1
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CONTENTS

SCIENCE AND PRACTICE

ORIGINAL RESEARCHES

REVIEWS

ANNIVERSARIES

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SCIENCE AND PRACTICE

Original Study Article
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Preparation of conjugates based on colloidal gold nanoparticles for application in rapid detection of antibodies to hepatitis E virus

Galina I. Alatortseva✉, Lyubov N. Nesterenko, Irina I. Amiantova, Lyudmila N. Pritvorova, Vera V. Dotsenko, Vitaly V. Zverev, Oksana A. Svitich

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Abstract

Relevance. Hepatitis E virus (HEV) is a common cause of viral hepatitis not only in areas with low levels of water supply and hygiene, but also in industrialized countries. Rapid tests development for the infection seromarkers detection in the absence of special equipment and trained staff remains the most important problem in improving the diagnosis of hepatitis E.

Aim. To produce conjugates of recombinant ORF2 antigen of HEV genotype 3 with gold nanoparticles (GNP) of varied sizes and to evaluate their applicability in the immunoassay for the detection of antibodies to HEV.

Materials and methods. Specific polyclonal and monoclonal antibodies, recombinant antigen ORF2 of hepatitis E virus genotype 3, blood serum samples of people diagnosed with acute hepatitis. Synthesis of GNPs and their conjugates with recombinant antigen, enzyme immunoassay, dot immunoassay, immunochromatographic analysis, transmission electron microscopy.

Results. Three samples of colloidal GNP were synthesized using citrate method with varied concentrations of reducing agent and were subsequently used for preparation of conjugates with recombinant antigen ORF2 of HEV genotype 3. Immunoreactivity of these conjugates was confirmed by dot-immunoassay with blood serum samples containing specific IgG. A conjugate based on a 41 nm GNP was chosen for use in immunochromatographic analysis (ICA). Optimal conditions for preparation of a multi-membrane composite, including formation of analytical and control lines and the conjugate area were identified, and test strips were developed. The obtained conjugate was tested by ICA using blood serum samples which had been subjected to preliminarily characterization by the content of the IgG antibody to HEV. High immunoreactivity of the conjugate was demonstrated. Antibodies to the virus were identified in 100% of the examined (n = 17) IgG-positive serum samples, while in negative samples (n = 17) they were absent.

Conclusion. The results demonstrated effectiveness of the obtained immunoreagents (recombinant antigen, antibodies, conjugate) for use in test-systems for rapid diagnosis of hepatitis E.

Keywords: *immunochromatographic assay, colloidal gold nanoparticles, recombinant antigen, hepatitis E virus, protein ORF2, IgG*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. 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. Mechnikov Research Institute of Vaccine and Sera (protocol No. 4, February 22, 2023).

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Оригинальное исследование
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Получение конъюгатов на основе наночастиц коллоидного золота для быстрого выявления антител к вирусу гепатита Е

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Аннотация

Актуальность. Вирус гепатита Е (ВГЕ) — частая причина вирусных гепатитов не только на территориях с низким уровнем водоснабжения и гигиены, но и в промышленно развитых странах. Важнейшей проблемой совершенствования системы диагностики гепатита Е остаётся разработка тестов быстрого определения серомаркеров инфекции для применения в условиях отсутствия специального оборудования и обученного персонала.

Цель работы — получение конъюгатов наночастиц коллоидного золота (НЧЗ) нескольких размеров с рекомбинантным антигеном ORF2 ВГЕ 3 генотипа и оценка возможности их применения в иммуноанализе для выявления антител к ВГЕ.

Материалы и методы. Исследовали специфические поликлональные и моноклональные антитела, рекомбинантный антиген ORF2 ВГЕ 3 генотипа, образцы сывороток крови людей с диагнозом острого гепатита. Осуществляли синтез НЧЗ и их конъюгатов с рекомбинантным антигеном, иммуноферментный анализ, дот-иммуноанализ, иммунохроматографический анализ, использовали метод просвечивающей электронной микроскопии.

Результаты. Цитратным методом с использованием различных концентраций восстановителя синтезированы 3 препарата НЧЗ. На их основе получены конъюгаты с рекомбинантным антигеном ORF2 ВГЕ 3 генотипа, иммунореактивность которых подтверждена методом дот-иммуноанализа с образцами сывороток крови, содержащими специфические иммуноглобулины класса G (IgG). Для применения в иммунохроматографическом анализе отобран конъюгат на основе НЧЗ диаметром 41 нм. Отработаны условия получения мультимембранного композита, включая формирование аналитической и контрольной линий и зоны конъюгата, изготовлены тест-полоски и проведены испытания полученного конъюгата методом иммунохроматографического анализа с образцами сывороток крови, предварительно охарактеризованными по содержанию IgG-антител к ВГЕ. Показана высокая иммунореактивность полученного конъюгата: антитела к вирусу выявлены в 100% обследованных IgG-положительных проб сывороток (n = 17) и не обнаружены в отрицательных пробах (n = 17).

Выводы. Получены иммунореагенты (рекомбинантный антиген, антитела, конъюгат), которые могут быть использованы при создании тест-систем для экспресс-диагностики ВГЕ.

Ключевые слова: *иммунохроматографический анализ, наночастицы коллоидного золота, рекомбинантный антиген, вирус гепатита Е, белок ORF2, иммуноглобулины класса G*

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Introduction

Hepatitis E virus (HEV) is a frequent cause of viral hepatitis not only in areas with low water quality and supply, but also in industrialized countries [1]. Specific IgM and IgG antibodies in serum or plasma of patients serve as serological markers of HEV infection. The European Association for the Study of the Liver recommends the following criteria for the clinical diagnosis of acute hepatitis E cases: the simultaneous presence of HEV RNA and specific IgM and/or IgG antibodies (anti-HEV AB) in the analyzed samples, increase of anti-HEV IgG AB titers in the presence of anti-HEV IgM AB, and the detection of HEV antigen (HEV AG). The diagnosis of chronic hepatitis E is confirmed by the detection of HEV RNA and/or HEV AG for more than 3 months. The past infection is indicated by the presence of IgG anti-HEV AB in the samples [2]. The main method for the diagnosis of acute and chronic hepatitis E is the polymerase chain reaction, which makes it possible to detect viral RNA at the earliest stages of the disease and in the absence of AB in chronic HEV patients with immunodeficiency. In some cases, serological methods for diagnosing hepatitis E are more reliable and specific [3], and in most cases have no alternative due to the short period of viremia in HEV infection [4]. Each method of hepatitis E serological diagnostics has limitations that make it difficult to accurately determine the stage of the disease. Due to problems associated with virus cultivation, kits for hepatitis E serological diagnostics most commonly utilize recombinant antigens, or less frequently, synthetic peptides.

