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ORIGINAL RESEARCHES

Original Study Article
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A study of the safety and immunogenicity of a new vaccine for the prevention of COVID-19 based on virus-like particles in phase I clinical trials

Tatiana V. Grebennikova✉, Olga N. Zaykova, Alexey A. Plotnikov, Lyudmila V. Kostina, Yana Yu. Chernoryzh, Olesia V. Eliseeva, Oleg E. Latyshev, Viktor F. Larichev, Irina T. Fedyakina, Milana A. Losich, Ilya M. Kirillov, Ilya E. Filatov, Marina V. Balandina, Valery V. Tsibezov, Kirill I. Yurlov, Ekaterina I. Lesnova, Valeria M. Kondratieva, Alina A. Kozlova, Marina S. Baranets, Aleksandr L. Gintsburg

National Research Center for Epidemiology and Microbiology named after N.F. Gamaleya, Moscow, Russia

Abstract

Introduction. One of the more promising developments in preventing the spread of infections, including COVID-19, is the production of vaccines based on virus-like particles (VLP). Currently, in the National Research Center for Epidemiology and Microbiology named after N.F. Gamaleya of the Ministry of Health of the Russia has developed a VLP-based vaccine against COVID-19.

The aim of this study is to evaluate the tolerability, safety and immunogenicity of a new vaccine for the prevention of COVID–19 based on VLP compared with placebo for 21 days after two intramuscular injections in phase I clinical trials.

Materials and methods. A double-blind, placebo-controlled study of the tolerability, safety and immunogenicity of a vaccine for the prevention of COVID-19 based on VLP was conducted with a dose of the drug containing 40 and 80 micrograms of antigen, the placebo being 0.9% NaCl. The presence or absence of adverse events (AEs) after vaccination was noted in 180 volunteers aged 18 to 55 years; clinical and biochemical blood parameters, the intensity of humoral and cellular immunity before and after vaccination were assessed using enzyme immunoassay, neutralization reactions, lymphocyte blast transformation reactions and flow cytometry.

Results. An analysis of the tolerability and safety of the new COVID-19 VLP-vaccine showed that most adverse events were registered within the first 10 days after vaccination, mainly after the first vaccination. In the period from 11 to 21 days after vaccination, AEs were observed in isolated cases. No deaths, serious or other AEs have been reported. The administration of the studied vaccine to the volunteers had no negative effect on the basic vital signs. A comparative analysis of immunogenicity indicators in volunteers showed that the administration of a vaccine with both an antigen content of 40 μg and an antigen content of 80 μg leads to a pronounced and significant increase in the level of specific immunoglobulins, virus neutralizing antibodies and activation of a cell-mediated immune response. As part of the phase I clinical trials, a dose of 80 μg was selected as optimal.

Conclusion. It has been shown that a new vaccine for the prevention of COVID-19 based on VLP with an antigen content of 40 and 80 μg when administered intramuscularly to volunteers does not cause serious adverse events and induces a tense humoral and cellular immune response.

Keywords: *COVID-19, SARS-CoV-2, virus-like particles, VLP*

Ethics approval. The study was conducted with the voluntary informed consent of the patients. The study protocol was approved at a meeting of the Ethics Council of the Department of Regulation of the Circulation of Medicines and Medical Devices of the Ministry of Health of the Russian Federation (No. 310, dated 05.31.2022).

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Оригинальное исследование
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Исследование безопасности и иммуногенности вакцины для профилактики COVID-19 на основе вирусоподобных частиц в рамках I фазы клинических испытаний

Гребенникова Т.В.✉, Зайкова О.Н., Плотников А.А., Костина Л.В., Чернорыж Я.Ю., Елисеева О.В., Латышев О.Е., Ларичев В.Ф., Федякина И.Т., Лосич М.А., Кириллов И.М., Филатов И.Е., Баландина М.В., Цибезов В.В., Юрлов К.И., Леснова Е.И., Кондратьева В.М., Козлова А.А., Баранец М.С., Гинцбург А.Л.

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Аннотация

Введение. Одним из перспективных направлений в предупреждении распространения инфекций, в том числе COVID-19, является получение вакцин на основе вирусоподобных частиц (virus like particles, VLP). В НИЦЭМ им. Н.Ф. Гамалеи разработана вакцина на основе VLP против COVID-19.

Цель работы — оценить переносимость, безопасность и иммуногенность новой вакцины для профилактики COVID-19 на основе VLP в сравнении с плацебо на протяжении 21 сут после двукратного внутримышечного введения в рамках I фазы клинических испытаний.

Материалы и методы. Двойное слепое плацебо-контролируемое исследование переносимости, безопасности, и иммуногенности вакцины для профилактики COVID-19 на основе VLP проводили с дозой введения препарата, содержащего 40 и 80 мкг антигена, плацебо — 0,9% NaCl. У 180 добровольцев в возрасте 18–55 лет отмечали наличие или отсутствие нежелательных явлений (НЯ) после вакцинации, оценивали показатели крови, напряжённость гуморального и клеточного иммунитета до и после вакцинации с помощью иммуноферментного анализа, реакции нейтрализации, реакции бласттрансформации лимфоцитов и проточной цитометрии.

Результаты. Анализ переносимости и безопасности новой вакцины против COVID-19 на основе VLP показал, что большинство НЯ регистрировались в течение первых 10 сут после вакцинации, преимущественно после 1-й вакцинации. В период с 11-х по 21-е сутки после вакцинации НЯ отмечались в единичных случаях. Летальных исходов, серьёзных и иных НЯ не зарегистрировано. Введение исследуемой вакцины добровольцам не оказало негативного влияния на основные жизненные показатели. Сравнительная характеристика показателей иммуногенности у добровольцев показала, что введение вакцины с содержанием антигена как 40, так и 80 мкг приводит к выраженному и достоверному росту уровня специфических иммуноглобулинов, вируснейтрализующих антител и активации клеточно-опосредованного иммунного ответа. В рамках I этапа клинических исследований доза 80 мкг была выбрана как оптимальная.

Заключение. Новая вакцина для профилактики COVID-19 на основе VLP с содержанием антигена 40 и 80 мкг при введении добровольцам внутримышечно не вызывает серьёзных НЯ и индуцирует напряжённый гуморальный и клеточный иммунный ответ.

Ключевые слова: *COVID-19, SARS-CoV-2, вирусоподобные частицы, вирусоподобные частицы*

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Introduction

The pandemic of a new coronavirus infection has posed serious challenges to public health services worldwide in the prevention, treatment and diagnosis of this disease. COVID-19, a severe acute respiratory infection caused by the SARS-CoV-2 virus (*Coronaviridae*, *Orthocoronavirinae*, *Betacoronavirus*, *Sarbecovirus*), is characterized by a high mortality rate, which, according to different studies, ranges from 0.5 to 15% [1–3].

The first cases of disease caused by SARS-CoV-2 virus were registered in December 2019 in China. The virus spread quite rapidly to all continents, and according to the World Health Organization (WHO), as of December 2021, there were more than 260 million confirmed cases of COVID-19 worldwide, including 5.2 million deaths[1].

The most severe manifestations of coronavirus infection are interstitial pneumonia with impaired respiratory function and multi-organ failure, which were often fatal [4–6]. The duration of post-infection immunity is not fully understood [7].

According to Rospotrebnadzor, as of May 12, 2024, about 785 million cases have been detected worldwide, the most unfavorable region being the Western Pacific. In Russia from 02.03.2020 to 05.05.2024 about 24 million cases were registered in 85 subjects[2]. Despite WHO statements that COVID-19 has moved into the category of seasonal infections and periodically causes outbreaks of the disease along with influenza and acute respiratory viral diseases, SARS-CoV-2 virus continues to infect people and claim their lives[3].

The variability of SARS-CoV-2, namely mutations in the receptor binding domain (RBD) of the S-protein, has led to the emergence of a variety of virus variants worldwide, of which Alpha (lineage B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) are the most epidemically significant and of current concern to WHO [8]. The emergence of new variants of the virus, including those with reduced sensitivity to virus-neutralizing antibodies and vaccination with already available vaccines, requires regular molecular genetic monitoring of SARS-CoV-2 and the development of new highly effective vaccines that promote the formation of intense and long-lasting immunity against topical strains of the causative agent of coronavirus infection [8].

An effective vaccine against COVID-19 should be safe, non-reactogenic, and induce the formation of virus-neutralizing antibodies in titers sufficient to prevent the development of the infectious process. Furthermore, the vaccine should promote an effective immune response with the least amount of antigen used. This reduces the cost of the vaccine and makes it affordable [9].

A vaccine based on virus like particles (VLP) for COVID-19 prophylaxis has been developed in the Gamaleya Research Center for Ecological Medicine. VLPs are formed from 4 recombinant structural proteins (S, M, E, N) and are similar in structure to SARS-CoV-2 virion, but without viral RNA. The surface S-protein (S-spike) of SARS-CoV-2 is responsible for binding to specific receptors on the surface of susceptible cells. Particles containing S-protein with consensus mutations of clades 19A, Delta and Omicron are used in the vaccine formulation. Thus, it is expected that antibodies to strains of these clades will be synthesized in the body after immunization.

The structural spike and membrane proteins undergo significant mutational changes, whereas the envelope and nucleocapsid proteins are highly conserved, indicating differential selection pressure to which SARS-CoV-2 has been subjected during evolution. At the same time, the contribution of viral M, E, and N proteins to the formation of B- and T-cell immunity is equally important, which is confirmed by the development of vaccines based on these proteins [10, 11]. It was shown that the vaccine under development stimulates the T-cell immune response in golden hamsters after the 1st immunization, which also provides protection against various SARS-CoV-2 strains [12].

Data on preclinical trials of the drug were submitted to the Ministry of Health of the Russian Federation.

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[1] WHO Director-General's opening remarks at the media briefing on COVID-19. 11 March 2020. URL: https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020
WHO. COVID-19 Epidemiological Update. 06.11.2024. URL: https://www.who.int/docs/default-source/coronaviruse/situation-reports/covid-19_epi_update_173.pdf?sfvrsn=457952e6_4&download=true

[2] The epidemiological situation and the spread of COVID-19 in the world as of 8 Moscow time on 05/12/2024 /FCUN ROSNIPCHI "Microbe". Federal Service for Consumer Rights Protection and Human Welfare. (In Russ.) URL: https://www.rospotrebnadzor.ru/12.05.2024%20г.%20Информация%20о%20случаях%20заболевания.docx

[3] COVID-19 Cases, World / WHO Coronavirus (COVID-19) dashboard. URL: https://data.who.int/dashboards/covid19/cases

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Permission to conduct clinical trials was obtained from 16.02.2022 No. 115.

The aim of this study was to evaluate the tolerability, safety and immunogenicity of a new VLP-based vaccine for COVID-19 prophylaxis compared to placebo for 21 days after double intramuscular administration within the framework of phase I clinical trials.

Study objectives:

1. To consistently evaluate the tolerability and safety of vaccine containing 40 and 80 μg antigen doses administered intramuscularly on the 10th day after single vaccination.

2. To evaluate the tolerability and safety of vaccine containing 40 and 80 μg antigen doses administered intramuscularly on the 21st day after double vaccination.

3. To evaluate the immunogenicity of the vaccine containing 40 and 80 μg antigen doses administered intramuscularly on the 21st day after double vaccination.

4. To determine the optimal vaccine dosage based on the immunogenicity and safety parameters obtained for the phase II clinical trial.

Materials and methods

Double-blind randomized placebo-controlled multicenter prospective study of Phase I clinical trials with dose-ranging to assess tolerability, safety and immunogenicity of the vaccine for COVID-19 prophylaxis based on VLP (containing particles similar to SARS-CoV-2) when administered intramuscularly to volunteers aged 18–55 years was conducted from February to December 2022 on the basis of two research centers: Elektrostal Central City Hospital and N.P. Bekhtereva Institute of the Human Brain of RAS in accordance with the principles of the Declaration of Helsinki (2013), the ICH guidelines on overdose, ICH Guidelines for Good Clinical Practice (version E6, approved by SRMR/135/95), Federal Law No. 61-FL "On Circulation of Medicines" dated 12.04.2010; Order of the Ministry of Health of the Russian Federation No. 200n "On Approval of the Rules of Good Clinical Practice" dated 01.04.2016; National Standard of the Russian Federation GOST R 52379-2005 "Good Clinical Practice" approved by Order of the Federal Agency for Technical Regulation and Metrology No. 497-st. from 04.06.2014, the Rules of Good Clinical Practice of the Eurasian Economic Union" approved by the Decision of the Council of the Eurasian Economic Commission from 03.11.2016 № 79 and other applicable requirements of national legislation.

Prior to inclusion in the study, volunteers were familiarized with information about the study and signed an informed consent form. Researchers recruited to participate in the clinical trial provided signed and dated resumes describing their research activities and certificates confirming their qualifications before the start of the study.

A total of 180 male and female volunteers aged 18–55 years were included in the study. All volunteers were healthy, met the inclusion criteria (**Appendix 1** on the journal's website: https://www.microbiol.crie.ru) and were divided into 3 groups:

- Group 1 — 60 volunteers who were immunized with vaccine containing 40 μg antigen twice at an interval of 21 days intramuscularly;
- Group 2 — 60 volunteers who were vaccinated with 80 μg antigen vaccine twice at an interval of 21 days intramuscularly;
- Group 3 — 60 volunteers who received placebo vaccine twice at an interval of 21 days intramuscularly.

The vaccine was purified recombinant SARS-CoV-2 virus-like particles synthesized in a baculovirus expression system. The S surface protein within the virus-like particles was represented by variants 19A, Alpha, Delta, and Omicron. The vaccine composition included a squalene-based adjuvant. The volume ratio of adjuvant to antigen was 1 : 1. Vaccine with 40 and 80 μg antigen content was investigated. A 0.9% NaCl solution was used as placebo.

Volunteers were examined at visits:

- visit 1 (hospitalization, randomization, the 1st vaccination);
- visits 2, 3 (2nd–3rd days after the 1st vaccination);
- visit 4 (10th day after the 1st vaccination);
- visit 5 (21st day after the 1st vaccination, hospitalization, 2nd vaccination);
- visits 6, 7 (2nd–3rd days after the 2nd vaccination);
- visit 8 (10th day after the 2nd vaccination);
- visit 9 (21st day after the 2nd vaccination).

We analyzed data from the electronic self-monitoring diary, performed physical examination, assessed vital signs, collected data on concomitant therapy, identified and recorded adverse events (AEs) and serious AEs, and evaluated inclusion/non-inclusion criteria. Blood was drawn to assess cellular and humoral immunity. Biochemical, clinical tests, determination of total IgE, coagulogram, and urinalysis were performed. During visits 4, 5, 8, 9, nasopharyngeal swabs were taken to determine the absence of SARS-CoV-2 virus RNA by polymerase chain reaction (PCR) method.

Lymphocyte Blast Transformation Reaction (LBTR). Blood was collected on the 10th day after the 1st and 2nd vaccinations (visits 4 and 8). Mononuclear cell fractions from peripheral blood were isolated by centrifugation on a one-step density gradient of Ficoll-Pak (PanEco), the isolated mononuclear cells were washed twice in pure RPMI-1640 medium and seeded in 96-well microculture plates at a concentration of 10^5 cells/well and stimulants were added in 100 μl to final concentrations. We used the medium separately (spontaneous proliferation) as a negative control; concanavalin A mitogen (5 μg/mL, PanEco) as a non-specific positive control; SARS-CoV-2 virus: PMVL-12, depo-

sition number EPI_ISL_572398 in the Gisaid database as a specific stimulant; Congo-Crimean hemorrhagic fever antigen as a nonspecific positive control; and Seppic adjuvant as a non-specific stimulant. Cells were cultured in RPMI-1640 medium containing 20% fetal calf serum, 2 mM glutamine, 4.5 g/L glucose, 50 μg/mL gentamicin, and 0.2 units/mL insulin at 37°C in a 5% CO_2 atmosphere. All of the mentioned processes were performed under sterile conditions.

Splenocyte proliferation was assessed by LBTR after 4 days using an inverted microscope (× 400). The results were expressed as proliferation stimulation index (PSI), calculated as the ratio of the mean number of lymphoblasts observed in the presence and absence of specific stimulants. A result was considered positive if the PSI > 2.

The obtained data were processed using the Prizm Graphpad v. 8.4.3 program (GraphPad Software). Statistical analysis was performed using the Statistica v. 12.6 program (StatSoft Inc.). Reliability of differences was evaluated by Student's t-criterion. A value of $p < 0.05$ was considered statistically significant.

Cell staining for flow cytometry. 1 million lymphocytes in a volume of 50 μl were transferred into centrifuge tubes and 5 μl each of anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies (Sorbent) were added and incubated for 45 min at 4°C. Washed twice in Hanks' solution (5 min at 200*g*). The supernatant was removed and cells were suspended in 200 μl of Hanks' solution and analyzed on a BD FACS Accuri C6+ flow cytometer. The obtained data were processed using the Cytoflex and Prizm Graphpad 8.0 programs. The results were analyzed using the FlowJo program (Three Star).

Humoral immunity was assessed by enzyme-linked immunosorbent assay (ELISA) and neutralization reaction (NR). Blood was collected on the 10th day after the 1st and 2nd vaccination (visits 5 and 9). ELISA was performed using a reagent kit for immunoenzymatic detection of IgG to RBD of the S surface glycoprotein S of SARS-CoV-2 coronavirus (SARS-CoV-2-RBD-ELA-Gamalei, RU No. RZN 2020/10393).

The levels of neutralizing antibodies were determined by titrating sera from 1 : 10 to 1 : 1280 against 100 $TCID_{50}$ of three SARS-CoV-2 strains from the Molecular Diagnostics Laboratory collection: Wuhan, Delta (line B.1.617.2) and Omicron (variant XBB 1.5). Neutralization reactions were performed by micromethod in 96-well plates on Vero E6 green monkey kidney cell culture. Serum dilutions were incubated with viruses for 1 h at 37°C in an atmosphere of 5% CO_2 and transferred to a plate with a cell monolayer. After 72 h, the reaction was counted by the presence of cytopathic action of the virus. The serum titer (the last neutralizing dilution) was considered to be the dilution at which 100% cell protection (no cytopathic effect) was ensured.

Cytokine concentrations in sera were measured by ELISA using commercial test systems (Vector-Best): Interleukin-2-ELISA-BEST "Reagent kit for immunoenzymatic determination of interleukin-2 concentration in serum"; Gamma-Interferon-ELISA-BEST "Reagent kit for immunoenzymatic determination of gamma-interferon concentration in serum"; Alpha-TNF-ELISA-BEST "Reagent kit for immunoenzymatic determination of tumor necrosis factor-alpha concentration in serum".

Statistical processing of data was performed using Microsoft Office Excel 2007-2016 and online statistical calculators (https://math.semestr.ru, https://medstatistic.ru). Mean, standard deviations, quartiles, minimum and maximum values, as well as frequencies, depending on the nature of the data, were used as descriptive characteristics of demographic and other baseline data parameters, as well as safety parameters and immunogenicity parameters.

To analyze quantitative indicators over time in each group, repeated-measures analysis of variance or Friedman analysis was used, depending on the nature of the data distribution. For a posteriori comparisons of values at screening and follow-up visits, Dunnett's criterion was used in the case of analysis of variance and Dunn's criterion with Bonferroni correction in the case of Friedman analysis.

Comparisons of groups with each other on qualitative features were performed using the χ^2 criterion or Fisher's exact test. Geometric mean titers (GMT) for each study group with 95% confidence intervals (CI) were estimated for each time point. Logarithmic transformation was applied to the raw data to analyze titers. For comparisons of the study groups with each other, analysis of variance (Bonferroni or Gates–Howell posterior comparisons if the variances were not equal) or Kraskell–Wallis analysis (Dunn posterior comparisons with Bonferroni correction) was applied to the logarithmic data, depending on the nature of the distribution. To assess the type of distribution, the Kolmogorov–Smirnov criterion was used, as well as the asymmetry and kurtosis indices.

Levene's criterion was used to test the homogeneity of dispersions, and Spearman's correlation was used to assess the relationship between neutralizing and specific antibody titers. 95% CI was calculated for the correlation coefficient. The frequencies with 95% CI calculated by the Clopper–Pearson method are given as descriptive characteristics for the efficacy parameters.

Results

In a safety and immunogenicity study of the novel VLP-based COVID-19 prophylaxis vaccine in a phase I clinical trial, screened volunteers (n = 180; 107 males and 73 females) were categorized into 3 groups, with 84% completing the study (n = 151: 52 in group 1,

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53 in group 2, and 46 in group 3). Demographic data are summarized in **Table 1**.

Evaluation of vaccine tolerability and safety

In the **Appendix 2** on the journal's website: https://www.microbiol.crie.ru, all AEs by groups are presented. During the entire period of post-vaccination follow-up within 21 days after twice-daily vaccination, 572 AEs were detected in 138 (76.7%) volunteers, including 216 AEs in 47 (78.3%) volunteers in Group 1, 226 AEs in 49 (81.7%) volunteers in Group 2, and 130 AEs in 42 (70%) volunteers in Group 3 (**Fig. 1**). The same volunteer could experience several different reactions simultaneously.

In 50.5% of cases, the most frequently reported general abnormalities and reactions at the injection site (in 37.7% — pain at the vaccination site and in 22.5% — fatigue), which in 95% of cases were observed in the first 10 days. Also, in 20.5% of cases, clinically significant abnormalities in laboratory tests were observed equally on both the 10th and 21st day after administration of the study drug, irrespective of vaccination. In 64.5% of cases, the registered AEs had a mild degree of severity, in 31.1% — moderate, in 4.4% — severe. In the majority of cases, the relationship between the development of AEs and vaccination was considered probable. The outcome in 95% of all cases was "recovery without consequences", while in 5% of cases the outcome was "not yet recovered". There were no fatalities, serious AEs, or other significant AEs that were considered to be of special interest due to their clinical significance.

The results of laboratory studies confirmed that administration of the investigated vaccine to volunteers aged 18–55 years had no negative effect on the main indicators of clinical and biochemical blood tests, IgE level and indicators of general urine analysis. Deviations of laboratory parameters from the norm were registered in all groups. The majority of deviations were regarded as clinically insignificant. The greatest number of clinically significant deviations was observed in the level of creatine phosphokinase and IgE. The results of electrocardiographic study before the beginning and after the completion of the administration of the preparations allow us to conclude that there was no effect of the administration of the studied vaccines on the work of the heart muscle, all results were within normal limits. Also, no development of vaccine-related neurologic disorders was detected in vaccinated volunteers. Physical examination of the volunteers before vaccination and at each visit did not reveal any abnormalities in the health status of the vaccinated volunteers in any of the groups, except for cases associated with the manifestations of AEs.

Study of humoral immune response in the neutralization reaction

At the screening in the research centers, all volunteers were negative in the rapid test for COVID-19, but antibodies were observed in ELISA and neutralization reactions. In the placebo group, GMT values fluctuated insignificantly, and an increase in GMT was observed in groups 1 and 2 against Wuhan, Delta and Omicron strains (**Fig. 2**).

Volunteers with low GMT (≤ 1/80) before vaccination showed a significant increase in antibodies in the NR after vaccination (**Fig. 3**). In the presence of high titers (> 1/80), lower rates of immunogenicity were observed in NR. In group 3, there was virtually no increase in antibody titers.

Study of humoral immune response by ELISA method

In the study of humoral immune response in dynamics by ELISA method it was shown that in group 1 the increase of IgG titers to S-protein SARS-CoV-2 on the 21st day after a single injection was observed in 100% of volunteers, after a 4-fold injection — in 70%, GMT amounted to 1600, and on the 21st day after a double injection GMT already amounted to 2177.26, seroconversion level — 77.8% (**Fig. 4**). In group 2, on the 21st day after a single vaccine administration, IgG titers increased in 100% of volunteers, and after a 4-fold vaccine administration — in 86%, the GMT was 4306.88. After the 2nd administration of the vaccine with 80 μg antigen content, the immunogenicity indices remained practically unchanged. In group 3, no increase in IgG titers was observed, and the GMT remained at the same level. The average indices of total IgA, IgM, IgG of volunteers during the dynamic observation underwent insignificant changes in all groups.

On the 21st day after the 1st vaccination, specific IgG to the S-protein of SARS-CoV-2 was detected in ELISA in all volunteers of group 1, with antibody titers of 1 : 400–1 : 12,800 and an GMT of 4201.57. The percentage of volunteers who showed an increase in antibody titer was 62.5%, of which those with

Table 1. Patient demographics, *M* ± *SD*

Groups	*n*	Age, years	Height, cm	Body mass, kg	Body mass index, kg/m^2
All study subjects	180	29.91 ± 10.36	171.82 ± 8.21	68.02 ± 10.58	22.94 ± 2.50
Group 1	60	31.62 ± 12.00	172.18 ± 8.50	67.94 ± 9.76	22.84 ± 2.22
Group 2	60	26.58 ± 7.74	172.05 ± 8.82	66.97 ± 11.33	22.49 ± 2.55
Group 3	60	31.53 ± 10.25	171.22 ± 7.32	69.16 ± 10.66	23.49 ± 2.65

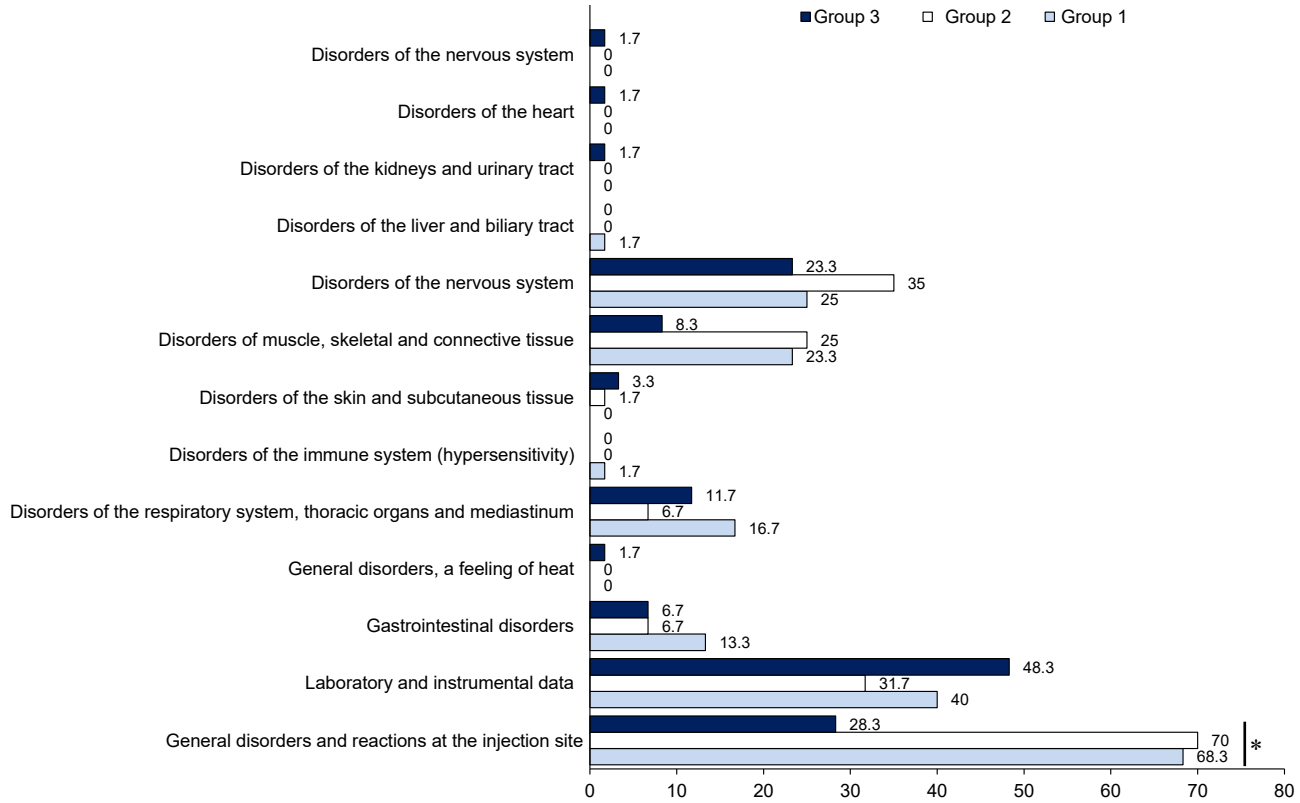

Fig. 1. Proportion of volunteers with AEs by organ system class.
* — statistically significant differences.

4-fold seroconversion were 19.6%. On the 21st day after the 2nd vaccination, the GMT was 4306.88 and the seroconversion rate was 20.4%. The multiplicity of increase in GMT relative to screening on the 21st day after the 1st vaccination was 2.1 and after the 2nd vaccination was 2.2.

In group 2, on the 21st day after the 1st vaccination, specific IgG to S-protein SARS-CoV-2 was detected in ELISA in 100% of volunteers, with antibody titers of 1 : 1600–1 : 12 800, and the GMT was 4950.94. The proportion of volunteers with an increase in antibody titer was 57.4%, including 14.8% with 4-fold seroconversion. On the 21st day after the 2nd vaccination, the GMT was 5261.46 and the seroconversion rate was 19.6%. The multiplicity of GMT increase relative to screening on the 21st day after the 1st vaccination was 1.7, after the 2nd vaccination — 1.8.

In group 3, specific IgG in ELISA on the 21st day after the 1st administration of placebo was detected in 91.7% of volunteers, with antibody titers of 0–1 : 12,800 and an GMT of 1241.36. The proportion of volunteers who showed an increase in antibody was 18.8%, with

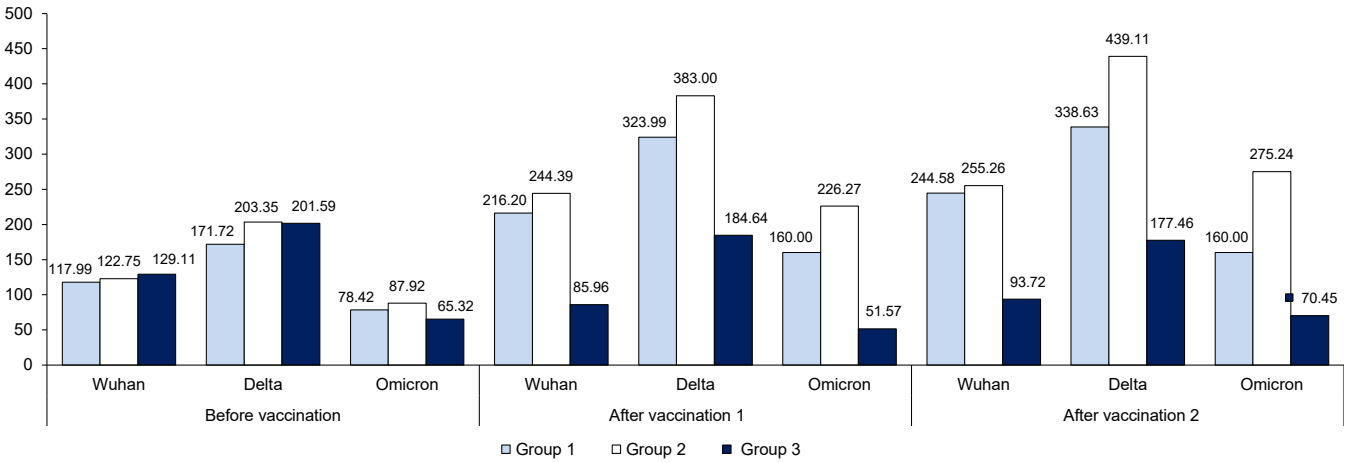

Fig. 2. GMT of neutralizing antibodies in all volunteers.

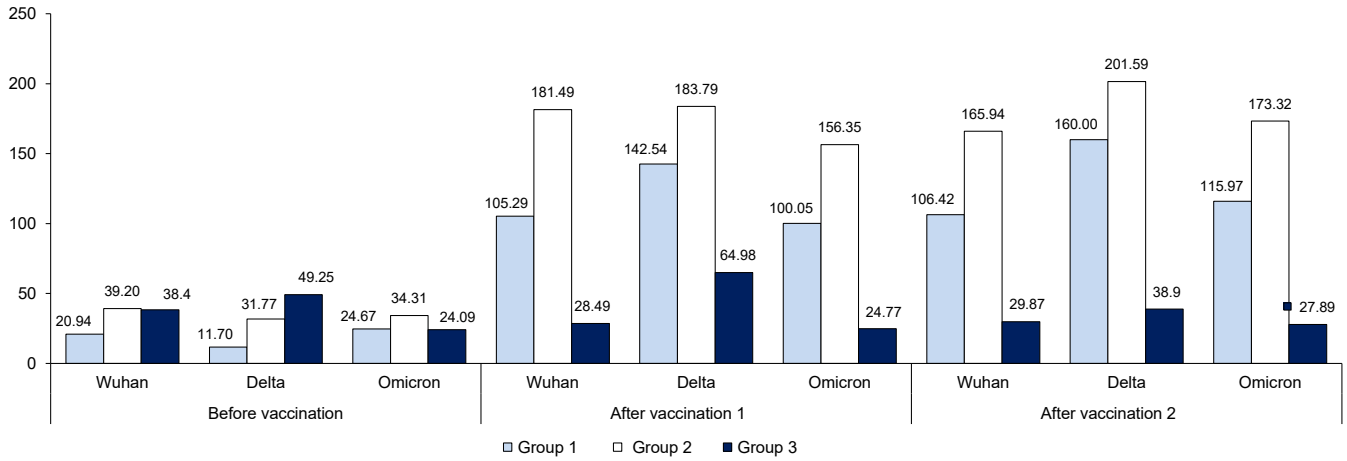

Fig. 3. GMT of neutralizing antibodies in volunteers with baseline low antibody titers ≤ 1/80.

4-fold seroconversion in 10.4%. On the 21st day after the 2nd administration, the GMT was 2311.60, with a seroconversion rate of 15.9%. At the same time, no COVID-19 cases were detected by PCR method.

Assessment of cellular immunity

The studies were carried out initially (screening V), as well as on the 10th day after the 1st and 2nd vaccination. When assessing the dynamics of PSI as a result of immunization with VLP-vaccine, a significant ($p < 0.05$) increase in the index in the studied groups compared to the screening was observed. As the number of vaccinations increased, a gradual increase in cellular response was noted ($p < 0.05$). The mean PSI level reached a maximum value on the 10th day after the 2nd immunization with a vaccine containing 40 μg antigen, per dose (2.17 [1.87; 3.05]) and with a vaccine containing 80 μg antigen, per dose (2.57 [1.98; 3.17]). In LBTR, the number of stimulated lymphocytes was examined, and if they are stimulated by a specific stimulant, it may indicate a prior encounter with antigen, which is possible either post-vaccination or post-infection. In group 3, there were volunteers with very high PSI values, which may suggest post-infection stimulation, as there were no such high PSI levels post-vaccination at any of the tested doses of antigen in the vaccine. Cellular and humoral immune response are not necessarily correlated. Both the presence of antibodies without the formation of cellular reactions and the absence of antibodies but with the formation of cellular immunity are frequently observed in COVID-19 convalescents. Therefore, despite the absence of seroconversion in the group of volunteers immunized with placebo, we cannot unequivocally state the absence of asymptomatic COVID-19 infection in volunteers with high levels of PSI with cellular response. It should be noted that such a high proliferative response to a specific antigen in the placebo group is only possible after an infection has occurred.

Examination of the $CD4^+/CD8^+$-lymphocyte ratio showed no abnormalities after vaccination with the VLP vaccine containing both 40 μg and 80 μg of antigen per dose, either toward helper or cytotoxic lymphocytes. This additional information about the absence of potential immunotoxicity can be observed after vaccination (according to ICH S8) (**Table 2**).

To study the immune status of volunteers, the dynamics of cytokine production during double immuni-

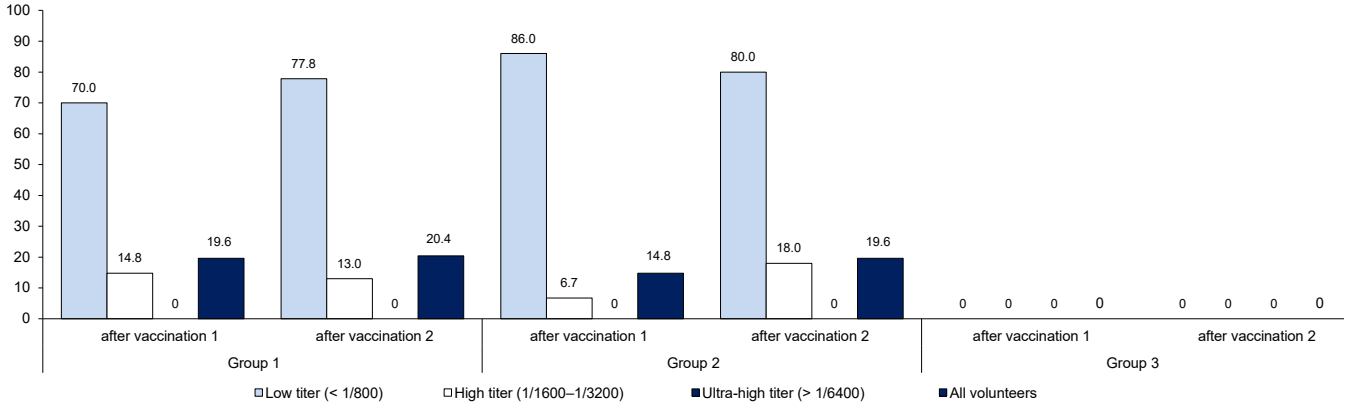

Fig. 4. Seroconversion rate at different study dates.
Y axis – proportion of volunteers with seroconversion (increase in specific antibody titer ≥ 4 times), %.

zation was also investigated. Sera from patients were taken before immunization and on the 10th day after the 1st and 2nd immunization (**Fig. 5**).

Analysis of statistical differences showed that in group 1, for the tumor necrosis factor-α (TNF-α) concentration, there was a statistically significant difference between the screening visit (Me = 4.6) and visit 4 (Me = 7.81). No significant differences were found for the rest of the data.

The results show that the developed VLP vaccine, containing both 40 and 80 μg of antigen per dose, can be an inducer of cell-mediated immune response, in which the $CD4^+/CD8^+$-lymphocyte ratio is not changed either toward helper or cytotoxic lymphocytes. And post-vaccination changes in cytokine levels are not sufficient for the development of immunopathologic conditions associated with excessive production of the proinflammatory cytokines under study.

Discussion

Inactivated and live vaccines are the most widely used for the prevention of infectious diseases. However, the emergence and spread of new infections, the variability of their causative agents and the recent pandemic that affected everyone show the need to develop and improve means of specific therapy and prophylaxis. In parallel with the development of mRNA-based, viral vector-based, and subunit vaccine production technology, the technology of VLP production is gaining popularity, which represents an alternative platform for vaccine development. The advantage of such a platform is the possibility of creating multivalent vaccines that are capable of inducing humoral and cellular immune response with the production of virus-neutralizing antibodies of a broad spectrum. This is important in the development of vaccines for the prevention of infections caused by viruses with high genetic variability, such as SARS-CoV-2. At the same time, the absence of genetic material in VLP-vaccines can guarantee an increased level of safety, which is confirmed by preclinical studies [13–15].

In the present study, the tolerability, safety and immunogenicity of a new vaccine against coronavirus infection caused by SARS-CoV-2 were investigated in phase I clinical trials in healthy volunteers. The drug is purified recombinant SARS-CoV-2 VLPs that are synthesized in a baculovirus expression system. The surface S-protein within the VLP is represented by variants 19A, Alpha, Delta and Omicron. The vaccine contains a squalene-based adjuvant.

The main objectives were to assess the tolerability, safety and immunogenicity of the vaccine compared to placebo for 21 days after double intramuscular administration, as well as to determine the optimal dose of antigen for further study of the safety and efficacy of the drug in phase II clinical trials.

When developing vaccines, including those for COVID-19 prophylaxis, special attention is paid to studies of safety, non-reactogenicity and tolerability of new vaccines, as these indicators directly affect the possibility of widespread use of these drugs and the level of public confidence in vaccination. In particular, studies of injectable forms of a number of mRNA vaccines from COVID-19 have revealed both local reactions, such as pain at the injection site, and serious adverse reactions, and some publications on vaccine safety have been withdrawn by journal editors despite the authors' objections. VLP-based vaccines have proven to be highly effective and safe, as demonstrated by vaccines against human papillomavirus (Gardasil, Gardasil9, Cervarix), hepatitis E (Hecolin) hepatitis B (Sci-B-Vac) and malaria (Mosquirix) [16–18].

Table 2. PSI data and $CD4^+/CD8^+$ ratios in the studied groups, Me [Q_1; Q_3]

No.	Immunization	Indicator	Group 1	Group 2	Group 3
1	Before vaccination	*n*	60	60	60
		PSI	1.4 [1.15; 1.69]	1.43 [1.07; 1.83]	1.25 [1.11; 1.59]
		$CD4^+/CD8^+$	2.15 [1.71; 2.81]	2.44 [1.77; 2.97]	2.7 [1.97; 3.25]
2	10th day after the 1st vaccination	*n*	54	52	49
		PSI	1.75 [1.31; 2.21]	2.06 [1.51; 2.3]	1.59 [1.22; 2.15]
		$CD4^+/CD8^+$	2.08 [1.65; 3.38]	2.23 [1.61; 3.38]	2.86 [2.06; 4.23]
3	10th day after the 2nd vaccination	*n*	47	49	45
		PSI	2.17 [1.87; 3.05]	2.57 [1.98; 3.17]	2.17 [1.45; 2.79]
		$CD4^+/CD8^+$	3.3 [2.2; 4.37]	3.18 [2.28; 4.42]	4.15 [2.51; 5.09]
Post-hoc analysis of PSI adjusted for multiple comparisons			p_{1-2} = 0.020 p_{1-3} < 0.0001 p_{2-3} = 0.020	p_{1-2} = 0.318 p_{1-3} < 0.0001 p_{2-3} = 0.004	p_{1-2} = 0.195 p_{1-3} < 0.0001 p_{2-3} = 0.006

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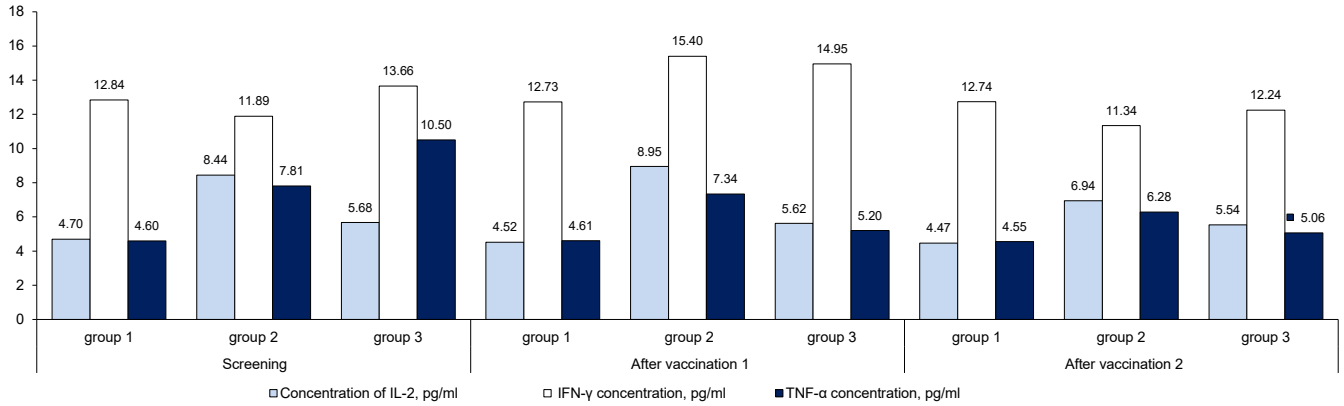

Fig. 5. Dynamics of cytokine levels in blood serum of volunteers.

According to the results of the presented study, AEs were observed both in volunteers vaccinated with the vaccine with 40 and 80 μg of antigen per dose and in the group immunized with placebo. At the same time, the majority of AEs were registered during the first 10 days after vaccination, mainly after the 1st vaccination. In the period from the 11th to the 21st day after vaccination, there were only a few cases of AEs.

The majority of the reported AEs with a "probable" and "possible" relationship were related to expected adverse events after immunization to the effect of the vaccine preparation. AEs of particular interest associated with COVID-19 vaccination (Guillain–Barré syndrome, generalized seizures, anaphylaxis, thrombocytopenia, coagulopathy, etc.) were not reported in any volunteers.

The detected fluctuations in the mean values of clinical blood and urine indices before vaccination and at different periods after vaccination do not allow us to speak about the influence of vaccination on these indices and can be explained by random factors, restructuring of the immune system of the body of the vaccinated in response to the introduction of antigen.

The average values of vital function indices in the study group were within the normal range, and the changes in these parameters according to the results of measurements after the beginning of the drug administration, compared to the initial values, were insignificant and within the reference values.

Significant changes in individual laboratory parameters relative to screening were observed in all study groups. Importantly, these abnormalities occurred both in volunteers who received the study vaccine at both doses and in the group of volunteers who received placebo. The absence of serious vaccine-related AEs or deaths during the study suggests good tolerability and safety of the vaccine with both 40 mcg and 80 mcg of antigen per dose.

Researchers at Radbaud University Medical Center in Nijmegen, the Netherlands, conducted a single-center clinical trial with dose-matched adjuvant ABNCoV2 vaccine based on VLP or capsid-like particles (cVLP). The RBD of the spike glycoprotein SARS-CoV-2 glycoprotein was covalently attached to the cVLP carrier. Forty-five healthy volunteers, aged 18-55 years, who were immunized intramuscularly, twice, were studied. Participants had a total of 249 possibly vaccine-related NIs within a week of vaccination (185 grade 1; 63 grade 2; 1 grade 3). Two serious events occurred; one was classified as a possible adverse reaction [19].

VLPs of the developed vaccine are capable of exerting a strong immunostimulatory effect on the organism, activating T- and B-lymphocytes, as they contain the main immunogenic proteins SARS-CoV-2 in native conformation. They easily penetrate into lymph nodes and are taken up by antigen-presenting cells, in particular dendritic cells, with subsequent antigen presentation by molecules of the major histocompatibility complex class II [19, 20].

Studies of immunogenicity of the developed vaccine against SARS-CoV-2 in the framework of phase I clinical trials showed that administration of the vaccine containing 40 and 80 μg of antigen to volunteers induced a significant increase in GMT compared to placebo. At the same time, the immune response was stronger in volunteers who initially had low antibody titers (NR — titer ≤ 1/80, ELISA — titer ≤ 1/800). When volunteers had high antibody titers (1/1600–1/3200 and ≥ 1/6400 in ELISA) at screening, these immunogenicity rates were lower and no seroconversion was observed. Of note, virally neutralizing antibodies were produced to various SARS-CoV-2 strains, including clades 19A, Delta, and Omicron. In group 3, an increase in antibody titers was noted in a number of volunteers, but no 4-fold increase was observed. It should be noted that COVID-19 was not detected in those included in the study.

In group 2, the immunogenicity indices in ELISA were insignificantly but superior to both the values in the general population and in volunteers with initially low and high antibody titers. Indicators of immune response intensity, antibody titers increase, including 4-fold, in NR were also higher in group 2, especially

for the current and predominant Omicron strain. Thus, in this group the GMT was 275.24, a multiple of 3.1, while in group 1 the GMT was 160, a multiple of 2.0.

High indices of immune response in group 3 most likely indicate a COVID-19 disease in a latent form, which did not manifest itself clinically and was not confirmed by PCR-test. It is important to note that this study was conducted at the height of the epidemic, and the main infectious agent at that time was the Omicron strain.

The insufficient sensitivity of some commercial ELISA test systems for detecting antibodies to SARS-CoV-2 should also be noted [21]. For example, in influenza, antibodies appear in 50–80% of unvaccinated adults without signs of disease [22]. A number of researchers note that up to 80% of COVID-19 infections may be asymptomatic [23, 24].

Researchers from the Netherlands studied the immune response of 45 volunteers who were immunized with ABNCoV2 vaccine based on cVLP with different amounts of antigen: 6, 12, 25, 50 or 70 μg [19]. A dose-dependent antibody formation was observed after the 2nd vaccination when immunized with vaccines containing 25–70 μg of antigen. Antibodies neutralized the major SARS-CoV-2 variants, but viral neutralizing activity was lower with the Omicron variant (BA.1), specific interferon-γ (IFN-γ)$^+$CD4$^+$ T cells were activated. The researchers' overall conclusion: immunization with the vaccine was well-tolerated, safe and resulted in a functional immune response.

SARS-CoV-2 infection induces immune responses that may have important implications for the development of vaccination strategies. T-cell immunity plays a central role in the control of SARS-CoV-2 infection. Antigen-specific CD4$^+$ and CD8$^+$ T cells and neutralizing antibodies play a protective role against SARS-CoV-2, whereas impairment of the adaptive immune response, namely a lack of naive T cells, can lead to adverse disease outcomes.

In the study of the immune response, it is important to assess the degree of cytokine imbalance and immune cell activation. Antigen mimicry between viral and human proteins can lead to the development of immune-mediated hemolysis, decreased leukocyte counts, cytokine storm, procoagulant state, and macrophage activation [25]. The synthesis of cytokines IFN-γ, interleukin-2, and TNF-α accounts for the Th1-type immune response [26]. IFN-γ and interleukin-2 activate macrophages, natural killer cells, and cytotoxic lymphocytes, which are crucial for virus elimination. IFN-γ is the most potent factor in macrophage activation. Full activation of macrophages can be achieved by low levels of IFN-γ. In vaccine development, it is important to avoid toxicity associated with its over-activation.

Abnormal stimulation of T cells and antigen-presenting cells (dendritic cells, macrophages and B cells) can lead to the development of a cytokine storm generated by suppressive release of cytokines, particularly TNF-α, which promotes migration of neutrophils from vessels and activation of clotting pathways. Hyperinflammatory reactions correlate with increased levels of serum interleukin-2, -6 and -7 [27, 28].

The developed VLP-vaccine can be an inducer of cell-mediated immune response, in which the CD4$^+$/CD8$^+$-lymphocyte ratio does not change either toward helper or cytotoxic lymphocytes. At the same time, the post-vaccination change in cytokine levels is not sufficient for the development of immunopathologic conditions associated with excessive production of the proinflammatory cytokines under study.

When the developed vaccine was used, antibody formation and cellular response increased markedly as the number of immunizations increased. However, the immunogenicity parameters obtained after immunization with the vaccine containing 80 μg antigen per dose were superior to those obtained after immunization with the vaccine containing 40 μg antigen per dose (especially for the Omicron strain); therefore, this dose was selected as the optimal dose for phase I clinical trials.

Conclusion

The tolerability, safety and immunogenicity of a new vaccine for COVID-19 prophylaxis based on VLP within the framework of phase I clinical trials on 180 volunteers were evaluated. It has been shown that vaccination with preparations containing 40 and 80 μg of antigen in the dose intramuscularly twice with an interval of 21 days does not cause serious AEs and induces humoral and cellular immune response. The CD4$^+$/CD8$^+$-lymphocyte ratio does not change either toward helper or cytotoxic lymphocytes. Post-vaccination changes in cytokine levels are not sufficient for the development of immunopathologic conditions associated with excessive production of the proinflammatory cytokines under study.

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Original Study Article
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Molecular and biological characterization of *Streptococcus pneumoniae* isolates from patients with pneumococcal meningitis

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Abstract

The aim of the study is to provide key characteristics of *Streptococcus pneumoniae* isolates circulating in Russia in 2015–2020 and isolated from pneumococcal meningitis patients based on high-throughput sequencing data, including global pneumococcal sequence clusters, serotypes, virulence factors and genetic determinants of resistance, in comparison with clinical data on antimicrobial susceptibility.

Materials and methods. We studied 68 invasive *S. pneumoniae* isolates from blood and cerebrospinal fluid of patients with bacterial meningitis in different regions of Russia in 2015–2020. Species identification was performed taking into account the morphology of colonies on blood agar, the presence of α-hemolysis, negative catalase reaction, sensitivity to optoquine, and positive latex-agglutination results. The sensitivity of isolates to antimicrobials was determined by microdilution in broth, and sensitivity categories were determined based on borderline values of minimum inhibitory concentrations (MICs). Whole genome sequencing of *S. pneumoniae* isolates, analysis of isolates for penicillin-binding protein signature, determination of global pneumococcal sequence clusters, MLST alleles, serotypes, sequence types and acquired resistance genes (*mefA*, *ermB*, *tetM*, *folA/P*, *cat*), identification of virulence genes were carried out.

Results. Twenty-eight GPSCs, 45 sequence types and 27 serotypes were identified. The coverage rates of PPV-23 and PCV-13 were 78% and 59%, respectively. Serotypes 3 (18%), 19F (9%), 23F (7%) and 15B (6%) were predominant. The GPSC12 lineage (serotype 3) was predominant (43%). Lineages expressing vaccine serotypes GPSC1(19F), GPSC6(14), GPSC13(6A), GPSC904(14) and GPSC10(19F) exhibited multiple antimicrobial resistance, including penicillin resistance. The resistant lineages expressing non-vaccine serotypes were GPSC230 (13) and GPSC177 (35F). In most cases, genotypic and phenotypic resistance to penicillin (increased MICs of β-lactams correlated with types of penicillin-binding proteins), erythromycin (*ermB*, *mefA*, *ermB*/*mefA*), clindamycin (*ermB*) and tetracycline (*tetM*), and trimethoprim-sulfamethoxazole (*folA*, *folP*) was found to be consistent. The virulence genes *cbpG*, *lytA*, *pce*/*cbpE*, *pavA*, *pfbA*, *ply*, *hysA*, *nanA* and *cps4A* were detected in all isolates. Zinc metalloproteinase C was detected in 13% of isolates.

Conclusion. A high diversity of serotypes and lineages among pneumococcal isolates from meningitis patients was revealed. Out of the 68 *S. pneumoniae* isolates from patients with bacterial meningitis, more than 17% belonged to non-vaccine serotypes. The results of phenotypic and genotypic antimicrobial resistance comparison were characterized by good concordance, which indicates the necessity for further study of the possibility of using whole-genome sequencing as a diagnostic tool to identify resistance mechanisms in clinical isolates of pneumococci.

Keywords: *Streptococcus pneumoniae, invasive pneumococcal infections, whole-genome sequencing, multilocus sequencing-typing; antimicrobial resistance, penicillin-binding proteins, global pneumococcal sequence cluster, serotypes*

Ethics approval. The study was conducted with the informed consent of the patients. The study protocol was approved by the Independent Interdisciplinary Committee for the Ethical Review of Clinical Trials (Protocol No. 1, January 17, 2020).

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Оригинальное исследование
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Молекулярно-биологическая характеристика изолятов *Streptococcus pneumoniae*, выделенных от больных пневмококковым менингитом

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Аннотация

Цель работы — дать ключевые характеристики изолятов *Streptococcus pneumoniae,* циркулирующих на территории России в 2015–2020 гг. и выделенных от больных пневмококковым менингитом, на основании данных высокопроизводительного секвенирования, включая глобальные кластеры пневмококковых последовательностей, серотипы, факторы вирулентности и генетические детерминанты резистентности, в сравнении с клиническими данными по чувствительности к антимикробным препаратам (АМП).

