic resistance to cephalosporins and aminoglycosides, and was susceptible to carbapenems. Its affiliation with ST5209 and K35 was established. *K. pneumoniae* 3253 was found to have 2 AMR determinants: $bla_{SHV-168}$ and *fosA*. The strain was susceptible to all groups of antibacterial drugs except aminopenicillins and was classified as ST441 and K62. In the genome of *K. pneumoniae* 3247, the bla_{NDM-1}, bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11} and bla_{OXA-1} genes were detected. The strain was phenotypically resistant to penicillins, cephalosporins, aminoglycosides, fluoroquinolones, all carbapenem group drugs (ertapenem, imipenem, meropenem), and susceptible to colistin, tigecycline, and ceftazidime-avibactam, and was classified as ST147 and K64.

Adhesin genes, a type VII secretion system, and gamma-hemolysins were identified in the genomes of all group 3 *S. aureus* strains.

Strains *S. aureus* 2226, 3092, 3110, 2211, 3196, 3197, and 3198 were characterized by an identical spectrum of virulence genes, which included, in addition to those listed above, the serine protease genes *splABCDE*, the immune evasion genes *sak*, *scn*, enterotoxin A *sea*, exfoliative toxin *eta*, and leukotoxins *lukDE*. SCC*mec* type IV cassettes were detected in strains 3196, 3197, 3110 and 3092, the *blaZ* beta-lactamase gene was identified in *S. aureus* strains 3198, 2211 and 2226, and the erythromycin and chloramphenicol resistance genes *ermC* and *cat* were found (**Fig. 2**). The strains showed phenotypic resistance to oxacillin and cefoxitin, which may indicate their belonging to the MRSA group. As a result of MLST typing and analysis of the protein A gene sequence repeats, their belonging to ST8 and spa-type (t) t008 was established. Also belonging to ST8, but to t024, was the *S. aureus* strain

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Molecular genetic characterization of *K. pneumoniae* strains isolated in the neonatal intensive care unit

Strain	Sequence type (ST)	Capsule type (K)	Pathogenicity genes				ABR genes
			hypermucoid phenotype regulator genes	colibactin synthesis gene	Yersiniabactin synthesis genes	type 3 fimbriae genes	
n = 3 *K. pn 849* JAVGJO000000000 *K. pn* 852 JAVHUD000000000 *K. pn* 862 JAVBWS000000000	3	3	*rmpA*	*clbABCDEFGHLMNOPQ*	*fyuA, irp1,2, ybtAEQPSTUX*	*mrkABCDFHIJ*	$bla_{SHV\text{-}1}$
n = 4 *K. pn* 850 JAVCZJ000000000 *K. pn* 854 JAVGJN000000000 *K. pn* 863 JAVBWT000000000 *K. pn* 893 JAVHUE000000000	3	3	–	*clbABCDEFGHLMNOPQ,*	*fyuA, irp1,2, ybtAEQPSTUX*	*mrkABCDFHIJ*	$bla_{SHV\text{-}1}$

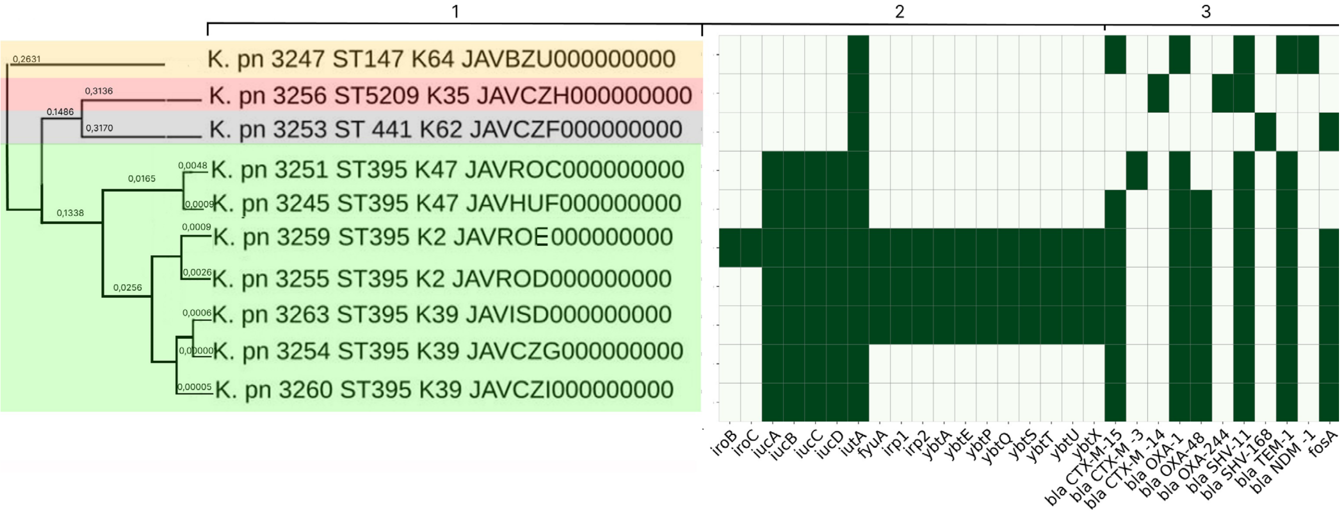

Fig. 1. Phylogenetic tree for genomes of *K. pneumoniae* strains of group 2.

1 — microorganism type, strain number, ST, K-type, GenBank database accession number; 2 — determinants of pathogenicity genes (green — presence of the trait, white — absence); 3 — determinants of ABR (green — presence of the trait, white — absence)

3107, which was distinguished by the absence of the SCC*mec* cassette.

Pathogenicity genes *splABCDE*, *eta* and *lukDE* were identified in the genomes of *S. aureus* strains 3086, 3087 and 3088, while *S. aureus* 2203 and 2213 were characterized by the presence of *sak* and *scn* genes (Fig. 2). The *blaZ* and *ermC* antibiotic resistance genes were found in *S. aureus* 3086, 3087, and 3088, while only *blaZ* was found in *S. aureus* 2213. All 5 isolates were resistant to amoxicillin, tetracycline and lincosamides. It was established that strains 3086, 3087, and 3088 belonged to ST1 t127, while *S. aureus* 2203 and 2213 belonged to ST1 t177.

As a result of analyzing the molecular genetic properties of *S. aureus* strains 3082, 3094, 3111, 3102, 2212, and 2204, the genes for exfoliative toxin *eta* and staphylokinase *sak* were identified, and the absence of serine protease determinants (*spl*) and leukotoxins D and E (*lukDE*) was established. Enterotoxin genes *sec* and *sell* were found in *S. aureus* 3082; *sec, sell* and *selo* were found in 3094. The *blaZ* gene was present in *S. aureus* 3082, 3111, 3094, and 3082; the *blaZ* gene and the aminoglycoside resistance determinant *aph(3')-III* were present in strain 3094; and *aph(3')-Ia* was present in *S. aureus* 2212. All 5 isolates were phenotypically resistant to amoxicillin and amikacin. The strains were identified as belonging to ST45, but to different spa-types: t102, t362, t8416, t5599, t5132 (Fig. 2).

S. aureus strains 3084, 3085, and 2219 were characterized by the presence of the pathogenicity genes *splABCDE*, *eta*, *lukDE* and *blaZ*. Genes for enterotoxins *sei, sej, selo* and *selr* were detected in 3085 strains.

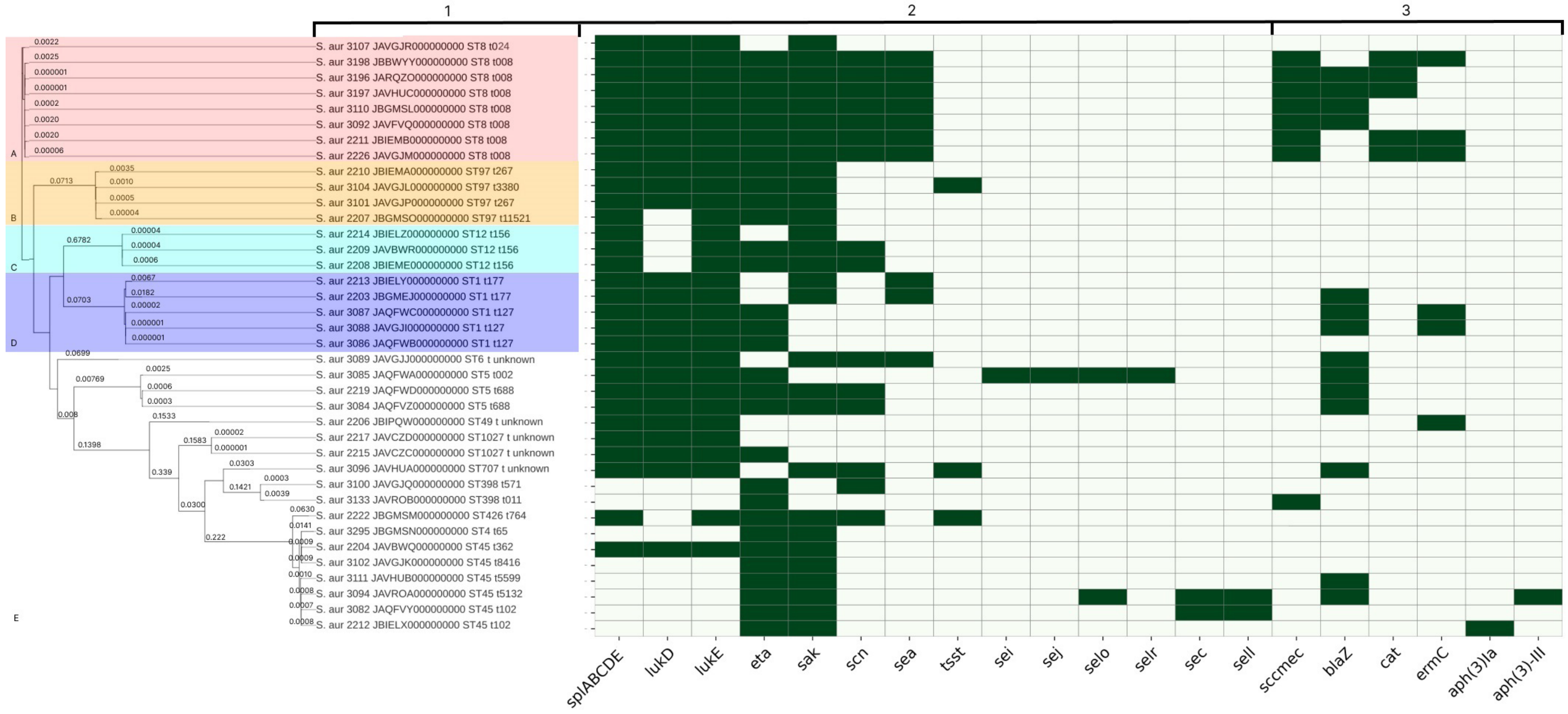

Fig. 2. Phylogenetic tree for genomes of *S. aureus* strains of group 3.

1 — microorganism type, strain number, GenBank database accession number, ST, spa type; 2 — pathogenicity gene determinants (green — presence of the trait, white — absence); 3 — antibiotic resistance determinants (green — presence of the trait, white — absence).

All strains were phenotypically resistant to amoxicillin and were identified as belonging to ST5, t002, and t688 (Figure 2). *S. aureus* strains 3096 and 3089 were characterized by the presence of the *splABCDE*, *lukDE*, *sak*, *scn*, and *blaZ* genes; strain 3096 contained the *tsst* gene, while strain 3089 contained the *sea* gene. They are also resistant to amoxicillin and were classified as ST707 and ST6, although the program used in the study did not allow their spa-type to be determined. *S. aureus* 3100 and 3133 were characterized by the absence of serine protease genes (*spl*) and leukotoxins D and E (*lukDE*); strain 3133 was resistant to oxacillin and cefoxitin, and a type IV SCC*mec* cassette was found in its genome. Both strains belonged to ST398, t571 and t011 (Fig. 2).

The remaining 12 strains belonged to different ST and spa-types. The genes *splABCDE*, *eta*, *lukDE* and *sak* were detected in *S. aureus* strains 2210, 3104, 3102 and 2207, and the toxic shock syndrome gene *tsst* was detected in *S. aureus* 3104. The strains belonged to ST97 and different spa types: t267, t3380, t11521 (Fig. 2). *S. aureus* strains 2208, 2209 and 2214 were characterized by the presence of the *splABCDE*, *lukDE*, *sak*, and *scn* genes and were assigned to ST12 and t156. Genes *splABCDE* and *lukDE* were identified in *S. aureus* 2206, 2217 and 2215, and their belonging to ST49 and ST1027 was established, but the program used in the study did not allow for the determination of their spa-types. Strain *S. aureus* 2222 was found to have the *splABCDE, lukE, sak, scn, eta* and *tsst* genes and was classified as ST426 and t764. *S. aureus* 3295 was characterized by the presence of *sak* and *eta* determinants and was classified as ST 4. All 12 strains lacked antibiotic resistance determinants and exhibited phenotypic susceptibility to antibacterial drugs from all groups.

In total, strains of *S. aureus* belonging to 13 different STs and 19 spa types were isolated from patients with CABSI, medical personnel and the external environment of the city and region.

Discussion

Circulation of the pathogen refers to its continuous and sequential transmission from one susceptible organism to another, ensuring its existence as a biological species, as well as the spread of the pathogen within healthcare facilities, characterized by the colonization of environmental surfaces and the involvement of patients and staff.

One of the most important criteria for a hospital strain is its belonging to a homogeneous (uniform in composition) population of circulating microorganisms [16]. The homogeneity of a population can be most reliably assessed by studying the genetic characteristics of the strains, which involves identifying and analyzing virulence genes, antibiotic resistance genes, and determining the ST through the analysis of alleles of housekeeping genes. For the genetic typing of *K. pneumoniae*, determining their K-type, which depends on the sequence of the *wzi* gene encoding a surface protein involved in capsule assembly on the cell's outer membrane, is of significant importance. Establishing the belonging of strains to one of the known pathotypes is also important [17].

In recent years, the existence of three *K. pneumoniae* pathotypes has been recognized: hypervirulent (hvKp), classical (cKp), and convergent (hv-MDRKp). The hypervirulent pathotype is associated with the development of serious invasive infections in healthy immunocompetent individuals. Currently, the main characteristic correlated with hypervirulence is the secretion of the siderophores aerobactin, salmochelin, yersiniabactin and the exotoxin colibactin [18]. The classical pathotype is globally widespread; these *Klebsiella* are representatives of the human microbiome, cause diseases in weakened patients, and are among the leading causes of nosocomial infections. Their genome invariably contains a complex of beta-lactamases and carbapenemases, and individual pathogenicity genes (excluding aerobactin and salmochelin) can also be detected. *K. pneumoniae* of the convergent pathotype combine the characteristics of hypervirulent and classical *Klebsiella*, meaning they have high pathogenic potential and multiple antibiotic resistance, and are capable of causing disease in both healthy immunocompetent individuals and immunocompromised patients [17, 18].

K. pneumoniae strains of group 1 isolated from sick children in the neonatal intensive care unit, as well as from equipment and care items, were similar in their virulence determinants and did not contain the aerobactin and salmochelin genes. However, the *rmpA* gene (hypermucoid phenotype) was detected in 3 strains, which was previously associated with hypervirulence. Currently, it is recognized that it is advisable to assess the entire complex of pathogenicity genes, and the presence of this determinant is not a significant indicator [5, 18]. The presence of the gene in only 3 out of the 7 strains studied (table) can be explained by its plasmid origin and high mobility within the microorganism population [19]. One beta-lactamase, bla_{SHV-1}, was identified in the genomes of all strains. Molecular typing revealed that the strain population belonged to a rare sequence type, ST3, and capsule type K3, which had not been previously isolated in Russia (only 16 isolates of this ST are registered in the BIGSdb-Pasteur database).

The homogeneity of the molecular-genetic and phenotypic properties of the strains isolated from patients and the external environment indicates the circulation of a population of classical *K. pneumoniae* strains within the hospital. The identification of this population is unfavorable from an epidemiological perspective and confirms the fact that cases of HAIs in the neonatal intensive care unit can be associated not only with hypervirulent strains of *K. pneumoniae* but also with classical strains that do not possess multiple antibiotic resistance and a wide range of pathogenicity genes [20]. This necessitates continuous molecular-epidemiological monitoring of *K. pneumoniae* strains belonging to different pathotypes in the microbiota of newborns, mothers, medical personnel, and surrounding objects in neonatal units of pediatric hospitals and perinatal centers, studying their properties, as well as integrating the data into the VGARus Russian database.

Analysis of *K. pneumoniae* strains from group 2 isolated in a multidisciplinary hospital revealed that strains *K. pneumoniae* 3254, 3260, and 3263 had an identical pathogenicity and antibiotic resistance gene spectrum, as well as the same antibiotic resistance phenotype. These strains belonged to ST395-K39 and were classified as a convergent pathotype due to the presence of aerobactin and a spectrum of beta-lactamase and carbapenemase genes. The strains were isolated from both patients and the external environment, indicating the circulation of *K. pneumoniae* ST395-K39 strains within the hospital. The presence of the yersiniabactin gene cluster in *K. pneumoniae* 3263 can be explained by horizontal gene transfer processes associated with a transposon, which has high mobility [21]. *K. pneumoniae* strains 3255 and 3259 (ST395-K2) and *K. pneumoniae* 3245 and 3251 (ST395-K47) were isolated in single cases only from patients, contained the aerobactin gene and AMR determinants in their genomes, and were also classified as a convergent pathotype.

Thus, 7 out of 10 strains isolated in a multidisciplinary hospital belonged to ST395, which could be considered evidence of their circulation. This is consistent with scientific literature data on the widespread distribution of this ST, among which strains with significant pathogenic potential are frequently found, capable of causing severe systemic infections [5, 17]. However, in-depth analysis of the strains' molecular genetic properties revealed their heterogeneity even within a single ST and led to the conclusion that only the *K. pneumoniae* ST395-K39 strain populations were circulating in the hospital.

All other *K. pneumoniae* strains: 3256 (ST5209-K35), 3253 (ST 441-K62), 3247 (ST147-K64) were classified as classical and were isolated only in isolated cases from patients. It should be noted that the *bla*$_{NDM-1}$ gene for New Delhi metallo-beta-lactamase was found in the genome of the *K. pneumoniae* 3247 strain, which explains this strain's high degree of antibiotic resistance. It is known that this determinant is associated with plasmids and is capable of active horizontal transfer [17], so the detection of such a strain is unfavorable from an epidemiological perspective, as it can lead to the rapid global spread of a polyresistant population within medical facilities.

Within the phylogenetic tree, all strains from group 2 clustered according to their ST and K-types. *K. pneumoniae* ST 395 strains formed 3 subclusters according to their K-types, and 3 *K. pneumoniae* ST395-K39 strains were included in a single cluster, uniting strains isolated from patients and the external environment of the medical organization (Fig. 1).

Analysis of pathogenicity gene spectra, antibiotic resistance profiles, MRSA or MSSA (methicillin-susceptible staphylococci) group affiliation, and ST and spa-typing [22] is crucial for studying the circulation of *S. aureus* strains. This is based on the analysis of the sequence of repeats in the gene for staphylococcal surface protein A (protein A) [4]. During investigations of local outbreaks of illness, determining the spa-type is an important step, as it allows for differentiation between strains belonging to the same ST.

Molecular genetic analysis of *S. aureus* group III strains associated with CABSI revealed that the genomes of 7 strains of *S. aureus* 2226, 3092, 3110, 2211, 3196, 3197 and 3198 contained a type IV SCC*mec* cassette, they had identical virulence gene profiles, the same resistance phenotypes, and belonged to ST 8 t008.

The strains were isolated from both a healthcare worker and patients diagnosed with CABSI who were receiving outpatient treatment at a hemodialysis center and inpatient treatment at medical institutions in the city, which confirms the fact of their circulation. The difference in strains based on the spectrum of *blaZ*, *ermC* and *cat* determinants (Fig. 2) can be explained by the fact that these genes are located on plasmids and have high mobility [23–25]. According to scientific literature, *S. aureus* ST8 t008 SCC*mec* IV strains are common, often associated with HAIs, and have been identified in Russia since the 1990s [4].

Strains *S. aureus* 3086, 3087 and 3088 are also identical in their pathogenicity genes, determinants, and antibiotic resistance phenotype. They belonged to the MSSA group, were typed as *S. aureus* ST1 t127, and were isolated from 3 patients (peritoneal catheter exit site). At the same time, strains of *S. aureus* of other sequence types were isolated from the peritoneal fluid of these same patients — ST5 (t688), ST97 (t267), and ST45 (t8416). This indicates that the population of *S. aureus* ST1 t127 strains is circulating in this medical organization.

All other group III *S. aureus* isolates were heterogeneous in their determinant spectrum, antibiotic resistance phenotype, sequence types, and spa types, which prevents an assessment of their epidemiological significance in this study.

Phylogenetic analysis of whole-genome sequences of the strains revealed the presence of five clusters, grouping strains belonging to the same sequence types and clonal complexes (CCs), which are groups of genetically closely related sequence types. *S. aureus* strains ST8, 97, 12, and 1 formed independent groups (A, B, C, D), while strains of various sequence types: ST6 and ST5 belonging to clonal complex 5 (CC5), *S. aureus* ST4 and ST45 belonging to CC45, as well as strains ST49, ST1027, ST707, ST398 and ST426 not belonging to specific clonal complexes but having phylogenetic relatedness to each other, were included in a single large cluster E (Fig. 2).

Thus, in the hemodialysis units of the city and region, 13 different sequence types and 19 spa-types of *S. aureus* strains were identified, and the circulation of populations of epidemic *S. aureus* MRSA (SCC*mec* IV) of molecular type ST8 t008 and *S. aureus* MSSA of molecular type ST1 t127 was demonstrated.

Conclusion

As a result of the study conducted in the Nizhny Novgorod region, a large genetic diversity of *K. pneumoniae* and *S. aureus* strains was identified. In-depth analysis revealed the circulation of a population of classical *K. pneumoniae* ST3-K3 strains in the neonatal intensive care unit of a pediatric hospital, isolated from the gastrointestinal tracts of children, from medical equipment and products, containing a number of virulence genes and the $bla_{SHV\text{-}1}$ beta-lactamase.

The circulation of strains of the convergent *K. pneumoniae* ST395-K39 pathotype was detected in a multidisciplinary hospital, and strains of *K. pneumoniae* ST395-K2, ST395-K47, ST5209-K35, ST441-K62, ST147-K64 were identified, containing a spectrum of pathogenicity genes and beta-lactamases in their genome, including the New Delhi metallo-beta-lactamase $bla_{NDM\text{-}1}$, which could lead to the formation and spread of a polyresistant clone in the hospital, capable of replacing the circulating pathogen and causing outbreaks of healthcare-associated infections.

Circulation of *S. aureus* MRSA (SCC*mec* IV) ST8 t008 and *S. aureus* MSSA ST1 t127 populations was identified in the hemodialysis units of the city and region, and other *S. aureus* strains belonging to 11 different STs- and 17 spa-types, potentially capable of forming hospital clones and spreading widely within the medical facility, were also detected. In this regard, to prevent the occurrence and spread of healthcare-associated infections (HAIs), it is necessary to conduct mandatory continuous microbiological monitoring in hospitals, an integral part of which should be molecular epidemiological monitoring aimed at obtaining up-to-date information on the genetic variants of circulating pathogens, including *K. pneumoniae* and *S. aureus*.

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DNA-protein COVID-19 combination vaccine containing multiepitope T-cell immunogen and receptor binding domain of the SARS-CoV-2 S protein

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Abstract

Introduction. During the COVID-19 pandemic, the development of preventive vaccines, including those based on new platforms, became extremely relevant. One such platform is vaccines, which combine, for example, DNA and protein components into a single vaccine.

The aim of this study was to investigate the immunogenicity of a DNA vaccine encoding a polyepitopic T-cell immunogen of the SARS-CoV-2 virus, combined with the recombinant RBD protein (the receptor-binding domain of the SARS-CoV-2 virus S protein, Wuhan-Hu-1 strain) conjugated to a polycationic carrier – polyglucin-spermidine (PGS), and to assess the contribution of individual components to the development of an immune response in BALB/c mice.

Materials and methods. To create the DNA vaccine (pBSI-COV-Ub), we used a strategy of designing an artificial polyepitope immunogen consisting of conserved immunodominant fragments of various structural proteins of the SARS-CoV-2 virus, containing a large number of T-lymphocyte epitopes: helper and cytotoxic. The recombinant RBD protein was conjugated with the polycation PGS, and upon mixing it with DNA, it formed the vaccine complex CCV–BSI, whose immunogenic properties were investigated in this work.

Results. Immunization of BALB/c mice with the CCV–BSI combined construct resulted in the induction of high antibody titers with neutralizing activity against live SARS-CoV-2 virus, as well as the formation of a virus-specific T-cell response, as demonstrated by ELISA, neutralization assay and ELISpot. It has been shown that the protein component contributes to the humoral immune response, while DNA contributes to the cellular immune response. Administration of the recombinant RBD protein led to the induction of only antibodies, administration of the DNA vaccine led to the induction of only a T-cell response, and administration of the combined preparation led to the induction of both a humoral immune response and specific T cells.

Conclusion. The unique combination of DNA and protein within a single vaccine construct allows for overcoming the limitations of each of these vaccine types and leads to the induction of both arms of immunity. The protein component can be replaced according to the current viral strain, and a universal T-cell immunogen can provide a response to a wide range of circulating variants. This platform can be further used to develop vaccines against various highly variable viruses.

Keywords: *SARS-CoV-2, DNA vaccines, subunit vaccines, immunogenicity, artificial T-cell immunogen*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the principles of humanity set out in the Directives of the European Community (86/609/EEC) and the Helsinki Declaration. The research protocol was approved by the Bioethical Commission of the State Research Center of Virology and Biotechnology «Vector» (protocol No. 1, March 21, 2023).

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Оригинальное исследование
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Комбинированная ДНК-белковая вакцина, содержащая полиэпитопный Т-клеточный иммуноген и рецептор-связывающий домен белка S вируса SARS-CoV-2

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Аннотация

Введение. В период пандемии COVID-19 стала чрезвычайно актуальной разработка профилактических вакцин, в том числе основанных на новых платформах. Одной из таких платформ являются комбинированные вакцины, сочетающие в одном препарате, например, ДНК и белковые компоненты.

Целью данной работы являлось исследование иммуногенности ДНК-вакцины, кодирующей полиэпитопный Т-клеточный иммуноген вируса SARS-CoV-2, в сочетании с рекомбинантным белком RBD (рецепторсвязывающий домен белка S вируса SARS-CoV-2, штамм Wuhan-Hu-1), конъюгированным с поликатионным носителем — полиглюкин-спермидином (PGS), а также оценка вклада отдельных компонентов в развитие иммунного ответа у мышей линии BALB/c.

Материалы и методы. Для создания ДНК-вакцины (pBSI-COV-Ub) мы воспользовались стратегией дизайна искусственного полиэпитопного иммуногена, состоящего из консервативных иммунодоминантных фрагментов различных структурных белков вируса SARS-CoV-2, содержащих большое количество эпитопов Т-лимфоцитов: хелперных и цитотоксических. Рекомбинантный белок RBD был конъюгирован с поликатионом PGS, в результате смешивания его с ДНК образовывал вакцинный комплекс CCV–BSI, иммуногенные свойства которого были исследованы в данной работе.

Результаты. Иммунизация мышей линии BALB/c комбинированной конструкцией CCV–BSI привела к индукции высоких титров антител, обладающих нейтрализующей активностью в отношении живого вируса SARS-CoV-2, а также к формированию вирус-специфического Т-клеточного ответа, что было показано с помощью иммуноферментного анализа, реакции нейтрализации и ELISpot. Показано, что вклад в гуморальный иммунный ответ вносит именно белковый компонент, а в клеточный — ДНК. Введение рекомбинантного белка RBD привело к индукции только антител, введение ДНК-вакцины — к индукции только Т-клеточного ответа, введение комбинированного препарата — к индукции и гуморального иммунного ответа, и специфических Т-клеток.

Выводы. Уникальная комбинация ДНК и белка в составе одной вакцинной конструкции позволяет преодолеть ограничения каждого из данных типов вакцин и приводит к индукции обоих звеньев иммунитета. Белковый компонент может быть заменён в соответствии с актуальным вирусным штаммом, а универсальный Т-клеточный иммуноген может обеспечить ответ на широкий спектр циркулирующих вариантов. Данная платформа в дальнейшем может быть использована для разработки вакцин против различных вирусов с высокой изменчивостью.

Ключевые слова: *SARS-CoV-2, ДНК-вакцины, субъединичные вакцины, иммуногенность, искусственный Т-клеточный иммуноген*

Этическое утверждение. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с принципами гуманности, изложенными в директивах Европейского сообщества (86/609/EEC) и Хельсинкской декларации. Протоколы были одобрены на заседаниях Биоэтической комиссии ГНЦ ВБ «Вектор» (протокол № 1 от 21.03.2023).

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Introduction

The COVID-19 pandemic led to the adoption of unprecedented measures in global healthcare. Specifically, for mass vaccination in emergency situations, vaccines based on innovative approaches have been approved: mRNA vaccines [1], DNA vaccines [2] and viral vector vaccines [3, 4]. It has been established that vaccines of these types are capable of inducing T-cell responses that are important for combating viral infection.

For related viruses SARS-CoV (the coronavirus that causes severe acute respiratory syndrome) and MERS-CoV (the coronavirus that causes Middle East respiratory syndrome), it has been shown that the virus-specific T cell response persists for decades, while neutralizing antibody titers decline significantly within six months after the illness [5, 6]. A decrease in the neutralizing activity of sera has also been shown with the emergence of new SARS-CoV-2 virus strains [7]. It should be noted that the long-lasting T cell response is specific not only to the main antigen – the S glycoprotein, the primary target of the humoral immune response – but also to other proteins that are not usually used as immunogens in subunit vaccines [8]. Since proteins N, M, E and others are less prone to accumulating mutations [9], their sequences are more conserved [10], and the T-cell immune response directed against their epitopes can be specific to emerging strains [11]. The promising strategy is creating artificial T-cell polyepitope immunogens containing immunodominant epitopes of viral/bacterial/tumor proteins recognized by $CD4^+$ and $CD8^+$ T cells. The DNA vaccine platform is capable of stimulating cytotoxic and T helper lymphocytes through intracellular synthesis and processing of the immunogen, followed by its presentation by antigen-presenting cells in complex with class I and II major histocompatibility complex (MHC) molecules.

However, in the case of SARS-CoV-2, the importance of the humoral immune response should not be forgotten, since high titers of neutralizing antibodies correlate with vaccine efficacy [12]. The most suitable immunogens for inducing antibody production are recombinant proteins [13]. They are limited in stimulating a specific cytotoxic response, but they activate B cells and T helper cells, especially well in the presence of adjuvants [14]. Such adjuvants can be aluminum salts, various emulsions and polysaccharides. One of the most studied α-glucans used for drug delivery is polyglucin (high molecular weight dextran) [15]. Polyglucin provides prolonged release of the active substance and is also an immunomodulator, which makes it work as an adjuvant. Thanks to the presence of hydroxyl groups, polyglucin is easily modified, particularly by the attachment of substances containing amino groups, such as proteins [16].

Combining different types of immunogens (DNA and protein) in a single construct appears to be a promising approach that can overcome the limitations of each type of vaccine and lead to the induction of both arms of immunity. In most studies investigating the possibility of combining different immunogens, immunization is performed using prime-boost strategies, where the first immunization is most often done with a DNA vaccine, and the booster is with a protein complexed with an adjuvant [17]. Certain studies have shown that co-administration of DNA and protein leads to increased immunogenicity compared to groups receiving the components separately, as well as groups receiving vaccines in a prime-boost system [18–21]. Thus, the administration of a mixture of DNA vaccine encoding the SARS-CoV-2 virus S protein and recombinant S protein, combined with an aluminum hydroxide adjuvant, resulted in the inducing of a strong protective immune response in Syrian hamsters, significantly exceeding the values in groups of animals immunized with individual components [21]. It was also shown that co-administering DNA and protein at the same site is more effective than administering them simultaneously but at different sites (e.g., in different paws) [19].

For other vaccines that induce a T-cell response, such as mRNA or vector vaccines, co-administration with protein is not found in the literature. However, there are studies dedicated to the positive effect of boosting the immune response to these types of vaccines with subunit drugs [22–25].

The aim of this study was to investigate the immunogenicity of a DNA vaccine encoding a polyepitopic T-cell immunogen of the SARS-CoV-2 virus, combined with the recombinant RBD protein (receptor-binding domain of the SARS-CoV-2 virus S protein) conjugated to a polycationic carrier — polyglucin-spermidine (PGS), and to assess the contribution of individual components to the development of an immune response in BALB/c mice.

Materials and methods

Construction of the pBSI-COV DNA vaccine

The DNA component was the plasmid pBSI-CoV-Ub, which we obtained earlier [26]. T-cell epitope prediction was performed using the NetMHCpan-4.1 program and the Immune Epitope Database 2.22, IEDB 2.22. The sequences of the S, N, M and E proteins were obtained from genome sequencing the SARS-CoV-2 virus strain Wuhan-Hu-1 (GenBank MN908947). The fragments selected for the study were analyzed for conservation using the GISAID database, then sequentially linked into a single construct, with ubiquitin added to the N-terminus, and the PADRE epitope (Pan DR Epitope, a universal T helper epitope that enhances the induction of B cell-regulating $CD4^+$ T cells and cytotoxic T lymphocytes), and the EPFRDYVDRFYKTLR marker epitope added to the C-terminus. The coding genetic sequence was optimized for efficient translation in mammalian cells using the Jcat program (http://www.jcat.de). The gene synthesis was performed by the DNA Synthesis company. Cloning into the pVAX1 vector was performed at the PspLI and ApaI sites.

Target gene expression was assessed using reverse transcription PCR and Western blotting [26].

Obtaining a combined DNA-protein vaccine

The RBD protein and its conjugate with PGS were obtained as previously described [27]. The RBD sequence was sourced from the genomic data of the Wuhan-Hu-1 isolate (GenBank: MN908947.3). A combined DNA-protein preparation was obtained by mixing 2 parts of plasmid DNA with 1 part of RBD protein conjugated to PGS (by mass ratio of DNA and protein), and then adding an excess of PGS at a mass ratio of 1:10 relative to the DNA. Complex formation was evaluated by the shift in electrophoretic mobility in a 1% agarose gel, as well as by electron microscopy: staining with a 2% aqueous solution of uranyl acetate, JEM-1400 microscope (Jeol).

Animal immunization, sample preparation

Work with laboratory animals was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" and the principles of humanity outlined in European Community directives (86/609/EEC) and the Helsinki Declaration. The protocols were approved by the Bioethics Committee of the State Research Center of Virology and Biotechnology "Vector" (protocol No. 1 dated March 21, 2023).

To assess the immunogenicity of the constructs, female BALB/c mice weighing 16–18 g, obtained from the nursery of the State Research Center of Virology and Biotechnology "Vector", were used. The animals were divided into groups of 6 mice and immunized intramuscularly twice, 3 weeks apart. Each mouse received 200 µL of the injectable drug into both quadriceps muscles of the hind legs (100 µL into each):

- CCV-BSI group — a combination vaccine containing 100 µg of DNA and 50 µg of protein;
- pBSI-COV-Ub group — 100 µg of plasmid;
- RBD-PGS group — 50 µg of RBD protein conjugated with PGS;
- intact group — non-immunized animals.