The problem of shortening HEV test turnaround time is solved by using rapid tests based on the immunochromatography assay (ICA) for detect HEV-specific IgG, IgM, total ABs and HEV AG using colloidal gold conjugated with recombinant AG or AB to viral proteins and/or o human or animal immunoglobulins. Along with quick results (15 minutes), the great advantage of the method is the possibility of testing at the patient's bedside in the absence of special equipment and trained personnel. The ICA test systems for the detection of HEV AG and anti-HEV AB developed and produced by foreign companies showed good reproducibility with sufficiently high sensitivity, specificity and good consistency with the results obtained using classical enzyme immunoassay (ELISA). A number of comparative studies have shown that ICA tests for detecting anti-HAV IgM AB manufactured in China ("IgM antibody to hepatitis E virus (HEV-IgM) rapid test", "Wantai", cat No. WJ-15) and Singapore ("MP Diagnostics ASSURE HEV IgM Rapid Test", "MP Biomedicals Asia Pacific Pte. Ltd.", cat. No. 43160-020), have even higher sensitivity than some traditional ELISA tests [5]. Canadian companies Citest Diagnostics Inc. and BiogateLabs produce ICA kits for simultaneous detection of IgG and IgM AB to HEV (cat No. IHE-302, cat No. RT-EV1112-C-1, respectively) in serum, plasma and whole blood. In the Russian Federation and the countries of the former Soviet Union, there is no manufacturing of tests for the rapid detection of HEV serological markers by the ICA method. Furthermore, there are no reports of ongoing research in this area.

Besides specific antigens or antibodies, the key components of such test systems are conjugates of colloidal nanoparticles with biological macromolecules which allow visual or digital recording of reaction results [6–9]. The unique optical properties of gold nanoparticles (GNP) provide visible surface plasmon resonance (SPR) peaks in synthesized GNP in the wavelength range of about 520 nm, depending on the size of the synthesized GNP [10]. The advantages of using GNP-based conjugates in rapid diagnostic tests combine the possibility of results visualization due to high molar absorption coefficients [10, 11] with their ability to fully restore properties after sorption on the membrane and subsequent drying [12, 13] in the analysis process. Diagnostic efficiency of ICA-tests is determined by the ability of the GNP conjugate to interact specifically with target molecules. It is related to physical and chemical properties of the GNP, in particular to their size and shape. It is also determined by antigenic specificity of the proteins adsorbed on them, which perform an additional function of secondary stabilization of colloidal particles in the conjugates.

All test systems for the detection of anti- HEV AB are based on the use of the capsid protein ORF2, which contains diagnostically significant epitopes, as the antigenic basis. Previously, we have obtained [14] the recombinant capsid protein ORF2 of HEV genotype 3 (recAg), which has the widest geographical distribution in the world [15, 16] and is dominant in Russia [17]. This recAg has been selected as the basis for the subsequent development of the rapid test for the simultaneous detection of anti-HEV IgG and IgM AB.

The objective of the present study was to synthesise conjugates of recAg with GNPs of varying sizes and to assess their potential application in an immunoassay for the detection of antibodies to HEV.

Materials and methods

Immunoreagents

Polyclonal goat AB against human IgG (GAHIss) («Imtek») (hereafter referred as AB), recAg (I.I. Mechnikov NIIVS) [18]; polyclonal rabbit IgG AB to recAg (hereafter referred PAB) obtained by immunizing rabbits and subsequent affinity purification (I.I. Mechnikov NIIVS); a conjugate of mouse monoclonal AB to recAg (hereafter referred MAB) with horseradish peroxidase (I.I. Mechnikov NIIVS) obtained by periodate oxidation method [19].

Blood sera of individuals diagnosed with acute viral hepatitis were provided by the National Institute of Public Health of the Ministry of Health of the

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Kyrgyz Republic and by the Belarusian State Medical University. According to the test results obtained with the DS-ELISA-ANTI-HEV-G reagent kit («Diagnostic systems») [20, 21] and with the developed at the I.I. Mechnikov NIIVS confirmatory test for the determination of anti-HEV IgG AB by the line blot method [22]; sera contained (n = 17) and did not contain (n = 17) anti-HEV IgG AB.

The study was conducted with the informed consent of the patients or their legal representatives. 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. Mechnikov Research Institute of Vaccine and Sera (protocol No. 4, February 22, 2023).

GNP colloidal solutions

GNPs with desired particle sizes were prepared by citrate method using tetrachloroauric (III) acid trihydrate $HAuCl_4 \times 3H_2O$ («Aldrich») as a precursor [11–13]. Taking into account that the average particle diameter reduces with an increase in citrate concentration in the reaction mixture [11], varying amounts of reducing agent allowed to obtain GNP samples with different particle sizes. One mL of a 1% $HAuCl_4 \times 3H_2O$ solution was added to 99 mL of deionized water; the solution was brought to boil with stirring, then various volumes of 1% aqueous solution of sodium citrate $C_6H_5Na_3O_7 \times 2H_2O$ («Merck») were added.

The solution was boiled with a reflux condenser for 20 min under stirring, then cooled to room temperature. Absorption spectra within the wavelength range from 400 to 700 nm were measured using a Multiskan GO tablet (flatbed) spectrophotometer («Thermo Scientific»). GNP structures were studied using «Jeol JEM ARM200F» atomic resolution scanning transmission electron microscope («HAADF-STEM») equipped with an energy dispersion analysis (EDX) system based on a «GIF Quantum» spectrometer with a «Centurio EDX» detector with 0.08 nm resolution at accelerating voltage of 200 kV in STEM mode («Jeol») [23, 24]. TEM samples for the study were prepared by depositing a sample of GNP solution pre-diluted to A_{520} = 1.0 absorbance onto carbon film-coated copper meshes.

Synthesis of conjugates

Optimal conditions for preparation of GNP conjugates with recAg were selected according to the described recommendations [25, 26]. Wells 1–11 of a 96-well polystyrene plate («Greiner») were filled with 10 µL of recAg solutions with concentrations varying from 1 to 50 µg/mL in phosphate buffer (PBS). Then, 100 µL of GNP solutions with pH from 5.5 to 9.0 were introduced into the wells A-H of the horizontal rows of the plate so that each column corresponded to a certain concentration of recAg, and each row corresponded to a pH value of the solution. After 15 min, 20 µL of 10% sodium chloride solution was added to all wells, and after further 10 minutes optical absorption of the solutions was measured at wavelengths of 520 and 580 nm (A_{520}, A_{580}). As absorbance of the solution at 580 nm increases with the loss of stability in the colloidal system, the stabilizing concentration of recAg was estimated by the difference «$A_{520} - A_{580}$» [25].

Conjugates of GNP with recAg were obtained by dropwise addition of 10 mL of GNP solution with pH 8.0 to the recAg solution until recAg concentration reached 25 µg/mL for GNP-1, 30 µg/mL for GNP-2, and 40 µg/mL for GNP-3. Subsequently, the mixtures were shaken at room temperature for 30 min using a Multi-Reax device («Heildorf»); then a 10% solution of bovine serum albumin (BSA) («Sigma») was added to a final concentration of 0.25%. Suspensions were incubated for 15 min under the same conditions and following that, centrifuged for half an hour at 4ºC at 8–11 × 10^3 rpm, depending on the size of the GNPs. The resultant precipitate was suspended in 1 mL of PBS with 0.1% BSA, 10% sucrose and 0.01% sodium azide, and absorption spectra were recorded as described earlier. The obtained conjugates of GNP with recAg were stored at 4ºC.