Материалы и методы. Исследовано 68 инвазивных изолятов *S. pneumoniae,* выделенных из крови и ликвора пациентов с бактериальным менингитом в разных регионах России в 2015–2020 гг. Видовую идентификацию проводили с учётом морфологии колоний на кровяном агаре, наличия α-гемолиза, отрицательной каталазной реакции, чувствительности к оптохину, положительных результатов латекс-агглютинации. Чувствительность изолятов к АМП определяли методом микроразведений в бульоне, категории чувствительности — на основании пограничных значений минимальных подавляющих концентраций (МПК). Проводили полногеномное секвенирование изолятов *S. pneumoniae*, анализ изолятов на сигнатуру пенициллинсвязывающих белков, определение глобальных кластеров пневмококковых последовательностей, аллелей MLST, серотипов, сиквенс-типов и генов приобретённой резистентности (*mefA, ermB, tetM*, *folA/P, cat*), идентифицировали гены вирулентности.

Результаты. Выявлены 28 GPSC, 45 сиквенс-типов и 27 серотипов. Степень охвата ППВ-23 и ПКВ-13 составила 78 и 59% соответственно. Доминировали серотипы 3 (18%), 19F (9%), 23F (7%) и 15B (6%). Преобладала (43%) линия GPSC12 (серотип 3). Линии, экспрессирующие вакцинные серотипы GPSC1(19F), GPSC6(14), GPSC13(6A), GPSC904(14) и GPSC10(19F), обладали множественной антимикробной резистентностью, включая резистентность к пенициллину. Резистентные линии, экспрессирующие невакцинные серотипы, — GPSC230 (13) и GPSC177 (35F). В большинстве случаев установлено соответствие генотипической и фенотипической резистентности к пенициллину (повышенные МПК β-лактамов коррелировали с типами пенициллинсвязывающих белков), эритромицину (*ermB, mefA, ermB/mefA*), клиндамицину (*ermB*) и тетрациклину (*tetM*) и триметоприму-сульфаметоксазолу (*folA, folP)*. У всех изолятов обнаружены гены вирулентности *cbpG, lytA, pce/cbpE, pavA, pfbA, ply, hysA, nanA* и *cps4A*. Цинковая металлопротеиназа С обнаружена у 13% изолятов.

Заключение. Выявлено высокое разнообразие серотипов и линий среди изолятов пневмококков, выделенных у больных менингитом. *И*з 68 изолятов *S. pneumoniae*, выделенных у пациентов с бактериальным менингитом, более 17% относились к невакцинным серотипам. Результаты сопоставления фенотипической и генотипической антимикробной резистентности характеризовались хорошей конкордантностью, что указывает на необходимость дальнейшего изучения возможности использования полногеномного секвенирования в качестве диагностического инструмента для выявления механизмов резистентности у клинических изолятов пневмококков.

Ключевые слова: *Streptococcus pneumoniae, инвазивные пневмококковые инфекции, полногеномное секвенирование, мультилокусное секвенирование-типирование, резистентность к антимикробным препаратам, пенициллинсвязывающие белки, глобальный кластер пневмококковых последовательностей, серотипы*

Этическое утверждение. Исследование проводилось при добровольном информированном письменном согласии пациентов. Протокол исследования одобрен Независимым междисциплинарным комитетом по этической экспертизе клинических исследований (протокол № 1 от 17.01.2020).

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

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

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Introduction

Streptococcus pneumoniae is a human respiratory pathogen and a major cause of morbidity and mortality worldwide. *S. pneumoniae* is the 4th most common cause of fatal infections, such as septicemia and meningitis, and is estimated by the World Health Organization (WHO) to cause 1.6 million deaths, of which 0.7–1.0 million occur in children under 5 years of age, mostly in developing countries [1–3]. Surface capsular polysaccharides of *S. pneumoniae* are one of the most important virulence factors and the basis of pneumococcal serotyping. Currently, more than 100 serotypes of *S. pneumoniae* are known [4]. Due to its ability to acquire exogenous DNA, pneumococcus can switch serotypes and acquire antibiotic resistance genes [5]. Uncontrolled use of antimicrobials, selective pressure of pneumococcal vaccines, high level of genetic recombination of *S. pneumoniae* inevitably lead to changes in pneumococcal population: emergence of new non-vaccine serotypes, emergence of isolates with multiple antimicrobial resistance, change in virulence profile. Due to the current situation in the world, in 2024 WHO included macrolide-resistant *S. pneumoniae* in the updated list of priority bacterial pathogens of intermediate level in the world[1].

Currently, whole-genome sequencing technologies allow obtaining information on genetic changes, serotypes, sequencing types determined both by the classical 7-locus scheme and by the core genome MLST, virulence profile, antimicrobial resistance status of pneumococci, which is important for epidemiologic surveillance [6–10].

The aim of the study: to provide key characteristics of *S. pneumoniae* isolates from pneumococcal meningitis patients circulating in Russia in 2015–2020, based on high-throughput sequencing data, including global pneumococcal sequence clusters, serotypes, virulence factors and genetic determinants of resistance, in comparison with clinical data on susceptibility to antimicrobials.

Materials and methods

The study of virulence factors and resistance genes in 68 invasive isolates of *S. pneumoniae* isolated from blood and cerebrospinal fluid of patients diagnosed with bacterial meningitis was performed. All isolates were obtained during different stages of the PeGAS multicenter study in 2015–2020 in different regions of Russia [11]. The isolation and primary identification of isolates were performed in local microbiological laboratories of the centers participating in the study as part of the standard procedure for bacteriological examination of biological material obtained from patients diagnosed with bacterial meningitis and in accordance with MG 4.2.1887-04 "Laboratory diagnosis of meningococcal infection and purulent bacterial meningitis". *S. pneumoniae* isolates were transported to the central laboratory of the Research Institute of Antimicrobial Chemotherapy (RIAC) on modified Dorset medium. The RIAC evaluated the compliance of the sent isolates with the inclusion criteria and performed their identification based on colony morphology on blood agar (NEM), the presence of α-hemolysis, negative catalase reaction, sensitivity to optoquine (Oxoid) and positive results of latex-agglutination using the DrySpot Pneumo kit (Oxoid). All isolates were stored in tubes with trypticase-soy broth (bioMerieux) supplemented with 30% sterile glycerol (Sigma) at –70°C until antimicrobial sensitivity was determined. Contaminated and non-viable isolates were excluded from the study. Sensitivity to antimicrobials was determined by broth microdilution in accordance with the requirements of ISO 20776-1:2020[2], and the sensitivity categories of isolates to antimicrobials were determined based on borderline values of minimum inhibitory concentrations (MIC) in accordance with EUCAST standards[3] and Russian recommendations. To control the quality of sensitivity determination, a reference strain of *S. pneumoniae* ATCC 49619 was tested in parallel with the isolates under study.

Whole-genome sequencing was performed at the Central Research Institute of Epidemiology of Rospotrebnadzor. Sample preparation was performed using the Nextera (Illumina) protocol. High-throughput sequencing was performed on a HiSeq 1500 instrument using HiSeq PE Rapid Cluster Kit v2 and HiSeq Rapid SBS Kit v2 (Illumina). Whole-genome nucleotide sequences were assembled using the SPAdes v. 3.13 program. A detailed description of the sample preparation methodology for whole-genome sequencing was given earlier [12].

The whole genomic nucleotide sequences of the studied isolates, data on serotypes and antibiotic sensitivity, as well as information on the sources of strains were deposited in the PubMLST database[4]: accession numbers: 51080–51125, 73010–73011, 73013–73015, 73017–73033.

Invasive pneumococcal isolates were analyzed for the PBP signature, where the combination of 3

[1] WHO bacterial priority pathogens list, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. URL: https://who.int/publications/i/item/9789240093461

[2] ISO 207761:2019 Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices. Part 1: Broth microdilution reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases.

[3] European Committee on Antimicrobial Susceptibility testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Ver. 14.0, 2024. URL: www. eucast.org/clinical_breakpoints/ (data of access: 01.11.2024).

[4] *Streptococcus pneumoniae* MLST Databases. URL: https://pubmlst.org/spneumoniae/ (data of access: 17.02.2020).

signatures (PBP1A, PBP2B, PBP2X) determines the level of resistance to β-lactams, global pneumococcal sequence clusters were identified, MLST alleles, serotypes, sequence types and acquired resistance genes (*mefA*, *ermB*, *tetM*, *folA/P*, *cat*) using a tool available on Pathogenwatch, a global genomic surveillance platform[5]. Virulence genes were identified using the online AMRseq program[6] and the BacWGSTdb program[7].

The study was non-interventional and did not involve comparison of groups. Descriptive statistics methods were used to present the results, with the determination of the absolute and relative number of observations.

Results

Molecular and biological characterization of invasive S. pneumoniae isolates

We identified 28 global pneumococcal sequence clusters (GPSCs), 45 sequence types and 27 serotypes (**Table 1**).

Analysis of whole genomic data showed that serotypes included in pneumococcal polysaccharide 23-valent vaccine (PPV-23) and pneumococcal conjugate vaccine 13 (PCV-13) were predominant among invasive isolates — 3 (18%), 19F (9%), 23F (7%) and 15B (6%). The coverage rate of PPV-23 was 79% and PCV-13 was 59%. Frequency of isolation of pneumococci of non-vaccine serotypes in meningitis in Russia: 28A and 35F — 3% each, 13, 37, 38, 10C, 15C and 15F — more than 1% (**Figure**).

Of the 28 GPSCs, 20 GPSCs were of vaccine serotypes and 6 GPSCs were of non-vaccine serotypes. Two lineages, GPSC212 (12F, 15F) and GPSC229 (15B, 15C) expressed both vaccine and non-vaccine serotypes. The GPSC12 lineage expressed only vaccine serotype 3 and accounted for more than 42%. In some cases, the same serotype was associated with different lineages. The second most frequent serotype 19F was expressed by lineages GPSC1, 10, 44 and a new lineage whose GPSC is undefined, serotype 23F by GPSC7 and GPSC49, and serotype 15B by GPSC11 and GPSC229. Non-vaccine serotypes were expressed by the lineages GPSC 365 (serotype 28A), GPSC177 (serotype 35F), GPSC212 (serotype 15F), GPSC229 (serotype 15C), GPSC123 (serotype 37), GPSC38 (serotype 38), and GPSC230 (serotype 13).

Antimicrobial sensitivity of invasive isolates of S. pneumoniae to antimicrobials

Analysis of sensitivity to antimicrobials showed that 6 (9%) invasive isolates were resistant to penicillin

[5] A Global Platform for Genomic Surveillance. URL: https://pathogen.watch
[6] AMRseq. URL: https://amrseq.net/ru/
[7] BacWGSTdb. URL: http://bacdb.cn/BacWGSTdb/index.php

Table 1. Global clusters of pneumococcal sequences associated with sequence types and serotypes

GPSC	Sequence types	Serotype	Number of isolates, *n* (%)
1	236	19F	2 (7)
2	15249	1	1 (4)
3	1012	11A	1 (4)
6	3418, 143	6E(6B),14	2 (7)
7	311, 152248, 16095, 311	23F	4 (14)
10	230	19F	1 (4)
11	1262	15B	2 (7)
12	505, 180, 15251, 15250, 2049	3	12 (43)
13	12493, 473	6A, 6B	2 (7)
16	66, 16098	9N	2 (7)
19	433	22F	2 (7)
32	3244, 11901,2824, 3544	7F, 8	4 (14)
38	393	38	1 (4)
43	239	9V	3 (11)
44	179	19F	1 (4)
49	440	23F	1 (4)
68	15252, 3187	18C	2 (7)
76	490	6A	1 (4)
98	1480	8	1 (4)
123	447	37	1 (4)
162	2361	4	2 (7)
177	2991	35F	2 (7)
212	6202	12F, 15F	4 (14)
229	1025	15B, 15C	3 (11)
230	2754	13	1 (4)
365	225	28A	2 (7)
376	9247	6E(6B)	1 (4)
904	782	14	1 (4)
Not assigned	15	19F	2 (7)
Not assigned	15247	10C	1 (4)
Not assigned	5205	8	1 (4)
Not assigned	13459	10A	1 (4)
Not assigned	16099	4	1 (4)

Note. Not assigned — global pneumococcal sequence cluster number is not assigned.

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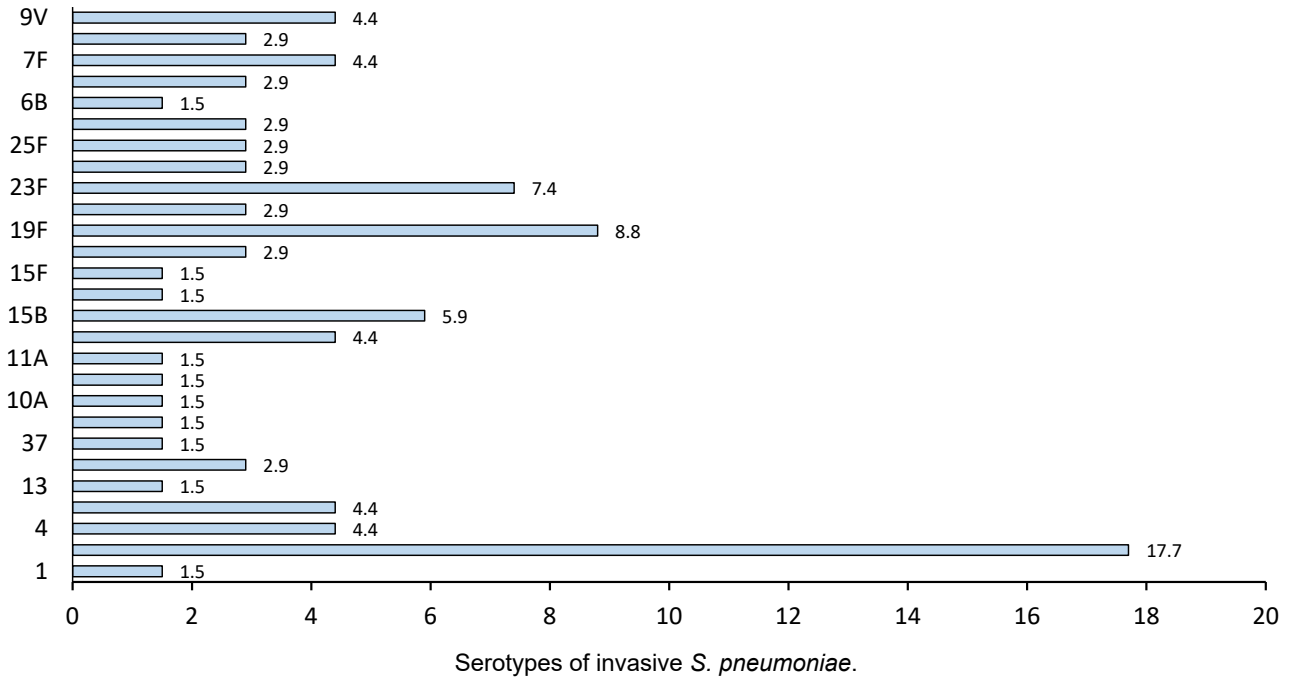

(**Table 2**), 11 (16%) to tetracycline, 5 (7%) to erythromycin, 2 (3%) to clindamycin, and 1 (1%) to respiratory fluoroquinolones. To trimethoprim-sulfamethoxazole 12 (18%) isolates were resistant and 12 (18%) were sensitive at increased exposure. Among the isolates of 28 GPSC lineages, isolates of 5 lineages expressing vaccine serotypes were resistant to 3 or more classes of antimicrobials simultaneously: GPSC1 (serotype 19F), GPSC6 (serotype 14) were resistant to penicillin, tetracycline, trimethoprim-sulfamethoxazole, erythromycin, and clindamycin; GPSC10 (serotype 19F), GPSC904;9 (serotype 14) — to penicillin, tetracycline and trimethoprim-sulfamethoxazole, GPSC6 (serotype 6E(B)) — to penicillin, tetracycline and trimethoprim-sulfamethoxazole, GPSC6 (serotype 6E(B)) — to penicillin, erythromycin, and trimethoprim-sulfamethoxazole. One lineage with vaccine serotypes was resistant to two classes of antibiotics simultaneously: GPSC44 (serotype 19F) to tetracycline and erythromycin. Two lineages expressing non-vaccine serotypes, GPSC177 (serotype 35F) and GPSC230 (serotype 13), were resistant to tetracycline and trimethoprim-sulfamethoxazole.

Genetic determinants of antimicrobial resistance

In clinical isolates of *S. pneumoniae*, resistance to β-lactams is primarily due to variations in amino acid sequences in the transpeptidase domains of penicillin-binding proteins (PBPs): PBP1a, PBP2b and PBP2x, which reduce the affinity of β-lactam antibiotics for these sites. The type of PBPs can predict the levels of resistance to β-lactams [13]. Analysis of the results of whole-genome sequencing of invasive *S. pneumoniae* isolates revealed that the most common type of PBP in sensitive isolates (PBP1a-PBP2b-PBP2x) was 2-0-2 — 29%, 11-4-0 — 4% and 12-0-6 — 3% were less common. All penicillin-resistant isolates had signatures: 13-16-47, 17-15-22, 24-53-77, 36-34-44, and 31-12-18. Several combinations of new PBP types were also identified: new-27-new, 34-11-new. Resistance to macrolides end lincosamides (erythromycin and clindamycin) was due to the presence of the methylase (*ermB*) gene in 5 (7%) isolates, while 1 (1%) isolate had both *ermB* and macrolide efflux pump (*mefA/E*) genes detected simultaneously. Resistance to trimethoprim-sulfamethoxazole was associated with substitution in *folA* I100L and/or insertion of 1 or 2 *foP* codons, whereas isolates categorized as sensitive at higher exposure often had insertion of 1 or 2 codons in *folP*. Chloramphenicol resistance was predicted by the presence of the chloramphenicol acetyltransferase gene (*cat*), while fluoroquinolone resistance was predicted by mutations in *gyrA* and *parC* genes.

Comparison of resistance phenotype and genotype

The concordance of resistance genotype and phenotype was generally high, but in some cases there were discrepancies between genotypic and phenotypic resistance.

Resistance to β-lactams showed good concordance. Six penicillin-resistant pneumococcal isolates had PBP signatures characteristic of resistant pneumococci. Interestingly, all 6 penicillin-resistant isolates had PBP1a(13-36)-PBP2b(12-53)-PBP2x(18-77) signatures, whereas all sensitive isolates had low-numbered PBP signatures. Thus, PBP transpeptidase signatures are reliable indicators of the MICs of various β-lactam antibiotics in clinical isolates of pneumococci and can serve as an alternative to phenotypic sensitivity testing.

Table 2. Resistance of pneumococcal lineages GPSC to antimicrobials

GPSC	Serotype	Number	Genotype, resistance, *n* (%)						
			PEN	TET	TS	CL	ERY	CHL	FX
1	19F	2	2 (3)	2 (3)	2 (3)	1 (1)	2 (3)	0	0
2	1	1	0	0	0	0	0	0	0
3	11A	1	0	0	1 (1)	0	0	0	0
6	6E(B),14	2	1 (1)	2 (3)	2 (3)	2 (3)	2 (3)	0	0
7	23F	4	0	0	4 (6)	0	0	0	0
10	19F	1	1 (1)	1 (1)	1 (1)	0	0	0	0
11	15B	2	0	0	2 (3)	0	0	0	0
12	3	12	0	1 (1)	0	0	0	0	1 (1)
13	6A, 6B	2	1 (1)	1 (1)	2 (3)	0	1 (1)	0	0
16	9N	2	0	0	1 (4)	0	0	0	1 (1)
19	22F	2	0	0	0	0	0	0	0
32	7F,8	4	0	0	0	0	0	0	0
38	38	1	0	0	0	0	0	0	0
43	9V	3	0	0	3 (4)	0	0	0	0
44	19F	1	0	1 (1)	1 (1)	1 (1)	1 (1)	0	0
49	23F	1	0	0	1 (1)	0	0	0	0
68	18C	2	0	0	2 (3)	0	0	0	0
76	6A	1	0	0	1 (1)	0	0	0	0
98	8	1	0	0	0	0	0	0	0
123	37	1	0	0	0	0	0	0	0
162	4	2	0	0	0	0	0	0	0
177	35F	2	0	2 (3)	2 (3)	0	0	0	0
212	12F, 15F	4	0	0	0	0	0	0	0
229	15B, 15C	3	0	0	3 (4)	0	0	0	0
230	13	1	0	1 (1)	1 (1)	0	1 (1)	0	0
365	28A	2	0	0	0	0	0	0	0
376	6E(6B)	1	0	0	1 (1)	0	0	1 (4)	0
904;9	14	1	1 (1)	1 (1)	1 (1)	0	0	0	0
Not assigned	10C, 19F, 8, 10A ,4	6	0	2 (3)	2 (3)	0	0	0	0
28	27	68	6 (9)	14 (21)	33 (49)	4 (6)	7 (10)	1 (1)	2 (3)

Note. PEN, penicillin resistance predicted based on PBP1a, PBP2b and PBP2x sequences; TET, tetracycline resistance predicted by the presence of the *tet* M gene; TS, trimethoprim-sulfamethoxazole resistance, associated with substitution in folA I100L and/or insertion of 1 or 2 codons in *folP_aa_insert_57-70*, CL — clindamycin resistance predicted of gene *erm B*; ERY — macrolide resistance predicted by the presence of the methylase gene (*ermB*) and macrolide efflux pump gene (*mefA/E*); CHL — chloramphenicol resistance predicted by the presence of the chloramphenicol acetyltransferase gene (cat); FX — fluoroquinolone resistance predicted by the presence of mutations in the *gyrA*, *parC* genes.

All 11 tetracycline-resistant pneumococci carried *tetM* genes. Three isolates containing *tetM* were categorized as sensitive, which may be due to mutations in *tetM* not considered in our study.

In the case of trimethoprim-sulfamethoxazole, a good correlation between the presence of resistance markers and phenotypic resistance was found for most isolates. Twelve isolates were double mutants with a

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substitution in *folA* I100L and an insertion of 1 or 2 codons in *folP_aa_insert_57-70* and were resistant to trimethoprim-sulfamethoxazole (Table 2). Twelve pneumococcal isolates containing insertions in *folP* had an IPC of 2 mg/L and were categorized as sensitive at increased exposure, and 9 isolates with single mutations in *folP* were categorized as sensitive (IPC ≤ 1 mg/L).

All 5 erythromycin-resistant isolates carried the *ermB* gene, 1 resistant isolate carried both *ermB* and *mefA*, while 2 isolates with the *mefA* gene remained phenotypically sensitive to erythromycin. The 2 clindamycin-resistant isolates carried *ermB* genes, but *ermB* was also detected in 2 phenotypically sensitive isolates.

Due to the fact that there are no criteria for determining the category of sensitivity to chloramphenicol for *S. pneumoniae*, the activity of this drug was assessed based on the epidemiological cut-off value. The MIC of chloramphenicol for all isolates tested was less than 8 mg/L, which corresponds to the wild-type population, but 1 isolate carried *cat* gene.

One isolate was found to be resistant to respiratory fluoroquinolones (levofloxacin and moxifloxacin); it had mutations in the *gyrA* and *parC* genes. At the same time, a mutation in the *parC* gene was detected in 1 isolate among fluoroquinolone-sensitive pneumococci.

Genetic determinants of pneumococcal virulence

To gain insight into the genetic features contributing to virulence, we examined the presence of the major protein virulence factors of pneumococcus. Choline-binding proteins (CbpG, LytA and Pce/CbpE), PavA and PfbA, known as fibronectin- and plasminogen-binding proteins, as well as hyaluronidase, pneumolysin, neuraminidase, and capsule-associated Cps4A were detected in all invasive pneumococcal isolates (**Table 3**). Zinc metalloproteinase C was detected in 9 (13%) isolates.

Discussion

In our study, pneumococci of serotype 3 prevailed among clinical isolates causing meningitis in Russia. A similar situation is observed in many other countries during the postvaccination period. In Austria, England, Canada, Sweden and Germany, a significant increase in invasive pneumococcal diseases of serotype 3 in adults has been observed over the last 3 years [14]. In Brazil, serotype 3 became the predominant cause of invasive disease in the post-PCV era among adults [15, 16]. The low efficacy of conjugate vaccines against serotype 3 pneumococci is related to the structure of the polysaccharide capsule, which is non-covalently bound to cell wall peptidoglycan [17–21]. It should be noted that isolates of the GPSC12 lineage (serotype 3), remaining the main cause of invasive forms of pneumococcal infection worldwide after the introduction of PCV13, usually retain sensitivity to antimicrobials [22–25]. The low incidence of antimicrobial resistance in serotype 3 isolates may be associated with the high invasiveness of this serotype and the relatively short duration of carriage, which, in turn, reduces the impact of antimicrobials in the treatment of infections of other etiologies [26]. At the same time, a serotype 3 study conducted in England [27] revealed that since 2018, GPSC12 lineage isolates resistant to penicillin, macrolides, chloramphenicol and tetracycline have been emerging [28, 29]. The increasing resistance of serotype 3 isolates indicates the circulation of more antibiotic-resistant clones [30]. In our study, all *S. pneumoniae* serotype 3 isolates were sensitive to penicillin; of 12 isolates isolated from cerebrospinal fluid, only 2 contained resistance genes to fluoroquinolones — *parC* and to tetracycline — *tetM*, while retaining phenotypic sensitivity to these drugs.

Pneumococci of serotype 19F were the second most frequently isolated in meningitis, which may be

Table 3. Characteristics of virulence genes

Virulence gene	Name of the encoded protein	% identity	Number of isolates	
			n	%
cbpG	Choline-binding protein G	99,30	68	100
lytA	Autolysine	98,75	68	100
pce/cbpE	Choline-binding protein E	99,18	68	100
ply	Pneumolysine	99,86	68	100
pavA	Fibronectin-binding protein	99,52	68	100
pfbA	Plasmin and fibronectin-binding protein A	99,72	68	100
hysA	Hyaluronidase	99,16	68	100
nanA	Neuraminidase A	98,77	68	100
cps4A	Capsule synthesis	96,54	68	100
zmpC	Zinc metalloproteinase C	99,96	9	13

due to the peculiarities of the capsular polysaccharide, which is more resistant to the deposition of the C3b component of complement and antibodies on the bacterial walls, which reduces the sensitivity to opsonophagocytosis [31, 32]. Our results indicate that the circulation of serotype 19F is associated with the spread of 3 lineages: GPSC10, GPSC1 and GPSC44. All lineages were characterized by resistance to various antimicrobials, with the GPSC10 and GPSC1 lineages showing multiple antimicrobial resistance. In Canada, 19F serotype dominance was associated with the spread of GPSC1, GPSC4, GPSC9, GPSC10, GPSC18, GPSC44 and GPSC119 lineages [33], in Sweden with the GPSC1 lineage [34], and in Asia, Europe, North America, and South America, 19F serotype was one of the dominant serotypes in the GPSC1 lineage [35]. In South Africa, the dominance of the 19F serotype was associated with the spread of the GPSC1 and GPSC21 lineages, and a high, about 50%, hospital-acquired mortality rate from pneumococcal meningitis was found to be associated with the 19F serotype [35, 36]. The increased incidence of 19F serotype after the introduction of PCV13 in India has been associated with the multidrug-resistant GPSC1 and GPSC10 lineages, with GPSC10 being of particular note as it expressed both vaccine serotypes, including 19F, and non-vaccine serotypes, and thus contributed most to the spread of non-vaccine serotypes among clinical isolates [37]. The GPSC10 lineage is capable of simultaneously expressing a wide range of serotypes, which facilitates its adaptation to selective vaccine pressure. An international dataset of the GPSC10 lineage showed that this lineage expresses 16 serotypes, of which only 6 are included in PCV13. Moreover, the GPSC10 lineage has a relatively high potential to develop invasive forms of infection and a propensity to cause meningitis, regardless of serotype [38]. It was found that it took about 3-5 years for pneumococci of the GPSC10 lineage to spread in France, and in Spain, Argentina, and Israel a rapid change in the serotype composition of this lineage occurred in the post-vaccination period. Thus, together with its transmissibility, GPSC10 should be considered as a high-risk lineage that may eventually reduce the efficacy of vaccines worldwide [39, 40]. In a study conducted by E. Egorova et al. found that in Russia in 2011-2018, serotype 19F isolates belonged to 8 different lineages (GPSC1, GPSC44, GPSC10 GPSC6, GPSC11, GPSC18, GPSC43 and GPSC591), of which the lineages GPSC1, GPSC6 and GPSC10 were characterized by resistance to antimicrobials [41].

According to the results of the present study, pneumococci of serotype 23F were the third most common serotype responsible for the development of bacterial meningitis in Russia. Four out of 5 isolates of serotype 23F belonged to the GPSC7 lineage, 1 — to the GPSC49 lineage. All isolates of serotype 23F were characterized by resistance to trimethoprim-sulfamethoxazole. In studies conducted in China and Iran, serotype 23F was one of the dominant serotypes isolated from the cerebrospinal fluid of patients [42, 43]. The results of studies performed in the UK showed that serotype 23F isolates were part of the GPSC7 lineage. While in 2002 this lineage was dominated by serotype 23F and had only a small number of serotype 23A, in 2009 isolates of serotype 23B appeared [29]. The GPSC7 lineage was one of the dominant lineages responsible for invasive disease during the PCV13 era in Hong Kong, Israel, Malawi, South Africa, Gambia and the USA [22].

The results of international studies indicate that the post-vaccination period is characterized by a decrease in the proportion of vaccine serotypes and an increase in non-vaccine pneumococcal serotypes among different age groups of the population [44, 45]. Lineages represented by vaccine serotypes in which non-vaccine serotypes were already present persist in the population [15]. It has been observed that these non-vaccine serotypes have a high potential to develop invasive forms of infection [46, 47]. The results of our study indicate that in Russia more than 17% of pneumococcal meningitis cases were caused by pneumococci of non-vaccine serotypes. The relatively high proportion of isolates with serotype 15B (6%) and the presence of isolates with non-vaccine serotypes 28A, 37 and 38, which were not previously associated with pneumococcal meningitis in Russia, are noteworthy. The level of antibiotic resistance was found to be lower in non-vaccine serotypes than in vaccine serotypes and differed depending on the GPSC lineage. In our study, lineages expressing non-vaccine serotypes were sensitive to all tested antimicrobials, except for 2 lineages (Table 2).

Different virulence factors are involved in the process of invasive infection at different stages. According to the results of our study, classical genes encoding virulence factors such as capsule synthesis, pneumococcal surface adhesin, autolysin, fibronectin binding protein and pneumolysin were found in all invasive isolates. An interesting observation was made regarding the *zmpC* gene. It was detected only in 9 invasive pneumococcal isolates of the pneumococcal lines GSPC229 (serotype 15C,15B), GSPC3 (serotype 11A), GSPC162 (serotype 4), GSCP904 (serotype 14) and the new line GSPC (serotype 8). Previously, in a study in the Netherlands, the *zmpC* gene was identified in invasive serotypes 8, 11A and 4 belonging to the GPSC3 lineage. It was found that cases of invasive pneumococcal infections caused by *zmpC*-positive pneumococci were more often accompanied by sepsis [48]. Japanese scientists suggested that the zinc metalloprotease *zmpC* suppresses the virulence of pneumococci by inhibiting bacterial invasion into the central nervous system [49]. Thus, zinc metalloprotease *zmpC* is of particular interest and requires additional research.

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Conclusion

1. 28 global pneumococcal sequence clusters (GPSC) and 45 *S. pneumoniae* sequence-types associated with invasive strains were identified in Russia. Of 68 *S. pneumoniae* isolates from patients with bacterial meningitis, more than 17% belonged to non-vaccine serotypes.

2. Antibiotic resistance of pneumococci of vaccine serotypes was higher than that of non-vaccine serotypes.

3. The emergence of non-vaccine serotype lineages of pneumococcus with determinants of high virulence, including resistance to antibiotics, necessitates further research into the molecular genetic characterization of isolates causing meningitis.

4. The results comparing phenotypic and genotypic antimicrobials were characterized by good concordance, indicating the need to further explore the possibility of using whole-genome sequencing as a diagnostic tool to identify resistance mechanisms in clinical isolates of *S. pneumoniae.*

In conclusion, the characterization of pneumococcal lineages and their genetic variations that influence resistance and invasiveness are highly informative for establishing a global strategy for continuous epidemiological surveillance of the pneumococcal population.

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Humoral immunity to adhesins and toxins of the pertussis pathogen in mice immunized with experimental acellular pertussis vaccines from biofilm and planktonic cultures of *Bordetella pertussis*

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Abstract

Introduction. Whooping cough remains an urgent health problem worldwide, including in countries with high vaccination rates, where, since the 1990s, there has been an increase in the incidence of whooping cough, an increase in the severity of the disease and mortality. In this situation, it is necessary to create a new generation of acellular pertussis vaccines (aPV) that can more effectively affect the colonization, persistence and transmission of *Bordetella pertussis*. One of the possible directions for improving the vaccine prophylaxis of pertussis infection is the creation of aPV based on protective antigens isolated from biofilm cultures of *B. pertussis*.

The aim of the study was to research the level of IgG antibodies to the antigens of the pertussis pathogen: adhesins — filamentous hemagglutinin (FHA), pertactin (PRN) and toxins — pertussis toxin (PT), lipopolysaccharide (LPS) in mice immunized with experimental aPV based on antigenic complexes (AC) isolated from biofilm and planktonic cultures of *B. pertussis*.

Materials and methods. Experimental aPV based on AC isolated from the culture medium of biofilm (aPV-B) and planktonic (aPV-P) cultures of *B. pertussis* strain No. 317 (serotype 1.2.3) were used in the experiments. IgG titers of antibodies to PT, FHA, PRN and LPS in blood sera of mice immunized with aPV-B and aPV-P was determined in ELISA.

Results. The titers of IgG antibodies to adhesins (FHA and PRN) in the aPV-B group were 8 and 4 times higher, respectively, compared with aPV-P, in the absence of significant differences in the titers of IgG antibodies to PT and LPS.

Conclusion. The higher ability of aPV-B to induce an immune response to *B. pertussis* adhesins compared to aPV-P, in the absence of significant differences between them in stimulating IgG antibodies to toxins, indicates the advantage of using antigenic complexes from biofilm cultures to create aPV of a new type.

Keywords: *Bordetella pertussis, pertussis toxin, filamentous hemagglutinin, pertactin, lipopolysaccharide, biofilms, planktonic cultures, electrophoresis, IgG antibodies, enzyme immunoassay.*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the I.I. Mechnikov Scientific Research Institute of Vaccines and Sera (protocol No. 15, December 25, 2024).

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Оригинальное исследование
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Гуморальный иммунитет к адгезинам и токсинам возбудителя коклюша у мышей, иммунизированных экспериментальными бесклеточными коклюшными вакцинами из биоплёночной и планктонной культур *Bordetella pertussis*

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Аннотация

Введение. Коклюш остаётся актуальной проблемой здравоохранения во всём мире, в том числе в странах с высоким уровнем вакцинации, где начиная с 1990-х гг. отмечается рост заболеваемости коклюшем, увеличение тяжести течения заболевания и летальности. В этой ситуации требуется создание нового поколения бесклеточных коклюшных вакцин (БКВ), способных более эффективно влиять на колонизацию, персистенцию и передачу *Bordetella pertussis*. Одним из возможных направлений совершенствования вакцинопрофилактики коклюшной инфекции является создание БКВ на основе протективных антигенов, выделенных из биоплёночных культур *B. pertussis*.

Цель работы — исследование уровня IgG-антител к антигенам возбудителя коклюша: адгезинам — филаментозному гемагглютинину (ФГА), пертактину (ПРН) и токсинам — коклюшному токсину (КТ), липополисахариду (ЛПС) у мышей, иммунизированных экспериментальными БКВ на основе антигенных комплексов, выделенных из биоплёночных и планктонных культур *B. pertussis*.

Материалы и методы. В опытах использовали экспериментальные БКВ на основе антигенных комплексов, выделенных из среды культивирования биоплёночной (БКВ-Б) и планктонной (БКВ-П) культур штамма *B. pertussis* № 317 (серовар 1.2.3). Титры IgG-антител к КТ, ФГА, ПРН и ЛПС в сыворотках крови мышей, иммунизированных БКВ-Б и БКВ-П, определяли в иммуноферментном анализе.

Результаты. Титры IgG-антител к адгезинам (ФГА и ПРН) в группе БКВ-Б были выше в 8 и 4 раза соответственно по сравнению с БКВ-П, при отсутствии значимых различий по титрам IgG-антител к КТ и ЛПС.

Заключение. Более высокая, по сравнению с БКВ-П, способность БКВ-Б индуцировать иммунный ответ к адгезинам *B. pertussis* при отсутствии существенных различий между ними в стимуляции IgG-антител к токсинам, указывает на преимущество использования антигенных комплексов из биоплёночных культур для создания БКВ нового типа.

Ключевые слова: *Bordetella pertussis, коклюшный токсин, филаментозный гемагглютинин, пертактин, липополисахарид, биоплёнки, планктонные культуры, электрофорез, IgG-антитела, иммуноферментный анализ*

Этическое утверждение. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с «Consensus Author Guidelines for Animal Use» (IAVES, 23.07.2010). Протокол исследования одобрен Этическим комитетом НИИВС им. И.И. Мечникова (протокол № 15 от 25.12.2024).

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

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Introduction

Pertussis remains a pressing public health problem worldwide, including in countries with a high level of vaccination, where there has been an increase in the incidence of pertussis, an increase in the severity of the disease course and mortality, including among vaccinated children, adolescents and adults since the 1990s [1, 2]. The continued circulation of virulent strains of *Bordetella pertussis* among the population is associated with the transition from whole-cell vaccines to acellular pertussis vaccines (aPV). aPVs provide protection against severe forms of pertussis, but protective immunity declines rapidly and does not prevent colonization of the respiratory tract and transmission of the pathogen, latent forms of the disease and asymptomatic carriage. Currently known aPVs contain 1 to 5 antigens derived from planktonic cultures of *B. pertussis*: pertussis toxin (PT) and adhesins: filamentous hemagglutinin (FHA), pertactin (PRN), fimbriae antigens Fim2 and Fim3. One of the probable reasons for the low efficacy of known aPVs is their inability to influence the formation of biofilm forms of *B. pertussis* in the respiratory tract [3]. The formation of biofilms by *B. pertussis* strains in the respiratory tract plays an important role in the pathogenesis of pertussis infection by increasing the virulence and persistence of *B. pertussis*. *B. pertussis* biofilms differ from planktonic cultures by an altered gene expression spectrum and production of numerous proteins, including adhesins and toxins. In this regard, vaccines from antigens of biofilm and planktonic cultures may differ in immunogenic activity [4].

In this situation, it is necessary to create a new generation of aPVs that can more effectively influence colonization, persistence and transmission of *B. pertussis*. One of the possible prospects for improving the vaccine prophylaxis of pertussis infection is the creation of aPVs based on protective antigens isolated from biofilm cultures of *B. pertussis* [5–8].

We have previously shown that the protective activity of aPV-B from biofilm culture was 2.5 times higher than that of aPV-P from planktonic culture during intracerebral infection of mice with a virulent strain of *B. pertussis* [9]. aPV-B also more effectively reduced the level of colonization by microbial cells of *B. pertussis* in the lungs of mice during intranasal infection with a virulent strain.

The aim of the study was to investigate the level of IgG antibodies to adhesins and toxins of the pertussis pathogen in mice immunized with experimental aPV based on antigenic complexes isolated from biofilm and planktonic cultures of *B. pertussis*.

Materials and methods

B. pertussis strain No. 317 (serotype 1.2.3) isolated in Russia from a pertussis patient in 2003, deposited in the Scientific Center for Examination of Medical Devices on 15.09.2017, patent No. 2689903, was used.

F1(CBA×C57Bl6) hybrid mice weighing 12–14 g were obtained from the Andreevka nursery, Moscow region. Animals were kept in vivarium conditions in accordance with the interstate standard for the maintenance and care of laboratory animals (GOST 33217-2014). The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the "Consensus Author Guidelines for Animal Use" (IAVES, 23.07.2010). The study protocol was approved by the Ethical Committee of the I.I. Mechnikov Research Institute (protocol No. 15, December 25, 2024)

Control of morphologic, serologic and culture properties of *B. pertussis* strain No. 317 was carried out in accordance with the Methodological Instructions. Cultivation of the strain in liquid synthetic nutrient medium, isolation of antigenic complexes (AC) from planktonic and biofilm cultures was carried out in accordance with the previously described method [10]. To characterize the composition of AC from biofilm and planktonic cultures, vertical electrophoresis in polyacrylamide gel (PAAG) under denaturing conditions according to Lammli [11] was used. Electrophoresis was performed in 10% Tris-glycine buffer at a current of 25 mA. At the end of the process, the gel was stained with Coomassie brilliant blue R-250, after which it was washed twice in an aqueous solution containing 10% acetic acid and 35% ethanol.

Detoxification of *B. pertussis* antigenic complexes was carried out with formalin to a concentration of 0.4% with the addition of sucrose (10%) for 20 days with periodic shaking at 37.0 ± 0.5°C. To obtain aPV, antigenic complexes were sorbed on 2% aluminum hydroxide gel (InvivoGen) in such a ratio that 1 ml of the mixture contained 50 μg of protein, 0.3 mg of aluminum hydroxide and PBS to 1 ml [10]. To study the level and dynamics of IgG antibodies to PT, PRN, FHA and lipopolysaccharide (LPS) (all from National Institute for Biological Standards and Control), mice of line F1(CBA×C57BL6) weighing 12–14 g were immunized intraperitoneally (n = 20 in each group) three times with an interval of 7 days with experimental aPV at a dose of 25 μg. Blood was drawn from mice at 7, 14, 21 and 28 days after the last immunization. Blood sampling (total) from mice was performed under ether anesthesia.

The level of IgG antibodies in the sera of immunized mice was detected by enzyme-linked immunosorbent assay. Sera of intact mice (n = 5) were used as a negative control. The concentration of antigens for adsorption on the plates was: PT — 2 μg/mL; FHA — 2 μg/mL; PRN — 2 μg/mL; LPS — 2.5 μg/mL. Peroxidase antispecies conjugate to mouse IgG (Invitrogen) and tetramethylbenzidine as substrate mixture were used in the experiments. The reaction results were counted using a Multiskan vertical scanning spectrophotometer (Thermo Scientific) at a wavelength of

450 nm. The values inverse to their maximum dilutions, at which the optical density (OD) values were 2 or more times higher than the OD values in the negative control wells, were taken as the serum titer.

Statistical analysis was performed using the Microsoft Office Excel application program package. Quantitative data are presented as $M \pm m$. Comparisons were performed using Student's t test. Differences were considered significant at $p < 0.05$.

Results

From the culture medium of biofilm and planktonic cultures of strain No. 317, ACs were isolated, which were used as the basis for the production of two variants of aPV: aPV-B and aPV-P.

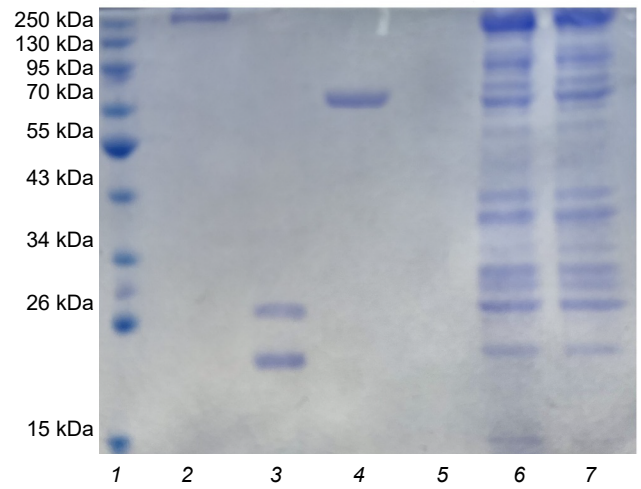

Fig. 1. Electrophoresis in PAAG from antigenic complexes of biofilm and planktonic cultures of *B. pertussis*.

The tracks on the electrophoregram are: *1* — molecular weight markers; *2* — FHA; *3* — PT; *4* — PRN; *5* — LPS; *6* — AC strain No. 317 (biofilm culture); *7* — AC strain No. 317 (planktonic culture).

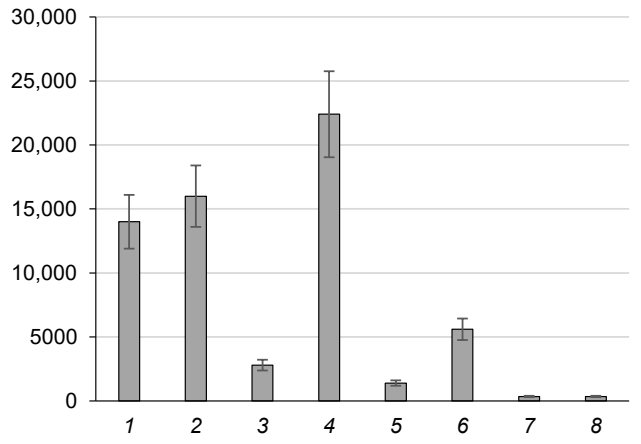

Fig. 2. Maximum titers of IgG antibodies to *B. pertussis* antigens in mice immunized with aPV-B and aPV-P.

On the Y-axis: antibody titers. On the X-axis: *1* — PT aPV-P; *2* — PT aPV-B; 3 — FHA aPV-P; *4* — FHA aPV-B; *5* — PRN aPV-P; *6* — PRN aPV-B; *7* — LPS aPV-P; *8* — LPS aPV-B. IgG antibody titers in intact mice < 100.

The results of analysis of crude and not adsorbed on aluminum hydroxide gel ACs isolated from biofilm and planktonic cultures of strain No. 317 by electrophoresis in PAAG are presented in **Fig. 1**. Proteins in the range of molecular masses from 15 to 220–250 kDa were detected in the AC composition of strain No. 317. At the same time, the intensity of protein bands with a molecular mass of 15 kDa was higher in AC from biofilm culture compared to AC from planktonic culture. FHA (220 kDa), PRN (69 kDa), and proteins with molecular masses of about 28 kDa, less than 26 kDa, and greater than 15 kDa corresponding to PT fragments were detected in both preparations. No protein components were detected on the LPS lane, indicating the immunochemical purity of the preparation used.

The results of IgG antibody levels to PT, PRN, FHA and LPS in mice immunized with aPV from biofilm and planktonic cultures of *B. pertussis* are shown in **Fig. 2**. The titers of IgG antibodies to PT in both groups reached maximum values on the 21st day with a subsequent decrease on the 28th day. Differences in IgG antibody titers to PT in groups aPV-B and aPV-P were statistically insignificant. The maximum titers of IgG antibodies to PRN in the aPV-B group were observed on the 14th and 21st days, and in the aPV-P group — on the 14th day with subsequent decrease in both groups. The titers of IgG antibodies to PRN in the aPV-B group were significantly higher than in the aPV-P group. The titers of IgG antibodies to FHA in aPV-B and aPV-P groups consistently increased and reached the maximum values on the 21st day. Further, a decrease in IgG antibody titers was noted on the 28th day. The maximum titers of IgG antibodies to FHA and PRN in the aPV-B group were 8 and 4 times higher than in the aPV-P group, respectively. The maximum titers of IgG antibodies to LPS in the aPV-B group were observed on the 14th day, and in the aPV-P group – on the 21st day with subsequent decrease. Differences in IgG antibody titers to LPS in both groups were statistically unreliable.

Discussion

B. pertussis produces a number of virulent factors that determine the pathogenetic mechanism of pertussis infection. They can be divided into adhesins (fimbriae, PRN, tracheal colonization factor, FHA) and toxins (PT, adenylate cyclase, tracheal cytotoxin, dermonecrotic toxin, LPS (endotoxin)). The adhesins provide fixation of the pathogen on the epithelial cells of the respiratory tract, and the toxins have a direct damaging effect. The main adhesin of *B. pertussis* is FHA, which is a protein with a molecular mass of 220 kDa, not associated with fimbriae [12]. PRN is a non-fimbrial protein (69 kDa) associated with the outer membrane of the microbial cell. PRN has no toxic properties and is an adhesin by its pathogenetic action and has immunomodulatory activity [13, 14].

PT is one of the main pathogenicity factors of *B. pertussis*, causes various biological effects *in vivo* and *in vitro* and accounts for a significant part of the disease symptoms in pertussis patients. PT is an exotoxin secreted by the microbial cell and is a protein with a molecular mass of 117 kDa, consisting of 5 structural units (S1, S2, S3, S4 and S5) with molecular masses ranging from 28 kDa for S1 to 9.3 kDa for S5 [15, 16].

LPS is a component of the outer part of the cell membrane of all Gram-negative bacteria, including *B. pertussis*. LPS molecules ensure the structural integrity of the bacterial cell and protect the membrane from aggressive environmental influences. Side effects of whole-cell pertussis vaccines are predominantly associated with *B. pertussis* LPS [17, 18].

Due to genotypic and phenotypic polymorphism, as well as depending on the culture conditions (biofilm or planktonic cultures), *B. pertussis* strains may differ in the levels of production of PT, FHA, PRN and other antigens. *B. pertussis* biofilms are formed as a result of complex coordinated interactions between microbial cells and biotic and abiotic substrates. In biofilm cultures, the expression of adhesins increases, which promotes attachment to the substrate and intercellular interactions.

We investigated the composition of *B. pertussis* planktonic and biofilm cultures and the level of IgG antibodies to adhesins (PRN, FHA) and PT and LPS toxins (endotoxin) in mice immunized with aPV from planktonic and biofilm cultures of *B. pertussis* strain No. 317. According to the data of electrophoresis in PAGE, the studied ACs had in their composition FHA, PRN and PT fragments — the main protective antigens of *B. pertussis*, which are part of aPV. In general, the electrophoregrams of both preparations were almost identical, except for the greater intensity of protein bands with a molecular mass of about 15 kDa in aPV-B. Increasing titers of IgG antibodies to PT, FHA and PRN were found in the sera of mice immunized with both preparations, which confirms the results of electrophoresis about the presence of these antigens in the composition of aPV-B. The dynamics of IgG antibody titers to PT, FHA, PRN and LPS in aPV-B and aPV-P groups in general had a similar character with the antibody titers increasing, reaching maximum values and subsequent decrease. At the same time, significant differences between aPV-B and aPV-P in the levels of antibodies to FHA and PRN adhesins were detected. The antibody titers to FHA and PRN in the aPV-B group were significantly higher than in the aPV-P group, which can be explained by the different specific content of these antigens in the composition of aPV due to a higher level of adhesin production by the biofilm culture or higher immunogenicity of FHA and PRN in the composition of aPV-B. There were no significant differences between aPV-B and aPV-P in antibody titers to PT and LPS.

Conclusion

The higher ability of aPV-B to induce an immune response to *B. pertussis* adhesins, which provide fixation of the pathogen on respiratory tract epithelial cells, compared to aPV-P, with no significant differences between both preparations in stimulation of IgG antibodies to PT, indicates the advantage of using antigenic complexes from biofilm cultures to create more immunogenic aPV of a new type.

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Original Study Article
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Dynamics of enzymatic activity in primary culture of Syrian hamster adherent leukocytes *ex vivo* infected with SARS-CoV-2

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Abstract

Introduction. The continued epidemic relevance of SARS-CoV-2, even after the end of the associated COVID-19 pandemic in 2023, necessitates further study of the interaction of this virus with the first line of cellular defense, neutrophils.

The aim of the study was to determine the enzymatic activity of peripheral blood leukocytes of Syrian hamsters (*Mesocricetus auratus*) in the dynamics of *ex vivo* SARS-CoV-2 infection, which characterizes the microbicidal potential of innate immunity cells.

Materials and methods. The SARS-CoV-2/Vladivostok/R-8726/2021 strain was used at infectious doses of 3 lg ($TCID_{50}$/mL) and 2 lg ($TCID_{50}$/mL) ($TCID_{50}$ is the 50% tissue cytopathic dose for the Vero E6 cell line); the contact time of the infecting virus-containing liquid with the cell culture was 1 h. The number of viable cells in the adherent leukocyte culture was counted using an inverted microscope equipped with a digital camera and MCView program. The specific (per 1 viable cell) activities of adenosine triphosphatase (ATPase), 5′-nucleotidase (adenosine monophosphatase, AMPase), lactate dehydrogenase (LDH), succinate dehydrogenase (SDH), myeloperoxidase (MPO) and cytochrome oxidase (CCO) were determined spectrophotometrically after incubation with specific substrates of infected and uninfected cell cultures 1, 16, 24, 48 h after virus inoculation.

Results. The enzymatic activity of leukocytes 1 h after virus inoculation, compared to uninfected leukocytes, was as follows: specific activity of ATPase, MPO was decreased, activity of AMPase, LDH, SDH was increased; 16 h after virus inoculation, activity of MPO was decreased, activity of AMPase, LDH, SDH was increased, activity of ATPase and CCO was at the initial level, i.e. approximately at the level of the uninfected control; 24 h after virus inoculation, AMPase activity was decreased, ATPase activity was increased, LDH, SDH, MPO, CCO activity was at the initial level; 48 h after virus inoculation, ATPase, LDH, SDH, MPO, CCO activity was increased, AMPase activity was at the initial level. Changes in enzymatic activity depend on the infecting dose and correlate with virus accumulation in the culture medium.

Conclusion. The revealed dynamics of enzymatic activity in the primary culture of adherent leukocytes *ex vivo* infected with SARS-CoV-2 indicates a decrease in the microbicidal potential of cells of innate immunity in the course of this infection.

Keywords: *SARS-CoV-2, leukocytes, neutrophils, ATPase, 5'-nucleotidase, lactate dehydrogenase, succinate dehydrogenase, myeloperoxidase, cytochrome oxidase, microbicidal potential*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the Somov Institute of Epidemiology and Microbiology (protocol No. 2, May 16, 2024).

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Оригинальное исследование
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Динамика ферментативной активности в первичной культуре адгезивных лейкоцитов сирийского хомячка, заражённых SARS-CoV-2 *ex vivo*

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Аннотация

Введение. Сохраняющаяся эпидемическая актуальность SARS-CoV-2 даже после завершения в 2023 г. связанной с ним пандемии COVID-19 определяет необходимость дальнейшего изучения взаимодействия этого вируса с клетками первой линии защиты — нейтрофилами.

Цель работы — определить ферментативную активность лейкоцитов периферической крови сирийских хомячков (*Mesocricetus auratus*) в динамике SARS-CoV-2-инфекции *ex vivo,* характеризующую микробицидный потенциал клеток врождённого иммунитета.

Материалы и методы. В работе использовался штамм SARS-CoV-2/Vladivostok/R-8726/2021 в заражающих дозах 3 lg ($ТЦД_{50}$/мл) и 2 lg ($ТЦД_{50}$/мл) ($ТЦД_{50}$ — 50% тканевая цитопатическая доза для линии клеток Vero E6); время контакта заражающей вируссодержащей жидкости с клеточной культурой — 1 ч. Количество жизнеспособных клеток в культуре адгезивных лейкоцитов подсчитывали с помощью инвертированного микроскопа, оснащённого цифровой камерой, и программы MCView. Удельную (в расчёте на 1 жизнеспособную клетку) активности аденозинтрифосфатазы (АТФазы), 5′-нуклеотидазы (аденозинмонофосфатазы, АМФазы), лактатдегидрогеназы (ЛДГ), сукцинатдегидрогеназы (СДГ), миелопероксидазы (МПО) и цитохромоксидазы (ЦХО) определяли спектрофотометрическим методом после инкубации со специфическими субстратами инфицированных и неинфицированных клеточных культур через 1, 16, 24, 48 ч после инокуляции вируса (п.и.в.).