Two weeks after the second immunization, blood was taken from the mice to analyze the humoral immune response, and spleens were taken to analyze the cellular response.

Sera were separated from cellular elements by centrifugation (9000*g*, 15 min), heated for 30 minutes at 56°C, and tested for the presence of antibodies that specifically bind to the RBD protein in an enzyme-linked immunosorbent assay (ELISA), and their virus-neutralizing activity was also analyzed.

Spleens were sequentially minced on 70 and 40 µm pore diameter nylon cell strainers (JET Biofil). Splenocyte preparation was performed as previously described [27].

Enzyme immunoassay

Recombinant RBD protein was adsorbed onto 96-well plates (Nunc) in 2M urea at 4°C overnight (1 µg/mL). The ELISA was performed as previously described [27]. The results were analyzed at a wavelength of 450 nm using a Feyond A-300 spectrophotometer (Allsheng).

Virus neutralization reaction

The neutralizing properties of blood serum antibodies were analyzed in a virus cytopathic effect inhibition assay on cell culture *in vitro*, as previously described [27]. The study used the SARS-CoV-2 strain nCoV/Victoria/1/2020, obtained from the State Collection of Viral Infection and Rickettsial Pathogens at the State Research Center of Virology and Biotechnology "Vector" of the Federal Service for Surveillance on Consumer Rights Protection and Human Well-being (Rospotrebnadzor). The neutralizing activity of the sera from immunized animals was assessed by serum dilution, at which the protection of cells from the cytopathic effect of the virus was recorded in 50% of the wells.

ELISpot

ELISpot was performed using the ELISpot Plus: Mouse IFN-γ (ALP) kit (Mabtech) according to the manufacturer instructions. Splenocytes were seeded at a density of 2.5×10^5 cells per well and were then treated with RPMI medium containing 10% fetal bovine serum (for the negative control), or a mixture of virus-specific peptides at a concentration of 20 µg/mL for each peptide, or concanavalin A (for the positive control). Spot counting was performed visually using an ELISpot reader (Carl Zeiss). The number of spot-forming units (SFU) per 10^6 cells was calculated by subtracting the values in the negative control wells.

Stimulating peptides were identified using the IEDB Analysis Resource tools and synthesized by AtaGenix Laboratories. The purity of the peptides was over 80%: VYAWNRKRI, FERDISTEI, CGPKKSTNL, RFASVYAWNRKRISN, VGGNYNYLYRLFRKS, GGNYNYLYRLFRKSN, YNYKLPDDFTGCVIA, KNKCVNFNFNGLTGT, QPTESIVRF, VSPTKLNDL, LLHAPATVCGPKKST, ASVYAWNRKRISN, YNYLYRLFRKSNL, AYSNNSIAI, QYIKWPWYI, LPPLLTDEM, SAPHGVVFL, WPWYIWLGF, YYRRATRRIRGGDGK, GTWLTYTGAIKLDDK, DDQIGYYRRATRRIR, VKPSFYVYSRVKNLN, CFVLAAVYRINWITG, YYRRATRRI, TPSGTWLTY, KHIDAYKTF, SPDDQIGYY.

Statistical processing

The data were analyzed using GraphPad Prism 6.0 software. Differences between groups were determined using the non-parametric Mann–Whitney method; differences were considered significant at $p < 0.05$.

Results

Design of an artificial polyepitopic T-cell immunogen

The T-cell immunogen was designed using an approach that allows for the selection of immunodominant epitopes and then combines them into a single sequence. Based on the analysis, 5 fragments from the S protein, 2 fragments each from the M and N proteins, and 1 fragment from the E protein were selected, which contain the highest number of $CD4^+$ and $CD8^+$ epitopes restricted by human and mouse MHC class I and II molecules. Homology analysis of these fragments for different virus strains, including variants relevant for 2024, showed a high degree of conservation (85.4–100%). A sequence encoding ubiquitin was added to the construct to increase the efficiency of intracellular processing of the synthesized protein. The amino acid sequences of the selected fragments and the overall structure of the immunogen are presented in **Fig. 1**. The designed immunogen was named BSI-COV-Ub.

The pBSI-COV-Ub plasmid (**Fig. 2**, *a*) was produced in preparative quantities and characterized previously [26]. The expression of the target gene encoding the artificial BSI-COV-Ub immunogen was determined at the RNA and protein levels using reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. According to RT-PCR data, the size of the amplified fragment was approximately 1470 bp, which corresponds to the theoretically calculated fragment when using specific primers (Fig. 2, *b*). Immunoblotting revealed discrete proteins, the largest of which corresponded to the theoretically calculated product of the BSI-COV-Ub gene (54.5 kDa; Fig. 2, *c*). The presence of a ladder of discrete proteins indicates effective processing of the T-cell immunogen within the cell.

The recombinant RBD protein and its conjugate with PGS were produced, purified and characterized previously [27].

Obtaining a DNA–protein complex

The combined complex of DNA and the RBD–PGS conjugate was obtained by mixing the components, and their interaction scheme in the mixture is presented in Fig. 3, *a*. Complex formation was determined by the change in the electrophoretic mobility of plasmid DNA in an agarose gel: the encapsulated plasmid showed significantly less mobility in an electric field compared to the naked plasmid (Fig. 3, *b*).

Electron microscopy (Fig. 3, *c*) showed that the particle sizes range is from 50 to 200 nm. A number of researchers suggest that nanoparticles of this size

Fig. 1. Schematic representation of the artificial polyepitopic T-cell immunogen BSI-COV-Ub, composed of fragments of SARS-CoV-2 virus proteins

are optimal for vaccine development because they accumulate in B-cell follicles and elicit a strong immune response [28].

Analysis of the humoral immune response

To assess the immunogenicity of the created vaccine constructs, BALB/c mice were immunized twice on days 0 and 21, and were sacrificed at the endpoint (day 35). The sera were tested for the presence of RBD-specific antibodies using ELISA, as well as for their ability to neutralize live virus. Sera from the mice immunized with pBSI-COV-Ub plasmid DNA and RBD protein conjugated to PGS, as well as sera from intact mice, were used as controls (**Fig. 4**, *a*).

According to the results of the RBD-specific ELISA two weeks after the second immunization, the average titers of specific antibodies in animals immunized with the CCV–BSI vaccine, which combines protein and DNA, were 1 : 1,557,215. In the group immunized with the RBD protein conjugated to PGS, the average titer was 1 : 391,951, which was not significantly different from the first group ($p = 0.2739$). In the sera of mice that received only the pBSI-COV-Ub plasmid and in the sera of intact animals, antibodies specifically binding to the RBD protein were detected at background levels (Fig. 4, *b*).

The results of the serum neutralization analysis using the nCoV/Victoria/1/2020 strain of the SARS-CoV-2 virus on cell culture *in vitro* also showed no significant differences between the groups immunized with the CCV–BSI combined construct and the RBD protein conjugated to PGS: the mean neutralizing titers were 1:238 and 1 : 263, respectively ($p = 0.7780$). Animal sera from those who received only the pBSI-COV-Ub plasmid and sera from intact animals did not show virus-neutralizing activity (Fig. 4, *c*).

Analysis of the cellular immune response

To assess the ability of the created constructs to induce cellular immunity in mice, spleens were taken at the end of the experiment, homogenized, and examined using the ELISpot method. Splenocytes from mice immunized with plasmid DNA pBSI-COV-Ub and RBD protein conjugated to PGS, as well as splenocytes from intact mice, were used as controls. The response was evaluated based on the ability of splenocytes to respond with interferon-γ secretion to specific stimulation, which was carried out using a pool of peptides from the RBD protein and the BSI-COV-Ub immunogen (**Fig. 5**, *a*).

ELISpot showed that the highest cellular immunity responses were achieved in the groups immunized with the DNA vaccine or the combined complex (Fig. 5, *b*). The average number of splenocytes responding to stimulation in both groups was 46 and 54 SFU per 10^6 cells, respectively ($p > 0.9999$ between groups and $p < 0.01$ compared to the other two groups). In the group receiving the RBD–PGS conjugate, a low response was recorded at the negative control level (2 SFU).

Discussion

The global spread of SARS-CoV-2 necessitated the rapid development of a vaccine. The global population vaccination campaign provided a unique opportunity to compare different strategies and platforms of the developed vaccines. Almost all existing approaches used by researchers were employed in the development, and as a result, not only classic vaccine types (inactivated, subunit) but also new-generation vaccines (mRNA, vector, DNA vaccines) have entered the world market.

It is not only the induction of a humoral immune response and neutralizing antibodies in particular that is critically important, but also the activation of a vi-

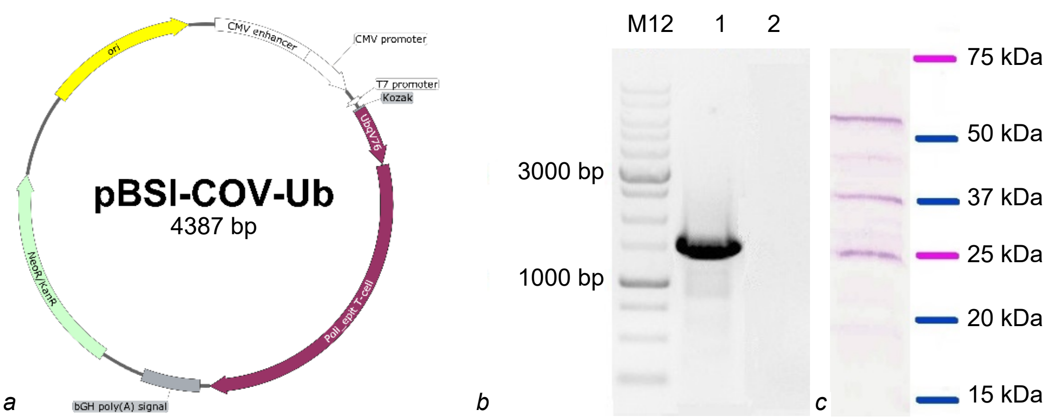

Fig. 2. Structure of plasmid pBSI-CoV-Ub (*a*) and target gene expression analysis after transfection of HEK293T cells.

b — RT-PCR products using total RNA from HEK293T cells transfected with plasmid pBSI-CoV-Ub (lane *1*) (electrophoresis in a 1% agarose gel). Lane *2*: PCR products obtained from total RNA preparations without reverse transcription.

c — protein products identified in HEK293T cells transfected with pBSI-CoV-Ub plasmid by Western blotting using monoclonal antibody 29F2 against the marker epitope.

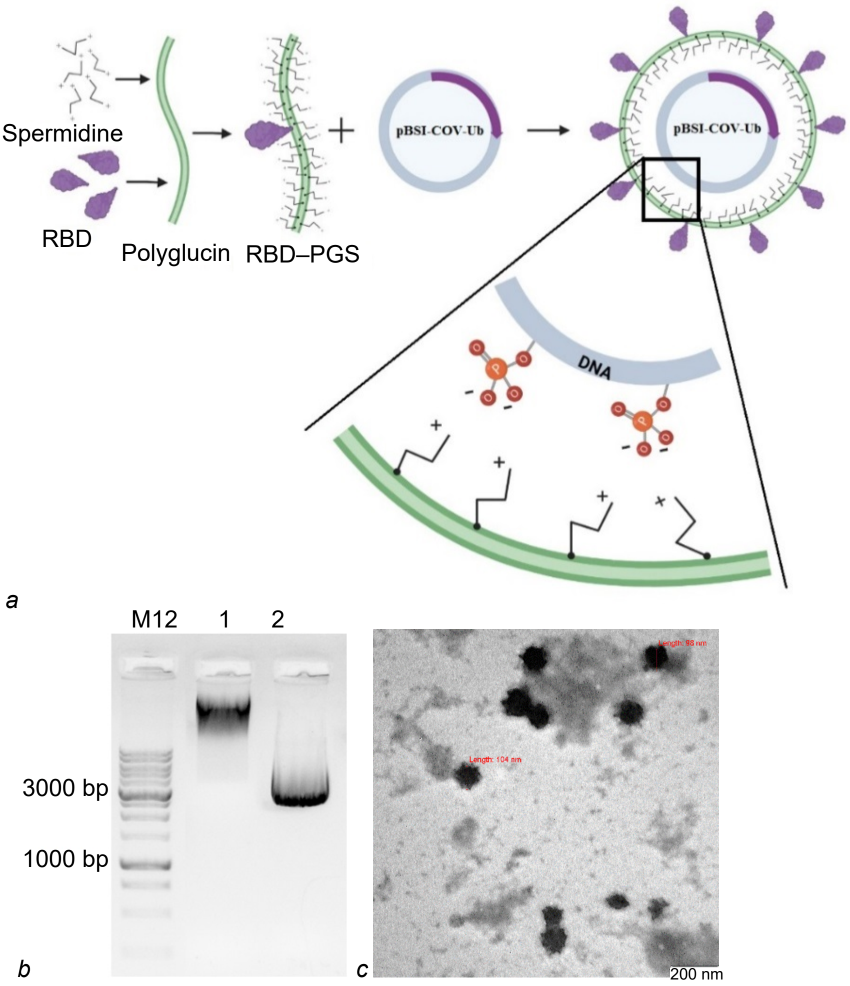

Fig. 3. DNA-protein complex.

a — schematic representation of the complex: the negative phosphate groups in the plasmid DNA electrostatically bind to the positive amino groups in spermidine conjugated with polyglucin.

b — confirmation of DNA encapsulation within the RBD–PGS shell by electrophoresis in a 1% agarose gel: *1* — CCV–BSI; *2* — pBSI-COV-Ub.

c — electron micrographs of CCV-BSI particles.

rus-specific T-cell response [29]. In this work, to create a vaccine construct, we combined two platforms: a DNA vaccine and a recombinant protein. The resulting mixture is DNA surrounded by positively charged PGS molecules conjugated to the RBD protein. The RBD was chosen as the antigen because this region of the S protein is the dominant target for the neutralizing response during COVID-19 infection.

Previously, we developed a polyepitope artificial immunogen containing CTL and Th epitopes from the SARS-CoV-2 virus proteins S, N, M and E (Fig. 1) and obtained the pBSI-COV-Ub DNA vaccine encoding this immunogen (Fig. 2) [26]. The fragments selected for inclusion in the final construct contain epitopes restricted by a wide range of human and mouse MHC class I and II molecules, which have been confirmed to be immunodominant in studies of SARS-CoV-2-specific cellular immunity and are conserved across different virus variants [30–32]. After the fragments were joined into a single sequence, ubiquitin was added to its N-terminus. The attachment of ubiquitin to a protein facilitates its targeting to the proteasome, leading to efficient processing and the release of peptide epitopes that are presented by MHC class I on the surface of an antigen-presenting cell and contribute to the activation of cytotoxic T lymphocytes [33], and through cross-presentation, they also activate T-helper cells.

The PGS conjugate used in this study was previously used to deliver candidate DNA vaccines against HIV-1, Ebola and COVID-19 [27, 34, 35]. In studies of these constructs, their safety and effective enhancement of the immunogenicity of DNA vaccines through the use of PGS have been proven. PGS protects DNA from the action of nucleases, promotes prolonged release of the immunogen, and has an immunomodulatory effect [15, 16]. PGS components are biodegradable and allow for the lyophilization of the vaccine preparation. The RBD–PGS conjugate is positively charged and, when DNA is added, begins to interact with its negatively charged phosphate groups, forming a complex (Fig. 3, *a*).

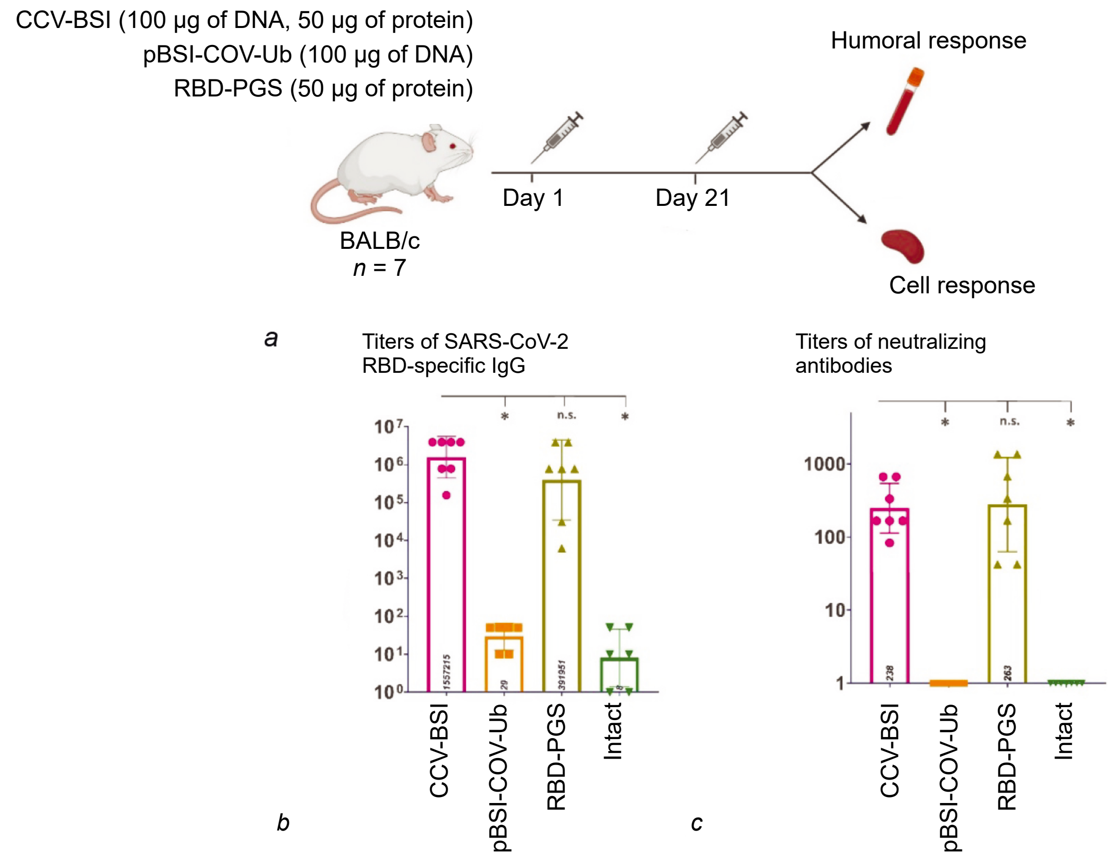

Fig. 4. Humoral immune response in BALB/c mice immunized with CCV–BSI, pBSI-COV-Ub, and RBD–PGS constructs. Intact, unvaccinated mice are the negative control.

a — schematic representation of the immunogenicity study experiment: mice were immunized twice with a 3-week interval, and samples were taken from the mice for analysis 2 weeks after the second immunization.
b — titers of SARS-CoV-2 RBD-specific IgG, determined by ELISA.
c — titers of neutralizing antibodies, determined using the SARS-CoV-2 strain nCoV/Victoria/1/2020.
The data for fragments *b* and *c* are presented as geometric means of the inverse titers with a geometric standard deviation, and individual values are marked with dots. The significance of the differences between the groups was calculated using the non-parametric Mann–Whitney method (*$p < 0.01$, **$p < 0.05$, n.s. — statistically non-significant).

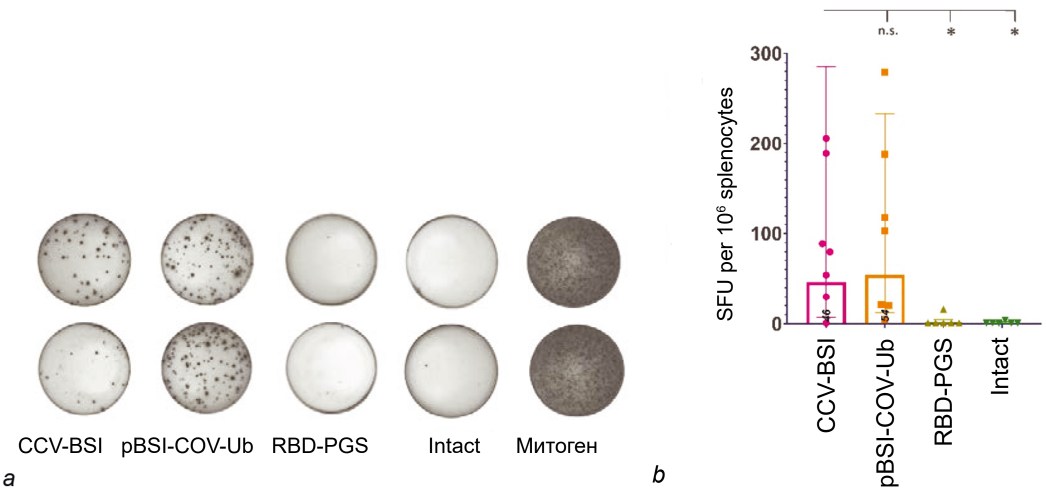

Fig. 5. Cellular immune response in BALB/c mice.

a — images of representative wells in ELISpot.
b — number of splenocytes releasing interferon-γ in response to specific stimulation with peptides from the RBD protein and the artificial BSI-COV-Ub immunogen, counted using ELISpot. Data are presented as geometric means with geometric standard deviation, individual values are marked with dots. The significance of the differences between the groups was calculated using the non-parametric Mann–Whitney method (*$p < 0.01$, **$p < 0.05$, n.s. — statistically non-significant).

Complex formation was confirmed by a decrease in DNA mobility in an agarose gel (Fig. 3, *b*).

The study of the humoral immune response in BALB/c mice after two intramuscular injections of the CCV–BSI complex showed that the combined construct, along with the RBD protein conjugated to PGS, induces the production of high titers of specific antibodies with neutralizing activity against the nCoV/Victoria/1/2020 strain of the SARS-CoV-2 virus in cell culture *in vitro* (Fig. 4, *a*, *b*). Recombinant RBD protein without an adjuvant has less pronounced immunogenicity compared to the conjugate [36]. The higher RBD-specific response in the group immunized with CCV–BSI compared to the group immunized with RBD–PGS (mean titer 1 : 1.5 million versus 1 : 0.3 million) may indicate the contribution of antigen multimerization resulting from DNA interaction with multiple molecules of the RBD–PGS conjugate, as well as the stimulation of a specific T-helper response by the DNA vaccine. A similar synergistic effect on the induction of a humoral immune response was noted by most researchers who studied the co-administration of DNA and subunit vaccines [18–20, 34].

When assessing the cellular response by determining the number of splenocytes producing IFN-γ in response to stimulation with viral peptides using the ELISpot method, the highest level of specific cellular immunity was found in the groups immunized with preparations containing the pBSI-COV-Ub DNA construct. This indicates that the DNA vaccine, both in its free state and when encapsulated, is capable of inducing a specific T-cell immune response against a wide range of viral strains, whereas immunization with the RBD protein conjugated to PGS does not induce a T-cell response (Fig. 5). In many studies, the use of DNA vaccines in conjunction with subunit vaccines leads to the induction of a T-cell response. Moreover, its development is only slightly dependent on the administration regimen, meaning that a DNA vaccine, whether used as a prime immunization or co-administered with protein, stimulates T-cells to a level comparable to that achieved by administering the DNA vaccine alone [20, 21, 34].

Conclusion

The unique combination of DNA and protein within a single vaccine construct allows for overcoming the limitations of each of these vaccine types and leads to the induction of both arms of immunity. The protein component can be replaced according to the current viral strain, and a universal T-cell immunogen can provide a response to a wide range of circulating variants. This platform can be further used to develop vaccines against various highly variable viruses.

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Criteria for assessment of the quality of *Pseudomonas aeruginosa* genome sequences

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Abstract

Introduction. With the development of sequencing technologies, the volume of genomic data is increasing, which necessitates the development of metrics for assessing the quality of genome assembly. Despite the unified nature of modern instruments (Plantagora, SQUAT, QUAST, BUSCO, CheckM2, etc.), they do not take into account the specific genome organization of particular species. The issue of import substitution of bioinformatics tools is particularly acute given limited access to foreign technologies. Furthermore, there are no specialized methods for assessing the quality of *Pseudomonas aeruginosa* genome assemblies, which is limited to general metrics (N50, number of contigs).

The aim of the study is to develop an algorithm and criteria based on a comprehensive approach for the specific assessment of the quality of whole-genome sequencing of *P. aeruginosa*.

Materials and methods. The study was conducted on 108 strains of *P. aeruginosa*. The proprietary software is developed in Java and Python languages.

Results. An algorithm for assessing the quality of *P. aeruginosa* whole-genome data has been developed based on the analysis of key housekeeping genes (*fur, algU, dinB*, etc.), genome size, GC content, and the N50 value. Genomes lacking key genes or with structural errors are classified as poor or medium, with the latter not recommended for phylogenetic analysis. The algorithm offers simple and clear parameters for assessing the quality of whole-genome data.

Conclusion. Based on the analysis of essential genes, genome size, GC content, and the N50 index, we have developed a classification of the quality of *P. aeruginosa* genome assemblies (good, medium, low). An algorithm and the Genomes Validator program have been created for rapid assessment.

Keywords: *Pseudomonas aeruginosa, whole-genome sequencing, housekeeping genes, quality assessment*

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Критерии оценки качества геномов *Pseudomonas aeruginosa*

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Аннотация

Введение. С развитием технологий секвенирования растёт объём геномных данных, что требует разработки показателей для оценки качества сборок геномов. Современные инструменты (Plantagora, SQUAT, QUAST, BUSCO, CheckM2 и др.) являются унифицированными, но при этом не учитывают особенностей организации генома конкретных видов. Особенно остро стоит вопрос импортозамещения биоинформационных инструментов в условиях ограниченного доступа к зарубежным технологиям. Кроме того, отсутствуют специализированные методы оценки качества сборок генома *Pseudomonas aeruginosa*, что ограничивается общими метриками (N50, количество контигов).

Цель работы — разработка алгоритма и критериев на основе комплексного подхода для специфической оценки качества полногеномного секвенирования представителей вида *P. aeruginosa*.

Материалы и методы. Исследование проводили на 108 штаммах *P. aeruginosa*. Авторское программное обеспечение разработано на языках Java и Python.

Результаты. Разработан алгоритм оценки качества полногеномных данных *P. aeruginosa* на основе анализа ключевых генов жизнеобеспечения (*fur, algU, dinB* и др.), размера генома, GC-состава и показателя N50. Геномы с отсутствием ключевых генов или структурными ошибками классифицируются как плохие или средние, последние не рекомендуются для филогенетического анализа. Алгоритм предлагает простые и понятные параметры оценки качества полногеномных данных.

Заключение. На основе анализа генов жизнеобеспечения, размера генома, GC-состава и показателя N50 нами разработана классификация качества сборок геномов *P. aeruginosa* (хорошее, среднее, низкое). Созданы алгоритм и программа «Genomes Validator» для оперативной оценки.

Ключевые слова: *Pseudomonas aeruginosa, полногеномное секвенирование, гены жизнеобеспечения, оценка качества*

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Introduction

With the development of high-throughput sequencing technologies and the decrease in their cost, the volume of genomic data produced is growing exponentially. Projects using large datasets of whole-genome sequencing (WGS) have many advantages: statistical power is increased, and it becomes possible to test various hypotheses about the micro- and macroevolution of genomes.

The continuous improvement of sequencing technologies and bioinformatics analysis has increased the significance of WGS in biology, medicine, pharmaceuticals, and agriculture, stimulating comparative genomic research. However, the growth in the number of sequencing projects and laboratories has led to an increase in the number of genome assemblies that are not always suitable for analysis. This highlighted the need to evaluate the quality of whole genome assembly data for researchers, who use it. This in turn created a necessity to develop standard metrics for comparing the quality of genome assemblies and annotations, as well as for evaluating the effectiveness of different methods used to obtain them.

Recent studies on genome assembly quality assessment have focused either on pre-assembly quality control or on the assembly evaluation in terms of contiguity and correctness. However, the assessment of correctness depends on the reference and is not applicable to *de novo* assembly projects. Therefore, it is worth studying methods that allow for quality assessment reports to be obtained both after and before assembly, to check the quality/correctness of *de novo* assembly and input data [1].

For genome assemblies, metrics such as the number of contigs, the number of scaffolds, and N50 (the maximum contig's length at which the total length of all contigs no shorter than this value accounts for at least 50% of the total length of all contigs in the assembly) provide only a brief overview of genome quality, not always reflecting its analytical suitability.

In turn, there are currently a sufficient number of resources and methods for the post-analysis stage of work, as well as for assessing genome quality: Picard [2], SQUAT [1], Plantagora [3], QUAST [4], CheckM1 [5], CheckM2 [6], GenomeQC [7], BUSCO [8]. However, they are unified and represent algorithms with different orientations, sometimes suitable for analyzing only eukaryotic organisms, while not taking into account the specific genome organization of a particular species. One of the most versatile and widely used instruments that utilizes genes to assess WGS data is BUSCO. Unlike the solutions mentioned above, BUSCO focuses on genome analysis using evolutionarily conserved orthologous genes, which are considered universal for certain taxonomic groups (bacteria, fungi, plants or animals). However, BUSCO does not provide an answer about the quality of the analyzed genome, only indicating the percentage of found/not found orthologous genes, and the final conclusion must be drawn by the specialist themselves. However, orthologous genes can be lost without affecting bacterial viability, unlike housekeeping genes, which can lead to an underestimation of genome quality.

Currently, WGS of infectious disease pathogens is widely used to study them, determine their origin, and track their spread. To assess the quality of such a large amount of data, domestic software tools are necessary. The latter is particularly important given that import substitution is becoming one of the strategic objectives in conditions where access to foreign technologies and foreign databases is difficult [9].

There are currently no evaluation criteria for WGS data for *Pseudomonas aeruginosa*. There are software services that perform assessments based on general (non-specific) criteria (N50, number of contigs, etc.) and do not take into account the characteristics of a specific microorganism.

The aim of the study is to create an algorithm and criteria for assessing the quality of WGS data from *P. aeruginosa* representatives, as well as to develop a domestic software capable of evaluating the quality of WGS data.

Materials and methods

The study used 108 genomes of *P. aeruginosa* strains: 24 strains were obtained from the Collection of Pathogenic Microorganisms of the Rostov-on-Don Antiplague Institute of Rospotrebnadzor (isolated in Rostov-on-Don, Khabarovsk, and Mariupol in 2022–2024), and 84 strains were obtained from the international NCBI database. WGS was conducted as part of the implementation of the federal project for the socio-economic development of the Russian Federation until 2030, "Sanitary Shield of the Country — Health Security (Prevention, Detection, Response)". Sequencing was performed on the MiSeq platform (Illumina) using the MiSeq Reagent Kit v2 (500-cycles) (Illumina). This method allows for reads 2 × 251 nucleotides long, with genome coverage ranging from 8 to 20.

The assessment of the primary sequencing data was performed using the FastQC program. The collected WGS data was analyzed using the QUAST program [4, 10]. The Trimmomatic [11] and Lighter [12] algorithms were used for trimming and for reads correction. Genome assembly from reads was performed using the Spades program [13]. All genomes have passed an initial assessment using the Kraken 2 program, which allows for the identification of DNA fragment belonging to various prokaryotic species [14]. The WGS data of strain PAO1 from the international NCBI database [15] were used as a reference genome.

The proprietary software was developed in the Java and Python programming languages. The algorithm for searching for gene sequences in the assembly was performed with the use of Smith-Waterman local alignment with a minimum similarity threshold of 80%.

The confidence interval was calculated, differences were considered significant at $p < 0.05$.

Results

It is known that the genome of the causative agent of *Pseudomonas aeruginosa* infection contains a number of genes that are critical for its viability. These genes are called housekeeping genes. It is evident that if any of these genes are missing from the WGS data, it is a sequencing and/or genome assembly error. This very feature was the basis of our proposed algorithm – all essential genes should be detected in a good-quality sequence. Of course, the selection of genes to be used for quality control is of great importance in this process.

One of the criteria we devised for the algorithm to assess genome quality is the selection of genes based on the following criteria:

- nucleotide sequences must be within 1000 bp;
- the gene must be a single-copy;
- the gene must be directly involved in the microorganism's physiological activity, performing essential functions for its life processes;
- the gene must present in all strains of *P. aeruginosa*.

The *oprI* gene was chosen for rapid species identification of *P. aeruginosa*. The main task is to assess the quality of sequencing data not only based on the identification of essential genes but also on translating their sequences. Taking in account that these genes are critical for the existence of a microbial cell, their absence from the genome or critical translation errors (stop codons) are considered as sequencing errors.

Housekeeping genes used for validating the selected whole-genome sequences of *P. aeruginosa*: *fur, algU, dinB, dnaQ, holA, holB, PA0472, fpvI, tonB1, cntL, sigX, capB, cspD, groES, rpoH*. The selected genes are essential for functioning and survival in the environment and in a macroorganism. The following parameters were chosen as criteria for evaluating the whole-genome sequences: the GC content of the *P. aeruginosa* genomes, the size of the *P. aeruginosa* whole-genome sequence, and the N50 scaffold value.

After conducting the research and selecting the quality assessment criteria for genomes, the Genomes Validator software was developed, which, for convenience, operates in "batch mode," analyzes an unlimited number of genomes, and presents the results in tabular form. For each genome, the original file name, species, quality (poor, average, good), length, N50 value, GC content, as well as the reason for the invalidity of the genome are indicated (**Fig. 1**).