The result of recAg seeding on the surface of GNP was controlled by enzyme-linked immunosorbent assay (ELISA) [25]. PAB solution with a concentration of 5 µg/mL in 0.1 M carbonate-bicarbonate buffer pH 9.6 was adsorbed in wells of a plate. The plates were blocked with 0.02 M phosphate-salt buffer pH 7.2 with 0.05% Tween-20 (FSB-T), 5% sucrose, 0.09% sodium caseinate. The wells were filled with 100 µL of supernatants obtained after centrifugation of the GNP conjugates with recAg. Horseradish peroxidase-labeled MAB was used as conjugate in ELISA; 0.5 mM 3,3',5,5'-tetramethylbenzidine solution was used as chromogen. Calibration samples were produced via serial dilutions of recAg (5, 2.5, 1.25, 0.625, 0.31 and 0.15 µg/mL) in the supernatant liquid obtained after centrifugation of colloidal gold solution. recAg concentration in the samples of interest was determined using a calibration plot of optical density values at 450 nm wavelength *vs* recAg concentration in calibration solutions.

Dot immunoassay reaction

To perform dot immunoassay in sandwich format, AB in the amounts of 0.1; 0.05; 0.025 and 0.0125 µg were sorbed onto 13 mm nitrocellulose membrane discs (0.45 µm). As a negative control, 0.1 µg of BSA solution in 0.02 M phosphate-salt buffer pH 7.2 (PSB) was applied. Free areas of the membranes were blocked with 0.09% sodium caseinate solution in PBS. The membranes were incubated with serum containing anti-HEV IgG AB for 1 hour in a thermo-shaker («Eppendorf») at 37ºC, followed by 30 min with solutions of GNP conju-

gates with recAg (A_{520} = 0.5 o.u.) at room temperature. Staining intensity of experimental and control spots on the membranes was evaluated visually.

Immunochromatographic analysis

AB solutions were applied to membranes for immunochromatography using an automatic IsoFlow dispenser («Imagene Technology») at a rate of 0.2 μL per 1 mm of membrane length. PAB was used in a control zone and AB — in analytical (test) zone. A conjugate of GNP with recAg was sorbed on the glass fiber membrane at the optimal concentration of A_{520} = 4 optical density (OD) units, which was determined by changing the dilutions of conjugates in the range of A_{520} values from 2 to 8 OD units with three positive serum samples tested for each dilution.

Assembly of a multimembrane composite and cutting of test strips was performed using an MTB 300 hand laminator («Kinbio Tech Co. Ltd.») and a guillotine type «Cutter ZQ2002» («Kinbio Tech Co. Ltd.»). When performing the ICA, 20 μL of blood serum was applied to the sampling area of an immunochromatographic test strip, then the edge of the strip was dipped into 80 μL of PBS; after 2–3 minutes the test strip was places horizontally and after further 15 minutes the result was evaluated. Result was considered positive when any intensity of staining of the control and analytical zones was observed, and as negative when there was no staining of the analytical zone. In the absence of staining of the control zone, the result was not taken into account.

For digital evaluation of the assay results, the test strips were scanned using a Gel DocTM XR device («Bio-Rad») with Image LabTM Software. Positivity coefficient (PC) was calculated as a ratio of staining intensity of an analytical area to background staining of a test strip.

Statistical data processing was performed using R and «Excel 2013» software.

Results

Methods for obtaining colloidal gold are well described in the scientific literature [11, 26–29]. In present work, we used a modified Frens citrate method for producing GNPs with particle diameters in the range from 15 to 40 nm, which is optimal for effective antibody sorption. Specifically, GNP samples with average particle sizes of 16 nm (GNP-1), 25 nm (GNP-2) and 41 nm (GNP-3) were synthesized. Synthesis of GNP was carried out via addition of 1% sodium citrate solution in volumes of of 1 mL, 0.75 mL and 0.5 mL to 50 mL of 0.01% tetrachloroauric(III) acid trihydrate solution. Mixing was conducted using magnetic stirrer at 300 rpm and temperature of 100ºC. Once solution color changed to red, stirring speed was increased to 500 rpm [27]. While the excess of citrate anions on the surface of nanoparticles stabilizes the gold sol, it is not preventing further interactions of GNPs with protein macromolecules during preparation of immunometric conjugates. It was demonstrated that the obtained colloidal solutions maintained their stable characteristic during storage at 4ºC in the absence of light for at least six months.

An important characteristic of the GNP is the position and configuration of the SPR band. Absorption spectra for all obtained GNP samples were recorded and results are presented in **Fig. 1.** As it can be seen in the figure, an increase in particle size led to an increase in color intensity of the solutions and a shift of absorption maximum to a region of longer wavelengths (518 nm, 522 nm and 528 nm for GNP-1, GNP-2 and GNP-3, respectively).

The average size and shape of nanoparticles were assessed using transmission electron microscopy (TEM) (**Fig. 2**). In high-resolution images, the relative homogeneity of particles in shape and size in each group is evident. The smaller particles have a predominantly spherical shape; the presence of nanoparticles deviating from the regular spherical shape is observed in the preparation of the GNP-3 sample, which is consistent with the classification of the structure of nanoparticles depending on their size [30].

The average particle sizes determined using TEM were 16 for GNP-1, 25 for GNP-2 and 41 nm for GNP-3 samples, respectively, which corresponded well with the values set by the synthesis conditions, and indicated that the method used was highly reproducible. The present study was focused on obtaining GNPs of three sizes in order to compare results obtained herein with the existing data [31–33].

Statistical difference between three groups of GNPs was assessed by constructing dispersion plots using the R software package Box Plot function (**Fig. 3**). The degree of dispersion was found to be highest for the

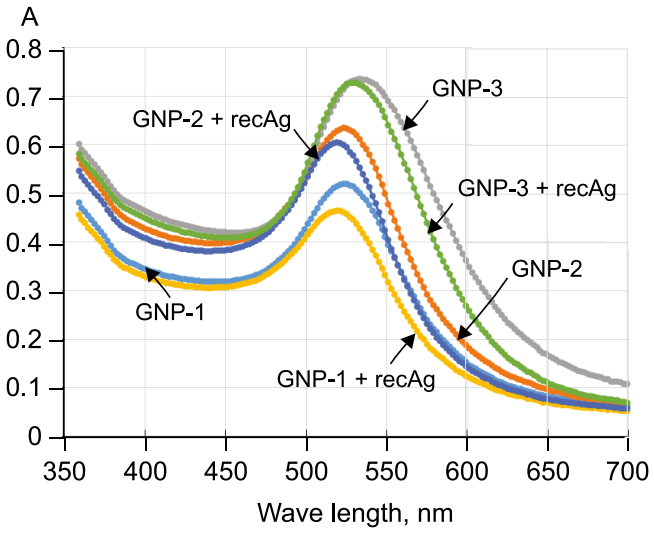

Fig. 1. Absorption spectra of GNP-1, GNP-2, GNP-3 solutions and their conjugates with recAg.