Результаты. Через 1 ч п.и.в. по сравнению с неинфицированными лейкоцитами была снижена удельная активность АТФазы, МПО, повышена активность АМФазы, ЛДГ, СДГ; через 16 ч п.и.в. снижена активность МПО, повышена активность АМФазы, ЛДГ, СДГ, на исходном уровне, т. е. примерно на уровне неинфицированного контроля находится активность АТФазы, ЦХО; через 24 ч п.и.в. снижена активность АМФазы, повышена активность АТФазы, на исходном уровне — активность ЛДГ, СДГ, МПО, ЦХО; через 48 ч п.и.в. повышена активность АТФазы, ЛДГ, СДГ, МПО, ЦХО, на исходном уровне — активность АМФазы. Изменения ферментативной активности зависят от величины заражающей дозы и коррелируют с накоплением вируса в культуральной среде.

Заключение. Выявленная динамика ферментативной активности в первичной культуре адгезивных лейкоцитов, инфицированных SARS-CoV-2 *ex vivo*, свидетельствует о снижении микробицидного потенциала клеток врождённого иммунитета в процессе этой инфекции.

Ключевые слова: *SARS-CoV-2, лейкоциты, нейтрофилы, аденозинтрифосфатаза, 5′-нуклеотидаза, лактатдегидрогеназа, сукцинатдегидрогеназа, миелопероксидаза, цитохромоксидаза, микробицидный потенциал*

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Introduction

Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) (Nidovirales: Coronaviridae, *Betacoronavirus*, subgenus *Sarbecovirus*) is the etiologic agent of the 2019 coronavirus disease (COVID-19), which pandemic (2020–2023) was the longest and one of the deadliest among acute respiratory diseases in recent human history [1]. After the end of the pandemic period, SARS-CoV-2 did not disappear from the human population, but became one of the components in the structure of the seasonal rise in the incidence of acute respiratory diseases [2]. For this reason, the study of the pathogenesis of SARS-CoV-2 infection does not lose its relevance, as a number of aspects of this process are not fully understood. In this regard, the process of virus interaction with peripheral blood cells, especially with cells of innate immunity (neutrophils and monocytes) is of particular interest.

The available literature contains theoretical speculations about the possible ability of SARS-CoV-2 virus to infect neutrophils. Thus, N. Rong *et al.* reported that the CD147 receptor, an alternative to the ACE2 receptor, which determines virus tropism, is expressed in neutrophils of healthy donors and activated in COVID-19 patients [3]. Another non-canonical receptor is the C-type lectin receptor, which mediates neutrophil extracellular trap formation in COVID-19 [4]. Based on this, it can be inferred that the virus is able to directly affect blood leukocytes.

The previously described morphological changes in peripheral blood leukocytes also indicate their significant involvement in the process of SARS-CoV-2 infection [5–8]. However, the scientific literature lacks detailed information on the nature and dynamics of enzymatic activity of leukocytes during infection with this virus. There are some reports about changes in the activity of myeloperoxidase (MPO) and lactate dehydrogenase (LDH) in the serum of patients diagnosed with COVID-19, and the severity of these changes correlates with the severity of the underlying disease [9–11]. For this reason, it is necessary to study not only morphological, but also morpho-functional changes in complex in order to judge the metabolic processes of innate immunity cells under the influence of SARS-CoV-2.

The aim of this study is to determine the enzymatic activity of peripheral blood leukocytes of Syrian hamsters in the dynamics of *ex vivo* SARS-CoV-2 infection, characterizing the microbicidal potential of innate immunity cells.

Materials and methods

Primary adhesive culture of leukocytes of the Syrian hamster (*Mesocricetus auratus*) was obtained from the blood of 15 individuals aged 4 months and weighing about 100 g. All procedures with animals were performed strictly in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of 18.03.1986. The study protocol was approved by the Ethical Committee of the Somov Research Institute of Epidemiology and Microbiology of Rospotrebnadzor (protocol No. 2 of 16.05.2024).

Blood was collected from the heart into glass tubes with heparin added to each tube at a rate of 5 U/mL. The tubes were placed in a thermostat at 45° and 37°C for 1 h, after which the top layer of plasma was carefully removed, and the leukocyte film was collected, adjusted to a concentration of 2×10^6 cells/mL with nutrient medium 199 (BioloT), and dispensed 100 μL into the wells of a flat-bottom 96-well plate (TFS), which was placed in a thermostat (5% CO_2, 37°C) for 40 min; then the medium with non-adherent cells was removed and the wells were washed three times with 150 μL of medium 199.

The number of live adherent cells in the well was determined using a MIB-R inverted microscope (LOMO) equipped with a MC-8.3 C digital camera (LOMO). Using the MCView program (LOMO-Microsystems), the area of the field of view, not including the edge of the well, was set equal to 0.26 mm^2, the number (n) of living (attached with an integral outer membrane) cells was counted in it; the cells crossing the outer boundary were counted on the left/upper edges of the square of the field of view and not counted on the right/bottom edges. Since the total area of the well is 35 mm^2, the total number of cells in the well (N) was estimated by the formula:

$$N = n \times \frac{35{,}00\ \text{мм}^2}{0{,}26\ \text{мм}^2} \approx 134{,}62 \times n. \qquad (1)$$

The final estimation of the number of live cells in each well was performed by 10 randomly selected fields of view.

Ex vivo infection of the primary culture of adherent leukocytes of Syrian hamster was performed by adding 100 μL of medium 199 with working dilution of supernatant of Vero E6 cell culture supernatant into wells with cell monolayer, infected with SARS-CoV-2 (in control samples — without virus-containing supernatant), followed by incubation for 1 h at 37°C, after which the wells were washed three times and filled with culture medium containing medium 199 with 15% fetal bovine serum (FBS) and 0.004% gentamicin K (BioloT). Two infectious doses were used: 3 lg (TCID50/mL) and 2 lg (TCID50/mL), where TCID50 is the 50% tissue cytopathic dose for the African green monkey (*Chlorocebus sabaeus*, ♀) kidney cell line (Vero E6).

The SARS-CoV-2/Vladivostok/R-8726/2021 strain was obtained from the Collection of Pathogenic Microorganisms of the Somov Research Institute of Epidemiology and Microbiology. This strain belongs to genotype Delta (AY.121) (VGARus ID:

prim000041; GenBank ID: OQ318430; GISAID ID: EPI_ISL_16643370) and was isolated from the nasopharyngeal wash of a COVID-19 patient in December 2021 on the Vero E6 cell line model [2].

SARS-CoV-2 RNA was detected by reverse transcription followed by real-time polymerase chain reaction (RT-qPCR) using the RT-qPCR-SARS-CoV-2 reagent kit (Syntol). RNA was isolated using the M-Sorb-NK reagent kit (Syntol). All manipulations were performed according to the manufacturer's protocols. The threshold cycle (threshold cycle, *CT*) of RT-qPCR was considered as a semi-quantitative characteristic of the content of viral particles in the medium: the higher their content, the lower the *CT*. The absence of virus corresponded to $CT \geq 36$.

ATPase and AMPase activity was determined after double washing of adherent leukocytes with growth medium without FCS by adding 50 μL of substrate for ATPase (8 mg/mL adenosine-5′-triphosphate in 10-fold diluted Tris-HCl-buffer, pH 7.8, containing 87 mg NaCl, 28.7 mg KCl, 5.2 mg $MgCl_2 \times 6\ H_2O$) and for AMPase (4 mg/mL adenosine-5′-monophosphate in the same buffer solution containing 87 mg NaCl and 70 mg $MgCl_2$). The samples were left at 37°C for 30 and 60 min, respectively. The reaction was stopped by adding 50 μL of a 1 : 1 mixture of ascorbic acid and molybdic acid. After 20 min, the absorbance of the solutions was measured[1] at a wavelength of 620 nm [12].

Myeloperoxidase (MPO) and cytochrome oxidase (CCO) activity was determined after double washing of adherent leukocytes with growth medium without FCS by adding 100 μL of orthophenylenediamine solution (Merck), 0,4 mg/mL for MPO or 3,3′-diaminobenzidine (Merck), 2 mg/mL for CCO in phosphate-citrate buffer pH 5.0 with 0.033% H_2O_2 and incubated for 10 min at room temperature. The reaction was stopped by adding 100 μL of 10% sulfuric acid solution. Optical density was measured at a wavelength of 492 nm [12].

Viral load in the dynamics of SARS-CoV-2 infection was determined by semi-quantitative method based on the change of threshold cycle in RT-qPCR ($C_T(0)$), after 1 h ($C_T(1)$) — in virus-containing fluid after contact with cells; 16 h ($C_T(16)$), 24 h ($C_T(24)$) and 48 h ($C_T(48)$) — in growth medium of infected cells.

The enzymatic activity of cells under the influence of SARS-CoV-2 infection was determined 1, 16, 24, 48 h after virus inoculation by comparing the ratios of specific (per 1 cell) enzymatic activities of infected and uninfected cells: for each time point t, the coefficient of change in specific enzymatic activity $\gamma(t)$ was calculated according to the formula:

$$\gamma(t) = \frac{\widetilde{D}(t)}{\widetilde{z}(t)} \times \frac{z(t)}{D(t)}, \quad (2)$$

where the optical density and the number of living cells for uninfected ($D(t)$ and $z(t)$) and infected ($D\sim(t)$ and $z\sim(t)$) samples, respectively, are taken into account [13, 14]. Of course, there is an a priori equation of:

$$\gamma(t) = 1. \quad (3)$$

Statistical processing of the results was based on the fact that at each time point t for each of the 6 enzymes, measurements were performed in 3 wells with uninfected cells and in 3 wells with infected cells. For this purpose, the maintenance medium was removed at the beginning in order to introduce media with appropriate substrates after washing. The enzymes are numbered in any order using the index f = 1, 2, ...6. Then at each time instant t there are $6 \times 3 = 18$ samples of virus-containing fluid: $C_{Tjf}(t)$, $j = 1, 2, 3$. Thus C_{Tjf} (1) represented the viral load in the samples as a result of virus accumulation in *de novo* medium: after the initial virus-containing fluid $C_{Tjf}(0)$ had been in contact with cells for 1 h; $C_{Tjf}(16)$, $C_{Tjf}(24)$, and $C_{Tjf}(24)$. Thus, for $t = 1, 16, 24, 48$ h, the sample mean $\langle C_T(t)\rangle$ and the standard deviation of the sample mean m_{CT} are determined using standard formulas as modified as follows::

$$\langle C_T(t)\rangle = \frac{1}{18} \times \sum_{f=1}^{6} \sum_{j=1}^{3} C_{Tjf}(t); \quad (4)$$

$$m_{C_T} = \frac{1}{3\sqrt{34}} \times \left(\sum_{f=1}^{6} \sum_{j=1}^{3} (C_{Tjf}(t) - \langle C_T(t)\rangle)^2\right)^{1/2}. \quad (5)$$

The initial sample was a single copy and its viral load was characterized by a single $C_T(0)$ value.

After the chemical reactions catalyzed by the studied enzymes, the optical density was measured in 3 wells with uninfected cells ($D_i(t)$, $i = 1, 2, 3$) and in 3 wells with infected cells ($D\sim_j(t)$, $j = 1, 2, 3$). Prior to this, the number of live cells was measured in each well: $z_i(t)$ ($i = 1, 2, 3$) and $z\sim_j(t)$ ($j = 1, 2, 3$). Each value of $z_i(t)$ and $z\sim_j(t)$ was evaluated by 10 fields of view according to (1)[2]: $z_{ik}(t)$, $k = 1, 2, ...10$ and $z\sim_{hk}(t)$, $h = 1, 2, ...10$. Thus all measurements $D_i(t)$, $z_{ik}(t)$, $D\sim_j(t)$, $z\sim_{jh}(t)$ are independent and equal at any values of the coefficients. There are 30 values of the fraction $D\sim_j(t)/z\sim_{jh}(t)$, 30 values of the fraction $z_{ik}(t)/D_i(t)$ and

[1] Hereinafter, photometric measurements were performed using a Multiscan RC spectrophotometer (LabSystems). Blanking was performed on a solution of equal amounts of medium without the corresponding substrates and cells.

[2] The counting of viable cells in suspension cultures is most easily performed in a Goryaev chamber, extracting a small volume of growth medium with cell suspension. This method is more convenient for a single measurement (which does not give sufficient statistical accuracy), but is difficult in case of several repetitions; moreover, the reliability of MTT-test results when working with suspension cell cultures is additionally reduced by artifactual capture of cells during washing, which has to be compensated by the use of pipette tips of special design (S-tips) [15].

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900 variants of their products of the form (2), i.e., the sample consists of 900 values of $\gamma(t)$. Therefore, the sample mean $\langle\gamma(t)\rangle$ and the standard deviation of the sample mean $m_{\gamma(t)}$ were calculated using standard formulas modified for this case:

$$\langle\gamma(t)\rangle = \frac{1}{900} \times \sum_{j=1}^{3} \sum_{k=1}^{10} \sum_{j=1}^{3} \sum_{h=1}^{10} \frac{\widetilde{D}_j(t)}{\widetilde{z}_{jh}(t)} \frac{z_{ik}(t)}{D_i(t)}; \quad (6)$$

$$m_{\gamma(t)} = \frac{1}{30\sqrt{899}} \times \left(\sum_{j=1}^{3} \sum_{k=1}^{10} \sum_{j=1}^{3} \sum_{h=1}^{10} \left(\frac{\widetilde{D}_j(t)}{\widetilde{z}_{jh}(t)} \times \right. \right.$$

$$\left. \left. \times \frac{z_{ik}(t)}{D_i(t)} - \langle\gamma(t)\rangle \right)^2 \right)^{1/2}; \quad (7)$$

The significance of differences between samples of 900 values for the time values, $\gamma(t_1)$ and $\gamma(t_2)$, and between samples of 18 values for the time values $C_T(t_1)$ and $C_T(t_2)$, where t_1 = 1, 16, 24, 48 h, t_2 = 1, 16, 24, 48 h, $t_1 \neq t_2$, was assessed using the Mann–Whitney–Wilcoxon test. This nonparametric test does not require a priori assumptions about the distribution function of random variables, the realization of which are the values of $\gamma(t)$ and $C_T(t)$. The assessment was considered reliable when the probability of realization of the alternative hypothesis was $p \leq 0.05$.

Results

The content of SARS-CoV-2 in the growth medium of adherent leukocyte culture is shown in **Fig. 1** (hereinafter, it should be kept in mind that a higher C_T value corresponds to a lower virus content in the test sample). During the 1st hour after virus inoculation, when the virus-containing fluid was in contact with the cells, the cells were infected. After removal of the virus-containing fluid, new particles in the medium accumulated as a result of virus replication in the infected cells. Taking into account the fact that these are different stages of the infection process, a break was made in the dynamic curves presented in Fig. 1.

One hour after virus inoculation (toward the end of the infection process), the ATPase activity of adherent leukocytes under SARS-CoV-2 infection *ex vivo* decreased relative to the uninfected control in a dose-dependent manner ($\gamma(1) < 1$), but then began to increase also in a dose-dependent manner: $\gamma(16) \sim 1$; $\gamma(24) \approx 1.2$; $\gamma(48) \approx 1.6$ (**Fig. 2**, *a*). The increase in ATPase activity between 16 and 48 h was nearly linear with a slight but reproducible excess of activity for a dose of 3 lg($TCID_{50}$) compared with 2 lg($TCID_{50}$).

AMPase activity changed differently from ATPase activity during infection (Fig. 2, *b*). During the initial period of infection, the level of 5′-nucleotidase increased rapidly compared with the uninfected control and remained at this level for at least 16 h after virus inoculation, then decreased by 24 h ($\gamma(24) \approx 0.8$ for both infecting doses) and increased slowly over the following 24 h ($\gamma(48) \sim 1$).

Changes in LDH (Fig. 2, *c*) and SDH (Fig. 2, *d*) activity during infection were similar: first a small sharp increase ($\gamma(1) \approx \gamma(16) \approx 1.2$), then a return to the activity value of the uninfected control ($\gamma(24) \sim 1$) and an increase over the following 24 h ($\gamma(48) \approx 1.8$). The decrease in dehydrogenase activity 24 h after infection is reproduced in all cases and is likely to be dose-dependent (most pronounced for ADH).

MPO activity in infected cells decreased rapidly in a dose-dependent manner compared with the uninfected control already within 1 h after virus inoculation (Fig. 2, *e*) and recovered to the previous level after 24 h ($\gamma(24) \sim 1$), and then it increased ($\gamma(48) \approx 1.4$).

CHO activity initially decreased in a dose-dependent manner (Fig. 2, *e*) but then returned to the level of the uninfected control as early as 16 h after virus inoculation ($\gamma(16) \approx \gamma(24) \approx 1.0$), and then it increased to $\gamma(48) \approx 1.2$ for dose 2 lg($TCID_{50}$) and to $\gamma(48) \approx 1.4$ for dose 3 lg($TCID_{50}$).

Discussion

Syrian hamsters (*Mesocricetus auratus*) are a convenient experimental model for reproducing SARS-CoV-2 coronavirus infection [1, 16, 17]. In this work, we used *ex vivo* infection of adherent leukocyte culture, which contains the main fraction of neutrophils undergoing a complex of morphofunctional changes upon contact with infectious agents [18]. Neutrophils, a very important innate immune cells, are rather short-lived leukocytes, and after 48 h their adhesive population is rapidly depleted (this, in particular, determines the chosen duration of the experiment).

It is known that AMPase (5′-nucleotidase) and ATPase are actively involved in the process of spatial transformation of the neutrophil plasma membrane during chemotaxis [18, 19]. In particular, 5′-nucleotidase is a regulator of the level of cyclic AMPase, which ensures the transmission of signals from the plasmalemma inside the cell and regulates the formation of extracellular adenosine, which mediates cytoprotection and a variety of physiological effects (suppression of inflammation, vasodilation, inhibition of thrombosis, anti-adrenergic, etc.) through specific receptors. [20]. When cells are damaged, they increase AMP and decrease ATP [21, 22]. Accordingly, an increase in AMPase activity and a decrease in ATPase activity were recorded during the first 16 h after virus inoculation. The early stages of coronavirus damage to target cells are associated with receptor-mediated fusion of virus-cell membranes and formation of special cisternae in the rough endoplasmic reticulum of the infected cells, in which virions are assembled [23, 24]. The monotonic increase in ATPase activity, start-

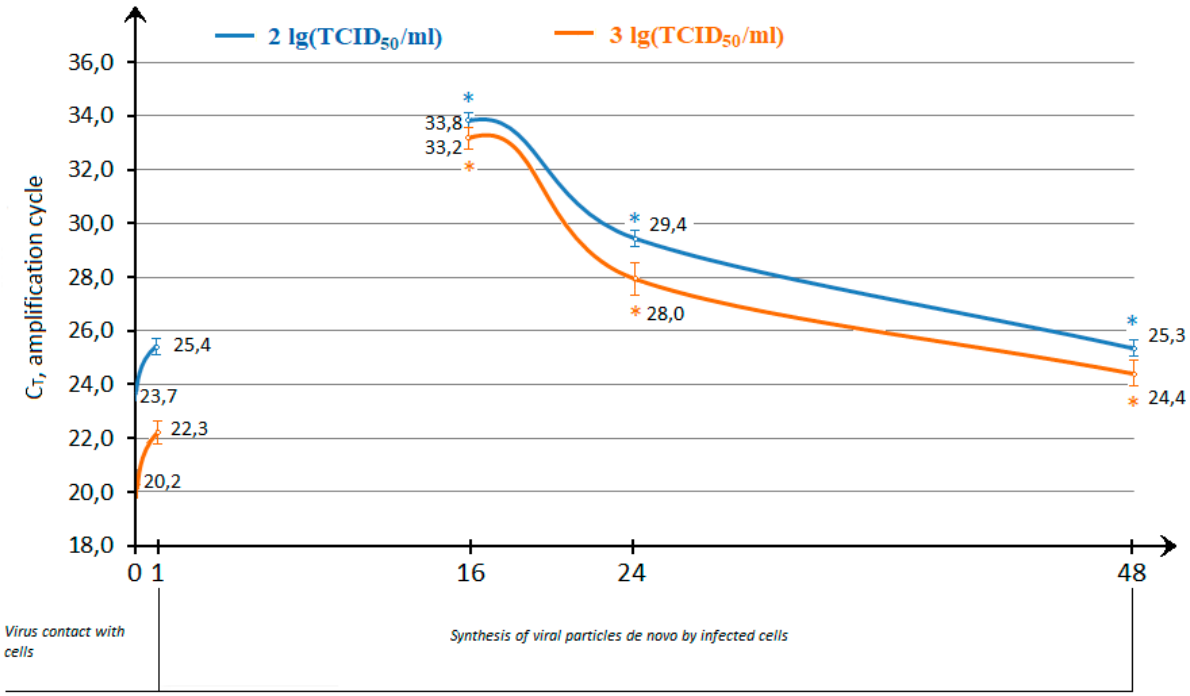

Fig. 1. Dynamics of viral load: lower C_T values correspond to higher virion concentration values, and vice versa.
During the 1st hour after virus inoculation, the concentration of virions decreases due to their penetration into target cells. After that, the medium changes and *de novo* accumulation of progeny virions produced by infected cells begins (*$p \le 0.05$ when compared to the C_T value at the previous time point).

ing at about 16 h after virus inoculation, is associated with active synthesis of viral proteins (both structural and regulatory) and virus-specific RNA. The repeated increase in AMPase activity later than 24 h after virus inoculation (Fig. 2, *a*, *b*) apparently reflects the process of secondary infection of leukocytes (including as a result of syncytium formation).

LDH is a zinc-containing intracellular enzyme that catalyzes the oxidation of lactic acid into pyruvate, takes part in glucose metabolism, is found in almost all cells of the body and is released when they are damaged [25]. Therefore, the level of serum LDH reliably marks the level of adverse effects of inflammatory reactions and other pathological processes. In particular, serum LDH levels have been found to be informative for assessing clinical severity and monitoring response to treatment in pneumonia in COVID-19 patients [26]. LDH belongs to coenzyme-independent flavoproteins and is part of the membrane-bound respiratory chain of membranes. The flavin group of this enzyme contains 4 iron atoms and is covalently bound to the protein, and the enzymatic activity of SDH depends on SH groups [25]. Mammalian SDH is not only involved in energy generation in mitochondria, but also plays a role in the oxygen sensitivity of the cells [27]. Dehydrogenase activity of infected cells first increases due to virus stimulation of replication processes and then decreases as a result of virus-induced cytodestruction: in the model of human immunodeficiency virus type 1 (Ortervirales: Retroviridae, *Lentivirus*) and immortalized cell lines of different origin, it was shown that the magnitude and rate of such dehydrogenase shift are proportional to the infecting dose and the level of pathogenicity of a particular strain (at the same infecting dose) [13, 14]. Under the conditions of the experiment described in this article, the dehydrogenase activity of SARS-CoV-2-infected primary culture of adherent leukocytes of the Syrian hamster (Fig. 2, *c*, *d*) has two maxima: on the 1st day, which is associated with virus entry into the cell, and after the 1st day – due to *de novo* virus production (Fig. 1, 2). Another explanation (related to the previous one): the first peak of dehydrogenase activity is associated with neutrophil viability, and the second peak with longer-lived monocytes (but the peak of the maximum was not reached due to the fact that the aim of the experiment was to study primarily the biochemistry of infected neutrophils).

MPO is a hemoprotein present in the azurophilic granules of neutrophils, released upon cell activation into the phagolysosome [28]. This enzyme is involved in the conversion of superoxide anion radical to hypochlorous acid, realizing the cell's defense against excessive reactive oxygen species mediators [29]. After phagocyte activation, degranulation occurs and MPO is secreted into the phagosome or into the extracellular space. MPO is an important part of antimicrobial activity of phagocytes, providing innate nonspecific immunity. *In vivo*, MPO is released into the extracellular fluid (specifically, into the blood) if for any reason the neutrophil is unable to phagocytize a pathogen, during

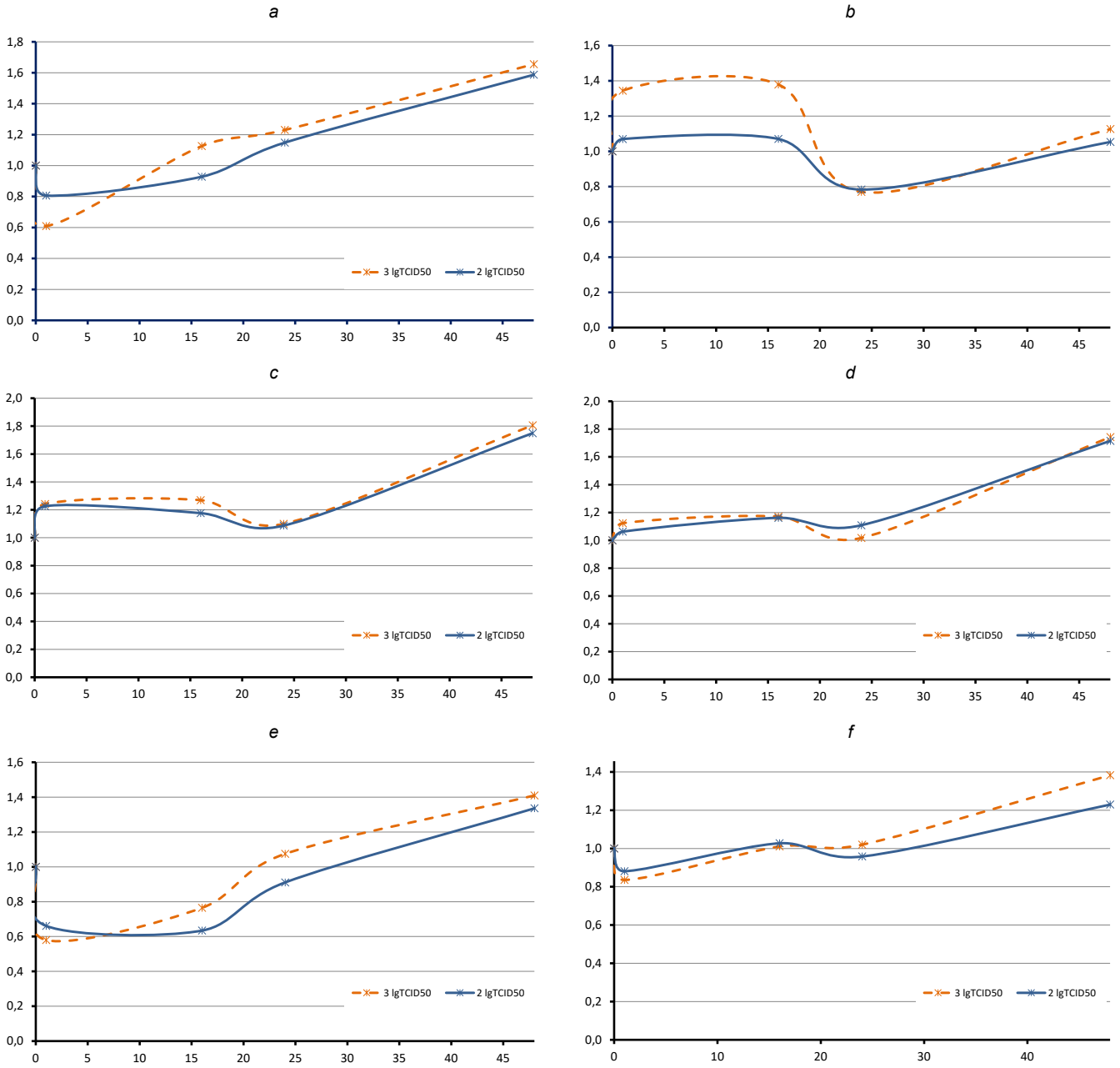

Fig. 2. Changes in enzyme activities as a result of SARS-CoV-2 infection: ATPase (*a*); AMPase (5'-nucleotidase) (*b*); LDH (*c*); SDH (*d*); MPO (*e*); CCO (*f*).

*$p \leq 0.05$ when compared with the γ value at the previous time point

cell lysis, or when the neutrophil is exposed to various soluble factors [28].

When using automated cytochemical blood cell counters in patients diagnosed with COVID-19, a decrease in MPO activity was observed [9]. At the same time, when neutrophil extracellular traps forming one of the lines of defense against pathogens (together with viruses, including SARS-CoV-2) are formed, an increase in MPO activity in the extracellular space is detected [30–32]. The decrease in MPO content in adherent leukocyte culture within 1 day after virus inoculation (Fig. 1, *e*) can be explained by the fact that under the influence of SARS-CoV-2 infection neutrophils excrete MPO into the extracellular space and form neutrophil-like extracellular trap structures *ex vivo*.

CCO localizes mainly on the inner mitochondrial membrane, where it captures protons from the intramitochondrial matrix and, by transferring electrons from cytochrome c to oxygen, reduces O_2 to H_2O. This enzyme plays an important role in the functioning of the aerobic link of the respiratory chain and energy production in eukaryotic cells [25]. Therefore, the decrease in CCO activity correlates with the decrease in ATPase

activity during the first hours after virus inoculation (see Fig. 2, *a*, *e*). Furthermore, in leukocytes, the activity of CCO serves as a reliable indicator of the level of oxidative metabolism, and its activity increases during cell death [25] — this is the effect observed in the culture of adherent leukocytes by the end of the 1st day after virus inoculation (Fig. 2, *e*).

The observed changes in the enzymatic spectrum of SARS-CoV-2-infected leukocytes are dose-dependent (Fig. 2): the modulus of such changes is proportional to the infecting dose of the virus. When analyzing the dynamics of enzymatic activity, it should be taken into account that by the end of the 1st day after virus inoculation, the cellular composition of the adherent leukocyte culture decreases at the expense of short-lived neutrophils, but at the same time, longer-lived cells remain in the culture.

Of course, we cannot exclude that SARS-CoV-2 virus-infected Vero E6 cells produce soluble exogenous factors that could affect cell physiology during infection, since virus-containing Vero E6 cell culture supernatant was used. However, it is known that Vero and Vero E6 cell lines do not produce type I interferon due to loss of the type I interferon gene cluster [33, 34] and are defective in the production of interferons-α-1/13, α-2, α-4, α-6, α-8, α-14, α-17, α-21, β-1 and ω-1 [33]. As for the remaining virus-containing soluble factors, they may be the subject of further studies.

Conclusion

The observed changes in enzymatic activity in neutrophils *ex vivo* infected with SARS-CoV-2 indicate a decrease in the microbicidal potential of these innate immunity cells, which is one of the causes of immune system dysfunction in COVID-19.

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Prediction of the incidence of lyme disease using mathematical modeling methods (using the example of the Kirov region)

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Annotation

Introduction. The Kirov region is an endemic region for Lyme disease (ixodic tick-borne borreliosis), which is caused by climatic conditions, an abundance of ticks and their feeders. The economic damage caused by Lyme disease includes the cost of treating patients and eliminating natural foci. Morbidity forecasting is necessary for planning preventive measures (acaricide treatments, awareness-raising activities with the population) and entomological monitoring. The effectiveness of such measures exceeds the above costs, which underlines the relevance of the study.

The **aim** of the study is to analyze the influence of various factors on the incidence of Lyme disease using mathematical modeling methods for further epidemiological forecasting using the example of the Kirov region.

Materials and methods. The data of the state reports «On the state of sanitary and epidemiological welfare of the population in the Kirov region» for 2006–2023 on the incidence of Lyme disease, the first and last reported cases of tick attachment to humans and the volume of acaricide treatments were studied. Hydrometeorological data: monthly and annual averages of air temperature, humidity, and precipitation. Spearman correlation analysis and multiple regression analysis were performed using the «Excel MS Office-2021» and «Statistica Advanced 12 for Windows RU» software. The level of $p < 0.05$ was chosen as a criterion of statistical significance.

Results. The interval forecast of incidence is up to 18.67 by 2024, 16.51 by 2025, and 14.36 per 100,000 population by 2026. Correlations between climatic factors and morbidity have been identified. A negative reliable correlation of moderate density was revealed between the incidence of Lyme disease in the Kirov region and the volume of acaricide treatments. Two forecasting models have been developed: based on the timing of the first and last reported cases of tick bites; based on hydrometeorological factors and the volume of acaricide treatment.

Conclusion. The incidence of Lyme disease in the Kirov region is characterized by a downward trend. Mathematical models for predicting morbidity in the Kirov region are proposed.

Keywords: *Lyme disease, morbidity, mathematical model, abiotic factors, biotic factors, anthropogenic factors*

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Оригинальное исследование
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Прогнозирование заболеваемости иксодовым клещевым боррелиозом с использованием методов математического моделирования (на примере Кировской области)

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Аннотация

Введение. Кировская область — эндемичный регион по иксодовому клещевому боррелиозу (ИКБ), что обусловлено климатическими условиями, обилием клещей и их прокормителей. Экономический ущерб от ИКБ включает затраты на лечение больных и ликвидацию природных очагов. Прогнозирование забо-

леваемости необходимо для планирования профилактических мероприятий (акарицидных обработок, информационно-разъяснительной работы с населением) и энтомологического мониторинга. Эффективность таких мер превышает вышеуказанные затраты, что подчёркивает актуальность исследования.
Цель исследования — изучение влияния различных факторов на заболеваемость ИКБ с использованием методов математического моделирования для дальнейшего эпидемиологического прогнозирования на примере Кировской области.
Материалы и методы. Изучены данные государственных докладов «О состоянии санитарно-эпидемиологического благополучия населения в Кировской области» за 2006–2023 гг. по заболеваемости ИКБ, первому и последнему зарегистрированным случаям присасывания клеща к человеку и объёму акарицидных работ. Гидрометеорологические данные: среднемесячные и среднегодовые значения температуры воздуха, влажности воздуха и объёма осадков. Проводили корреляционный анализ по Спирмену и множественный регрессионный анализ, в качестве критерия статистической значимости был выбран уровень $p < 0,05$.
Результаты. Интервальный прогноз заболеваемости: к 2024 г. — до 18,67 на 100 тыс. населения, к 2025 г. — 16,51, к 2026 г. — 14,36. Выявлены корреляции между климатическими факторами и заболеваемостью ИКБ, отрицательная достоверная корреляционная связь умеренной тесноты между заболеваемостью ИКБ в Кировской области и объёмом акарицидных работ. Разработаны две модели прогнозирования: на основе сроков первого и последнего зарегистрированных случаев присасывания клещей; на основе гидрометеорологических факторов и объёма акарицидных работ.
Заключение. Заболеваемость ИКБ в Кировской области характеризуется тенденцией к снижению. Предложены математические модели для прогнозирования заболеваемости ИКБ в Кировской области.

Ключевые слова: *иксодовый клещевой боррелиоз, заболеваемость, математическая модель, абиотические факторы, биотические факторы, антропогенные факторы*

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

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

Для цитирования: Утенкова Е.О., Княжев И.С., Щур Н.С. Прогнозирование заболеваемости иксодовым клещевым боррелиозом с использованием методов математического моделирования (на примере Кировской области). *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2025;102(2):179–189.
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Introduction

Lyme disease is a natural focal zoonotic disease with a vector-borne mechanism of transmission caused by bacteria of the genus *Borrelia* of the family *Spirochaetaceae*, transmitted by ticks of the genus *Ixodes*. In the epidemiologic aspect, the *I. persulcatus* and *I. ricinus* ticks are of significance, the role of other species is not excluded [1–3]. The Kirov region is located in the north of the Volga Federal District and is characterized by the highest level of Lyme disease incidence not only in the district, but also in Russia [4]. Most of the territory of the subject under study is located in the middle and southern taiga zone, which is characterized by favorable conditions for the activity of ticks – vectors of Borrelia and their feeders [5]. Natural focal infections, which include Lyme disease, are climate-dependent, as the variation of weather conditions not only affects the habitat of ticks, expanding their range, but also affects their vital activity. High average annual temperature, warm interseasons, abundant precipitation, increased air and soil humidity, increased intensity of solar radiation contribute to the survival, early activation, reproduction, and prolongation of the period of activity of mites [6–9]. Analysis of the influence of abiotic, biotic and anthropogenic factors on the tick population plays a key role in epidemiological surveillance, as it allows predicting the activity and distribution of ticks, optimizing preventive measures and reducing the risks of mass infection of the population [4, 10–16]. Taking into account the above facts, it becomes clear that tick-borne infections are a serious threat to both public health and the economy of the region. The high intensity of the epidemic process of Lyme disease can lead to significant financial losses due to the costs of medical care, increased temporary disability, long-term complications and reduced efficiency of the production sector [17–19].

All of this makes it important to forecast the level of Lyme disease incidence. Predicting the next rise in the incidence of Lyme disease will allow timely implementation of a set of preventive measures: acaricide treatment of territories and informing the population about protection measures. The economic benefit and efficiency of such measures significantly exceeds the costs of eliminating natural foci and treating patients [10, 14–16, 20].

The aim of the study is to investigate the influence of various factors on the incidence of Lyme disease using mathematical modeling methods for further prediction of Lyme disease incidence, using the example of the Kirov region.

Materials and methods

For retrospective analysis of Lyme disease incidence in the Kirov region we used the data of state reports of the Department of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare in the Kirov region "On the state of sanitary-epidemiological well-being of the population in the Kirov region" for 2006–2023 by indicators: Lyme disease incidence rates (per 100 thousand population) in the Kirov region, the first registered case of tick bite (February, March and April) from 2006 to 2023, the last registered case of tick bite on a person (September, October and November) from 2006 to 2023, and the volume of acaricide treatment (ha).

As hydrometeorological data we used data from the weather archive of Pobedilovo airport by months (January-December) for 2006–2023: mean monthly and mean annual values of air temperature (°C), air humidity (%) and precipitation volume (mm) for 2006–2023. The results were processed using methods of mathematical statistics using Excel MS Office-2021 and Statistica Advanced 12 for Windows RU standard software packages. Spearman correlation analysis was used to establish relationships between the studied variables. Multiple regression analysis was used to assess the possibility of prediction. The level of $p < 0.05$ was chosen as the criterion of statistical significance, which corresponds to a 5% probability of error of the first kind.

Results

In 2006–2023, the epidemic process in Kirov region was characterized by a downward trend in Lyme disease morbidity (**Fig. 1**). Thus, there is a decrease in Lyme disease incidence in Kirov region – annually on average by 2.2 per 100 thousand population ($R^2 = 0.660$).

Indicators of the dynamic series of Lyme disease incidence (per 100 thousand population) in Kirov region are shown in **Table 1**.

Forecasting of Lyme disease incidence in the Kirov region till 2026 has been carried out. With an error probability of 5%, it can be stated that the Lyme disease incidence rate on average for 2024 will be up to 18.67 per 100 thousand population, for 2025 — up to 16.51, for 2026 — up to 14.36 (**Fig. 2**).

To study the influence of the beginning and end of the epidemic season (the period of high activity of ticks — vectors of *Borrelia*) on the Lyme disease incidence in the Kirov region, a multiple linear regression model was built, where the variable was: y_1 — incidence of Lyme disease in the Kirov region per 100 thousand population. Factors: x — time factor (the period from 2006 to 2023), the first registered case of tick bites to a person (February, March and April) from 2006 to 2023, the last reported case of tick to human (September, October and November) from 2006 to 2023. Since the model used qualitative attributes, dummy variables were introduced: z_1 (1 — for the factor value March, 0 — for the rest of the factor values); z_2 (1 — for the factor value April, 0 — for the rest of the factor values); h_1 (1 — for the factor value September, 0 — for the rest of the factor values); h_2 (1 — for the factor value October, 0 — for the rest of the factor values). The mathematical model is: $y_1 = 4433.314 - 2.190x + 9.614z_1 + 4.629z_2 - 19.565h_1 - 3.617h_2$ (**Table 2**). The coefficient of determination is $R^2 = 0.718$. This means that the Lyme disease incidence rate is 71.8% determined by factors x, z_1, z_2, h_1, h_2 and 28.2% by unaccounted factors. Fisher's coefficient < 0.05, at 5% level the model is recognized as significant. At the 5% level, the factor x is significant. It can be said that every year the Lyme disease incidence in the Kirov region decreases on average by 2.19 per 100 thousand population.

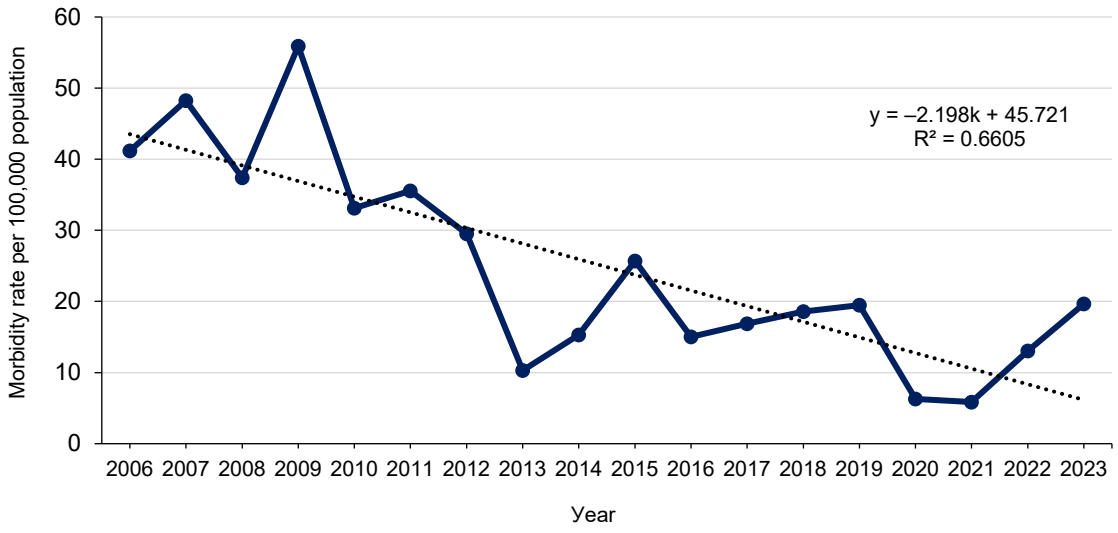

Fig. 1. The dynamics of the Lyme disease incidence in the Kirov region in 2006–2023.

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Table 1. Indicators of the dynamic range of Lyme disease incidence per 100,000 population of the Kirov region

Year	The registered Lyme disease incidence per 100,000 population of the Kirov region	Absolute increase	Growth rate, %	The value of a 1% increase	Growth rate, %
2006	41.19	–	–	–	–
2007	48.24	7.1	17.1	41.19	117.1
2008	37.42	–10.8	–22.4	48.24	77.6
2009	55.9	18.5	49.4	37.42	149.4
2010	33.11	–22.8	–40.8	55.90	59.2
2011	35.57	2.5	7.4	33.11	107.4
2012	29.52	–6.1	–17.0	35.57	83.0
2013	10.3	–19.2	–65.1	29.52	34.9
2014	15.31	5.0	48.6	10.30	148.6
2015	25.71	10.4	67.9	15.31	167.9
2016	15.03	–10.7	–41.5	25.71	58.5
2017	16.88	1.9	12.3	15.03	112.3
2018	18.58	1.7	10.1	16.88	110.1
2019	19.48	0.9	4.8	18.58	104.8
2020	6.29	–13.2	–67.7	19.48	32.3
2021	5.86	–0.4	–6.8	6.29	93.2
2022	13.04	7.2	122.5	5.86	222.5
2023	19.68	6.6	50.9	13.04	150.9
2023/2006	–	–21.5	–52.2	41.19	47.8

Since qualitative features were used in the model: the first registered case of tick-borne infections (February, March and April) from 2006 to 2023, the last registered case of tick-borne infections (September, October, November) from 2006 to 2023, correction factors were introduced before dummy variables (**Table 3**). The Lyme disease incidence in the Kirov region per 100 thousand population in the system varies: with the first registered

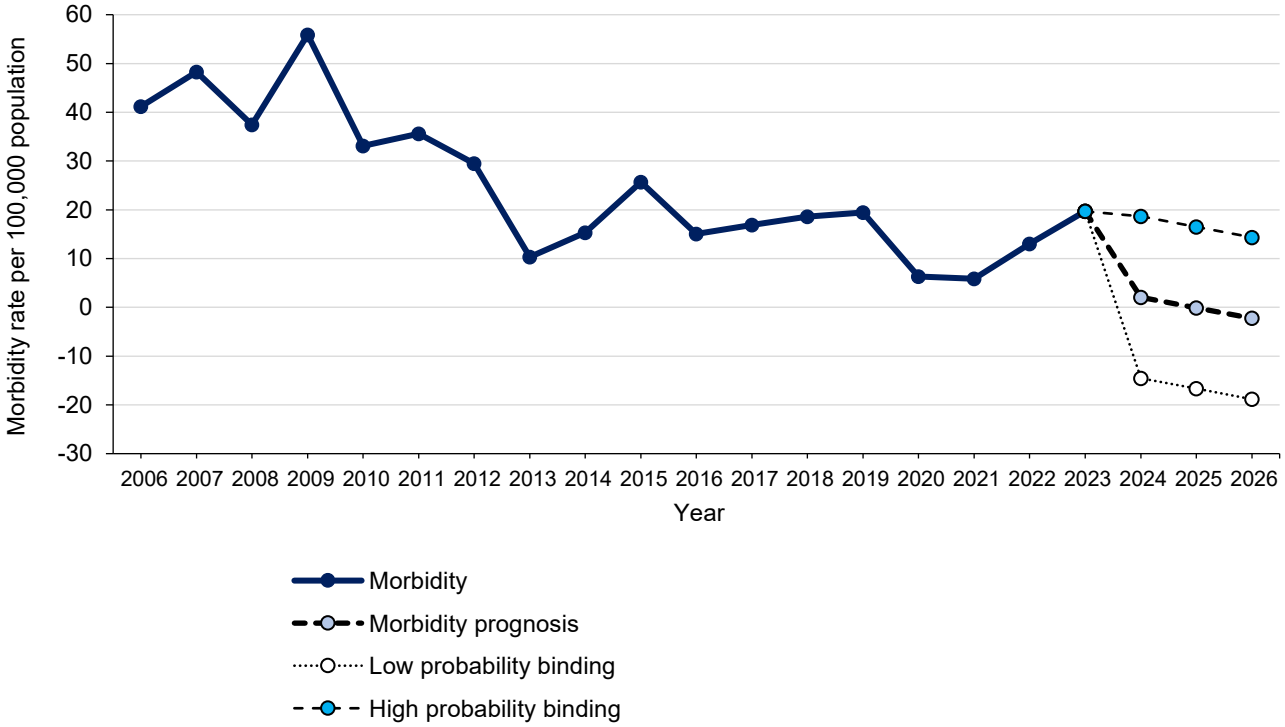

Fig. 2. Interval forecast chart for the Lyme disease incidence in the Kirov region until 2026.

Table 2. Standardized β-coefficients for the multiple regression model

Factor	β*	SE of β*	β	SE of β	t(69)	*p*-value
Intercept	–	–	4433,314	904,802	4,900	0,000
x	–0.810	0.165	–2.190	0.447	–4.905	0.000
z_1	0.340	0.316	9.614	8.925	1.077	0.303
z_2	0.165	0.314	4.629	8.804	0.526	0.609
h_1	–0.319	0.149	–19.565	9.155	–2.137	0.054
h_2	–0.115	0.174	–3.617	5.443	–0.664	0.519

Note. Intercept is a free term of the regression equation; β* — standardized regression coefficient; SE of β* — standard error of standardized regression coefficient; β — regression coefficient; SE of β — standard error of regression coefficient; t (69) — calculated value of T-criterion in assessing the significance of regression coefficient; p-value — the significance level.

Table 3. Correction coefficients of regression

The value of the factor	β	$\beta^{попр} = \beta - \Delta$	The value of the factor	β	$\beta^{попр} = \beta - \Delta$
The first recorded case of a tick bite of a human was in February	0.000	–4.750	The last recorded case of a bite of a human was in September	–19.565	–11.840
The first recorded case of a tick bite of a human in March	9.614	4.870	The last recorded case of a tick bite of a human was in October	–3.617	4.110
The first recorded case of a tick bite of a human in April	4.629	–0.120	The last recorded case of a tick bite of a human was in November	0.000	7.730
The amount	14.24	–	The amount	–23.18	–
Δ	4.75	–	Δ	–7.73	–

Note. β — standardized regression coefficient; $\beta^{попр}$ — standardized correction for regression coefficient.

case of tick bite in February the incidence of Lyme disease is below average, in March — above average, in April — below average; with the last registered case of tick bite in September the Lyme disease incidence is below average, in October and November — above average.

In the Kirov region there is a tendency to increase the areas subject to acaricide treatments (**Fig. 3**). Based on the regression coefficient value, it can be concluded that every year the volume of acaricide treatments in the Kirov region increases on average by 125.87 ha. Indicators of the dynamic series of acaricide treatment volume in the Kirov region are shown in **Table 4**.

The correlation matrix of dependence of Lyme disease incidence in the Kirov region per 100 thousand population from 2006 to 2023 on hydrometeorological factors (by months) and the volume of acaricide treatments was constructed (**Table 5**). A strong negative relationship between Lyme disease incidence and time factor, as well as negative relationships of medi-

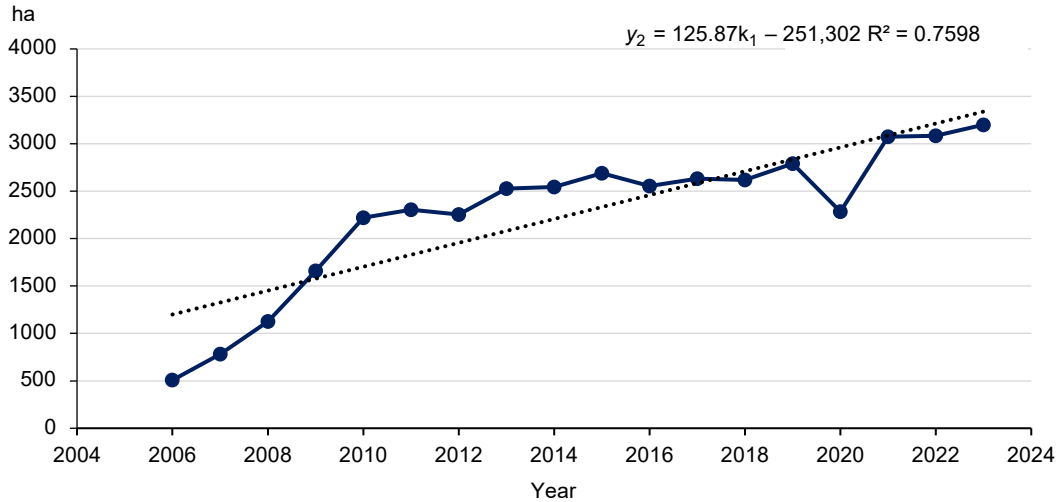

Fig. 3. The volume of acaricide treatments in the Kirov region in 2006–2023.

um strength between Lyme disease incidence and the volume of acaricide treatments were revealed. A medium positive correlation was found between the Lyme disease incidence and air temperature in September ($r = 0.51$; $p < 0.05$). Weak positive correlations were found between air humidity in June and August and Lyme disease incidence ($r = 0.47$; $p < 0.05$ and $r = 0.47$; $p < 0.05$, respectively). We found weak positive correlations between December precipitation volume and Lyme disease incidence ($r = 0.49$; $p < 0.05$), medium positive correlations between June, August and November precipitation volume and Lyme disease incidence ($r = 0.52$; $p < 0.05$; $r = 0.68$; $p < 0.05$ and $r = 0.66$; $p < 0.05$, respectively), and a strong positive association between October precipitation volume and Lyme disease incidence ($r = 0.75$; $p < 0.05$).

The correlation matrix of dependence of Lyme disease incidence (per 100 thousand population) in Kirov region on average annual values of hydrometeorological factors (air temperature and humidity, precipitation for 2006-2023) and the volume of acaricide treatment has been constructed (**Table 6**).

For multiple regression analysis of the resultant variable we used the indicator y_3 — incidence of Lyme disease (per 100 thousand population) in the Kirov region. Sample correlation coefficients are presented in Table 6. Factor signs and model: v_1 — volume of acaricide treatment (ha); v_2 — average annual air temperature (°C); v_3 — average annual air humidity (%); v_4 — average annual precipitation (mm); $y_3 = -70.117 - 0.003v_1 - 1.304v_2 + 0.993v_3 + 93.133v_4$. The regression equation is statistically valid at the significance level of $p < 0.05$. The 75.1% of the variation in the incidence of Lyme disease is explained by the variation in factor attributes ($R^2 = 0.751$).

Based on the standardized β-coefficients, the influence of factors on the dependent variable was assessed (**Table 7**). The incidence of Lyme disease in Kirov region per 100 thousand population in the system is changing:

- the average incidence of Lyme disease decreases when the volume of acaricide treatments is increased;
- an increase in average annual air temperature leads to a decrease in the incidence of Lyme disease on average;
- an increase in average annual air humidity leads to an increase in the incidence of Lyme disease on average;
- with an increase in average annual precipitation, the incidence of Lyme disease increases on average.