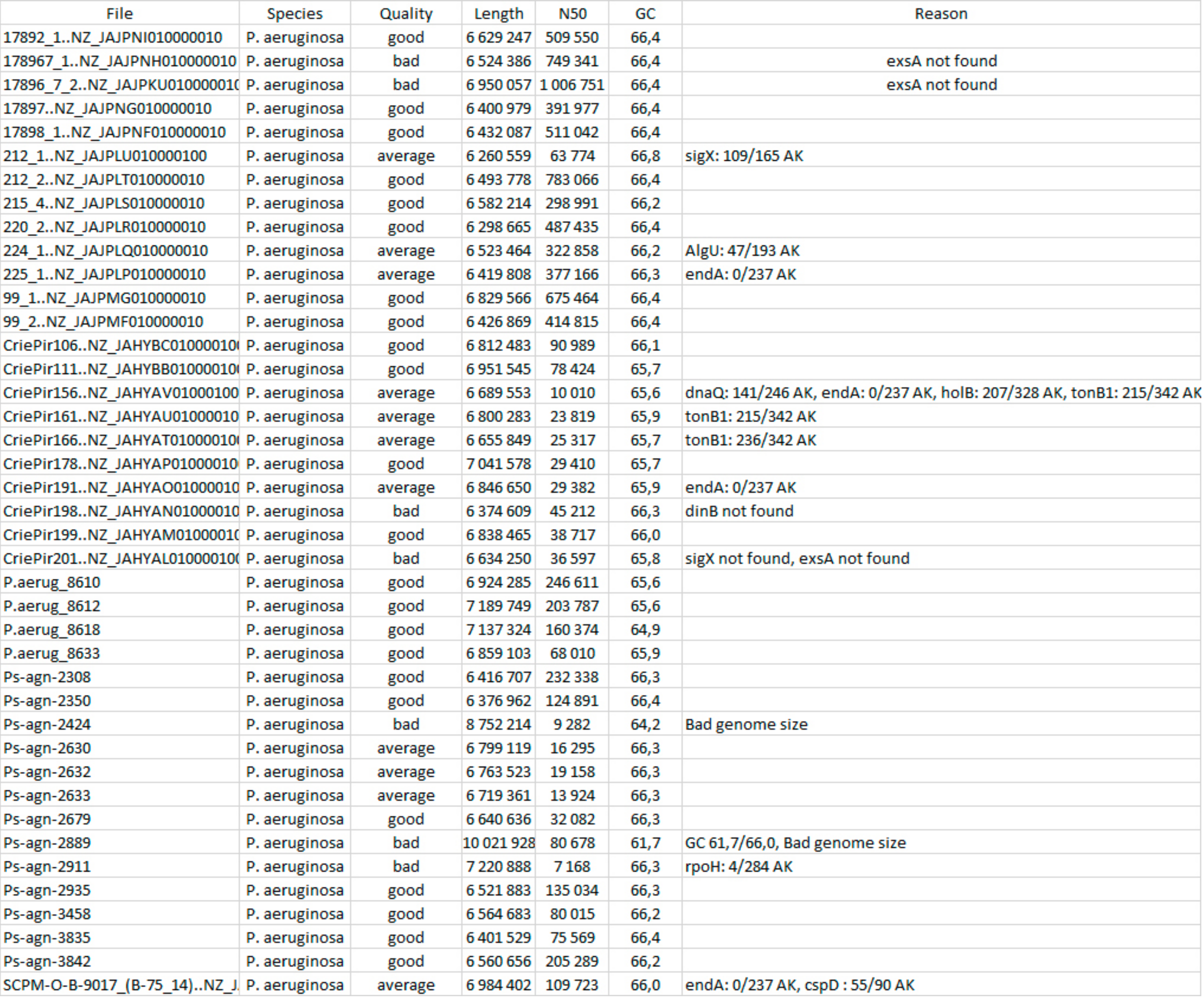
The developed program Genomes Validator is a cross-platform, which has a graphical interface, does not require installation, allows to analyze multiple genomes at the same time, and is available for downloading at https://github.com/alexeyvod/GenomesValidator. It has an intuitive interface and is user-friendly for those without programming skills.

The program was validated on a sample of 108 whole-genome sequences of *P. aeruginosa* strains. Following validation, genomes of good (63%), medium (29%), and poor (8%) quality were identified. Further analysis identified 37% of the genomes analyzed (of medium and poor quality), which can help avoid errors in subsequent calculations using bioinformatics methods. The average N50 value among the sample was 1,250,527.

The parameters N50, genome length, and GC content were identical to the values obtained from the programs used for comparison: CheckM2 and QUAST. However, these programs do not provide genome quality assessment metrics.

When assessing the quality of bacterial genomes using CheckM2 software, we found out that genomes with a Completeness value of 100 showed significant variability in the Contamination index. At the same time, the use of the Genomes Validator made it possible to estimate additionally the size of the whole-genome sequence, which may be more informative for practical analysis of WGS data. (**Fig. 2**). This parameter allows for a preliminary assessment of the presence of extrachromosomal elements in the genome of the strain under study. It should be noted that contamination with foreign DNA usually affects the overall GC content and causes a significant change in genome size, whereas the presence of plasmids or other mobile genetic elements does not lead to significant changes in this parameter.

Based on the statistical analysis of the Completeness and Contamination parameters (**Table**), it was found that Contamination values in the range of 2 to 8 may indicate a possible low reliability of the obtained WGS data. However, such results could also be due to specific characteristics of the clinical isolate's genome. Thus, the genome of strain Ps-agn-2889, analyzed in the CheckM2 program, has a Completeness score of 100 with a Contamination score of –35.65, but the reason for the contamination is not clear from the data obtained. Analysis in the program Genomes Validator revealed a 1.5-fold increase in genome size and GC content, indicating clear contamination with foreign bacterial DNA. The genome of clinical strain 44269, analyzed using the CheckM2 program, has a Completeness score of 100 with a Contamination score of –12.04, which casts doubt on its quality. Nevertheless, when using the Genomes Validator program, the genome size and GC content indicate the clear presence of extrachromosomal elements that affect the Contamination score, rather than contamination with foreign DNA, as evidenced by the research of the strain authors [16].



File	Species	Quality	Length	N50	GC	Reason
17892_1..NZ_JAJPNI010000010	P. aeruginosa	good	6 629 247	509 550	66,4	
178967_1..NZ_JAJPNH010000010	P. aeruginosa	bad	6 524 386	749 341	66,4	exsA not found
17896_7_2..NZ_JAJPKU01000001	P. aeruginosa	bad	6 950 057	1 006 751	66,4	exsA not found
17897..NZ_JAJPNG010000010	P. aeruginosa	good	6 400 979	391 977	66,4	
17898_1..NZ_JAJPNF010000010	P. aeruginosa	good	6 432 087	511 042	66,4	
212_1..NZ_JAJPLU010000100	P. aeruginosa	average	6 260 559	63 774	66,8	sigX: 109/165 AK
212_2..NZ_JAJPLT010000010	P. aeruginosa	good	6 493 778	783 066	66,4	
215_4..NZ_JAJPLS010000010	P. aeruginosa	good	6 582 214	298 991	66,2	
220_2..NZ_JAJPLR010000010	P. aeruginosa	good	6 298 665	487 435	66,4	
224_1..NZ_JAJPLQ010000010	P. aeruginosa	average	6 523 464	322 858	66,2	AlgU: 47/193 AK
225_1..NZ_JAJPLP010000010	P. aeruginosa	average	6 419 808	377 166	66,3	endA: 0/237 AK
99_1..NZ_JAJPMG010000010	P. aeruginosa	good	6 829 566	675 464	66,4	
99_2..NZ_JAJPMF010000010	P. aeruginosa	good	6 426 869	414 815	66,4	
CriePir106..NZ_JAHYBC01000010	P. aeruginosa	good	6 812 483	90 989	66,1	
CriePir111..NZ_JAHYBB01000010	P. aeruginosa	good	6 951 545	78 424	65,7	
CriePir156..NZ_JAHYAV01000100	P. aeruginosa	average	6 689 553	10 010	65,6	dnaQ: 141/246 AK, endA: 0/237 AK, holB: 207/328 AK, tonB1: 215/342 AK
CriePir161..NZ_JAHYAU01000010	P. aeruginosa	average	6 800 283	23 819	65,9	tonB1: 215/342 AK
CriePir166..NZ_JAHYAT01000010	P. aeruginosa	average	6 655 849	25 317	65,7	tonB1: 236/342 AK
CriePir178..NZ_JAHYAP01000010	P. aeruginosa	good	7 041 578	29 410	65,7	
CriePir191..NZ_JAHYAO01000010	P. aeruginosa	average	6 846 650	29 382	65,9	endA: 0/237 AK
CriePir198..NZ_JAHYAN01000010	P. aeruginosa	bad	6 374 609	45 212	66,3	dinB not found
CriePir199..NZ_JAHYAM0100001	P. aeruginosa	good	6 838 465	38 717	66,0	
CriePir201..NZ_JAHYAL01000010	P. aeruginosa	bad	6 634 250	36 597	65,8	sigX not found, exsA not found
P.aerug_8610	P. aeruginosa	good	6 924 285	246 611	65,6	
P.aerug_8612	P. aeruginosa	good	7 189 749	203 787	65,6	
P.aerug_8618	P. aeruginosa	good	7 137 324	160 374	64,9	
P.aerug_8633	P. aeruginosa	good	6 859 103	68 010	65,9	
Ps-agn-2308	P. aeruginosa	good	6 416 707	232 338	66,3	
Ps-agn-2350	P. aeruginosa	good	6 376 962	124 891	66,4	
Ps-agn-2424	P. aeruginosa	bad	8 752 214	9 282	64,2	Bad genome size
Ps-agn-2630	P. aeruginosa	average	6 799 119	16 295	66,3	
Ps-agn-2632	P. aeruginosa	average	6 763 523	19 158	66,3	
Ps-agn-2633	P. aeruginosa	average	6 719 361	13 924	66,3	
Ps-agn-2679	P. aeruginosa	good	6 640 636	32 082	66,3	
Ps-agn-2889	P. aeruginosa	bad	10 021 928	80 678	61,7	GC 61,7/66,0, Bad genome size
Ps-agn-2911	P. aeruginosa	bad	7 220 888	7 168	66,3	rpoH: 4/284 AK
Ps-agn-2935	P. aeruginosa	good	6 521 883	135 034	66,3	
Ps-agn-3458	P. aeruginosa	good	6 564 683	80 015	66,2	
Ps-agn-3835	P. aeruginosa	good	6 401 529	75 569	66,4	
Ps-agn-3842	P. aeruginosa	good	6 560 656	205 289	66,2	
SCPM-O-B-9017_(B-75_14)..NZ_J	P. aeruginosa	average	6 984 402	109 723	66,0	endA: 0/237 AK, cspD : 55/90 AK

Fig. 1. Practical demonstration of the program Genomes Validator; the results of genome analysis are presented in table format.

Discussion

The *oprI* gene was chosen as the species-defining gene for several reasons: its nucleotide sequence is 253 bp long, which allows for species identification even with very poor WGS data quality; the OprI protein plays an important role in binding to peptidoglycan, participates in immunological reactions, and is responsible for susceptibility to antimicrobial peptides [17–19]. This gene was chosen because one meta-analysis showed that it is successfully used to identify the *P. aeruginosa* species with high accuracy [20].

Housekeeping genes (*fur, algU, dinB, dnaQ, holA, holB, PA0472, fpvI, tonB1, cntL, sigX, capB, cspD, groES, rpoH*) were selected for validation of the chosen whole-genome sequences of *P. aeruginosa* based on their functional significance, as determined by a literature data analysis.

The *fur* gene is the main regulator of iron uptake in prokaryotic organisms, is essential for *P. aeruginosa* to cause pathogenesis, and for survival under iron-deficient conditions [21].

The sigma factor *algU* is a key stress response regulator that controls the expression of over 300 genes, plays a crucial role in virulence factor synthesis and pathogenesis thru quorum sensing, and enhances alginate production by increasing the expression of the *algD* operon [22].

SOS-mediated mutagenesis involves the products of the *dinB* gene, which perform translesion DNA synthesis, TLS (through damage), exhibiting low accuracy but helping to rapidly replicate DNA in response to various damaging agents. However, mutations accumulate, which in turn help acquire adaptive mechanisms in response to antibacterial drugs [23].

The DNA polymerase III ε subunit, encoded by the *dnaQ* gene, is very important and provides 3'-5' exonuclease activity, correcting mismatches encountered during DNA repair, which allows for the removal and correction of mismatched base pairs. Mutations in the *dnaQ* gene can disrupt these processes, leading to more than 1000-fold increase in the mutation rate in the genome [24]. The DNA polymerase III holoenzyme consists of δ and δ' subunits, which are encoded by the *holA* and *holB* genes, forming a complex with the ε subunit of the *dnaQ* gene and jointly participating in DNA repair [25]. The PA0472 gene encodes the RNA polymerase σ factor. It's difficult to judge what role a specific σ factor plays in the *P. aeruginosa* genome, but it is known that RNA polymerase σ factors perform a huge range of vital functions: promoter recognition, double-stranded DNA unwinding, binding to RNA polymerase, and transcription control. They are also involved in the transcription of specific regulons associated with the response to environmental changes and are included in iron transport [26].

Comparison of the quality metrics of the CheckM2 and Genomes Validator programs

«Genomes validator»	«CheckM2»	
	completeness	contamination
Good/High	99.99 ± 0.001	0.98 ± 0.203
Average	83.89 ± 1.865	2.23 ± 0.222
Poor/Low	81.01 ± 6.667	8.72 ± 3.708

Note. The confidence interval is indicated at $p < 0.05$

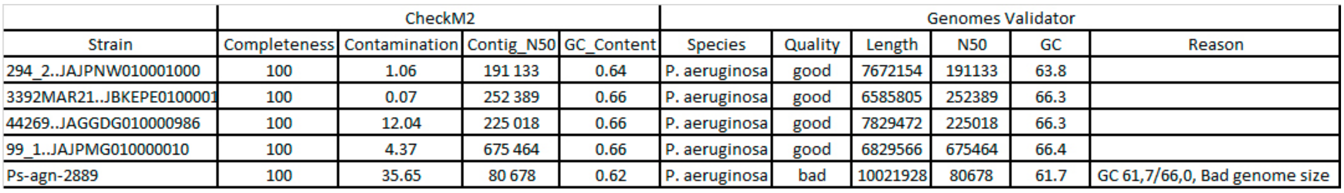
One of the RNA polymerase σ-factors involved in iron assimilation processes is the FpvI protein, encoded by the *fpvI* gene, which is involved in regulating the uptake of the high-affinity siderophore pyoverdine, an important virulence factor as it can displace iron from the iron–transferrin complex [27].

P. aeruginosa has 3 genes in its genome that encode TonB proteins (*tonB1*, *tonB2*, and *tonB3*), and only the TonB1 protein, encoded by *tonB1*, interacts with TonB-dependent transporters involved in iron or heme uptake [28].

In addition to the main siderophores, *P. aeruginosa* produces another metallophore encoded by the *cntL* gene, called pseudopalin, which is essential for the uptake and utilization of zinc, cobalt, and nickel in its pathogenesis. Urease, which is a nickel-dependent enzyme, is produced by *P. aeruginosa*, while cobalt is essential for the cobalamin-dependent ribonucleotide reductase (NrdJab), which functions in biofilm formation under oxygen-limited conditions [29].

It is known that in *P. aeruginosa*, *sigX* is involved only in the transcription of its own gene and is largely responsible for the transcription of *oprF*, which encodes the major outer membrane protein OprF, which in turn is involved in several crucial functions: maintaining cell structure, outer membrane permeability, and recognition by the host immune system [30]. Deletion or knockout of the *algU* and *sigX* genes in the PAO1 genome disrupts biofilm formation [31].

The *capB* and *cspD* genes are responsible for encoding cold shock proteins involved in adaptation to cold in the environment [32].



	CheckM2				Genomes Validator					
Strain	Completeness	Contamination	Contig_N50	GC_Content	Species	Quality	Length	N50	GC	Reason
294_2..JAJPNW010001000	100	1.06	191 133	0.64	P. aeruginosa	good	7672154	191133	63.8	
3392MAR21..JBKEPE0100001	100	0.07	252 389	0.66	P. aeruginosa	good	6585805	252389	66.3	
44269..JAGGDG010000986	100	12.04	225 018	0.66	P. aeruginosa	good	7829472	225018	66.3	
99_1..JAJPMG010000010	100	4.37	675 464	0.66	P. aeruginosa	good	6829566	675464	66.4	
Ps-agn-2889	100	35.65	80 678	0.62	P. aeruginosa	bad	10021928	80678	61.7	GC 61,7/66,0, Bad genome size

Fig. 2. Fragment of a table comparing the performance characteristics of the CheckM2 and Genomes Validator programs.

The *groES* gene encodes a heat shock protein that helps the microorganism survive at 42°C [33]. As is known, these resistance mechanisms are an integral part of the physiology of *P. aeruginosa* cells [33].

The σ^{32} factor, encoded by the *rpoH* gene, is the main regulator of the heat shock response, controlling the function of *groES*, among others [34].

The selected housekeeping genes confirmed their relevance in terms of their key role in the viability of *P. aeruginosa*, demonstrating the importance of their functioning for the biological processes of this microorganism. Furthermore, gene identification is not only based on the nucleotide sequence but is also translated into an amino acid sequence. This method was chosen to detect the stop codon in the gene and demonstrate not only its location in the genome but also its functionality. Thus, when assessing data quality, the absence of one or more of the selected genes will be considered a criterion for poor genome quality. If the N50 values for the genome selected for analysis are 10,000 or higher, but one of the candidate genes has a stop codon, it can be classified as a medium-quality genome. However, in our opinion, using a genome of this quality for phylogenetic analysis, SNP typing, or MLST analysis is not recommended. At the same time, searching for certain genes in the genome is possible, but without using them for typing the analyzed strain.

Despite the high N50 score and other evaluation parameters, the absence of two or more genes indicates poor WGS data quality. The N50 parameter is used to assess and compare the quality of genome assembly, allowing for the selection of the best among good/high-quality options.

The next criterion used to assess the quality of the WGS data was the GC content of the *P. aeruginosa* genomes. Analyzing the genomes of the strains using the CheckM2 program, we observed that genomes with Completeness scores > 97% and Contamination < 3% have a GC content ranging from 63.8% to 66.6%, which led us to establish threshold values of 65.2 ± 2.5%. The range was chosen wider to account for possible changes in the genomic composition. This criterion was supported by a literature review, which did not contradict our results and allowed us to include this parameter in a comprehensive quality assessment criterion for genome assemblies [35, 36]. This criterion demonstrates whether there is contamination of foreign DNA or reads from related species in the selected genome(s) for subsequent analysis.

WGS data validation using this criterion works as follows: if the genome selected for analysis falls within the established GC content values, it is considered a high-quality genome. If the selected genome does not fall within the established GC content values, it is considered a low-quality genome.

The quality of the genome is also assessed by the size of the *P. aeruginosa* whole-genome sequence. Thus, to evaluate the WGS data, the criteria for the minimum and maximum permissible genome sizes were used. The minimum genome size was 5.84 Mb, and the maximum was 8.26 Mb. The decision to use these values was based on literature data: for example, studies have reported that the auxiliary genome can vary within the range of 6.9–18.0% [38, 39]. The standard value for the length of the *P. aeruginosa* whole-genome sequence was taken as 6–7 Mb [37, 39].

Based on the above, the criterion for assessing the good quality of the *P. aeruginosa* genome will be a genome ranging in size from 5.84 to 8.26 Mb. If the analyzed genome falls outside the specified values, its quality will be assessed as poor or average, or the option of a more detailed and thorough analysis of this genome should be considered to exclude its structural features.

Genomes with an average level can be used limitedly for phylogenetic analysis, SNP typing, or MLST analysis, but they can be used to search for specific genes (without typing them) or for INDEL analysis.

Genomes with a low quality level are recommended not to be included in bioinformatics analysis and should be corrected by re-sequencing.

In addition to the CheckM2 and QUAST programs, which were selected as comparison tools, there are the SQUAT and Plantagora programs, but they do not meet the criteria of our research objects, as they are primarily developed for eukaryotic organisms. At the same time, CheckM2 is a tool developed for assessing the quality of prokaryotic genomes, while QUAST is a universal program. In developing our evaluation criteria, we tried to move away from complex tables with mathematical parameters assessing the quality of the WGS data provided by QUAST after the analysis. This involves the participation of bioinformatics specialists in the analysis and, in our opinion, does not fully reflect the quality of the WGS data, but rather assesses how well the genome assembly was performed [4]. At the same time, CheckM2 provides digital data on the parameters of the analyzed genome across various metrics, without drawing clear conclusions about the quality of the genome or whether it can be used for further research. The Contamination index does not always reflect the quality of the genomes of clinical isolates containing extrachromosomal elements.

Thus, we have tried, on the one hand, to select clear and concise parameters for evaluating WGS data, and on the other hand, to simplify the process for the user to obtain a specific result without resorting to in-depth bioinformatics analysis or using command linesк.

Conclusion

A comprehensive study was conducted in which we selected housekeeping genes that allow us to assess the quality of the *P. aeruginosa* WGS data. Quality assessment criteria have been defined: genome length

and GC content, which allow for the evaluation of the *P. aeruginosa* genome assembly.

Based on validated assessment criteria tested on a sample of genomes, the assembly of the *P. aeruginosa* genome can be classified into three categories based on the quality level of the source material: good, medium, and low. Good quality — the genome length is within the average genome size for the species ± 18%, the GC content is ± 2.5% of the average for *P. aeruginosa*, all essential genes have been found, and their protein product translation is not disrupted by a stop codon. Average quality – all essential genes found, but errors in their translation were detected due to the formation of a stop codon as a result of a sequencing error. Low quality — at least one gene in the life support system is missing, or the genome size or GC content does not match the value characteristic of the species.

An algorithm and a publicly available program for rapid analysis based on WGS data of *P. aeruginosa*, Genomes Validator, have been developed.

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Genetic diversity of mutations affecting the hemolytic activity of *Bordetella pertussis* bacteria during *in vitro* cultivation

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Abstract

Introduction. The two-component BvgAS system regulates the transcription of the pathogen's virulence genes and plays a key role in the pathogenesis of whooping cough, an anthroponotic infectious disease. Currently, the factors affecting the BvgAS system of *Bordetella pertussis* bacteria in the human organism have not been studied in practice. It is known that disruption of the *bvgAS* operon structure leads to phase modulations and changes in virulence. IS-elements intergation into the *bvgAS* operon and other virulence genes of *B. pertussis* bacteria is important mechanism in regulating their expression, potentially leading to the long-term persistence of this pathogen in the human body.

Aim. Identification and description of spontaneous IS-elements insertions in *B. pertussis* into *bvgAS*, *cya* and *fhaB* virulence genes responsible for hemolytic activity during *in vitro* bacterial cultivation.

Materials and methods. *B. pertussis* strains from the collection of the N. F. Gamaleya National Research Center for Epidemiology and Microbiology were used: virulent *B. pertussis* 475 strain and its isogenic attenuated strain *B. pertussis* 4MKS; virulent *B. pertussis* strain Tohama I and its avirulent mutant *B. pertussis* 347. Bacteria were cultivated on casein-charcoal agar with blood addition. The formation of haemolysis zones was assessed visually. PCR, real-time PCR and sequencing were used for the genetic characterization of the obtained insertion mutants.

Results. *B. pertussis* mutants containing insertions of IS-elements in *fhaB* and *bvgAS* genes with impaired hemolytic activity (Hly$^-$ phenotype) have been isolated *in vitro*, as well as *B. pertussis* mutants that retained hemolytic activity (Hly$^+$ phenotype), containing IS-elements in a previously undescribed orientation in the *bvgAS* gene. The frequency of insertional mutant formation depended on conditions of cultivation and bacteria genotype.

Conclusion. Arguments are made for the hypotheses about the IS-elements involvement in *B. pertussis* bacteria transition to a state of reduced virulence, which provides the possibility of long-term persistence of this pathogen in the human body.

Keywords: *Bordetella pertussis, IS-elements, regulation of transcription, virulence, persistence, hemolytic activity, Hly$^-$ phenotype*

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Оригинальное исследование
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Генетическое разнообразие мутаций, влияющих на гемолитическую активность бактерий *Bordetella pertussis* при культивировании *in vitro*

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Аннотация

Введение. Ключевую роль в патогенезе коклюша, антропонозного инфекционного заболевания, играет двухкомпонентная система BvgAS, регулирующая транскрипцию генов вирулентности возбудителя. Факторы, оказывающие воздействие на работу системы BvgAS бактерий *Bordetella pertussis* в организме человека, практически не изучены. Известно, что нарушение структуры оперона *bvgAS* приводит к фазовым переходам и изменению вирулентности. Перемещение IS-элементов в оперон *bvgAS* и другие гены вирулентности возбудителя коклюша является важным механизмом в регуляции их экспрессии, потенциально приводящим к длительной персистенции бактерий в организме человека.

Цель работы — поиск и характеристика спонтанных инсерционных мутантов бактерий *B. pertussis* по генам вирулентности *bvgAS*, *cya* и *fhaB,* ответственным за гемолитическую активность при культивировании бактерий *in vitro*.

Материалы и методы. Использованы штаммы *B. pertussis* из коллекции НИЦЭМ им. Н.Ф. Гамалеи: вирулентный штамм *B. pertussis* 475 и изогенный ему аттенуированный штамм *B. pertussis* 4MKS, вирулентный штамм *B. pertussis* Tohama I и его авирулентный мутант *B. pertussis* 347. Бактерии культивировали на казеиново-угольном агаре с добавлением крови. Формирование зон гемолиза оценивали визуально. Для молекулярно-генетической характеристики полученных инсерционных мутантов использовали методы ПЦР, ПЦР-РВ, секвенирования.

Результаты. *In vitro* выделены мутанты *B. pertussis* с нарушенной гемолитической активностью (фенотип Hly$^-$) с инсерциями IS-элементов в генах *fhaB* и *bvgAS*, а также мутанты, сохранившие гемолитическую активность (фенотип Hly$^+$), содержащие IS-элементы в ранее не описанной ориентации в гене *bvgAS*. Частота образования инсерционных мутантов зависела от условий культивирования и генотипа бактерий.

Заключение. Приведены аргументы, свидетельствующие о возможной роли IS-элементов в переходе бактерий *B. pertussis* в состояние пониженной вирулентности, что обеспечивает длительную персистенцию возбудителя коклюша в организме человека.

Ключевые слова: *Bordetella pertussis, IS-элементы, регуляция транскрипции, вирулентность, персистенция, гемолитическая активность, Hly$^-$-фенотип*

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

The Gram-negative bacterium *Bordetella pertussis* causes an acute contagious infectious disease in humans called whooping cough. The genus *Bordetella* is traditionally divided into "classical" species — *B. pertussis, B. parapertussis* and *B. bronchiseptica* — and "new" species — *B. avium, B. petrii, B. holmesii, B. hinzii, B. trematum* and *B. ansorpii*. In recent years, 3 more species have been described: *B. bronchialis, B. flabilis* and *B. sputigena* [1].

Among the virulence factors of the whooping cough pathogen, two main groups are distinguished: toxins and adhesins. Toxins include pertussis toxin, adenylate cyclase toxin (ACT), tracheal cytotoxin, dermonecrotic toxin and lipopolysaccharide endotoxin. Adhesins include filamentous hemagglutinin, agglutinogens, or fimbriae 2 and 3, the outer membrane protein pertactin, the BrkA protein and other components of the *Bordetella* type III secretion system. It is believed that the hemolysin in the bacterium *B. pertussis* is ACT [2], however, there is currently data on the influence of the FhaB protein on erythrocyte hemolysis *in vitro* when interacting with ACT [3].

An important feature of the genomes of bacteria of the genus *Bordetella* is the presence of repeated insertion sequences — IS elements — in the chromosome [4]. The number and diversity of IS elements differ among different members of the genus. The maximum number of IS481 and IS1002 is registered in the *B. pertussis* chromosome [4]. It is believed that IS elements were involved in the evolution of bacteria of the genus *Bordetella* from a common ancestor, *B. bronchiseptica* [5].

The two-component system BvgAS, consisting of the histidine kinase BvgS and the transcription regulator protein BvgA, plays a special role in the pathogenesis of whooping cough. The BvgAS system acts as a key regulator, controlling the transcription process of genes responsible for the virulence of the bacterium *B. pertussis* [6–8]. Furthermore, the BvgAS system regulates many intracellular processes, including *B. pertussis* metabolism and microbe-host interaction [8]. The level of BvgA~P production and phosphorylation determines the level of transcription of Bvg-dependent genes. The level of BvgA~P phosphorylation is determined by the activity and quantity of the BvgS phosphokinase, which depends on a number of factors, including culture conditions. Depending on the functioning of the BvgAS system, *B. pertussis* bacteria can be in a virulent state — Phase I (Bvg$^+$), an avirulent state — Phase IV (Bvg$^-$), or an intermediate phase Bvg^i, each of which contributes to the pathogenesis of whooping cough, the persistence of bacteria in the human body, and their transmission to a new host.

Despite the lack of information about factors affecting the BvgAS system's function in a living organism, certain conditions that alter the virulence of *B. pertussis* bacteria have been described when cultured *in vitro* on solid media [6–8]. Thus, lowering the cultivation temperature to 27°C, adding 50 mM magnesium sulfate or nicotinic acid to the nutrient medium promotes the bacteria's transition to an avirulent phase.

Changes in the phase state of *B. pertussis* bacteria can occur as a result of disruption of the bvgAS virulence operon structure. Two types of induced mutations that cause changes in the phenotype of *B. pertussis* are described. The first mutants without hemolytic activity (lacking zones of hemolysis on blood agar plates, Hly$^-$ mutants) were selected by culturing the laboratory virulent strain *B. pertussis* Tohama I in the presence of erythromycin [9]. The main phenotypic characteristic of Hly$^-$ mutants was the absence of zones of hemolysis around individual colonies grown on Bordet–Gengou medium supplemented with sheep blood. The frequency of Hly$^-$ mutant detection was characterized by the authors as 10^{-5}–10^{-6}. In 1989, a study by S. Stibitz et al. was published, in which the region of the *B. pertussis* Tohama I mutant responsible for hemolytic activity was mapped and sequenced. It has been shown that Hly- mutants contained a *frameshift* (f.s.) mutation associated with the acquisition of a cytosine in a sequence of 6 cytosines in the *bvgS* gene [10]. Another type of *B. pertussis* mutant in the Tohama I strain was isolated by S. Stibitz in 1998 [11]. Mutants with the bvgAS operon were found to be viable under selective conditions characterized by the overproduction of the mutant BvgA protein cloned within a plasmid. The author characterized 15 *B. pertussis* insertion mutants that survived under the specified conditions, and corresponding sequences were identified in 7 of them. Five mutants contained IS481, and 2 mutants contained IS1002 at the *bvgAS* operon *ctag* site [12]. The research has shown that *B. pertussis* mutants similar to those obtained in *in vitro* experiments have been found in late-stage whooping cough convalescents, in asymptomatic carriers in contact with whooping cough patients [13], and in experimental whooping cough in Old World monkeys at a late stage of the infectious process [14].

In recent decades, various types of knockout and regulatory mutants for the *fhaB, katA, prn* and *brkA* virulence genes of *B. pertussis*, containing IS element insertions, have been isolated from patients with whooping cough. The accumulation of such *B. pertussis* mutants in the population is thought to be linked to the widespread use of acellular pertussis vaccines in several countries [15–18].

We have proposed a hypothesis that IS elements are involved in the regulation of the expression of virulence genes and housekeeping genes of the whooping cough pathogen, which is one of the mechanisms for the long-term persistence of *B. pertussis* bacteria and the formation of persistence in the human body, and which determines the circulation and maintenance of anthroponotic infection foci.

The aim of this study was to identify and characterize spontaneous insertion mutants of *B. pertussis* bacteria in the virulence genes *bvgAS, cya* and *fhaB*, responsible for the Hly$^-$ phenotype, which are formed during *in vitro* bacterial cultivation.

Materials and methods

The study used *B. pertussis* strains from the Gamaleya Research Institute of Epidemiology and Microbiology collection: virulent *B. pertussis* 475 and its isogenic attenuated *B. pertussis* 4MKS Str^{R}Nal^{R}Cm^R [19]; virulent *B. pertussis* laboratory strain Tohama I and its avirulent mutants *B. pertussis* 347 *bvg AS::Tn5* Str^{R}Km^R.

B. pertussis bacteria were cultured on casein-charcoal agar (Medgamal) supplemented with 15% sheep blood at 35°C for 24–36 hours for culture growth and for 72–96 hours for the formation of colony-forming units (CFU). The number of colonies, their size, and shape were counted and characterized visually. The absence of extraneous microflora was monitored using light microscopy after Gram staining.

DNA from *B. pertussis* bacteria was extracted using the Sorb-B DNA extraction kit (AmpliSens). For purification of amplification products, a DNA extraction and purification kit from an agarose gel (Eurogen) was used.

For real-time polymerase chain reaction (qPCR), a CFX96 Touch thermocycler (Bio-Rad) was used, and for PCR, a device from the company Tercik was used.

The nucleotide sequence of the amplification products was determined using a 3130 Genetic Analyzer (Applied Biosystems/Hitachi).

Results

Method for registering the integration of IS elements into the bvgAS operon

Among the described mutations in the virulence genes of the whooping cough pathogen, insertion mutations — the integration of IS elements into the specific site of the *B. pertussis bvgAS* operon — deserve special attention, according to our hypothesis. In previous studies, we described a method and test systems for detecting mutations in the *bvgAS* operon, which contains IS elements 481 and 1002 integrated in a specific, at that time the only known, orientation, which we conditionally designated as orientation (1) [13].

To identify *B. pertussis* mutants containing IS elements 481 and 1002 integrations in a previously undescribed opposite orientation relative to the known orientation (1), we have modified a previously developed real-time PCR system. The new orientation is provisionally designated as orientation (2).

Integration of IS elements 481 and 1002 in orientation (2) — IS481 (2) and IS1002 (2), the positions of the primers and probes are schematically represented in **Fig. 1**. **Table 1** presents the nucleotide sequences of the probes and primers, including those flanking the specific NctagN sites located within the structure of the *cya* and *fhaB* genes.

The temperature-time profile for real-time PCR and PCR to detect IS481 and IS1002 integrations in orientation (2) and at the NctagN site of the *bvgAS* operon and *cya* and *fhaB* genes is presented in **Table 2**.

The specificity of the selected primers and probes was confirmed by determining the nucleotide sequence of the corresponding amplification products. The num-

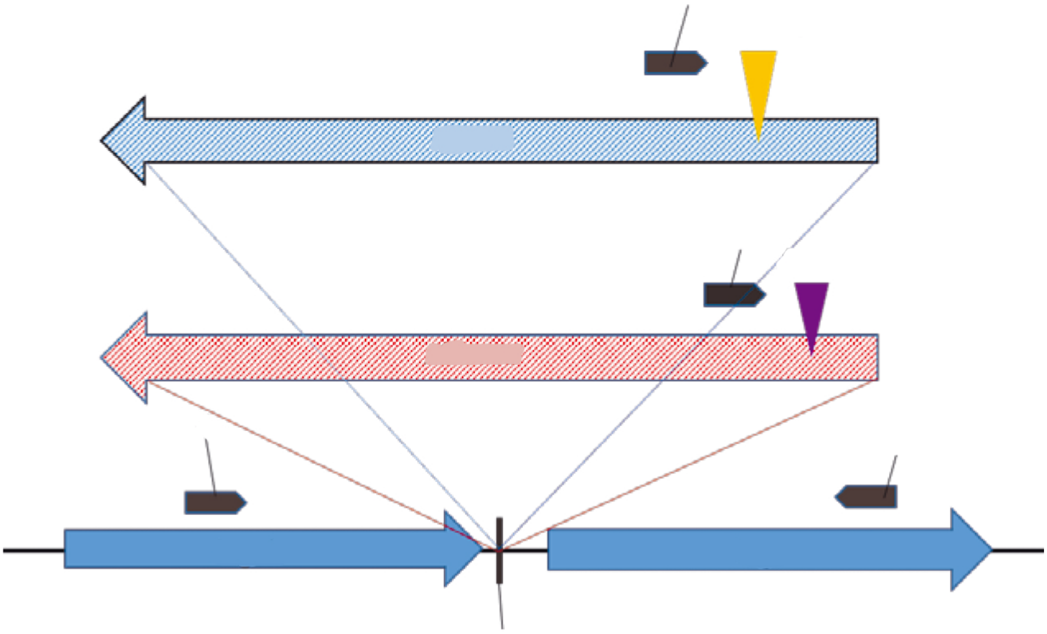

Fig. 1. Structure of the *B. pertussis* chromosome region containing the integration of IS 481 (2) or IS 1002 (2) at the *cctagg* site of the *bvgAS* operon.