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ORIGINAL RESEARCHES

GNP-3 sample with the largest particle sizes ranging from 30 to 56 nm. On contrary, dispersion was minimal (14 to 18 nm) for GNP-1 and the size range for GNP-2 was 20 to 37 nm. Quantitative data was confirmed to be normally distributed by using Shapiro–Wilk criterion ($p \leq 0.05$), and together with the position of the medians for GNP-1, GNP-2 and GNP-3 a conclusion was drawn that for all GNP samples, the size distribution is close to normal.

Most common method for production of GNP-based markers for use in immunochemical reactions is based on non-covalent (adsorption) conjugation of proteins on the surface of nanoparticles. This approach is technologically simple and preserves the native structure and properties of macromolecules in resulting complexes, in particular their immunoreactivity [27, 34, 35]. In this case, non-covalent bonds formed as a result of events such as electrostatic interactions between negatively charged nanoparticles and positively charged sites on protein surface, as well as hydrophobic attraction between protein and metal surface, donor-acceptor interactions between metal and electrons of nitrogen and sulfur atoms in protein composition [26]. It is also confirmed, that in the process of formation of GNP complexes with proteins a stabilizing protein shell is formed around them [35, 36]. In the present work, passive immobilization technique was also employed to obtain conjugates of three GNP samples with recAg.

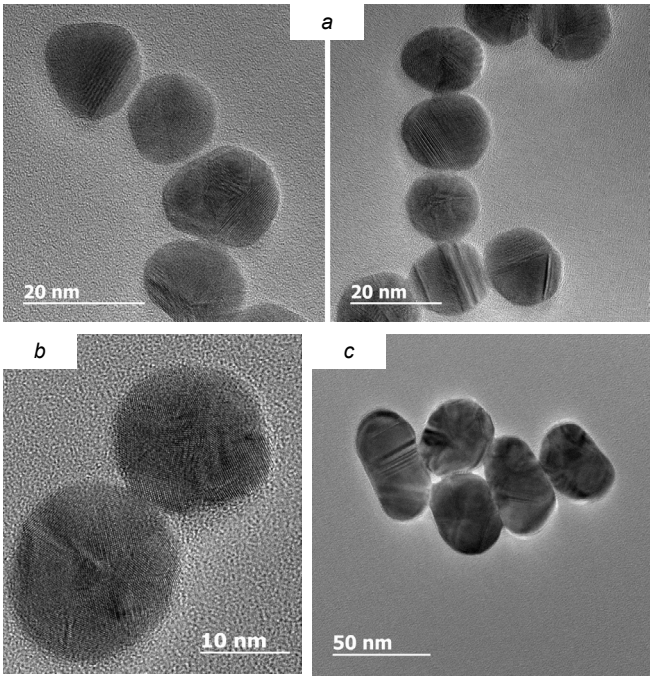

Fig. 2. Photographs of GNP obtained by means of TEM.
a — GNP-1; *b* — GNP-2; *c* — GNP-3.

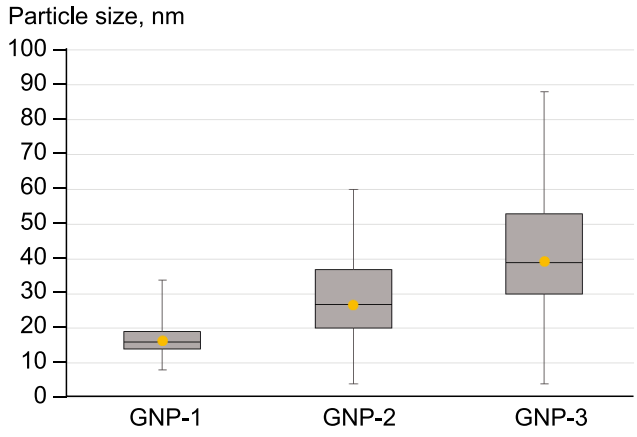

Fig. 3. Size distribution diagrams for GNP-1, GNP-2, and GNP-3 (Box Plot functions).

In order to obtain a stable sol, conditions of recAg adsorption on the surface of GNP were optimized taking into consideration physicochemical properties of the protein important for stabilization of colloidal system, such as molecular weight, solubility, isoelectric point (pI = 5.43) and its concentration. Stabilizing concentration of recAg was determined in pH range from 5.5 to 9.0 for all three sizes of GNP. **Figure 4** demonstrates the dependence of indicator «A_{520} –A_{580}» on protein concentration at different pH values for GNP-2.

The addition of sodium chloride to GNP solutions with low recAg concentrations led to aggregation of protein-unstabilized GNPs, resulting in an increase of absorption at λ = 580 nm. At the same time, a solution color change from red to grey-blue was observed. Reaching stabilizing concentrations of recAg led to an increase and further plateauing of the «A_{520} – A_{580}» index value, as well as sol stability and solution color in the presence of electrolyte.

It was further determined that the system was less stable in the acidic medium. A possible reason for that might be lower stability of sols in solutions with pH close to isoelectric point of recAg (5.36). At pH ≥ 7, the difference between the obtained curves was negligible, therefore conjugation of GNP with recAg was carried out at pH 8. Stabilizing concentrations of recAg were determined by the values at which the curves reached a plateau (20 μg/mL for GNP-1, 25 μg/mL for GNP-2, and 30 μg/mL for GNP-3). Concentration of recAg, required for stabilization of the colloidal system increased with an increase in the GNP particle size. Subsequently, recAg concentrations higher than the stabilizing concentration by 10–15% were used to obtain conjugates, as it had been recommended elsewhere [33].

To investigate interaction of protein with GNP in the samples of conjugates, the absorption spectra of GNPs and their conjugates with reqAg were compared. Binding of nanoparticles from the solution to protein molecules immobilized on the gold surface results in a noticeable shift in the SPR peak. The magnitude of this shift is determined by the particle size and can be used as a parameter for assessing binding activity of the adsorbed protein [35]. A method for determining composition of GNP conjugates with proteins that is based on evaluation of intrinsic fluorescence of unbound protein

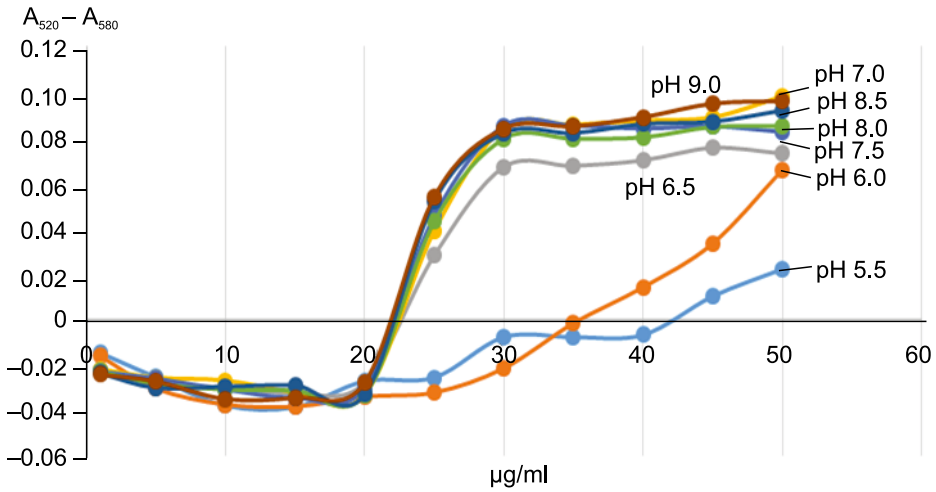

Fig. 4. Dependence between the optical absorption difference $A_{520} – A_{580}$ of the GNP-2 solution (25 nm) and the concentration of recAg at different pH values of the solution.