Discussion

The Lyme disease incidence in the Kirov region in 2006–2023 had an uneven tendency. When comparing the incidence in 2023 with 2006, there is a decrease in the intensity of the epidemic process manifestation

Table 4. Indicators of the dynamic range of acaricide treatments in the Kirov region

Year	The volume of acaricide treatments in the Kirov region, ha	Absolute increase, ha	Growth rate, %	The value of a 1% increase	Growth rate, %
2006	507.0	–	–	–	–
2007	782.7	275.7	54.4	507.00	154.4
2008	1125.0	342.3	43.7	782.70	143.7
2009	1660.5	535.5	47.6	1125.00	147.6
2010	2220.6	560.1	33.7	1660.50	133.7
2011	2303.4	82.8	3.7	2220.60	103.7
2012	2253.0	–50.4	–2.2	2303.40	97.8
2013	2526.3	273.3	12.1	2253.00	112.1
2014	2545.7	19.5	0.8	2526.27	100.8
2015	2688.4	142.7	5.6	2545.73	105.6
2016	2553.7	–134.7	–5.0	2688.44	95.0
2017	2633.2	79.5	3.1	2553.72	103.1
2018	2617.7	–15.5	–0.6	2633.20	99.4
2019	2790.8	173.1	6.6	2617.70	106.6
2020	2284.3	–506.5	–18.1	2790.80	81.9
2021	3075.3	791.0	34.6	2284.30	134.6
2022	3083.0	7.7	0.3	3075.26	100.3
2023	3200.7	117.7	3.8	3083.00	103.8
2023/2006	–	2693.7	531.3	507.00	631.3

Table 5. Correlation matrix of the dependence of the Lyme disease incidence in the Kirov region per 100000 population from 2006 to 2023 on the average monthly values of hydrometeorological factors and the volume of acaricide treatments

Indicator	Lyme disease incidence in the Kirov region per 100000 population	
	r	*p*
The time factor, years	–0.77	< 0.05
Volume of acaricide treatments, ha	–0.64	< 0.05
Air temperature, °C		
in January	–0.28	> 0.05
in February	–0.41	> 0.05
in March	0.18	> 0.05
in April	–0.29	> 0.05
in May	–0.10	> 0.05
in June	–0.15	> 0.05
in July	–0.16	> 0.05
in August	–0.19	> 0.05
in September	0.51	< 0.05
in October	0.15	> 0.05
in November	–0.11	> 0.05
in December	0.24	> 0.05
Air humidity, %		
in January	0.22	> 0.05
in February	–0.28	> 0.05
in March	0.36	> 0.05
in April	0.15	> 0.05
in May	–0.15	> 0.05
in June	0.47	< 0.05
in July	0.35	> 0.05
in August	0.47	< 0.05
in September	–0.04	> 0.05
in October	0.05	> 0.05
in November	0.45	> 0.05
in December	0.20	> 0.05
Precipitation, mm		
in January	0.50	< 0.05
in February	0.03	> 0.05
in March	0.43	> 0.05
in April	0.36	> 0.05
in May	0.47	> 0.05
in June	0.52	< 0.05
in July	0.24	> 0.05
in August	0.68	< 0.05
in September	0.40	> 0.05
in October	0.75	< 0.05
in November	0.66	< 0.05
in December	0.49	< 0.05

(per 100 thousand population) by 52.2%. The most significant changes are observed in 2009 (increase by 49.4%) and in 2013 (decrease by 65.1%). The growth of Lyme disease morbidity in 2009 (by 49.4%) can be explained by the lengthening of the ticks activity season: it amounted to 208 days in the region on average, which is 14 days more compared to 2008 (194 days), by the increase in the number of ticks in the region, by the increase in the number of ticks active in the region (194 days), increase in the number and activity of vectors, which is indirectly evidenced by the increase in the number of visits to medical and preventive institutions for tick bites by 1.6 times from 13,432 people in 2008 to 21,477 people in 2009, and insufficient volume of acaricide treatments (2 times lower than required)[1]. The decrease in the incidence of Lyme disease in 2013 (by 65.1%) can be explained by a 1.5-fold increase in the volume of acaricide treatments (2526.3 ha in 2013 compared to 1660.5 ha in 2009), a decrease in the number and activity of vectors, which is indirectly evidenced by the decrease in the cases of seeking health care after tick bites by almost 3 times from 21,477 people in 2009 to 7219 people in 2009, and rodent control measures[2]. Despite the overall downward trend, the incidence dynamics remain unstable. After a significant drop in 2013, there is a gradual increase in the following years, followed by a decrease in 2020, which may be related to the COVID-19 pandemic[3]. The dynamics of incidence in 2005–2009 is explained by the lengthening of the tick activity season due to the warm and long autumn period, in which people were more often engaged in collecting wild plants in natural biotopes, continued work on garden plots, which led to an increase in contact of the population with ticks [7–9, 21, 22].

The interval forecast of Lyme disease incidence in the Kirov region for the 2024–2026 period demonstrates a downward trend. The observed decrease in the intensity of manifestations of the epidemic process can be explained by an increase in the volume of acaricide treatments, changes in weather conditions (a tendency to decrease the average annual precipitation). It should be noted that the influence of other factors cannot be excluded.

Early or, on the contrary, late onset of ixodid tick activation can lead to changes in the timing of the epidemic season [10–13]. The lengthening of the tick ac-

[1] The State report "On the sanitary and epidemiological situation in the Kirov region in 2009". URL: https://www.43.rospotrebnadzor.ru/documents/gosregdoklad/publications/svoddokl2009.pdf

[2] The State report "On the sanitary and epidemiological situation in the Kirov region in 2013". URL: https://www.43.rospotrebnadzor.ru/documents/gosregdoklad/publications/svoddokl2013.pdf

[3] The State report "On the sanitary and epidemiological situation in the Kirov region in 2020". URL: https://www.43.rospotrebnadzor.ru/documents/gosregdoklad/publications/gosudarstvennyy-doklad-2020.pdf

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Table 6. Correlation matrix of the dependence of Lyme disease incidence in the Kirov region per 100000 population on the average annual values of hydrometeorological factors and the volume of acaricide treatment

Correlated indicators	Incidence of Lyme disease	Volume of acaricide treatment, ha	Air temperature, °C	Air humidity, %	Precipitation, mm
Incidence of ixodic tick-borne borreliosis	1.00	–0.64	–0.03	0.35	0.74
Volume of acaricide treatment, ha	–0.64	1.00	–0.20	–0.10	–0.69
Air temperature, °C	–0.03	–0.20	1.00	0.19	–0.06
Air humidity, %	0.35	–0.10	0.19	1.00	0.40
Precipitation, mm	0.74	–0.69	–0.06	0.40	1.00

Table 7. Standardized β coefficients of the regression model

Factor	β*	SE of β*	β	SE of β	t(69)	*p*-value
Intercept	–	–	–70,117	56,530	–1,240	0,237
v_1	–0.147	0.218	–0.003	0.004	–0.675	0.512
v_2	–0.083	0.125	–1.304	1.955	–0.667	0.517
v_3	0.167	0.128	0.993	0.758	1.310	0.213
v_4	0.715	0.224	93.133	29.210	3.188	0.007

Note. Intercept is a free term of the regression equation; β* — standardized regression coefficient; SE of β* — standard error of standardized regression coefficient; β — regression coefficient; SE of β — standard error of regression coefficient; t(69) — calculated value of T-criterion in assessing the significance of regression coefficient; *p*-value — the significance level.

tivity season is a consequence of the warm and long fall period [21]. Using the method of multiple linear regression, we proposed a mathematical model of Lyme disease incidence in Kirov region per 100 thousand population, for prediction of which we used such indicators as the first and the last registered cases of ticks biting a person for 2006–2023. With the first registered case in February, the incidence of Lyme disease is below average by 4.75 per 100 thousand population, in March — above average by 4.87, in April — below average by 0.12. With the last reported case of a tick biting a person in September, the incidence of Lyme disease is below average by 11.84 per 100,000 population, and above average by 4.11 and 7.73 in October and November, respectively. It should be noted that any forecast cannot be absolute, as the influence of other factors can never be completely excluded. An example can be 2020, when the first victim of tick bites was registered in February, but the incidence of Lyme disease in the Kirov region in this year was low. In this case, the influence of anti-epidemic measures related to the COVID-19 pandemic cannot be excluded.

The area of acaricide treatments in 2010 compared to 2006 increased 4.4 times — from 507 to 2220.5 ha [9]. Our statistical analysis for 2006–2023 showed that the volume of acaricide treatments in the Kirov region increased annually by an average of 125.87 ha. We found a negative reliable correlation of moderate closeness between Lyme disease incidence in 2006–2023 and the volume of acaricide treatments during this period.

Biotic factors play no less significant role in the life cycle of ticks. Many vertebrates: hares, ungulates, rodents, birds serve as feeders for ticks and reservoirs for tick-borne pathogens. The increase in the number of animals directly correlates with the growth rate of the population of ixodid ticks [4, 23–25]. Anthropogenic factors should be considered from the position of economic activity and human behavior in nature. Active logging of forest areas leads to dynamic changes in microclimatic conditions. The replacement of old forest by young growth causes an increase in exposure to sunlight, changes in temperature, air humidity and upper soil layers. The cleared areas are populated with small-leaved tree species, shrubs, and grasses, creating a favorable niche for the reproduction of tick feeders and vectors [24, 25]. Reduction of species biodiversity of a particular ecosystem, resulting in disruption of food chains, is associated with an increased probability of tick-borne infection. For example, the decline of a key predator of small mammals, the red fox, in the northeastern and western United States has led to an increase in the incidence of Lyme disease in this region. In turn, active recreation or labor activity in open spaces during the warm season increases the probability of human contact with infected arthropod vectors of Borrelia [26–29]. These factors directly influence the risk of human contact with ticks, which is necessary to build an accurate predictive model of the epidemiologic risk of population morbidity.

Tick activity can be influenced by weather conditions [10]. Abiotic factors have a significant impact

on the survival and reproduction of ticks in a particular biogeocenosis. It has been proved that mean annual temperatures and seasonality of their fluctuations play a critical role in the life cycle of arthropods [23, 25]. Thus, mild winters contribute to higher survival rates of individuals, and warm interseasonal periods promote early activation and prolong the period of mite activity, which leads to habitat expansion [24, 25]. The abundance of precipitation, high relative air humidity and the degree of soil moisture are critical at all stages of the tick life cycle. The amount of solar radiation and daylight hours are positively related to mite activity [23]. From 1984 to 2010, a significant increase in the average annual air temperature and relative air humidity was recorded in the Kirov region. The main changes occurred in the autumn months, which had a favorable effect on the life activity of ixodid ticks [27, 30]. Russian scientists proposed a regression equation, where the dependent variable is the incidence of tick-borne encephalitis, and the factors are the temperature of May in season n in the medium-term cycle, the temperature of August in season n in the medium-term cycle, the temperature of October of the previous season ($n - 1$) and the temperature of November. According to the authors, the course of change in the incidence of tick-borne encephalitis in Irkutsk agrees quite well with the course of climate change in its macrocyclical and trend components. A study conducted in 2011 found that for three natural foci of tick-borne encephalitis (located in the territory of Novosibirsk, Irkutsk and Gorno-Altaisk), the common significant factors in predicting the incidence of this natural focal infection are: air humidity in November of the previous year, humidity in April and June, and temperature in June of the current year [31,32]. These factors were identified both in the construction of the logical solving function and in the regression equation. The positive influence of solar activity on the incidence of tick-borne encephalitis in Novosibirsk and Irkutsk was shown. Using the method of multiple linear regression, we proposed a mathematical model indicating the dependence of morbidity of tick-borne encephalitis on climatic indicators and the volume of acaricide treatments. Based on the values of regression coefficients the incidence of Lyme disease (per 100 thousand population) in the Kirov region in the system changes:

- increase in the volume of acaricide treatments per 1 hectare decreases the incidence of Lyme disease on average by 0.003 per 100 thousand population;
- an increase in the average annual air temperature by 1°C leads to an average decrease in Lyme disease morbidity by 1.304 per 100 thousand population;
- an increase in average annual air humidity by 1% leads to an average increase in the incidence of Lyme disease by 0.993 per 100 thousand population;
- an increase in the average annual precipitation by 1 mm leads to an average increase in the incidence of Lyme disease by 93.133 per 100 thousand population.

It should be noted that with an error probability of 5%, the average annual precipitation is significant, while the other factors are insignificant. Based on the values of β-coefficients, the greatest contribution to the change in the incidence of Lyme disease is made by the average annual precipitation volume.

Conclusion

Today, mathematical modeling methods are successfully used to predict the incidence of various nosological forms of infectious diseases. Lyme disease has long been an urgent problem for Russia as a whole and, especially, for regions with high morbidity, such as the Kirov region. The authors for the first time made an attempt to predict the level of morbidity of infectious diseases on the territory of this subject depending on some hydrometeorological and anthropogenic factors using mathematical modeling methods. A mathematical model for forecasting the incidence of Lyme disease in the Kirov region depending on the average annual values of climatic indicators and the volume of acaricide treatments was proposed. An interval forecast of Lyme disease incidence in the Kirov region for the 2024–2026 is made, which demonstrates a tendency to decrease the intensity of epidemic process manifestations. For a more accurate prediction of the level of Lyme disease incidence in the Kirov region, a set of factors is required, including the number and species diversity of tick feeders, changes in the structure of forest ecosystems due to anthropogenic impacts, human activity in natural areas during the season of increased tick activity, as well as geographical peculiarities of tick distribution in the administrative-territorial units of the region, taking into account hydrometeorological indicators. The study requires further development, including determination of the prospects for practical application of the proposed forecasting models. This will help to substantiate the necessity for further research and develop strategies to stabilize the epidemiological situation in the Kirov region.

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Possibilities and prospects of using biofluorescent proteins at the stage of preclinical evaluation of live vaccines, using the example of the *Yersinia pestis* vaccine strain EV NIIEG pTURBOGFP-B

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Abstract

Introduction. Currently, studies aimed at finding the most informative and optimized method for assessing the survival rate of the plague microbe vaccine strain in the body of animals vaccinated against plague are relevant.

Aim — to evaluate the feasibility of using biofluorescent proteins using the example of the *Yersinia pestis* vaccine strain EV NIIEG pTurboGFP-B (EVGFP) in combination regimens with immunomodulators at the stage of preclinical evaluation of live vaccines.

Materials and methods. Guinea pigs were immunized with EVGFP grown at 28ºC and 37ºC (EVGFP28 and EVGFP37, respectively), in combination with immunomodulators (azoximer bromide, AB, and human recombinant interferon gamma, HRI).

Results. Fluorescence microscopy revealed seeding (up to 600 m.c. in one field of view) with EVGFP cells at the site of culture introduction in all experimental groups on the 1st day. *In vivo* flow cytometry showed that on the 1st day in all experimental groups the phagocytic index (PI) averaged 94.5%, with a subsequent decrease by the 4th day by an average of 4.4 times (21.2%). On the 4th day of the study in the EVGFP37+AB group the PI exceeded the similar indicator in the EVGFP37 group by 1.8 times. On the contrary, in the EVGFP28+HRI group the PI decreased by 2.2 times relative to the similar indicator in the EVGFP28 group. In addition, in the EVGFP37+AB and EVGFP37+HRI groups, on day 4, the PI was 2 times higher than in the EVGFP28+AB and EVGFP28+HRI groups, respectively. In the EVGFP37 group, the phagocytic number was on average 1.5 times higher than in the EVGFP28 group.

Conclusion. The obtained data confirm the dependence of the outcome of *in vivo* interaction of the plague microbe with spleen phagocytes on the temperature of bacterial growth, as well as on the presence of AB and HRI. The use of biofluorescent proteins, as shown by the example of the EVGFP strain and the flow cytometry method, expands the possibilities for assessing live plague vaccines in preclinical studies.

Keywords: *biofluorescent Y. pestis vaccine strain EV NIIEG pTurboGFP-B, azoximer bromide, human recombinant interferon gamma, phagocytosis, macrophages, neutrophils, fluorescence microscopy, flow cytometry*

Ethics approval. The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the Consensus Author Guidelines for Animal Use (IAVES, 07/23/2010). The study protocol was approved at a meeting of the Bioethics Commission of the Russian Research Anti-Plague Institute "Microbe" (protocol No. 5 of May 25, 2023).

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Оригинальное исследование
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Перспектива применения биофлуоресцентных белков на этапе доклинической оценки живых вакцин на примере вакцинного штамма *Yersinia pestis* EV НИИЭГ pTURBOGFP-B

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Аннотация

Введение. Актуальны исследования, направленные на поиск наиболее информативного и оптимизированного метода оценки приживаемости вакцинного штамма чумного микроба в организме животных, привитых против чумы.

Цель работы — оценить целесообразность использования биофлуоресцентных белков на примере вакцинного штамма *Yersinia pestis* EV НИИЭГ pTurboGFP-B (EVGFP) в схемах сочетанного применения с иммуномодуляторами на этапе доклинической оценки живых вакцин.

Материалы и методы. Морских свинок иммунизировали EVGFP, выращенным при 28°С и 37°С (EVGFP28 и EVGFP37 соответственно), в сочетании с иммуномодуляторами: азоксимера бромидом (АБ) и интерфероном-γ человеческим рекомбинантным (ИЧР).

Результаты. Методом люминесцентной микроскопии выявлено обсеменение (до 600 м.к. в одном поле зрения) клетками EVGFP места введения культуры во всех опытных группах на 1-е сутки. Методом проточной цитометрии *in vivo* установлено, что на 1-е сутки во всех опытных группах фагоцитарный индекс (ФИ) составлял в среднем 94,5% с последующим снижением к 4-м суткам в среднем в 4,4 раза (на 21,2%). На 4-е сутки исследования в группе EVGFP37+АБ ФИ превосходил в 1,8 раза аналогичный показатель в группе EVGFP37. Напротив, в группе EVGFP28+ИЧР ФИ снижался в 2,2 раза относительно аналогичного показателя в группе EVGFP28. Кроме того, в группах EVGFP37+АБ и EVGFP37+ИЧР на 4-е сутки ФИ в 2 раза превышали показатели в группах EVGFP28+АБ и EVGFP28+ИЧР соответственно. В группе EVGFP37 фагоцитарное число превосходило в среднем в 1,5 раза показатель в группе EVGFP28.

Заключение. Получены данные, подтверждающие зависимость исхода взаимодействия *in vivo* чумного микроба с фагоцитами селезёнки от температуры выращивания бактерий, а также от присутствия АБ и ИЧР. Применение биофлуоресцентных белков, как показано на примере штамма EVGFP и метода проточной цитометрии, расширяет возможности оценки живых вакцин против чумы на доклиническом этапе.

Ключевые слова: *биофлуоресцентный вакцинный штамм Y. pestis EV НИИЭГ pTurboGFP-B, азоксимера бромид, интерферон-γ человеческий рекомбинантный, фагоцитоз, макрофаги, нейтрофилы, люминесцентная микроскопия, проточная цитофлуориметрия*

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Introduction

In recent years, various fluorescent proteins have been used as easily detectable markers of microorganisms in biological and medical research. One of such proteins is the green fluorescent protein GFP (green fluorescent protein) found in the jellyfish *Aequorea victoria* [1, 2]. The advantages of using GFP protein compared to other reporter proteins are its ability to fluoresce in the absence of any cofactor or substrate, prolonged signal, low metabolic toxicity, and absence of background fluorescence. To date, a wide variety of different plasmids engineered with GFP are available and have been successfully used. For example, the commercial vector pTurboGFP-B provides stable expression and rapid maturation of fluorescent protein [2].

Engineered strains of *Y. pestis* producing GFP are used to study the conditions of symbiosis formation with protozoa and/or modeling of interaction with host cells (mammals — rodents) [3, 4], with a vector (fleas) [5]. The fluorescent protein GFP as a biosensor has been successfully used to detect *Y. pestis* in mixed cultures, tracking bacteria *in vitro* and *in vivo*, establishing intracellular localization of cells of recombinant strains of *Y. pestis*, assessing phagocytosis of *Y. pestis* by macrophages of BALB/c mice [6–9]. Genomic technologies have made it possible to use GFP as a molecular vector for the development of vaccines against various bacterial and viral infections of humans and animals [1, 10].

One of the main tasks of preclinical studies in the development of live vaccines against plague is to determine the degree of residual virulence (harmlessness) of the vaccine strain of the plague microbe in terms of engraftment and spread in the macroorganism. Traditionally, live cell engraftment of plague microbe strains has been recorded using the bacteriological method. Labeling of strains of *Y. pestis* strains with fluorescent proteins opens new possibilities that allow *in vivo* monitoring microorganisms using a number of other techniques, including luminescence microscopy, which provides visualization of the plague pathogen by the presence of a specific fluorescent glow [3, 4], and flow cytometry, which helps to determine the number of live microbial cells by light scattering and fluorescence intensity of each individual cell in a suspension of organs [11].

It is known that the reference *Y. pestis* vaccine strain EV NIIEG takes root and reproduces in the organs of laboratory animals, which contributes to the development of active plague immunity [12]. One of the most significant factors influencing the engraftment of the plague pathogen, which is able to multiply in various conditions similar to its habitat in the bodies of warm-blooded mammals (37°C) and poikilothermic fleas (20–28°C), is temperature [13]. The temperature-dependent adaptation strategy of *Y. pestis* is an essential part of the concept of the bacterium overcoming innate immunologic defense mechanisms, such as phagocytosis, and the induction of an inflammatory response by macrophages and neutrophils [13].

The use of various immunomodulators in the scheme of plague vaccination allows to reduce the antigenic load on the body without reducing the intensity of the immune response. Thus, azoxymer bromide (AB) in combination with the *Y. pestis* vaccine strain EV NIIEG has a stimulating effect on the reactivity of cells of the phagocytic system of laboratory animals, increases the protective activity of live plague vaccine almost 3 times and, as well as human recombinant interferon-γ (HRI), stimulates antibody formation and cytokine response [14–16].

Taking into account all of the above facts, an attempt was made to expand the possibility of assessing the effectiveness and duration of the non-sterile phase of the immune response, which is an obligatory stage in the formation of specific plague immunity, through the use of fluorescent proteins in testing various schemes of live plague vaccine administration.

The aim of the study is to evaluate the feasibility of using biofluorescent proteins on the example of the *Y. pestis* vaccine strain EV NIIEGP pTurboGFP-B in schemes of its combined use with immunomodulators at the stage of preclinical evaluation of live vaccines.

Materials and methods

To create a GFP-producing strain, the *Y. pestis* vaccine strain EV NIIEG was used. The gene encoding TurboGFP synthesis was introduced as part of the commercial vector plasmid pTurboGFP-B (4103 bp, "Evrogen") by electroporation according to the recommendations [17]. Electroporation was performed on a Gene Pulser Xcell device ("Bio-Rad") using a standard protocol for prokaryotic cells. The presence of commercial plasmid was determined by antibiotic resistance marker by seeding the strain on LB agar plates with 50 μg/mL ampicillin. Colonies were viewed with the naked eye, as well as on a "Axio Lab.A1" fluorescent microscope ("Carl Zeiss Microscopy GmbH"), registering intense fluorescent glow in the ultraviolet region of the spectrum. The constructed recombinant strain was designated *Y. pestis* EV NIIEG pTurboGFP-B (EVGFP) and was deposited under the number KM 2115 in the State Collection of Pathogenic Bacteria of the Russian Anti-Plague Institute "Microbe" of Rospotrebnadzor.

The *Y. pestis* strain EV NIIEG was grown on LB agar (pH 7.2), and the biofluorescent strain EVGFP was grown on LB agar (pH 7.2) with ampicillin (50 mg/mL) for 48 h at 28°C. Furthermore, EVGFP cell culture with increased resistance to leukocyte phagocytosis was used [18]. For this purpose, EVGFP cells were seeded from a 2-day 28°C culture at a final concentration of 1×10^7 CFU/mL on LB broth (pH 7.2) and cultured under aeration conditions at 37°C for 18 h. Suspensions were prepared from EVGFP cultures grown at 28°C (EVGFP28) and 37°C (EVGFP37) using the turbidity

standard CCA 42-28-59-85P at a concentration of 4 × 10^9 CFU/mL.

The experimental model was guinea pigs weighing 250-350 g obtained from the Experimental animals department with a vivarium of the Russian Anti-Plague Institute "Microbe". The animals were divided into 6 experimental groups of 12 animals each. Guinea pigs were subcutaneously immunized with the EVGFP strain grown at 28°C (groups 1–3) and 37°C (groups 4–6) at a dose of 2 × 10^9 CFU/0.5 mL. Guinea pigs from groups 2 and 5 were additionally subcutaneously injected with AB ("PetrovaxPharm") at a concentration of 60 μg (EVGFP28+AB and EVGFP37+AB, respectively) 1 h before immunization. Biomodels of groups 3 and 6 additionally received HRI ("Pharmaclone") at a concentration of 2000 IU (EVGFP28+HRI and EVGFP37+HRI, respectively). The control group 7 consisted of intact guinea pigs (4 individuals). All manipulations with animals were carried out in accordance with the legislation of the Russian Federation and international principles. Animal immunization experiments were performed in accordance with Sanitary Rules and Standards 3.3686-21 "Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases". The program of experimental work with animals was approved by the Bioethics Commission of the Russian Anti-Plague Institute "Microbe" (Protocol No. 5 of 25.05.2023).

The biochemical properties of *Y. pestis* was studied using API standard biochemical test strips ("bioMérieux SA") according to the manufacturer's instructions.

Morphology of bacterial cells was evaluated using an Olympus CX41light microscope ("Olympus") and a VZ-C31S digital camera ("VideoZavr") in the VideoZavr v. 1.5 program at magnifications of 40 and 100.

The engraftment of EVGFP strain in guinea pig organs was evaluated by microscopic and flow cytometric methods. On the 1st, 4th, 7th and 14th days of immunogenesis, animals were sacrificed using chloroform, dissected and smears of internal organs (injection site, inguinal lymph nodes, blood, spleen, liver, lungs) were taken. The smears-imprints of animal organs were viewed in an Axio LAB.A1fluorescent microscope ("Carl Zeiss") with A-Plan 100x/1.25 OiI objective lens at a magnification of × 1000. The specimens were examined under the microscope and photographed at 5 fields of view per preparation. Photographs were taken using an AxioCam ERc5s camera ("Carl Zeiss").

To investigate the engraftment of *Y. pestis* EV NIIEG pTurboGFP using flow cytometry, a suspension of spleen cells at a concentration of 10^6 cells/mL was prepared in RPMI-1640 medium with gentamicin (100 μg/mL) using the standard method. Then to 100 μL of the obtained suspension was added 1 mL of BD FACS Lysing Solution ("BD Biosciences") diluted 10 times (according to the instructions) for lysis of erythrocytes and fixation of leukocytes and samples were incubated for 20 min.

Cell suspensions were analyzed on a CyAn ADP flow cytofluorimeter in the Summit v.4.3 Built 2445 program. Detection of EVGFP bacteria in phagocytes (macrophages and neutrophils) of guinea pig spleen was performed according to the previously developed protocol for determination of phagocytic reaction indices in human blood and animals with FITC-labeled *Y. pestis* EV NIIEG cells [15, 19]. In spleen cell suspensions, phagocytes were differentiated by light scattering parameters (size and degree of granularity) from lymphocytes (Fig. 3, *a*), and then by gating the phagocytic population the percentage of fluorescent phagocytes (macrophages, neutrophils) was determined in the spleen, which corresponded to the relative content of active phagocytes (PI — phagocytic index, %) that absorbed *in vivo* live cells of the tested biofluorescent strain of the plague microbe. The average number of bacteria absorbed by phagocytes (PN — phagocytic number) was estimated by the average value of their luminescence intensity in the green region of the spectrum (Mean), measured in conventional units of fluorescence intensity (cytofluorimeter channels) [14, 19–21].

Statistical processing of the obtained results was performed using the Microsoft Office Excel 2016, Statistica 10.0 ("StatSoft Inc.") standard software package. The data were presented as median (Me) and quartile deviations (Q_{25}; Q_{75}) with calculation of reliability of differences in the studied groups using Mann–Whitney U-criterion.

Results

Comparative culture analysis of the original vaccine strain and biofluorescently labeled vaccine strain

The culture, morphological and biochemical properties of recombinant EVGFP and the original *Y. pestis* EV NIIEG strains were compared. It was found that the recombinant strain had cell morphology typical of the plague pathogen. Cells of the strain EVGFP carrying the GFP fluorescent protein gene were identified by the presence of green fluorescent glow of the bacteria. On LB agar with ampicillin, strain EVGFP formed characteristic R-type colonies of yellow-green color (**Fig. 1**).

The characteristic features of the growth of the plague pathogen in liquid nutrient medium were a clear broth and a loose, powdery or flake-like sediment at the bottom of the test tube, which easily disintegrated when shaken. Based on the analysis of biochemical properties, it was found that the recombinant strain EVGFP did not differ in its characteristics from the original *Y. pestis* strain EV NIIEG. Both strains fermented glucose, mannitol, arabinose, and did not degrade inositol, sorbitol, rhamnose, sucrose, melibiose and amygdalin.

Administration of the EVGFP strain to guinea pigs at a massive dose of 2 × 10^9 CFU did not cause death of the biomodels during the entire observation period.

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Thus, the studied EVGFP strain did not differ from the original *Y. pestis* strain EV NIIEG in its cultural, morphological and biochemical properties, and also corresponded to the reference vaccine strain in terms of harmlessness.

Evaluation of the engraftment of Y. pestis strain EV NIIEG pTurboGFP by luminescence microscopy

During microscopic examination, EVGFP cells were detected in smear-prints only from the site of culture administration in all groups of immunized guinea pigs and only on the 1st day of immune response (**Fig. 2**). Cells of the fluorescent strain were visualized as bright green sticks. The number of bacteria was 400 ± 100 m bacteria in one field of view, indicating abundant EVGFP cell infiltration of the injection site. On further observation on the 4th, 7th and 14th days of immunogenesis, no cells of the recombinant strain were registered in the smear-prints of all examined organs of guinea pigs.

Evaluation of the engraftment of Y. pestis strain EV NIIEG pTurboGFP using flow cytofluorimetric technology

Taking into account the fact that spleen neutrophils and macrophages are one of the main objects for assessing phagocytosis and engraftment of *Y. pestis* [11], it was reasonable to use guinea pig spleen cells for cytometric studies.

The intensity of the fluorescence signal of GFP protein was used to determine the presence of EVGFP in the population of spleen phagocytes (macrophages and neutrophils), and the indices of the *in vivo* phagocytic reaction were taken into account. The histograms

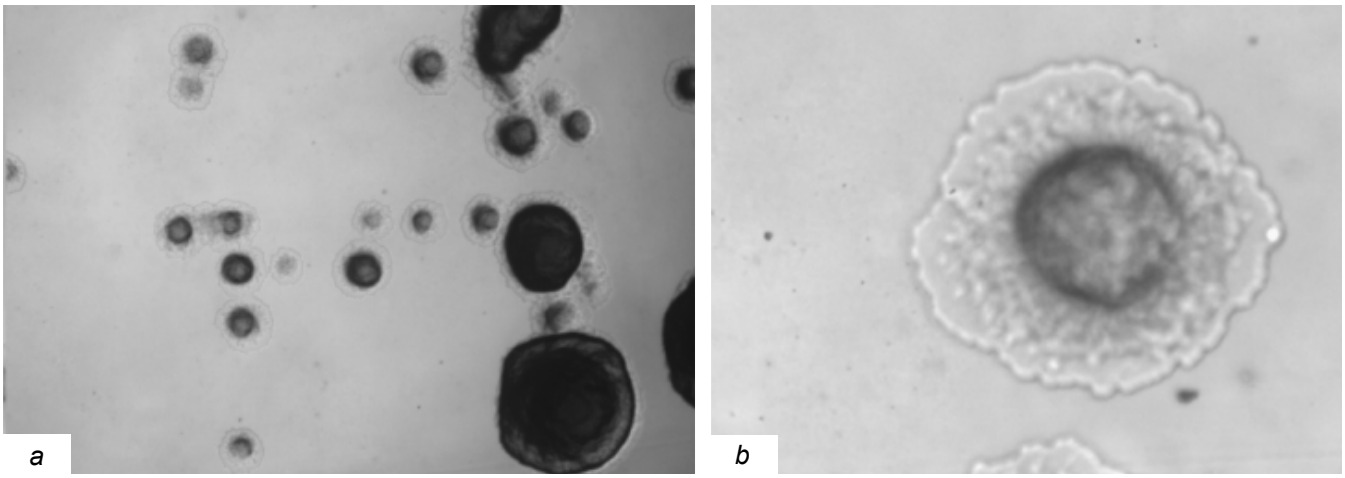

Fig. 1. R-type of colonies of *Y. pestis* strain EV NIIEG pTurboGFP-B, ×40 (*a*) and ×100 (*b*).

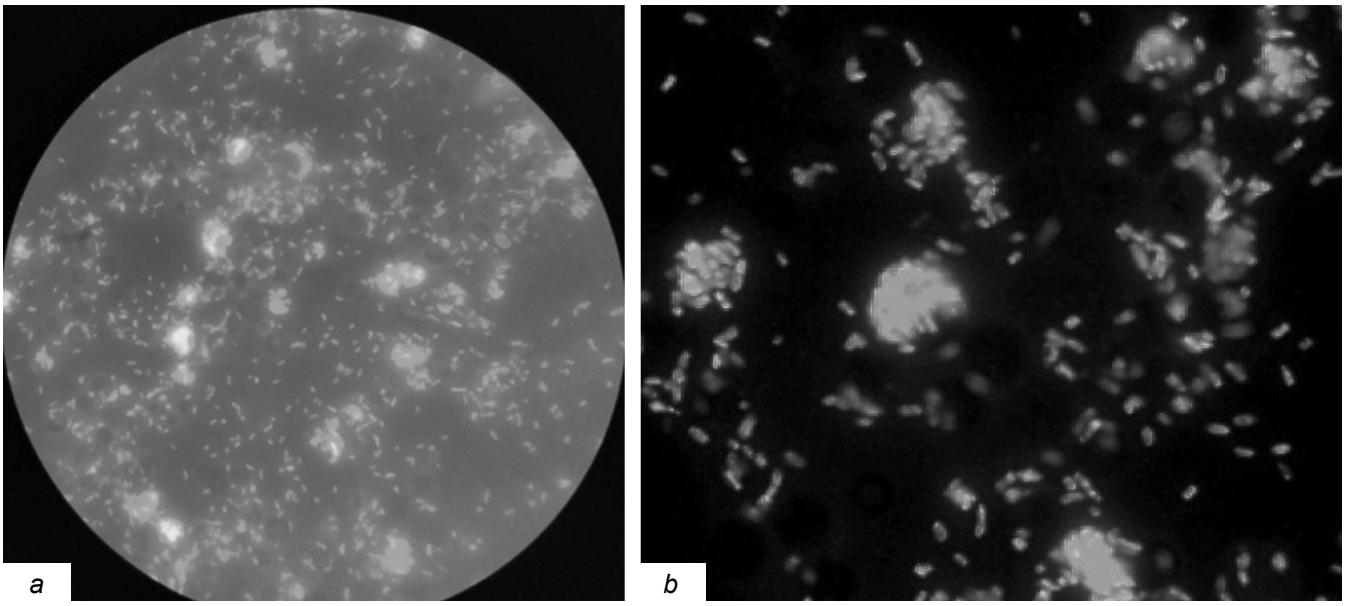

Fig. 2. Detection of cells of the biofluorescent *Y. pestis* vaccine strain EV NIIEG pTurboGFP-B in imprint smears of the subcutaneous tissue of the guinea pig thigh (injection site) on day 1 of the immune response at ×1000 magnification.
a — image in the microscope eyepiece; *b* — computer monitor image.

in **Fig. 3**, *b–d* demonstrate the dynamics of the decrease in the relative number of phagocytes in the spleen characterized by intense green fluorescence due to the uptake of cells of EVGFP strain grown at 37°C.

Fig. 3 clearly shows that in the interval from 1 to 4 days the proportion of fluorescent phagocytes decreased from 94.54 to 21.23%, and by the 14th day — to 0, i.e. to the values characteristic of spleen phagocytes of intact (control) animals, which do not have fluorescent luminescence in the green region of the spectrum (Fig. 3, *e*).

Comparative evaluation of the effect of immunomodulatory drugs in combined use with biofluorescent strain and conditions of its cultivation on phagocytic activity of spleen cells

At the next stage, we analyzed the efficiency of drug application on the outcome of interaction of the studied EVGFP strain with guinea pig spleen phagocytes under *in vivo* conditions. On the 4th day of immunogenesis, a significant decrease in PI in the EVGFP28+HRI group was found compared to the same index in the EVGFP28 group (**Table**).

In the EVGFP37+HRI group, the PI was registered at the level of the analogous index in the EVGFP37 group. At the same terms (4th day) in the EVGFP37+AB group the PI was significantly increased ($p < 0.05$) in comparison with the analogous index in the EVGFP37 group. A significant increase of the PN in EVGFP28+AB group on the 4th and 7th days, and in EVGFP28+HRI group — on the 7th day in comparison with this index in EVGFP28 group was revealed ($p < 0.05$).

Next, we determined the effect of EVGFP cultivation temperature on the ability of guinea pig spleen phagocytes to engulf and digest cells of the fluorescent strain. In the EVGFP37+AB and EVGFP37+HRI groups at day 4, the PI values were 2-fold higher ($p < 0.05$) than in the EVGFP28+AB and EVGFP28+HRI groups, respectively. In the EVGFP37 group on the 4th day the PN was 1.5 times higher ($p < 0.05$) than in the EVGFP28 group on average.

Discussion

The preclinical stage of evaluation of live plague vaccines should not only demonstrate that the vaccine is immunogenic and has protective efficacy, but also substantiate the safety of the preparation, primarily related to the degree of residual virulence (harmlessness) of the vaccine strain of the plague microbe, characterized by its ability to spread and prolonged persistence in the organs of biomodels.

Cell engraftment of the biofluorescent strain was studied in guinea pig organs by microscopic and flow cytometric methods for 14 days, which corresponds to the acceptable period of cell reproduction of the *Y. pestis* vaccine strain EV NIIEG, which causes immunological reorganization of the organism (non-sterile phase of immunity) [12].

The method of fluorescence microscopy, one of the main optical methods of fluorescence visualization [22], makes it possible to clearly and efficiently assess the stage of virulent strain spreading in organs and tissues of white mice [4]. In our studies, the established visualization of EVGFP in smear-prints from the site of culture administration in all experimental groups on the 1st day of the immune response is consistent with the results of earlier quantitative bacteriological studies, in which the highest number of cells of the *Y. pestis* vaccine strain EV NIIEG were found at the injection site (2.3×10^7 m.c./g) after 24 h [12].

The absence of fluorescent microbial cells on the 4th, 7th, and 14th days of immunogenesis is probably due to the low concentration of EVGFP in the smear-prints ($< 10^5$ m.c./ml), lying beyond the lower threshold of 10^5 m.c./mL sensitivity of fluorescent microscopy [23].

New opportunities were opened by the use of a more sensitive and productive technology of flow cytofluorimetric analysis, which allows to control *in vivo* survival of bacteria in phagocytes, as well as to monitor their distribution in the host organism in dynamics [11, 24]. Phagocytes containing fluorescent bacterial cells were recorded for 4 days. The decrease in the number of fluorescent phagocytes (by the 4th day on average 4.4 times as compared to the 1st day) could be associated with both intracellular digestion of ingested bacteria and a decrease in the absorption capacity of phagocytes towards plague microbes in the active growth stage *in vivo* at the temperature of the host organism. Intracellular localization of EVGFP in macrophages and neutrophils of spleen was confirmed by similar results obtained by foreign scientists by flow cytometry in experiments with another fluorescent strain of *Y. pestis* (pAGFP) expressing GFP [11]. The sample preparation procedure we used (Lyse/No-wash) without washing splenocytes from the erythrocyte lysing and leukocyte fixing FACS Lysing Solution actually completely excluded bacterial adhesion on the cell surface, since the solutions used for erythrocyte lysis remove bacteria adsorbed on the surface of formaldehyde-fixed leukocytes [19].

The use of biofluorescent strain and flow cytofluorimetric technology allowed us to register the different effect of immunomodulatory drugs used in this work on the outcome of interaction of the studied EVGFP strain with phagocytes of guinea pig spleen.

The obtained data on the combined use of AB with the vaccine strain, based on the analysis of phagocytic activity of macrophages and neutrophils of guinea pig spleen in our experiment, confirmed the previously revealed fact of the stimulating effect of AB on the phagocytic activity of blood leukocytes of BALB/c mice in relation to *Y. pestis* EV NIIEG in *in vitro* tests [15].

According to the literature data on immunological similarity in IFN-γ genetic expression patterns and

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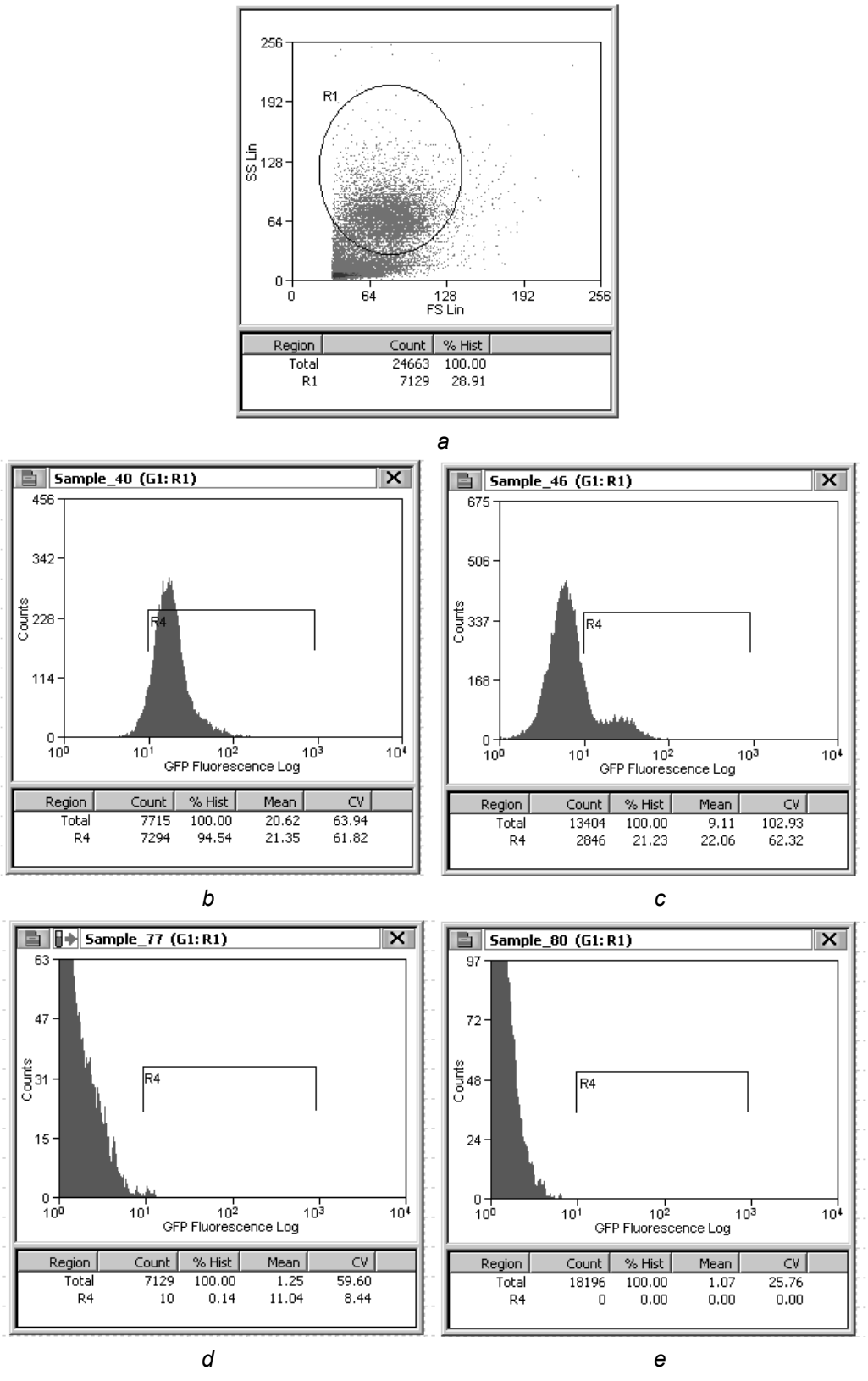

Fig. 3. Fluorescence intensity of phagocytes of the spleen of guinea pigs immunized with the biofluorescent *Y. pestis* vaccine strain EV NIIEG pTurboGFP-B (37°C) in the dynamics of the immune response.

Cytogram *a*: area R1 corresponds to the total number of phagocytes FS/SS dot plot of a sample of guinea pig spleen cells inoculated with the original *Y. pestis* vaccine strain EV NIIEG (control). The characteristic distribution of individual spleen phagocytes by size (FS) and degree of granularity (SS) is presented. Spleen phagocytes (28.91% of cells) are localized in the R1 region of the cytogram.
Active phagocytes are localized in the histograms in the R4 region: *b*–*e* — days 1, 4, 7 and 14 respectively, after immunization of guinea pigs with the *Y. pestis* EV NIIEG pTurboGFP-B (37°C).

Detection of the biofluorescent *Y. pestis* vaccine strain EV NIIEG pTurboGFP in guinea pig spleen phagocytes during immunization in combination with immunomodulators according to flow cytometry data, Me (Q_{25}; Q_{75})

Group	Immunization schedule	Day	Phagocytic index, %	Phagocytic number
1	EVGFP28	1	89.2 (83.0; 95.4)	19.3 (17.0; 21.5)
		4	19.8 (16.6; 22.0)	12.6 (11.8; 13.4)
		7	0.07 (0; 0.1)	1.6 (0.9; 2.3)
		14	0	0.7 (0.5; 0.8)
2	EVGFP28+AB	1	99.1 (98.0; 99.4)	24.8 (20.7; 28.9)
		4	17.5 (15.4; 19.6)	19.2 (17.5; 20.9)*
		7	0.02 (0; 0.05)	10.0 (8.9; 11.2)*
		14	0	0
3	EVGFP28+HRI	1	97.4 (95.5; 99.4)	22.1 (18.0; 26.2)
		4	9.0 (8.4; 9.6)*	18.2 (15.7; 20.8)
		7	0.06 (0; 0.1)	8.7 (7.9; 9.5)*
		14	0	1.5 (1.3; 1.7)
4	EVGFP37	1	92.5 (87.8; 97.2)	19.6 (16.2; 23.0)
		4	21.2 (18.4; 24.0)	20.1 (15.4; 24.8)#
		7	0.1 (0.05; 0.20)	10.0 (8.3; 11.7)
		14	0.1 (0.05; 0.20)	1.0 (0.9; 1.1)
5	EVGFP37+AB	1	98.5 (96.8; 99.2)	25.2 (20.0; 30.4)
		4	37.1 (37.0; 41.2)*#	17.5 (14.4; 20.6)
		7	0.2 (0.1; 0.3)	10.7 (8.1; 13.3)
		14	0	0.3 (0.1; 0.5)
6	EVGFP37+HRI	1	90.6 ± 5.2 (85.4; 95.8)	21.6 (17.1; 26.2)
		4	19.4 (17.6; 21.2)#	21.9 (18.0; 25.8)
		7	0.5 (0.2; 0.8)	10.5 (8.4; 12.6)
		14	0.2 (0.1; 0.3)	2.3 (1.9; 2.7)
7	Control (EV)		0	0

Note. * — significant differences ($p < 0.05$) with group 1 on the corresponding day; # — significant differences ($p < 0.05$) between groups 1 and 4, 2 and 5, 3 and 6 on the corresponding day.

amino acid sequence homology between guinea pig and human [25, 26], HRI had a different effect on the phagocytosis process, decreasing the PI and increasing the PN (number of microbial cells per phagocyte) at 28°C. This increase could be explained by the assumption of the ability of HRI to inhibit intracellular digestion of bacteria in phagocytes. However, this explanation is contradicted by the known ability of recombinant IFN-γ to activate macrophages and cause the death of intracellular microorganisms [27]. Consequently, this fact is explained by the effect of IFN-γ on the properties of the vaccine strain, leading to a decrease in its engraftment in the macroorganism.

In experiments with the use of biofluorescent strain cultures grown in different temperature regimes for vaccination, the known fact of longer survival and digestion of 37°C *Y. pestis* culture cells in macrophages was confirmed [28, 29], associated with the induction of expression of Caf1 (F1) and other antiphagocytic factors that block phagocytosis: outer membrane protein Ail, plasminogen activator Pla, PsaA (pH6 antigen) [11, 30, 31].

Conclusion

On the example of application of biofluorescent *Y. pestis* vaccine strain EV NIIEG pTurboGFP-B and the method of flow cytometry showed the possibility of increasing the efficiency of assessment of strain engraftment in the macroorganism, and the use of cytofluorimetric technology increases its informativity and objectivity, including due to the speed of such analysis, allowing to perform additional studies that reveal the mechanisms of interaction between the micro- and macroorganism.

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Experience of applying the metagenomic sequencing method on fragments of the 16S rRNA gene for the detection and identification of natural focal infection pathogens

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Abstract

Introduction. Metagenomic sequencing is one of the most promising methods for both the detection and identification of natural focal infection (NFI) pathogens and for determining the species composition of various bacterial communities.

The aim is to detect and identify the NFI pathogens in samples of field and clinical material using metagenomic sequencing of *16S* rRNA gene fragments, and to analyze the taxonomic composition of endosymbiotic microorganisms in the samples.

Materials and methods. Samples of field (14 samples) and clinical (2 samples) material with varying loads of DNA from NFI pathogens, determined by PCR (*Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Francisella tularensis*, *Rickettsia* spp., *Coxiella burnetii*), were investigated. Amplification of fragments of the gene encoding *16S rRNA* was performed using primers flanking the variable regions of the gene.

Results. In 14 out of 16 studied samples, target NFI pathogens were detected. The species identified included *R. aeschlimannii* (in 57.1% of positive samples), *B. valaisiana* (in 16.6%), *F. tularensis* (in 75%), *C. burnetii* (in 100%), and borreliae — pathogens of relapsing fevers (*B. turcica*, *B. hispanica*) were also found in one sample. The taxonomic structure of the microbiome of *Ixodes ricinus*, *Dermacentor reticulatus*, *Rhipicephalus annulatus, Hyalomma aegyptium, Dermacentor marginatus* ticks collected in the southern regions of the Russian Federation was studied. It was shown that the predominant microorganisms are representatives of the genera *Flavobacterium, Pseudomonas, Serratia, Aeromonas, Pedobacter, Bradyrhizobium, Shingomonas*. DNA markers of microorganisms — endosymbionts of ticks *Candidatus Midichloria mitochondrii*, representatives of the genera *Rickettsiella*, *Coxiella*, non-pathogenic and conditionally pathogenic species of the genus *Francisella* were found in pools of *Ixodes* ticks.

Conclusion. The effectiveness of the method of metagenomic sequencing of fragments of the *16S* rRNA gene for the detection and identification of NFI pathogens in samples of clinical and field material was demonstrated. Metagenomic sequencing of *16S rRNA* gene regions can be recommended as an additional laboratory method for detecting and identifying NFI pathogens.

Keywords: metagenomic sequencing, *16S rRNA*, natural focal infections, detection, identification, microbiome.

Ethical approval. The study was conducted with the informed consent of the patients. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The study protocol was approved by the Local Ethics Committee of the Stavropol State Medical University (protocol No. 112, May 5, 2023).

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Оригинальное исследование
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Опыт применения метода метагеномного секвенирования по фрагментам гена *16S* рРНК для детекции и идентификации возбудителей природно-очаговых инфекций

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Аннотация

Введение. Метагеномное секвенирование — один из наиболее перспективных методов как для детекции и идентификации возбудителей природно-очаговых инфекций (ПОИ), так и для определения видовой структуры различных бактериальных сообществ.

Цель работы — выполнить детекцию и идентификацию возбудителей ПОИ в образцах полевого и клинического материала методом метагеномного секвенирования фрагментов гена *16S рРНК*, проанализировать таксономический состав эндосимбиотических микроорганизмов в образцах.

Материалы и методы. *И*сследованы образцы полевого (14 проб) и клинического (2 пробы) материала с различной нагрузкой ДНК возбудителей ПОИ, определённой методом полимеразной цепной реакции (*Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, Francisella tularensis, Rickettsia* spp., *Coxiella burnetii*). Амплификацию фрагментов гена, кодирующего *16S рРНК*, осуществляли с помощью праймеров, фланкирующих вариабельные участки гена.

Результаты. В 14 из 16 исследуемых образцов детектированы целевые возбудители ПОИ. До вида идентифицированы *R. aeschlimannii* (в 57,1% положительных образцов)*, B. valaisiana* (в 16,6%)*, F. tularensis* (в 75%), *C. burnetii* (в 100%), также в одном образце выявлены боррелии — возбудители возвратных лихорадок (*B. turcica, B. hispanica*). Исследована таксономическая структура микробиома клещей *Ixodes ricinus, Dermacentor reticulatus, Rhipicephalus annulatus, Hyalomma aegyptium, Dermaceptor marginatus*, собранных в южных регионах России. Выявлено, что преобладающие микроорганизмы — это представители родов *Flavobacterium, Pseudomonas, Serratia, Aeromonas, Pedobacter, Bradyrhizobium, Shingomonas*. В пулах иксодовых клещей обнаружены ДНК-маркеры микроорганизмов — эндосимбионтов клещей *Candidatus Midichloria mitochondrii*, представителей родов *Rickettsiella, Coxiella*, непатогенных и условно-патогенных для человека видов родов *Francisella*.

Заключение. Показана эффективность метода метагеномного секвенирования фрагментов гена *16S* рРНК для детекции и идентификации возбудителей ПОИ в пробах клинического и полевого материала. Метагеномное секвенирование по участкам гена *16S рРНК* может быть рекомендовано в качестве дополнительного метода лабораторного исследования образцов с целью детекции и идентификации возбудителей ПОИ.

Ключевые слова: *метагеномное секвенирование, 16S рРНК, природно-очаговые инфекции, детекция, идентификация, микробиом*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с «Consensus Author Guidelines for Animal Use» (IAVES, 23.07.2010). Протокол исследования одобрен локальным этическим комитетом Ставропольского государственного медицинского университета (заключение № 112 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

Natural focal infections (NFI) are widespread in the world and represent an important medical and social problem, the importance of which has been steadily increasing in recent years with the identification of new pathogens, the sources and vectors of which are blood-sucking arthropods, small mammals and birds [1–3]. Continued activity and expansion of the territories of natural foci, as well as high anthropogenic load on the environment lead to a constant increase in the number of people in contact with pathogens and exposed to the risk of infection [4]. It has been proved that simultaneous infection of carriers and vectors with different NFI pathogens is a natural and widespread phenomenon, which, in turn, determines the possibility of combined pathology in humans [5–7].

Currently, molecular genetic methods, primarily polymerase chain reaction (PCR), are widely used for laboratory diagnostics of NFI, which allows detecting the presence of DNA/RNA of NFI pathogens in the material in a short period of time. Most of the developed PCR test systems for detection of pathogens are designed for detection of one or more pathogens [8]. Detection of all potential pathogens requires the use of a set of test systems, which is time-consuming and labor-intensive.

Metagenomic sequencing (MGS) methods are modern approaches that allow simultaneous detection and identification of all microorganisms, both known and new, contained in a sample, and do not require culturing [9]. The use of MGS methods for the identification of infectious agents, including NFI, seems to be especially demanded in cases when traditional laboratory tests do not allow identifying the etiologic agent in atypical course of the disease, as well as in cases of mixed infection with different pathogens [10–12]. Furthermore, MGS of field material samples (ectoparasites, organs of small mammals, birds, etc.) collected during epizootological survey of the territory can be useful for obtaining new comprehensive data on the species composition of pathogenic and endosymbiotic microorganisms associated with different types of carriers and vectors of infections [13].

There are several variants of MGS: targeted sequencing of genome regions encoding evolutionarily conserved genes (*16S rRNA*, etc.) and whole-genome MGS. An approach based on target sequencing of variable regions of the *16S rRNA* gene has been widely used to analyze the taxonomic composition of bacteria in samples and to detect pathogenic bacterial species. The advantages of this method include the possibility of taxonomic classification of a wide range of bacteria, the presence of a stage of preliminary specific enrichment of the target region of the bacterial genome before sequencing, and the relative simplicity of bioinformatics analysis of the results compared to the method of sequencing the complete metagenome [14, 15].

The aim of the study was to perform detection and identification of pathogens in samples of field and clinical material by MGS of *16S rRNA* gene fragments, analyze the taxonomic composition of endosymbiotic microorganisms in samples.

Materials and methods

Sixteen samples of field (collected during the epizootologic survey) and clinical material with different load of PCR-determined DNA of pathogens of bacterial etiology (*Borrelia burgdorferi sensu lato*, *Anaplasma phagocytophilum*, *Francisella tularensis*, *Rickettsia* spp., *Coxiella burnetii*) were studied. The samples contained genetic material of one and several pathogens (**Table 1**).