The shaded arrows indicate the position of the integrated element IS 481 (blue arrow) or IS 1002 (red arrow) within the *bvgAS* operon in the *cctagg* site (blue solid arrows). The position of the primers used for amplification is indicated by dark pentagons. Colored triangles indicate the position of the DNA probes used in the real-time PCR hybridization reaction.

Table 1. Nucleotide sequences of primers and probes used to detect integrations of IS elements 481 and 1002 in orientation (2) — IS481 (2) and IS1002 (2) — into the *bvgAS, cya* and *fhaB* genes

Primer/probe	Nucleotide sequence
	qPCR
Bp111	GGTCAATCGGGCATGCTTATGG
BVG300	ACGTCGAACGGAGGAATGTC
R6G481, probe	(R6G)TCGCCGACCCCCCAGTTCACTCAAG(BHQ1)
Rox1002, probe	(ROX)ACCACGCCATCGCAACTCAGGGCA(BHQ2)
	PCR *bvgAS*::IS; PCR *cya*::IS; PCR *fhaB*::IS
CyaF	CCATGTCGAGCTGGCCCGTG
CyaR	GGCCACTTCTCGACCGTGCC
FhaB1-F	GGCTGAGCCGTTTCGACCTT
FhaB1-R	CACGGTCGTTCAGCGCAACA
S_F	GTCGCTGGTGGAACTGATAG

Table 2. Temperature-time profiles of real-time PCR and PCR for detecting IS element integrations in the NctagN site of the *bvgAS, cya* and *fhaB* operons

Target	Праймеры/зонд	Temperature, °C	Time, s	Number of cycles
qPCR *bvgAS*::IS481 (2)	Bp111-BVG300/R6G481	95	300	1
		95	20	40
		60	50	
qPCR *bvgAS*::IS1002 (2)	Bp111-BVG300/Rox1002	95	300	1
		95	20	40
		60	50	
PCR *bvgAS*::IS	S_F-BVG300	95	300	1
		95	20	40
		63	30	
		72	30	
PCR *cya*::IS	CyaF-CyaR	95	300	1
		95	20	40
		67	30	
		72	30	
PCR *fhaB*::IS	FhaB1-F-FhaB1-R	95	300	1
		95	20	40
		67	30	
		64	30	

ber of genome equivalents (GE) of *B. pertussis* DNA and IS 481 (1) and IS 1002 (1) integrations were determined using the real-time PCR test systems (BpIS-Q) and real-time PCR BpIS-INT1 [13], respectively. The number of IS 481 (2) and IS 1002 (2) integrations was estimated based on the values of the reaction threshold cycle. The corresponding set of reagents, analogous to the set for quantifying IS element integrations in orientation (1), is designated as BpIS-INT2. The frequency of integrations was calculated as before using the ratio N_{IS}/N_{GE}, where N_{IS} is the number of integrations and N_{GE} is the number of GE in 5 μL of the DNA solution being studied.

Phenotype of B. pertussis bacteria 4MKS, 475, Tohama I, and Tohama 347 and molecular genetic characterization of their populations

Within the framework of this study, populations of the laboratory strain *B. pertussis* Tohama I and its Bvg$^-$ mutant *B. pertussis* Tohama 347, as well as virulent bacteria *B. pertussis* 475 and isogenic attenuated bacteria *B. pertussis* 4MKS, were analyzed to identify insertion mutants in the *bvgAS, cya* and *fhaA* virulence genes, which are responsible for the Hly$^-$ phenotype that develops when *B. pertussis* bacteria are cultured *in vitro*.

B. pertussis are slow-growing, fastidious bacteria. For the phenotypic differentiation of the phase states of *B. pertussis* bacteria when cultured on solid media with blood added, the following characteristics of the colonies can be used: size, shape, and the presence of hemolysis zones. Bvg$^+$ colonies are convex, smooth, shiny, small (1–2 mm), and form distinct zones of hemolysis; Bvg$^-$ colonies are flat, rough, larger (2.0–2.5 mm), and do not form zones of hemolysis.

Colonies of *B. pertussis* bacteria grown on casein-charcoal agar (CCA) blood agar were analyzed for the listed parameters. *B. pertussis* culture from an ampoule was streaked onto a Petri dish containing CCA agar with added blood (primary culture), and after incubation at 35°C for 24–36 hours, it was re-streaked in a dense line onto the same Petri dish (first passage). After checking for microbiological purity using light microscopy, another subculture was performed (2nd passage). The 3rd passage culture was used to seed the ampoule culture into individual colonies by the titration method (ampoule culture analysis). Simultaneously, individual colonies from the second passage, grown on CCA medium with blood, were analyzed for morphology and the presence of hemolysis zones. Three CFUs of each strain meeting the criteria for virulent *B. pertussis* bacteria were streaked onto fresh blood agar plates, DNA was extracted from the resulting culture, and the bacteria were simultaneously serially diluted to form CFUs on the blood agar medium. Colony morphology and the presence of hemolysis zones were analyzed (culture analysis from individual colonies). This study characterized 500–1000 CFU of each strain of virulent and attenuated *B. pertussis* bacteria, grown from ampoule cultures and individual colonies.

Phenotypic and molecular biological analysis was performed on *B. pertussis* bacteria dried at different times in different laboratories. The vast majority of *B. pertussis* 4MKS and *B. pertussis* 475 ampoule cultures from the 3rd passage contained bacteria in the virulent Bvg$^+$ phase; IS element integration into the *bvgAS* operon was detected by real-time PCR at a low frequency — less than 10^{-4} per bacterial cell. In the ampoule culture spreads, all the colonies that grew had a Bvg$^+$ phenotype.

In certain cases, in *B. pertussis* 4MKS bacteria from the 3rd passage of an ampoule culture dried more than 4 years ago, the total frequency of insertion mutants in both orientations was (2–5) × 10^{-2} (**Table 3**). During sieving, approximately 2–3% of Hly-CFU were recorded. The colonies were flat and rough in shape and 2.0–2.5 mm in size, while Hly^{+} colonies were 1–2 mm.

Similar results were obtained in a study of ampoule cultures of *B. pertussis* Tohama I. The frequency of IS481 (1) integrations into the *bvgAS* operon, detected by real-time PCR, ranged from 10^{-4} per bacterium to several percent. No integrations of other IS elements were found in the *B. pertussis* Tohama I bacteria. The maximum number of non-hemolytic CFU, reaching 95% of the total number of colonies, was identified when bacteria dried on April 13, 1974 (30 years ago) were streaked. The frequency of IS element integrations into the *bvgAS* operon was 4 × 10^{-2} per bacterium (Table 3).

B. pertussis Tohama 347 *bvgAS::Tn5* strain was expected not to form zones of hemolysis, and the size and shape of the colonies corresponded to the Bvg^{-} phenotype. The frequency of integrations in all *B. pertussis* Tohama 347 cultures analyzed by real-time PCR was less than 10^{-6} per bacterium.

A similar study was conducted with cultures grown from individual Hly^{+} colonies of *B. pertussis* 475, *B. pertussis* 4MKS, and *B. pertussis* Tohama I. When each strain of bacteria was streaked from individual colonies onto CCA medium with added blood from 10^{3} CFU, no colonies that did not form zones of hemolysis were observed. The size and shape of the colonies corresponded to the Bvg^{+} phenotype described above. The frequency of IS element integration into the *bvgAS* operon did not exceed 10^{-4}–10^{-5} per bacterium.

Colonies of Hly^{-} strains of *B. pertussis* 4MKS and *B. pertussis* Tohama I were selectively subjected to further analysis. 46 colonies of each strain were transferred to Petri dishes containing CCA with blood and antibiotics to test their antibiotic resistance and hemolytic ability. As a result, all individual *B. pertussis* 4MKS colonies were resistant to streptomycin, nalidixic acid, and chloramphenicol, while *B. pertussis* Tohama I bacteria were sensitive to all antibiotics tested. The identified antibiotic resistance is fully consistent with the characteristics of the parent strains *B. pertussis* 4MKS and *B. pertussis* Tohama I. Colonies of both strains maintained a pronounced Hly^{-} phenotype in the replicates.

In PCR with SF-BVG300 primers on DNA extracted from the 3rd passage of ampoule cultures and the CFU of the tested strains, no large fragments were detected, despite the presence of IS element integration in some bacteria within the population. The absence of amplification products with the insert is due to the significantly lower efficiency of amplifying large fragments compared to small fragments without an insert.

Thus, in the cultures of virulent and attenuated *B. pertussis* strains analyzed, grown from ampoule cultures, Hly^{-} mutants lacking hemolytic activity and containing IS element integrations in the *bvgAS* operon are detected with varying frequency. Cultures grown from single bacterial colonies are homogeneous and contain insertion mutants at a frequency of less than 10^{-4} per bacterium. Bacterial populations grown from individual colonies contain non-hemolytic bacteria colonies of the Hly^{-} phenotype in insufficient numbers for phenotypic analysis.

Molecular genetic characterization of Hly- mutants of B. pertussis 4MKS and Tohama I

DNA was extracted from 40 replicas of each strain of *B. pertussis* 4MKS and *B. pertussis* Tohama I, with an Hly^{-} phenotype. The obtained samples were analyzed using PCR with the primers SF-BVG300, FhaB1-F-FhaB1-R, and CyaF-CyaR.

The size of the amplification products from the SF-BVG300 primer-investigated Hly mutants of *B. pertussis* 4MKS was approximately 1300 bp in 25% (10 clones) and approximately 300 bp in 75% (30 clones).

Table 4 presents the results of the PCR product analysis of some Hly^{-} clones of *B. pertussis* 4MKS with primers SF-BVG300, FhaB1-F-FhaB1-R, and CyaF-CyaR.

Integration into the *cya* gene was not detected in any of the clones, whereas integration into the *fhaB* gene was recorded in 26 out of 30 Hly^{-} mutants of *B. pertussis* 4MKS that did not contain IS element integration

Table 3. The proportion of Hly^{-} colonies and the registration of IS element integration events in the *bvgAS* operon of *B. pertussis* 475, *B. pertussis* 4MKS and *B. pertussis* Tohama I strains when cultured on CCA blood agar

Strain	Ampoule culture		Culture from CFU	
	Hly^{-},%	*bvgAS* ::IS*	Hly^{-}, %	*bvgAS* ::IS*
B. pertussis 475	N/A	5 × 10^{-5}	N/A	3 × 10^{-5}
B. pertussis 4MKS	2	(2–5) × 10^{-2}	N/A	2 × 10^{-4}
B. pertussis Tohama I	95	4 × 10^{-2}	N/A	5 × 10^{-4}
B. pertussis Tohama 347	100	≤ 10^{-6}	100	≤ 10^{-6}

Note. *The total integration frequency for all IS elements is given. DNA extracted from the 3rd passage of the ampoule culture or colonies was used for PCR; N/A — not found.

in the *bvgAS* operon. For 3 Hly mutants, the nucleotide sequence of the amplification products of the *fhaB* gene fragment, 1300 bp in size, has been determined. In all cases, IS481 (1) was found integrated at a specific site, *fhaB*.

Clone #33, which did not contain integrations in the *bvgAS* or *fhaB* genes, was found to have a 2-nucleotide deletion near the *cctagc* site in the *bvgS* gene (**Fig. 2**). In 3 Hly clones (No. 32-2, 15-2, and 17-2), no IS element integrations or other nucleotide sequence disruptions were detected in the analyzed amplicons.

Out of 10 insertion Hly$^-$-BvgAS mutants with a specific nucleotide sequence, 5 contain an IS481 insertion (2), and 5 mutants contain an IS1002 insertion (1). **Fig. 3** shows a fragment of the sequence containing IS481 (2) at a specific site in the *bvgAS* operon.

Among the analyzed Hly clones, no integrations of IS1002 (2) and IS481 (1) were detected, whereas the corresponding integrations are recorded in the population when analyzed by real-time PCR.

The PCR products of 40 analyzed Hly- mutants of *B. pertussis* Tohama I, using primer pairs SF-BVG300, FhaB1-F-FhaB1-R, and CyaF-CyaR, had a size close to the calculated one — approximately 300 bp, 388 bp, and 261 bp, respectively. Nucleotide sequencing of 3 amplicons: *bvgAS, cya* and *fhaB* confirmed the absence of IS element integrations in them and did not reveal any structural alterations compared to the native sequence.

Given the presence of IS element integrations in the *bvgAS* operon in 4% of the *B. pertussis* Tohama I bacterial population and their absence in the DNA of bacteria with the Hly$^-$ phenotype, we searched for IS element integrations in the *bvgAS* operon in bacteria with the Hly$^+$ phenotype. PCR with SF-BVG300 primers on DNA extracted from 12 CFU with the Hly$^+$ phenotype revealed products approximately 1300 bp in size in 5 (41.7%) CFU, presumably containing an IS element insertion at the analyzed site. qPCR analysis confirmed that all of them contain an IS481 (1) integra-

a

GGAACTGATAGACCTCGCCAAACGCAACAATCTCGCCTAGCGCCGCGC**ATG**CCCGCCCCGCACCG
CCTGTACCCCCGCAGTCTGATCTGCCTGGCTCAGGCGCTATTGGCATGGGCTTTGC**tg**GCATGGGC
GCCCGCGCA

b

GGAACTGATAGACCTCGCCAAACGCAACAATCTCGCCTAGCGCCGCGC**ATG**CCCGCCCCGCACCG
CCTGTACCCCCGCAGTCTGATCTGCCTGGCTCAGGCGCTATTGGCATG

Fig. 2. Sequence fragment of the wild-type *bvgAS* operon of *B. pertussis* (*a*) and the Hly mutant *B. pertussis* No. 33 (*b*).

Nucleotide sequences of the *bvgAS* operon are shown in capital letters; ATG is the methionine codon of the *bvgAS* gene; **tg** is a deletion; CCTAGC is a specific integration site for IS elements.

Table 4. Results of PCR analysis of DNA from *B. pertussis* 4MKS clones with the Hly$^-$ phenotype (selectively)

Clone number on strain templates	Genes, mutation types		
	*bvgAS::*IS	*fhaB::*IS	*cya::*IS
2	IS481 (2)*	N/A*	N/A
3	IS481 (2)*	N/A	N/A
49	IS481 (2)*	N/A	N/A
48	IS481 (2)*	N/A	N/A
22	IS481 (2)*	N/A	N/A
9	IS1002 (1)*	N/A	N/A
10	IS1002 (1)*	N/A	N/A
32	IS1002 (1) *	N/A	N/A
34	IS1002 (1) *	N/A	N/A
43	IS1002 (1) *	N/A	N/A
31	N/A*	IS481 (1)*	N/A
33	∆TG*	HO	N/A
3-2	N/A*	IS481 (1) *	N/A
32-2	N/A*	N/A*	N/A
15-2	N/A*	N/A	N/A
17-2	N/A*	N/A	N/A
28-2	N/A	IS481 (1) *	N/A

Note. *The structure has been confirmed by sequencing. N/A — integration was not found by PCR.

a

*atttccagtttctcaaatccggttcggatgaaccatgcatacaacctattgaatct**T**cacaCCTAGCGCCGCGC**ATG**CCCG*
CCCCGCACCGCCTGTACCCCCGCAGTCTGATCTGCCTGGCTCAGGCGCTATTGGCATG

b

*tgaaccatgcatacaacctattgaatct**T**cacacctaacgagcacggacttctccgcgccacgcgggacgcgaagaacgccggac*
aggccgcggttaccaacatggtatccggcgggctggttgccgtcgcccgcgcgccgcatccggccatcgccgccccaaccacgga
*ggacccgcac**atg** ttgagcaac*

Fig. 3. Sequence fragments of the *bvgAS* operon of *B. pertussis* 4MKS Hly$^-$ mutant, clone 49 (*a*), and an insertion mutant of *B. pertussis* with IS481 integrated into a degenerate *cctaac* site located upstream the start of transcription of the *bteA* gene (*b*) [16].

The sequence of the *bvgAS* operon is indicated in capital letters, and the specific integration site CCTAGC is underlined; the sequence of the *bteA* gene is italicized, and the specifically degenerate integration site *cctaac* is underlined; the methionine start codons of the proteins VvgS and BteA are in bold; the 3' end sequence of IS481(1) is indicated in lowercase letters; and the presumed transcription start is indicated by a bold capital letter **T**.

tion into the *bvgAS* operon. Sequencing of the 1300 bp PCR products of SF-BVG300 confirmed the presence of the IS481 insertion in 3 of them (1) and the preservation of the native sequence around the integration site.

Thus, the emergence of *B. pertussis* bacteria with the Hly$^-$ phenotype when cultured on solid media is not only due to previously known frameshift mutations in *bvgS*, deletions and insertions in the *bvgA* gene, integration of IS481 (2) and IS1002 (1) into the intergenic space of the *bvgAS* operon, or deletions in the *bvgS* gene, which were found in this study, but also to newly identified insertions of IS elements into the *fhaB* gene. The insertion of IS481 (1) into the *bvgAS* operon of *B. pertussis* Tohama I does not lead to the formation of a pronounced Hly$^-$ phenotype in the bacteria. PCR analysis of virulence gene fragments *bvgAS, cya* and *fhaB*, containing specific NctagcN sites, did not reveal any visible structural changes in the analyzed gene regions in Hly$^-$ mutants of *B. pertussis* Tohama I.

Discussion

A previous study conducted by us showed that a population of virulent *B. pertussis* bacteria cultured on solid CCA medium is not homogeneous; it contained a certain proportion of mutants characterized by IS element insertions at a specific site in the *bvgAS* operon. Such mutants were not only described by us, but were also obtained and characterized under selective conditions by S. Stibitz et al. In our studies, insertion mutants of BvgAS were found in convalescents and contacts of whooping cough patients, and in laboratory animals in the late stages of whooping cough infection. The qPCR test system we developed earlier, used in the experiments, allowed for the detection of IS1002 and IS481 integration events in only one orientation, which we designated as (1). We hypothesized that IS elements are capable of moving and integrating into a specific site within the *bvgAS* operon in reverse orientation (2). Therefore, this study aims to develop a method for identifying integration events of IS elements into the *bvgAS* operon in orientation (2) and to register the corresponding insertion mutants of *B. pertussis* when cultured *in vitro*.

The same primers and probes complementary to the IS element sequences were used for PCR and the registration of amplification products resulting from the integration of IS elements in both orientations, but in pairs with primer SF for orientation (1) and with BVG300 for orientation (2) (Fig. 1). To estimate the number of integrations in the orientation (2) in this study, the values of the reaction threshold cycle were used instead of calibration curves, as in previous experiments, which slightly reduces the accuracy of the integration quantification.

In independent experiments, when screening bacteria of all strains grown from CFU, except for *B. pertussis* Tohama 347, the frequency of integration events detected by real-time PCR did not differ significantly from what we had previously determined and was 10^{-4}–10^{-5} per bacterium.

In this study, we analyzed *B. pertussis* 4MKS and *B. pertussis* 475 cultures dried at different times in our laboratory, as well as *B. pertussis* 475 cultures obtained at different times from various sources, including the L.A. Tarasevich State Research Institute of Standardization and Control of Medical Biological Preparations collection. IS element integrations were identified in all ampoule cultures in both orientations (1) and (2). During the analysis of 16 preparations, IS1002 integration into the *bvgAS* operon was absent in 7 of them, either in one or both of the orientations studied. IS481 integration was present in the *bvgAS* operon in orientation (1) in 75% of cases and in orientation (2) in 25% of cases. Integration of IS 481 was not detected in only one ampoule culture of *B. pertussis* strain 475. In 25% of cases, IS1002 (1) integration was not detected, and in 3 (16%) samples, neither IS1002 (1) nor IS1002 (2) integrations were detected. Thus, the frequency of IS element detection in the *bvgAS* operon of two isogenic strains is reduced in the sequences IS481 (1), IS481 (2), IS1002 (1) and IS1002 (2). The reliability and significance of the identified patterns for other *B. pertussis* strains remain to be determined in subsequent studies.

Among the analyzed Hly$^-$ clones of *B. pertussis*

4MKS ampoule cultures, no integrations of IS1002 (2) and IS481 (1) were detected (Table 4), while they are identified by real-time PCR in the overall population of *B. pertussis* 4MKS. Presumably, such insertion mutants are among the bacteria with the Hly^+ phenotype. Analysis of 10 colonies forming zones of hemolysis did not reveal any changes in the size of the corresponding amplicon, indicating a low percentage of possible insertion mutants in the Hly^+ clone population. The results of qPCR analysis of the bacterial population, including the studied $Hly^–$ and Hly^+ clones, support this conclusion: the frequency of detection of IS1002 (2) and IS481 (1) integrations is 10 times lower than that of IS1002 (1) and IS481 (2), which are detected at a frequency of approximately $10^{–2}$ per bacterium. However, finding integrants without phenotypic markers at the expected frequency of less than $10^{–3}$ per bacterium appears difficult. For this reason, Hly^+ clones with possible IS1002 (2) and IS481 (1) integrations were excluded from this study.

In the *B. pertussis* Tohama I bacterial population, only IS481 (1) integration was detected in the *bvgAS* operon, and no IS1002 integrations were found. When streaking the test cultures grown from CFU on blood agar, no colonies with the $Hly^–$ phenotype were detected, which is consistent with the frequency of integration events identified by real-time PCR. The fact that IS481 (1) is predominantly integrated into a specific site within the *bvgAS* operon of *B. pertussis* Tohama I bacteria, as well as the different integration frequencies of IS481 and IS1002 in isogenic variants of *B. pertussis* strain 475, requires further study and may be related to the specific movement characteristics of IS elements in different strains.

The frequency of IS481 integration in *B. pertussis* Tohama 347 bacteria cultures grown from ampoules and colonies is approximately 100 times lower ($10^{–6}$–$10^{–7}$ per bacterium) than in isogenic virulent *B. pertussis* Tohama I bacteria. This fact was noted by us in previous studies [20, 21]. Analysis of the *B. pertussis* Tohama 347 DNA amplification product with SF-BVG300 primers showed that the Tn5 insertion in the *bvgAS* operon is located outside the analyzed fragment of the virulence operon and is likely unable to prevent the movement of IS elements into the analyzed site. This circumstance indicates the dependence of transposition frequency on the integrity of the *bvgAS* operon. It should be noted that we were unable to register a dependence of the IS element transposition frequency in the *bvgAS* operon on modulating conditions when culturing *B. pertussis* Tohama I, 4MKS, and 475 bacteria in the presence of $MgSO_4$ and at reduced temperature. On the one hand, this observation does not support the dependence of transposition on modulating conditions, and on the other hand, considering the identified decrease in integration frequency in *B. pertussis* Tohama 347, it can be suggested that the BvgA and BvgS proteins are not directly involved in the regulation of transposition. The empirical result obtained requires further study.

The high frequency of *B. pertussis* insertion mutants we found in certain ampoule cultures suggests a strong dependence of the transposition frequency on the bacterial culture conditions, including, likely, within the host organism. This circumstance may be responsible for the instability in obtaining frameshift mutants and their revertants, as noted by A. Weiss et al. [9].

Thus, the presented results show that our proposed real-time PCR test systems allow for the detection of IS element integration events in both orientations within the *bvgAS* operon. The frequency of their transposition depends on the genotype, including the integrity of the virulence operon, and the conditions under which the bacteria are cultured.

Analysis of the structure of the virulence gene fragments *bvgAS, cya* and *fhaB* in Hly- mutants showed that they contain the integration of IS481 (2) or IS1002 (1) at a specific cctagc site in the *bvgAS* operon (*B. pertussis* 4MKS), or IS481 (1) at a similar site in *fhaB* (*B. pertussis* Tohama I). Four Hly mutants were also found whose chromosomes do not have insertions in the virulence gene fragments tested. A 2-nucleotide deletion was found in the *bvgS* gene of one of them, which disrupts the expression of the BvgS phosphokinase involved in the regulation of a large number of pertussis pathogen genes, including all virulence genes. In 3 out of 4 Hly mutants, the methods used did not reveal any abnormalities in the structure of the *bvgAS* or *fhaB* genes. There were no integrations into the *cya* gene in any of the analyzed clones. It is likely that these 3 mutants contain the IS element insertions in the *bvgA* gene characterized by S. Stibitz, or other mutations, such as frameshift mutations in the *bvgS* gene, which were not identified using the methods employed.

The presence of an IS element integrated into the intergenic space of the *bvgAS* operon, blocking the transcription of the *bvgS* gene, appears to be a perfectly expected reason for the formation of the $Hly^–$ mutant phenotype of *B. pertussis* 4MKS. However, there are several examples of IS481 integration into specific sites located upstream of the *brkA, kat* and *bvgS* genes, at least, not accompanied by the termination of their expression [16, 18, 20, 22]. In one study, not only was the hypothesis formulated that transcription from a promoter located at the end of IS481 regulates the expression of the *brkA* gene product, but the start of the corresponding transcript was also determined (Fig. 2). The promoter identified by the authors is located at the same end of IS481 (2) in the genome of clone 49 of *B. pertussis* 4MKS, but it apparently does not provide a sufficient level of *bvgS* gene transcription, resulting in the formation of the $Hly^–$ phenotype of clone 49 (Fig. 2). It can be assumed that the disruption of the *bvgS* gene transcription in mutants containing the IS1002 insertion (1) occurs by the same mechanism or as a result of its complete cessation. The lack of experimental data on the presence of promoters within the IS1002 structure currently prevents a definitive conclusion. Apparently, in this way, the IS481 insertion ensures the transcription of the *bvgS* gene and the differential expression of virulence genes in selected S. Stibitz mutants, manifested by the preservation but significant reduction in the expression of the *ptx* genes and a less pronounced reduction in *fha* [20]. An interesting case of transcription regulation is discussed by A. D'Halluin et al. [22]. Regulation of *fim2* gene transcription/translation is shown as a result of antisense RNA synthesis from the IS481 internal promoter integrated upstream of the *fim2* gene. It is suggested that the described changes in the regulation of *B. pertussis* virulence genes, particularly the *bvgAS* operon, may be one of the "triggering" mechanisms for the formation of *B. pertussis* persistence in the body of its only host — humans. Or they can increase the viability of bacteria outside the body, facilitating the transmission of infection to a new susceptible organism.

In all Hly$^-$ mutants containing an insertion in the *fhaB* gene, IS481 integration was detected (1). Because the integrations are located within the coding sequence of the *fhaB* gene, they disrupt its transcription and translation, regardless of the direction of the IS element integration. While the link between mutations in the *bvgAS* operon and the Hly$^-$ phenotype appears obvious, the effect of a knockout mutation in the *fhaB* gene on *B. pertussis* hemolytic activity requires further investigation. According to current understanding, the FhaB protein is not directly related to the hemolysis reaction of blood erythrocytes. This function is attributed to ACT, specifically its C-terminal region. However, recent data suggest that the FhaB protein interacts with ACT. Most likely, the hemolytic activity of ACT *in vitro* is realized after interaction with filamentous hemagglutinin on the surface of the bacterial cell and delivery of ACT into the eukaryotic cell [3].

The absence of the described *bvgAS::IS481* or *bvgAS::IS1002* insertion mutants among the bacteria isolated from whooping cough patients is noteworthy, while clinical isolates containing IS element insertions in the *fhaB, prn* genes and those involved in the regulation of *katA* and *brkA* have been described in the literature [16, 18, 20, 22]. In the experiments of this study, such mutant bacteria *bvgAS::IS481* (1) were detected in populations of *B. pertussis* bacteria persisting in the bodies of whooping cough convalescents and asymptomatic carriers. It can be assumed that this state of the whooping cough pathogen is optimal for persistence in the human body and survival in the external environment, and upon entering a susceptible organism, precise excision of the IS element (or inversion) occurs, the bacteria regain their virulence, the organism is infected, and the disease develops. A similar role is assigned to bacteria in a state of reduced virulence by M.R. Farman et al., who studied the transcription profiles of a large group of genes in *B. pertussis* bacteria inside macrophages [23]. Our demonstration of the existence (accumulation) of Bvg$^-$-mutants of *B. pertussis* at late stages of infection suggests a regulatory role for IS element integrations in the formation of persistent bacteria in the human body and possibly their involvement in bacterial transmission to a new host. It can be expected that the precise excision of an IS element from a virulence operon restores its structure and the ability of the bacteria to cause disease. The mechanisms and conditions that typically cause rare events of precise exclusion remain unclear. It is also unclear at what stage of pathogen persistence or transmission it occurs. The ability of the IS element to be precisely excised was previously demonstrated by us in the *Escherichia coli* model [21, 24].

The results of the analysis of Hly$^-$ mutants of *B. pertussis* Tohama I, which we registered in approximately 95% of the analyzed individual colonies grown from one of the series of lyophilized cultures from the Gamaleya Research Institute of Epidemiology and Microbiology Museum, appear unexpected. Among 40 Hly$^-$ colonies, no *B. pertussis* mutants containing integrations in the specific NctagN sequence in the *bvgAS, cya* and *fhaB* genes were found. The mutations responsible for the genotype formation of the Hly$^-$ clones of *B. pertussis* Tohama I isolated by us remain unidentified. It is quite likely that they, like the 3 unidentified mutations in Hly$^-$ clones of *B. pertussis* 4MKS, belong to the class of reading frame shift mutations in the *bvgS* gene, insertions in the specific site of *bvgA*, or contain other unidentified disruptions in the structure of the *bvgAS* operon, the *fhaB* gene or the *cya* gene.

The absence of integrations at the *cctagg* site of the *bvgAS* operon analyzed in Hly$^-$ colonies against the background of their reliable detection by PCR in the population before its dispersion (up to 4% IS481 integrations (1)) allowed us to suggest that integrations at the corresponding site may be present in the genome of some bacteria that have retained hemolytic activity (with the Hly$^+$ phenotype). Indeed, among the Hly$^+$ phenotype bacteria, 41.7% of insertion mutants containing IS481 (1) in the *bvgAS* operon were found, while the native sequence of the operon around the integration site was preserved. Considering the analysis above and the orientation of IS481, it can be expected that an effective promoter, which ensures the transcription of the *bvgS* gene and the Hly$^+$ phenotype in *B. pertussis* insertion mutants, is located at the end of the IS481 element opposite to that described by H. Han et al. [18] and in a study by S. Stibitz, who showed a differential decrease in the expression of the *fhaB* and *ptx* genes and less pronounced zones of hemolysis in *B. pertussis* insertion mutant bacterial colonies [12].

For a more detailed characterization of the *bvgAS::IS481*(1) Hly$^+$ phenotype mutant, we have planned a comparative study of the toxic activity of pertussis toxin and dermonecrotic toxin, agglutination titers, and electron microscopic analysis of the morphology and structure of *B. pertussis* Tohama I and its mutant genotype, as well as a bioinformatics analysis of the IS481 sequence aimed at identifying and comparing the putative promoters located at the ends of the IS elements.

Thus, during *in vitro* cultivation of *B. pertussis* bacteria, IS481 integrations (1) are more frequently detected in the *bvgAS* operon, which we previously identified in the genomes of *B. pertussis* bacteria isolated from whooping cough convalescents and experimental animals. Previous data on the analysis of the integration events of IS elements in one orientation (1) largely reflect the accumulation dynamics of all *bvgAS::IS* insertion mutants in *B. pertussis* bacteria.

Conclusion

The data obtained allow for several significant conclusions to be drawn regarding the characteristics of *B. pertussis* IS elements:

- IS elements are capable of moving between specific sites on the *B. pertussis* chromosome, causing gene inactivation or changes in their transcriptional regulation when the bacteria are cultured *in vitro*;
- the frequency of IS element movement and the types of spontaneous mutations they induce depend on the genotype and culture conditions of *B. pertussis* bacteria;
- factors influencing the frequency of IS element movement and the formation of spontaneous, insertion, or other mutations induced by IS elements require further study;
- during the cultivation and storage of *B. pertussis* bacteria, conditions may arise that induce the formation of a population heterogeneous in the structure of *fhaB*, *bvgAS* and likely other bacterial virulence genes;
- the movement of IS elements to a specific site in the intergenic space of the *bvgAS* operon leads to changes in the regulation of virulence genes and other *bvg*-dependent genes, possibly ensuring the long-term persistence of *B. pertussis* bacteria in the host organism.

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Original Study Article
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Monitoring for *Clostridioides difficile*-associated infection in hospital

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Abstract

Introduction. *Clostridioides difficile* — an anaerobic, spore-forming, Gram-positive bacteria — is a component of the normal intestinal microflora. *C. difficile*-associated infection develops during its overcolonisation, when vegetative forms produce exotoxins that cause inflammation n the colon wall. Toxigenic strains of *C. difficile* are the main cause of healthcare-associated infections in hospitals.

The aim of the study is to investigate the frequency of detecting *C. difficile* (both toxigenic and non-toxigenic strains) in patients admitted to the gastroenterology department of the Yu.M. Lopukhin Federal Scientific Clinical Center for Physical and Chemical Medicine of the Federal Medical Biological Agency of Russia in 2021–2023 with diarrhea syndrome and other established diagnoses.

Materials and methods. The study included 547 patients aged 19–88 years (46.6% male, 53.4% female). Real-time polymerase chain reaction was used to detect *C. difficile* DNA and its toxin A and B genes, and a bacteriological examination of stool was also performed. Upon detection of clinical signs of bacterial infection, a C-reactive protein (CRP) test was performed.

Results. *C. difficile* DNA was detected in 65 (11.9%) patients, and toxins A and B genes were found in 32 (5.9%) patients. Non-toxigenic strains were more frequently detected in men (55%) under 40 years, while toxigenic strains were equally frequent in both sexes under 40. CRP analysis indicated that inflammatory processes were more likely in patients over 40 years old. The predominant diagnosis in toxigenic strain carriers with high CRP was C. difficile-associated enterocolitis (ICD-10: A04.7), whereas in the non-toxigenic group, it was ulcerative colitis (ICD-10: K51). Extended bacteriological analysis revealed significant gut microbiota imbalances in all patients.

Conclusion. Over the three-year surveillance period, the prevalence of *C. difficile*-positive patients increased from 6.6 to 7.9%, and the proportion of samples positive for toxin A/B genes rose from 5.2% to 7.9%. These findings underscore the necessity for enhanced preventive measures to mitigate risk factors for CDI in hospital settings. Therefore, preventive measures are necessary to reduce the impact of risk factors for the development of *C. difficile*-associated infection in hospitals.

Keywords: *diarrhea syndrome, Clostridioides difficile, fecal microbiota transplantation, hospital epidemiological monitoring, hospital microbiological monitoring*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Local ethics committee of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine (protocol No. 2022/05/31, May 31, 2022).

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Оригинальное исследование
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Мониторинг *Clostridioides difficile*-ассоциированной инфекции в условиях стационара

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Аннотация

Введение. *Clostridioides difficile* — вид анаэробных спорообразующих грамположительных бактерий — является составной частью нормальной микрофлоры кишечника. *C. difficile*-ассоциированная инфекция развивается при избыточной колонизации *C. difficile*, вегетативные формы которой способны продуцировать экзотоксины, вызывающие воспаление в стенке толстой кишки. Токсигенные штаммы *C. difficile* являются основным возбудителем инфекций, связанных с оказанием медицинской помощи в стационаре.

Целью исследования является изучение частоты выявления *C. difficile* (как токсигенных, так и нетоксигенных штаммов) у пациентов гастроэнтерологического отделения ФНКЦ ФХМ им. Ю.М. Лопухина ФМБА России, госпитализированных в 2021–2023 гг. с синдромом диареи и другими установленными диагнозами.