molecules is described in the literature [36]. Depending on the size of GNPs, the orientation of proteins on the surface of GNP may vary, which in turn, can lead to different biological activities of conjugates. Thus, the nanoparticles size can affect reactivity of adsorbed proteins. In the present study, the absorption spectra of solutions of GNP conjugates with recAg demonstrated a shift of the absorption maximum to the long-wavelength region, which was more pronounced for smaller GNPs as can be seen in Fig. 1. The efficiency of protein macromolecules' binding to the surface of the GNPs was evaluated by measuring the amount of residual protein remaining in the supernatants after conjugate samples' centrifugation [25]. Concentrations of residual protein in the supernatant liquids measured after two cycles of centrifugation averaged 2.0 and 1.0 μg/mL for GNP-1, 3.0 and 0.8 μg/mL for GNP-2, and 4.6 and 2.2 μg/mL for GNP-3. The amount of bounded recAg was 88% for GNP-1, 87% for GNP-2, 84% for GNP-3, indicating a high efficiency of the process.

The immunoreactivity of conjugates was confirmed by dot immunoassay with human serum samples containing IgG antibodies to HEV. Staining of spots with sorbed AB was observed on all membranes, with the intensity of staining decreasing slightly with serial dilutions of sorbed AB (0.1; 0.05; 0.025 and 0.0125 μg). These results confirmed reactivity of all of the obtained conjugates as well as the possibility of their application using a nitrocellulose membrane as a solid phase (**Fig. 5**). The specificity of conjugates was confirmed by the absence of staining in negative control spots (BSA).

Among the tested samples, GNP-3 conjugate with recAg demonstrated the most stable bright staining of the reaction zone in dot immunoassay. Thus, it was selected for conducting model experiments on ICA on both positive (n = 17) and negative (n = 17) blood serum samples which had been pre-tested in ELISA. It is also worth noting that ELISA commonly has a higher sensitivity compared to ICA. The «DS-IFA-ANTI-NEV-G» reagent kit («Diagnostic Systems»), one of the best kits for the diagnostics of HEV worldwide [37], was chosen as the «gold» standard.

Further, the conditions for formation of control and analytical zones of the test strip have been selected. This included varying parameters such as concentrations of sorbed AB and PAB, the speed of their application and conditions for drying the membranes. Based on the obtained results, a dilution of the conjugate with A_{520} = 4 was chosen, as it demonstrated sufficiently bright coloring of the test and control bands and there was no background staining of the membrane in reactions with blood serum samples. Increasing the AB concentration in the analytical zone (0.25, 0.5, 1 mg/mL) and PAB concentration in the control zone (0.5, 1, 2 mg/mL) resulted in an increase of staining intensity. At AB concentration of 0.5 mg/mL and PAB concentration of 2 mg/mL, the intensity of the control and test bands was sufficient for visual detection, so these concentrations were selected as optimal. It should also be mentioned that with a significant increase in the concentration of sorbed antibodies, there is a possibility of their partial desorption and migration with the flow across the membrane [26, 28].

The analytical characteristics of the obtained immunochromatographic test strips were evaluated in reactions with blood serum samples containing (n = 17) and not containing (n = 17) IgG antibodies to HEV (Fig. 5). The study demonstrated 100% sensitivity and 100% specificity of the test strips on the 34 samples that were analyzed. All results were divided into three groups according to the strength of staining of the analytical zone: «1+» (7.8–15.1; Me = 9.6); «2+» (26.7–35.1; Me = 31.0); «3+» (58.3–77.7; Me = 62.8). For the majority (73%) of the positive samples staining intensity was «2+» and «3+» making it applicable for visual detection.

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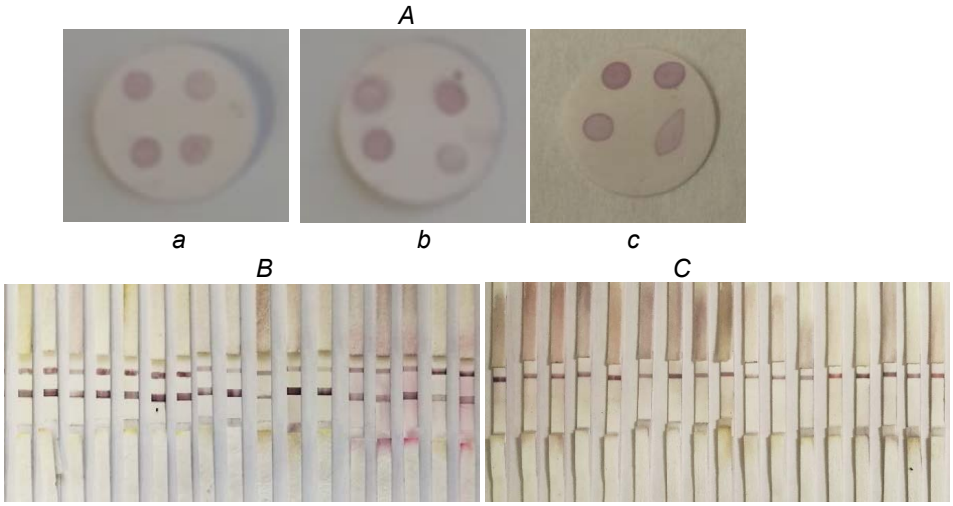

Fig. 5. Results of dot immunoassay using (*A*) GNP conjugated with recAg (*a* — GNP-1; *b* — GNP-2; *c* — GNP-3) and immunochromatographic analysis of positive (*B*) and negative (*C*) serum samples using GNP-3 conjugated with recAg.

Discussion

The principle underlying ICA is the movement of the analyte across the membrane, which results in the formation of specific immune complexes in designated areas of the membrane. These are then detected by staining. In the majority of cases, conjugates of antigens and antibodies with GNP are employed in ICA tests. The extant literature provides comprehensive coverage of methodologies for synthesizing GNP with varying particle sizes, as well as conjugating these particles with biological macromolecules [9, 12, 13]. The present study concentrated on the synthesis of GNPs of varying sizes, with the understanding that their optical properties are contingent on their size and shape. A range of GNP sizes were synthesized and their physico-chemical properties investigated. Conjugates of GNPs with HEV recAG were then obtained, and the reactivity of the obtained conjugates was evaluated by dot immunoassay and ICA.

As the diameter of the particles increased, the wavelength of maximum absorption has shifted slightly to longer wavelengths (518 and 522 and 528 nm for GNP-1, GNP-2 and GNP-3, respectively), that is consistent with the extant literature [23]. The color of the solutions changed from orange-red at the smallest size particles up to purple in a colloidal gold solution with a particle size of about 41 nm. As demonstrated by extant literature data, an augmentation in the particle size to 50 nm or more has the potential to result in a diminution of the analytical signal [32, 33]. Therefore, the present study was limited to the acquisition of three sizes of GNP: 16, 25 and 41 nm. The synthesized GNPs have sizes according to scanning electron microscopy are comparable to those calculated using the Frens method [11].