Work with clinical material was performed with voluntary informed consent of patients. The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the "Consensus Author Guidelines for Animal Use" (IAVES, 23.07.2010). Materials from animals used in the study was obtained according to the Plan of epizootological survey of the Stavropol Territory for NFI and particularly dangerous infections for 2024 (agreed by the Head of the Department of Rospotrebnadzor in Stavropol Krai on 21.12.2023, approved by the Chief Physician of the Center of Hygiene and Epidemiology in Stavropol Krai on 21.12.2023). The study protocol was approved by the Local ethical committee of Stavropol State Medical University (conclusion No. 112 dated 19.05.2023).

Ixodid ticks were collected from April through June from animals and vegetation (flagging), species identification of ticks was performed by morphological method [16]. The ticks were used to make pools of 10 specimens each according to Methodological Recommendations 3.1.0322-23[1]. Sample preparation of clinical and field material was performed in accordance with Methodological guidelines 1.3.2569-09[2].

Ticks were treated with 70% ethanol and washed in phosphate-buffered saline. Homogenization parameters for the obtained samples were selected based on the genus of the ticks. The obtained suspension was centrifuged in 300 μL of sterile physiological solution.

Extraction of nucleic acids from human blood serum samples, homogenates of tick pools and flush from the thoracic cavity of the common vole was performed using the RIBO-prep reagent kit (InterLabService).

[1] Methodological recommendations MP 3.1.0322-23 "Collection, accounting and preparation for laboratory examination of blood-sucking arthropods in natural foci of infectious diseases" (approved by the Head of Rospotrebnadzor on 04/13/2023).

[2] Methodological guidelines MU 1.3.2569-09 "Organization of work of laboratories using methods of nucleic acid amplification when working with material containing microorganisms of pathogenicity groups I-IV" (approved by the Head of Rospotrebnadzor on 12/22/2009).

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Table 1. Data on samples used for MGS analysis

No.	Sample type	Sample data, location of extraction	PCR-confirmed pathogen	Ct
1	Tick suspensions	*Ixodes ricinus*, from vegetation, Krasnodar, Sochi	*B. burgdorferi* s.l.	21.8
2		*I. ricinus*, from vegetation, Krasnodar, Sochi	*B. burgdorferi* s.l.	22.1
3		*I. ricinus*, from vegetation, Krasnodar, Sochi	*B. burgdorferi* s.l.	21.1
4		*I. ricinus*, cattle, Republic of South Ossetia	*A. phagocytophilum*	31.4
5		*I. ricinus*, cattle, Republic of South Ossetia	*A. phagocytophilum*	23.4
6		*Dermacentor marginatus*, from vegetation, Stavropol	*Rickettsia* spp.	17.2
7		*Rhipicephalus annulatus*, cattle, Republic of South Ossetia	*Rickettsia* spp.	23.3
8		*D. marginatus*, from vegetation, Stavropol	*F. tularensis*	26.6
9	Flush from chest cavity	*Microtus arvalis*, Stavropol	*F. tularensis*	10.1
10	Tick suspensions	*Hyalomma aegyptium* from Mediterranean turtle, Republic of Dagestan	*B. burgdorferi* s.l.	20.2
			Rickettsia spp.	16.3
11		*I. ricinus* from vegetation, Krasnodar, Sochi	*B. burgdorferi* s.l.	25.4
			Rickettsia spp.	17.0
12		*I. ricinus* from vegetation, Krasnodar	*B. burgdorferi* s.l.	25.7
			Rickettsia spp.	18.8
13		*Dermacentor reticulatus* from vegetation, Stavropol	*F. tularensis*	25.5
			Rickettsia spp.	17.2
14		*D. reticulatus* from vegetation, Stavropol	*F. tularensis*	12.6
			Rickettsia spp.	21.1
15	Blood serum	Human, Stavropol	*C. burnetii*	21.4
16		Human, Stavropol	*C. burnetii*	21.3

The presence of DNA of NFI pathogens in the samples was determined by PCR using the following reagent kits: AmpliSens *Coxiella burnetii*-FL, AmpliSens *TBEV*, *B. burgdorferi s.l.*, *A. phagocytophilum*, *E. chaffeensis/E. muris-FL* (Central Research Institute of Epidemiology of Rospotrebnadzor), *Francisella tularensis*-RGF gene (Russian Anti-Plague Institute "Microbe"). DNA of rickettsiae in field samples was detected according to the protocol described by O. Mediannikov et al. [17].

Amplification of microbial *16S rRNA* gene fragments contained in the samples for MGS was performed using primers described by I. Abellan-Schneyder et al. [18] (**Table 2**). A separate reaction mixture was prepared for amplification of each variable fragment of the *16S rRNA* gene (V1–V2, V1–V3, V3–V4, V4, V4–V5, V6–V8, V7–V9). The composition of the reaction mixture: primer F (C = 7.2 pmol/μL) — 1.25 μL, primer R (C = 7.2 pmol/μL) — 1.25 μL, BioMaster HS-Taq PCR-Color (2×) PCR mixture (Biolabmix) — 12.5 μL, sample DNA — 10 μL. PCR products were amplified according to the thermocycling program: 95°C — 5 min; 95–20 s, Ta — 30 s, 72°C — 40 s (40 cycles); 72°C — 5 min; 4 — ∞.

The size and purity of the obtained PCR products were assessed by electrophoresis in 1% agarose gel. The procedure for purification of PCR products from excess primers and components of the reaction mixture was performed using the CleanMag DNA kit (Eurogen). Equivalent amounts of amplification products of *16S rRNA* V1–V9 gene fragments were taken for library preparation. The final concentration of target DNA was measured on a Qubit fluorimeter using the Qubit 1X dsDNA High Sensitivity (HS) kit (Invitrogen).

DNA fragment libraries were prepared according to the Ion Xpress Plus gDNA Fragment Library Preparation protocol (Revision K.0) using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific Inc.). Sequencing of libraries prepared from amplicon mixtures was performed on the GeneStudio S5 Plus platform (Thermo Fisher Scientific Inc.).

The Fastp Qs[3], Kallisto[4], STAR [19], Bowtie2[5] programs were used for bioinformatics analysis of MGS data on *16S rRNA* gene regions. The quality of fastq-files was assessed using Fastp Qc and Kallisto programs; reads with quality index Q < 20 were excluded from the analysis. Sequence alignment and filtering were performed using STAR and Bowtie2 software.

[3] URL: https://github.com/OpenGene/fastp
[4] URL: https://github.com/Roslin-Aquaculture/RNA-Seq-kallisto
[5] URL: https://bowtie-bio.sourceforge.net/bowtie2/index.shtml

Table 2. Primer sequences for amplification of fragments of the gene encoding *16S rRNA*

No.	Fragment marking	Fragment length, bp	Annealing temperature, °C	Primer	Sequence 5'–3'
1	V1–V2	311	57	27F	AGAGTTTGATYMTGGCTCAG
				338R	GCTGCCTCCCGTAGGAGT
2	V1–V3	507	57	27F	AGAGTTTGATYMTGGCTCAG
				534R	ATTACCGCGGCTGCTGG
3	V3–V4	404	54	341F	CCTACGGGNGGCWGCAG
				785R	GACTACHVGGGTATCTAATCC
4	V4	293	54	515F	GTGCCAGCMGCCGCGGTAA
				806R	GGACTACHVGGGTWTCTAAT
5	V4–V5	429	54	515F	GTGCCAGCMGCCGCGGTAA
				944R	GAATTAAACCACATGCTC
6	V6–V8	439	57	939F	GAATTGACGGGGGCCCGCACAAG
				1378R	CGGTGTGTACAAGGCCCGGGAACG
7	V7–V9	377	51	1115F	CAACGAGCGCAACCCT
				1492R	TACGGYTACCTTGTTACGACTT

Assembly of short *de novo* read sequences into longer sequences (contigs) was performed using SPAdes assembler. Taxonomic affiliation of genomic sequences was determined by comparing them with the NCBI database (RefSeq and GenBank using Rapsearch2[6]).

The results of species identification of microorganisms (Borrelia, rickettsiae) obtained by MGS using *16S rRNA* gene sections were confirmed by Sanger sequencing method.

Results

MGS was performed on the *16S rRNA* gene regions of 16 samples of clinical and field material containing DNA of bacterial pathogens (**Table 3**). Nucleotide sequences obtained by MGS were deposited in the GenBank database (BioProject PRJNA1227530; SAMN46987881-SAMN46987896).

The number of reads satisfying the Q20 parameter for the studied samples was 1127–40,969. The GC value for all samples varied in the range of 49.7–52.4%, which corresponds to the exome regions of *16S rRNA* gene fragments used for MGS analysis. During the processing of the data obtained, it was found that the highest amount of reads after filtration was obtained for sample No. 13 (93,789,000 K). A reduction in the number of reads was observed for samples Nos. 9 and 14 (29,314,000 K and 28,704,000 K). The total number of reads after the filtering step for the 4 samples (Nos. 2, 5, 7 and 10) ranged between 9,172,000–16,651,000 K. The number of filtered reads for the remaining samples ranged 488–7,633,000 K. The least amount of filtered data (Q < 20) after bioinformatics processing was observed for samples Nos. 4, 8 and 11. The highest number of poor quality data (Q < 20) was obtained for samples Nos. 9, 13, and 14. The result of data quality assessment is shown in **Fig. 1**.

NFI pathogens identified by MGS using 16S rRNA gene variant regions

In the study of suspension samples of ixodid ticks (Nos. 1–8, **Table 3**) with PCR-confirmed mono-infection with *Borrelia* genetic complex *B. burgdorferi* s.l., *A. phagocytophilum*, rickettsiae and *F. tularensis,* using the MGS method of *16S rRNA* gene, detected representatives of the *Borrelia* (samples Nos. 1–3), *Francisella* (sample No. 8) (microorganisms identified to genus) genera, as well as *R. aeschlimannii* (samples Nos. 6, 7, microorganism identified to species). The pathogen of human granulocytic anaplasmosis could not be detected by MGS (samples Nos. 4, 5). The results of species identification of *R. aeschlimannii* in samples Nos. 6, 7 were confirmed by Sanger sequencing of a genome fragment.

Mixed-infected samples of ixodid ticks (samples Nos. 10–14, **Table 3**) with a combination of two tick-borne pathogens (*B. burgdorferi* s.l and *Rickettsia* spp.; *F. tularensis* and *Rickettsia* spp.) were studied. All target microorganisms were detected in the samples by the MGS method. *R. aeschlimannii* (samples Nos. 10, 13), *B. valaisiana* (sample No. 12), *F. tularensis* (samples Nos. 13, 14) were identified to species, also microorganisms of the *Rickettsia* (samples Nos. 11, 12, 14) and *Borrelia* (samples Nos. 11, 12, 14) genera whose species could not be identified were detected in the samples. Genetic markers (DNA) of Borrelia, pathogens of relapsing fevers (*B. turcica*, *B. hispanica*) were detect-

[6] URL: https://github.com/zhaoyanswill/RAPSearch2

Table 3. Comparison of results obtained by PCR and MGS methods of *16S rRNA* gene fragments

Sample No.	PCR method		MGS method of *16S rRNA* gene fragments	
	identified pathogens	Ct	Q20, % (number of reads)	pathogens identified (number of reads corresponding to the target pathogen, %)
			Mono-infected samples	
1	*B. burgdorferi* s.l.	21.80	91.50 (5822)	*Borrelia* spp. (2,90)
2	*B. burgdorferi* s.l.	22.10	91.90 (20,236)	*Borrelia* spp. (3,10)
3	*B. burgdorferi* s.l.	21.10	92.20 (10,627)	*Borrelia* spp. (3,20)
4	*A. phagocytophilum*	31.40	87.50 (12,922)	Unidentified
5	*A. phagocytophilum*	23.40	91.80 (6618)	Unidentified
6	*Rickettsia* spp.	17.20	91.60 (8239)	*R. aeschlimannii* (8,90)
7	*Rickettsia* spp.	23.30	92.10 (22,324)	*R. aeschlimannii* (0,80)
8	*F. tularensis*	26.60	91.00 (1127)	*Francisella* spp. (2,60)
9	*F. tularensis*	10.10	91.60 (40,161)	*F. tularensis* (9,90)
			Mixed-infected samples	
10	*B. burgdorferi* s.l. *Rickettsia* spp.	20.20 16.20	90.60 (163,336)	*B. turcica* (27,00) *B. hispanica* (7,60) *R. aeschlimannii* (9,50)
11	*B. burgdorferi* s.l. *Rickettsia* spp.	25.40 17.00	91.90 (11,506)	*Borrelia* spp. (2,40) *Rickettsia* spp. (2,60)
12	*B. burgdorferi* s.l. *Rickettsia* spp.	25.70 18.80	92.40 (5952)	*B. valaisiana* (7,60) *Rickettsia* spp. (2,60)
13	*F. tularensis* *Rickettsia* spp.	25.50 17.20	91.00 (11,506)	*F. tularensis* (9,90) *R. aeschlimannii* (11,30)
14	*F. tularensis* *Rickettsia* spp.	12.60 21.10	91.70 (40,696)	*F. tularensis* (9,90) *Rickettsia* spp. (4,10)
			Clinical material	
15	*C. burnetii*	21.40	90.60 (8926)	*C. burnetii* (5,30)
16	*C. burnetii*	21.30	90.00 (7223)	*C. burnetii* (5,00)

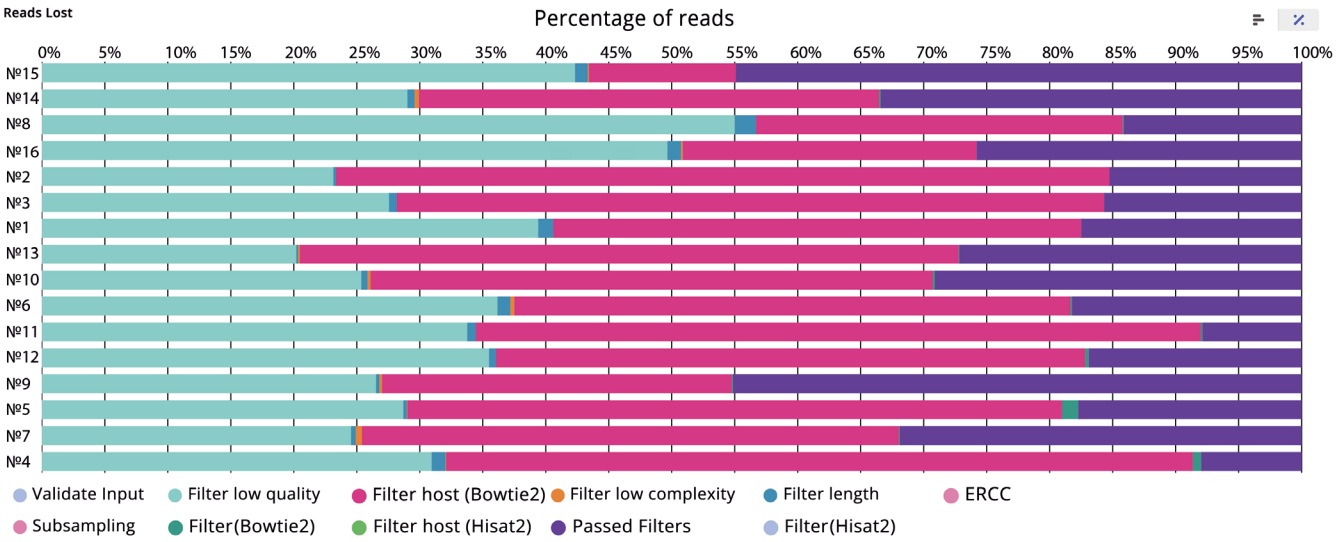

Fig. 1. Histogram showing the result of MGS data quality assessment by *16S rRNA* gene regions.
The color of the sectors in the histogram reflects the number of reads for each sample that passed quality filtering (in %, top).
For a color version of the figure, see the journal website.

ed in sample No. 10 by MGS. It was not possible to confirm the results of Borrelia species identification in sample No. 10 by Sanger sequencing, which is due to the mixed-infection of the sample with *H. aegyptium* tick suspension. The results of species identification of the remaining microorganisms detected in the samples were confirmed by sequencing of pathogen genome fragments.

By MGS method in 3 samples containing DNA of the tularemia pathogen (Ct 10.1; 12.6; 25.5) *F. tularensis* was identified to species, in 1 sample (Ct 26.6) the presence of microorganisms of the *Francisella* spp. genus was found, species identification could not be performed.

C. *burnetii* was identified by MGS results using the *16S rRNA* gene region in 2 obviously positive blood plasma samples from patients with Q fever (Ct values 21.3–21.4). The presence of *C. burnetii* DNA was detected in clinical samples 15, 16 from Q fever patients by MGS, the proportion of target reads was 5.0–5.3%. Furthermore, nucleotide sequences of *Methylophilus medardicus* bacteria, as well as representatives of the *Acinetobacter* and *Shingomonas* genera were detected in clinical samples, which may indicate possible contamination of samples at the stages of collection, storage and laboratory examination [20].

We compared the results of the study of field and clinical samples with different DNA load of pathogens of NFI obtained by MGS methods using *16S rRNA* gene regions and PCR. It is shown that as a result of MGS of 6 samples, positive for the presence of borreliae DNA of the *B. burgdorferi* s.l. genetic complex, identification of borreliae to genus (*Borrelia* spp. Ct 21.8; 22.1; 21.1; 25.4) was carried out in 4 samples, while the identification to species (*B. valaisiana* Ct 25.7, *B. turcica*, *B. hispanica* St 20.2) was carried out in 2 samples.

According to MGS results, fragments of *Rickettsia* spp. genome were detected in all obviously positive samples, in 4 samples (Ct values 16.2; 17.2; 17.2 and 23.3) the rickettsia species (*R. aeschlimannii*) was identified, in 3 samples (Ct values 17.0; 18.8 and 21.1) species identification of rickettsia could not be performed. The presented results of identification of *Borrelia* and rickettsiae in the studied material (Table 3) are confirmed by the literature data on the difficulty of species identification by MGS of representatives of the *Rickettsia* and *Borrelia* genera [8, 15]. Accurate species identification of *Rickettsia* and *Borrelia* using MGS is difficult due to high homology of nucleotide sequences of *16S rRNA* gene for these bacterial pathogens [12, 13]. In the case of detection of microorganisms of the *Rickettsia* and *Borrelia* genera by MGS of the *16S rRNA* gene, further identification to species by Sanger sequencing may be necessary.

The only pathogen that could not be confirmed by MGS was *A. phagocytophilum*.

Taxonomic composition of the microbiome of ixodid ticks

The study of the taxonomic structure of the microbiome of ticks was carried out in accordance with their species affiliation, place and territory of collection (**Fig. 2**).

Main taxonomic groups of the tick microbiome:

- for representatives of *I. ricinus* (samples Nos. 1–3): *Flavobacterium* spp. (57–81%), *Pseudomonas* spp. (7–27%), *Serratia* spp. (2–4%), *Pedobacter* spp. (2–4%);
- for representatives of *I. ricinus* (samples Nos. 4, 5): *Candidatus Midichloria mitochondrii* (31–87%), *Clostridium* spp. (6–61%), *Sphingomonas* spp. (3%), *Staphylococcus* spp. (1–10%), *Bradyrhizobium* spp. (1%);
- for representatives of *I. ricinus* (samples Nos. 11, 12): *Pseudomonas* spp. (7–49%), *Serratia* spp. (4–12%), *Rickettsiella endosymbiont of Pandinus imperator* (3–19%), *Rhodobacterales* spp. (3%);
- for representatives of *D. reticulatus* (samples Nos. 13, 14): *Flavobacterium* sp. Nj (25–53%), *Cardinium endosymbiont of Bemisia tabaci* (19%), *Clostridium* spp. (15%), *Francisella-like endosymbiont of Dermacentor reticulatus* (9–21%), *Francisella persica* (2%), uncultured *Francisella* spp. (1–6%), *Bradyrhizobium* spp. (1–3%), *Dyadobacter* spp. (1–3%);
- for representatives of *R. annulatus* (samples No. 7): *Rickettsiella endosymbiont of Pandinus imperator* (5%), uncultured *Coxiella* spp. (10%), *Wolbachia pipientis* (9%), *Candidatus Coxiella mudrowiae* (6%), *Coxiella endosymbiont of Rhipicephalus microplus* (3%), *Coxiella endosymbiont of Rhipicephalus geigyi* (1%), *Coxiella endosymbiont of Rhipicephalus turanicus* (3%), *Shingomonas* spp. (5%), *Staphilococcus* spp. (5%), *Bradyrhizobium* spp. (3%), *Flavobacterium* spp. (2%), *Leptotrichia wadei* (2%);
- for representatives of *H. aegyptium* (samples No. 10): *Rickettsia endosymbiont of Bemisia tabaci* (3%), *Flavitalea flava* (1%), uncultured *Borrelia* spp. (9%), *Blastopirellula marina* (4%), *Dyadobacter alkalitolerans* (2%), *Bradyrhizobium* (1%);
- for representatives of *D. marginatus* (samples Nos. 6, 8): *Pseudomonas* spp. (9–33%), uncultured *Arsenophonus* spp. (11%), uncultured *Alteromonas* spp. (2%), *Alphaproteobacteria bacterium* (2%), *Coxiella endosymbiont of Dermacentor marginatus* (2%).

Discussion

In this study, we applied the MGS method using *16S rRNA* gene regions for detection and identification

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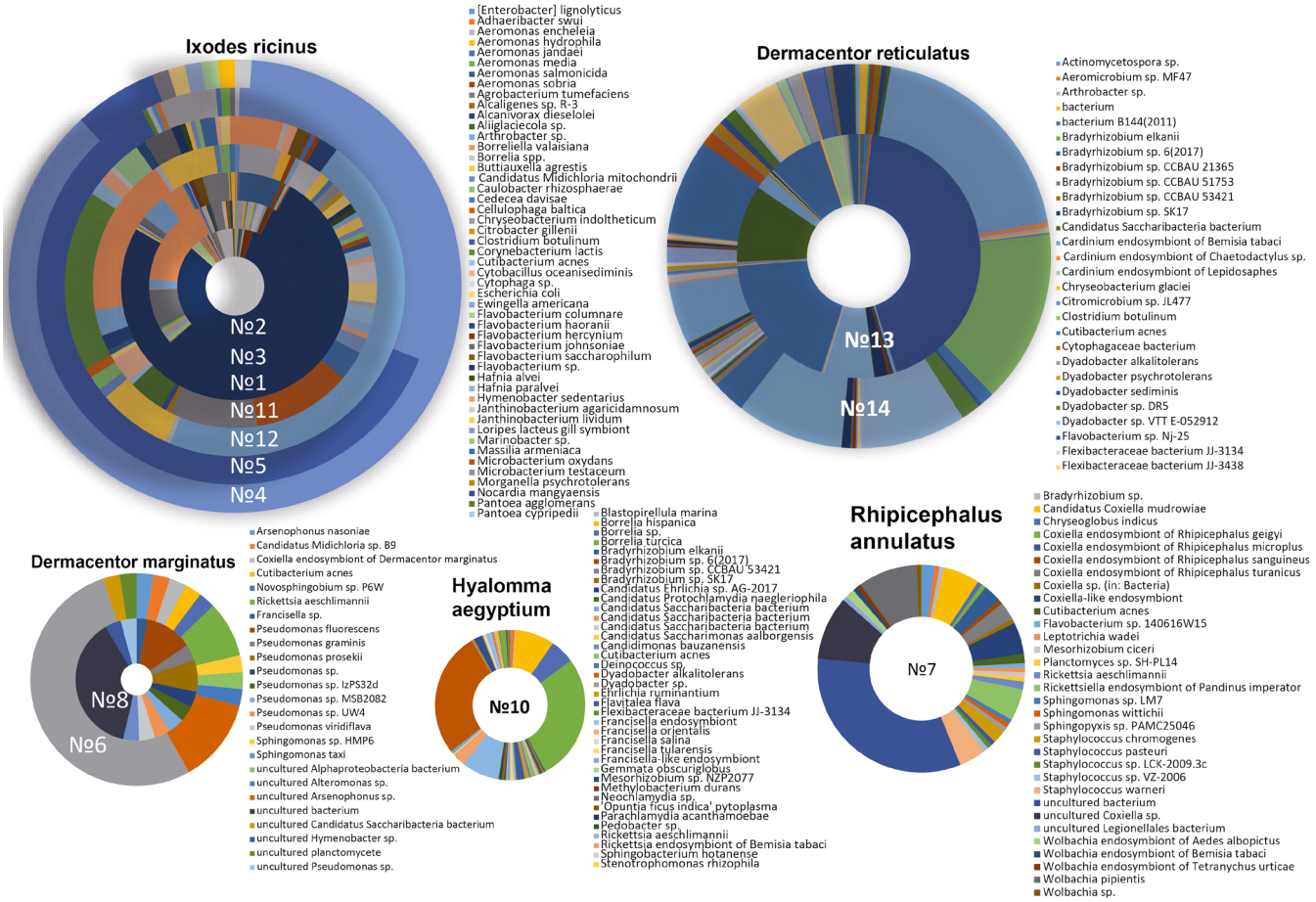

Fig. 2. Taxonomic composition of microbiomes of ixodid ticks (sample numbers are indicated by numbers).
Due to the availability of a large amount of data, only the most represented taxa are marked with color markers.
For a color version of the figure, see the journal's website

of known pathogens of bacterial etiology in samples of clinical and field material, and investigated the possibility of its use in simultaneous detection of different types of pathogens. Mixed infection with two pathogens of NFI (borreliosis, tick-borne rickettsiosis, tularemia) in several pools of ixodid ticks was determined. The negative result in the detection of *A. phagocytophilum* may be due to low concentration of the bacterial pathogen in the tested material, as well as insufficient quality and quantity of data obtained after bioinformatics processing.

The results of using the method of targeting MGS by *16S rRNA* gene regions to detect pathogens of NFI in samples of clinical and field material are presented in a number of publications. Thus, L. Kingry et al., using the MGS method at the *16S rRNA* gene region in clinical samples from febrile patients, detected tick-borne pathogens: *B. burgdorferi* s.l., *B. mayonii*, *B. miyamotoi*, *B. hermsii*, *A. phagocytophilum*, *Ehrlichia chaffeensis*, *E. muris* subsp. *eauclarinsis*, *E. ewingii*, and *F. tularensis* [8]. R. Takhampunya et al. detected microorganisms of the *Anaplasma*, *Bartonella*, *Coxiella*, *Leptospira*, *Orientia* genera in the blood of patients with fever of unclear genesis [15]. Furthermore, other authors have obtained the results of the study using the *16S rRNA* MGS method of samples of ixodid ticks for the entire spectrum of tick-borne pathogens [20–22].

One of the demanded areas of application of the MGS method for the *16S rRNA* gene region is the study of clinical samples from patients with fevers of unclear genesis in cases when traditional methods of research (PCR, enzyme immunoassay, serologic methods, etc.) failed to identify the pathogen. Detection of microorganisms belonging to genera including pathogens of NFI in the material from febrile patients will allow further in-depth molecular genetic analysis to confirm the presence of DNA of the detected pathogens in the sample.

In the literature, there are numerous reports of human cases of combined forms of NFI caused by associations of microorganisms, the clinical course of which is significantly more severe compared to mono-infections, and laboratory confirmation of the diagnosis is difficult [23, 24]. In the etiologic interpretation of such cases, the metagenomic approach acquires special relevance and clearly demonstrates its advantage.

As a result of bioinformatics processing of MGS data using variable fragments of the *16S rRNA gene*, the taxonomic composition of the microbiome associated

with *I. ricinus*, *D. reicinus*, *R. annulatus*, *H. aegyptium*, *D. marginatus* ticks collected in the southern regions of Russia (Fig. 2) was determined. The microbiome of all ticks was dominated by the following microorganisms: *Flavobacterium* spp., *Pseudomonas* spp., *Serratia* spp., *Aeromonas* spp., *Pedobacter* spp., *Bradyrhizobium* spp. and *Shingomonas* spp. Probably, some of these bacteria entered the organism of mites in the process of their vital activity or inhabit their chitinous exoskeleton and digestive system, while not being symbionts of arthropods [25].

Furthermore, DNA markers of microorganisms — endosymbionts of ticks, including *Candidatus Midichloria mitochondrii* (samples Nos. 4, 5), representatives of genera *Rickettsiella*, *Coxiella*, *Candidatus Coxiella mudrowiae* (sample No. 7), non-pathogenic and conditionally pathogenic for human species *Francisella* spp. (*F. frigiditurris*, *F. philomiragia*, *F. persica*) (sample No. 13).

It is interesting to note that the composition of the bacterial community of the ixodid tick pool of sample No. 10 based on the data of MGS sites of the *16S rRNA* gene differed significantly from the other samples, which may be related to the peculiarities of the tick feeder and the species of the vector of tick-borne infections — the Mediterranean turtle. Bacteria of the *Bradyrhizobium* genus — symbiotic microorganisms of plants, *Blastopirellula marina* and *Dyadobacter alkalitolerans*, being natural inhabitants of saline water bodies and sandy soils, were detected in small amounts. The obtained results, presented in Table 3 and Fig. 2, are consistent with the literature data on bacterial pathogens carried by *H. aegyptium* ticks and found in the blood of reptiles (pythons, lizards and turtles) [26, 27]. Information has been published on the detection of markers of pathogens (borreliosis, tick-borne rickettsiosis) during the study of biological material from reptiles and ticks removed from reptiles: *R. aeschlimannii* [28], *B. turcica* [29, 30], *B. hermsii* [31], *B. crocidurae* [32] and *B. hispanica* [33]. The above data on the high occurrence of *Borrelia* — pathogens of relapsing fevers in animals confirm the wide distribution of these bacterial pathogens in a number of regions and have almost ubiquitous character.

The use of MGS in the study of ixodid ticks can obviously be effective in obtaining comprehensive information on the species spectrum of NFI pathogens, as well as endosymbionts associated with different species of ixodid ticks inhabiting different regions. As a consequence, new perspectives in the study of the species spectrum of pathogens, as well as the selection of microorganisms to assess the specificity of existing and developing PCR test systems for the study of field samples [34]. The information obtained in this work about the species of endosymbiotic microorganisms detected in ixodid tick pools is consistent with previously published data [34].

It is necessary to take into account the limitations of the method when determining the species affiliation of closely related microorganisms, including for a number of Borrelia and Rickettsia species based on MGS data [35, 36]. It has been shown that the results of taxonomic classification may differ depending on the variation regions used [37–39]. In this case, the use of a mixture of primers targeting different hypervariable regions of the *16S rRNA* gene [40–42] contributes to increasing the discriminatory power of the method, which was applied in the present study.

Conclusion

We analyzed the taxonomic composition of microorganisms, as well as the detection and identification of pathogens in samples by MGS method using *16S rRNA* gene sections, and experimentally confirmed the effectiveness of this method for the detection of pathogens in clinical and field samples. Microorganisms belonging to *Rickettsia* spp., *Borrelia* spp., *Francisella* spp. were detected, including human pathogenic species, as well as species identification of pathogens with different DNA load in the studied material, in particular, *R. aeschlimannii* (Ct at PCR up to 23.3), *C. burnetii* (Ct < 21.4), *F. tularensis* (Ct < 26.6), *Borrelia* spp. *burgdorferi* s.l. (*B. valaisiana*, Ct < 25.7), borreliae of the pathogens of relapsing fevers (*B. turcica*, *B. hispanica* Ct < 20.2). The taxonomic structure of the microbiome of *I. ricinus*, *D. reticulatus*, *R. annulatus*, *H. aegyptium*, *D. marginatus* ticks collected in the southern regions of Russia was studied. It is shown that microorganisms from genera *Flavobacterium*, *Pseudomonas*, *Serratia*, *Aeromonas*, *Pedobacter*, *Bradyrhizobium* and *Shingomonas* predominate. DNA markers of microorganisms — endosymbionts of ticks *Candidatus Midichloria mitochondrii*, representatives of genera *Rickettsiella*, *Coxiella*, non-pathogenic and conditionally pathogenic for human species of the genus *Francisella* — were found in the pools of ixodid ticks.

Continued work in this area will allow a more accurate assessment of the resolution of the method for the detection and identification of pathogens. The study of patterns of existence of pathogens in the structure of the tick microbiome is a promising area for further research.

The main advantage of the MGS method using the *16S rRNA* gene region in the study of field and clinical samples is the possibility to perform simultaneous detection and identification of all bacteria in the sample, including known pathogens, without the necessity for several diagnostic tests. Targeted MGS can be used for etiologic interpretation in case of atypical course and abbreviated clinical picture of the disease, in case of mixed-infection with several pathogens of bacterial etiology, when there is a difficulty with the diagnosis using traditional laboratory methods of investigation. MGS can also be used to obtain information on the tax-

onomic composition of the bacterial microbiome in the organism of different species of carriers and vectors of NFI pathogens.

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Production and purification of recombinant proteins VP2 and VP3 of the *Alongshan* virus of the *Jingmenvirus* group and evaluation of their immunochemical properties

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Abstract

Introduction. *Alongshan* virus is a representative of the unclassified group of *Jingmenviruses* (*Flaviviridae*), which is detected in Ixodes persulcatus, Ixodes ricinus ticks and various mosquito species in Russia, China, Finland and France. Unlike traditional orthoflaviviruses, the *Alongshan* virus genome is represented by 4 positive-sense RNA segments. The first and third segments of the genome encode proteins homologous to proteins of the replicative machinery of orthoflaviviruses, the remaining segments encode putative structural proteins that have no known homologues: segment 2 — VP1a (envelope protein), VP1b and NuORF; segment 4 — VP2 (capsid) and VP3 (membrane). Human cases of Alongshan virus-associated disease have been described.

The **aim** of this study is to develop a system for expression and purification of recombinant VP2 and hydrophilic site VP3 proteins to test their antigenic properties.

Materials and methods. Miass527 strain of *Alongshan* virus was used to produce hyperimmune mouse sera and recombinant proteins in a bacterial expression system. Bioinformatic analysis of sequences encoding target proteins and genetically engineered cloning were carried out in this study. Western blotting and enzyme immunoassay (ELISA) were performed to control the results.

Results. Recombinant proteins of *Alongshan* virus have been used in a laboratory diagnostic test system to determine the presence of antibodies to the virus. The obtained recombinant VP2 protein is able to detect antibodies in all tested sera of infected mice, as well as antibodies in human sera both in Western blotting and in enzyme immunoassay. At the same time, antibodies to the recombinant region of VP3 protein were detected irregularly in antiviral immune sera.

Conclusion. The detection of antibodies to *Alongshan* virus in patients confirms the necessity for further investigation of this group of viruses.

Keywords: *Jingmenvirus group, Alongshan virus, diagnostics, recombinant proteins*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products of Russian Academy of Sciences (protocol No. 200923-1, September 20, 2023).

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Оригинальное исследование
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Получение и очистка рекомбинантных белков VP2 и VP3 вируса *Alongshan* группы *Jingmenvirus* и оценка их иммунохимических свойств

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Аннотация

Введение. Вирус *Alongshan* — представитель неклассифицированной группы *Jingmenvirus* (*Flaviviridae*), обнаружен в клещах *Ixodes persulcatus*, *Ixodes ricinus* и различных видах комаров в России, Китае, Финляндии и Франции. В отличие от классических ортофлавивирусов, геном вируса Alongshan представлен 4 сегментами РНК положительной полярности. Первый и третий сегменты генома кодируют белки, гомологичные белкам репликативной машинерии ортофлавивирусов, остальные сегменты кодируют предположительно структурные белки, не имеющие известных гомологов: сегмент 2 — VP1a (белок оболочки), VP1b и NuORF; сегмент 4 — VP2 (капсидный) и VP3 (мембранный). Описаны случаи заболевания людей, связанные с вирусом Alongshan.

Цель работы — разработка системы экспрессии и очистки рекомбинантных белков VP2 и гидрофильного участка VP3 для проверки их антигенных свойств.

Материалы и методы. Для получения гипериммунных сывороток мышей и рекомбинантных белков в бактериальной системе экспрессии был использован штамм Miass527 вируса *Alongshan*. Произведены биоинформатический анализ последовательностей, кодирующих целевые белки, генно-инженерное клонирование. Для контроля результатов осуществлены иммуноблотинг и иммуноферментный анализ.

Результаты. Рекомбинантные белки вируса *Alongshan* использованы в лабораторной диагностической тест-системе для определения наличия антител к вирусу. Полученный рекомбинантный белок VP2 способен выявлять антитела во всех исследованных сыворотках инфицированных мышей, а также антитела в сыворотках людей как в вестерн-блоттинге, так и в иммуноферментном анализе. При этом антитела к рекомбинантному участку белка VP3 в противовирусных иммунных сыворотках выявлялись нерегулярно.

Заключение. Выявление антител к вирусу *Alongshan* у пациентов подтверждает необходимость дальнейшего исследования данной группы вирусов.

Ключевые слова: *группа Jingmenvirus, вирус Alongshan, диагностика, рекомбинантные белки*

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

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Introduction

Members of the genus *Orthoflavivirus* (family *Flaviviridae*) infect vertebrates and invertebrates. Such important human pathogens as dengue virus, yellow fever virus, West Nile virus, tick-borne encephalitis virus (TBEV), Japanese encephalitis virus, etc. belong to this genus. Recently, a new group of viruses named *Jingmenvirus*, which is related to the *Orthoflavivirus* genus, has been characterized [1]. Unlike classical flaviviruses, *Jingmenviruses* have a segmented RNA genome [2]. The first and third segments of the *Jingmenvirus* genome encode proteins homologous to helicase and RNA-dependent RNA polymerase of orthoflaviviruses [3, 4]. The second and fourth segments encode unique proteins: envelope proteins VP1a and VP1b, presumably capsid protein VP2 and membrane protein VP3.

The Jingmenvirus group includes viruses such as *Jingmen* tick virus, *Alongshan* virus (ALSV), Yang-

gou tick virus and Takachi virus [1, 5, 6]. They have a wide geographical distribution and are detected in blood-sucking arthropods (especially ticks) and mammals, including those found in human sera [3, 7–13]. ALSV and *Jingmen* tick viruses apparently can cause acute infection in humans accompanied by fever [1, 14–16]. ALSV was first detected and isolated from the blood of a patient with febrile illness in China [17]. Later, ALSV was detected by polymerase chain reaction (PCR) in other patients: 86 people with a history of fever, headache, and tick bites between May and July 2017 were examined [17]. The virus was also detected in *Ixodes persulcatus* ticks and various mosquito species (*Anopheles yatsushiroensis*, *Aedes vexans*, *Culex pipiens pallens* and *Culex tritaeniorhynchus*) in China.

In Russia, the virus was detected in ticks in the Kaliningrad, Chelyabinsk and Ulyanovsk oblasts and in the Republics of Altai, Tatarstan, Karelia and Tyva [5, 18–20]. In connection with the described cases of human disease, epidemiological studies of *Jingmenvirus* group representatives are essential.

Currently, it is not known to which protein antibodies are produced during *Jingmenvirus* infection. Recombinant VP2 protein was successfully used to detect ALSV-specific antibodies in sheep and cattle in China by enzyme-linked immunosorbent assay (ELISA) [17]. ALSV-specific antibodies were detected in 9.2% (22/240) of the examined sheep and 4.6% (11/240) of the examined cattle. In 2019, Finnish scientists used constructs encoding the ALSV envelope proteins VP1a, VP1b, VP2 and VP3 transfected into Vero E6 mammalian cells. These cells were used to detect antibodies in the sera of 900 patients by immunofluorescence [21].

The aim of this study is to obtain recombinant proteins of ALSV and use them in a laboratory diagnostic test system to determine the presence of antibodies to the virus.

Materials and methods

Virus and cells

To obtain recombinant proteins, we used the Miass527 strain of ALSV isolated from *I. persulcatus* ticks collected in 2014 in Miass, Chelyabinsk region (NCBI access code: MN648770-MN648773) [1]. The recombinant sE TBEV protein was kindly provided by V.S. Baryshnikova [22].

Escherichia coli cells, strain TOP10 (Promega) were used for cloning, and strains JM109 and BL21 (Promega) were used for expression of recombinant proteins.

Production of hyperimmune blood sera

To obtain hyperimmune sera, mongrel mice ICR (Scientific Center of Biotechnology) were used, immunized under the skin with ALSV (strain Miass527) with Freund's adjuvant (BD) 3 times (once a week), 10 days after the last injection the blood was collected in total (decapitation). The obtained sera from 3 mice to ALSV were used in immunoblotting and ELISA. Serum from 1 mouse to TBEV (strain KE-328) was kindly provided by V.S. Baryshnikova [22]. Serum from an unimmunized mouse was used as a negative control.

The study protocol was approved by the Ethical Committee of the M.P. Chumakov Federal Scientific Center for Research and Development of Immunobiological Products of the Russian Academy of Sciences (Poliomyelitis Institute) (Protocol No. 200923-1 of September 20, 2023).

Sera of the conditionally healthy population

Sera of conditionally healthy individuals with antibodies to TBEV from Moscow and Moscow region were kindly provided by the Center for Hygiene and Epidemiology in Moscow region.

Bioinformatics analysis

To determine signal peptides, hydrophilic and hydrophobic parts of proteins, the SignalP 4.1 Server[1] program was used. DNA to Protein program on the Zbio.net website was used to determine protein mass.

Cloning of VP2 and VP3 protein genes of Alongshan virus (strain Miass527)

Reverse transcription was performed using Invitrogen SuperScript III reverse transcriptase (Thermo Fisher Scientific).

PCR was performed with a Veriti 96 Well Thermal Cycler (Applied Biosystems) using Platinum Super Fi II polymerase (Thermo Fisher Scientific).

PCR mix composition: SuperFi buffer 10 µl, 2.5 mM nucleotides 1 µl, primers (forward and reverse) 1 µl each, Platinum SuperFi II polymerase 1 µl, cDNA 2 µl, water 32 µl. Total volume 50 µl.

PCR program for Platinum Super Fi II polymerase is as follows:

- 98°C 30 s;
- 98°C 10 s;
- 60°C 10 s;
- 72°C 30 s/30 cycles;
- 72°C 5 min.

The following restriction endonucleases were used in this study:

BamHI and *HindIII (*Thermo Fisher Scientific) for plasmid pQE32 (Qiagen) and amplicons VP2 and VP3;

BamHI and *XhoI* (Thermo Fisher Scientific) for plasmid pet28a+ (plasmid collection of M.P. Chumakov Federal Scientific Center for Research and Development of Immunobiological Products of Russian Academy of Sciences) and the VP2 amplicon.

[1] SignalP 4.1 Server».
URL: http://www.cbs.dtu.dk/services/TMHMM-2.0

DOI: https://doi.org/10.36233/0372-9311-612

After restriction, the obtained vectors and inserts were run in 1% agarose gel (PanEco) based on 1xTBE buffer: 0.09 M Tris (DiaM), 0.09 M H_3BO_3 (Pushchino Laboratories), 2 mM EDTA (DiaM) and purified using centrifuge columns (Qiagen).

DNA concentration was measured by optical density on a NanoDrop One instrument (Thermo Fisher Scientific). The ligation reaction was performed using T4 DNA ligase (Thermo Fisher Scientific). The vector and insert were taken in a 5 : 1 ratio.

E. coli TOP10 cells were transformed with the obtained ligase mixtures by heat shock method and grown in LB medium (**Table 1**) at 37°C. The presence of the insert was verified by PCR with primers for cloning. The Sanger sequencing method was used to confirm the presence of polyhistidine-tag, start and stop codons, and the absence of nonsynonymous substitutions. Sequencing was performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an Applied Biosystems 3500 Genetic Analyzer (Waltham) instrument.

Transformation and cultivation of E. coli cells upon expression

During transformation, the following strains were used for protein expression: strain JM109 for plasmid pQE32 and strain Bl21 for pet28a+. LB, SOB and TB media were used for cell cultivation (Table 1).

One cell colony was taken from a Petri dish and grown in 5 mL of medium supplemented with 100 ng/mL antibiotic (ampicillin for pQE32, kanamycin for pet28a+) for 18 hours. The cell suspension was then transferred to 250 mL of medium with 100 ng/mL ampicillin or 50 ng/mL kanamycin. When the cell mass reached an optical density of 0.5–0.8 at a wavelength of 600 nm (9.6×10^9 cells/mL), isopropyl-β-D-1-thiogalactopyranoside (IPTG; Helicon) was added. The cells were incubated at different temperatures and times with stirring. The cell mass was then precipitated by centrifugation at 1700g, 4°C for 30 min, and the resulting precipitate was washed with 50 mL of phosphate-salt buffer (PBS; Sigma-Aldrich).

Table 1. Composition of media for induction of expression of the target protein in *E. coli* JM109 and Bl21 cells

Medium	Ingredients	Concentration, g/L
LB	Tripton	10
	Yeast extract	5
	NaCl («Fluka»)	10
SOB	Tripton	20
	Yeast extract	5
	NaCl	0,585
	KCl («Fluka»)	0,185
TB	Tripton	12
	Yeast extract	24
	Glycerol 99%	20*

Note. *Concentration of glycerol is given in mL/liter.

The conditions under which recombinant proteins expression was performed, unless otherwise specified: incubation time of cells — 12 h in LB medium at 37°C and IPTG concentration 0.5 mM.

Cell disruption by ultrasound

The cell mass was resuspended in 20 mL of lysis buffer (HEPES 100 mM (DiaM), NaCl 0.15 M, pH 8.5) and treated with ultrasound (Soniprep 150 device, MSE) as follows: 3 times for 1 min each with a 7 ms pulse on ice. Then the cells were centrifuged (7800g, 4°C, 30 min), the precipitate was resuspended in 20 mL of lysis buffer with 8 M urea (to release the protein into the soluble fraction) and treated with ultrasound once for 30 s, followed by centrifugation (7800g, 4°C, 30 min).

Separation and purification of recombinant proteins

Recombinant proteins were purified by affinity chromatography using a ready-to-use Ni-NTA Fast Start kit (Qiagen).

Buffer change to PBS and concentration were performed using Amicon Ultra-15 10 kDa centrifuge ultrafilters (Merck).

The resulting recombinant proteins were separated by electrophoresis in 15% polyacrylamide gel (PAGE) under denaturing conditions (SDS-PAGE). A calibration plot constructed from known concentrations of BSA (Genesystool) was used to determine the concentration of target proteins. Further, the concentration of the target protein was measured using the GBox instrument (Syngene) in the GeneTools program (Syngene).

Obtaining negative control (Mock)

To obtain a negative control (Mock), the same manipulations were performed with pQE-32 and pet28a+ plasmids without the insert as with the constructs with the insert: transformation, cultivation of the corresponding bacterial cells, expression, isolation and purification. Controls were further used for visualization in PAGE, immunoblotting and ELISA.

Immunoblotting

The obtained recombinant proteins were separated by electrophoresis in 15% PAGE-SDS and transferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated with 5% skimmed cow's milk (Best Value) in Tris-buffered saline (TBS: 25 mM Tris, 0.15 M NaCl, pH 8.3) for 1 h.

The membrane was then incubated with target serum for 1 h: mouse, human or to histidine tag. Next, the membrane was washed with TBS with 0.05% Tween-20 (TBS-T) and incubated with horseradish peroxidase (HRP)-labeled antibodies against mouse or human IgG, respectively (Abcam) for 1 h. If HRP-labeled antibod-

ies against histidine tag (Abcam) were used, incubation with secondary labeled antibodies was not required.

Before imaging, the membrane was washed again with TBS-T, then imaging was performed using the ECL kit (Bio-Rad) in a Genesys gel-documentation system (Genesys).

Enzyme immunoassay

80 ng of protein diluted in PBS was added to a well of a 96-well plate and incubated overnight at 4°C. The plate was washed with PBS, incubated with 4% skimmed cow's milk (Best Value) in PBS for 1 h, then with mouse sera in PBS with 0.05% Tween-20 for 1 h. The plate was washed, incubated with HRP-conjugated antibodies against mouse IgG (Abcam) for 1 h, respectively, followed by washing and addition of TMB substrate (Sigma-Aldrich), after 30 min the reaction was stopped with 2 M sulfuric acid (Lenreactiv). The results were detected at a wavelength of 450 nm on a spectrophotometer (Thermo Fisher Scientific).

Results

Selecting and obtaining targets for cloning

In the current study, the capsid protein VP2 was presumably chosen because Chinese scientists have already detected antibodies to this protein in cattle [13], as well as the membrane protein VP3, which was cloned for the first time in our study. The proteins are encoded in segment 4 of the ALSV genome.

The sequence of the signal peptide (19 aa) was determined for the VP2 protein; it has no hydrophobic sites; the 243 aa fragment lacking the signal peptide was chosen for cloning because it could complicate the subsequent purification of the protein. VP3 protein contains 9 transmembrane hydrophobic domains, thus, hydrophilic regions 1–89 aa and 244–389 aa were chosen for cloning (**Fig. 1**). Presumed protein sizes were 25 kDa for VP2 is, and 10 and 18 kDa for hydrophilic regions of VP3-1 and VP3-2 proteins, respectively.

Then, based on the nucleotide sequences of VP2 protein and hydrophilic regions of VP3 protein, primers were selected (**Table 2**) and the corresponding PCR products were obtained (**Fig. 2**).

Obtaining vector constructions

The construct with the insertion of the 244-389 aa fragment of VP3 protein based on the pQE-32 vector was successful (**Figs. 3, 4**). However, with other inserts based on the pQE-32 vector, the expression of recombinant proteins either did not occur (in the case of the VP2 insert) or led to the death of bacterial cells at 3 h of cultivation (in the case of the insertion of the 1–267 aa fragment of VP3 protein), which indicates the possible toxicity of the protein for these bacterial cells. There-

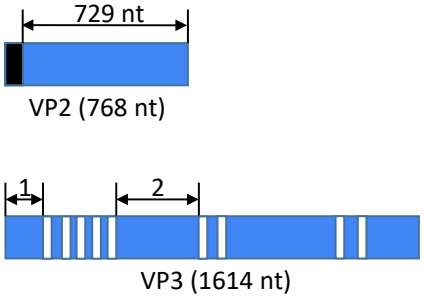

Fig. 1. Schematic representation of VP2 and VP3 target regions chosen for cloning.

Black fragment – signal peptide of VP2 protein, blue – transmembrane domains of VP3 protein. 1 — VP3 protein region 1–267 aa, 2 — VP3 protein region 244–389 aa.

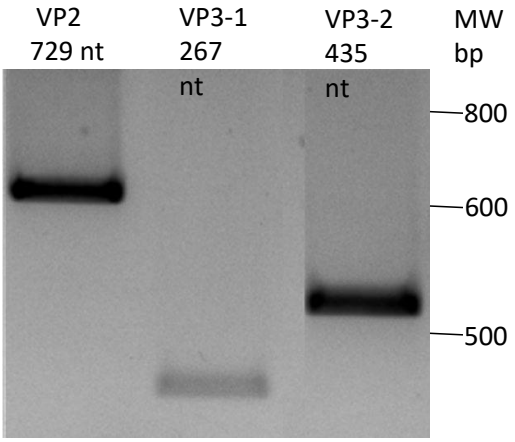

Fig. 2. Electrophoretic analysis of amplicons of target regions of VP2 and VP3 protein genes.

MW, DNA length marker. VP2, fragment encoding 1–89 aa; VP3, fragment encoding 244–389 aa VP3.

Table 2. Oligonucleotides for cloning the VP2 protein and the hydrophilic regions of the VP3 protein

Primer	Sequence	Genome regions based on the ALSV sequence (GenBank #MN648773.2)
VP2-28s	GAGCTAGGATCCAAGCCAAACGGAGCCCCAGAT	168–188
VP2-28as	GAGCTACTCGAGCTACTGAAAAACCTGGTAGTTG	857–872
VP3s 244–389	GAGCTAGGATCCGACAAGGATCAAGCCTACCTC	1576–1597
VP3as 244–389	TAGCTCAAGCTTCCATTGGGTGTAGACCAGGT	1998–2017
MiVP3s 1–89	GCTAGGATCCGTGCGACCCCAACTACCAGGT	848–868
MiVP3as 1–89	TAGCTCAAGCTT TCTCTCCTCCAGTCGCC	1095–1114

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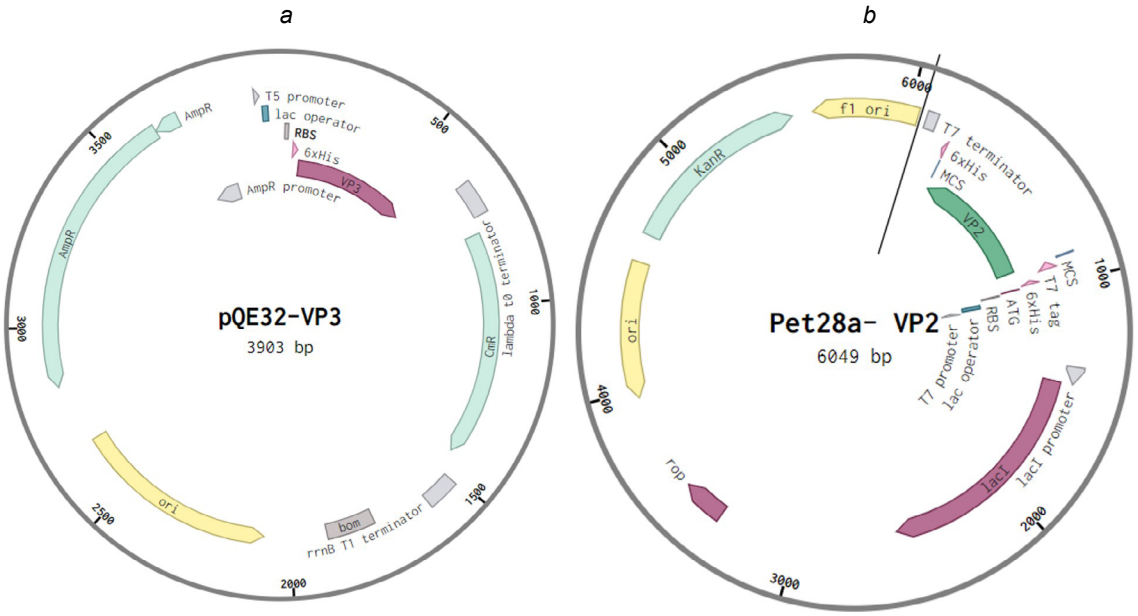

Fig. 3. Genetically engineered constructs which demonstrated expression of target proteins.
a — pQE-32 plasmid construct and VP3 protein fragment; *b* — pet28a plasmid construct and VP2 protein fragment.
AmpR — ampicillin resistance gene; lac operator — lactose operator; bp — base pairs; KanR — kanamycin resistance gene; RBS — ribosome entry site; 6xHis — 6-histidine site; MCS — restriction sites.