Материалы и методы. В исследование были включены 547 пациентов в возрасте 19–88 лет (46,6% мужчин, 53,4% женщин). Для выявления ДНК *C. difficile* и генов её токсинов A и B применяли метод полимеразной цепной реакции в реальном времени, дополнительно проводили бактериологическое исследование кала. При обнаружении клинических признаков бактериальной инфекции проводили анализ на С-реактивный белок (СРБ).

Результаты. ДНК *C. difficile* выявлена у 65 (11,9%) пациентов, гены токсинов A и B обнаружены у 32 (5,9%) пациентов. Нетоксигенные штаммы *C. difficile* чаще детектировались у мужчин (55%) в возрасте до 40 лет. Токсигенные штаммы *C. difficile* с одинаковой частотой встречались у мужчин и женщин в возрасте до 40 лет. Анализ на СРБ показал, что воспалительные процессы чаще развиваются у пациентов старше 40 лет. Преобладающее заболевание с высоким показателем СРБ у пациентов с токсигенным штаммом — энтероколит, вызванный *C. difficile* (кодирование по МКБ-10: A04.7), в группе с нетоксигенным штаммом — у пациентов с диагнозом: язвенный колит (кодирование по МКБ-10: K51). У всех пациентов наблюдались выраженные нарушения баланса кишечной микрофлоры согласно развёрнутому бактериологическому анализу кала.

Заключение. В результате трёхлетнего мониторинга установлено увеличение доли пациентов с *C. difficile* с 6,6 до 7,9%. Доля образцов, положительных на гены токсинов A/B *C. difficile,* также увеличилась с 5,2 до 7,9%. Таким образом, необходимо проводить профилактические мероприятия для уменьшения влияния факторов риска развития *C. difficile*-ассоциированной инфекции в стационаре.

Ключевые слова: *синдром диареи, Clostridioides difficile, трансплантация фекальной микробиоты, эпидемиологический мониторинг в условиях стационара, микробиологический мониторинг в условиях стационара*

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Introduction

Ensuring a safe environment for patients and healthcare workers and maintaining quality control of medical care are top priorities for healfcare institutions. Reducing morbidity and mortality associated with nosocomial infections is of particular importance. A key factor in mitigating the risk of healthcare-associated infections (HAIs) is establishing an effective epidemiological surveillance system. Such a system enables the timely detection of infectious diseases in both patients and healthcare workers, thereby improving the safety and quality of medical care. Furthermore, preventive measures aimed at reducing HAIs can help mitigate social risks and the likelihood of insurance claims for patients and healthcare workers[1].

The causative agents of HAIs are diverse, including microorganisms, protozoa, helminths, viruses, and prions. Among them, toxigenic strains of *Clostridioides difficile* are the most common. Numerous factors contribute to the development of *C. difficile* infection, the primary one being the use of antibacterial therapy and the associated alteration of gastrointestinal microflora. The highest risk is associated with antibacterial drugs from the penicillin, cephalosporin, fluoroquinolone, and clindamycin groups. Other risk factors include age over 65, theuse of proton pump inhibitors in treatment regimens, and comorbidities. The transmission mechanism for *C. difficile*-associated infection is fecal-oral, occurring through contact with contaminated hands of healthcare personnel, used medical equipment, and surfaces in hospital rooms. *C. difficile* is part of the normal intestinal microflora, and carriers of *its* spores can be both patients and healthcare workers. The emergence of vegetative forms can be associated with individual host factors, disruption of the intestinal microflora composition, its stability, and a decrease in the abundance of *Firmicutes* and *Bacteroides*.

The clinical manifestation of *C. difficile* infection is due to the pathogenic properties of the vegetative forms, which can produce exotoxins: enterotoxin A, cytotoxin B and binary toxin CDT. Certain strains produce both toxins A and B, others only toxin A or binary toxin, and less commonly, only toxin B. The presence of a high titer of antitoxic antibodies and colonization resistance of the normal intestinal microflora can result in clinically asymptomatic forms of the disease.

Etiological identification of *C. difficile*-associated infection is relevant in the presence of clinical forms of the disease. Laboratory diagnosis should employ multiple methods to improve accuracy. In Russia, according to clinical guidelines[2], and considering the ACG clinical guidelines [1], a three-stage algorithm for laboratory confirmation is recommended for diagnosing *C. difficile* infection:

1. Isolation of a toxigenic *C. difficile* using bacteriological methods and determination its susceptibility to antibacterial drugs.
2. Detection of glutamate dehydrogenase in fecal samples using serological methods (immunochromatographic assay, enzyme-linked immunosorbent assay) or polymerase chain reaction (PCR)..
3. Detection of toxins A and B in fecal samples using serological methods and PCR [2].

The gold standard for establishing disease etiology is the bacteriological method of isolating a pure culture and determining its cytotoxicity on cell culture. However, assesing toxin production by isolated *C. difficile* strains via cell culture assay is burdensome for practical laboratories in medical organizations, despite its high sensitivity and specificity. Furthermore, the isolation of a pure *C. difficile* culture from patients with signs of acute intestinal infection is not routinely performed and is not included in the standards of medical care for intestinal infections of unknown etiology.This significantly hinders timely diagnosis when diarrheal syndrome occurs in a hospital setting within 48 hours of admission or when a clinical episode of diarrhea is linked to a hospital stay within the previous 4 weeks. Such cases should be considered healthcare-associated infections (HAIs) regardless of whether the infection is exogenous or endogenous [3, 4].

Enzyme-linked immunosorbent assay (ELISA) for the detecting *C. difficile* toxins A and B in fecal samples is the most widely used method in multidisciplinary hospitals in Russia.

Real-time PCR (qPCR) is a highly sensitive and prioritized method due to its rapid turnaround time. It is used in medical organizations with PCR capabilities for the simultaneous detection and identification of *C. difficile* DNA and genes for toxin A and B in biological material (feces). Combining PCR with other methods can increase the predictive value of a positive result [5].

The aim of the study was to investigate the frequency of *C. difficile* detection (both toxigenic and non-toxigenic strains) in patients admitted to the gastroenterology department of a multidisciplinary hospital.

Materials and methods

A retrospective observational, open, descriptive study was conducted in the gastroenterology department of the Y.M. Lopukhin Federal Scientific Clinical

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[1] Methodological recommendations "Epidemiological diagnosis of infectious diseases related to the provision of medical care, based on standard definitions of the case." 2024.
URL: https://nasci.confreg.org/libs/files/mr_sos.pdf (data of access: 08.06.2025). (In Russ.)

[2] Clinical recommendations "Clostridial infection: diagnosis, treatment and prevention". 2022. URL: https://www.rnmot.ru/public/uploads/RNMOT/clinical/2022/ФИНАЛЬНЫЕ%20КЛИНИЧЕСКИЕ%20РЕКОМЕНДАЦИИ%20Клостридиальная%20инфекция%209112022.pdf (data of access: 08.06.2025). (In Russ.)

Center of Physical and Chemical Medicine from January 1, 2021 to December 31, 2023.

Inclusion criteria were: male and female patients aged 18–89 years hospitalized in the gastroenterology department, presenting with clinical signs of diarrhea syndrome, and diagnosed with various conditions including irritable bowel syndrome (ICD-10 code: K58), inflammatory bowel diseases (ulcerative colitis (K51), Crohn's disease (K50)), *C. difficile*-associated enterocolitis (A04.7), pseudomembranous colitis (K52.8) and megacolon (K59.3).

Exclusion criteria were: patients with diarrhea without a confirmed diagnosis; the presence of mental or behavioral disorders preventing proper history taking; immunodeficiency conditions (HIV, bone marrow or organ transplantation, primary immunodeficiency); a concomitant illness requiring hospitalization/surgery or considered life-threatening within 30 days prior to or at enrollment, as assessed by the investigator; and a history of severe alcohol, drug, or narcotic dependence.

The study was conducted with voluntary informed written consent from all patients. The study protocol was approved by the local ethics committee of the Y.M. Lopukhin Federal Scientific Clinical Center of Physical and Chemical Medicine of the Federal Medical Biological Agency of Russia (protocol No. 2022/05/31 dated May 31, 2022).

All included patients underwent a bacteriological stool examination and PCR detection of *C. difficile* DNA and the genes encoding toxins A and B (*tcdA*, *tcdB*). Patients with clinical signs of bacterial intestinal infection (persistent fever > 3 days despite antipyretic, leukocytosis with neutrophilic shift presence of pathological impurities in the stool — blood, mucus) also had blood test for C-reactive protein (CRP).

Stool analysis for C. difficile DNA and detection of toxin A and B genes

Stool samples for *C. difficile* testing were collected within the first 24 hours of admission to ensure standardization. DNA extraction from feces was performed using the NK-SORBENT nucleic acid isolation kit (NPF Lytech). Extracted DNA was stored at -20°C prior to PCR.

Detection and differentiation of *C. difficile* DNA and toxins A and B genes were performed using the Fluoroplex *Clostridioides difficile* + Toxin A + Toxin B reagent kit (NPF Lytech) via real-time PCR on a CFX96 thermocycler (Bio-Rad Laboratories). An internal exogenous control (detected in the HEX channel) was included to monitor DNA extraction quality and prevent false-negative results. PCR conditions were: 80°C for 2 minutes, 95°C for 1 minute 30 seconds, followed by 40 cycles: 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 40 seconds. A total of 547 DNA samples were tested.

Bacteriological stool culture

Analysis was conducted according to the methodological guidelines "Bacteriological Diagnosis of Dysbacteriosis" (approved on April 14, 1977) and OST 91500.11.0004-2003 "Patient Management Protocol." Intestinal dysbacteriosis. One gram of the native stool sample was homogenized in 9 mL of physiological saline (1/10) and left at room temperature for 10–15 minutes. From the original dilution (10^1), a series of subsequent dilutions were prepared up to 10^8. The resulting suspension was inoculated onto culture media for cultivating various groups of microorganisms.

Bacteriological analysis was evaluated:

- after 20–22 hours on Endo, blood agar, SS agar and XLD agar;
- after 48 hours on Sabouraud's medium, yolk salt agar and milk-inhibitory;
- after 72 hours on Blaurock, MRS-2 and iron sulfite agar.

Statistical analysis

The sample size was not calculated a priori. Statistical analysis was perfomedusing SPSS Statistics v. 27 (IBM). To analyze the prevalence assessment of toxigenic and non-toxigenic strains, indicators such as the incidence rate and the structure of morbidity (proportion) were used. Quantitative data were assessed for normality. Frequency data within groups are presented as percentage. Descriptive statistics (absolute values, percentages) were used. Given the descriptive nature of the analysis of trends from 2021–2023, the statistical significance of differences was not calculated.

Results

Research subjects (participants)

The study included 547 individuals aged 19–88 years, comprising 255 (46.6%) men and 292 (53.4%) women. The average hospital stay was 9.44 days.

Main research findings

Real-time PCR detected C. difficile DNA in 65 patients (34 male, 31 female), representing 11.9% of the sample. Toxin A and B genes were found in 32 (5.9%) patients. Non-toxigenic *C. difficile* strains were predominantly found in men (55%) under 40 years old. Toxigenic strains were found with equal frequency in men and women under 40.Analysis from 2021 to 2023 showed an increase in patients with *C. difficile* from 6.6% to 7.9%. The detection rate of toxigenic *C. difficile* strains also increased from 5.2% to 7.9% (**Table**).

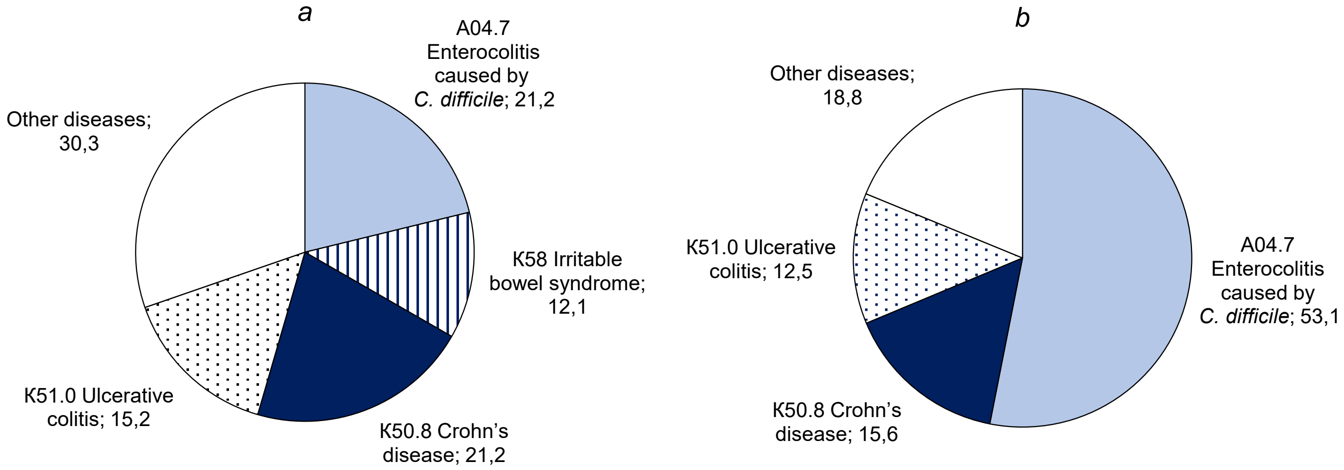
The frequency of various diseases among patients with confirmed *C. difficile* infection is shown in the **Figure**.

Non-toxigenic *C. difficile* strains (*n* = 33) were identified in patients with *C. difficile*-associated entero-

Detection rate of toxigenic and non-toxigenic *C. difficile* strains in patients during the observation period 2021–2023 (*n*=547)

Analysis	2021		2022		2023		Total patients
	number of patients	%	number of patients	%	number of patients	%	
Non-toxigenic strains of *C. difficile* have been identified	14	6.6	6*	3.5	13	7.9	33
Toxigenic strains of *C. difficile* (toxins A and B) were identified	11*	5.2	8	4.7	13	7.9	32
C. difficile strains were not identified	186	88.2	157	91.8	139	84.2	482
Total patients	211		171		165		547

Note. *One patient was hospitalized twice: the first time in 2021, then independently a second time in 2022.



Distribution of confirmed diagnoses in patients with diagnosed non-toxigenic (*a*) and toxigenic (*b*) *C. difficile* strains.

colitis (7, 21.2%), Crohn's disease (5,15.2%), ulcerative colitis (5, 15.2%), and irritable bowel syndrome (4, 12.1%). In 10 (30.3%) cases, non-toxigenic strains were detected in patients with gastrointestinal diseases (e.g., chronic pancreatitis, steatohepatitis, stomach ulcers).

Toxigenic *C. difficile* strains (*n* = 32) were detected in patients diagnosed with: *C. difficile*-associated enterocolitis (17, 53.1%), Crohn's disease (5, 15.6%), ulcerative colitis (4,12.5%), and other ulcerative lesions in the large intestine (6,18.8%).

Blood biochemistry parameters

CRP were measured in 49 of the 65 patients with detected *C. difficile* strains. CRP levels were elevated (>5 mg/L) in 22 patients (group with toxigenic *C. difficile* strains — 11 people, including 8 men and 3 women; group with non-toxigenic *C. difficile* strains — 11 people, including 6 men and 5 women), ranging from 6.53 to 240.9 mg/L. High CRP levels were more common in male patients. Among men with toxigenic strains, the predominant diagnosis (5 out of 11, 45%) was C. difficile-associated enterocolitis (all over 40). In the non-toxigenic group, the largest proportion of men (4 out of 11, 36.4%) had ulcerative colitis (aged 40–60). These results suggest a higher likelihood of inflammatory processes in patients over 40 with various forms of colitis.

Bacteriological stool analysis

A comprehensive bacteriological analysis for intestinal dysbiosis was performed on 36 (55.4%) patients with positive PCR for *C. difficile* based on clinical indications. All exhibited significant disturbances in intestinal microflora (normoflora): reduction in obligate anaerobes (bifidobacteria and lactobacilli were significantly reduced in some cases), a shift in the ratio of lactose-positive and lactose-negative *Escherichia coli* toward the latter, the appearance of hemolytic *Escherichia coli*, and a decrease in the total number of enterococci. Contamination with sulfite-reducing *Clostridium* spp. above normal (≤ 10^6) was detected in 20 (55.6%) patients. In 18 (50%) patients, increased colonization with opportunistic microflora was detected, including *Proteus* spp., *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Acinetobacter* spp., and *Staphylococcus aureus*. At the same time, increased presence of *Candida fungi* was detected in 13 (36%) patients, indicating imbalances in the intestinal microflora, caused in part by antibiotic therapy.

Treatment of C. difficile-associated infection

Among the 65 PCR-positive patients, 24 (36.9%) received first-line antibiotic therapy with oral vancomycin (1 g/day for 10–14 days). This group included 18 patients with toxigenic strains and 6 with non-toxigenic strains.

For recurrent *C. difficile* infection, 6 patients (4 women, 2 men under 60 years old) diagnosed with *C. difficile*-associated enterocolitis underwent fecal microbiota transplantation (FMT) into the terminal ileum via an endoscopic instrument channel.

Recurrences (relapses of the disease)

Re-hospitalization within the 2021-2023 period for patients with previously detected *C. difficile* DNA was recorded in 9 (13.8%; 6 women, 3 men) of the 65 patients. Initial detection was non-toxigenic strains in 5 patients and toxigenic in 4. Re-hospitalization occurred in patients with Crohn's disease (3 non-toxigenic, 1 toxigenic), ulcerative colitis (2 non-toxigenic, 2 toxigenic), and enterocolitis from a toxigenic strain (1 patient).

Discussion

Summary of Research Findings

Including gastroenterology patients with diarrhea syndrome allowed us to cohort with signs of active *C. difficile* infection. This approach aligns with international hospital testing practices for *C. difficile* infection. According to ACG guidelines, testing should be considered for individuals with ≥3 loose stools in 24 hours, those with high comorbidity indices (oncology, hematology, transplant patients), and patients with signs of intestinal obstruction [6, 7]. Our results indicate an increase in *C. difficile* infections in the gastroenterology department from 2021 (6.6%) to 2023 (7.9%), including an increase in toxin-positive from 5.2% to 7.9%. This rise may be linked to the COVID-19 pandemic. T. Zuo et al. concluded that COVID-19 adversely affects gut microbiota, reducing colonization resistance against *C. difficile* [8].

The inclusion of *C. difficile* in the ESCAPE pathogen group and its ability to form hospital strains (clones) is concerning. Besides primary *C. difficile* diseases (enterocolitis, pseudomembranous colitis, megacolon), superinfection can complicate other conditions like Crohn's disease and ulcerative colitis [9]. Therefore, epidemiological and microbiological monitoring for *C. difficile* in gastroenterology department, both in symptomatic patients and those with risk factors is highly relevant. The development of *C. difficile* infection in patients can be associated with endogenous causes (the development of clinical signs of the disease in asymptomatic carriers due to excessive colonization with *C. difficile* and toxin production), as well as with medical care [10]. M. Sartelli et al. report an increase in community-acquired cases over the past 20 years (up to 25–28% of *C. difficile*-associated infections) [11].

Identification of toxigenic and non-toxigenic strains

As part of the normal gut flora, *C. difficile* may not cause disease. In this study, since all patients had diarrhea, the identification of only non-toxigenic *C. difficile* strains (without toxin genes) was interpreted as the diarrheal episode likely not being caused by *C. difficile*, but rather by other factors (other infectious agents, exacerbation of the underlying disease, drug effects, etc.). While detection of non-toxigenic strains is not always associated with the presence of an infectious disease, but there is evidence of asymptomatic carriage of *C. difficile* as a predisposing factor for the development of clinically manifest *C. difficile* infection [12]. Geographic variation in asymptomatic *C. difficile* carriage rates among hospitalized adults exists, ranging from 4.4% to 23.2% [13]. Detection of toxigenic *C. difficile* strains upon admission is associated with an almost 6-fold higher risk of developing clinical infection. The detection rate of toxigenic *C. difficile* strains in hospitalized patients in a multicenter study by I.M. Zacharioudakis *et al.* was 8.1% [14]. In our study, the rate in the gastroenterology department in 2023 was 7.9%.

CRP level test

Using standard laboratory tests like CRP, a systemic inflammatory response marker, in diagnosing *C. difficile* infection and assessing severity helps identify patients at risk for severe disease and poor prognosis.

Bacteriological stool analysis

This study involved patients with an adult gut microbiome, characterized by diversity, stability, and resilience. Significant reductions in diversity and stability along with decreased abundance of obligate members (*Firmicutes, Bacteroidetes, Actinobacteria*), have been reported in *C. difficile* infection [15, 16]. A predominance of Proteobacteria (especially *Enterobacteriaceae*) is also noted. Our bacteriological findings confirmed intestinal dysbiosis, characterized by an imbalance with a predominance of opportunistic pathogens like *Proteus* spp., *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Acinetobacter* spp. and *Staphylococcus aureus.*

Treatment of C. difficile-associated infection in the hospital

The choice of anticlostridial therapy according to Russian clinical guidelines depends on disease severity. For a first mild episode in patients with a favorable prognosis (young age, no immunodeficiency/chronic diseases), metronidazole (500 mg three times daily for 10 days.) can be used. For other patient groups with a first mild episode of *C. difficile* infection, vancomycin (250 mg four times daily for 10 days) is recommended.

FMT currently has a low evidence level and weak recommendation, but is suggested for severe/ fulminant *C. difficile* infection refractory to standard antibiotic therapy. Observational study results support the use of FMT protocols [17]. For example, if severe/fulminant infection is refractory to vancomycin combined with IV metronidazole after 5 days, FMT via colonoscopy is indicated. Results from a randomized clinical trial by G. Ianiro et al. show effective use of this algorithm combined with a 14-day vancomycin course, achieving cure rates of 75% after one FMT and 100% after multiple procedures [18]. However, FMT equires access to a bank of frozen fecal microbiota.

In our study, 24 (36.9%) out of 65 patients PCR-positive patients received vancomycin (1 g/day orally). In the absence of positive clinical and laboratory dynamics, FMT was performed on 6 patients for the treatment of recurrent *C. difficile* infection. Clinical effect was achieved in all patients, and there were no readmissions to the inpatient department. Our data confirm FMT as a highly promising method for treating *C. difficile* infections, consistent with the literature [19–21].

Conclusion

The identification of *C. difficile* infection across various patients group underscores the need to test all patients with diarrhea syndrome for *C. difficile*. To improve diagnostic accuracy and establish a definitive etiology, a combination of identification methods should be employed. PCR, being highly sensitive and rapid, is currently the preferred standalone method for diagnosing C. difficile infection. Incorporating PCR into clinical guidelines and quality criteria for managing *C. difficile* infection will facilitate timely diagnosis, appropriate selection of anticlostridial therapy, and reduce the overall incidence and mortality from HAIs.

A separate issue for epidemiological and microbiological monitoring in medical organizations is screening healthcare workers for asymptomatic carriage or latent infection, as well as testing environmental surfaces (diagnostic and treatment equipment, feeding tubes, etc.) for *C. difficile* contamination. This is particularly relevant for gastroenterology departments and will be a primary focus of our future, more detailed research on the epidemiology and prevention of *C. difficile* infection in hospitals.

Eliminating risk factors for *C. difficile*-associated infection, primarily through the rational use of antibiotic, is the main preventive measure. Supporting the host's dispositional properties, particularly by enhancing the colonization resistance of the colon, is the primary strategy for preventing recurrence.

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Improvement of the MLVA typing scheme for *Burkholderia mallei* strains

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Abstract

Introduction. The registration of sporadic cases of glanders in horses in Russia, caused by *Burkholderia mallei*, highlights the importance of developing genotyping algorithms for this pathogen. The MLVA method (multilocus-variable tandem repeat analysis), based on a comparative analysis of the number of variable tandem repeats (VNTRs), remains a promising genotyping tool. As the number of whole-genome sequences in international databases increases, the informative value of VNTR loci changes, necessitating a revisiting of existing typing schemes.

The aim of this study was to assess the feasibility of including the VNTR locus BPSS1974$^{\#I}$ in the MLVA-6 scheme for intraspecies differentiation of *B. mallei*.

Materials and methods. The study of 64 strains of *B. mallei* was conducted *in silico* and *in vitro* using MLVA, differentiating region amplification, whole-genome sequencing, and bioinformatic analysis.

Results. Genotyping *B. mallei* using the MLVA-6 scheme failed to determine *in silico* VNTR profiles of 13 strains from the GenBank NCBI database for one or more loci due to low read coverage of the corresponding genomic regions or their complete absence (null alleles). The effective number of alleles (ne) and the polymorphic information content (PIC) index for the MLVA-6 scheme loci ranged from 3.842–8.103 and 0.740–0.877, respectively. The potential for including the VNTR locus BPSS1974$^{\#I}$ in this scheme was determined based on the molecular stability of the motif within it and a high values for ne and PIC, which were 4.299 and 0.767, respectively. VNTR profiles of the collection strains at locus BPSS1974$^{\#I}$ were identical to the corresponding strains in the GenBank database. The results of the cluster analysis using a combined MLVA-6 scheme and the BPSS1974$^{\#I}$ locus were consistent with the phylogenetic reconstructions obtained using other molecular genetic methods.

Conclusion. The VNTR locus BPSS1974$^{\#I}$ can be considered a marker, the inclusion of which in the MLVA-6 scheme will improve the accuracy of genotyping and the determination of the regions of origin of newly isolated *B. mallei* strains.

Keywords: *Burkholderia mallei, glanders, genotyping, MLVA, tandem repeats*

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Оригинальное исследование
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Совершенствование схемы MLVA-типирования штаммов *Burkholderia mallei*

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Аннотация

Введение. Регистрация на территории России отдельных случаев сапа у лошадей, возбудителем которого является *Burkholderia mallei*, определяет важность разработки алгоритмов генотипирования этого па-

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тогена. Перспективным остается метод MLVA (multilocus-variable tandem repeat analysis), основанный на сравнительном анализе количества вариабельных тандемных повторов (VNTR — variable number tandem repeats). По мере увеличения числа полногеномных последовательностей в международных базах данных изменяется информативная ценность VNTR-локусов, что требует пересмотра существующих схем типирования.

Цель работы — оценить перспективность включения VNTR-локуса BPSS1974$^{\#I}$ в схему MLVA-6 для внутривидовой дифференциации *B. mallei*.

Материалы и методы. Исследование 64 штаммов *B. mallei* проводили *in silico* и *in vitro* с помощью методов MLVA, амплификации дифференцирующих регионов, полногеномного секвенирования и биоинформатического анализа.

Результаты. Генотипирование *B. mallei* при использовании схемы MLVA-6 не позволило определить *in silico* VNTR-профили 13 штаммов из базы данных GenBank NCBI по одному или нескольким локусам ввиду низкого покрытия ридами соответствующих областей генома или их полного отсутствия (нуль-аллели). Эффективное число аллелей (ne) и индекс полиморфного информационного содержания (PIC) для локусов схемы MLVA-6 варьировали в диапазонах 3,842–8,103 и 0,740–0,877 соответственно. Перспективность включения в эту схему VNTR-локуса BPSS1974$^{\#I}$ определена на основе молекулярной стабильности мотива в его составе и высоких показателей ne и PIC, составивших 4,299 и 0,767 соответственно. VNTR-профили коллекционных штаммов по локусу BPSS1974$^{\#I}$ были идентичны соответствующим штаммам из базы данных GenBank. Результаты кластерного анализа при сочетанном использовании схемы MLVA-6 и локуса BPSS1974$^{\#I}$ соответствовали филогенетическим реконструкциям, полученным с использованием других молекулярно-генетических методов.

Заключение. VNTR-локус BPSS1974$^{\#I}$ можно расценивать как маркер, включение которого в схему MLVA-6 позволит повысить точность генотипирования и установления регионов происхождения вновь выделенных штаммов *B. mallei*.

Ключевые слова: *Burkholderia mallei, сап, генотипирование, MLVA, тандемные повторы*

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Introduction

The Gram-negative bacterium *Burkholderia mallei* is the causative agent of glanders, a potentially fatal anthropozoonosis that primarily affects equids: horses, mules and donkeys [1, 2]. Human illness is linked to their professional activities and occurs through close contact with infected animals, for example, among farm and veterinary workers [3]. There are known cases of infection among employees of bacteriological laboratories who work with *B. mallei* cultures [4, 5]. In Russia *B. mallei* is classified as pathogenicity group II (hazard) agent, while abroad it is considered a potential group B bioterrorism agent, which is due to the possibility of a public health emergency arising from the use of *B. mallei* as a biological weapon [6–8].

Currently, outbreaks or sporadic cases of glanders occur in several regions of Asia and the Middle East, North Africa, as well as Central and South America [9, 10]. The registration of imported cases of glanders in countries free of this disease poses a threat of infection to local livestock, humans, as well as the possibility of reintroduction of the infection [11, 12].

Cases of glanders are being reported in countries bordering Russia, which necessitates vigilance from veterinary services regarding this disease, especially in the regions of Siberia and the Far East. The outbreak of glanders in horses in 2023 at the "Chita State Stud Farm and Hippodrome named after Khosaen Khakimov," registered by the World Organization for Animal Health[1], highlights the importance of epidemiological monitoring of glanders in Russia.

Modern methods of intraspecies typing allow obtaining a detailed genetic characterization of the pathogen to address issues such as investigating cases of human or animal disease, and determining the evolutionary and phylogenetic relationships between microbial strains [13–15]. However, in the case of glanders, the search for effective DNA targets is complicated by the high conservation of the *B. mallei* genome. The high density of microsatellite and minisatellite repeats (Variable Number Tandem Repeats, VNTR) in *B. mallei* genome determines the prospects of using strain differentiation schemes based on multilocus variable number tandem repeat analysis (MLVA). In foreign sources, a

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[1] Russia — Burkholderia mallei (Inf. with) (Glanders) — Follow up report 1 [FINAL].
URL: https://wahis.woah.org/#/in-review/4915

scheme of 23 VNTR loci is most often used for this purpose, it was originally proposed for the causative agent of melioidosis — *Burkholderia pseudomallei*, a clone of which is supposed to be the ethilogical agent of glanders [16]. However, this scheme is not optimized for the *B. mallei*. A large number of typing loci increases the overall cost of the analysis.

To reduce the labor intensity and cost of the MLVA method, the development of shortened schemes is relevant, allowing the maintenance of the discriminatory power and reliability of molecular epidemiological investigations using fewer loci. The combination of 6 VNTR loci we previously selected allowed the differentiation of *B. mallei* strains with high discriminatory power [17]. However, the informative value of VNTR loci changes as the number of new whole-genome sequences in international databases increases, which necessitates a review of existing typing schemes.

In a recent study, we demonstrated the effectiveness of using a scheme of 5 VNTR and 4 SNP loci for establishing the geographical origin of *B. pseudomallei* strains and determining the clonality of isolates in melioidosis cases [18]. The new VNTR locus BPSS1974$^{\#I}$, proposed in this study, located within the BPS_RS29560 gene region, was characterized by stable amplification and the presence of amplicons in all *B. pseudomallei* strains examined. The tandem repeat sequence (motif) consisted of 9 nucleotide pairs and had a degenerate structure that was under stabilizing selection. The presence of an orthologous gene in *B. mallei* genome determines the possibility of using the BPSS1974$^{\#I}$ locus to improve the 6-locus MLVA typing scheme.

The aim of the study was to assess the feasibility of including the VNTR locus BPSS1974$^{\#I}$ in the MLVA-6 scheme for genetic typing of *B. mallei* strains.

Materials and methods

The research objects were whole-genome sequences of 56 *B. mallei* strains from the GenBank NCBI database[2]. Our sample included nucleotide sequences of *B. mallei* strains isolated in endemic countries (India, Pakistan, Brazil), certain parts of Asia and the Middle East where outbreaks or cases occur sporadically (Turkey, China, Myanmar, Bahrain), as well as in USA and some European countries (Hungary, Yugoslavia).

To assess the stability of DNA loci within genotyping systems, 7 strains of *B. mallei* from the collection of the Volgograd Plague Control Research Institute were used. Duplicates of the strains P-1, Muksuwar-11, V-120, C-4 and C-5, deposited in the State Collection of Pathogenic Microorganisms and Cell Cultures under the numbers SCPM-O-B-4688, SCPM-O-B-7093, SCPM-O-B-7146, SCPM-O-B-4682 and SCPM-O-B-4683, corresponded to the collection strains. In turn, the collection strains Muksuwar-11, Bogor-37 and Zagreb were matched by strains whose nucleotide sequences have been deposited in GenBank NCBI under accession numbers GCA_033870375.1, GCA_033870395.1 and GCA_033870355.1. The study also included the collection strain *B. mallei* 16050, isolated from a horse in 2023 during an outbreak of glanders at a state stud farm in Chita. The genome sequence of *B. mallei* strain 16050 has been deposited in the VGARus database (id vnip002404). A total of 64 strains of *B. mallei* were analyzed in the study.

For setting up polymerase chain reactions for MLVA typing and amplification of differentiating regions of the genome (Different Region Analysis, DFR), DNA was extracted using the RIBO-prep reagent kit (Central Research Institute of Epidemiology, Moscow). Genomic DNA for sequencing was extracted using the Biolabmix-DU-250 kit (Biolabmix) according to the manufacturer instructions.

Primers and amplification parameters for typing using the MLVA-6 scheme and the BPSS1974$^{\#I}$ locus were described in earlier studies [17, 18]. The VNTR profiles of the strains were determined as the set of allelic variants at each locus and are presented as a numerical pattern in the format "L933k/L3145k/L3652k/L20k/L1217k/S2862k/BPSS1974$^{\#I}$".

DFR genotypes were determined by real-time hybridization-fluorescence PCR (real-time PCR) for 9 fragments: BmVAT1, BmVAT2, BmVAT3, BmVAT4, BmVAT5, BmVAT6, BmVAT7, BmVAT8, BmVAT9, using the oligonucleotide sequences and genotyping scheme described by O.S. Bondareva et al. [19]. The DFR typing results were converted into a binary matrix, where the presence of an amplicon was denoted by "1" and its absence by "0".

Library preparation for whole-genome sequencing was performed using the Nextera XT library prep kit, and sequencing was carried out on the MiSeq platform using the MiSeq Reagent Kit v3 (all from Illumina Inc.). The raw nucleotide reads were processed using the Trimmomatic utility with standard parameters for Illumina. Processed reads were assembled using SPAdes v3.15.4 with the "-isolate" option and standard command-line parameters [20].

Cluster analysis and dendrogram construction were performed using FAMD v. 1.31 [21] and Mega v. 11.0.11 [22] software, employing the nearest neighbor joining algorithm and the Jaccard genetic distance coefficient. The Hunter–Gaston discriminatory index (HGDI) [23] was used to assess the discriminatory power of the typing schemes. To analyze the informativeness of the microsatellite loci, the number of alleles, the effective number of alleles (ne) and the polymorphic information content (PIC) index were determined.