A plethora of methodologies have been proposed for the experimental estimation of the number of molecules adsorbed on the surface of a nanoparticle [35, 38] and for the calculation of the protein concentration for planting macromolecules on GNP. Synthesis of a conjugate of GNP with antigen molecules is contingent on the resolution of two principal tasks: the generation of a stable sol and the preservation of the antigenic activity of protein molecules. The synthesis conditions that have been determined as optimal in this study take into account the effect of antigen concentration and pH of the solution on the sol stability. The minimum protein concentration at which the color of the mixture did not change was considered to be stabilizing, i.e. determining the limits of sol stability. The protein shell surrounding the colloidal nanoparticles is hydrated, which ensures that after application to a porous glass fiber membrane and subsequent drying, the particles, when interacting with the sample and buffer, are returned to the solution without compromising their reactivity. Consequently, antigen conjugates with GNP of three sizes were obtained, demonstrating stability over a period of six months.

A preliminary evaluation was conducted in order to ascertain the capacity of the synthesized conjugates to interact specifically with AB. This investigation employed the dot immunoassay technique in the capture format, a method that facilitated the determination of the products' remarkable activity and suitability for utilization in assays employing nitrocellulose membranes as a solid phase. In the ICA reactions, an increase in the size of the GNP resulted in a significant increase in the analytical signal, and the brightest band in the test zone was observed when using a conjugate with a particle size of 41 nm. This confirmed an increase in the extinction coefficient of GNP solutions with an increase in particle size. The enhancement of the analytical signal upon utilization of large-diameter GNP conjugates for preparation may be attributed to the higher sensitivity of the human eye to the more contrasting purple hue.

Conclusion

During the course of this work, GNPs with three particle sizes (16, 25, and 41 nm) were obtained and characterized, and optimal conditions for preparation of GNP conjugates with recAg were determined. The resultant GNP conjugates demonstrated high efficiency and specificity of their interaction with anti-HEV IgG AB. Also, a possibility of application of GNP-3 conjugate with recAg in ICA was demonstrated.

The resulting conjugate is intended for use in the development of a test system for the rapid simultaneous detection of IgG and IgM AB to HEV. The appearance of IgG AB in the patient blood occurs a few days after the appearance of IgM AB. Anti-HEV IgM AB has been observed to persist for six to nine months. By way of contrast, serum anti-HEV IgG AB has been shown to persist for decades following the virus exposure [16]. In the early stages of the disease, the simultaneous detection of anti-HEV IgG and IgM AB is an additional tool of the diagnostic algorithm. Detection of anti-HEV IgG AB alone may indicate both acute or past infection and post-vaccination immunity. A significant area of utilization for the rapid anti-HEV IgG test pertains to epidemiological studies, particularly in scenarios where laboratory equipment is absent.

The results obtained in this study represent novel data and could be used as a foundation for the development of a new efficient ICA test for rapid diagnostics of hepatitis E.

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ORIGINAL RESEARCHES

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Study of the protective properties of immunodominant proteins of orthopoxviruses in various methods of immunization

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Abstract

Introduction. In 2022, the World Health Organization declared monkeypox a public health emergency. The monkeypox virus (MPV) is part of the *Orthopoxvirus* genus within the *Poxviridae* family. During replication, orthopoxviruses produce two distinct forms of viral particles: the extracellular enveloped virion (EEV), released via exocytosis, and the intracellular mature virion (IMV), released through cell lysis. These forms differ in surface proteins composition, influencing their immunogenicity and infectivity.

Aim. To evaluate the immunogenic and protective activity of nine surface antigens of vaccinia virus.

Materials and methods . Recombinant human adenoviruses type 2 (rAd2) carrying surface antigens of vaccinia virus were obtained using homologous recombination in bacteria, followed by adenoviral particle assembly in HEK293 cells. The immunogenic and protective properties of these adenoviruses were tested in BALB/c mice. The presence of antibodies to the vaccinia virus was assessed using ELISA, and survival rates were evaluated in a lethal infection model after intranasal challenge with the vaccinia virus strain Western Reserve.

Results. The most immunogenic and protective antigens of the vaccinia virus within rAd2 were glycoprotein B5 of the EEV and membrane-associated protein H3 of the IMV, both showing 100% protective efficacy after intranasal immunization.

Conclusion. Using a panel of recombinant adenoviruses carrying genes of vaccinia virus surface proteins, it was shown that optimal protection is achieved using a combination of enveloped and mature virion antigens. This method could be used for development of new multivalent preparations against various viral infections.

Keywords: *monkeypox virus, vaccinia virus, recombinant adenovirus, immunogenicity, protective activity*

Ethical 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 Biomedical Ethics Committee of the N.F. Gamaleya National Research Center for Epidemiology and Microbiology (protocol No. 34, January 16, 2023).

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Оригинальное исследование
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Исследование протективных свойств иммунодоминантных белков ортопоксвирусов при различных способах иммунизации

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Аннотация

Введение. Всемирная организация здравоохранения в 2022 г. объявила оспу обезьян чрезвычайной ситуацией в области здравоохранения. Вирус оспы обезьян (MPV) принадлежит к роду *Orthopoxvirus* семейства *Poxviridae*. Ортопоксвирусы во время репликации образуют 2 отдельные формы вирусных частиц: экзоцитозом выделяется оболочечный внеклеточный вирион (EEV), а зрелый внутриклеточный вирион (IMV) высвобождается в результате лизиса клеток. У этих двух форм вирионов отличаются наборы поверхностных белков, что обусловливает их различия в иммуногенности и инфекционности.

Цель работы — оценка иммуногенной и протективной активности 9 поверхностных антигенов вируса осповакцины.

Материал и методы. Геномы рекомбинантных аденовирусов человека 2-го серотипа, несущих поверхностные антигены вируса осповакцины, получали методом гомологичной рекомбинации в бактериальных клетках с последующей сборкой аденовирусных частиц в клетках HEK293. Иммуногенные и протективные свойства полученных рекомбинантных аденовирусов изучали на мышах BALB/c. Сыворотки крови после иммунизации животных исследовали методом иммуноферментного анализа на наличие антител к вирусу осповакцины. Протективные свойства оценивали на летальной модели инфекции мышей после интраназального заражения вирусом осповакцины штамма Western Reserve.

Результаты. Наиболее иммуногенными и протективными антигенами вируса осповакцины в составе рекомбинантных аденовирусов человека 2-го серотипа были гликопротеин B5 оболочечного внеклеточного вириона и мембранно-ассоциированный белок H3 зрелого внутриклеточного вириона. При изучении защитной эффективности антигенов показана 100% эффективность B5 и H3 при интраназальной иммунизации.

Заключение. При использовании панели рекомбинантных аденовирусных векторов, несущих гены поверхностных белков вируса осповакцины, показано, что оптимальная защита может быть достигнута с помощью комбинации антигенов оболочечного и зрелого вирионов. Данный подход может быть использован при разработке новых поливалентных вакцин против различных вирусных инфекций.