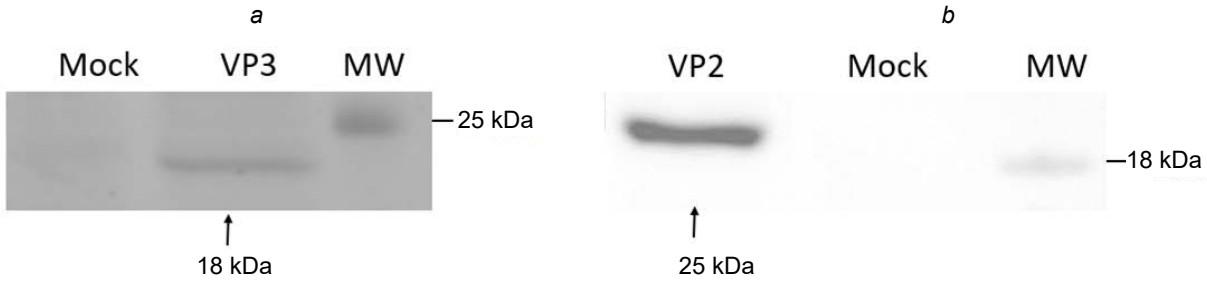

Fig. 4. Immunoblotting results of recombinant VP3 (*a*) and VP2 (*b*) proteins with antibodies to histidine.

fore, we decided to assemble a new genetically engineered construct of pET28a+ plasmid with VP2 protein insertion (Fig. 3).

The successful protein expression in pQE-32-244-389 and pet28a-VP2 constructs was confirmed using histidine-tag antibodies. Both recombinant proteins showed positive results in western blot (Fig. 4). Cells were cultured under the following conditions: LB medium, 37°C, IPTG concentration 0.5 mM, 12h.

Optimization of conditions for recombinant protein expression

A number of experiments were undertaken to increase protein yield. The following parameters were optimized: cell culture medium, duration of incubation with IPTG, IPTG concentration and cell growth temperature. **Fig. 5** shows that the highest concentration of VP3 target protein was achieved when 0.5 mM IPTG was added during bacterial growth for 12 h at 37°C. The use of different LB, SOB and TB media did not affect the expression of the partial recombinant VP3 protein (Fig. 5, *c*).

In this regard, we determined the working conditions of target protein expression – cell cultivation for 12 h with the concentration of lactose operator inducer 0.5 mM at 37°C in LB medium.

Changes in the medium conditions, IPTG concentration and temperature did not affect the expression of recombinant VP2 protein in *E. coli* cells of strain BL21 (**Fig. 6**), but with increasing time of bacterial cell growth the expression increased.

Since the bacterial medium, growth temperature and IPTG concentration did not affect the expression of recombinant VP2 protein, we chose the working conditions for expression: culturing cells for 12 h at 37°C with a concentration of 0.5 mM lactose operator inducer in LB medium.

Purification of recombinant proteins

One of the important steps for further purification of proteins is to determine their solubility. For the partial recombinant VP3 protein, it was determined to be in the soluble fraction without urea (**Fig. 7**). The target VP2 protein is in the insoluble fraction (with 8 M urea),

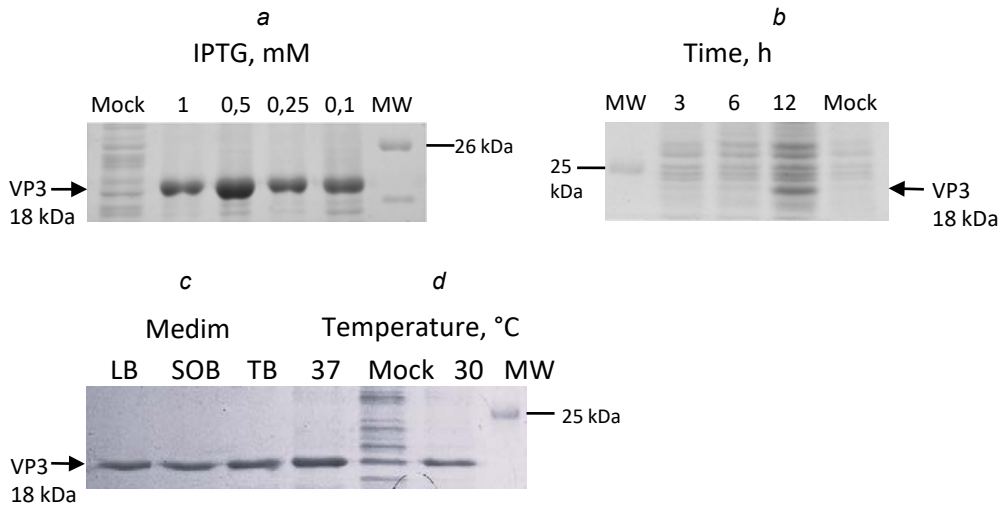

Fig. 5. PAGE-electrophoretic analysis of partial recombinant VP3 244-389 aa protein expressed at different IPTG concentrations (*a*), time (*b*), medium (*c*) and temperature (*d*).

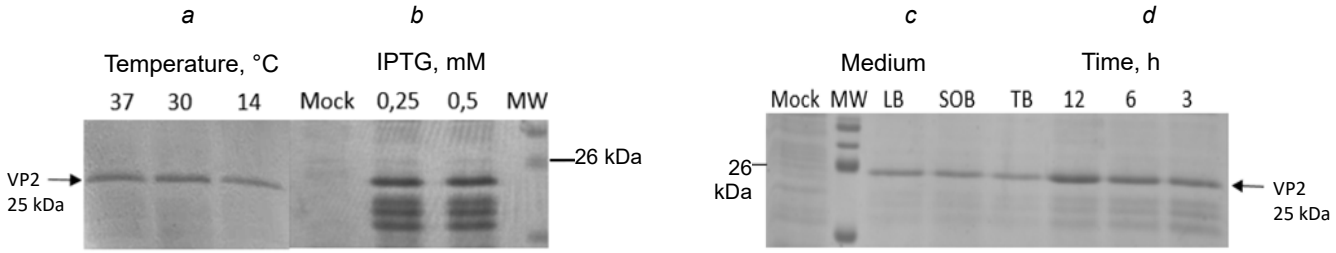

Fig. 6. PAGE-electrophoretic analysis of partial recombinant VP2 protein expressed at different IPTG concentrations (*a*), time (*b*), medium (*c*) and temperature (*d*) of expression for the target protein.

which could make it difficult to further obtain and purify. The addition of serine protease inhibitors, which may affect the solubility of the protein, was not successful — urea addition is necessary for the purification of recombinant VP2 protein.

The recombinant proteins were further purified on a gravity column by affinity chromatography (**Fig. 8**). After desalting and concentration on centrifuge filters, the concentration of proteins was measured by the Bradford method: for the partial recombinant VP3 protein it was 70 μg/mL, for VP2 protein — 120 μg/mL (when the proteins were expressed in 250 mL of medium with cell mass).

The ability of the recombinant VP2 protein to interact with antiviral antibodies was demonstrated by Western blot and the use of hyperimmune mouse sera to ALSV. Three hyperimmune mouse sera were tested — the VP2 protein interacted with all of them, while the partial recombinant VP3 protein was detected with only one. As an example, **Fig. 9** shows an immunoblot of recombinant VP2 and VP3 proteins with hyperimmune mouse serum. Serum from an unimmunized mouse was used as negative serum in the Western blot.

To prove the specificity of recombinant VP2 protein in Western blot, mouse sera to TBEV were also used (**Fig. 10**). Recombinant TBEV sE protein served as a positive control [22]. The VP2 protein we obtained did not bind to TBEV antibodies.

The results of Western blot analysis were confirmed in ELISA with hyperimmune mouse serum to ALSV (**Fig. 11**). Different protein concentrations (20, 40, 80 and 120 ng/well) and serum dilutions (1 : 180 and 1 : 360) were tested in ELISA to determine the

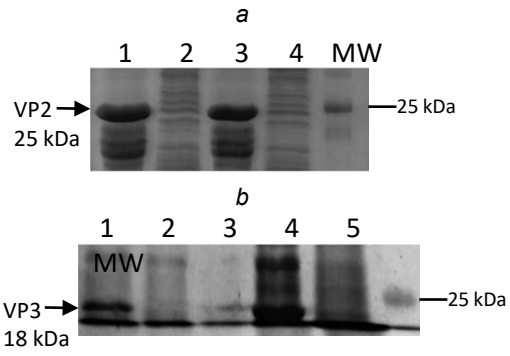

Fig. 7. Determination of VP2 (*a*) and VP3 (*b*) target protein fractions in PAGE.

a — cell lysate after sonication in lysis buffer: *1* — with 8 M urea; *2* — without additives; *3* — with 8 M urea and serine protease inhibitors; *4* — with serine protease inhibitors. *b* — cell lysate after 12 h of cultivation (*1*), Mock (*2*), cell lysate before IPTG addition (*3*), cell lysate after sonication in lysis buffer (*4*), precipitate fraction with 8 M urea (*5*)

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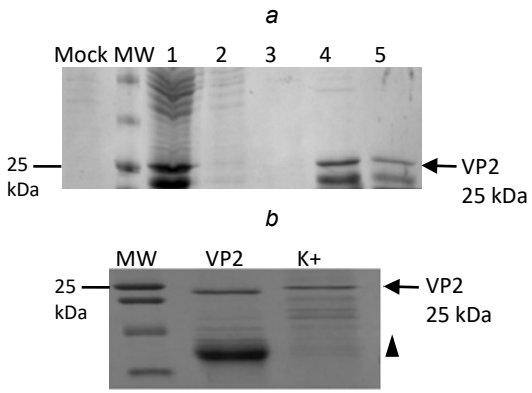

Fig. 8. Purification of target protein VP2 in PAGE using Qiagen Ni-NTA Fast Start kit (*a*) and desalting with amicones (*b*).

a: *1* — VP2 protein in lysis buffer with 8 M urea; *2* — flow-through fraction; *3* — wash-buffer column washing fraction; *4* — eluate containing target protein; *5* — eluate containing protein degradation products. *b* — recombinant VP2 protein and its degradation products (label: triangle); K+ — positive control.

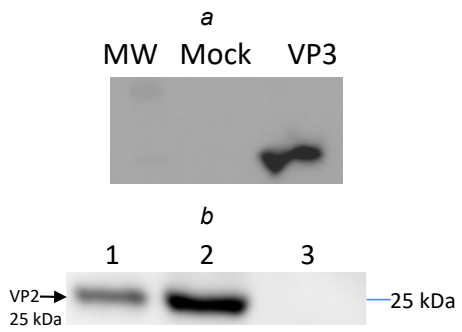

Fig. 9. Results of immunoblotting of target proteins with serum of mouse immunized with ALSV (strain Miass527).

a — expression of recombinant VP3 protein; *b* — cell lysate after expression of recombinant VP2 protein (*1*), isolated and desalted recombinant VP2 protein (*2*), Mock (*3*). HRP-labeled antibodies against mouse IgG (Abcam) were used for detection.

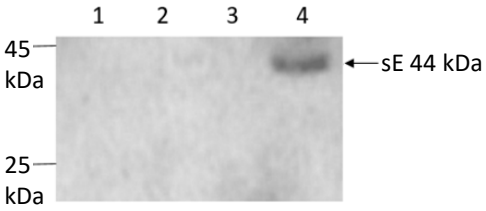

Fig. 10. Results of immunoblotting of the target protein with hyperimmune mouse serum to TBEV (strain KE-328).

HRP-labeled antibodies against mouse IgG (Abcam) were used for detection. *1* — isolated and desalted recombinant VP2 protein; *2* — Mock; *3* — lysate of SPEV cells without TBE virus; *4* — recombinant sE protein with the size of 44 kDa [22].

working concentration of recombinant proteins. A protein concentration of 80 ng/well was found to be optimal. Serum from an unimmunized mouse was used as a negative control. To further confirm the results, the hyperimmune mouse serum to ALSV was also tested in ELISA, where a plasmid was used as a substrate, with which all the same manipulations were performed as with the insertion plasmid and recombinant protein during purification (Mock).

Positive ELISA and Western blot results allow the use of VP2 protein for detection of antibodies against ALSV in sera.

Analysis of sera from conventionally healthy subjects

Using the recombinant proteins obtained by us, 30 sera of conditionally healthy people from Moscow and Moscow region with antibodies to TBEV were tested. In ELISA analysis, samples of target protein at a concentration of 80 ng/well were used, Mock (which had the same concentration of total protein) was used as a negative control in the substrate, and serum of a conditionally healthy person who did not have antibodies to TBEV was used as a negative serum. Thus, 1 serum containing antibodies to the VP2 protein of ALSV was identified. No antibodies were detected to partial recombinant 244-389 aa VP3 protein.

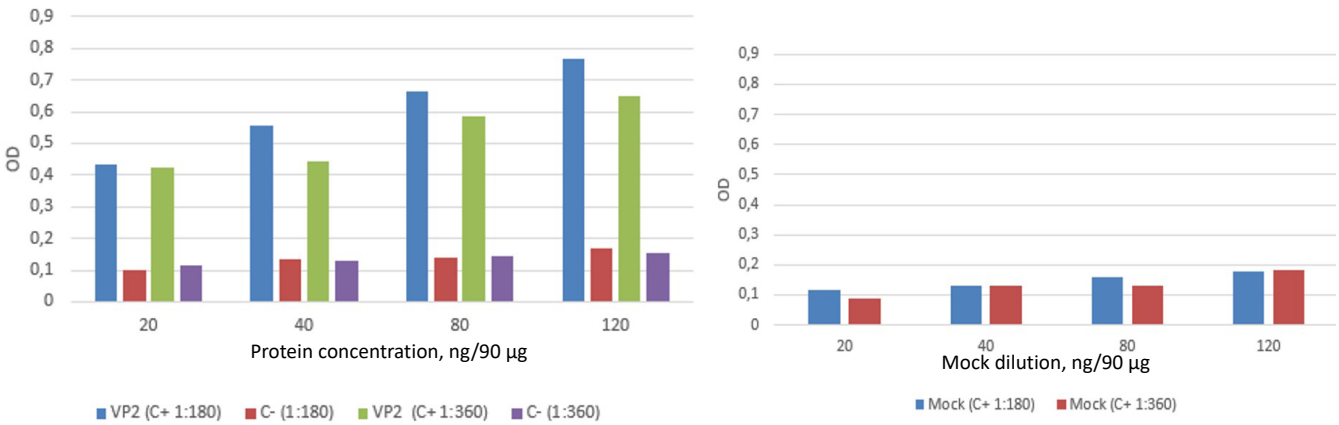

Fig. 11. Analysis of mouse hyperimmune serum to ALSV Miass527 with recombinant VP2 protein (*a*) and Mock (*b*).

The wells of a 96-well plate were sensitized with VP2 protein solution; hyperimmune mouse serum to ALSV (C+) and serum from unimmunized mouse (C–) with the dilutions indicated in the figure were used in the assay. HRP-labeled antibodies against mouse IgG (Abcam) were used for detection.

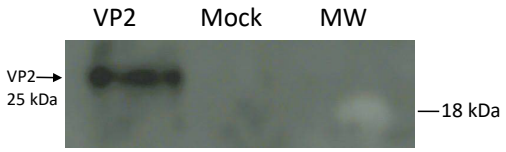

Fig. 12. Results of immunoblotting of target protein with serum of conditionally healthy human.
HRP-labeled antibodies against human IgG (Abcam) were used for detection.

To confirm the ELISA results, we performed a Western blot of the patient's ELISA-positive serum (**Fig. 12**).

Discussion

Previously, the VP2 protein was already obtained by Chinese scientists using the pET30a vector, which was expressed at 15°C in BL21 (DE3) *E. coli* cells [17]. In the current study, it was shown that VP2 protein was successfully expressed using plasmid pet28a(+), low temperature of cell cultivation was also tested, but it did not affect the expression level of recombinant protein. Efficient isolation of recombinant VP2 protein takes place upon addition of 8 M urea. At the same time, it was shown that the partial recombinant VP3 protein (vector pQE-32), which we obtained for the first time, is soluble. The yield of the target product is higher with the pet28a(+) vector than with pQE-32, and the obtained concentrations of both proteins are sufficient for multiple ELISAs.

Recombinant VP2 protein was used to detect antibodies in cattle in China [17]. In the currently study, we confirmed that VP2 protein has antigenic properties in immunoblot and ELISA. For the first time, we showed that the recombinant VP2 peptide of ALSV strain Miass527 obtained by us has no antigenic cross-reaction with TBEV in Western blot. It has been shown that when mice are immunized with live ALSV, antibodies to the recombinant VP2 protein are produced more regularly and less regularly to the partial recombinant VP3 protein. This may be due to the different spectrum of antibodies in the mouse sera obtained – the VP2 protein interacts with a large spectrum of antibodies produced at different stages of infection. The ELISA also detected antibodies to recombinant VP2 peptide in a conditionally healthy person with a history of tick bite, while no antibodies to recombinant VP3 peptide were detected in human sera.

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SCIENCE AND PRACTICE

Review
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Streptococcal effective molecules as promising anticancer agents: *pros* and *cons*

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Abstract

Oncological diseases remain the main cause of death and disability of the population worldwide. The most malignant types of cancer include of pancreas, liver and brain tumors. Modern methods of therapy (including chemoradiation, targeted and immune therapy) do not allow achieving the desired effectiveness in this group of patients. In this regard, new approaches to the treatment of oncological diseases are needed.

The **aim** of this review was to discuss the mechanisms of anticancer action of *Strepococcus pyogenes* and other streptococcal species, as well as to consider their cancer-stimulating effects and their limitations.

The review considers the current state of the problem of using bacterial oncotherapy involving streptococci A group (in particular, *S. pyogenes*). The involvement of *S. pyogenes* pathogenicity factors is discussed: M-protein, exotoxins: streptolysins S, O, superantigens, arginine deiminase, etc., as well as molecular mechanisms mediated by the host immune system cells. The authors' own data show that *S. pyogenes* exhibits selective M-protein-mediated cytolytic activity against C6 glioma and pancreatic adenocarcinoma (Panc) tumor cells and no activity against normal fibroblasts. The data on preclinical and clinical application of the streptococcal-based medicine OK-432 for the therapy of oncological diseases are briefly summarized. Tumor-associated properties of streptococci (induction of cytokine storm, proliferation, migration and angiogenesis of vascular epithelial cells, formation of neutrophil extracellular traps in the tumor microenvironment) caused by their interaction with immune cells of the tumor-bearing organism are also discussed.

Conclusion. The research data presented in this review convincingly demonstrate that *S. pyogenes*, with the participation of pathogenicity factors, can directly exert an antitumor effect on cancer cells. However, it should be noted the streptococcal vaccine should be used with caution, taking into account the individual characteristics of the patient's immune system, since *S. pyogenes* can also have effects of the opposite nature, requiring further research.

Keywords: *review, Strepococcus pyogenes, antitumor mechanisms, pathogenicity factors, immune system, cancer-associated mechanisms, oncolytic effects, bacterial antitumor drugs*

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Научный обзор
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Эффекторные молекулы стрептококков как перспективные противоопухолевые средства: плюсы и минусы

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Аннотация

Онкологические заболевания остаются одной из основных причин инвалидности и смертности населения во всём мире. К наиболее злокачественным типам рака относятся опухоли поджелудочной железы, печени и головного мозга. Современные методы терапии (в том числе химиолучевая, таргетная, иммунная терапия) не позволяют добиться желаемой эффективности у этой группы пациентов. В этой связи необходимы новые подходы для терапии онкологических заболеваний.

Целью обзора явилось обсуждение механизмов противоопухолевого действия *Strepococcus pyogenes* и других видов стрептококков, а также рассмотрение их опухольстимулирующих эффектов и существующих при этом ограничений.

В обзоре рассмотрено современное состояние проблемы использования бактериальной онкотерапии с участием стрептококков группы А (в частности, *S. pyogenes*). Обсуждается участие факторов патогенности *S. pyogenes:* М-белка, экзотоксинов: стрептолизинов S, O, суперантигенов, аргининдеиминазы и др., а также молекулярные механизмы, опосредованные клетками иммунной системы организма-носителя. Собственные данные авторов показывают, что для *S. pyogenes* обнаружена селективная опосредованная М-белком цитолитическая активность в отношении опухолевых клеток глиомы С6 и аденокарциномы поджелудочной железы и её отсутствие на нормальных фибробластах. Кратко суммируются данные по доклиническому и клиническому применению полученного на основе стрептококков препарата ОК-432 для терапии онкологических заболеваний. Обсуждаются и опухоль-ассоциированные свойства стрептококков (индукция секреции цитокинов, пролиферации, миграции и ангиогенеза эпителиальных клеток сосудов, образование внеклеточных ловушек нейтрофилов из микроокружения опухоли), обусловленные их взаимодействием с клетками иммунной системы организма-опухоленосителя.

Заключение. Представленные в обзоре научные данные убедительно показывают, что *S. pyogenes* могут при участии факторов патогенности непосредственно оказывать противоопухолевое действие на раковые клетки. Однако стрептококковую вакцину следует применять с осторожностью, учитывая индивидуальные особенности иммунной системы пациента, поскольку *S. pyogenes* могут оказывать и эффекты противоположного характера, требующие дальнейших исследований.

Ключевые слова: *обзор, Strepococcus pyogenes, противоопухолевые механизмы, факторы патогенности, иммунная система, опухоль-ассоциированные механизмы, онколитические эффекты, бактериальные противоопухолевые препараты*

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Introduction

Cancer diseases are one of the main causes of disability and mortality worldwide, second only to cardiovascular pathology [1]. According to the World Health Organization and the International Agency for Research on Cancer (Globocan), 19,976,499 new cases of cancer were globally registered in 2022, of which 9,743,832 were death (48.8%)[1]. The World Health Organization predicts that by 2045, cancer incidence and mortality will increase by 55% to reach 31.0 million and 16.7 million cases respectively.

Recent scientific achievements in biology, medicine and oncology have been marked by the discovery of cancer stem cells, horizontal transfer of genetic information (mobile genetic elements), and the emergence of new genetic diagnostic methods: whole-genome and whole-exome sequencing. These and other advances contributed to a paradigm shift from cohort-based to individualized treatment, which led to the emergence of new scientific directions: targeting and immunotherapy, which gave hope for the cure of many types of cancer [2, 3].

On the other hand, all these successes in oncology caused new problems associated with the establishment of molecular and cellular heterogeneity of tumors, the development of multiple drug resistance of cancer cells to therapy, its high toxicity to normal tissues and cells, and the participation of tumor microenvironment cells in cancer progression [4, 5]. These obstacles required reconsideration of existing treatment methods and search for new therapeutic approaches to oncologic diseases.

The use of live bacteria that selectively colonize the tumor represents a promising prospect for cancer therapy that overcomes many problems [6]. Unlike most therapeutic agents, bacteria have multiple mechanisms targeted at inhibiting tumor growth. Microorganisms selectively colonize tumors and proliferate within them, where they initiate anticancer immune responses, which may ultimately increase survival after systemic infection in tumor models of animals [7]. For example, a *Salmonella typhimurium* strain VNP20009 attenuated more than 10,000-fold compared to the wild-type strain has a tumor : liver colonization ratio > 1000 : 1 and exhibits at titers of $1 \times 10^4 – 3 \times 10^6$ CFU/mouse) a strong inhibitory effect (57-95%) on the growth of Lox, DLD-1, A549, WiDr, HTB177, MDA-MB-231 and B16F10 human tumors and the development of pulmonary metastases in mouse tumor models [8, 9]. Furthermore, such bacteria can be further programmed using simple or complex genetic and bioengineering methods for the synthesis and tumor-selective delivery of anticancer drugs [10]. For example, the use of bacteria as transport vectors can increase the poor tumor penetration and activity of preventive drugs for chemotherapy while reducing their systemic toxicity to the organism. Bacteria can be used for targeted delivery of chemotherapeutics, cytokines, immunomodulators, enzymes, prodrugs, or small interfering RNAs to tumors [6]. Furthermore, bacteria themselves can synthesize anticancer enzymes, for example, L-asparaginase and methionine-gamma-lyase [11, 12].

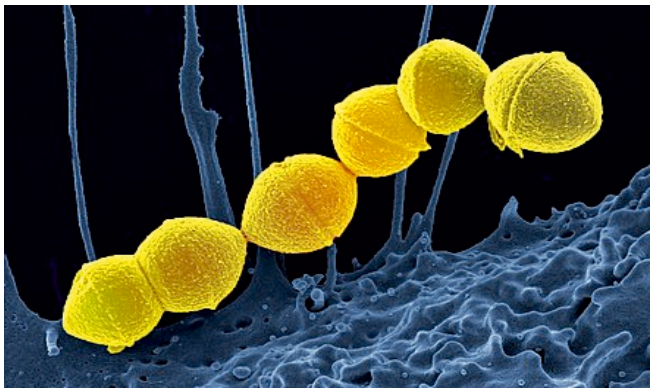

Fig. 1. Electron micrograph of *S. pyogenes*.
Source: Streptococcus pyogenes.
URL: https://en.wikipedia.org/wiki/Streptococcus_pyogenes

Motility is an important property and allows bacteria to penetrate deeper into tumor tissue. In contrast to the passive distribution and limited penetration of chemopreventive agents, bacteria are complex living organisms that can obtain energy from the environment and convert it into kinetic energy of movement, which allows them to move independently deeper into the tumor [13]. Microorganisms are able to influence the tumor microenvironment and produce their own oncolytic metabolites (peptides, bacteriocins), which makes these microbes a promising approach for cancer therapy [14]. Treatment with live bacteria can be used either as monotherapy or in combination with other cancer therapies. Oncolytic bacteria include *Bifdobacteria, Clostridium*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Streptococcus bovis* and *S. pyogenes* (**Fig. 1**) [14].

Since *S. pyogenes* are a group of microorganisms that cause the development of a frequent pathogenic form of group A streptococcal infection in humans and one of the objects studied at the Department of molecular microbiology of the Institute of Experimental Medicine, these bacteria were chosen as the key object for discussion of its anticancer properties in this review.

The aim of the review was to discuss the mechanisms of anticancer action of *S. pyogenes* and other streptococcal species, as well as to consider their tumor-stimulating effects and the limitations that exist in this regard.

Antitumor mechanisms of *Streptococcus pyogenes*

Since 1891, when Dr. W. Coley first used a vaccine based on live *S. pyogenes* and *Serratia marcescens* to treat 10 patients with terminal osteosarcoma and sug-

[1] International Agency for Research of Cancer (Globocan). URL: https://globocan.iarc.fr

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gested an association between the development of fever and tumor regression [15], several bacterial strains have been studied and selected for testing on patients. In order to understand the pathogenetic mechanisms of the effect of *S. pyogenes* on tumor cells, it is necessary to consider the associated pathogenicity factors of these microorganisms.

Pathogenicity factors

More than 40 pathogenicity factors have been found for *S. pyogenes* [16]. All of them can be divided into microbial cell-associated and extracellular ones.

The streptococcus cell is surrounded by a cell wall formed by the peptidoglycan polymer consisting of N-acetylglucosamine and N-acetylmuramic acid and covalently linked to them by teichoic and lipoteichoic acids [17]. Protein and polysaccharide components are connected to the cell wall (**Fig. 2**) [18].

The cell wall polysaccharides also include carboxyhydrate antigens formed by group-specific polysaccharide-A, the differences in the structure of which are the basis for the classification of streptococci [20]. Among cell wall proteins, the main pathogenicity factor is M (Emm) protein, which provides resistance of *S. pyogenes* to phagocytosis and multiplication in the blood [21].

The authors of the review showed that both wild-type *GUR* and M-protein mutant *GURSA1* strains (10^6 CU/mL) exerted *in vitro* cytotoxic effect on C6 glioma cells using the xCELLigence system (**Fig. 3**) [22, 23].

The data of Fig. 3 show that streptococcal strains *GUR*, *GURSA1* demonstrated rapid and strong oncolytic effect against C6 glioma cells. *S. pyogenes* strains *GUR* and *GURSA1* had the slowest cytotoxic effects on C6 glioma cells with the highest cell inhibition rates of 81.8 and 79.3% after 4 and 6 h, respectively. Moreover, the cytotoxic effects of *S. pyogenes* strains *GUR* and *GURSA1* slightly increased over time, reaching growth inhibition rates of 85.0 and 81.5% after 8 h, respectively [22]. Similar results were obtained when *S. pyogenes* strains *GUR* and *GURSA1* were tested on mouse pancreatic adenocarcinoma (Panc) cells in real time (**Fig. 4**) [23].

The results in Figs. 3, 4 show that both strains inhibited the growth of C6 glioma and Panc cells, and the effect of *GURSA1* strain was weaker than that of *GUR*, which confirms the presence of a cytotoxic effect due to the presence of M-protein as a pathogenicity factor. At the same time, both strains had no cytotoxic effects on normal fibroblast cells in real time (**Fig. 5**) [23].

S. pyogenes do not synthesize a single M-protein, but there are at least up to 4 groups of M-proteins: FG I (*Mrp*); FG II, M-protein and H-protein; FG III (*Enn*); and FG IV (M-protein). The M and Enn proteins form two groups with 9 subgroups, and the Mrp proteins form 4 groups with 10 subgroups (**Fig. 6**) [24].

M-proteins are known to consist of α-helical fibrils with a diameter of 50-60 nm located on the bacterial cell wall. These proteins have a super-spiral structure and can form dimers with different polypeptide chain lengths (**Fig. 7**) [25].

M-proteins as pathogenicity factors contribute to the resistance of *S. pyogenes* to phagocytosis by macrophages and host antibodies [26]. M-proteins can bind blood plasma proteins: immunoglobulins G and A (Fc-fragments), fibrinogen, fibronectin, albumin, plasminogen, C4b-binding protein (C4BP), factor-H and complement proteins [19]. The interaction of M-protein with plasma factors affects the adhesive and invasive properties of *S. pyogenes*, and through alteration of blood coagulation capacity indirectly affects cancer cell viability. This is confirmed by the fact that the state

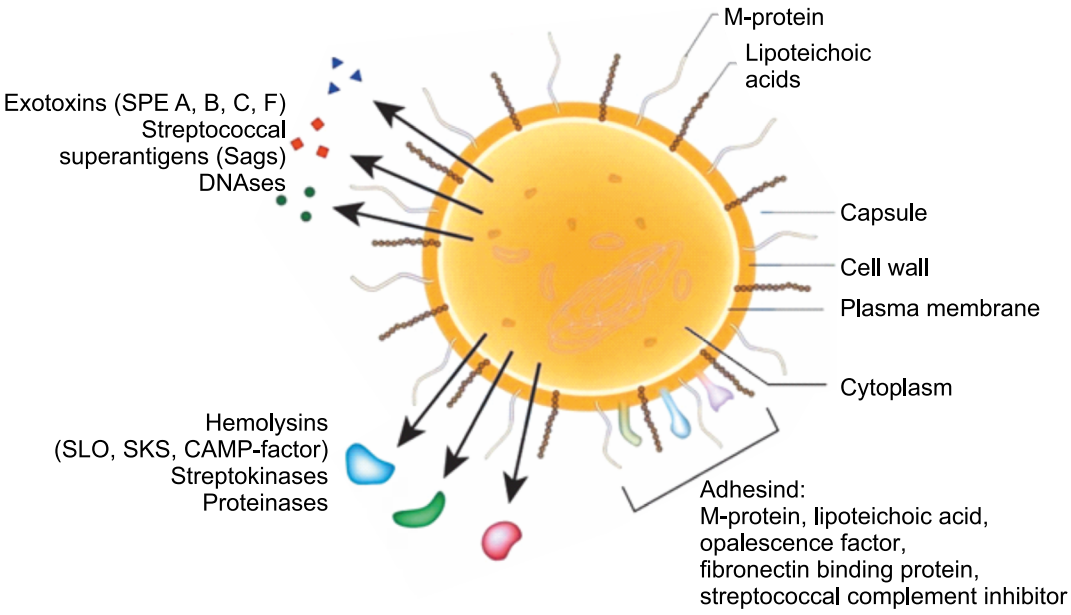

Fig. 2. Schematic representation of the streptococcal cell and the main biologically active products of *S. pyogenes* [19].

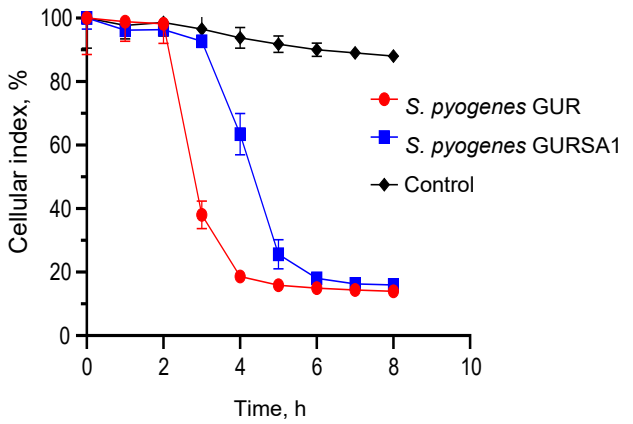

Fig. 3. Effect of *S. pyogenes* strains *GUR, GURSA1* on C6 cells in real time using xCELLigence system [23].

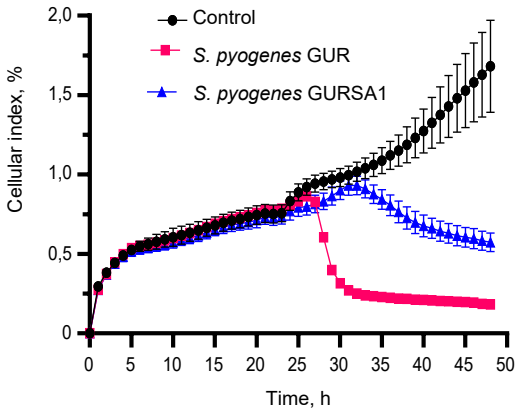

Fig. 4. Effect of *S. pyogenes* strains *GUR, GURSA1* on Panc cells in real time using xCELLigence system [23].

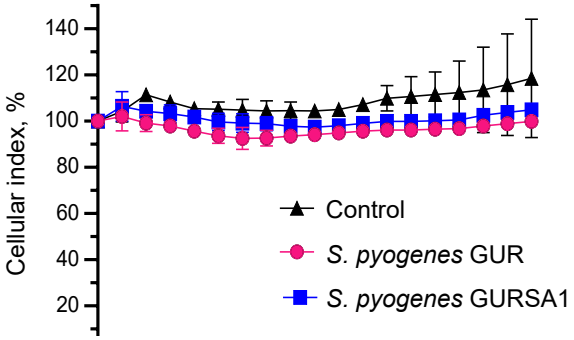

Fig. 5. Effect of *S. pyogenes* strains *GUR, GURSA1* on normal fibroblast cells in real time using xCELLigence system [23].

of blood hypercoagulability as a component of tumor microenvironment promotes cancer progression [27].

The other pathogenicity factors of *S. pyogenes* have been studied to a lesser extent. They include: exotoxins streptolysins S, O, superantigens, serine and cysteine proteinases, streptokinase, arginine deiminase, endo-β-N-acetylglucosaminidase, nicotinamidine dinucleotidase, etc. [19]. For example, arginine deaminase (ADI) consists of 2 domains, the first of which is formed by 5 ββαβ sites repeating around a pseudo-5-fold axis containing the active center. The second domain is a 4-domain helical chain (**Fig. 8**).

ADI was first isolated from the *Su* strain (3 μg/mL), which is capable of inhibiting the growth of transformed BALB/3T3 fibroblast cells [29]. ADI is localized in the cell wall of *S. pyogenes*, whereas in *S. suis* the same enzyme is present in the membrane fraction of cells. Its activity is associated with the ability to convert arginine to ammonia and citrulline [30]. On the other hand, arginine utilization enhances the proliferation and growth of some types of tumors such as melanoma, hepatocellular carcinoma and prostate cancer. Since arginine deficiency induces cycle arrest in G_1-phase cancer cells, activation of mTOR- and GCN2-kinase genes in them, which trigger autophagy and apoptosis [31, 32], T. Fiedler et al. studied the efficacy of ADI therapy (3.5–350.0 IU/mL) and its combinations with cytostatic drugs: chloroquine (5 and 20 μM), hydroxamic acid suberoylanilide (SAHA; 0.25 and 0.5 μM) or palomide 529 (dual TORC1/TORC2 inhibitor; 7.5 μM) to inhibit the growth of 12 glioblastoma (GBM) cell lines *in vitro* and *in vivo* on male *NMRI Foxn1nu* mice weighing 20–

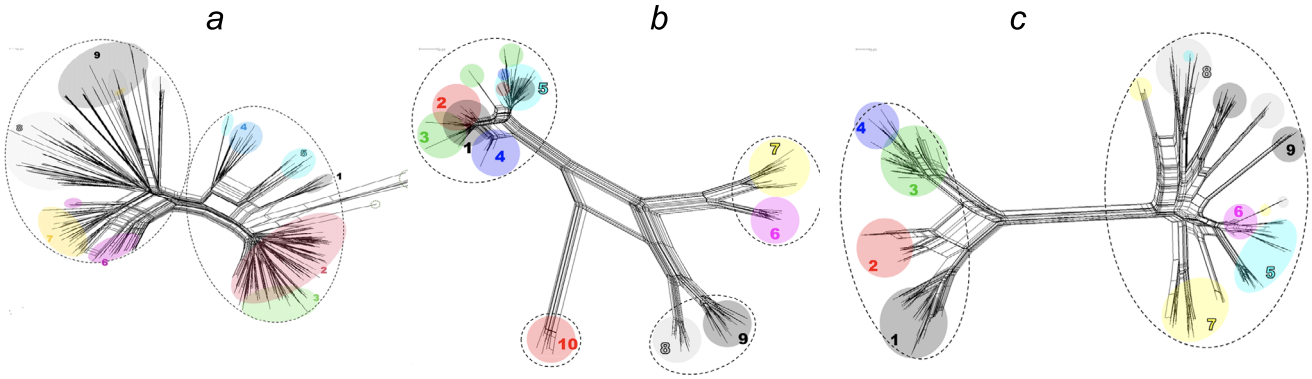

Fig. 6. Genetic network analysis of SplitsTree, including 537 genetic sequences with the allocation of clusters of M proteins [24].

a — M protein; *b* — Mrp (221 sequences); *c* — Enn proteins (262 sequences). Black dotted ellipses denote two groups of M proteins, green dotted ellipses denote chimeric M proteins, and colored ellipses denote subgroups of M proteins.

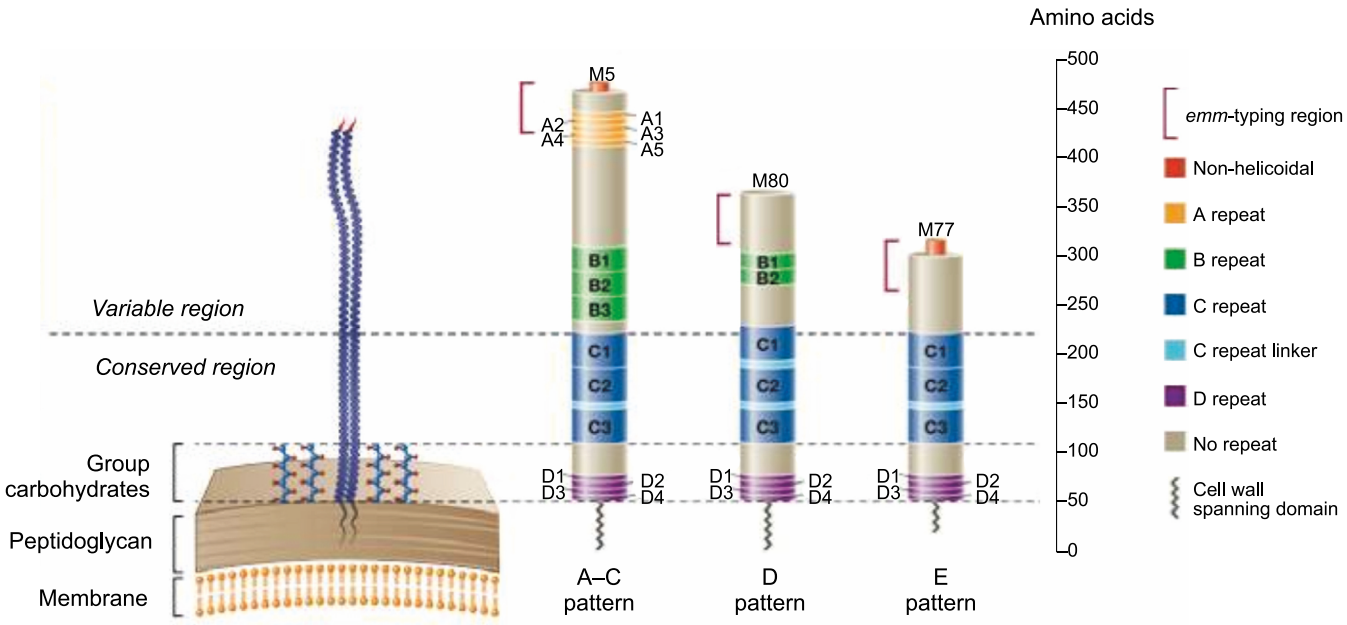

Fig. 7. Three M (emm) proteins (M5, M80, and M77) [25].

The length of the M protein and the size of the repeat and non-repeat domains are shown to the scale. The A–C emm patterns are the longest M proteins with a hypervariable domain of 230 amino acids. The D and E structured M proteins have hypervariable domains consist from 150 and 100 residues, respectively. The "A" repeats are absent from the vast majority of M proteins belonging to the D and E pattern groups. The "B" repeats are present in most A–C and D M proteins, but are absent from the E–M subtypes of M proteins

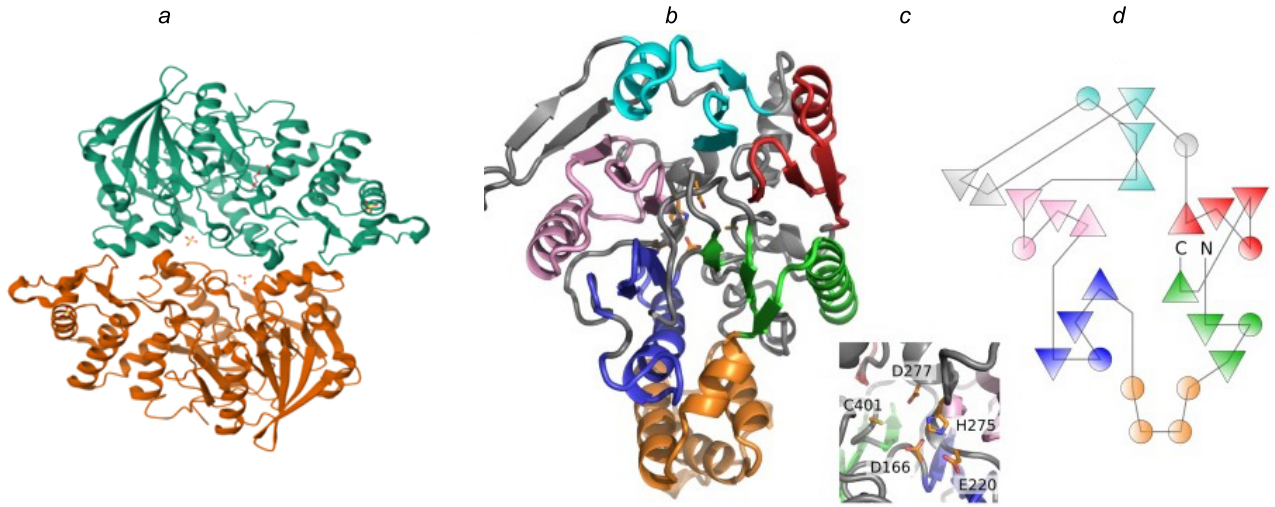

Fig. 8. Structure of arginine deaminase from *S. pyogenes* (*a*); crystal structure illustrating the connecting regions (*b*): green, blue, pink, light blue and red colors, respectively; residues of the ADI active site (*c*), and the ADI topological diagram (*d*) [28].

25 g [33]. ADI administration inhibited GBM growth in 50% of tested lines (**Fig. 9**).

In cell lines for which ADI reduced cell proliferation, its combinations with chemotherapy agents were tested. Synergistic effects were observed on HROG02, HROG05 and HROG10 66% GBM cells with growth inhibition of up to 70% using the combination of ADI with Palomid 529. Similar anticancer effects (60% cell death) were established after adding chloroquine to ADI. The reason for the anticancer effects of ADI was epigenetic suppression of arginine synthesis pathway genes (argininosuccinate synthetase *ASS1* and argininosuccinate lyase *ASL*). In an *in vivo* model, ADI (250 U/kg wt) and its combination with SAHA (25 mg/kg wt) inhibited 70% of HROG05 heterograft tumor size compared to control (Fig. 9, *b*) [32].

C. Maletzki et al. found that ADI (35 mU/mL) induced the expression of heat shock and arginine metabolism protein genes (*ASS1, ASL, ArgI, CPSI, OTC*) in HROG02, HROG05, HROG52, HROG63 cells of GBM patients (**Fig. 10**) [34]. As in a previous study, ADI administration stimulated autophagy and senescence of tumor cells. Combinations of ADI with curcumin, resveratrol, quinacrine and sorafenib for 3 days enhanced the cytotoxicity of the enzyme by activating autophagy.

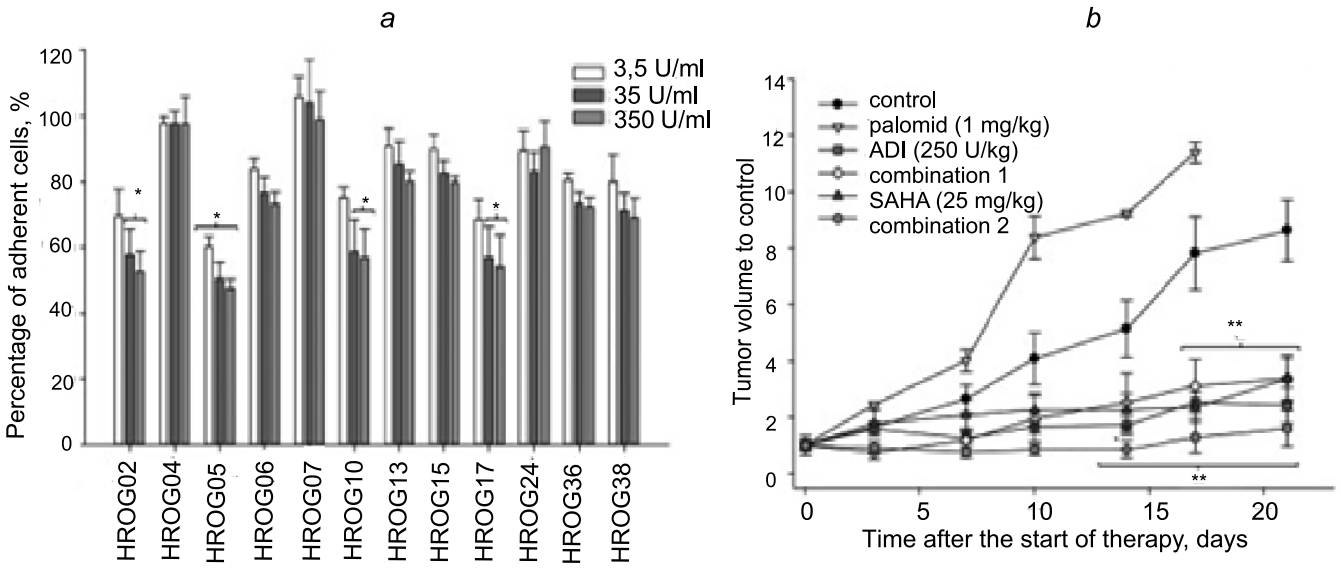

Fig. 9. Crystal violet staining of the GBM cells after ADI incubation during 72 h (*a*) and tumor growth curve (*b*).
Tumor volumes are given as x-fold increase compared to day 0 [33].

Other pathogenicity factors of *S. pyogenes* include: cholesterol-dependent cytolysins and streptolysin O (SLO). S.C. Feil et al. determined the three-dimensional structure of this enzyme (**Fig. 11**) [35].

The SLO molecule consists of 571 amino acids, which form 4 domains having a β-folded structure. The first N-terminal domain (70 amino acids) is cleaved off by proteolysis by streptococcal proteases after secretion. In addition to the domains, SLO contains 2 transmembrane regions, TMH1 (residues 259–288) and TMH2 (residues 359-386), which have an α-helical structure. SLO includes the 11-amino acid sequence ECTGLAWEWWR, rich in tryptophan residues, which promotes the incorporation of the molecule into cell membranes [35]. As a result of oligo- and polymerization, SLOs form large pores in cell membranes. These mechanisms lead to structural damage, severe ATP depletion and cell death by necrosis. Consequently, this mechanism of action of SLO can be used for cytolysis of cancer cells. In this regard, C. Gruber et al. using RNA trans-splicing developed 3'-pre-trans-splicing molecules (PTM) containing an embedded *SLO* gene in the target gene of matrix metalloproteinase-9 (*MMP9*) in highly aggressive squamous cell carcinoma cells [36]. This technique allowed the replacement of a tumor-specific transcript with a peptide/toxin encoding gene, resulting in cell death. A fluorescent tag was used to visualize the transcript (**Fig. 12**).

Trans-splicing between the 50th and 30th nucleotides of *MMP9* and *PTM* genes resulted in the formation of mRNA encoding a full-length GFP-protein containing a DsRed tag. Transfection of HEK293 human squamous cell carcinoma and embryonic kidney HEK293 cancer cells with *SLO-PTM* induced death in them due to SLO expression (**Fig. 13**).

Thus, the use of SLO is a new approach in bacterial therapy of human malignant tumors [37]. In other *Streptococcus* species, such as *S. bovis* those belonging to group D, bacteriocin bovicin HC5 synthesized in ribosomes has been found [37]. This cationic peptide has a cytolytic effect against MCF-7 breast adenocarcinoma and HepG2 hepatocellular carcinoma cells [38].

Mediated mechanisms of S. pyogenes' anticancer action

In addition to direct cytolytic action on tumor cells, *S. pyogenes* in the body can also exert cytostatic anticancer effects mediated by immune cells and factors. Once in the body, streptococci are perceived by the immune system as foreign and trigger an inflammatory response. During this reaction, a cytokine and chemokine storm develops due to increased levels of tumor necrosis factor-α (TNF-α), interferon-γ, interleukins (IL-1β, IL-6, IL-10), monocyte chemoattractant protein-1 [38]. In addition to these changes, Y.H. Liu et al. found that during infection with the A20 strain of *S. pyogenes*, the patient's brain cells expressed glial fibrillary acidic protein, inducible nitric oxide synthase, components of nicotinamideadenine dinucleotide phosphate oxidase, all at a high level, which stimulated the production of reactive oxygen species. Myeloperoxidase secreted by activated macrophages and neutrophils was also expressed in the cortex and hippocampus of infected mice (**Fig. 14**) [39].

Increased levels of IL-10 and IL-12 in blood activate $CD18^{+}$-monocytes and $CD11c^{-}CD123^{+}$-plasmacytoid and $CD11c^{+}CD123^{-}$-myeloid dendritic cells (DCs) through the Toll-like receptor (TLR) signaling pathway [40]. T.G. Loof et al. showed that when KTL3 was stimulated by *S. pyogenes* strain through TLR4 activation

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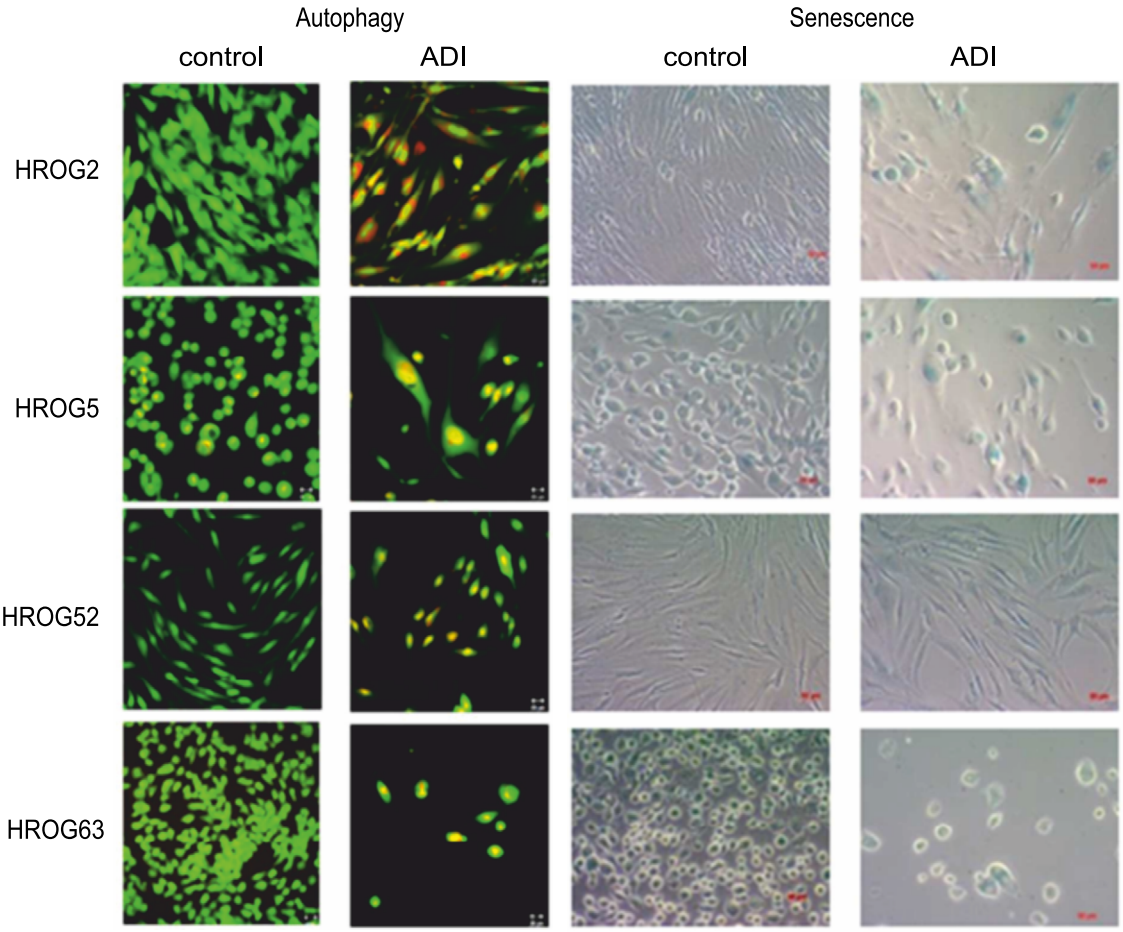

Fig. 10. ADI treatment induces autophagy and senescence in GBM cells [34].
Acridine orange (orange) and calcein AM (green) staining. Analysis was conducted on a laser scanning microscope (Zeiss) using a 20x objective.

in MyD88$^-$DCs, the expression of costimulatory molecules CD40, CD80, CD86 and the production of inflammatory cytokines were decreased in them [41, 42]. Activation of receptors and CD54, CD70, CD83, CCR7 molecules triggers p38MAPK, ERK, JNK, PI3K/Akt/NF-κB signaling cascades in DCs that enhance their proliferation and differentiation [43, 44]. Recently, X. Chen et al. found that TLR4 also stimulates the cGAS-STING/NF-κB signaling pathway, which mediates DC maturation and IL-6 secretion [45]. These results suggest that *S. pyogenes* through stimulation of monocytes, macrophages, may exhibit their anticancer effects.