Results

In silico analysis showed that the entire 692 bp amplicon of the BPSS1974$^{\#I}$ locus, located at positions

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[2] URL: http://www.ncbi.nlm.nih.gov/genome

1627686–1628377 in the genome of the reference strain *B. mallei* ATCC 23344 (GCA_033956065.1), corresponded to the 1755 bp BMA_RS16575 gene, located on the second chromosome. Much like in the *B. pseudomallei* genome, the BMA_RS16575 gene is annotated in the NCBI GenBank database as a collagen-like protein containing a triple helix repeat (WPJ46631.1, 584 bp). The forward primer site occupied positions 1627686–1627707 in the *B. mallei* ATCC 23344 genome and 28–49 in the BMA_RS16575 gene sequence, while the reverse primer site occupied positions 1628359–1628377 and 701–719, respectively. As in the *B. pseudomallei* genome, positions 3, 6 and 9 of the motif within the BPSS1974$^{\#I}$ locus were under stabilizing selection.

In silico typing of 56 *B. mallei* strains from the NCBI GenBank database using primers to the BPSS1974$^{\#I}$ locus revealed 10 allelic variants, the most common of which were repeats with copy numbers 56 and 54 — each accounting for 17 strains. 55 and 53 repeats were found in 9 and 5 strains respectively; the number of repeats was 58 for 2 strains, and the remaining 5 variants were unique. In *B. mallei* SCPM-O-B-4686, primer binding sites were absent (null allele).

Using the MLVA-6 scheme, null alleles were identified at the L1217k locus in 14 strains from the GenBank NCBI database. Analysis of the molecular nature of the null alleles showed that the absence of amplification products at loci BPSS1974$^{\#I}$ and L1217k is due to the deletion of the corresponding genome fragments. Null alleles at the L1217k locus, identified in 4 complete genomes of strains *B. mallei* 6, 34, BMQ and 23344, were considered as one of the allelic variants in further work. However, the genomes of *B. mallei* strains BM 1, BM 5, BM 6, Turkey5, NCTC3709, SCPM-O-B-4682, SCPM-O-B-4683, SCPM-O-B-4684, SCPM-O-B-4688, and SCPM-O-B-4686 are represented by a set of contigs of varying lengths, which does not exclude the absence of a PCR product in *in silico* analysis due to an incomplete nucleotide sequence. Therefore, these strains were excluded from further analysis.

The MLVA-6 scheme did not allow the *in silico* determination of the genotypes of *B. mallei* strains BM-1, BM-3, BM-6 and BM-9 at locus L20k and *B. mallei* 3076 at locus L3652k, as the target VNTR region was located on different contigs. Due to this, these strains were also removed from the study.

Excluding 13 strains from the sample resulted in the loss of 3 unique allelic variants at locus BPSS1974$^{\#I}$ and one at locus L20k. **Table 1** presents the *B. mallei* strains that were subsequently used for cluster analysis.

As a result of VNTR loci amplification of *B. mallei* collection strains, PCR products were obtained that appear as single bands on the electropherogram. At the same time, the amplification product for the L1217k locus was absent in 3 strains from the collection (*B. mallei* P-1, C-4 and C-5). Subsequent amplicon sequencing allowed us to determine the corresponding number of repeats for each locus (Table 1). Subsequently, when calculating the polymorphism indicators of VNTR loci, the MLVA profiles of strains from GenBank NCBI and the collection of the Volgograd Plague Control Research Institute were combined.

When calculating the effective number of alleles (ne) and the polymorphic information content (PIC) index for the BPSS1974$^{\#I}$ locus, the values were found to be 4.299 and 0.767, respectively. In the adjusted sample of strains, the number of alleles per locus in the MLVA-6 scheme was distributed as follows: the minimum number of alleles was 8 and 10 in loci L3145k (ne = 4.257; PIC = 0.765) and L933k (ne = 7.087; PIC = 0.859), respectively; 11 alleles in loci L3652k (ne = 7.368; PIC = 0.864) and L1217k (ne = 3.842; PIC = 0.74); 13 alleles in locus S2862k (ne = 7.628; PIC = 0.869); and the maximum number of alleles was 16 in locus L20k (ne = 8.103; PIC = 0.877). The HGDI did not change when locus BPSS1974$^{\#I}$ was added to the MLVA-6 scheme and remained at 0.981.

For the analysis of genetic polymorphism in strains whose VNTR profiles were reliably determined, the method of amplifying differentiating regions of the genome was additionally used. As a result, 51 strains of *B. mallei* were distributed into 19 DFR types, of which 6 were identified for the first time (DFR20 — 001110011, DFR21 — 000111110, DFR22 — 111110110, DFR23 — 110110111, DFR24 — 010111010, DFR25 — 111101100).

When comparing dendrograms constructed based on typing results using the MLVA-6 scheme (**Figure**, *a*) and when including the BPSS1974$^{\#I}$ locus in this scheme (Figure, *b*), differences were observed in the arrangement of root clusters and individual strains. Thus, when using the MLVA-6 scheme and the BPSS1974$^{\#I}$ locus in combination, a new cluster was formed, which we designated as G and which consists of subclusters G1, G2 and G3. Subcluster G1 formed the *B. mallei* strain Bahrain1. Subcluster G2 included strains BM-4 and *B. mallei* 11, while subcluster G3 included strains *B. mallei* NCTC 10229, 2002734299, and Ivan, isolated by researchers at the Pecs Institute in Hungary in 1961, and *B. mallei* BM_campo 2.1. For the G3 subclade strains, a common DFR type (DFR02) was established, and their MLVA profiles differed by 1 repeat at loci L3145k and L3652k, while the VNTR genotypes of strains BM_campo 2.1 and Ivan were completely identical.

The adjacent branches of the dendrogram contain strains from clusters F and E. For strains from cluster F — *B. mallei* 34 of unknown origin and NCTC 10247 — the same number of repeats were determined at VNTR loci L3145k, L20k, and BPSS1974$^{\#I}$, and their DFR profiles were unique and differed at loci BmVAT1 and BmVAT6. Cluster E included strains from India (2000031066 and SAVP1) with identical MLVA profiles at loci L933k, L3145k and L20k, while their DFR

Table 1. Results of MLVA and DFR typing of *B. mallei* strains

B. mallei strain name (GenBank or VGARus accession number)	Location and year of isolation	MLVA-profile*	MLVA-profile/ cluster number**	DFR-type***
6 (GCA_000755845.1)	Turkey, 1950	14/7/8/4,86/–/7/50	1/A	17
Ц-4 (vnip002737)****	Mongolia, 1967	11/7/6/17/–/8/54	2/A	06
Ц-5 (vnip002738)****	Mongolia, 1967	11/7/6/17/–/8/54	2/A	06
BMQ (GCA_000755885.1)	India, 1932	2/4/7/9/–/5/54	1/B	12
23344 (GCA_000755865.1)	Myanmar, 1944	2/4/7/29/–/5/54	2/B	12
NCTC 3708 (GCA_003590195.1)	India, 1932	2/6/10/5/7,9/10/55	1/C	24
16050 (vnip002404)****	Russia, Chita, 2023	5/6/11/17/5/10/54	2/C	06
mongolia_1	Mongolia, 2022	9/5/16/17/5/6/55	3/C	06
B-120 (vnip002736)****	Russia, Ulan-Ude, 1985	10/6/8/17/5/6/55	4/C	06
SCPM-O-B-7146 (GCA_003627695.1)	Russia, Ulan-Ude, 1985	10/6/8/17/5/6/55	4/C	06
BM-2 (GCA_028621615.1)	China, Hebei, no data	6/8/10/19/4,9/6/55	1/D	16
2002721277 (GCA_003590185.1)	USA, 1956	10/8/7/17/4,9/12/55	2/D	16
China5 (GCA_000757315.2)	China, 1956	10/8/8/16/4,9/10,4/55	3/D	16
2000031063 (GCA_000756025.2)	Hungary, no data	10/8/8/16/4,9/12/55	4/D	16
P-1 (vnip002735)****	Yugoslavia, no data	6/5/11/7/-/6/55	1/E	09
SAVP1 (GCA_000015465.1)	India, no data	4/5/9/7/9,9/11/55	2/E	04
2000031066 (GCA_003590125.1)	India, no data	4/5/7/7/13,9/12/54	3/E	21
NCTC 10247 (GCA_000762285.1)	Turkey, Ankara, 1960	5/9/12/7/4,9/7/54	1/F	03
34 (GCA_939576165.1)	no data,1972	10/9/16/7/–/6/54	2/F	20
Bahrain1 (GCA_001729545.1)	Bahrain, 2011	2/10/14/15/5,9/6/54	1/G1	22
BM-4 (GCA_028621665.1)	China, Hebei, no data	6/10/10/18/8/13/54	1/G2	23
11 (GCA_000959405.1)	Turkey, 1949	8/10/10/8/4,9/3/54	2/G2	19
NCTC 10229 (GCA_000015605.1)	Hungary, Budapest, 1961	10/10/14/9/4,9/8/54	1/G3	02
2002734299 (GCA_000959165.1)	Hungary, 1961	10/11/14/9/4,9/8/54	2/G3	02
Ivan (GCA_000986905.1)	Hungary, 1961	10/10/13/9/4,9/8/54	3/G3	02
BM_campo 2.1 (GCA_905359435.1)	Brazil, 2016	10/10/13/9/4,9/8/54	3/G3	02
UFAL2 (GCA_905359425.1)	Brazil, 2017	8/4/9/14/4,9/4/27	1/H	06
PRL-20 (GCA_000169875.1)	Pakistan, Lachor, 2005	5/4/6/6/3,9/7/58	1/I	25
India86-567-2 (GCA_000959465.1)	India, no data	6/4/8/6/8,9/11/58	2/I	01
Turkey1 (GCA_002345985.1)	Turkey, no data	7/4/11/6/6,9/7/56	1/J	15
Turkey2 (GCA_002346025.1)	Turkey, no data	7/4/10/6/6,9/7/56	2/J	15
Turkey3 (GCA_002346065.1)	Turkey, no data	7/4/7/6/6,9/6/56	3/J	15
Turkey4 (GCA_002346085.1)	Turkey, 1960	7/4/11/6/6,9/6/56	4/J	15
Turkey6 (GCA_002346125.1)	Turkey, no data	7/4/11/6/6,9/6/56	4/J	15
Turkey7 (GCA_002346145.1)	Turkey, no data	7/4/11/6/6,9/7/56	1/J	15
Turkey8 (GCA_002346165.1)	Turkey, no data	7/4/11/6/6,9/7/56	1/J	15
Turkey9 (GCA_002346185.1)	Turkey, no data	7/4/12/6/6,9/7/56	5/J	15
Turkey10 (GCA_002346005.1)	Turkey, no data	7/4/11/6/6,9/7/56	1/J	15
Muksuwar-11 (vnip002733)****	India, 1979	5/4/8/17/6,9/12/53	1/K	11
SCPM-O-B-7093 (GCA_003627585.1)	India, 1979	5/4/8/17/6,9/11/53	2/K	07
Mukteswar (GCA_033870375.1)	India, 1996	5/4/8/17/6,9/11/53	2/K	11
Zagreb (vnip002734)****	Yugoslavia, no data	5/4/8/15/6,9/6/53	3/K	07
Bogor-37 (vnip002732)****	Indonesia, 1979	5/4/8/15/6,9/11/53	4/K	07
Zagreb (GCA_033870355.1)	Yugoslavia, 1996	5/4/8/15/6,9/11/53	4/K	07
Bogor (GCA_033870395.1)	Indonesia, 1995	5/4/8/15/6,9/11/53	4/K	07

End of the Table 1.

B. mallei strain name (GenBank or VGARus accession number)	Location and year of isolation	MLVA-profile*	MLVA-profile/ cluster number**	DFR-type***
Kweiyang#4 (GCA_001608335.1)	China, 1942	9/7/4/24,71/6,9/15/56	1/L	22
2002721274 (GCA_002522985.1)	USA, 1956	9/7/4/19/6,9/16/56	2/L	01
ATCC 23344 (GCA_033956065.1)	Myanmar, 1942	8/7/4/31/6,9/15/56	3/L	01
JHU (GCA_002346205.1)	USA, Maryland, 2000	8/7/4/27/6,9/15/56	4/L	01
FMH 23344 (GCA_000755785.1)	Myanmar, 1944	8/7/4/27/6,9/15/56	4/L	01
FMH (GCA_002346045.1)	USA, Maryland, 2000	8/7/4/27/6,9/15/56	4/L	01

Note. *The MLVA profile of the strains is recorded as a numerical pattern of the number of repeats in the scheme "L933k/L3145k/L3652k/L20k/L1217k/S2862k/BPSS1974$^{\#I}$".
** Each MLVA profile is assigned a serial number within the corresponding cluster formed during the construction of the dendrogram.
***DFR-type numbers have been assigned according to previously published data [19].
****Strains from the collection of the Volgograd Plague Control Research Institute.

types were also unique and differed by the absence of locus BmVAT6 in *B. mallei* SAVP1. A separate branch within cluster E was formed by the *B. mallei* P-1 strain from Yugoslavia.

The inclusion of locus BPSS1974$^{\#I}$ in the typing scheme allowed strain *B. mallei* BM-2 to be placed as a separate branch within cluster D, which consists of strains *B. mallei* 2002721277, China5, and 2000031063. All strains in this cluster have the same number of repeats at loci L3145k, L1217k and BPSS1974$^{\#I}$, as well as an identical DFR profile (DFR16).

The genotype at locus BPSS1974$^{\#I}$, corresponding to 58 repeats, was identified only in the Indian strain *B. mallei* India86-567-2 and PRL-20, which allowed these strains to be grouped into a separate cluster I. Strain India86-567-2 belonged to type DFR01, while PRL-20 belonged to the newly identified type DFR25, which differed from DFR01 by the absence of the BmVAT5 and BmVAT8 loci.

The position of the strain *B. mallei* 16050, which we isolated and identified, is particularly interesting. It formed a common cluster with *B. mallei* mongolia_1 and *B. mallei* V-120, which are genetically identical in their MLVA profile, and its duplicate SCPM-O-B-7146. All 4 strains were found to have identical DFR profiles (DFR06) and VNTR profiles at loci L20k and L1217k. At the same time, *B. mallei* mongolia_1 and V-120 were also found to have the same number of repeats at loci S2862k and BPSS1974$^{\#I}$, and they differed by 1 repeat at loci L933k and L3145k, which allowed these strains to be placed in a separate branch within cluster C. For *B. mallei* 16050, a closer relationship was established with the *B. mallei* V-120 strain, as their MLVA profiles contained 6 repeats at the L3145k locus.

A separate cluster group was formed by the UFAL2 strain (Brazil, 2017) with a unique number of repeats at loci L20k (n = 14), S2862k (n = 4), and BPSS1974$^{\#I}$ (n = 27). At the same time, *B. mallei* UFAL2, along with strains from Russia and Mongolia, belonged to type DFR06.

MLVA profiles of duplicate strains from different collections were identical or differed by only 1 locus, allowing them to remain within the same cluster. Thus, differences of 3 repeats were found at locus L933k between *B. mallei* C-4 and SCPM-O-B-4682, and of 1 repeat between *B. mallei* C-5 and SCPM-O-B-4683. The Muksuwar-11 strain differed from the SCPM-O-B-7093 and Mukteswar (GCA_033870375.1) strains by 1 repeat at the S2862 locus. The Zagreb strain from our institute collection differed from Zagreb (GCA_033870355.1) by 5 repeats at the S2862 locus (**Table 2**).

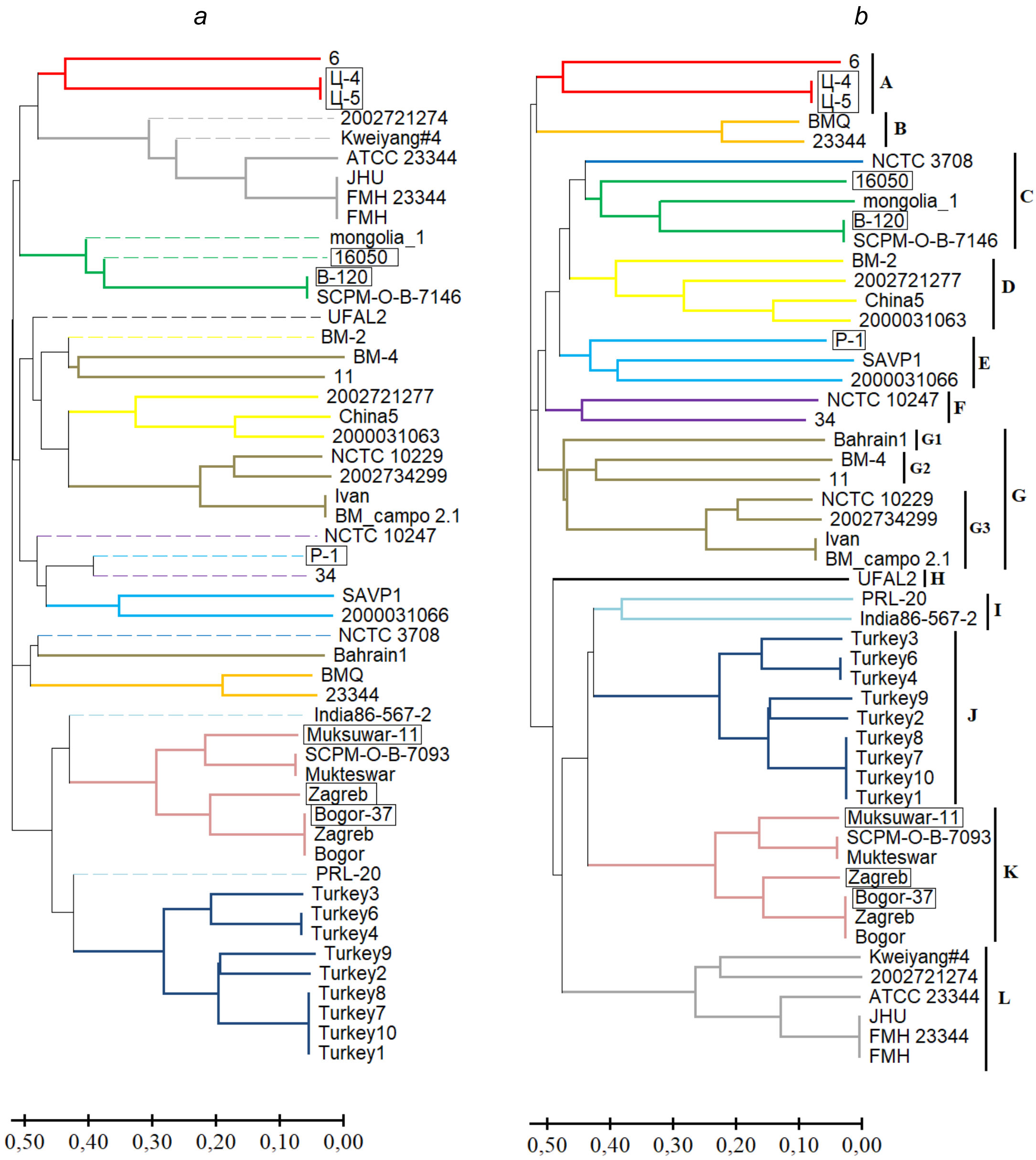
As a result, the addition of locus BPSS1974$^{\#I}$ to the MLVA-6 scheme allowed to identify a closer genetic relationship among a number of strains and the formation of new clusters that correlate with their DFR profiles.

Discussion

The effectiveness of intraspecies typing of infectious agents is largely determined by the availability of their genome nucleotide sequences. Whole-genome sequencing and subsequent assembly of *B. mallei* genome are complicated by a large number of repetitive sequences and a high GC content. As a result, of the 113 *B. mallei* genomes deposited in NCBI GenBank as of May 2025, only 33 were complete, while most of the remaining genomes are represented by dozens or hundreds of contigs. At the same time, certain strains were not unique.

For certain strains in the sample, it was impossible to determine the VNTR genotype at individual loci *in silico*. Low read coverage of the genome regions corresponding to the L20k and L3652k loci prevented the target VNTR region from being localized within a single contig in 5 strains. Insufficient coverage during sequencing can be attributed to the GC content of these regions, which was over 70% in the reference strain *B. mallei* ATCC 23344.

Another problem when searching for target VNTR loci in incomplete genomes is the absence of corre-



Comparative analysis of the typing results of 51 *B. mallei* strains using the MLVA-6 scheme (*a*) and the combined use of the MLVA-6 scheme and the BPSS1974#I locus (*b*).

Identical root clusters are marked in the same color. *a*: Strains whose positions changed upon inclusion of the BPSS1974$^{\#I}$ locus are marked with a dashed line. The strains from the collection of the Volgograd Plague Control Research Institute are highlighted by a frame

sponding genome regions for these loci (null alleles). In this case, there is no reliable way to determine the cause of the null alleles: it could be the result of both natural evolutionary changes and errors in genome assembly. We detected null alleles at the L1217k locus in 14 strains from the sample, 10 of which were represented by incomplete genomes, leading to their exclusion from further study. When performing PCR with DNA from collection strains, the amplification product for the L1217k locus was absent in 3 strains. However, null alleles in complete genomes increase the risk of false homoplasy, which is why such loci are excluded from analysis in many population studies. However, the probability of registering a null allele at each locus increases with the sample size, which can lead to the exclusion of a large number of loci, and this in turn will negatively impact the quality of the research.

As a result, 13 strains were excluded from the initial sample, and the remaining 51 *B. mallei* strains were distributed into 39 MLVA types using a 6-locus VNTR typing scheme (HGDI = 0.981). We included the BPSS1974$^{\#I}$ locus in this scheme because of the structure of the motif within it, the degeneracy of which reduces the likelihood of DNA polymerase errors during replication and sequencing. At the same time, the sequence of the motif itself is under the influence of stabilizing selection, which contributes to its preservation in the genome.

Table 2. MLVA profiles of *B. mallei* duplicate strains from various collections

B. mallei strain number (GenBank accession number)	Number of repeats in the loci						
	L933k	L3145k	L3652k	L20k	L1217k	S2862k	BPSS1974$^{\#I}$
P-1*	6	5	11	7	–	6	55
SCPM-O-B-4688 (GCA_003627635.1)**	6	5	11	7	–	6	55
Muksuwar-11*	5	4	8	17	6,9	12	53
SCPM-O-B-7093 (GCA_003627585.1)**	5	4	8	17	6,9	11	53
Mukteswar (GCA_033870375.1)***	5	4	8	17	6,9	11	53
B-120*	10	6	8	17	5	6	55
SCPM-O-B-7146 (GCA_003627695.1)**	10	6	8	17	5	6	55
Ц-4*	11	7	6	17	–	8	54
SCPM-O-B-4682 (GCA_003627705.1)**	14	7	6	17	–	8	54
Ц-5*	11	7	6	17	–	8	54
SCPM-O-B-4683 (GCA_003627655.1)**	10	7	6	17	–	8	54
Bogor-37*	5	4	8	15	6,9	11	53
Bogor (GCA_033870395.1)***	5	4	8	15	6,9	11	53
Zagreb*	5	4	8	15	6,9	6	53
Zagreb (GCA_033870355.1)***	5	4	8	15	6,9	11	53

Note. * *B. mallei* strains from the collection of the Volgograd Plague Control Research Institute. ***B. mallei* strains deposited in the State Collection of Pathogenic Microorganisms and Cell Cultures.
****B. mallei* strains from the Friedrich Loeffler Institute, the German Federal Research Institute for Animal Health

In the current study, we found that all VNTR loci had a PIC > 0.5, indicating their high discriminatory power, with the effective number of alleles ranging from 3.842 to 8.103 per locus and averaging 6.381. Based on these indicators, locus BPSS1974$^{\#I}$ occupied an intermediate position between VNTR loci L3145k and L933k, represented by perfect and degenerate minisatellite repeats, respectively.

Successful amplification of the BPSS1974#I locus during PCR with DNA from *B. mallei* strain 16050, isolated from a sick horse in 2023, indicated the stability of the areas flanking the VNTR region. In turn, the full-length nucleotide sequence of the BPSS1974$^{\#I}$ locus, identified by whole-genome sequencing data analysis of the *B. mallei* 16050 strain, showed its potential for in silico MLVA typing.

Our cluster analysis results of *B. mallei* strains based on the determination of repeat numbers in 7 VNTR loci were compared with the core genome multilocus sequence typing (cgMLST) data conducted by S. Appelt et al. [13]. The comparison showed that the composition and mutual arrangement of the clusters had many common features. Thus, according to cgMLST data, strains from China were grouped into two main clusters, corresponding to clusters D and L in the MLVA dendrogram we constructed. These clusters included strains for which the geographical region of origin in GenBank is listed as the USA: cluster L included strains *B. mallei* 2002721274, JHU and FMH, while cluster D included *B. mallei* 2002721277. Our clustering was consistent with the cgMLST results and indicated a likely Chinese origin of these strains. Within cluster D, a close relationship was confirmed between strains 2000031063 (Hungary) and China5 (China), whose MLVA profiles differed only at the S2862k locus. In addition to the cgMLST data, the genetic proximity of the strains within MLVA clusters D and L was also confirmed by the results of our DFR typing. Thus, with the exception of *B. mallei* Kweiyang#4, which lacked an amplification product at the BmVAT6 locus, a common DFR profile was determined for all strains within each cluster.

The strains from Turkey investigated in this study were classified in 3 different clusters. The MLVA-6 scheme, supplemented with locus BPSS1974$^{\#I}$, allowed for the distribution of strains from these clusters according to their position on a minimum spanning tree constructed based on cgMLST data [13]. Thus, based on typing results using only the MLVA-6 scheme, strain *B. mallei* PRL-20 was included in the same cluster as strains from Turkey. Determining the number of repeats at the BPSS1974$^{\#I}$ locus allowed *B. mallei* PRL-20 to be placed on the dendrogram in a separate branch with the *B. mallei* India86-567-2 strain from India, while the strains from Turkey formed a separate, most numerous cluster J. Two other strains from Turkey, *B. mallei* 11 and NCTC 10247, were included in sub-cluster G2 and cluster F, respectively.

The inclusion of locus BPSS1974$^{\#I}$ in the MLVA-6 scheme, along with strain *B. mallei* India86-567-2, allowed us to clarify the position of another Indian strain, *B. mallei* 3708, which formed a separate branch within cluster C. The clustering of Indian strains established using the developed MLVA-7 scheme was consistent with the data obtained in the study by H. Singha et al. [24]. In the presented study, based on MLVA typing at 23 loci, the strains *B. mallei* India86 567-2, Mukteswar, SAVP1, BMQ and NCTC 3708 were found to belong to different clusters, which was consistent with the results obtained in this study.

The MLVA and DFR profiles of the Brazilian strains we established confirmed the previously proposed hypothesis of two episodes of introduction of the glanders into Brazil [25]. Therefore, the close genetic relationship we identified within sub-cluster G3 between strains of European origin and BM_campo 2.1 suggested a possible introduction of glanders into Brazil from Europe, likely during colonization or trade.

The inclusion of the BPSS1974$^{\#I}$ locus in the MLVA-6 scheme allowed to localize of *B. mallei* strain 11 in the neighboring G2 sub-cluster, which was consistent with the data from M.V.D. Falcão et al. [25]. The authors performed typing of strains based on 15 phylogenetically informative single nucleotide polymorphisms using PCR followed by high-resolution melting analysis, which identified the L3B3sB3 branch, including strains from MLVA clusters G2 and G3 formed in this study. Analysis of single nucleotide polymorphisms [25] also showed that strain *B. mallei* UFAL2 belongs to the L3B2 branch, which includes strains isolated in Brazil. Due to the lack of nucleotide sequences from other Brazilian strains in Genbank, we were unable to determine the MLVA and DFR profiles of the L3B2 lineage strains. At the same time, the unique VNTR profile of strain *B. mallei* UFAL2 allowed it to be placed in a separate MLVA cluster H, which corresponds to the division of Brazilian strains into different phylogenetic lineages.

Based on the results of MLVA and DFR analysis, a close genetic relationship was established between *B. mallei* strains V-120 and 16050, isolated 18 years apart from diseased animals in Eastern Siberia, and *B. mallei* strain mongolia_1, isolated from a diseased horse in Mongolia in 2022. Belonging to the same DFR type (DFR06) and sharing a unique VNTR profile at loci L20k and L1217k, along with the geographical proximity of the territories of these countries where glanders outbreaks were reported, suggests a common source of origin of these strains. At the same time, *B. mallei* strains C-4 and C-5, isolated in Mongolia in 1967, formed a separate MLVA cluster. The DFR type (DFR06) was also identified in the Brazilian strain *B. mallei* UFAL2. Common DFR profiles of strains from different MLVA clusters have been identified in several other cases, which may indicate a decrease in the specificity of the selected DFR loci as the number of *B. mallei* genome sequences in genetic databases increases.

The observed changes in the VNTR profiles of duplicate *B. mallei* strains from different collections may be related to storage conditions, particularly the methods used for maintaining bacterial cultures. In a study by J.M. U'Ren et al. [26], when studying the stability of 32 VNTR loci proposed for typing melioidosis strains using the serial passage method on plates, changes were found in 12 loci. Mutations were identified in the L933k, L3145k and S2862k loci, which were included in our developed typing scheme for glanders causative agent. The variability of loci L933k and S2862k in the genomes of duplicate strains of *B. mallei*, as established in this study, indicated a similar pattern of variation in these VNTR loci among pathogenic *Burkholderia*. The stability of the molecular structure of locus BPSS1974$^{\#I}$ has been determined in strains from different collections, which is important for epidemiological investigations and evolutionary analysis.

Thus, the VNTR locus BPSS1974$^{\#I}$ can be considered a marker, the inclusion of which in the MLVA-6 scheme will improve the accuracy of genotyping and the determination of the regions of origin of newly isolated *B. mallei* strains. Alongside the improvement of schemes for differentiating *B. mallei* strains for more effective intraspecies typing, there is a necessity to increase the number of available nucleotide sequences of *B. mallei* genomes, combined with the addition of metadata about their exact geographical origin.

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REVIEWS

Review
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Next-Generation Protective Gloves: Current Trends in Technological Solutions and Application Prospects (Scientific Review)

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Abstract

Introduction. Protective gloves are widely used in medicine to provide biological protection for patients and medical staff. However, if gloves are used improperly, there is a risk of healthcare-associated infections (HAIs) for both staff and patients. A significant problem is the resulting growth in medical waste and its disposal. Therefore, developing new approaches to ensure maximum protection for staff and patients, and minimize the risk of infection, by creating protective gloves based on biodegradable polymer materials with an antimicrobial coating, is an urgent epidemiological and environmental objective.

This paper discusses modern technologies and emerging issues in the creation of new materials for protective gloves with antimicrobial properties. These materials can be made using guanidine derivatives, quaternary ammonium compounds (QACs), chlorinated phenols, essential oils, iodine compounds, silver salts, metal oxides and metal nanoparticles and oxides, vegetable oil extracts, aniline dyes. The introduction of biofillers such as starch and nanocellulose will help improve biodegradability. They will also help maintain the necessary physical and chemical characteristics. The problem of synthetic rubber waste disposal can be solved by the development of new composite materials with improved biodegradation characteristics. These materials are in the form of thermoplastic elastomers (TPE), polylactide (PLA) and polylactone (PLC).

Conclusion. A review of the scientific literature revealed a significant global interest in the creation of protective gloves with antimicrobial properties made from biodegradable materials. However, in addition to directly suppressing the growth of pathogenic microflora, their use may also pose a number of problems related to their impact on human health and the ecosystem. The successful implementation of this direction hinges on the continuation of scientific research on imparting the declared properties to gloves. This research should use effective, reliable and safe technologies, as well as the development of unified methods and protocols for assessing antimicrobial activity. Once these are in place, the research can be implemented widely in practice. The production of biodegradable protective gloves offers significant potential, as it will help to reduce the risk of infection spreading in healthcare organizations and contribute to environmental protection.

Keywords: *protective gloves, antimicrobial properties, functional materials, review, biodegradable components*

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Научный обзор
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Защитные перчатки нового поколения: актуальные тенденции технологических решений и перспективы применения (научный обзор)

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Аннотация

Введение. Защитные перчатки широко используют в медицине, они обеспечивают уровень биологической защиты пациентов и медицинского персонала. Вместе с тем существует риск развития инфекций, связанных с оказанием медицинской помощи, у сотрудников и пациентов при их ненадлежащем применении. Значимой проблемой является рост медицинских отходов и их утилизация. В этой связи разработка новых подходов к обеспечению максимальной защиты персонала и пациентов, минимизации риска инфицирования, благодаря созданию защитных перчаток на основе биоразлагаемых полимерных материалов с антимикробным покрытием, является актуальной эпидемиологической и экологической задачей.

В статье обсуждаются современные технологии и возникающие проблемы при получении новых материалов для создания защитных перчаток с антимикробными свойствами при использовании производных гуанидина, четвертичных аммониевых соединений, хлорированных фенолов, эфирных масел, соединений йода, солей серебра, частиц и наночастиц металлов и их оксидов, экстрактов растительных масел, анилиновых красителей. Введение таких бионаполнителей, как крахмал и наноцеллюлоза, будет способствовать улучшению биоразлагаемых свойств при сохранении необходимых физико-химических характеристик. Разработка новых композитных материалов с улучшенными характеристиками биодеградации в форме термопластичных эластомеров, полилактида и поликапролактона позволит решить проблему утилизации отходов из синтетического каучука.

Заключение. Анализ современной научной литературы определил высокий интерес в мире к созданию защитных перчаток с антимикробными свойствами на основе биоразлагаемых материалов. Однако их применение, кроме непосредственного подавления роста патогенной микрофлоры, может дополнительно нести ряд проблем, связанных с влиянием на здоровье человека и экосистему. Для успешной реализации данного направления важно продолжать научные исследования по приданию перчаткам заявленных свойств с использованием эффективных, надёжных и безопасных технологий с разработкой унифицированных методик и протоколов оценки антимикробной активности для последующего широкого внедрения в практику. Создание биоразлагаемых защитных перчаток имеет большие перспективы, поскольку будет способствовать снижению риска распространения инфекций в медицинских организациях и внесёт существенный вклад в охрану окружающей среды.

Ключевые слова: *защитные перчатки, антимикробные свойства, функциональные материалы, научный обзор, биоразлагаемые компоненты*

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

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Introduction

The development of human potential and environmental protection are important objectives that define the domestic policy of the country and require the improvement of a set of measures to create conditions for strengthening the health of citizens, increasing life expectancy, reducing mortality and preventing infectious diseases, including healthcare-associated infections (HAIs), which have been identified as a biological threat by a Decree of the President of the Russian Federation[1].

Healthcare-associated infections (HAIs) are one of the key problems in global healthcare [1, 2]. Every day, 7 to 15 out of 100 patients acquire them in the hospital, and 1 in 10 cases result in death[2]. Issues of HAI prevention are traditionally of greatest significance in maternity hospitals [3, 4] and surgical departments [5]. Its relevance is increasing due to the complex demographic situation, as well as the rise in natural disasters, technological accidents and military conflicts. In the formation of HAI outbreaks, in addition to bacterial pathogens, whose resistance to antimicrobial drugs and biocides has become global, the significance of viruses is growing, including respiratory [6] and intestinal viruses [7]. The significance of the pathogens of blood-borne viral infections remains high [8]. The above suggests strengthening non-specific measures for HAI prevention, particularly improving personal protective equipment.

Modern issues in the use of protective gloves in medicine

Protective gloves are an essential attribute for every healthcare worker. As single-use personal protective equipment (PPE), they are designed to create a barrier between the patient and the healthcare worker while performing professional duties. Medical gloves, in addition to their basic protective properties (impermeability to microorganisms, airtightness, strength, safety for patient and staff health), should also have satisfactory consumer properties (comfort, high-quality packaging and labeling, ease of disposal, functionality)[3].