Ключевые слова: *вирус оспы обезьян, вирус осповакцины, рекомбинантный аденовирус, иммуногенность, протективность*

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Introduction

Orthopoxviruses belong to the *Poxviridae* family, *Chordopoxvirinae* subfamily, *Orthopoxvirus* genus, which contains 13 species: *Orthopoxvirus abatinomacacapox*, *Orthopoxvirus akhmetapox*, *Orthopoxvirus camelpox*, *Orthopoxvirus cowpox*, *Orthopoxvirus ectromelia*, *Orthopoxvirus monkeypox*, *Orthopoxvirus raccoonpox*, *Orthopoxvirus skunkpox*, *Orthopoxvirus taterapox*, *Orthopoxvirus vaccinia*, *Orthopoxvirus variola*, *Orthopoxvirus volepox*, and the unofficially classified Alaska smallpox virus[1]. Species are divided into 2 related clades: Old World (Eurasian and African) and North American orthopoxviruses [1]. Many of these viruses can cause severe disease in domestic animals as well as zoonotic infections in humans. Human infection most commonly occurs through livestock rearing, contact with domestic animals, and trade in exotic animals as a result of direct contact with them. When introduced into non-endemic regions, enzootic orthopoxviruses may pose a threat to native and endemic species [2–4].

Orthopoxviruses of different species are antigenically and immunologically close to each other. Vaccination against smallpox, for example, provides cross-protection against other members of the genus [5]. However, after the global eradication of smallpox in 1980, many countries began to phase out routine vaccination against the disease [6]. As a result, a significant proportion of the population currently lacks immune protection to orthopoxviruses. This explains the increasing frequency of outbreaks in various regions of the world, among which monkeypox is particularly notable. The global outbreak that occurred in 2022 was recognized by the World Health Organization (WHO) as a public health emergency of international concern [7]. The ongoing epidemic of monkeypox in African countries may lead to accelerated viral evolution and adaptation to human-to-human transmission of the zoonotic disease [8]. Despite existing vaccines, WHO has recommended the development of less reactive vaccines to improve the efficacy and duration of protection to get the current outbreak under control.

Orthopoxviruses have a large and complex proteome containing more than 200 proteins, of which more than 30 are structural proteins [9, 10]. During infection, the virus exists in two antigenically distinct infectious forms [11–13]. Intracellular mature virions (IMV) are the main infectious viral particles and play a key role in human-to-human transmission. The surface membrane of this form contains at least 11 proteins: A14.5 (~10 kDa), E10 (12 kDa), I5 (13 kDa), A13 (14 kDa), A27 (14 kDa), A9 (~18 kDa), A14 (17–25 kDa), A17 (23–29 kDa), L1 (25–29 kDa), D8 (34 kDa) and H3 (35 kDa) [9–13]. Extracellular enveloped virions (EEVs) are formed from IMVs and have an additional lipoprotein membrane responsible for virus dissemination within the body [12]. EEV and IMV surface proteins ensure the infectivity of orthopoxviruses and serve as major targets for the induction of protective immunity [14, 15]. Because antibodies that neutralize IMV do not neutralize EEV, it is believed that immunization with antigens from both of these forms is necessary for maximal protection [16].

The immunodominant proteins to which antibodies are produced, including virus-neutralizing antibodies, are two EEV glycoproteins, A33 and B5, as well as IMV proteins: L1, H3, D8 and a complex of 3 proteins of immature virion A14, A17 and D13 or a complex of A14, A17 and A27 of a mature virion [14, 17–19]. Proteins A33 and B5 play a role in envelope virion formation and subsequent infection [14]. Among the surface proteins of the mature virion, 3 proteins mediate the attachment of the virus to the host cell. Protein D8 forms dimers on the viral membrane and ensures virion infectivity by binding to chondroitin sulfate on a cell membrane [20]. A27 and H3 proteins bind heparan sulfate. A27, a major membrane protein, is required for the formation of the enveloped extracellular virion and is involved in the attachment of the virus to the cell and subsequent fusion of the viral and cell membranes. A27 forms a complex with the transmembrane proteins A14 and A17, which are important structural elements of the mature virion membrane. A17 is also required for virus entry into the cell and serves as an anchor for A27 [21]. H3 binds to the cell surface via heparan sulfates and, like D8 and A27 proteins, is involved in virus entry into the cell. H3 is a major protein in the development of immune response in humans [22]. L1 is a transmembrane protein that is essential for the formation of mature virions and is involved in virus entry into the cell [23].

Although most of these antigens (A27, L1, B5 and A33) have been studied as part of various polyvalent vaccines [18, 24, 25], certain immunodominant proteins were not included in these studies. Furthermore, while orthopoxviruses are highly immunogenic, the antigens themselves have low immunogenicity. Vaccination with purified proteins or DNA encoding proteins of vaccinia virus requires multiple immunizations for induction of a protective immune response [24, 26, 27]. In contrast to DNA vaccines and subunit vaccines, vaccination with recombinant adenoviruses (rAd) has been shown to promote both a robust humoral and cellular immune response and protective immunity after a single immunization [28–30]. Given the importance of rapid induction of protective immunity in a potential outbreak, we studied the possibility of a single immunization with rAd expressing the EEV and IMV antigens of the vaccinia virus.

The aim of this study was to evaluate the immunogenic and protective activity of 9 surface antigens of vaccinia virus. To achieve this goal, rAd carrying genes

[1] ICTV. Subfamily: Chordopoxvirinae. Genus: Orthopoxvirus. URL: https://ictv.global/report/chapter/poxviridae/poxviridae/orthopoxvirus

of surface proteins of mature (D8, H3, L1, A14, A17, A27 and D13) and extracellular (A33 and B5) virions of smallpox virus were constructed. Furthermore, individual immunogenic and protective properties of the obtained adenoviruses were investigated by intranasal and intramuscular methods of immunization. This approach can be used in the development of new polyvalent vaccines against various viral infections.

Materials and methods

Cell lines and viruses

The Vero E6 cell line (green monkey kidney) was cultured in DMEM medium (Cytiva) containing 4% fetal bovine serum (Gibco), 25,000 units of penicillin and 25 mg of streptomycin (Paneco) at 5% CO_2. The HEK293 cell line (human embryonic kidney) was cultured in DMEM medium (Cytiva) containing 10% fetal bovine serum (Gibco), 25,000 units of penicillin and 25 mg of streptomycin (Paneco) at 5% CO_2.

Western Reserve strain of vaccinia virus (VACV WR) (GenBank #OP584857.1) was grown in Vero E6 cells. Aliquots of virus-containing medium were stored at –80ºC. The biological activity of the virus was determined by standard titration method on cell culture by counting plaques [31].

Production of recombinant adenoviruses

rAd was constructed using the technology described previously [32]. Vaccinia virus antigen genes were amplified from the VACV WR genome (GenBank #OP584857.1) using primers indicated in **Table 1** and cloned under the control of the human cytomegalovirus promoter. Plasmid vectors with the human rAd genome of type 2 (rAd2) and the target antigen were obtained by homologous recombination in bacterial cells. The rAds were produced and grown in HEK293 cells. Viruses were purified and concentrated by ultracentrifugation in cesium chloride density gradient according to standard methods [33]. The number of virus particles was determined by the standard spectrophotometric method [34].