Furthermore, *S. pyogenes* stimulate a subpopulation of interferon-producing DCs and natural killers. Activation of these cells enhances processing, tumor antigen presentation, TNF-α-induced TRAIL- and Fas-ligand-dependent apoptosis [46]. In turn, plasmacytoid myeloid DCs activate tumor-specific cytotoxic CD8$^+$-, CD4$^+$-T-lymphocytes and their secretion of cytokines [40, 47]. According to the study of W. Li et al. factors involved in DC activation in hepatocellular carcinoma are presented in **Fig. 15**.

The key pathogenicity factor, M-protein or acidic streptococcal glycoprotein (SAGP) binds to an IAP-specific coupled receptor, which activates proteintyrosine phosphatase dephosphorylates epidermal growth factor and blocks the p42/44MAPK-signaling cascade, thereby inhibiting proliferation of A431 epidermoid carcinoma cells [48]. *S. pyogenes* secret exotoxins SpeB, SpeA, which have cysteine protease ac-

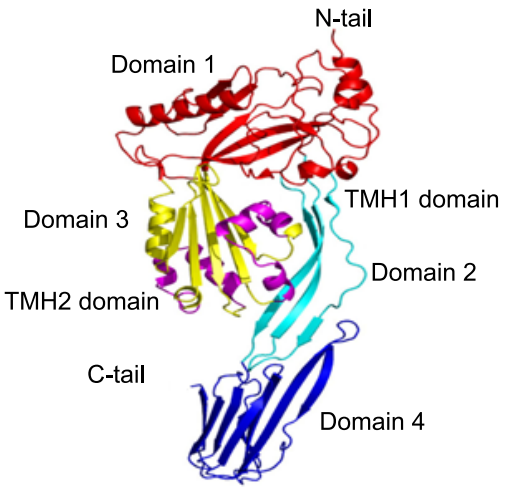

Fig. 11. Strepolisin O molecular structure [35].

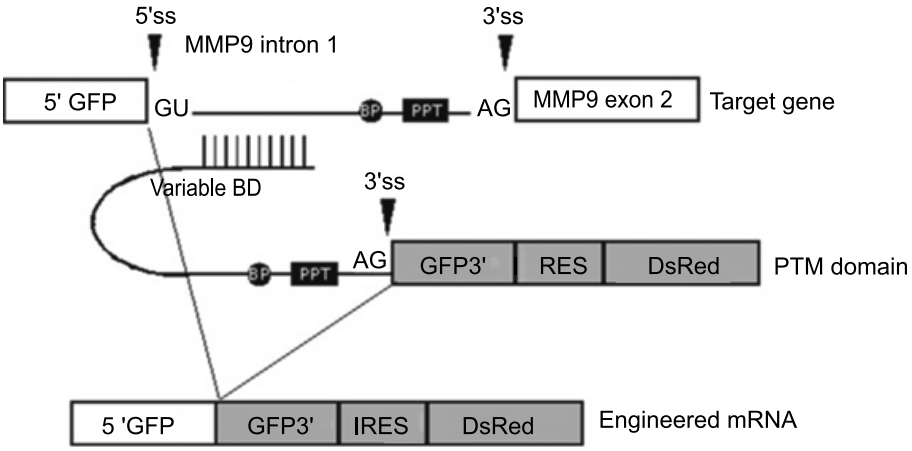

Fig. 12. Schematic representation of the fluorescent trans-splicing assay system [36].
PTM is the engineered mRNA linked via an antisense BD domain to *MMP9* intron 1. BP is the branch point; PPT is the polypyrimidine sequence.

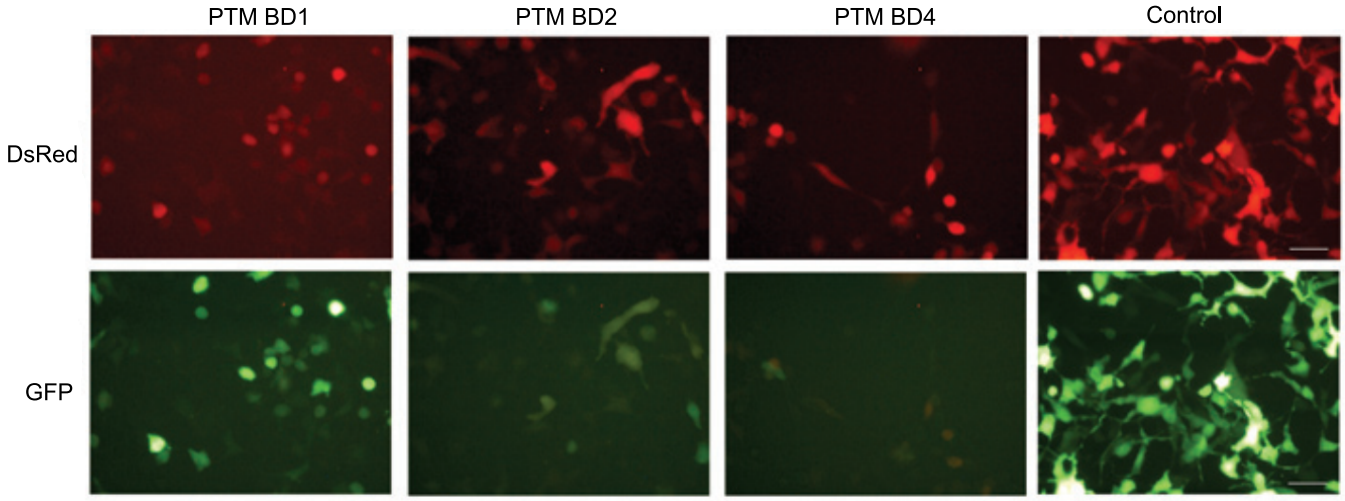

Fig. 13. Fluorescence microscopy of transfected HEK293 cells [36].
Control was transfected with a construct containing DsRed dye and full-length cDNA of GFP. Cells expressing GFP indicate trans-splicing in the target gene and the PTM carrying one of the three BD domains (BD1, BD2, and BD4). Scale bar is 20 mm.

tivity and inactivate inflammatory factors IL-8, C5a, cathelicidin LL-37, and inhibit neutrophil chemotaxis [26, 47, 49, 50]. DNAases SpnA and SdaI of *S. pyogenes* suppress the formation of neutrophil extracellular traps, SpeB suppresses the activity of antimicrobial chemokines (**Fig. 16**). Furthermore, SpeB promote the formation of gazdermin D, which forms pores, induces pyroptosis, inhibits IL-1β and H-kininogen, which exert potent proinflammatory effects [47]. Consequently, secretion of streptococcal exotoxins can inhibit tumor growth and development.

Not only live streptococci have cytotoxic activity, but also the preparation OK-432 (picibanil, NSC-B116209; Fig. 16) obtained on their basis, the administration of which (0.001–1.0 μg/mL) caused $CD56^{+}$NK activation. This resulted in growth inhibition by 7–14 days of MADB106 mammary carcinoma in an F344 rats` intraperitoneal model [51]. OK-432 administration promoted the activation of T-helper lymphocytes and leukocytes by increasing the number of neutrophils [26]. In the review by Y. Ryoma et al. summarize the mechanisms of anticancer activity of OK-432, showing that picibacil induces direct anticancer action by inhibiting RNA synthesis and cell cycle arrest. Also OK-432 stimulates the synthesis of TNF-α, perforin and interferon-γ, which activate the expression of intercellular adhesion molecules (ICAM-1), HLA-DR and eventually apoptosis of cancer cells [52]. OK-432 stimulates the production of IL-8, macrophage factors (G-CSF, GM-CSF) that enhance proliferation and maturation of granulocytes, monocytes, lymphocytes and platelets [52]. Recent results convincingly demonstrate that collagen-like protein-1 (Scl1) of *S. pyogenes* inhibits the formation of neutrophil extracellular traps in a rat model with Panc02 pancreatic ductal adenocarcinoma [53].

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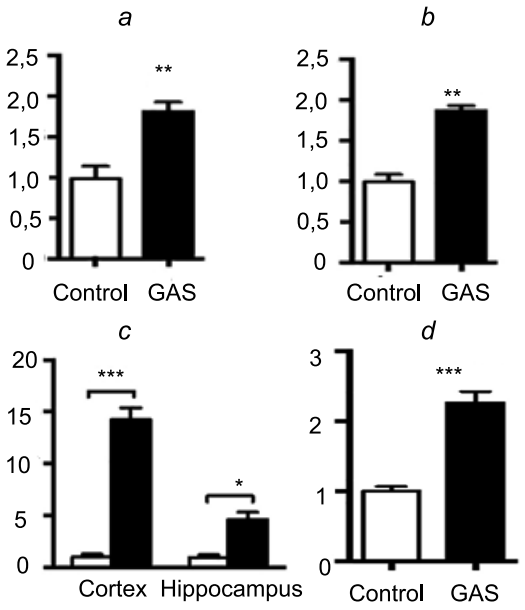

Fig. 14. Changes in GFAP (*a*), iNOS (*b*), MPO (*c*) and MMP-9 (*d*) levels in the brain during infection with *S. pyogenes* strain A20 in a patient [39].

In this regard, it is interesting to note that streptococci can infect the tumor, forming biofilms on it. Streptococcal infection activates the body's immune system, which increases the number of neutrophils and lymphocytes in the focus of infection [14, 54]. Thus, bacterial components can enhance the interaction between the tumor and the immune system, acting as adjuvants, promoting the presentation of tumor antigens and activation of the immune system [55].

Preclinical and clinical studies

The results of *in vitro* and *in vivo* experiments with *S. pyogenes*, which established their anticancer effects and mechanisms, stimulated further preclinical and clinical trials with these microorganisms. For example, T. Iwai et al. recently conducted a preclinical study to evaluate the efficacy of intratumoral administration of OK-432 or its combination with radiofrequency ablation on 4–5 weeks old male Sprague-Dawley rats (n = 145; 80–90 g) with metastatic osteosarcoma [55]. The animals were divided into 4 groups to evaluate overall survival and tumor size: control (no treatment), radiof-

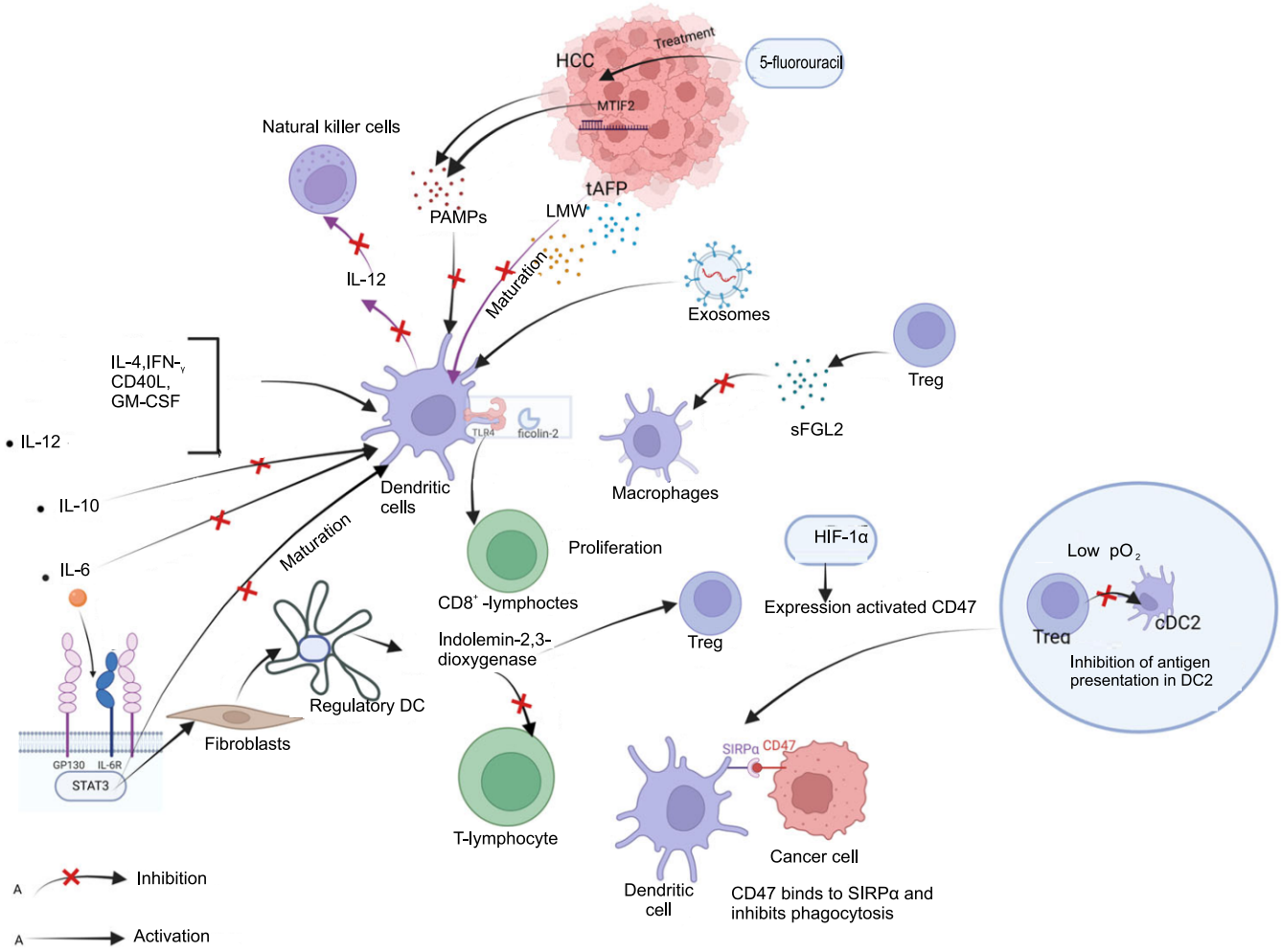

Fig. 15. Mechanisms of streptococcal activation of DCs targeted at inhibiting of hepatocellular carcinoma growth [47].

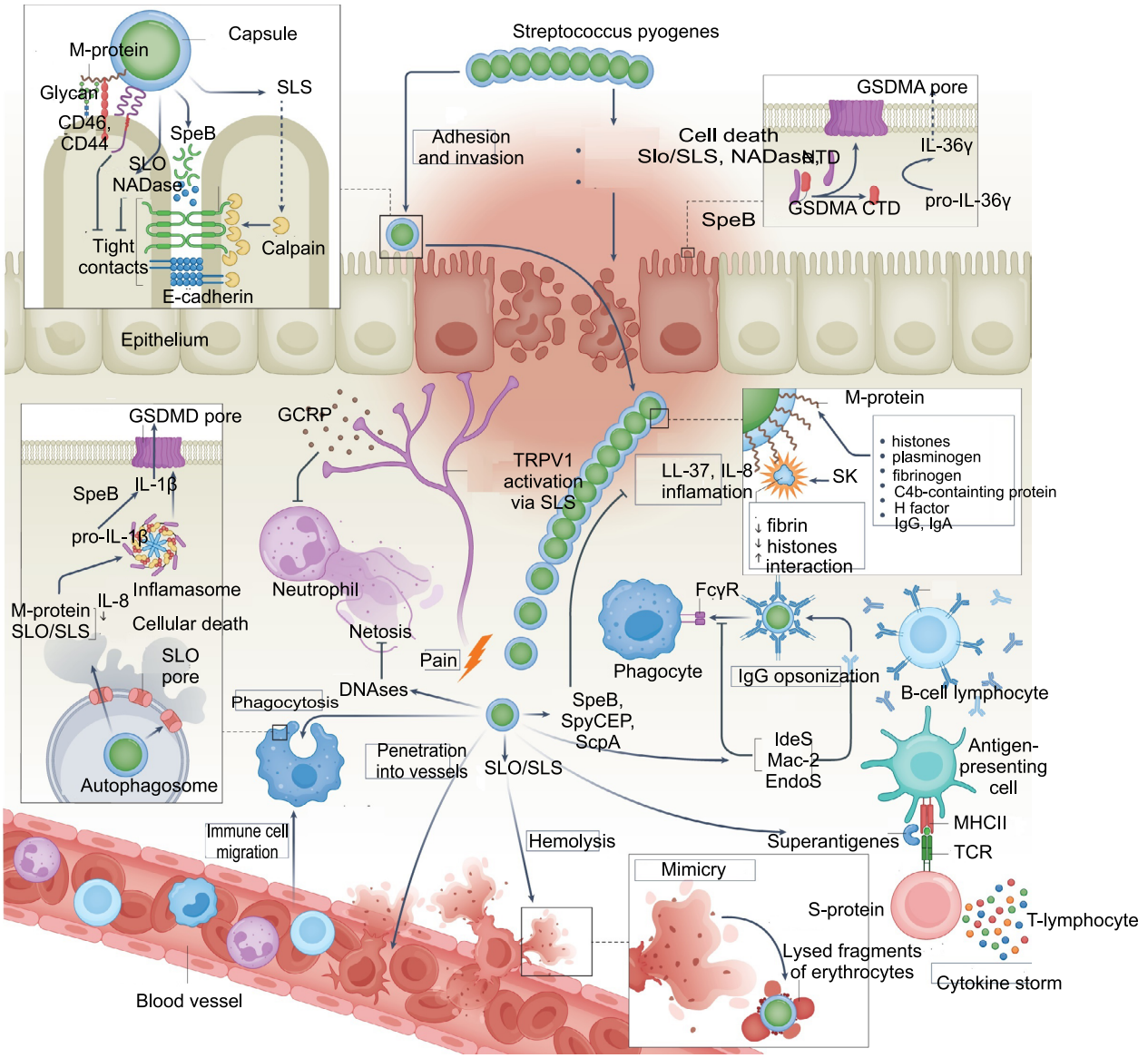

Fig. 16. Molecular and cellular mechanisms of *S. pyogenes* pathogenesis [26].

requency ablation, OK-432, and RFA + OK-432. The median survival of rats in the control group was 28.4 days (11–51 days) and 38.4 days (10–51 days) in OK-432 groups, 40.0 days (12–51 days; $p = 0.14$) in RFA and 47.3 days (15–51 days; $p = 0.084$) in RFA + OK-432 groups ($p = 0.046$) [56].

Japanese scientists M.S. Oba et al. conducted a meta-analysis including 14 studies involving 796 patients with stage III or IV gastric cancer after surgical resection to evaluate the efficacy of OK-432 immunochemotherapy compared to standard chemotherapy. The primary endpoint was overall survival [57]. The control and OK-432 groups included 726 and 796 patients, respectively. The median overall survival was 42.6 months for the OK-432 group and 32.3 months for the control group. The overall hazard ratio was 0.88 (95% confidence interval 0.77-1.00; $p = 0.050$; **Fig. 17**) [57]. The authors conclude that immunochemotherapy

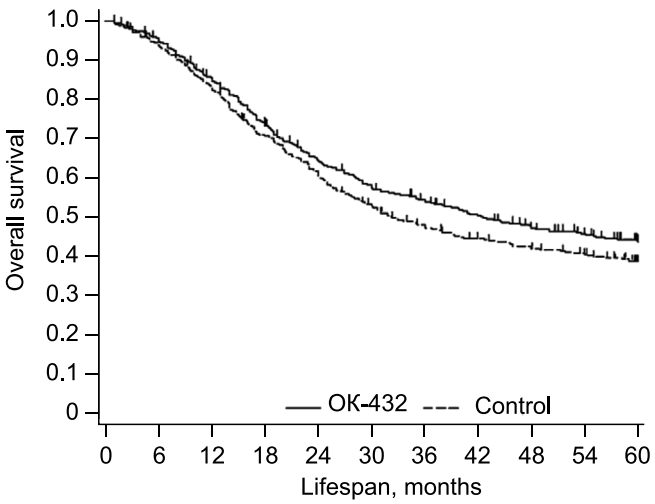

Fig. 17. Overall survival of gastric cancer patients who received OK-432 immunotherapy [57].

with OK-432 may be effective for patients with gastric cancer in stages III or IV after surgery [57].

In an earlier study, the same authors, summarizing 6 randomized trials involving 1522 patients with gastric cancer, showed that systemic administration of OK-432 had a significant effect on increasing life expectancy (OR = 0.81; 95% CI = 0.65–0.99; $p = 0.044$) and 3-year survival. This rate was 67.5 and 62.6% in the immunochemotherapy and chemotherapy groups, respectively [58].

It should be noted that mild adverse reactions such as: fatigue, anorexia, local inflammation, hyperemia, chest and abdominal pain were observed during clinical use of OK-432. The most frequent symptom was fever controlled with antipyretic therapy. Intracranial pressure was rarely reported. Only a few patients experienced fatal outcomes due to the development of embolism, acute nephritis, hemorrhage, and improperly dosed injections of the drug [59]. Serious side effects require identification of their causes and relationship to the current therapy, perhaps they were due to incorrect dosage or development of individual intolerance to the drug.

Mechanisms of cancer-stimulating effects of *S. pyogenes*

In the process of studying the anticancer effect of *S. pyogenes*, data of the opposite nature were gradually accumulated. For example, D. Kong et al. studied the effects of OK-432 (sapilin) in patients with breast cancer and found increased secretion of cytokines IL-1a, IL-6, fibroblast growth factor-β, vascular endothelial growth factor and transforming growth factor-β1 in drainage fluids. Furthermore, its administration enhanced proliferation, migration and angiogenesis of HUVEC, HFL1 epithelial cells, fibroblasts and collagen deposition [60]. These results indicate that *S. pyogenes* through activation of proliferation, migration and angiogenesis of epithelial cells and fibroblasts can stimulate the development of breast cancer. Exotoxins SpnA, SpeB and serine protease SpyCEP of *S. pyogenes* take part in

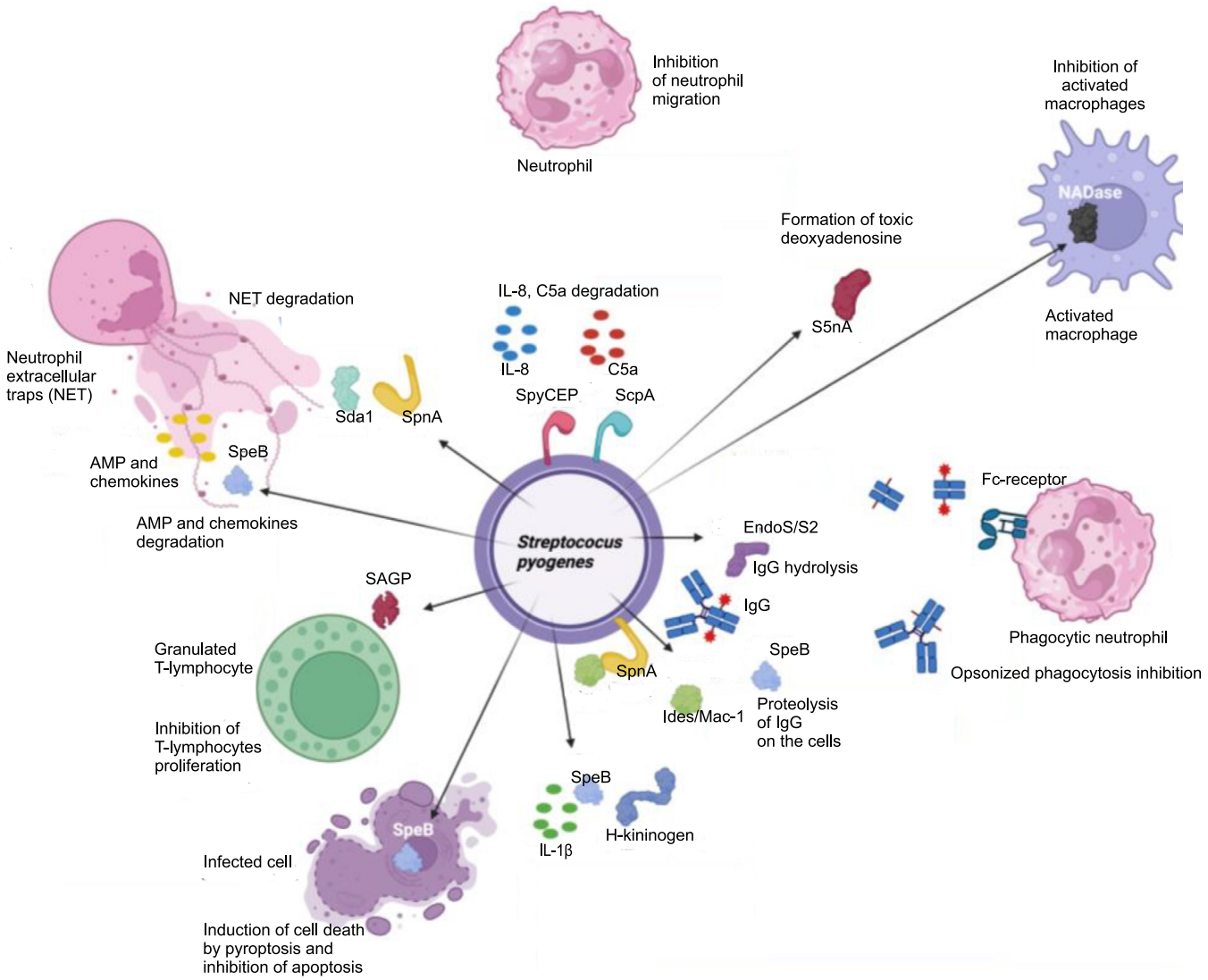

Fig. 18. Total scheme of the *S. pyogenes* effects to immune cells [61].

the degradation of IL-8, IL-1β, complement factor C5a, which inhibit the migration (chemotaxis) of neutrophils (**Fig. 18**). In turn, SpeB induces IgG proteolysis, degradation of antimicrobial peptides, chemokines, which promotes the formation of neutrophil extracellular traps and cancer development [61].

NADase S5nA inhibits phagocytic activity of macrophages against tumor cells, inhibits IgG proteolysis and antibody-mediated killing of cancer cells by neutrophils (opsonophagocytosis). The peptidases EndoS/S2, IdeS/Mac-1 and Mac-2, SpeB are involved in the hydrolysis of glycans and ubiquitin-binding proteins that inhibit apoptosis, and M-protein (SAGP) suppresses T-lymphocyte proliferation [61]. All these events indirectly stimulate the cell cycle, proliferation, migration of tumor cells observed predominantly in chronic streptococcal infection in cancer patients.

Conclusions

Contrary to established views, streptococcal infection or fever may not always be undesirable in cancer patients and does not always lead to a decrease in immune system reserves in the cancer patient. The research evidence presented in that review strongly suggests that *S. pyogenes* can, with the involvement of pathogenicity factors, directly exert anticancer effects on cancer cells. Furthermore, these bacteria stimulate interleukins, chemokines of the host immune system cells (neutrophils, T-, B-lymphocytes, macrophages, dendritic cells), which, in turn, participate in anticancer immune responses.

Modern oncological therapeutic methods (chemotherapy, radiation therapy) have a number of disadvantages associated with non-selectivity of action on cancer cells, as well as, due to cellular and molecular-genetic heterogeneity of tumors, the development of therapeutic resistance, which leads to the inability to monitor the effectiveness of treatment in dynamics. All these problems cause high oncological morbidity and mortality, progression of oncological diseases with the emergence of more resistant forms of tumors with a high rate of metastasis.

Thus, it is necessary to improve cancer therapy regimens using new approaches that include selective anticancer activity combined with minimal toxicity to normal tissues of the body, as well as the ability to overcome the constant dynamic molecular and genetic changes in neoplastic cells. The use of *S. pyogenes* as a therapeutic strategy in cancer patients meets all of these criteria. However, it should be noted that streptococcal vaccine should be used with caution, taking into account the individual characteristics of the patient's immune system, because *S. pyogenes* may have opposite effects that require further research.

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REVIEWS

Systematic review
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Current status of developments in the field of respiratory syncytial virus vaccines

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Abstract

Respiratory syncytial virus (RSV) is the leading cause of upper respiratory tract infections in children and the elderly. The only specific treatment approved for RSV is the monoclonal antibody Palivizumab for passive immunoprophylaxis in high-risk infants. Sixty years after the virus was discovered, several safe RSV vaccine candidates have been licensed. This was facilitated by research to identify the structure of RSV, to study the basic functions of RSV components as well as the mechanisms of innate and acquired immune responses to infection. The negative result of a clinical trial of formalin-inactivated RSV vaccine in children, which resulted in the death of several vaccinated individuals, was taken into account.

The aim of the study was to summarize data from studies of RSV vaccine candidates in laboratory animals and in clinical trials on different age groups.

Articles for the analysis of preclinical and clinical trials of RSV vaccines were found, using the PubMed search engine with “respiratory syncytial virus and vaccine” as the keywords. The selection criteria were that original articles should contain information on preclinical and clinical studies, the latter including phase I–IV randomized controlled trials. From 1967 to the present year, 296 articles summarizing data from studies of RSV vaccine candidates and 1788 articles summarizing data from animal trials of vaccine candidates were found. The review summarizes data from preclinical studies of vaccine candidates and their developers, vaccine formulations, animal models on which the studies were conducted, as well as a brief description of the main findings. Data on clinical trials of vaccine candidates are presented, including target populations, clinical trial number and sources where the results of these trials were published.

Keywords: *respiratory syncytial virus, vaccine, preclinical studies, clinical studies, literature review*

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Современное состояние разработок в области создания вакцин против респираторно-синцитиального вируса

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Аннотация

Респираторно-синцитиальный вирус (РСВ) является основной причиной инфекций верхних дыхательных путей у детей и пожилых. В отношении РСВ одобрено единственное специфическое средство лечения — препарат моноклональных антител паливизумаб для пассивной иммунопрофилактики у младенцев из группы высокого риска. Спустя 60 лет с момента обнаружения вируса были лицензированы несколько без-

опасных вакцинных кандидатов против РСВ. Этому способствовали исследования по выявлению структуры РСВ, изучению основных функций компонентов РСВ, механизмов врождённого и приобретённого иммунного ответов на инфекцию. Был учтён отрицательный опыт клинического испытания формалин-инактивированной вакцины против РСВ на детях, закончившийся смертью нескольких вакцинированных.
Цель исследования — обобщить данные исследований вакцинных кандидатов против РСВ на лабораторных животных и в клинических испытаниях на различных возрастных группах.
Поиск статей для анализа доклинических и клинических испытаний вакцин против РСВ осуществляли с использованием электронно-поискового ресурса PubMed по ключевым словам «respiratory syncytial virus and vaccine». В качестве критериев отбора указывали, что оригинальные статьи должны содержать сведения о доклинических и клинических исследованиях, причём последние включали в себя I–IV фазы рандомизированных контролируемых испытаний. За 1967–2025 гг. обнаружено 296 статей, обобщающих данные по исследованию вакцинных кандидатов против РСВ, и 1788 статей, в которых суммированы данные об испытаниях вакцинных кандидатов на животных. В обзоре суммированы данные доклинических исследований вакцинных кандидатов с указанием разработчиков, состава вакцинных препаратов, животных моделей, на которых проводились исследования, с кратким описанием основных результатов. Представлены данные о клинических испытаниях вакцинных кандидатов с указанием целевых групп населения, номера клинического исследования и источников, где опубликованы результаты этих исследований.

Ключевые слова: *респираторно-синцитиальный вирус, вакцина, доклинические исследования, клинические исследования, обзор литературы*

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Introduction

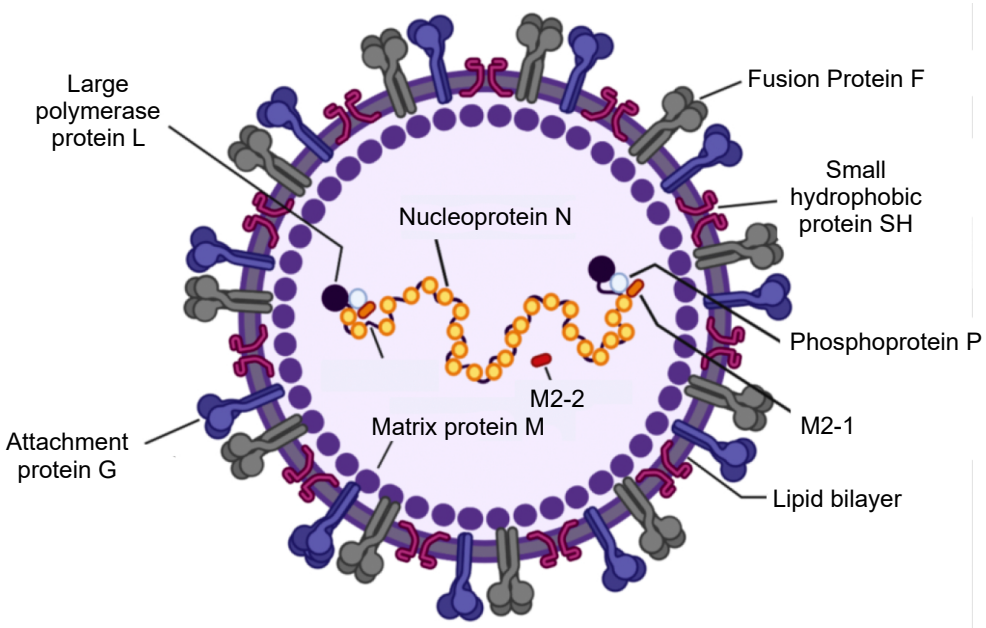
Among severe acute human respiratory diseases, respiratory syncytial virus (RSV) accounts for approximately 22% of cases. The number of deaths annually ranges from about 55,000 to 199,000, including 50,000–75,000 among children under 5 years of age [1, 2]. After RSV disease, no lasting immunity is formed, due to which the body is not resistant to subsequent infections [3, 4]. The reasons why this happens have not yet been established. In 2023, GlaxoSmithKline (UK) and Pfizer (USA) licensed subunit vaccines for the prevention of RSV infection in people over 60 years of age. Developer Moderna (USA) has submitted a messenger RNA (mRNA) vaccine for the prevention of RSV infection in the elderly. Many alternative vaccine candidates are in various stages of clinical trials.

The aim of the review is to summarize data on the development of RSV vaccines using different platforms and methodological approaches, the results of their preclinical studies in different animal models and clinical trials in different age groups.

General characterization of respiratory syncytial virus

RSV belongs to the order *Mononegavirales*, family *Pneumoviridae* (pneumoviruses), genus *Orthopneumovirus*. Until 2016, the virus was referred to the family *Paramyxoviridae* [5]. Another representative of the pneumovirus family is human metapneumovirus. Both pneumoviruses occupy a leading position in the structure of morbidity in children from birth to one year of age, the elderly and immunocompromised persons [6]. RSV infection is accompanied by severe pneumonias and bronchiolitis. According to expert estimates, in 2019, there were about 3.6 million human hospitalizations due to RSV infection and 101,400 related deaths among children aged 0–60 months worldwide [7].

RSV virions are pleomorphic: spherical forms with a diameter of 100–350 nm and filamentous forms with a length of up to 10 μm and a diameter of 60–200 nm are distinguished [8, 9]. The genetic material of RSV is represented by single-stranded RNA with negative polarity with a size of 15.2 kb. The RSV genome is unsegmented and contains 10 open reading frames encoding 9 structural and 2 non-structural proteins. The *M2* gene contains 2 open reading frames and encodes 2 proteins: M2-1 and M2-2. The viral RNA is packaged inside a nucleocapsid of helical symmetry type, which is a ribonucleoprotein complex (RNP) (**Figure**). Replication occurs with the participation of an RNA-dependent RNA polymerase consisting of a large L protein and phosphoprotein P (cofactor L). P-, L- and M2-1-proteins participate in the transcription process [10, 11]. RSV refers to enveloped viruses whose genetic material is surrounded by a protein envelope and a lipid bilayer acquired from the host cell during virus assembly and budding. Matrix protein M is located between the capsid and the outer lipid bilayer. The integral mem-



RSV structure.

The RSV genome is unsegmented with a length of 15.2 bp, has a negative polarity, and encodes 11 viral proteins. The RSV viral envelope contains 3 transmembrane glycoproteins: attachment protein G, fusion protein F, and small hydrophobic protein SH. On the inner side of the viral envelope is the matrix protein M. The viral RNA is encapsulated by a nucleoprotein (N) and a large protein (L). Phosphoproteins P and M2-1 mediate transcription of viral RNA. M2-2 regulates viral RNA synthesis. The figure was adapted from [16].

brane fusion protein F, attachment protein G, and small hydrophobic SH protein are embedded in the outer lipid bilayer. The F protein belongs to class I glycoproteins. Its main function is to allow virus entry into the target cell. Prior to interaction with the target cell, protein F is located on the surface of RSV in the "pre-F" conformation and acquires the "post-F" conformation due to the fusion of the viral and cell membrane [12].

The M2-2 protein is involved in replication and transcription [13]. NS1 and NS2 proteins are not part of the virion; their main role is to act as antagonists of α-/β-interferons, which are produced by the human body in response to viral infection [14, 15].

Prototypes of RSV vaccines

RSV was first discovered in 1956 [17], and for more than 60 years, work has been underway to develop a vaccine against RSV. The successful experience with formalin-inactivated vaccines against poliomyelitis, measles and parainfluenza was also applied to the first RSV vaccines, but the attempts were unsuccessful and new approaches to this problem were required.

Most RSV vaccines are developed on the basis of one of the most conserved RSV proteins, F, because this surface protein is the antigenic target for virus-neutralizing antibodies [18]. Approaches in which F-protein is stabilized in pre-F or post-F form have been developed [19, 20]. However, more often developers choose pre-F because virus-neutralizing antibodies are induced in greater amounts to this form of F-protein under conditions of natural infection or immunization [21].

History of RSV vaccines

In the 1950s, formalin inactivation of the virus was recognized as a successful technique [22]. In the United States in the 1960s, pilot trials of formalin-inactivated RSV vaccine (FI-RSV) were first conducted in infants and then large-scale clinical trials of the vaccine in children of different ages. The youngest cohort included children aged 2–7 months. Children in the experimental group (aged 4 months to 10 years) received 2 doses of FI-RSV intramuscularly, while those in the control groups received trivalent parainfluenza vaccine [23]. A 4-fold increase in RSV antibody levels was observed in 68% of those vaccinated with FI-RSV compared with control groups [23]. During the RSV circulation season, children receiving FI-RSV were more severely ill (7.9%) than controls (4.7%) [23]. An increased course of the disease was observed in children younger than 2 years of age [23]. Another study involved children aged 2–7 months; after FI-RSV vaccination, a 6-fold increase in neutralizing antibodies was observed compared with the control group [24]. In the winter period following the vaccination (1966/1967), 21 out of 30 children in the group of FI-RSV-vaccinated children became ill from natural exposure to RSV, whereas in the control group, it only happened in 5% of cases. Sixteen children in the FI-RSV group required hospitalization, while 2 infants died [24]. When comparing the results of trials among different age groups, it was found that children in the youngest group were at the most risk of severe disease progression when immunized with FI-RSV in the case of natural RSV infection.

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The development of vaccine-induced disease progression was found to be due to the fact that children were initially immunized with FI-RSV. One argument was that children who had previously been ill with RSV did not develop a state of immunopathology following immunization with FI-RSV after RSV infection. According to another argument, intramuscular injection of live RSV to RSV-naïve children did not show a protective effect of vaccination, but also did not develop a state of immunopathology. Thus, when developing RSV vaccine candidates, it is necessary to characterize in detail the phenotype of emerging memory T cells, as well as memory B cells with certain antigenic specificity.

After the unsuccessful FI-RSV clinical trials, attempts to develop a live attenuated vaccine began. The logic of this strategy is that during immunization, RSV will replicate exclusively in the upper respiratory tract, as a result of which the synthesized viral antigen will undergo intracellular processing and presentation on the surface of antigen-presenting cells, which will contribute to the formation of a balanced T- and B-cell immune response. When infants are vaccinated, the immune response will be formed locally in the respiratory mucosa where low levels of maternal IgG antibodies are observed [25]. The main advantage of using such technology is the method of administration by nasal spraying, which is recommended for use in pediatrics.

At the initial stages, certain difficulties were encountered in the creation of live attenuated vaccines due to the peculiarities of RSV cultivation, thermolability and its low viability. Live attenuated RSV can be obtained in several ways: by repeated passaging of the virus at reduced temperature, or by mutagenesis, which involves treatment of viruses with special mutagens, or by introducing mutations into the wild-type virus. To attenuate RSV, manipulations in SH or G proteins are commonly performed [26]. However, clinical trials of vaccine candidates obtained by mutagenesis have shown that a balance between sufficient viability of the vaccine strain and its immunogenicity cannot be achieved. In the 1980s, under the leadership of N.P. Leshchinskaya, a low-temperature strain obtained from Prof. P.M. Chanok (USA) was modified by conducting an additional 16 consecutive passages at a reduced temperature. During intranasal immunization with this vaccine candidate in children aged 1–2 years, clinical reactions of moderate severity were observed, which correlated with a 4-fold increase in specific anti-RSV antibodies. High antibody titers persisted only for 6-8 weeks after vaccination and then decreased to baseline levels [27]. Thus, due to attenuation mechanisms, it was not possible to obtain a ready-made vaccine preparation providing maintenance of long-term protective immunity. At the same time, the development of vaccine-induced pathology has not been shown with the use of live attenuated vaccines. Due to the failure of classical methods, alternative methods of producing RSV vaccines began to be developed.

Current approaches to the development of RSV vaccines

After the unsuccessful FI-RSV clinical trial, vaccine development in this area slowed down as new candidates underwent rigorous safety testing. On the other hand, the involvement of new approaches and technologies to combat RSV has led to notable advances in vaccine prophylaxis.

Vaccine candidates against RSV that have undergone clinical trials have been presented in detail in many reviews [28–31]. Note that such reviews should be updated every year due to the relevance and importance of the issue of vaccination coverage in populations that require RSV vaccine. It is important to select relevant animal species for preclinical trials to test the efficacy and safety of vaccines. In the case of RSV vaccines, a model of vaccine-induced enhancement of disease progression needs to be specifically studied. This problem is discussed in detail in the review by G. Zhang et al. [32]. The totality of a number of indicators characterizing innate and acquired immunity, which are associated with the severity of RSV infection in different animals, is presented in the review by S.B. Drysdale et al. [33]. This review collected data on trials of RSV vaccine candidates in laboratory animals, which were subsequently tested in humans.

mRNA vaccines

Nucleic acid-based vaccines, particularly mRNA vaccines, are an alternative to traditional vaccines. They consist of synthetic mRNA molecules with a structure that allows synthesizing the target protein of the antigen when it enters the target cell. When developing mRNA vaccines, special importance is given to mRNA delivery systems, since mRNA molecules are unstable and are subject to degradation by extracellular ribonucleases. Lipid nanoparticles are more commonly used, but other delivery strategies are being developed. For example, cell-penetrating peptide technology that allows mRNA to be delivered targeted to antigen-presenting cells for induction of an effective immune response [34].

The vaccine candidate mRNA-1345 (Moderna Inc., USA) encodes surface antigen F in a pre-F conformation, which is delivered using lipid nanoparticles (LNPs) [35]. The Spikevax vaccine (Moderna Inc.), used to immunize patients 12 years of age and older against COVID-19, was developed based on the results obtained from mRNA-1345 studies [36]. In 2024, a mRNA-1345 vaccine candidate called mResvia was approved by the U.S. Food and Drug Administration (FDA) for immunization of patients over 60 years of age. The developer initially created several vaccine candidates containing the mRNAs required to elicit expression of protein F in the pre-F conformation

(mRNA-1777 and mRNA-1172) [37, 38]. The vaccine candidate mRNA-1345 was derived from genetic modification of the mRNA within mRNA-1172 performed to increase F protein expression and alter its distribution in infected cells [39, 40]. In 2023, the results of a phase III clinical trial (ConquerRSV, NCT05127434) of mRNA-1345 (subsequently mResvia) were announced, according to which the efficacy of this vaccine was 83.7%. The study evaluated the clinical effect of immunization against two symptoms associated with lower respiratory tract disease in humans caused by RSV [41]. Clinical trials of the vaccine candidate mRNA-1345 in children aged 5 months to 2 years are currently underway (NCT05743881). Clinical trials are underway for the safety and efficacy of mRNA-1345 in immunized pregnant and postpartum infants (NCT06143046).

In 2024, phase I clinical trial studies of the world's first bivalent mRNA vaccine IN006 (InnoRNA, China), approved not only by China's National Drug Administration but also by the FDA, began. This mRNA vaccine targets the expression of F-protein in the pre-F conformation of both RSV serotypes, A and B. Lipid nanoparticles are used as delivery system[1].

Phase I and II clinical trial studies of mRNA vaccine candidates LNP CL-0059 and LNP CL-0137 mRNA vaccines against RSV (Sanofi, France) are currently underway. The study description reports that two different lipid nanoparticles are being tested for mRNA delivery (NCT05639894).

Vector vaccines

Vector vaccines are based on carrier vectors for delivery of RSV antigens to target cells and induction of an immune response against the virus antigens in the insert. RSV vaccines using modified Ankara smallpox vaccine viruses (MVA), adenoviruses, bovine parainfluenza viruses, Sendai virus and influenza viruses as vectors are currently undergoing clinical trials.

In studies at the Institute of Experimental Medicine (Russia), live influenza vaccine (LIV) serves as the vector. Three polyepitope cassettes of RSV were selected for integration into the genome of attenuated influenza virus [42]. The first cassette contains the fragment F243-294, with the antigenic site II of the RSV F-protein to which the monoclonal antibody Palivizumab binds. The other two cassettes are targeted primarily at the activation of cytotoxic T lymphocytes during vaccination and are represented by epitopes within the M2-1 protein of RSV.

Experiments on laboratory mice revealed that recombinant vaccine strains of RSV encoding a polyepitope T-cell cassette induce the development of a stable, fully functional RSV-specific systemic and local $CD8^+$ T-cell immune response that protects immunized animals from RSV reproduction in the lungs. Immunization with a T-cell vector vaccine has been shown to induce the formation of tissue-resident memory T cells to the built-in immunodominant $CD8^+$ T-cell epitope [43]. At the same time, the incorporation of the RSV F243-294 fragment into the HA molecule of influenza virus was found to be insufficient for induction of protective levels of RSV-specific antibodies in mice. However, immunization with such a chimeric virus prevents the development of RS-induced pulmonary pathology [44].

At the Smorodintsev Research Institute of Influenza (Russia), the developers use a modified influenza virus A/PR/8/34, weakened by shortening the NS1 protein to 124 amino acid residues, as a vector for delivery of the RSV F-protein transgene containing antigenic sites II and IV [45]. The resulting vaccine candidate RSV/Flu-01E has undergone phase I clinical trials (NCT05970744) in volunteers of two age groups — 18–59 years of age and elderly over 60 years of age and phase II clinical trials in volunteers over 60 years of age.

The vaccine candidate MVA-BN-RSV (Bavarian Nordic A/S, Denmark) is engineered based on modified Ankara vaccinia virus (MVA). The recombinant vaccinia strain MVA expresses F- and G-proteins of both subtypes (A and B) of RSV, as well as N- and M2-proteins of RSV. The results of phase I clinical trials showed that immunization with MVA-BN-RSV induced the production of humoral and cellular immune response against RSV in elderly (50–65 years old) and in adults (18–49 years old) [46]. In a phase II clinical trial (NCT02873286), MVA-BN-RSV was found to be well tolerated by immunized individuals over 55 years of age and resulted in a durable immune response that persisted for at least 6 months after a single vaccine administration. The results of a second immunization one year after the first immunization showed that the level of T-cell immune response was higher or similar to that observed after the first immunization [47]. However, in a phase III clinical efficacy trial in individuals over 60 years of age, it was recognized that MVA-BN-RSV immunization did not meet the study endpoint and was ineffective[2].

In the RSV001 study (NCT01805921), two recombinant vaccines were researched: PanAd3-RSV and MVA-RSV (ReiThera, Italy). PanAd3-RSV was developed based on monkey adenovirus, while MVA-RSV was based on modified Ankara vaccinia virus [48, 49]. The following proteins were used as RSV antigens: F (F_0 ΔTM — full-length, F — without transmembrane domain), N and M2-1, which are delivered to the target cell by replication-defective monkey adenovirus vector

[1] Innorna Announces First Participant Dosed in Phase 1 Clinical Trial of Investigational Bivalent RSV mRNA Vaccine IN006; 2024. Available at: https://innorna.com/news/330.html

[2] Bavarian Nordic. Bavarian Nordic Provides Update on RSV Vaccine Program. URL: https://www.bavarian-nordic.com/investor/news/news.aspx?news=6808

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(PanAd3) or MVA vector [50]. The vaccine candidates PanAd3-RSV and MVA-RSV administered in different combinations by different routes (intramuscular and intranasal administration of PanAd3, intramuscular administration of MVA) were well tolerated and immunogenic in adult humans (18–50 years of age) [51]. In the RSV001 study (NCT01805921), induction of humoral and cellular immune response after immunization was recorded in elderly individuals (60–75 years) [52].

The vaccine candidate VXA-RSV-f (Vaxart, USA) was developed based on adenovirus subtype 5 expressing RSV F-protein and a molecular adjuvant in the form of double-stranded RNA [53]. A phase I clinical trial (NCT02830932) was conducted in humans aged 18–49 years and ended in 2018. Results to date have not been published[3].

The vaccine candidate Ad26.RSV.Pre-F (Janssen, Belgium) was designed on the basis of adenovirus type 26 expressing F-protein in the pre-F conformation [54]. In phase I and II clinical trials (NCT03502707), different vaccine administration regimens, including combination with recombinant F-protein, were tested on the elderly (over 60 years of age). Those immunized with the combined regimen showed a more intense production of humoral and cellular immune response compared to the group injected with Ad26.RSV.preF alone [55]. A phase II (NCT03339713) clinical trial evaluated the combined administration of Ad26.RSV.preF and seasonal influenza vaccine (Fluarix, GSK). The vaccine was shown to demonstrate an acceptable safety profile and no evidence of immune interference in the elderly (over 60 years of age) [56]. A phase I/a (NCT03303625) clinical trial (NCT03303625) of Ad26.RSV.Pre-F showed that immunization in RSV-seropositive children aged 12–24 months and adults (18–50 years) produced RSV-specific neutralizing antibodies that persisted for 7 months. In addition, those immunized with Ad26.RSV.Pre-F were less susceptible to RSV infection *in vivo* [57].

ChAd155-RSV (GlaxoSmithKline, UK) is based on chimpanzee adenovirus-155 and encodes RSV proteins F, N and M2-1 [58]. The vaccine candidate was pre-tested on calves, in which the mechanisms of immune response to RSV are closest to children. After calf vaccination, neutralizing antibodies to RSV were induced, there was no evidence of vaccine-mediated disease enhancement, and protection against bovine RSV was demonstrated [59]. In a phase I/II clinical trial (NCT02927873), RSV-seropositive children aged 12–23 months were immunized with ChAd155-RSV and followed for 2 years after vaccination. As a result, a dose-dependent increase in RSV neutralizing antibody titers was observed [60].

The published results of phase I/II clinical trials (NCT03636906) of ChAd155-RSV in children aged 6–7 months showed that immunization caused induction of the humoral immune response in immunized individuals, and no signs of vaccine-mediated disease enhancement developed [61].

The vaccine candidate MEDI-534 (MedImmune, currently AstraZeneca, USA) is based on a chimeric bovine and human parainfluenza virus type 3 (PIV3) expressing the RSV F-protein [62]. In a phase I clinical trial (NCT00345670) involving children with previous RSV, this live attenuated intranasal vaccine was safe but immunogenicity was very low [63]. However, when tested in RSV seronegative infants, the target population for this vaccine, MEDI-534 was well tolerated by the immunized and induced an immune response against RSV in approximately 50% of cases and against the PIV3 vector in all cases [64].

The vaccine candidate SeVRSV (NIAID, USA) is a replication-capable Sendai virus, a strain of murine parainfluenza virus type 1 (PIV-1) that expresses the F-protein of RSV. Based on a phase I clinical trial (NCT03473002), it was found that immunization with SeVRSV in humans aged 18–45 years results in a low level of immune response to RSV. This fact is explained by the presence of pre-existing immunity to the vector in this age group [65].

Live attenuated vaccine

There are several approaches to the development of live attenuated RSV vaccines. The traditional approach is based on the sensitivity of the virus to certain reproductive temperatures or chemical agents. An alternative approach is based on the use of reverse genetics techniques to produce attenuated replication-competent virus. Vaccines developed using reverse genetics techniques have been tested on infants as young as 4 weeks of age [66, 67]. Live attenuated vaccines are considered safe for children who have not previously had RSV, as their use does not cause a vaccine-induced enhancement of the disease course after RSV infection [24, 68]. Furthermore, live attenuated vaccines are usually available as nasal drops. Once in the respiratory tract, they replicate there, regardless of the presence of maternal antibodies, ensuring the formation of both humoral and cellular immune response [69, 70].

One way to obtain an attenuated strain of RSV is deletion of the *SH* gene. Several vaccine variants have been developed in this way.

The vaccine candidate rA2cp248/404/1030ΔSH was designed with several temperature-sensitive mutations, with mutations obtained by passaging the virus at low temperature, and with a deletion of the *SH* gene. In an immunogenicity study of rA2cp248/404/1030ΔSH on RSV-seronegative children 1–2 months of age, high immunogenicity of the vaccine was observed. The vac-

[3] ClinicalTrials.gov. Dose-Ranging Trial of Safety & Immunogenicity of an Oral Adenoviral-Vector Based RSV Vaccine (VXA-RSV-f). URL: https://clinicaltrials.gov/study/NCT02830932

cine candidate was weakly immunogenic in RSV-seropositive children and adults [67].

The vaccine candidate MEDI-559 (MedImmune, currently AstraZeneca, USA) differs from the previous candidate by the presence of 39 synonymous nucleotide substitutions [71]. Both vaccine candidates, rA2cp248/404/1030ΔSH and MEDI-559, were tested on healthy children aged 5–24 months who had no previous history of RSV. However, the results of the studies revealed insufficient genetic stability of both candidates, a tendency to reversion of individual point mutations leading to decreased temperature sensitivity, which was observed in more than one third of the vaccine virus isolates [71, 72].

Vaccine candidate RSVcps2 (NIAID, NIH/Wyeth, USA) is a stabilized version of the MEDI-559 vaccine. According to the results of phase I clinical trials (NCT01852266 and NCT01968083) in children aged 6–24 months, RSVcps2 was well tolerated and had moderate immunogenicity [73].

An attenuated live vaccine phenotype can be achieved by deletion of the *M2-2* RSV gene. Deletion of *M2-2* results in a shift in the viral RNA synthesis program, which increases the level of viral RNA transcription and viral antigen expression, but decreases viral genome replication [74].

Two vaccine candidates MEDI/ΔM2-2 (NIAID, USA) and LID/ΔM2-2 (NIAID, USA) were evaluated [75, 76]. Both variants contain the *M2-2* deletion and both are derived from different RSV cDNA derivatives of the same A2 subtype, differing by 21 nucleotide sequences located at different sites in the genome. In the vaccine candidate LID/ΔM2-2, non-translated regions of the *SH* gene were deleted and synonymous substitutions (silent mutations) were added to the SH protein of RSV. The inserted mutations do not affect the phenotype of the assembled virus, as confirmed by experiments in mice [77]. Vaccination with both candidates stimulated the production of RSV-neutralizing antibodies, with LID/ΔM2-2 considered more effective because it provided a slight increase in replication. On average, the peak titer of virus secreted by vaccinated individuals was 100-fold higher in those who received LID/ΔM2-2 [75, 76]. Therefore, it was proposed to modify the vaccine candidate LID/ΔM2-2 by introducing either an additional mutation in the L protein causing temperature sensitivity or 5 point mutations in the N, F, and L proteins, which were previously found in the attenuated RSV strain and associated with adaptation to reduced temperature. Because of active replication, the LID/ΔM2-2 candidate was modified. However, the vaccine candidate LID/cp/ΔM2-2 was subsequently found to be hyperattenuated, had a low infectious titer, and stimulated antibody production in only a fraction of clinical trial participants [76].