Depending on the degree of invasiveness of medical procedures and the risk of patient infection, protective gloves are divided into surgical and diagnostic/examination gloves. Surgical gloves must be sterile. Diagnostic/examination gloves can be sterile or non-sterile. Non-sterile gloves are used as standard precautions when working with patients infected and/or colonized with resistant strains of microorganisms and individuals infected with viruses that cause blood-borne infections (hepatitis B and C viruses, HIV, etc.).

Depending on the material, protective gloves are divided into latex, synthetic, and combined. Currently, most manufacturers of protective gloves use natural or synthetic latex as a base [9]. Natural latex (rubber) products are made from the sap of the *Hēvea brasiliēnswas* plant and are more comfortable [10–12]. In Russia, latex gloves are predominantly used. The material feels comfortable against the skin of the hands, which is ensured by its high elasticity and ability to absorb sweat. Despite its positive properties, natural latex often causes allergic reactions – up to 25% of nurses who use latex gloves report symptoms of dermatitis [13]. Due to this, most healthcare workers prefer gloves made of synthetic materials: vinyl, neoprene, polyurethane and nitrile. These materials are more durable and resistant to chemicals, while also being thinner, which provides high sensitivity for your fingertips. Nitrile gloves are used most often, followed by polyvinyl chloride and polychloroprene gloves [14, 15]. These gloves are pore-free, but they have less strength and elasticity than latex gloves; unlike latex gloves, their integrity is not restored after minor damage.

One type of high-strength gloves is chainmail gloves. They are still used in our country, but they only protect against cuts, not punctures. In this regard, the use of double gloves is being introduced in European medical institutions and some Russian hospitals. In this case, the risk of the surgeon's hands coming into contact with the patient's blood is reduced from 70% to 2%, and in 87% of cases where the outer glove is perforated, the inner glove remains intact [16]. When using two pairs of gloves worn one over the other, the skin is significantly better protected from blood exposure than when using a single pair of gloves. However, detecting damage while wearing double gloves is just as difficult as with single gloves.

In 1993, double gloves with puncture indication appeared on the European market, which have now become widespread. The concept behind them is quite simple, but effective. The lower glove is different in color from the upper one. When the outer glove is damaged, liquid (blood, rinse water, etc.) gets between the gloves, and a contrasting spot forms in the area of the puncture, signaling a perforation. Thus, up to 97% of punctures are detected when using indicator gloves, while only 8% are detected when using a single layer of gloves [16].

Depending on the type of material, gloves must meet different standards. The ASTM D6319-19 "Stan-

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[1] Decree of the President of the Russian Federation dated 11.03.2019 No. 97 "On the Fundamentals of the State Policy of the Russian Federation in the field of chemical and biological safety for the period up to 2025 and beyond".

[2] WHO launches first ever global report on infection prevention and control. 2022. URL: https://www.who.int/news/item/06-05-2022-who-launches-first-ever-global-report-on-infection-prevention-and-control (дата обращения: 06.05.2022).

[3] MP 3.5.1.0113-16 "The use of gloves for the prevention of infections related to the provision of medical care in medical organizations" (approved by the Head of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare, Chief State Sanitary Doctor of the Russian Federation A.Yu. Popova 02.09.2016).

dard Specification for Nitrile Examination Gloves for Medical Application" applies for nitrile examination gloves, the ASTM D3578-19 "Standard Specification for Rubber Examination Gloves" applies for natural rubber examination gloves and the ASTM D5250-19 "Standard Specification for Poly(vinyl chloride) Gloves for Medical Application" applies for polyvinyl chloride gloves. The EN 374-2:2014 standard "Protective gloves against dangerous chemicals and microorganisms — Part 2: Determination of resistance to penetration, IDT)" presents a method for assessing resistance to penetration by microorganisms, providing a minimum protection level of 2 (a glove that is air and water-tight, having passed a leak test, is considered resistant to microorganisms). The updated standard introduced new requirements for testing to assess virus protection (in addition to fungi and bacteria), and ISO 16604 for assessing protection against contact with blood and biological fluids for both the finished product and the material itself.

No type of glove is completely impermeable to microorganisms. Infection can penetrate gloves through micro-injuries and pores, the sizes of which are comparable to that of microorganisms. The liquid that gets inside gloves is most often localized in the thumb area and at the fingertips, and only in 30% of cases are these defects noticed by users. The average frequency of such unnoticed punctures during surgical procedures is 34.7–92% [17]. The frequency of surgical glove damage during orthopedic surgeries can reach 26.1%, with 82% of these damages going unnoticed [18]. There are differences in the frequency of glove punctures among doctors and nurses. Thus, out of 1457 doctor's gloves examined, 17.3% were damaged, and out of 325 nurse's gloves, 23.7% were damaged [19].

Despite modern technology significantly improving the mechanical properties of gloves, even wearing two gloves on one hand cannot eliminate the penetration of microorganisms in case of punctures or other mechanical damage. M.N. Bardorf et al. evaluated the impact of punctures on the protective properties of surgical gloves made from materials such as latex, nitrile, and neoprene [20]. It was found that the penetration of microorganisms depended on the rigidity or elasticity of the material. In the study by A.N. Goldman et al., over 50% (out of 33 pairs) of outer gloves used during orthopedic surgeries had macro- or micro-perforations due to contact with surgical rotating instruments (drills) [21]. A. Wolfensberger et al. noted that in 14% of cases, microorganisms were transferred from gloves to hands during their removal [22]. In this regard, when choosing the type of surgical gloves, it is necessary to take into account the specific conditions of the types of surgical interventions, which can be met through the additional properties of the gloves.

Medical PPE manufacturers have proposed the introduction of gloves with an internal antibacterial coating as a highly effective measure for practical application. The protective mechanism of one type of such gloves is based on active coating technology containing chlorhexidine gluconate, which has a broad spectrum of antimicrobial action [23].

A serious mistake that medical staff often make when working with gloves (especially powdered ones) is treating them with alcohol solutions. According to surveys (questionnaires), the proportion of employees who do not change gloves after treating them with alcohol-based sanitizers, or who only change gloves when in contact with blood, is 6.5% [24]. It is known that alcohols destroy the upper protective layer of gloves, making them permeable to microorganisms and forming a compress of powder in the form of a mixture of skin antiseptic and "glove juice", which can lead to postoperative adhesions in the patient and negatively affect wound healing. Powder from laboratory staff gloves getting into a biological sample can also lead to false-negative results when testing blood serum for HIV [16].

It is not recommended to treat gloves with sanitizers and disinfectants, which has been confirmed by experimental studies. Penetration of *Escherichia coli* and *Staphylococcus aureus* through latex medical gloves without antiseptic was established after 30 minutes in 15% of cases, after 60 minutes in 25%, and through gloves of the same types but treated with sanitizer, in 29.2% and 45.5% respectively [25].

A significant problem remains the practice of staff washing their hands with soap while wearing gloves between assisting different patients or between procedures on the same patient, which is not recommended but occurs quite frequently in practice. Hands can also come into contact with the potentially contaminated outer surface of the gloves during removal. Study conducted by A.R. Tenorio et al. on vancomycin-resistant enterococci found that despite the use of gloves, these pathogens were detected on the hands of staff after glove removal in 30% of cases [26].

It can be concluded that the hand protection provided by modern types of protective gloves is insufficient for medical staff and patients, and that antimicrobial agents need to be added to the PPE. The increase in the number of antibiotic-resistant microorganisms dictates the necessity to search for new antimicrobial fillers against which resistance will develop to a lesser extent. First and foremost, this concerns inpatient departments with high circulation of nosocomial strains of microorganisms, where the use of antimicrobial protective gloves will be most in demand.

The Growth of Medical Waste as a Leading Environmental Problem

In medical facilities, single-use gloves are subject to disinfection/decontamination after use as medical waste of the corresponding class (usually classes B or

C, in certain cases D or E)[4]. Presenting an epidemiological hazard, medical waste is a far more significant threat than most other production and consumption waste [27]. Changes in patient diagnostic and treatment methods, and a sharp increase in the demand for single-use medical instruments have significantly impacted the morphological composition of hospital waste, its generation rate and accumulation.

Medical waste is complex and diverse, and many of its components are hazardous in terms of their microbiological composition. Not only is sanitary and epidemiological safety important, but also the economic efficiency of their disinfection/neutralization. Ensuring the epidemiological and environmental safety of medical waste management requires active interdepartmental cooperation and legal regulation [28].

According to the WHO, the generation of medical waste worldwide is increasing annually and by 2021 had already reached 2.27 kg per person per day. Developing countries generate 2–3 times more medical waste than developed countries [29]. According to the US Environmental Protection Agency, approximately 20% of medical waste is generated in hospitals, 15% in laboratories, 35% in nursing homes, and 30% in urgent care clinics and other healthcare facilities. Alongside this, in 2020, as noted in the UN report, 30% of healthcare facilities in developed countries and 60% in the least developed countries lacked waste disposal equipment [30]. In Russia in 2021, approximately 8.448 billion tons of waste were generated [31], of which 3.5 million tons were medical waste [32].

The application of PPE based on synthetic materials (polypropylene, polyurethane, and polyacrylonitrile in masks, as well as latex, vinyl, polyethylene, and nitrile in gloves) poses a serious environmental problem of pollution in the form of not only solid waste but also plastic particles formed as a result of their fragmentation. These particles are non-biodegradable, can persist in the environment for a long time, and pose a serious threat to aquatic and terrestrial fauna and flora. For example, according to the estimates of experts, the number of disposable face masks that ended up in the World Ocean in 2020 alone was estimated at 1.56 billion units [33].

Progress in medical technology is inevitable, but it leads to a significant increase in medical waste. In this regard, research is being conducted on both new technologies for neutralization and destruction, as well as principles for waste quantity management. Open burning of medical waste or burning in any other improper manner leads to the release of dioxins, furans and particulate matter into the air, polluting the environment [34].

In this regard, the objective of giving protective gloves not only antimicrobial properties but also the maximum possible biodegradable components is one of the most important preventive and environmental measures for minimizing the risks of infectious diseases arising and spreading, and for preserving public health in a safe and comfortable living environment.

New materials for producing gloves with antimicrobial properties

Antimicrobial components that could be incorporated into the material of protective gloves made of natural or synthetic rubber include guanidine derivatives (chlorhexidine salts and polyhexamethylene biguanide), quaternary ammonium compounds, chlorinated phenols (triclosan), essential oils (farnesol, phenoxyethanol, octoxyglycerin), iodine compounds, silver salts, metal nanoparticles and their oxides, as well as extracts from certain plant oils (purple gentian), aniline dyes (brilliant green), chitosan-based compounds, turmeric and similar substances [35]. By covalently bonding an antibacterial agent to polymer surfaces, a lasting effect and self-disinfection of the material can be achieved. Metals and their oxides, particularly silver particles, zinc oxide, copper, as well as composite materials based on them, are considered as potential antimicrobial components [36–38].

In a study by X. Chen et al., calcium carbonate whiskers (CaCOw), tetrapod-like zinc oxide whiskers (T-ZnOw), and magnesium oxysulfate whiskers in the form of needles and irregularly shaped particles (MgOSw) were added to natural latex (before vulcanization) to impart antimicrobial properties [39]. Antimicrobial activity was studied using the Oxford cup method and the suspension method. *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* were used as test microorganisms. The best results for all microorganisms were obtained for samples containing zinc oxide. Antimicrobial activity was noted in materials containing calcium carbonate. Samples based on magnesium oxysulfate showed an antimicrobial effect only against *P. aeruginosa*. The results obtained confirmed the fact that T-ZnOw activates the active oxidizing group (OH), leading to the death of microorganisms.

E. Smiechowicz et al. proposed using cellulose fiber to create protective gloves [40]. Silicon nanoparticles with immobilized silver nanoparticles were introduced into the fibers. The authors limited themselves to only the results of the material's properties (strength, vapor permeability, resistance to mechanical impact and pH changes), without indicating its antimicrobial activity.

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[4] Section X of SanPiN 2.1.3684-21 "Sanitary and epidemiological requirements for the maintenance of territories of urban and rural settlements, for water bodies, drinking water and drinking water supply, atmospheric air, soils, residential premises, operation of industrial and public premises, organization and conduct of sanitary and anti-epidemic (preventive) measures" (approved by the Head of the Federal Service for on supervision in the sphere of consumer rights protection and human well-being, by the Chief State Sanitary Doctor of the Russian Federation A.Yu. Popova on January 28, 2021).

In a study by T. Arpornwichanop et al., a partial antimicrobial effect of an additive to natural latex in the form of N,N,N-trimethylchitosan, adsorbed on poly(methyl methacrylate) and stabilized with silica nanoparticles, was established against Gram-positive and Gram-negative microorganisms [41]. The pronounced antimicrobial effect of this composition was established only for the Gram-positive *S. aureus*.

Graphene oxide, as well as its derivatives, have a broad spectrum of antiviral activity against DNA and RNA viruses, including enveloped and non-enveloped viruses. Laser-induced graphene-based materials exhibited antibacterial properties [42]. Studies on their effectiveness have been conducted exclusively in laboratory settings to date, and there is no literature data confirming their effectiveness when tested in practical conditions.

Strong antibacterial properties were found when silver particles, deposited on graphene oxide, were introduced into natural latex. The antimicrobial activity of the composite materials by T. Li et al. was evaluated based on the area of the growth inhibition zones of the test microorganisms *E. coli* and *S. aureus* in an experiment with these additives [43]. The synthesized materials demonstrated high antibacterial activity, but the authors were unable to establish a correlation between the size of the growth inhibition zones and the concentration of the synthesized additive. The mechanism of antimicrobial action was explained by the active release of silver ions (Ag^+) and their interaction with thiol groups (SH) within the protein molecules of microorganisms.

L.A. Alshabanah et al. conducted research on a polymer composite material that also contained silver nanoparticles with a size of 17–51 nm [44]. Polyvinyl alcohol or thermoplastic polyurethane were used as the matrix. Antimicrobial properties were evaluated similarly based on the zones of inhibition of microbial growth (*S. aureus* and its methicillin-resistant variant (MRSA), *E. coli*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*). High antimicrobial activity of the polyurethane-based material containing 4% silver nanoparticles was established against all tested strains.

M. Kahar Bador et al. described a method for imparting antimicrobial properties to nitrile gloves by applying polyhexamethylene biguanide hydrochloride to their surface [45]. The effectiveness assessment was conducted with the involvement of medical staff from the intensive care unit. The number of colony-forming units (CFU/mL) of microorganisms that grew on nutrient agar was determined as a result of seeding samples (glove swabs) after staff performed four procedures (intravenous administration of solution, oral care for the patient, physiotherapy, changing bed linen). The studies were conducted in parallel using standard nitrile gloves without polyhexamethylene biguanide hydrochloride (control). In the first three out of four types of manipulations, antibacterial gloves had less pronounced bacterial contamination compared to the control. No significant difference in bacterial contamination of gloves was observed between the groups when changing bed linen.

S. Ali et al. studied the antimicrobial effect of polyhexamethylene biguanide hydrochloride on the survival of *Streptococcus pyogenes*, carbapenem-resistant *E. coli*, MRSA and *K. pneumoniae* [46]. 15 minutes after applying the suspension (10 μL) containing 10^4 CFU/mL of bacterial culture to untreated gloves (1 cm^2), regardless of the type of contamination, the listed bacteria maintained their initial concentration level. Gloves treated with polyhexamethylene biguanide hydrochloride reduced the level of microbial contamination by 99.99% within 10 minutes of contact. However, the antimicrobial properties of such gloves were strongly dependent on the presence of biological contaminants (blood, organic compounds, etc.).

In a study by M. Suchomel et al., the effect of chlorhexidine bigluconate on the survival of microorganisms on the hands of surgeons after wearing gloves for 3 hours was investigated [47]. The hands were pre-treated with a skin antiseptic containing 60% (v/v) *n*-propanol. Wearing surgical gloves containing chlorhexidine bigluconate resulted in a 2.67 log10 reduction in the number of microorganisms. With untreated gloves, the degree of reduction was less pronounced (from 1.96 log10 to 1.68 log10, $p \leq 0.01$).

In a similar study (with chlorhexidine biguanide), J. Leitgeb et al. found a high degree of antimicrobial hand protection after wearing gloves for 2 hours [48]. The experiment involved 16 healthy adult volunteers. They wore a glove with chlorhexidine biguanide on one hand (experimental) and one without it (control) on the other. After wearing them for 2 hours, they performed specific finger movements and then removed the gloves from their hands. Subsequently, test microorganisms *S. aureus* (ATCC 6538) or *K. pneumoniae* (ATCC 4352) were applied to the inner surface of the cut-off fingers of the gloves at a concentration of 10^8 CFU/mL. The main evaluation criterion was considered to be the average number of viable forms, expressed in CFU, after 5 minutes of contact with the test microorganisms. In glove samples treated with chlorhexidine biguanide, the average number of *S. aureus* was 6.24 log10 lower compared to the control group, and *K. pneumoniae* was 6.22 log10 lower.

Quaternary ammonium compounds are an effective additive to natural latex. A. Arakkal et al. found that the addition of quaternary poly(4-vinylpyridine) to the polymer precursor provided the necessary degree of protection (99.99%) against clinical isolates of *P. aeruginosa* (PAO1) and *A. baumannii* (C80) [49].

In several scientific papers, W. Moopayak et al. used mangosteen rind to give gloves antimicrobial properties [50]. The active components were xanthones – secondary metabolites also found in plants, fungi and lichens. Xanthones are attributed with antioxidant, anticancer, anti-inflammatory, antiallergic, antibacterial, antifungal and antiviral properties. Natural rubber was used as a biofiller. Adding mangosteen rind powder improved the antimicrobial properties of rubber gloves without compromising their physical and mechanical characteristics. At the same time, the toxicity of the material was significantly lower compared to samples with silver nitrate.

A composite material based on mangosteen powder and zinc oxide is described by M. Luengchavanon et al. [51]. The antimicrobial component was applied to the surface of standard nitrile gloves. The minimum inhibitory concentration for MRSA was 160 µg/mL. High inhibitory activity against *K. pneumoniae* was established. Despite the high effectiveness of the method presented by the authors, achieving the effect required prolonged contact of the microorganisms with the surface — at least 30 minutes, which is difficult to achieve in real-world conditions.

Aloe extract is considered one of the promising antimicrobial components of plant origin. In an experiment, H. Khanzada et al. established the high antimicrobial effectiveness of polyvinyl acetate fibers with aloe extract against *S. aureus* and *E. coli* using the electrospinning method [52].

The introduction of antimicrobial additives into the composition of gloves can, in some cases, contribute to expanding their functionality. Thus, C. Salvadores Fernandez et al. applied a composite material containing zinc oxide to nitrile gloves and integrated flexible electrical contacts into the glove material, creating a highly sensitive layer on the fingertips that, according to the authors, can be used as a diagnostic tool [53]. They successfully used such gloves to study pig sphincter damage in an *ex vivo* experiment.

A new approach to creating self-disinfecting protective gloves, based on the action of an electric current generated by friction between nylon and silicone rubber, has been proposed by S. Bayan et al. [54]. A small bioelectric device (2 × 2 cm) made of silicone rubber and nylon (applied to nitrile) generated over 20 V of transient voltage or 41 µW of output power, which was sufficient to charge a capacitor to 65 V in just ~50 seconds. The energy released was used to destroy microorganisms that land on the surface. Thus, to reduce the number of *P. aeruginosa* and *E. coli*, the method's effectiveness was 50–70%.

E.M. Klupp et al. studied the antimicrobial activity of examination gloves with light-activated properties [55]. The experiment was conducted under practical conditions on Gram-positive microorganisms. Glove surfaces were contaminated according to a standardized method (ASTM D7907) with a suspension of *Enterococcus faecium* (ATCC 6057) and its vancomycin-resistant variant, as well as MRSA (ATCC 43300). The results indicated insufficient activity of the applied method against the strains used. The level of contamination reduction was less than 1 $\log_{10}$ after 10 minutes of light exposure. The authors concluded that the method was ineffective.

In a study by D. Patil et al., a method for imparting antimicrobial properties to a nitrile without adding an active substance is described [56]. A specific nanotopographic pattern resembling a cicada wing was created on the surface of the gloves using reactive ion etching. The altered surface structure led to the death of over 85% of *P. aeruginosa* isolates, with the effectiveness of the material's antimicrobial properties showing a clear dependence on the surface structure.

Modern approaches to creating biodegradable protective gloves

Polymeric materials, including synthetic rubber, take a significant amount of time to decompose naturally — around 3 years [57]. One of the reasons for their long persistence in the environment in an unchanged form is the synthetic origin of rubbers, for the production of which raw materials from fossil fuels are used. The variety of monomers used for rubber synthesis has led to the creation of numerous types of synthetic rubbers, but their disposal remains difficult. Managing rubber waste at the end of its life cycle is a global environmental problem of our time.

The rate of decomposition of gloves in nature, like any other material, is influenced by numerous factors, including the types of soil microorganisms present, temperature, pH of the environment, and the presence of chemical additives in the material composition. It is known that synthetic rubber can be combined with thermoplastics. The resulting thermoplastic elastomers will possess both thermoplastic and elastomeric properties. Their thermoplastic properties will allow for more effective processing.

Certain thermoplastics — polylactic acid (PLA) and polycaprolactone (PCL) — contain hydrolyzable ester bonds, which allow materials based on them to exhibit good hydrolytic degradation [58]. Adding these compounds into synthetic rubber can enhance the product's biodegradability. Currently, PLA, being a biobased polymer (produced from corn or sugar beets), is widely used in the development of eco-friendly plastics, 3D printing filaments, and as a component of polymer blends. A thermoplastic poly(ether urethane) derived from PCL, hydrogenated 4,4'-methylene diphenyl diisocyanate, and chain extenders of varying lengths (e.g., 2-ethynyl urea diol obtained from an amino acid) completely degrades within 100 days in an alkaline solution [59].

Polyesters of 1,4-butanediol, 1,3-propanediol, and/or 2,3-butanediol can also be used as materials for biodegradable films, in combination with various organic acids such as lactic, sebacic, itaconic, and succinic acid [60].

G.Y. Yew et al. pointed out the possibility of using other biological supplements derived from food products and plants (algae) [61]. Works have been published on the application of biofillers in elastomers made from cellulose, starch, chitosan, PLA or polyglycolic acid [62].

Starch has been proposed as a biofiller for latex. S. Daud et al. conducted experimental studies with sago starch to improve the biodegradability of natural rubber films [63]. Sago starch with sulfate-ester groups was obtained by treating it with an aqueous solution of sulfuric acid for 7 days at room temperature. The initial starch particle size was 1.233 μm, and after the acid hydrolysis process, it decreased to 0.313 μm, which allowed for the production of latex films with uniformly incorporated starch particles. The biodegradability of such films was up to 25% within 3 weeks.

R. Blanchard et al. described the use of nanocrystalline cellulose as a biofiller for natural rubber [64]. However, the authors did not present convincing data on the antimicrobial activity of such films.

Conclusion

Protective gloves are an essential piece of PPE widely used in various fields, primarily in medicine. The choice of glove type depends on the specific tasks being performed and the requirements of quality standards. The most common materials for making gloves are nitrile, natural latex, and polyvinyl chloride, which meet their respective safety and effectiveness standards. Modern international standards, such as ASTM and ISO, provide a high level of protection against the penetration of microorganisms, including viruses, bacteria, and fungi. However, research shows that even when all precautions are taken and modern technology is used, there is a risk of microorganisms penetrating damaged gloves, especially during complex procedures involving sharp instruments.

Despite significant advancements in the production of protective gloves, there remains a necessity for further improvement in quality control methods and the development of new approaches to ensure maximum worker protection. This includes raising staff awareness of safe glove use guidelines and implementing innovative solutions to minimize the risk of infection.

The problem of synthetic rubber waste disposal is becoming increasingly relevant due to its difficulty in decomposing naturally. Despite the variety of synthetic rubbers, their long-term presence in the environment poses serious ecological problems. Modern research is focused on developing materials with improved biodegradation characteristics, such as thermoplastic elastomers containing hydrolyzable ester bonds, which will significantly accelerate the decomposition process. The use of biopolymers like PLA and PCL opens up prospects for creating environmentally friendly materials suitable for a wide range of applications, including the production of disposable gloves. The adding of biofillers such as starch and nanocrystalline cellulose will improve the biodegradability of materials while ensuring the preservation of the necessary physical and mechanical characteristics. Research in the field of modifying the composition and structure of synthetic rubbers to increase their biodegradation rate is necessary to address the global environmental problem associated with the management of rubber waste. The use of composite materials that include both traditional synthetic components and biodegradable additives appears to be a promising scientific direction for the development of the polymer materials industry.

An analysis of current scientific literature has demonstrated a significant increase in interest in the development of protective gloves with antimicrobial properties. Despite the obvious appeal of the idea of giving gloves antibacterial properties, its implementation faces a number of serious technical and medical challenges. It is important to consider not only the effectiveness of antimicrobial components but also their potential impact on the health of medical staff, patients and the environment. The need to develop unified criteria for assessing antimicrobial activity and identifying risks requires a comprehensive risk-based approach that takes into account the interests of all stakeholders. The lack of standardized testing methods makes it difficult to compare the results of different studies and hinders the development of general recommendations for the use of antimicrobial additives.

Thus, for the successful promotion of the idea of creating antiseptic gloves, it is important to conduct further scientific research aimed at developing reliable and safe methods for introducing active substances, and to develop standard protocols for assessing antimicrobial activity for subsequent implementation in healthcare practice.

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Review
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Immunological and epidemiological effectiveness of pediatric vaccination against hepatitis A using a single dose of inactivated vaccine

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Abstract

Hepatitis A is an acute liver disease caused by the hepatitis A virus (HAV), which can be prevented by means of vaccination. The standard hepatitis A vaccination schedule consists of two doses of inactivated vaccine, but for economic reasons and the purpose of improving vaccination coverage, universal pediatric single-dose vaccination programs have been implemented in certain regions of the world.

The **aim** of this review is to summarize and analyze published data on the duration of protective antibody levels after a single-dose pediatric immunization, as well as hepatitis A incidence in countries where hepatitis A vaccination is carried out using a single dose of inactivated vaccine.

Recent data from different regions of the world, including the Russian Federation, support the effectiveness of the single-dose hepatitis A vaccination strategy, both in terms of the duration of the immune response and the impact on the incidence rates. However, further studies on the long-term effectiveness of single-dose immunization with inactivated vaccine, as well as continuous hepatitis A surveillance, are necessary to assess the duration of protection and the necessity for booster vaccination later in life.

Keywords: *hepatitis A, hepatitis A vaccine, single-dose immunization*

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Обзор
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Иммунологическая и эпидемиологическая эффективность вакцинации детей против гепатита А с применением одной дозы инактивированной вакцины

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Аннотация

Гепатит А — это острое заболевание печени, вызываемое вирусом гепатита А, которое можно предотвратить с помощью вакцинации. Стандартная схема вакцинации против гепатита А предполагает введение двух доз инактивированной вакцины, однако по экономическим соображениям и для повышения уровня охвата вакцинацией в некоторых регионах мира внедрены программы массовой вакцинации детей с при-

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менением одной дозы вакцины.

Целью обзора является обобщение и анализ опубликованных данных о длительности сохранения протективных уровней антител после однократной иммунизации детей, а также о заболеваемости в странах, в которых проводится вакцинопрофилактика гепатита А с использованием одной дозы инактивированной вакцины.

Данные последних лет, полученные в разных регионах мира, в том числе в России, подтверждают эффективность стратегии вакцинации против гепатита А с применением одной дозы вакцины как с точки зрения длительности сохранения иммунологического ответа, так и с точки зрения влияния на уровни регистрируемой заболеваемости. Однако для оценки продолжительности защиты и необходимости ревакцинации на более позднем этапе жизни необходимы дальнейшие исследования по долгосрочной эффективности стратегий вакцинации с применением одной дозы инактивированной вакцины, а также непрерывный надзор за гепатитом А.

Ключевые слова: *гепатит А, вакцина против гепатита А, однократная иммунизация*

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

Hepatitis A is an acute liver disease caused by the hepatitis A virus (HAV) that can be prevented through vaccination. There are two types of vaccines against hepatitis A: live attenuated vaccines used in China [1] and inactivated vaccines available worldwide [2]. Targeted immunization of at-risk groups is effective in preventing sporadic cases and small outbreaks of hepatitis A, but it does not lead to the formation of a significant herd immunity; therefore, it is ineffective in terms of reducing the incidence of hepatitis A among the general population [3]. It has been proven that the strategy of universal vaccination of toddlers provides significant epidemiological and economic benefits in regions with transitional endemicity from high to medium regarding hepatitis A. In such regions, the HAV circulation among children decreases due to improved sanitary conditions, resulting in an increased proportion of susceptible adolescents and adults. Since HAV infection in children under 5 years old is usually asymptomatic, and the proportion of clinically apparent cases increases with age, this leads to an overall increase in the number of clinically significant and severe cases of the disease. According to the recommendations of the World Health Organization (WHO), a significant number of countries have included universal two-dose vaccination against HAV in their national pediatric immunization programs (Israel, Panama, Turkey, Greece, Kazakhstan, Mongolia, South Korea, Australia, Saudi Arabia, Qatar, and Bahrain) or have implemented regional programs for specific parts of the country (Russia, the USA, China, Italy) [4].

The standard immunization schedule for inactivated vaccines against hepatitis A consists of two doses administered at 6-month intervals and ensures the production of protective levels of antibodies to HAV (anti-HAV) that persist for decades [5]. For economic reasons and for the purpose of increasing vaccination coverage in certain countries, a single-dose vaccination against hepatitis A was implemented. For the first time, universal single-dose vaccination of toddlers was conducted in Argentina in 2005 [6]. In 2013, the Republic of Korea also introduced a single-dose vaccination for military personnel [7]. In 2014, Brazil began mass vaccination of children over 12 months old using a single dose of the vaccine [8]. Subsequently, this lead to more countries preferring single-dose immunization when conducting universal vaccination of children against hepatitis A (Chile, Paraguay, Colombia, Mexico, Canada (Quebec), Tunisia, Turkmenistan) [4].

Based on the experience of Argentina and in accordance with WHO recommendations, since August 2012, the Republic of Tuva (Russian Federation) has been conducting universal vaccination against hepatitis A for children aged 3 years and older with single-dose immunization [9]. Since the duration of immune protection with the administration of only a single dose of the vaccine remains a concern, countries that have implemented single-dose immunization against the disease continue to monitor the epidemiological and immunological effectiveness of this vaccination strategy.

The aim of this review is to summarize and analyze published data on the duration of protective antibody levels after a single immunization of children, as well as on the incidence of disease in countries where vaccination against hepatitis A is conducted with single-dose immunization.

Duration of protective immunity after administration of a single dose of inactivated hepatitis A vaccine in individual cohorts

Data on the immunogenicity of inactivated vaccines against hepatitis A support the possibility of a single-dose immunization. Thus, 2–4 weeks after the administration of the first dose of inactivated vaccine, anti-HAV IgG antibodies at titers above 20 mIU/ml (the level considered protective) are produced in 100% of immunocompetent children and young adults [10]. Moreover, a single administration of inactivated vaccine successfully suppresses outbreaks of hepatitis A and prevents the disease in contact individuals [11]. The notion of the necessity of administering a booster dose of inactivated vaccine against hepatitis A was initially based on assumptions about the decline of antibody levels over time. However, long-term observations conducted among Europeans vaccinated before traveling to endemic regions have demonstrated the maintenance of protective anti-HAV levels for at least 4–11 years after the administration of the first dose of the vaccine [12]. The first randomized trials of immunization using a single dose of inactivated vaccine against hepatitis A were conducted in 2003 in Nicaragua [13]. The study included 239 children aged 1.5 to 6.0 years, negative for anti-HAV antibodies, with a follow-up period of 18 months. Cases of hepatitis A infection, confirmed by the detection of anti-HAV IgM antibodies, were recorded in 4 children in the vaccinated group and in 22 children who did not receive the vaccine. All 4 cases of hepatitis A infection in vaccinated children occurred within the first 6 weeks after immunization, indicating infection before vaccination or in the first few days after the procedure. Thus, the protective efficacy of vaccination with single dose was 85% in the first 6 weeks and 100% 6 weeks post-immunization. In a study conducted in Thailand, the immunogenicity of a single dose of the vaccine was evaluated after 18 months of observation among 193 healthy children, with the proportion of individuals with protective levels of anti-HAV antibodies being 98%, and the geometric mean titer (GMT) of antibodies not differing from the level observed 6 months after the administration of the first dose of the vaccine [14]. In a study conducted in Argentina involving 546 healthy children, protective levels of anti-HAV antibodies were observed in 99.7% of participants three years after a single-dose immunization, and in 100% after the standard two-dose immunization schedule [15]. It should be noted that in this study, a protective concentration of anti-HAV antibodies was accepted as 10 mIU/ml, rather than 20 mIU/ml, in accordance with the revised stance of the expert community on the minimum protective concentration of anti-HAV antibodies [16].

In two studies, a direct comparison of immunogenicity and the duration of protective antibody levels was conducted when administering 1 or 2 doses of inactivated vaccine against hepatitis A to children. In the first study, 8 years after vaccination, differences were noted both in the frequency of detecting protective levels of anti-HAV in one- and two-dose immunization schemes (74.3% vs. 97.7%) and in GMT values (123.9 vs. 40.2 mIU/ml) [17]. In the second study, 100% and 96.7% of participants who received a double and single dose of inactivated vaccine against hepatitis A, respectively, had protective levels of anti-HAV seven years after vaccination, but the GMT levels were higher among those who received 2 doses of the vaccine (712.5 vs. 125.6 mIU/ml) [18].

Thus, data on the duration of anti-HAV persistence in individual cohorts after a single administration of the inactivated HAV vaccine against hepatitis A allowed for the consideration of the possibility of applying such a single-dose strategy in the implementation of mass vaccination programs against hepatitis A.

Immunological efficacy of a single-dose vaccination against hepatitis A in universal immunization programs

Argentina was the first country in the world to implement universal vaccination against hepatitis A using a single dose of the vaccine in 2005, after a nationwide outbreak in 2003–2004. Vaccination is carried out among children aged 12 months and older, and since 2006, vaccination coverage has exceeded 90%, with the average coverage rate across the country from 2006 to 2011 being 96.8% (77–100%). Only in 3 out of 24 provinces during this period did coverage not exceed 90% [19, 20]. After the campaign began, the Ministry of Health of Argentina organized continuous and intensified monitoring for hepatitis A incidence and post-vaccination immunity levels [21]. Among children examined 4 years after a single immunization at 12 months of age, a protective antibody level (10 mIU/ml and above) was found in 93% of cases [20]. Based on these results, in April 2012, the WHO Strategic Advisory Group of Experts on Immunization concluded that national immunization programs could consider including single-dose immunization schedules using inactivated vaccine against hepatitis A in their immunization schedules as an acceptable alternative to the standard two-dose regimen, while regional health authorities should regularly monitor long-term immunological protection with the single-dose vaccination schedule [22]. In accordance with this recommendation, the Ministry of Health of Argentina conducted a second study in 2013 to assess the long-term prevalence of protective antibodies among 1,088 children on average 7.7 years (6.3–9.2 years) after single-dose immunization at 12 months of age. In 97.4% of the study participants, the concentration of anti-HAV antibodies was ≥ 10 mIU/ml, and the GMT was 170.5 mIU/ml [23]. Currently, based on the experience in Argentina, there is data on the maintenance of protective levels of anti-HAV antibodies for up to 12 years after a single immunization. The level of seroprotection after 12 years was 93%, although these data were obtained from a very small cohort of 27 children [24].