The vaccine candidate LID/ΔM2-2/1030s was created by adding the genetically stabilized mutation 1030s. The results of phase I clinical trials (NCT02237209, NCT02040831) on children aged 6–24 months established a high titer of neutralizing antibodies in immunized individuals, which was clinically equivalent to the antibody titer produced by natural RSV infection, according to expert estimates [78].

A modified LID-based vaccine candidate, D46/NS2/N/ΔM2-2-HindIII, was engineered to achieve a similar phenotype to the MEDI/ΔM2-2 candidate. Overall, it was more attenuated than LID/ΔM2-2 but exhibited high viral titers [76].

In a study by S.S. Stobart et al., a recombinant strain of RSV OE4 (RSV-A2-dNS1-dNS2-ΔSH-d_{Gm}-G_{snull}-line19F) with increased levels of antigen F in the pre-F conformation was shown to exhibit thermostability and immunogenicity despite strong attenuation in the airways of cotton rats [79].

Another promising strategy involves NS1 and NS2 deletions that disrupt the host cell TGFβ signaling pathway, transforming the immune response to enhance viral replication [80, 81].

Phase I phase I clinical phase trials (NCT03227029 and NCT03422237) of the RSV/ΔNS2/Δ1313/I1314L vaccine candidate (Sanofi, France) have established the immunogenicity and protective efficacy of the vaccine in RSV-seronegative children aged 6-24 months [82]. A phase III clinical trial (NCT06252285) of the RSV/ΔNS2/Δ1313/I1314L vaccine candidate is currently underway with 6300 children aged 6–22 months.

Subunit vaccines

Most subunit RSV vaccines under development contain a surface F-protein in the pre-F conformation [83]. The immunogenicity of such vaccines is enhanced by the use of adjuvants or multiple immunizations [84]. Given the composition of subunit vaccines and the failure of FI-RSV clinical trials, special attention is paid to safety. Immunization with FI-RSV has been found to result in insufficient production of antibodies with neutralizing activity. Subsequent signs of vaccine-mediated disease enhancement are attributed, among other things, to poor stimulation of Toll-like receptors in B-cells, which entails a lack of affinity maturation of antibodies [85]. Thus, affinity maturation due to Toll-like receptor stimulation is a key factor in helping to prevent a vaccine-induced disease amplification state. Subunit vaccines are currently being developed for pregnant women, the elderly, and adolescents who have previously had RSV to reduce the risk of vaccine-mediated disease enhancement [30, 86].

The vaccine candidate DS-Cav1 (NIAID, USA) is based on the F-protein in the pre-F conformation [87]. In a phase I clinical trial of DS-Cav1 in adults aged 18-50 years (NCT03049488), it was found that immunized individuals showed an increase in RSV-F-specific antibodies with neutralizing activity. Moreover, the level

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of neutralizing antibodies was above the baseline level for 10 months after immunization [88]. In this study, aluminum hydroxide was used as an adjuvant.

The vaccine candidate DepoVax-RSV or DPX-RSV (ImmunoVaccine Technologies Inc., Canada) was developed based on the ectodomain of the SH (SHe) protein of RSV. This peptide is administered with DepoVax adjuvant, which ensures prolonged exposure of the antigen to the immune system. According to the results of Phase I clinical trials (NCT02472548), an increase in anti-SHe-specific antibodies was recorded after DPX-RSV administration in immunized people aged 50–64 years old [89].

In May 2023, the FDA approved the first Arexvy vaccine (GlaxoSmithKline, UK) for people over 60 years of age. This vaccine contains RSV F-protein in a pre-F conformation and is administered together with adjuvant AS01E to provide a protective effect against RSV subtypes A and B. According to the results of phase I clinical trials (NCT03814590 and NCT04090658) of Arexvy on the elderly aged 60–80 years, it was found that immunization promoted the formation of RSV-specific IgG antibodies, neutralizing antibodies, and $CD4^+$ T cells which were detectable in the immunized after one year [90, 91]. The results of a phase II clinical trial (NCT04657198) documented an increase in immunization efficacy after revaccination of study participants 1.5 years after the first immunization [92]. A 2b randomized open-label efficacy and safety study of Arexvy was announced between 2023 and June 2025. Participants in the study included people aged 18 years and older who had undergone lung or kidney transplantation and were at increased risk of lower respiratory tract disease compared to healthy people aged 50 years and older (NCT05921903). One study in a phase III clinical trial (NCT04732871) reported an increase in neutralizing antibody titers to RSV and cases of immunization-induced adverse events [93]. The most recently published data from a phase III clinical trial (NCT04886596) evaluated the efficacy of Arexvy immunization in 24,967 participants during 2 epidemiologic seasons of RSV circulation. The efficacy of Arexvy immunization was about 67.2% for lower respiratory tract disease and about 78.8% for severe lower respiratory tract disease caused by RSV [94].

Based on the interim results of a phase III clinical trial (NCT05035212), another subunit vaccine for the prevention of RSV infection in the elderly, Abyrsvo (Pfizer, USA), which contains RSV F-protein in the pre-F conformation, was approved. According to the results of phase I clinical trials of Abyrsvo, immunized people produced RSV-specific IgG antibodies to A- and B- strains of RSV [95]. In phase II clinical trials Abyrsvo was tested in non-pregnant and pregnant women (NCT04071158 and NCT04032093). A phase III clinical trial (NCT04424316) in pregnant women has recently been completed. Infants born to mothers immunized during pregnancy were found to have a lower incidence of RSV-associated diseases than those born to unimmunized mothers [96]. The phase III clinical trial RENOIR (NCT05035212) determined that the efficacy of prevention of RSV-induced lower respiratory tract disease was about 66.7% [97]. The MONET (NCT05842967) phase III clinical trial immunized people aged 18-59 years with chronic diseases at risk for severe RSV. The vaccine was well tolerated, safe and immunogenic [101]. Overall, the rates of RSV-specific neutralizing antibodies in immunized individuals from the MONET study were not lower than those in immunized older adults over 60 years of age from the RENOIR study (NCT05035212).

Vaccines based on virus-like particles

Vaccines based on virus-like particles are considered to be a subclass of subunit vaccines. These vaccines are synthesized by self-assembly of nanoparticles that exhibit multiple copies of a selected viral antigen on their surface. A high level of humoral and cellular immune response is achieved not only due to the multiple repetition of antigen sites, but also due to the immunostimulatory properties of the matrix for immobilization of nanoparticles [99]. The absence of viral genome in the composition of these vaccine candidates makes them safe due to their inability to reproduce viable virions.

The vaccine candidate ResVax (Novavax, USA) is based on the F-protein of RSV and is a vaccine based on virus-like particles. The vaccine is being developed to protect infants by vaccinating expectant mothers, children aged 6 months to 5 years and elderly individuals over 60 years of age. A phase II clinical trial (NCT02247726) found that immunization of healthy pregnant women induced the production of neutralizing antibodies to RSV that were effectively transmitted to the newborn [100]. The success of this phase of research led to the inclusion of the vaccine candidate ResVax in the PREPARE study program (a multicenter, randomized, placebo-controlled phase III study, NCT02624947). The clinical trial involved follow-up of infants born at the very beginning of the epidemic season of RSV circulation from mothers immunized with ResVax in the third trimester [101]. It turned out that ResVax immunization was not effective. Despite this, it was stated that ResVax reduced hospitalizations in children associated with RSV-related lower respiratory tract infections by 44%. Furthermore, a 39.4% reduction in RSV-specific lower respiratory tract infections and a 58.8% reduction in RSV-associated hypoxemic respiratory failure in infants younger than 3 months of age have been reported [101].

The vaccine candidate SynGEM (Virtuvax, The Netherlands) is a mucosal vaccine containing an F-protein in a pre-F conformation bound to bacteria-like particles derived from *Lactococcus lactis*. Due to the bacteria-like particles, the RSV vaccine antigen appears in

a more natural conformation and an enhanced immune response against RSV is observed [102]. Clinical trials of the vaccine without adjuvant were ineffective in the elderly group. Vaccination contributed to a 61% reduction in hospitalizations in the elderly with chronic obstructive pulmonary disease. According to the results of phase I clinical trials, no increase in neutralizing antibodies in sera, which recognize epitopes within the unique Ø site of the F-protein, was observed in immunized individuals [103, 104]. Nevertheless, the vaccine induced the production of palivizumab-like antibodies. Currently, clinical trials of this vaccine have been suspended.

Vaccine candidate V-306 (Virometrix, Switzerland) contains a peptide consisting of mimetics of the F-protein RSV mimicking antigenic site II. The peptide is conjugated to synthetic nanoparticles made of self-organizing lipopeptides. In a Phase I clinical trial (NCT04519073), the V-306 vaccine was found to be safe and immunogenic in healthy women aged 18–45 years old [105].

Conclusion

RSV is the cause of severe respiratory diseases in children of the 1st year of life, immunocompromised people and the elderly [106, 107]. The clinical presentation of RSV varies from mild upper and lower respiratory tract infections to pneumonias and bronchiolitis in children.

To date, the FDA has approved the use of 3 vaccines for the prevention of RSV infection in humans. The bivalent subunit vaccine Abrysvo (Pfizer, USA) is approved for use in pregnant women and the elderly. Arexvy subunit vaccine (GlaxoSmithKline, UK) and mRESVIA mRNA vaccine (Moderna, USA) are approved for use in elderly people over 60 years of age.

The development and efficacy studies of RSV vaccines for children, who represent the main target group, are ongoing. In this case, the main problems include the need to vaccinate infants at an early stage (2-4 months), the revealed effect of enhancing the course of the disease associated with the introduction of FI-RSV with alum adjuvant, and difficulties in achieving the required indicators of prophylactic efficacy. For a long time, the only specific treatment for RSV infection was the monoclonal antibody drug Palivizumab, usually prescribed to people at high risk of severe forms of the disease. In this case, a significant factor is the high cost of treatment with this drug, which makes it unaffordable in countries with the highest incidence of RSV infection [108].

In 2022–2023, the monoclonal antibody drug Nirsevimab (AstraZeneca, UK; Sanofi, France) was approved and recommended for children in cases of severe RSV infection and its complications [109].

Vaccines are currently being developed for women of reproductive age and pregnant women to protect a cohort of naïve infants, as well as to protect the elderly and children. Vaccines based on virus-like particles, vector, subunit and live attenuated vaccines are in various phases of clinical trials.

Up-to-date information on vaccine candidates in preclinical studies is presented in **Table 1**. Information on candidates studied in different phases of clinical trials is summarized in **Table 2**.

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Table 1. Results of preclinical trials of RSV vaccine candidates

Vaccine (developer)	Vaccine composition (adjuvant)	Animal models (method of administration)	Research and results	Source
LIVE ATTENUATED VACCINES				
RSV-MinL4•0 (Codagenix, USA)	RSV-Min L – vaccine candidate obtained by introducing silent mutations (codon deoptimization) (without adjuvant)	African green monkeys (i.n. + i.t.).	RSV-MinL4•0 is more attenuated than RSV A2 when injected with 2 × 10^6 PFU (10^6 i.n. and i.t. each): the value of peak viral titers in tracheal smears in the group immunized with RSV-MinL4 is 100 times lower than in the wt RSV group. When RSV A2 was infected with RSV at a dose of 2 × 10^6 PFU on day 104 of the experiment, the protective potential of RSV-MinL4•0 was revealed: the peak values for virus isolation on day 6 in swabs from the trachea and from the oropharynx were 1000-fold and 100-fold lower than in the naive monkey group, respectively	[110]
IT-RSVΔG (Intravacc, Netherlands)	RSV isolate of strain 98-25147-X (pRSV-X) in which the gene encoding the G-protein (ΔG) has been deleted (without adjuvant)	Cotton rats (i.n.).	pRSV-X ΔG was more attenuated compared to the pRSV-X vector when injected with 10^5 $TCID_{50}$: peak titers in the lungs and noses were 4-5 times lower than in the pRSV-X group. A single vaccination with rRSV-X ΔG protected rats against RSV-X virus infection at a dose of 10^6 $TCID_{50}$: on day 5 after the challenge trial, virus was undetectable in the lungs of immunized animals, whereas in the placebo group the virus multiplied up to 5 $\log_{10}$ $TCID_{50}$ in the lungs. Vaccination with pRSV-X ΔG did not result in the development of bronchopulmonary pathology, and histopathologic signs of lesions were significantly reduced compared to the naive control group	[111]
MV-012–968 (Meissa, USA)	RSV-A2-dNS1-dNS2-ΔSH-dGmGsnull-line19F codon deoptimization of NS1, NS2 genes; deleted SH gene (ΔSH). F protein in a “pre-fusion”	BALB/c mice (i.n.)	When MV-012-968 was administered at a dose of 10^6 focal forming units (FFU), the viral load in the lungs was significantly lower than in mice infected with RSV A2 and RSV A2-19F. MV-012-968 vaccination protected mice from RSV A2-line19F infection at a dose of 10^5 FFU: no virus was isolated in the MV-012-968 group 5 days after the challenge trial. Lung mucus production in candidate MV-012-968 was significantly lower than in RSV A2-line 19F and RSV A2-del-M2 at 5 days after the challenge trial.	[79]
		Cotton rats (i.n.).	Replication of MV-012-968 (OE4) administered at a dose of 10^5 FFU was absent in the noses and lungs of cotton rats. MV-012-968 (OE4) completely protected rats from RSV-A2-line19F infection at a dose of 10^6 FFU on day 42 after vaccination. In contrast to FI-RSV, vaccination with MV-012-968 (OE4) did not induce histopathologic changes in the lungs after RSV infection	[19]
RSV ΔNS2/Δ1313/I1314L (NIAID/NIH, Sanofi, USA)	Candidate with ts mutations in the L-protein (deletion at position 1313, compensatory mutation *I1314L* that results from passaging the virus at progressively higher temperatures); NS2 deletion (ΔNS2) (without adjuvant)	BALB/c mice (i.n.)	Candidate RSV ΔNS2/Δ1313/I1314L is hyperattenuated in mice. The RSV/Δ1313 variant multiplied significantly weaker in the respiratory tracts of mice when administered at a dose of 10^6 PFU compared with wt RSV. This animal model is not suitable for evaluating the immunogenicity and protective efficacy of hyperattenuated vaccine candidates	[112, 113]
		Juvenile chimpanzees (i.n. + i.t.)	When chimpanzees were immunized with RSV ΔNS2/Δ1313/I1314L at a dose of 10^6 PFU each (i.n. + i.t.), peak viral load values were 3-25-fold lower compared with the highly attenuated candidates MEDI-559 (rA2 cp248/404/1030ΔSH) and cps2 (a genetically stabilized version of MEDI-559) in nasal flushes. In contrast, RSV ΔNS2/Δ1313/I1314L virus titer in bronchoalveolar lavage (BAL) and tracheal washings was 20-fold higher than in the comparison groups. The vaccine induced high levels of RSV virus-neutralizing antibodies; however, experiments on protection against RSV infection were not performed in this study	[113]
RECOMBINANT VECTOR VACCINES				
MVA-BN RSV (Bavarian Nordic BN, Denmark)	MVA (modified Ankara vaccine), which includes genes encoding F, G, N, and M2-1 (RSV A) and G (RSV B) (without adjuvant)	BALB/c mice (i.n.)	Mice were immunized with MVA-BN RSV at a dose of 1 × 10^8 $TCID_{50}$ at 3 week intervals and 2 weeks later were infected with RSV A2 at a dose of 1 × 10^6 PFU. No virus was detected in the lungs 4 days after the challenge trial. In the groups in which selective depletion of CD4 and CD8 T cells was used, virus replication in the lungs was 600- to 2,000-fold weaker than in the control group	[114]

Continuation of the Table 1

Vaccine (developer)	Vaccine composition (adjuvant)	Animal models (method of administration)	Research and results	Source
PanAd3-RSV и MVA-RSV (ReiThera, Italy)	PanAd3 (monkey adenovirus) and MVA, which include genes encoding F, N, M2-1 (RSV) (without adjuvant)	Cotton Rats (i.n., i.m.).	PanAd3-RSV (5×10^8 viral particles) and MVA-RSV (1×10^7 PFU) were administered in different combinations at an interval of 4 weeks. In all groups, rats were protected against RSV administered at a dose of 1×10^5 PFU: the virus did not replicate in the lungs on the 5th day. Challenge virus did not reproduce in the nares in the PanAd3-RSV/MVA (i.m.) and PanAd3-RSV/PanAd3-RSV groups. Pathologic changes in the lungs after the challenge trial were less pronounced in the PanAd3-RSV/MVA (i.m.) group compared to the FI-RSV group, whereas the other groups showed histopathology similar to the naive control group after the challenge trial.	[115]
		Calves (2-4 weeks) (i.n., i.m.)	PanAd3-RSV (5×10^{10} viral particles) and MVA (2×10^7 PFU) were administered to calves in different combinations at 4 week intervals. On day 6 after the challenge trial, RSV administered at a dose of 10^4 PFU did not replicate in the respiratory tract of calves from the PanAd3-RSV/MVA (i.m.), PanAd3-RSV (i.m.)/PanAd3-RSV (i.m.), PanAd3-RSV/PanAd3-RSV (i.m.), and PanAd3-RSV (i.m.)/MVA (i.m.) groups. In the PanAd3-RSV/PanAd3-RSV group, the virus reproduced in the lungs. In the PanAd3-RSV/MVA (i.m.) group, virus was not detected in the nares and no macroscopic lung lesions were found. Calves immunized with the heterologous prime-boost scheme developed less massive infiltration of polymorphonuclear neutrophils in the lungs and BAL after the challenge trial compared to calves from the homologous prime-boost groups	[115]
		Macaque (i.n., i.m.).	PanAd3-RSV (5×10^{10} viral particles) and MVA (2×10^7 PFU) were administered to macaques in different combinations at 8-week intervals. Production of interferon-γ and interleukin-4 in peripheral blood mononuclear cells was increased 6-fold after boost. Interleukin-4 production in mononuclear cells was higher in the PanAd3-RSV/MVA (i.m.) group compared with the PanAd3-RSV (i.m.)/MVA (i.m.) group. 1 week after the 2nd immunization, the mononuclear cells were dominated by a population of $CD4^+$ T cells. Only macaques immunized with PanAd3-RSV produced RSV-specific IgA. Macaques immunized with PanAd3-RSV (i.m.) had a 40-fold higher titer of neutralizing antibodies compared with PanAd3-RSV. The protective effect of immunization was not evaluated	[50]
Ad26.RSV.F and Ad35.RSV.F (Janssen Vaccines, Netherlands)	Adenoviruses of types 26 and 35 that include the gene encoding F in the pre-F conformation (without adjuvant)	BALB/c mice (i.m.)	Double immunization of mice at 4-week intervals with candidate Ad26.RSV.F and Ad.35.RSV.F in different combinations at a dose of 10^{10} viral particles stimulated the production of RSV F-specific T-cell immune response at week 12: high levels of interferon-γ, tumor necrosis factor-α and interleukin-2 CD8+ T-cell production was in the Ad.35.RSV.F/Ad26.RSV.F and Ad.26.RSV.F/Ad35.RSV.F groups. The immune response in mice in all groups developed Th1 type: high IgG2a/IgG1 ratios were recorded compared to the FI-RSV group. High level of neutralizing antibodies in Ad.35.RSV.F/Ad26.RSV.F and Ad.26.RSV.F/Ad35.RSV.F groups	[54]
		Cotton rats (i.m.)	Double immunization with Ad26.RSV.F and Ad.35.RSV.F variants according to the scheme of homologous and heterologous prime-boost at a dose of 10^{10} viral particles induced high levels of virus-neutralizing antibodies and fully protected animals from RSV replication in the respiratory tract without causing pathological changes in lung tissues after the challenge trial. The possibility of reducing the vaccine dose was evaluated by immunizing animals with Ad26.RSV.F and Ad.35.RSV.F once at doses of 10^6–10^8 viral particles and exposing them to RSV A2 infection 7 weeks later. All immunized animals were completely protected from virus replication in the lungs, and replication in the nares was significantly reduced in all groups compared with controls. Histopathology scores were similar in both groups and did not differ from the naive control group. The duration and cross-protective potential of the vaccine were evaluated by immunizing rats twice with homologous and heterologous prime-boost candidates taken at doses of 10^8 and 10^{10} viral particles and infecting the animals with RSV A2 or RSV B15/9 30 weeks after immunization. Challenge viruses did not replicate in the lungs in all vaccine groups, with the Ad26.RSV.F/Ad.35.RSV.F combination (10^{10} viral particles) better protecting animals from RSV B15/9 replication in the nares. In contrast to FI-RSV, the tested vaccines did not cause histopathologic changes in lung tissues after the challenge trial.	[54]

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Continuation of the Table 1

Vaccine (developer)	Vaccine composition (adjuvant)	Animal models (method of administration)	Research and results	Source
		Cotton rats (i.m.).	The protective efficacy of single immunization with Ad26.RSV.preF at a dose of 10^5–10^8 viral particles and double immunization with RSV. preF protein was compared. On day 49, rats were injected with RSV A2 at a dose of 10^5 PFU. In the Ad26.RSV.preF group, the challenge virus did not reproduce in the noses and lungs in contrast to the RSV. preF group. A correlation was established between the production of neutralizing antibodies and protection of animals from RSV infection	[116]
VXA-RSV f oral (Vaxart, USA)	Ad-RSVF is a type 5 adenovirus that contains a gene encoding a full-length F protein (without adjuvant)	Cotton rats (or.)	Double immunization of rats with Ad-RSVF at different doses (10^8-10^{10} PFU) protected rats from RSV A2 infection at a dose of 1 × 10^5 PFU on day 70: the virus did not reproduce in the noses and lungs in the group where the vaccine dose was higher (dose-dependent protective effect of immunization). The histopathology rate in the Ad-RSVF group was much lower than in the FI-RSV group. The level of induction of pro-inflammatory cytokines was minimal in the Ad-RSVF group	[117]
ChAd155-RSV GS (GSK, UK)	Chimpanzee adenovirus containing genes encoding F-protein (deletion of transmembrane region), N, M2-1 (without adjuvant)	Calves (i.m.)	Calves were immunized with ChAd155-RSV at a dose of 5 × 10^{10}. Challenge trial was performed shortly (4 weeks) or long (16 weeks) after immunization. In contrast ChAd155-RSV unimmunized calves showed fever, high fever, respiratory distress syndrome, high levels of pulmonary consolidation, and histopathologic changes in lung tissue after the challenge trial. When the challenge trial was performed after 4 weeks, virus replication in BAL and nasopharyngeal washings in the unimmunized was 500-1000 times and 10 times higher, respectively, than in the ChAd155-RSV group. When the challenge trial was performed at week 16, peak viral titers in BAL and nasopharyngeal washings were approximately the same in the ChAd155-RSV and PBS groups, but viral clearance occurred earlier. In order to establish the role of pre-existing immunity to RSV, calves were fed milk containing antibodies to RSV prior to immunization with ChAd155-RSV or PBS. In the ChAd155-RSV group, calves with RSV antibodies showed no signs of RSV infection (malaise, fever, respiratory rate) in contrast to the PBS group, where calves had RSV antibodies. In the ChAd155-RSV groups with RSV antibodies, the challenge virus reproduced 100 and 10 times less in the BAL and nasal cavity than in the PBS group	[118]
SeV/RSV (NIAID, USA)	Sendai virus (parainfluenza virus type 1), which contains a gene encoding full-length F (RSV) (without adjuvant)	African green monkeys (i.n. + i.t.)	SeV/RSV was attenuated when administered at a dose of 2 × 10^6 EID_{50}. The virus was not secreted in nasal washings and was 1000-fold more attenuated than b/h PIV-3 RSV F2. On day 28, monkeys were infected with RSV A2 at a dose of 1.4 × 10^6 EID_{50}. Challenge virus was secreted in the upper respiratory tract to a lesser extent in the SeV/RSV group compared with controls and was not isolated from the BAL. No adverse effects were observed in the SeV/RSV group after the challenge trial. Lung slices showed small foci of lymphohistiocytic inflammation around terminal and respiratory bronchioles	[119]
rBCG-N-hRSV (Catholic University of Chile, Chile)	Bacillus Calmette-Guerin (BCG) bacterium that contains the gene encoding the N protein of RSV (without adjuvant)	BALB/c mice (s.c.)	Mice were injected with rBCG-N-hRSV or BCG-WT at a dose of 3 × 10^5 PFU. After 3 weeks, mice were infected with RSV at a dose of 1 × 10^7 PFU. The BCG-WT and unimmunized mice had approximately 10% weight loss, whereas weight loss was negligible in the rBCG-N-hRSV group. The viral load in the BAL was 7- and 300-fold lower in the rBCG-N-hRSV group than in the BCG-WT and unimmunized mice groups, respectively. In histological sections of lungs, inflammation was less pronounced in the rBCG-N-hRSV group than in the groups of unimmunized mice and BCG-WT. In the long-term protection study, the challenge trial was performed 50 days after immunization. Mice in the rBCG-N-hRSV group had less weight loss compared to the other groups, the number of infiltrating cells in the BAL and the viral load were lower than in the groups of unimmunized mice and BCG-WT mice	[120]
		BALB/c mice (s.c.)	Mice were immunized with BCG-N, BCG-M2 at a dose of 1 × 10^8 PFU. Mice were infected intranasally with RSV at a dose of 1 × 10^7 PFU. After the challenge trial, mice significantly lost weight in the control groups (BCG-WT, UV-RSV, BCG-OVA). No weight loss occurred in the BCG-N and BCG-M2 groups. Computed tomography demonstrated no evidence of pneumonia and inflammation in the lung tissue in BCG-N, BCG-M2 groups, in contrast to the group of unimmunized mice after the challenge trial. No active neutrophil infiltration in the BAL was recorded in the BCG-N, BCG-M2 groups after the challenge compared to the unimmunized mice. After the challenge trial, BCG-N and	[121]

Continuation of the Table 1

Vaccine (developer)	Vaccine composition (adjuvant)	Animal models (method of administration)	Research and results	Source
			BCG-M2 groups were assigned low pulmonary histopathology scores equivalent to a naive control group that was not infected with RSV. BCG-N and BCG-M2 immunization was found to stimulate primarily T-cell immunity: T cells with a Th1-like pattern of cytokine secretion were activated	
		BALB/c mice (RAG-1 deficient) (s.c.)	Mice were immunized 2 times 10 days apart with BCG-N, BCG-M2, and BCG-WT at a dose of 1×10^8 PFU. After 21 days, mice were infected with RSV at a dose of 1×10^7 PFU. Mice in the BCG-N group actively lost weight (on par with unimmunized RSV-infected mice). After RSV infection, the BCG-N and BCG-WT groups had large numbers of infiltrating cells such as neutrophils and eosinophils in the BAL. On day 6 after challenge, the viral load in the lungs was almost the same in all groups. On day 12, a decrease in RS viral load was observed in the group of BALB/c mice (not RAG-1). These results suggest that T cells are required for RSV clearance and that the mechanisms of innate immunity are insufficient to protect against RSV infection.	[122]
		Holstein calves (s.c.)	Calves were injected with rBCG-N-hRSV or WT-BCG at a dose of 10^6 PFU 2 times with an interval of 2 weeks. After 2 weeks, calves were infected with RSV at a dose of 10^4 $TCID_{50}$. All calves developed signs of RSV (fever, lethargy, nasal and ocular discharge, mild dyspnea), but clinical condition scores were lower in the rBCG-N-hRSV and WT-BCG groups than in the unimmunized group. There were no differences in pulmonary lesion scores between the rBCG-N-hRSV, WT-BCG, and unimmunized calf groups after the challenge trial and no evidence of vaccine-mediated disease enhancement	[123]
MVA-F and MVA-G (National Heart and Lung Institute, UK) (Institute of Molecular Virology, Germany)	MVA vector carrying genes encoding F- and G-proteins of RSV	BALB/c mice (i.p.)	Double immunization of mice with MVA-F and MVA-G variants at a dose of 10^8 infectious units resulted in the formation of pronounced humoral and T-cell immunity to RSV. Infection of immunized mice with RSV at a dose of 1.2×10^6 PFU showed accelerated virus elimination in the MVA-F and MVA-G groups compared with the group of mice immunized with the MVA vector. Signs of eosinophilia after challenge were absent in the MVA-F and wtMVA groups and were small in the MVA-G group. However, lymphocytosis was pronounced in the MVA-F group (~50%) compared with the MVA-G group (~20%). There were more polymorphonuclear cells in the MVA-G group, which was comparable to the FI-RSV group. After challenge, mice actively lost weight in the MVA-G (up to 15%), MVA-F (~12%) and FI-RSV (~10%) groups compared with the group of control animals after RSV challenge, indicating an exacerbation of the disease course after immunization and RSV infection	[124]
rVV-G и rVV-F (University of Pittsburgh School of Medicine, USA)	Cowpox viruses containing genes encoding F- or G-protein in different forms: rVVGsol (soluble, secreted form), rVVGmem (form anchored in membrane), rVVG (both forms)	BALB/c mice (i.p., scar.)	Immunization of rVVF, rVVG and rVVGmem mice resulted in the induction of high levels of antibodies, regardless of the route of administration. In the rVVGsol group, the level of RSV-specific antibodies was ~550-fold higher with i.p. administration than with immunization by scarification. When immunized mice were infected with RSV A2 strain, virus was not isolated from the lungs in the rVVF, rVVG, rVVGmem, and rVVGsol groups. When immunization by scarification was performed in the rVVG and rVVGsol groups, an increase in the number of eosinophils in the BAL after the challenge trial was observed. The rVVGmem group had 10-fold higher eosinophil counts than the rVVG and rVVGsol groups. No eosinophils were detected in the BAL during i.p. administration. The method of vaccine administration influenced the development of pulmonary pathology	[125]
LAIV + NA/RSV and LAIV + NS1/RSV (Institute of Experimental Medicine, Russia)	Recombinant LDHV vaccine strains encoding the PCV M2-1 polyepitope T-cell cassette (70-101+114-146)	BALB/c mice (i.n.)	Mice were immunized 2 times 3 weeks apart with vaccine variants LAIV + NA/RSV or LAIV + NS1/RSV at a dose of 10^6 EID_{50} per mouse. Three weeks after the 2nd immunization, mice were infected intranasally with RSV strain A2 at a dose of 2×10^5 PFU/individual. RSV was found to be detected at insignificant levels in the LAIV + NA/RSV and LAIV + NS1/RSV groups, in contrast to the PBS and LAIV groups in lungs collected at day 5. In contrast to the FI-RSV group, vaccine variants LAIV + NA/RSV and LAIV + NS1/RSV did not induce pulmonary eosinophilia and lung pathology after RSV infection	[126]

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Continuation of the Table 1

Vaccine (developer)	Vaccine composition (adjuvant)	Animal models (method of administration)	Research and results	Source
LAIV + NA/RSV and LAIV + NS1/RSV (Institute of Experimental Medicine, Russia)	Recombinant LDHV vaccine strains encoding the PCV M2-1 polyepitope T-cell cassette (70-101+114-146)	BALB/c mice (i.n.)	Mice were immunized 2 times with an interval of 3 weeks with vaccine variants LAIV + NA/RSV or LAIV + NS1/RSV at a dose of 10^6 EID_{50}. Seven days after the 2nd immunization, lungs were harvested from mice to identify subpopulations of resident T cells. Immunization with LAIV + NA/RSV or LAIV + NS1/RSV was found to produce high levels of tissue-resident memory T cells to an embedded immunodominant $CD8^+$ T-cell epitope. Moreover, the levels of influenza-specific memory $CD8^+$ T-cells in the LAIV + NA/RSV and LAIV + NS1/RSV groups were higher than in the LAIV group	[43]
LAIV-HA + G-RSV and LAIV-HA + A-RSV (Institute of Experimental Medicine, Russia)	Incorporation of the target fragment F243–294 of RSV into the HA molecule, which is the target fragment of LAIV	BALB/c mice (i.n.)	Vector vaccine candidates differed in the composition of the chimeric *HA* + RSV gene: the RSV insert was linked to the HA1 subunit using either AAAPGAA (A) or G4SG4S (G) linker. Mice were immunized 2 times 3 weeks apart with vaccine variants LAIV-HA + G-RSV or LAIV-HA + A-RSV at a dose of 10^6 EID_{50}. Three weeks after the 2nd immunization, mice were infected intranasally with RSV strain A2 at a dose of 5×10^5 PFU/specimen. On the 5th day after infection, the extent of RSV replication in lung tissue was assessed. Immunization with LAIV-HA + G-RSV and LAIV-HA + A-RSV failed to prevent the development of RSV. Nevertheless, there was a significant difference between the virus titer values in the LAIV-HA + G-RSV group compared to PBS. Evaluation of the bronchial epithelium of mice revealed that the degree of damage was significantly lower in the LAIV-HA + G-PSV group compared to the PBS group, whereas no significant difference was found for the vaccine variant LAIV-HA + A-PSV. Histopathological evaluation of mouse lung segments after RSV infection showed a high degree of protection in the groups immunized with the vector vaccines LAIV-HA + A-RSV and LAIV-HA + G-RSV, in contrast to FI-RSV	[45]
PR8-NS-F, PR8-sF-NS (A.A. Smorodintsev Research Institute of Influenza, Russia)	Influenza virus A/PR/8/34 truncated to 124^{th} amino acid (NS_{124}), which includes an immunogenic F-protein cassette (fragment $F_{248–290}$ and $F_{409–451}$) with or without IgGκ signal peptide (for extracellular delivery).	BALB/c mice (i.n.)	Mice were immunized once with vaccine candidates NS-2AF (contains a 2A site at the N-terminus of the RSV insert), NS-2AsF (NS-2AF, which contains IgGκ), NS-F (shortened NS1), sF-NS (NS-F, which contains IgGκ) at a dose of 6 log10 $TCID_{50}$. On day 28, RSV A2 was infected at a dose of 6 $\log_{10}$ PFU. After challenge, viral load levels in immunized mice were 1.5 $\log_{10}$ PFU/mL and 2.26 $\log_{10}$ PFU/mL lower in the NS-2AsF and NS-2AF groups, respectively, compared with the group of non-immunized mice. In the 2nd experiment, in the sF-NS group, the viral load was 2 log10 PFU/mL less after the shuttle challenge compared with the group of unimmunized mice. In the NS-F group, the reproduction of the challenge virus was comparable to the group of unimmunized mice. Mice in the NS-2AsF group showed low levels of inflammation and minimal lymphocytic infiltration. Minimal changes in lung tissue morphology were observed in the sF-NS group	[46]
			SUBUNIT VACCINES	
DS-Cav1 (NIAID, USA)	Soluble version of F protein in pre-F conformation with antigenic site Ø (poly I:C)	Mice CB6F1/J (♀BALB/cJ + ♂ C57BL/6J (i.m.)	Mice were injected with RSV pre-F in different variants (DS, Cav1, TriC, DS-Cav1) at a dose of 10 μg at an interval of 3 weeks. In all groups, the titer of neutralizing antibodies against RSV in sera was 4 times higher than in the RSV post-F group and 20 times higher than the established protective threshold	[127]
	Soluble version of F protein in pre-F conformation with antigenic site Ø (poly I:C)	Rhesus macaques (i.m.).	Macaques were administered RSV pre-F in different variants (DS, DS-Cav1) at a dose of 50 μg with an interval of 4 weeks. In the DS and DS-Cav1 groups, the neutralizing antibody titer was 5–10 times higher in sera than in the RSV post-F group. By week 8, the neutralizing antibody titer was higher in the DS-Cav1 group than in the DS group and 60-fold higher than in the RSV post-F group	[127]
	Soluble version of F-protein in the pre-F conformation with antigenic site Ø (nanoemulsion, Adju-Phos)	African monkeys	Monkeys were immunized three times with DS-Cav1 (125 μg) in different volumes with different adjuvants. On day 70, animals were infected with RSV at a dose of 2×5.5 $\log_{10}$ PFU. In the DS-Cav1 group (administered in a volume of 0.25 ml), the value of peak RSV titers was 3 $\log_{10}$ and 2 $\log_{10}$ lower in the lungs and nasal cavity than in the control group. A correlation was found between the protective effect in the nasal cavity and IgA stimulation, and protection in the lungs and Fc-mediated antibody activity	[128]

Continuation of the Table 1

Vaccine (developer)	Vaccine composition (adjuvant)	Animal models (method of administration)	Research and results	Source
	Soluble version of F protein in pre-F conformation with antigenic site Ø (Alum, Poly I :C, Poly (IC:LC), MPLA, SAS, Alum + MPLA, SAS + Carbopol, Adjuplex, AddaVax).	CB6F1/J mice (i.m.)	Mice were injected with RSV F DS-Cav1 in combination with different adjuvants 2 times with an interval of 3 weeks at a dose of 10 μg. No side effects were observed in all groups. RSV F DS-Cav1 groups with SAS + Carbopol adjuvants had the highest neutralizing antibody titers, which were 15-fold higher than in the Alum + MPLA and Alum groups, and 5-fold higher than in Poly (I:C). In the groups with MPLA, Alum + MPLA, AddaVax and Adjuplex, IgG1-immune response was recorded in sera. Vaccination in groups with a combination of SAS, SAS + Carbopol, Poly (I:C) and Poly (IC:LC) adjuvants induced IgG1- and IgG2a-immune response. The neutralizing antibody titer in the RSV F DS-Cav1 group without adjuvant was below the protective threshold value	[129]
	Soluble version of F-protein in the pre-F conformation with antigenic site Ø (ISA 71 VG, or ISA 71 VG + Carbopol))	Calves (i.m.)	Calves were injected with bRSV F DS-Cav1 with different adjuvants 2 times at an interval of 4 weeks at a dose of 50 μg. No difference was observed between neutralizing titers in the ISA 71 VG and ISA 71 VG + Carbopol adjuvant groups. This adjuvant combination slightly enhances the immune response in cattle	
DPX-RSV (Immunovaccine, USA)	The extracellular SH domain of the RSV protein SHe (subtype A) incorporated into the DepoVax oil-based platform (Pam3CSK4 and Alum)	CD-1 mice (i.m.)	Mice were injected with SHe at a dose of 25 μL mixed with alum (Alum-SHe) or as part of DPX (DPX-SHe) once or twice (3 weeks apart). Mice immunized with 1 or 2 doses of DPX-SHe had higher IgG antibody titers than the Alum-SHe group and persisted for 20 weeks after immunization. Immunization with Alum-SHe did not result in adverse reactions (decreased activity, cyanosis, decreased body temperature, and hunched posture), unlike the DPX-SHe group. A decrease in complement proteins (C3 and C4) and hypersensitivity reactions were observed in the Alum-SHe group, unlike in the DPX-SHe group	[130]
BARS13 (Advaccine) (Biopharmaceuticals Suzhou Co, China)	BARS13 (or CSA+G consists of CSA - cyclosporine A (can induce Treg) and recombinant G-PSV (shortened)	BALB/c mice (i.m.)	Mice were injected with BARS13 at a dose of 10 μg 2 times with an interval of 2 weeks. After 2 weeks, mice were infected with RSV A2 at a dose of 5×10^7 PFU. In the BARS13 group, the viral load in the lungs after challenge was 10-fold lower than in the FI-RSV and G-protein groups. In the BARS13 group, the dynamics of weight change in mice were comparable to the group of mice that were not infected with RSV. There were no signs of histopathology in the lungs of mice from the BARS13 group. The protective effect in the BARS13 group was explained by the contribution of Treg cells, which were high in BAL and lymph nodes after the challenge trial	[131]
			BARS13 was modified by inclusion in the vaccine formulation and administered with RSV pre-F. Mice were immunized with 10 μg of BARS13 or BARS13 + pre-F 2 times with an interval of 2 weeks. After 2 weeks, mice were infected with RSV at a dose of 2×10^6 PFU. Serum IgG antibody levels were higher in the BARS13 + pre-F group than in the BARS13 group. There were high levels of Treg cells in the BARS13 + pre-F group compared with the FI-RSV and pre-F groups. Neutralizing antibody levels were 6-fold higher in the BARS13 + pre-F group than in the BARS13 group. After MS challenge trial, the pulmonary tissue burden in the BARS13 + pre-F group was 10% lower than in the BARS13 group. The degree of pulmonary inflammation in the BARS13 + pre-F group was minimal	[132]

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End of the Table 1

Vaccine (developer)	Vaccine composition (adjuvant)	Animal models (method of administration)	Research and results	Source
RSVPre-F3/ (GSK3844766A (Glaxo-SmithKline, UK)	Peptide F stabilized in a pre-fusion conformation (AS01)	Rabbits (i.m.)	Rabbits were injected with RSVPre-F3 alone or in combination with Boostrix vaccine three times. In RSVPreF3, RSVPreF3/AS01, RSVPreF3 +*Boostrix* groups, IgG antibody titers to RSVPre-F3 were high. Immunization with RSVPre-F3 had no effect on body weight, vision, skin, appetite and body temperature. Clinical and pathological changes (increase in leukocytes, neutrophils, fibrinogen and C-reactive protein concentration, decrease in albumin) were recorded on the next day after vaccine administration, and the indices returned to normal within 4 weeks. Rabbits were injected with RSVPre-F3 several weeks before mating, during pregnancy and during lactation. Passive transfer of antibodies from immunized females to rabbits was observed	[133]
		Rats (i.m.)	Rats were injected with RSVPre-F3 several weeks before mating, during pregnancy and lactation. In most rats, IgG to RSVPre-F3 was detectable before mating (31/48), during pregnancy (14/31), and during lactation (16/31), and the antibodies were transmitted to the offspring. Immunization of females with RSVPre-F3 had no effect on fertility, pregnancy, lactation, survival, or changes in external signs of visceral and skeletal development in the offspring	
PCB-F (NIAID, USA)	Purified RSV F protein	Cotton rats (i.m.)	Double immunization with protein at doses of 0.05-5.00 μg resulted in the formation of high levels of RSV F-specific antibodies that circulated for 6 months after vaccination, but their neutralizing activity was low. Infection of rats with RSV 3 months after immunization showed a 100-fold decrease in viral load in the lungs in the RSV-F group compared with the control group. At 6 months after immunization, protection against RSV was practically absent – in the FI-RSV and RSV-F groups. In the RSV-F group (5 μg), a vaccine-mediated disease enhancement after RSV challenge comparable to FI-RSV was observed	[134]
VACCINES BASED ON VIRUS LIKE PARTICLES				
V306-SVLP (Virometix, Switzerland)	A mimetic of a peptide that binds to motavizumab V-306 (alum for 15 μg dose of V-306)	BALB/c mice (i.m.)	Mice were injected with V306-SVLP at different doses twice with an interval of 3 weeks. On day 31, mice were infected with RSV at a dose of 10^6 PFU. Immunization with V306-SVLP stimulated the production of neutralizing antibodies to RSV. On day 5 of the challenge trial, the virus was not reproduced in the V306-SVLP (50–300 μg) groups, in contrast to the PBS, FI-RSV, and V306-SVLP (15 μg) groups. No histopathologic changes were observed in the lungs in all V306-SVLP groups, in contrast to FI-RSV groups	[135]
		Rabbits (i.m.)	Rabbits were injected with V306-SVLP at a dose of 140 μg three times with an interval of 28 days. High levels of neutralizing antibodies to 8 different strains of RSV subtypes A and B were observed	
		Cotton rats (i.m.)	Cotton rats were injected with 10D11 anti-V-306 monoclonal antibody preparation and palivizumab at different doses. After 1 day, rats were infected with RSV at a dose of 10^4 PFU. High titers of RSV in the lungs were observed in the PBS group. A dose-dependent reduction in replication was observed in the 10D11 and palivizumab groups. Vaccination with V-306 stimulated the production of antibodies with palivizumab-like properties	
VX-121 (Icosavax, USA)	I53-50 nanoparticle with multiple DS-Cav1 trimers (trimer F stabilized in a “pre-fusion” conformation) on the surface (AddaVax)	BALB/c mice (s.c.)	Mice were injected with DS Cav1-I53-50 or DS Cav1 at a dose of 5 μg three times with an interval of 2 weeks. DS Cav1-I53-50 induced a stronger antigen-specific and neutralizing immune response. Immunization with DS Cav1-I53-50 induced 5.3-fold higher follicular T helper formation in immunized mice than DS Cav1. Since follicular T-helpers control the proliferation of B-lymphocytes in the follicles of lymphoid organs, the number of B-cells in the lymph nodes of mice from the DS Cav1-I53-50 group was higher than in the DS Cav1 group	[136]
	I53-50 nanoparticle with multiple DS-Cav1 trimers on the surface (SWE)	Macaques (Indian) (s.c.)	Macaques were immunized twice with DS-Cav1 (50 μg) and DS-Cav1-I53-50 (96 μg) 28 days apart. DS-Cav1-I53-50 induced 5-fold stronger antigen-specific 25-fold more intense neutralizing antibody production than DS-Cav1. Specific antibodies in macaque sera were found to target the F-protein of RSV in a pre-F conformation	

Note. i.n. — intranasally; i.t. — intratracheally; i.m. — intramuscularly; s.c. — subcutaneously; or. — orally; i.p. — intraperitoneally; scar. — scarification

Table 2. Overview of vaccines in various stages of clinical trials

Vaccine (developer)	Clinical trial phase (target group)	Period	Test No. in the registry	Source
RECOMBINANT VECTOR VACCINES				
RSV001 (PanAd3-RSV and MVA-RSV) (ReiThera, Italy)	Phase I (A.; E.)	2013–2015	NCT01805921	Yes [48, 49]
VXA-RSV f (Vaxart, USA)	Phase I (A.)	2016–2018	NCT02830932	None
ChAd155-RSV GS (GSK, UK)	Phase I (A.) Phase I/II (141) Phase I/II (C.) Phase II (A. ♀)	2016–2017 2016–2021 2018–2022 2015–2018	NCT02491463 NCT02927873 NCT03636906 NCT02360475	Yes [58] Yes [60] None Yes [138]
MVA-BN RSV (Bavarian Nordic BN, Denmark)	Phase I (A. E.) Phase I (A.) Phase II (E.) Phase 2a (A.) Phase III (E.)	2015–2016 2016–2018 2016–2018 2021–2021 2022–2024	NCT02419391 NCT02864628 NCT02873286 NCT04752644 NCT05238025	Yes [46] Yes [46] Yes [47] Yes [139] Yes [140]
SeV/RSV (NIAID, USA)	Phase I (A.)	2018–2019	NCT03473002	Yes [65]
Ad26.RSV.preF (Janssen Vaccines & Prevention B.V., Netherlands)	Phase I (A.; E.) Phase I (C.) Phase I/2a (A.; C)	2016–2019 2022 2017–2022	NCT02926430 NCT03606512 NCT03303625	Yes [141] Yes [57] Yes [57]
Ad26.RSV.preF (Janssen Vaccines & Prevention B.V., Netherlands)	Phase II (E.) Phase II (A.; E.)	2017–2021 2017–2021	NCT03339713 NCT03334695	Yes [56] Yes [142]
rBCG-N-hRSV (Catholic University of Chile, Chile)	Phase I (A. ♂)	2017–2018	NCT03213405	Yes [143]
RSV/Flu-01E (A.A. Smorodintsev Research Institute of Influenza, Russia)	Phase I (A.; E.) Phase II (E.)	2022–2023 2023–2024	NCT05970744	None None
Ad26.RSV.FA2, Ad35.RSV.FA2 (Crucell Holland BV, Netherlands)	Phase I (A.) Phase I (A.)	2015–2016 2015–2016	NCT02561871 NCT02440035	None None
LIVE ATTENUATED VACCINES				
MV-012-968 (Meissa, USA)	Phase I (A.) Phase I (141)	2020–2020 2021–2023	NCT04227210 NCT04909021	None None
RSV MEDI ΔM2-2 (NIAID/NIH, MedImmune (AstraZeneca), USA)	Phase I (141)	2011–2015	NCT01459198	Yes [75]
RSV cps2 (NIAID/NIH, MedImmune (AstraZeneca), USA)	Phase I (141) Phase I (141)	2013–2015 2013–2016	NCT01968083 NCT01852266	Yes [73]
RSV LID ΔM2-2, NIAID (Sanofi Pasteur, France)	Phase I (141) Phase I (141)	2014–2016 2014–2015	NCT02040831 NCT02237209	[76] [76]
RSV LID/ΔM2-2/1030s (NIAID, USA, Sanofi Pasteur, France)	Phase I (141) Phase I (141) Phase I (141)	2020–2023 2016–2018 2015–2017	NCT04520659 NCT02952339 NCT02794870	None Yes [78] Yes [78]
RSV LID cp ΔM2-2 (NIAID, USA)	Phase I (141) Phase I (141)	2015–2018 2015–2018	NCT02890381 NCT02948127	Yes [144] Yes [144]
RSV D46/NS2/N/ΔM2-2-HindIII (NIAID, USA)	Phase I (141) Phase I (141)	2017–2018 2017–2018	NCT03102034 NCT03099291	Yes [145] Yes [145]
RSV ΔNS2/Δ1313/I1314L (NIAID, USA)	Phase I (141) Phase I (141)	2013–2018 2017–2022	NCT01893554 NCT03227029	Yes [82] Yes [146]
RSV 6120/ΔNS2/1030s (NIAID, USA)	Phase I (141)	2017–2022	NCT03387137	None
RSV 6120/ΔNS1 and RSV 6120/F1/G2/ΔNS1	Phase I (141)	2018–2023	NCT03596801	None
RSV ΔNS2/Δ1313/I1314L; RSV 6120/ΔNS2/1030s; RSV 276, (NIAID, USA)	Phase I and II (141)	2019–2022	NCT03916185	None
RSV ΔNS2/Δ1313/I1314L; RSV 276 (NIAID, USA)	Phase I and II (141)	2017–2020	NCT03422237	Yes [146]

Continuation of the Table 2

Vaccine (developer)	Clinical trial phase (target group)	Period	Test No. in the registry	Source
RSV D46 cpΔM2-2, (NIAID, USA)	Phase I (141)	2015–2019	NCT02601612	None
MEDI-559, (MedImmune) (AstraZeneca, USA)	Phase I and II (141)	2008–2011	NCT00767416	Yes [72]
SUBUNIT RECOMBINANT VACCINES				
GSK3888550A RSVPreF3 (GSK, UK)	Phase I (A. ♀) Phase II (A. ♀) Phase II (A. ♀) Phase III (A. ♀)	2018–2019 2019–2022 2019–2020 2020–2023	NCT03674177 NCT04126213 NCT04138056 NCT04605159	Yes [147] Yes [148] None None
GSK3844766A RSVPreF3, Arexvy (GSK, UK)	Phase I/II (A., E) Phase I (E.) Phase II (E.) Phase III (E.) Phase III (A., E) Phase III (E.) Phase III (E.) Phase III (E.) Phase III (E.) Phase III (E.) Phase III (E.)	2019–2021 2019–2022 2020–2021 2021–2024 2023–present 2021–present 2021–2022 2021–2022 2022–2023 2022–2023 2023–2024 2023–2024	NCT03814590 NCT04090658 NCT04657198 NCT04886596 NCT05921903 NCT04732871 NCT04841577 NCT05059301 NCT05559476 NCT05568797 NCT05590403 NCT05879107	Yes [91] Yes [90] Yes [92] Yes [94, 149] None Yes [93] Yes [150] Yes [151] Yes [152] Yes [153] Yes [154] None
RSVPreF3 (GSK, UK)	Phase I (A.)	2014–2018	NCT02298179	Yes [155]
DPX-RSV(A) (Immunovaccine, USA and Dalhousie University, Canada)	Phase I (A.)	2015–2017	NCT02472548	Yes [89]
RSV F DS-Cav1 (NIH/ NIAID/VRC, USA)	Phase I (A.)	2017–2020	NCT03049488	Yes [86]
RSVpreF Abrysvo (Pfizer, USA)	Phase II (A. ♀) Phase I/II (A., E) Phase 2b (A. ♀) Phase II (E.) Phase III (C., A. ♀) Phase Ib (E.) Phase II (A. ♀) Phase II (A.) Phase III (E.) Phase III (A.) Phase III (E.) Phase III (A., E.)	2019–2019 2018–2021 2019–2022 2018–2021 2020–present 2023–2023 2019–2019 2020–2021 2021–present 2021–2022 2022–2022 2023–2024	NCT04071158 NCT03529773 NCT04032093 NCT03572062 NCT04424316 NCT05788237 NCT04071158 NCT04785612 NCT05035212 NCT05096208 NCT05301322 NCT05842967	Yes [156] Yes [95, 157] Yes [158] Yes [159] Yes [160] None Yes [156] Yes [161] None Yes [162] Yes [163] None
RSV F vaccine (RSVpreF) (Pfizer, USA)	Phase I/II (E.)	2018–2020	NCT03572062	Yes [159]
BARS13, (Advaccine) (Suzhou) Biopharmaceuticals Co., Ltd, China)	Phase I (A.) Phase II (A., E.) Phase II (A.)	2019–2019 2020–2023 2018–2019	NCT04851977 NCT04681833 ACTRN12618000948291	Yes [164] None None
VACCINES BASED ON VIRUS LIKE PARTICLES				
V306-SVLP (Virometix AG, Switzerland)	Phase I (A. ♀)	2020–2022	NCT04519073	Yes [105]
SynGEM (Mucosis B.V., Netherlands)	Phase I (A. ♀♂)	2016–2017	NCT02958540	Yes [104]
RSV F (ResVax) (Novavax, USA)	Phase I (A.) Phase II (A. ♀) Phase II (A. ♀) Phase II (A. ♀) Phase II (A. ♀)	2010–2011 2012–2013 2013–2014 2014–2016 2015–2020	NCT01290419 NCT01704365 NCT01960686 NCT02247726 NCT02624947	Yes [165] Yes [166] Yes [167] Yes [100] Yes [102]
RSV F nanoparticle, (Novavax, USA)	Phase I (E.) Phase II (E.) Phase II (E.) Phase III (E.) Phase II (E.) Phase I (141)	2012–2014 2014–2016 2015–2016 2015–2016 2017–2018 2014–2016	NCT01709019 NCT02266628 NCT02593071 NCT02608502 NCT03026348 NCT02296463	Yes [168] None None None None None

End of the Table 2

Vaccine (developer)	Clinical trial phase (target group)	Period	Test No. in the registry	Source
VACCINES BASED ON MESSENGER RNA				
mRNA-1345	Phase I (C.) Phase II (C., A., E.) Phase II (A. ♀) Phase II-III (E.)	2023–present 2020–2024 2023–present 2021–present	NCT05743881 NCT04528719 NCT06143046 NCT05127434	None Yes [35] None Yes [41, 169]
RSV mRNA LNP CL-0059; LNP CL-0137	Phase I-II (A., E.)	2022–present	NCT05639894	None

Note. C. — Children; E. — Elderly (lower threshold 50, 55 or 60 years); A. — Adults (from 18 years, upper threshold varies).

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