The data obtained in Brazil as a result of the implementation of a child immunization program using single dose of inactivated vaccine against hepatitis A, initiated in 2014, also indicate the immunological effectiveness of this approach. Thus, 6–7 years after immunization, the level of seroprotection was 64.2% [25], while the proportion of seropositive individuals in the cohort examined 1 month after immunization was 93.6% [26].

Priority data on the duration of anti-HAV antibody persistence after a single immunization were obtained in one of the regions of the Russian Federation — the Republic of Tuva. Since 2012, in the Republic of Tuva, where the incidence of hepatitis A during the pre-vaccination period exceeded the national average by 10–15 times, a universal single vaccination against hepatitis A for children aged 3 years and older has been conducted. By the end of 2012, 65,097 children received a single immunization, resulting in a coverage rate of 87.4% for children aged 3 to 8 years [9]. Since then, vaccination against hepatitis A has been included in the regional vaccination schedule of Tuva for children aged 3 years and older. The immunological efficacy of a single immunization of children in Tuva was assessed in 5 independent cohorts, examined 1 month, 1, 5, 9 and 11 years after vaccination [9, 27]. The results obtained in these studies are summarized in **Fig. 1**.

Protective concentrations of anti-HAV antibodies (10 mIU/ml and above) were detected in 98.0% of those examined 1 month after a single immunization, in 93.5%, 91.1%, 99.4%, and 75.4% of children 1, 5, 9 and 11 years after a single immunization, respectively [9, 27]. Differences in seroprotection rates between cohorts examined 1, 5 and 9 years after vaccination were not statistically significant, although the observed decrease in the frequency of detecting protective levels of anti-HAV antibodies was statistically significant compared to the cohort examined 1 month after vaccination. In the cohort examined 11 years after a single-dose immunization, the decrease in the frequency of detecting protective antibody levels was statistically significant compared to previous years. The values of anti-HAV GMT were also similar in cohorts examined at 1, 5 and 9 years (449.6, 577.3, and 1446.3 mIU/ml, respectively; $p > 0.05$), but a significant decrease was noted in the cohort examined 11 years after vaccination (282.6 mIU/ml; $p < 0.05$) [27].

Data on the duration of protective anti-HAV antibody levels after single-dose immunization within mass vaccination programs are summarized in **Table 1**. Taken together, these data suggest that humoral immunity to HAV after a single-dose vaccination may decline more rapidly compared to the standard two-dose vaccination schedule, which provides seroprotection in over 90% of vaccinated children for up to 15 years [2, 28]. However, it should be noted that a decrease in humoral immunity does not necessarily indicate a lack of potential protection against hepatitis A. A study of the HAV-specific T-cell response in children under 12 years of age after a single-dose vaccination demonstrated the presence of $CD4^+$ and $CD8^+$ memory T-cells in 53.8% and 26.9% of seronegative children, respectively [24]. Similarly, the production of interferon-γ in peripheral blood mononuclear cells stimulated by the VP1 antigen of HAV was observed in 32.4% of seronegative children 6–7 years after a single dose of the inactivated vaccine, indicating the presence of cell-mediated immune memory [25].

Epidemiological effectiveness of single-dose vaccination against hepatitis A in universal immunization programs

The epidemiological effectiveness of universal vaccination programs for children against HAV, implemented using the standard two-dose immunization schedule, assessed by the reduction in registered incidence rates, number of hospitalizations, HAV-associated mortality, and the number of HAV-positive environmental samples, is well-known and documented in all

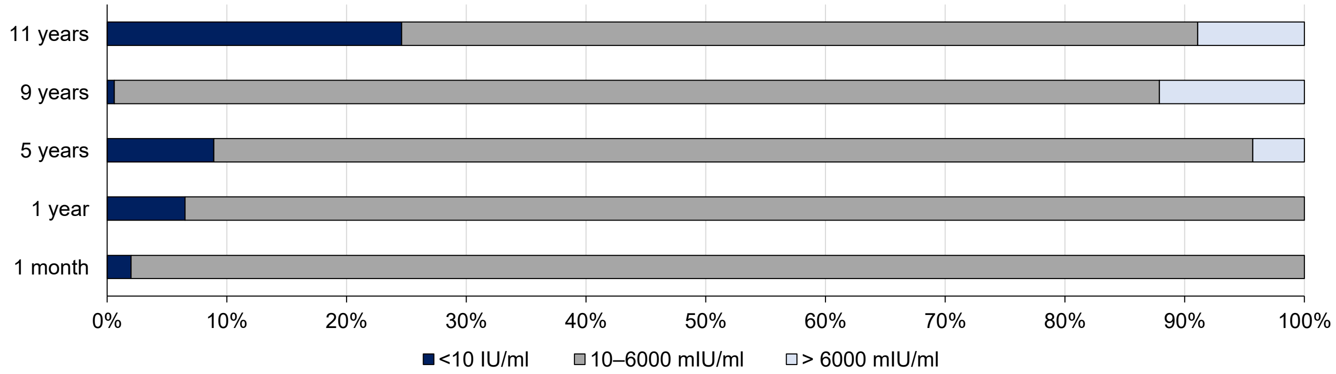

Fig. 1. The proportion of children with different concentrations of anti-HAV antibodies after a single-dose vaccination against hepatitis A in the Republic of Tuva [27].

Table 1. Persistence of protective levels of antibodies to HAV after a single dose of inactivated vaccine during mass immunization of children

Country/Region	Maximum duration of surveillance, years	Proportion of individuals with protective levels of anti-HAV antibodies, %	Reference
Argentina	4 years	93.0	[20]
	7,7 years	97.4	[23]
	12 years	93.0	[24]
Brazil	7 years	64.2	[25]
Russian Federation (Tuva Republic)	5 years	91.1	[9]
	9 years	99.4	[27]
	11 years	75.4	[27]

countries that have implemented such programs [2, 4]. Data on the epidemiological effectiveness of vaccination programs against HAV using single-dose immunization are presented in **Table 2**.

In Argentina, which introduced single-dose vaccination in 2005, the incidence of HAV decreased by ≥ 88.0%, from 66.5 (in 2000–2002) to 7.9 (in 2006–2011) cases per 100,000 population, with an average vaccination coverage of 96.8% in 2006–2011. Moreover, from 2007 to 2022, no cases of fulminant hepatitis or liver transplantation associated with HAV were recorded, despite HAV being the main cause of fulminant hepatitis and liver transplantation in children during the pre-vaccination period [29]. In Brazil, where universal vaccination of children with a single dose of the hepatitis A vaccine was implemented in 2014, vaccination coverage varied from 60.1% to 97.1% in 2014–2017. The recorded annual incidence rates of hepatitis A decreased from 3.02–3.48 cases per 100,000 people in 2010–2013 to 0.47–1.46 cases per 100,000 population in 2015–2017. From 2014 to 2016, the overall reduction in hepatitis A cases in Brazil was 85.5%. The most significant decrease in the incidence of hepatitis A was observed among children under 14 years of age, but a decrease was also observed in other age groups [8].

In the Republic of Korea, single-dose immunization against hepatitis A was implemented for military personnel starting in 2013. To evaluate the effectiveness of this vaccination program, the incidence of hepatitis A among vaccinated and unvaccinated military personnel during the implementation period of the vaccination, from 2013 to 2016, was analyzed. Among vaccinated military personnel, the incidence was 3 cases per 603,550 people per year, while among unvaccinated personnel, it was 21 cases per 1,020,450 people per year (which corresponds to 0.5 versus 2.06 per 100,000 population). Thus, the effectiveness of single-dose immunization against HAV was 75.85% [7].

The incidence of hepatitis A in Tuva during the pre-vaccination period (2001–2012) was the highest in Russia, with the majority of cases being registered among children and adolescents under 18 years old. In this group, peak incidence rates reached 450–860 cases per 100,000 population (**Fig. 2**). After the introduction of single-dose immunization for children in 2012, the incidence sharply decreased to 7.5 cases per 100,000 people among children and adolescents under 18 years old and to 3.2 cases per 100,000 people in the general population one year after the start of the vaccination program. Since 2016, no cases of hepatitis A have been registered in the region, not only among vaccinated children but also among unvaccinated adults.

It should be noted that from 2013 to 2023, against the background of practically nonexistent incidence of hepatitis A in Tuva, neighboring regions recorded sig-

Table 2. Epidemiological effectiveness of vaccination programs using a single dose of inactivated vaccine against hepatitis A

Year of implementation	Country /Region	Groups under vaccination	Efficiency	Reference
2005	Argentina	Children ≥ 12 months of age	Reduction in incidence by 88% from 2006 to 2011; disappearance of cases of fulminant hepatitis A	[29]
2012	Russian Federation (Tuva Republic)	Children ≥ 3 years of age	Reduction in incidence in 2013 by an average of 96.7% and by 96.9% among children. The absence of registered cases of the disease since 2016	[9, 27]
2013	Republic of Korea	Military personnel	Reduction in incidence by 75.9% after 1.5–2.0 years of implementation	[7]
2014	Brazil	Children 15-24 months of age	The reduction in incidence during 2014–2016 was on average by 85.5%, and by 96.8% among children.	[8]

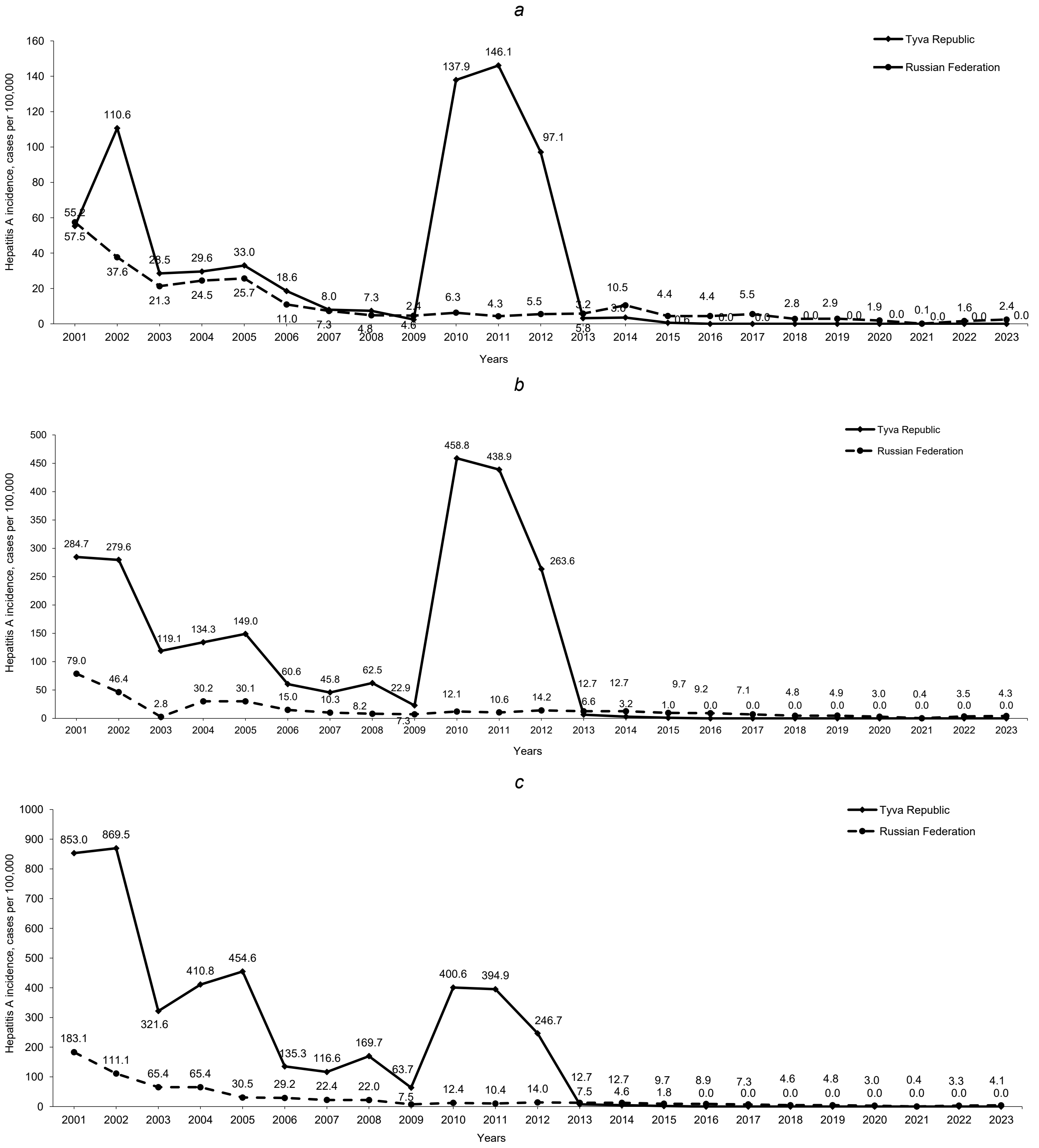

Fig. 2. The incidence of hepatitis A in the Republic of Tuva and the national average for 2001–2023 among the general population (*a*), children aged 0–14 (*b*) and 0–17 years (*c*) [27].

nificant incidence rates of hepatitis A, which confirms that the decrease in incidence in Tuva is not a result of reduced HAV circulation throughout the Siberian Federal District. Thus, in 2013–2023, the incidence of hepatitis A ranged from 0.1 to 6.48 per 100,000 population in the Republic of Buryatia, from 0.19 to 34.55 in the Republic of Khakassia, from 0.04 to 9.83 in the Irkutsk region, and from 0.0 to 34.93 in the Krasnoyarsk Krai [27]. Moreover, the high annual incidence rates of enterovirus infections and shigellosis recorded in Tuva from 2013 to 2023 indicate the persistence of sanitary unsafety in the region [27]. These data indicate that the decrease in the hepatitis A incidence observed in Tuva from 2013 to 2023 was not related to improvements in sanitary conditions, but was a result of the vaccination prevention program.

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The frequency of detecting HAV RNA in environmental samples (wastewater, open water bodies) is an important indicator reflecting the intensity of virus circulation and, therefore, represents a crucial criterion for the epidemiological effectiveness of HAV vaccination. It is expected that a significant reduction in the number of symptomatic hepatitis A infections will be accompanied by a decrease in the detection rates of hepatitis A in wastewater. However, the monitoring of HAV RNA in wastewater and various open water bodies in Tuva, conducted in 2021–2023, confirmed the continued presence of the virus in the environment [27]. Moreover, phylogenetic analysis confirmed that the sequences of the virus isolated from water samples in Tuva in 2021 and 2022 belonged to a group of genetic variants that were isolated from patients in this region in 2008 (pre-vaccination period), suggesting the stable circulation of this particular variant of the virus. However, in 2023, another variant of HAV, closely related to sequences isolated in various regions of Russia from 2019 to 2023, including the Irkutsk region bordering Tuva, was detected in wastewater and environmental samples [27]. This finding confirms the presence of a new strain in Tuva.

Based on the presented data, it cannot be definitively stated that the single vaccination strategy led to a decrease in the frequency of detecting HAV RNA in wastewater samples in Tuva, as such monitoring had not been conducted in the region before. However, in 2002–2004, wastewater samples in Tuva were tested for the HAV antigen, resulting in a positive rate of 12.5–63.3% [30]. Considering that the average proportion of samples positive for HAV RNA in 2021–2023 was below 2.5%, it can be assumed that vaccination has indeed led to a significant reduction in virus shedding in the region.

During the monitoring of HAV RNA in environmental objects in Argentina, conducted in 2009–2010 and 2017–2022, a constant presence of HAV RNA in wastewater was also detected [31, 32]. However, unlike Tuva, the detection of HAV RNA in wastewater samples in Argentina correlates with cases of acute hepatitis A [33].

A possible explanation for the continued shedding of the virus in Tuva and its detection in environmental samples and wastewater, despite the absence of reported cases of acute hepatitis A, may be related to the age of the vaccinated children. It is evident that the detection of the virus in the absence of registered cases of the disease is most likely indicative of the presence of an asymptomatic infection, as HAV infection in children under 5 years old most often occurs asymptomatically [23]. In most countries that have implemented universal vaccination against hepatitis A, immunization of children is carried out at the age of 12 months and older [2]. However, in Tuva, a single-dose vaccination for children aged 3 years and older has been implemented, primarily in order to be able to use a domestic vaccine against hepatitis A, licensed for children aged 3 years and older. Thus, children under 3 years of age are not immunized and remain susceptible to HAV.

The most likely hypothesis explaining the persistence of HAV circulation in the absence of symptomatic cases is the transmission of the virus among children under 3 years of age. Confirmation of the hidden circulation of HAV in Tuva is also evidenced by the increasing proportion over time of individuals with high (above 6000 mIU/ml) concentrations of anti-HAV antibodies [27], which indicates the boosting of the post-vaccination humoral response upon encountering the virus.

The accumulated experience to date of using single immunization in the implementation of hepatitis A vaccination programs has demonstrated the high epidemiological effectiveness of this approach and the preservation of immunological effectiveness for at least a decade. At the same time, those who received a single dose of inactivated vaccine against hepatitis A in childhood may require revaccination in adulthood, considering the lack of actual data or mathematical modeling results on the preservation of humoral immunity for decades after such a vaccination scheme. Moreover, although it is believed that the standard two-dose immunization provides immunity to HAV that lasts for decades or even a lifetime, rare cases of infection in at-risk adults who were vaccinated in childhood have been described. In such patients, the presence of low levels of post-vaccination antibodies did not protect against infection with a high dose of the virus, but it did provoke the selection of viral genetic variants carrying changes in immunogenic epitopes, indicating immune evasion [35]. These cases, rare and atypical for hepatitis A, nevertheless suggest that the combination of waning post-vaccination immunity and a high dose of the virus may lead to an outbreak of hepatitis A infection and the emergence of mutant virus variants. In this regard, the consequences of the accumulation of individuals with low levels of anti-HAV antibodies in the population as a result of single-dose immunization during childhood are not entirely clear. Nevertheless, these potential risks do not outweigh the obvious advantages of mass vaccination programs against hepatitis A with single-dose immunization: the ability to quickly control the incidence of hepatitis A, as well as the economic and logistical efficiency of such an approach.

Conclusion

Mass vaccination programs against hepatitis A, based on the standard two-dose immunization schedule, have long proven their effectiveness, the ability to induce long-lasting immunity lasting at least 15 years, and have led to the control of hepatitis A in many countries. Significantly less data is available on the immunological and epidemiological effectiveness of universal vaccination programs against hepatitis A using a single dose of inactivated vaccine. Data from recent years, obtained in various regions of the world, including Russia, confirm the effectiveness of such a vaccination strategy against hepatitis A both in terms of the immunological response and in terms of its impact on the levels of reported morbidity. However, to assess the duration of protection and the need for revaccination at a later stage in life, further studies on the long-term effectiveness of vaccination strategies with single-dose immunization, as well as continuous surveillance of hepatitis A, are necessary.

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ЮБИЛЕИ

К 70-летию академика РАН Александра Николаевича Куличенко

23 сентября 2025 года отметил 70-летие Александр Николаевич Куличенко, доктор медицинских наук, профессор, академик Российской академии наук, директор Ставропольского научно-исследовательского противочумного института Роспотребнадзора.

Александр Николаевич Куличенко — известный российский учёный, микробиолог, академик РАН, более 45 лет своей жизни посвятил проблемам борьбы с особо опасными инфекциями. За годы научной и научно-организационной деятельности А.Н. Куличенко внёс неоценимый вклад в развитие молекулярной диагностики особо опасных инфекций в нашей стране.

Александр Николаевич в 1978 г. с отличием окончил Саратовский медицинский институт. В течение 29 лет работал в Российском научно-исследовательском противочумном институте «Микроб» (Саратов), где прошёл путь от аспиранта до заместителя директора по научной работе. Здесь он сформировался как учёный и в составе авторского коллектива был удостоен Государственной премии Российской Федерации в области науки и техники (2003 г.) за заслуги в области разработки новых методов диагностики и профилактики сибирской язвы.

Научная деятельность академика А.Н. Куличенко охватывает широкий круг задач современной молекулярной биологии и эпидемиологии. На основании анализа структуры геномов возбудителей чумы, сибирской язвы, бруцеллёза им были разработаны и внедрены в практику первые ПЦР-тест-системы для детекции этих особо опасных патогенов, научно обоснованы принципы пробоподготовки при ПЦР-анализе, нашедшие отражение в действующей методической базе по генной диагностике инфекционных болезней. В работах Александра Николаевича развито современное научное направление — алгоритмы применения методов молекулярного анализа в эпидемиологии природно-очаговых и других зоонозных инфекций, разработаны приёмы последовательного генотипирования при эпидемиологическом надзоре, предложен комплексный подход к мониторингу зоонозных инфекций на основе анализа генома патогенов и технологий с использованием геоинформационных систем в реальном времени. А.Н. Куличенко впервые проведены исследования по генетическому профилированию современных вариантов возбудителей природно-очаговых и особо опасных инфекций в регионах Южного и Северо-Кавказского федеральных округов, вскрыты особенности процессов микроэволюции возбудителей чумы, Крымской геморрагической лихорадки, сибирской язвы, территориальной приуроченности штаммов. Под его руководством получены приоритетные данные по филогенетике возбудителей бруцеллёза и сибирской язвы. Предложены и внедрены в практику новые современные подходы к прогнозному моделированию природно-очаговых инфекционных болезней с применением информационных технологий, дистанционного зондирования Земли из космоса и с помощью беспилотных летательных аппаратов.

В июле 2007 г. Александр Николаевич Куличенко принял руководство Ставропольским научно-исследовательским противочумным институтом Роспотребнадзора, и сегодня его имя неразрывно связано с новейшей историей института. За это время Ставропольский противочумный институт стал развивающимся научно-практическим комплексом, решающим задачи в области эпидемиологического надзора, диагностики и профилактики особо опасных инфекций. Благодаря организаторскому таланту и большому труду А.Н. Куличенко, был введён в эксплуатацию (2013 г.) новый основной лабораторный корпус института, отвечающий современным достижениям науки и оснащённый передовым оборудованием. В 2023 г. продлены на очередной срок полномочия сотрудничающего с Всемирной организацией здравоохранения (ВОЗ) Научно-исследовательского центра по чуме, функционирующего на базе Ставропольского научно-исследовательского противочумного института. С именем А.Н. Куличенко связано развитие инновационных направлений в институте: постгеномных технологий, метагеномного анализа, применение молекулярного анализа в эпидемиологии.

Важной вехой в жизни института в этот период было участие в военной операции в Южной Осетии в 2008 г. Александр Николаевич лично возглавил специализированную противоэпидемическую бригаду института, которая приступила к работе уже на третий день военных действий. Итогами работы бригады были обеспечение эпидемического благополучия населения и воинских контингентов, восстановление централизованного водоснабжения г. Цхинвала, восстановление деятельности санитарно-эпидемиологической службы Республики Южная Осетия.

Другие важные достижения института под руководством Александра Николаевича — обеспечение эпидемиологического благополучия во время зимней Олимпиады в Сочи 2014 г. и текущей и референсной лабораторной диагностики во время пандемии COVID-19, за которую 44 сотрудника института были награждены орденами Пирогова и медалями Луки Крымского.

Сегодня институт стал мощным многопрофильным центром. Активно функционируют 3 референс-центра и сотрудничающий с ВОЗ центр по чуме, Проблемная комиссия Учёного совета Роспотребнадзора «Профилактика болезней, общих для человека и животных».

А.Н. Куличенко ведёт активную общественную и научную деятельность. Он является членом Президиума Всероссийского научно-практического общества эпидемиологов, микробиологов и паразитологов, заместителем председателя Координационного научного совета по санитарно-эпидемиологической охране территории Российской Федерации, членом редакционного совета журналов «Проблемы особо опасных инфекций» и «Бактериология», членом редакционной коллегии изданий: «Журнал микробиологии, эпидемиологии и иммунобиологии», «Медицинский вестник Северного Кавказа».

Большое влияние на становление Александра Николаевича как учёного оказало творческое руководство академика РАН Г.Г. Онищенко, существенное значение в формировании его научного потенциала имели постоянные деловые контакты с учёными-академиками А.Л. Гинцбургом, В.В. Кутыревым, И.А. Дятловым и др.

А.Н. Куличенко — автор (соавтор) 35 монографий, более 700 опубликованных научных работ, 62 патентов Российской Федерации на изобретение. Под руководством А.Н. Куличенко сформирована научная школа по молекулярному анализу возбудителей особо опасных инфекций. При его научном руководстве и консультировании защищены 3 диссертации на соискание учёной степени доктора наук и 22 — на соискание учёной степени кандидата наук.

А.Н. Куличенко — лауреат Государственной премии Российской Федерации в области науки и техники (2003 г.), имеет государственные награды: «Орден Пирогова» (2021 г.), медали ордена «За заслуги перед Отечеством» I и II степени (2018 и 2009 гг.), награждён грамотами Президента Российской Федерации, удостоен нагрудного знака «Почётный работник Роспотребнадзора», а также «Орденом Дружбы» Республики Южная Осетия (2020).

Научная деятельность академика РАН, доктора медицинских наук, профессора Александра Николаевича Куличенко вносит значительный вклад в развитие отечественной науки.

Многотысячный коллектив членов Общероссийской общественной организации «Всероссийское научно-практическое общество эпидемиологов, микробиологов и паразитологов», редакция «Журнала микробиологии, эпидемиологии и иммунобиологии» поздравляют Александра Николаевича Куличенко с 70-летним юбилеем. Примите искренние пожелания успехов в Вашем важном деле! Пусть этот знаменательный рубеж станет предпосылкой для новой, яркой страницы Вашей жизни. От всей души желаем Вам здоровья, творческих сил и вдохновения для новых побед и великих свершений!

ХРОНИКА

РЕЗОЛЮЦИЯ
Конгресса с международным участием «Эпидемиология — 2025» (Москва, 15–16 октября 2025 г.)

15–16 октября 2025 г. в соответствии с Приказом Руководителя Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека от 21.05.2025 № 388 состоялся Конгресс с международным участием «Эпидемиология — 2025». Конгресс был организован Центральным научно-исследовательским институтом эпидемиологии Роспотребнадзора при поддержке Российской академии наук, Всероссийского научно-практического общества эпидемиологов, микробиологов и паразитологов, Национальной ассоциации специалистов по инфекционным болезням имени академика В.И. Покровского, Федерации лабораторной медицины и Ассоциации медицинских микробиологов.

В заседаниях Конгресса приняли участие более **800** человек очно (в том числе более **20** членов РАН) и около **3000** онлайн из **78** субъектов Российской Федерации и **17** зарубежных стран (Абхазия, Азербайджан, Армения, Белоруссия, Ботсвана, Бурунди, Гвинейская Республика, Вьетнам, Казахстан, Киргизия, Нигерия, Никарагуа, Таджикистан, Уганда, Узбекистан, Швейцария, Южная Осетия). В работе Конгресса приняли участие специалисты Роспотребнадзора, Минздрава России, Минобрнауки России, ФМБА России, Росздравнадзора, Минобороны России, других ведомств и медицинских организаций, сотрудники научно-исследовательских институтов, студенты и преподаватели высших учебных заведений, члены профессиональных научных сообществ.

Реализация приоритетных задач в сфере обеспечения санитарно-эпидемиологического благополучия населения и биобезопасности Российской Федерации регламентируется рядом государственных документов: Федеральным законом от 30.12.2020 № 492-ФЗ «О биологической безопасности в Российской Федерации», Указом Президента Российской Федерации от 07.05.2024 № 309 «О национальных целях развития Российской Федерации на период до 2030 года и на перспективу до 2036 года», в соответствии с которыми целью государственной политики является поддержание допустимого уровня риска негативного воздействия опасных факторов на население и окружающую среду.

Кроме того, в Российской Федерации реализуются и направлены на обеспечение биологической безопасности следующие федеральные проекты и государственные программы:

- Федеральный проект «Санитарный щит страны — безопасность для здоровья (предупреждение, выявление, реагирование)» на 2022–2030 гг.;
- Федеральная научно-техническая программа развития генетических технологий на 2019–2030 гг.;
- Государственная программа Российской Федерации «Обеспечение химической и биологической безопасности Российской Федерации» на 2021–2027 гг. и др.

Научная повестка Конгресса открылась масштабным пленарным заседанием, которое началось с приветствия Руководителя Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека А.Ю. Поповой.

В приветственном слове А.Ю. Поповой было отмечено, что эпидемиология инфекционных болезней в России является одной из быстро развивающихся областей медицинской науки и практики. Глобализация современного общества, изменение климата, миграция населения способствуют формированию благоприятных условий для распространения многих инфекционных болезней, что требует совершенствования системы управления эпидемическим процессом на основе разработки и внедрения новых технологий эпидемиологического надзора и контроля, и предложенная организаторами научная программа Конгресса всесторонне охватывает проблему эпидемиологии с позиции приоритетных междисциплинарных исследований.

Приветствия в адрес участников Конгресса поступили также из Совета Федерации Федерального собрания Российской Федерации, Государственной Думы Российской Федерации, Российской академии наук, Отделения медицинских наук Российской академии наук.

Работа Конгресса началась научным докладом директора ФБУН Центральный НИИ эпидемиологии Роспотребнадзора академика РАН В.Г. Акимкина. В докладе была подчёркнута историческая значимость вклада отечественных учёных в развитие теоретических основ эпидемиологии как общемедицинской науки. Автором были отмечены современные эпидемиологические угрозы для мирового здравоохранения и тренды в области эпидемиологии, актуальные направления эпидемиологического надзора за возбудителями инфекционных болезней, включая геномный эпидемиологический надзор. В.Г. Акимкиным были рассмотрены пути совершенствования эпидемиологического надзора с применением цифровых технологий, современных методов диагностики и профилактики инфекционных болезней на основе инновационных биоинженерных и генетических технологий. Отмечено, что геномный эпидемиологический надзор за возбудителями болезней, обладающими пандемическим и высоким эпидемическим потенциалом, направлен на мобилизацию усилий по быстрому выявлению и изучению свойств патогенов с целью своевременной оценки эпидемиологической обстановки и организации эффективных профилактических и противоэпидемических мероприятий.

Особый интерес вызвали доклады известных отечественных учёных: профессора Р.Г. Василова, академиков РАН О.А. Свитич, В.В. Кутырева, И.А. Дятлова, А.А. Тотоляна, В.М. Говоруна, Н.И. Брико и профессора И.В. Ямпольского. В рамках Конгресса проведены пленарное и **23** секционных заседаний, заслушано **165** научных докладов. Проведено совместное заседание научных обществ (Всероссийского научно-практического общества эпидемиологов, микробиологов и паразитологов, Национальной ассоциации специалистов по инфекционным болезням имени академика В.И. Покровского, Федерации лабораторной медицины, Ассоциации медицинских микробиологов), комиссии Научного совета по микробиологии, эпидемиологии и инфекционным болезням Отделения медицинских наук РАН, комиссии Научного совета по генетике и селекции Отделения биологических наук РАН, Проблемной комиссии Учёного Совета Роспотребнадзора по Федеральному проекту «Санитарный щит страны — безопасность для здоровья (предупреждение, выявление, реагирование)», Проблемной комиссии Учёного Совета Роспотребнадзора по профилактике инфекций, связанных с оказанием медицинской помощи, посвящённое актуальным аспектам фундаментальных и прикладных исследований в области эпидемиологии и совершенствования эпидемиологического надзора с целью обеспечения санитарно-эпидемиологического благополучия населения Российской Федерации.

Ключевой темой Конгресса являлись вопросы совершенствования эпидемиологического надзора, профилактики заноса и распространения на территорию Российской Федерации возбудителей инфекционных болезней и реализации экстерриториального мониторинга за возбудителями инфекционных болезней, совершенствования системы управления эпидемическим процессом и обеспечения биологической безопасности Российской Федерации. В прозвучавших докладах была подчёркнута важность контроля рисков, связанных с появлением новых инфекций, вызываемых неизвестными патогенами, преодолением микроорганизмами и вирусами межвидовых барьеров в сочетании с возникающими под воздействием внешней среды изменениями генотипа и фенотипа макроорганизмов человека и животных, распространением антимикробной резистентности и др.

Отдельные секционные заседания были посвящены вопросам эпидемиологии, диагностики и профилактики различных нозологических форм инфекций (ВИЧ-инфекция, природно-очаговые инфекции, вирусные гепатиты, туберкулёз, острые респираторные вирусные инфекции, инфекции желудочно-кишечного тракта и др.), антибиотикорезистентности, современным достижениям в области биотехнологических разработок и цифровизации для решения значимых эпидемиологических задач.

Участники Конгресса отметили высокий научный уровень представленных сообщений и глубокий профессиональный интерес участников к различным направлениям современной эпидемиологии. В рамках заседаний секций участниками была развёрнута активная творческая дискуссия по рассматриваемым научным и практическим вопросам.

Таким образом, участниками Конгресса была отмечена важность эпидемиологии как общемедицинской науки, актуальность современных направлений совершенствования эпидемиологического надзора, включая геномный эпидемиологический надзор за возбудителями инфекционных болезней, цифровизацию, активное использование достижений в области биоинженерии и инновационных генетических технологий с целью решения актуальных вопросов обеспечения санитарно-эпидемиологического благополучия и биобезопасности Российской Федерации.

Учитывая исключительную важность развития эпидемиологической науки и практики в современных условиях, принимая во внимание результаты научных дискуссий и представленных докладов на Конгрессе, участники Конгресса постановили:

Признать деятельность Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека и других министерств и ведомств по обеспечению санитарно-эпидемиологического благополучия населения и биологической безопасности в Российской Федерации важнейшим направлением государственной политики.

Отметить исключительную важность консолидации усилий научного сообщества в области обеспечения биологической безопасности государства и предотвращения угроз пандемического распространения возбудителей инфекционных болезней.

Развивать междисциплинарное взаимодействие учёных в области эпидемиологии, биотехнологии, генетики и инфекционных болезней для обеспечения национальной безопасности и технологической независимости Российской Федерации.

Признать приоритетным научным направлением развитие геномного эпидемиологического надзора как современного уникального инструмента управления эпидемическим процессом.

Рекомендовать активное применение цифровых технологий и результатов анализа больших данных в практику эпидемиологического надзора.

Развивать подходы по совершенствованию лабораторной диагностики возбудителей инфекционных болезней с разработкой тест-систем для индикации и идентификации возбудителей на основе молекулярно-биологических методов исследования.

Расширить инновационные технологии редактирования генома с целью разработки новых диагностических, лечебных и вакцинных препаратов.

Развивать направления профессиональной образовательной деятельности по актуальным направлениям научных исследований в области эпидемиологии, в том числе по соблюдению требований биологической безопасности, предупреждению формирования и распространения устойчивости микроорганизмов к противомикробным препаратам, продолжить практики проведения интерактивных семинаров и дистанционного обучения.

Признать работу Конгресса успешной и результативной, способствующей развитию эпидемиологической науки и укреплению системы биологической безопасности государства.

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