Animal models

Mice of the inbred line BALB/c, males and females, body weight 16–18 g, were obtained from the nursery of the Pushchino Nursery of laboratory animals, branch of the Institute of Bioorganic Chemistry of RAS. The animals were kept in the vivarium of N.F. Gamaleya NRCEM in accordance with the requirements for keeping laboratory animals and had free access to food and water. 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 Biomedical Ethics Committee of the N.F. Gamaleya NRCEM of the Ministry of Health of Russia (protocol # 34 from 16.01.2023).

Immunization and infection of mice

Five-week-old BALB/c mice were immunized intranasally under mild inhalation anesthesia or intra-

Table 1. Primer sequences for amplification of vaccinia virus genes

Name	Sequence
A33-F	CACCGGCGGTCGACAGATCTGCCACCATGATGACACCAGAAAACGACG
A33-R	GATATCTCTAGATTAACAAAAATACTTTCTAACTTCTTGTG
H3-F	ACTAAGCTTATATGGCGGCGGCGAAAACT
H3-R	ATCTAGATATCTG TTAGATAAATGCGGTAAC
B5-F	CACCGGCGGTCGACAGATCTGCCACCATGAAAACGATTTCCGTTGTTACG
B5-R	TCTAGATTACGGTAGCAATTTATGGAACTTATA
D8-F	CACCGGCGGTCGACAGATCTGCCACCATGCCGCAACAACTATCTCCTATTAA
D8-R	GATATCGCTAGCTTACTAGTTTTGTTTTTCTCGCGAATATCG
A14-F	CACCGGCGGTCGACAGATCTGCCACCATGGACATGATGCTTATGATTGG
A14-R	GATATCTCTAGATTATTAGTTCATGGAAATATCGCTATG
A27-F	CACCGGCGGTCGACAGATCTGCCACCATGGACGGAACTCTTTTCCC
A27-R	TCTAGATTATTACTCATATGGGCGCCGTC
L1-F	CACCGGCGGTCGACAGATCTGCCACCATGGGTGCCGCGGCAAGCAT
L1-R	GATATCTCTAGATTATCAGTTTTGCATATCCGTGGTAGC
A17-F	CACCGGCGGTCGACAGATCTGCCACCATGAGTTATTTAAGATATTACAATATG
A17-R	GATATCTCTAGATTATTAATAATCGTCAGTATTTAAACTG
D13-F	CACCGGCGGTCGACAGATCTGCCACCATGAATAATACTATCATTAATTCTTTG
D13-R	ACTAGTTTATTAGTTATTATCTCCCATAATCTTG

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muscularly with rAd at a dose of 2×10^{10} viral particles. The same adenoviruses were re-administrated at the same dose 3 weeks later. Serum samples were collected on day 28 after the 1st immunization. Immunized mice were subjected to intranasal infection with vaccinia virus strain WR at a dose of 16 LD_{50} (5×10^4 PFU) 35 days after the 1st immunization. During 14 days after infection, the animals were examined daily and clinical symptoms of smallpox infection (decreased locomotor activity, hunched, ruffled hair, conjunctivitis), changes in body weight, and death of mice were recorded. Animals were sacrificed if they lost more than 25% of their body weight.

Enzyme immunoassay

The titer of specific IgG antibodies to vaccinia virus in serum samples was determined by enzyme-linked immunosorbent assay. 96-well plates were coated with vaccinia virus WR (10^5 PFU/plate) diluted in carbonate-bicarbonate buffer (pH 9.6) overnight at 4ºC. Serum samples were serially diluted twice to 1 : 102,400, added to blocked 96-well plates and incubated at 37ºC for 1 h. The plates were then washed and secondary antibodies conjugated to a complex of recombinant streptavidin with horseradish peroxidase were added to each well. The plates were incubated for 1 h at 37ºC. Thereafter, tetramethylbenzidine hydrochloride was added to the plates. The reaction was stopped by adding H_2SO_4 and the absorbance (450 nm) was read using a Multiskan FC tablet photometer (ThermoFisher). The highest serum dilution with an optical density value 2 or more times higher than the value in the corresponding serum dilution of intact (unimmunized) mice was taken as the final titer.

Statistical processing of data

Statistical processing of data and construction of diagrams were performed in the GraphPad Prism 8 program. Statistical significance of differences between the studied groups was determined using the Wilcoxon T-test for dependent samples or Mann–Whitney U-test for independent samples. Differences were considered reliable at $p < 0.05$.

Results

Design and production of rAd expressing surface antigen genes

The main immunodominant proteins of the vaccinia virus are 2 glycoproteins of the enveloped extracellular virion, A33 and B5, as well as proteins of the mature intracellular virion: D8, H3, L1, A14, A17, A27 and D13. These antigens have a high degree of homology between different orthopoxvirus species (**Table 2**) [35–37].

The genes of the proteins described above were obtained from the genome of vaccinia virus and cloned into the rAd2 genome under the control of the human cytomegalovirus promoter (**Fig. 1**). The rAd2 genome carries a deletion of the E1 region that renders the adenovirus replication-defective (an expression cassette with the target gene is cloned into this deletion site). To increase the packaging capacity, the E3 region was also deleted.

Thus, we obtained 9 rAd2, 2 of which carried surface antigens of the enveloped virion (rAd2-B5 and rAd2-A33) and 7 of which carried antigens of the mature virion (rAd2-H3, rAd2-L1, rAd2-D8, rAd2-D13, rAd2-A14, rAd2-A17, and rAd2-A27).

rAd2 vectors induce humoral immune response in vivo

To evaluate the immunogenicity of the obtained rAd (rAd2-A33, rAd2-B5, rAd2-H3, rAd2-L1, rAd2-D8, rAd2-D13, rAd2-A14, rAd2-A17, rAd2-A27), mice were immunized intramuscularly or intranasally with adenoviruses at a dose of 2×10^{10} viral particles once or twice. Titers of specific IgG antibodies to vaccinia virus were determined by enzyme-linked immunosorbent assay on the 28th day after immunization (**Figs. 2, 3**).

Table 2. Homology between proteins of different orthopoxvirus species

VACV antigen	Antigen (% homology)		
	Orthopoxvirus monkeypox	Orthopoxvirus cowpox	Orthopoxvirus variola
L1	M1 (98,4%)	CPVX099 (98,4%)	M1 (99,2%)
H3	H3 (93,83%)	CPVX112 (94,46%)	I3 (93,85%)
D8	E8 (94,41%)	CPVX125 (97,37%)	F8 (93,09%)
D13	E13 (98,91%)	CPVX131 (98,73%)	N3 (98,91%)
A14	A15 (100%)	CPVX146 (100%)	A14 (97,78%)
A17	A18 (97,55%)	CPVX150 (95,59%)	A17 (97,55%)
A27	A29 94,55%)	CPVX162 (95,45%)	A30 (93,64%)
A33	A35 (92,47%)	CPVX168 (91,49%)	A36 (89,25%)
B5	B6 (96,53%)	CPVX199 (93,69%)	B7 (92,74%)

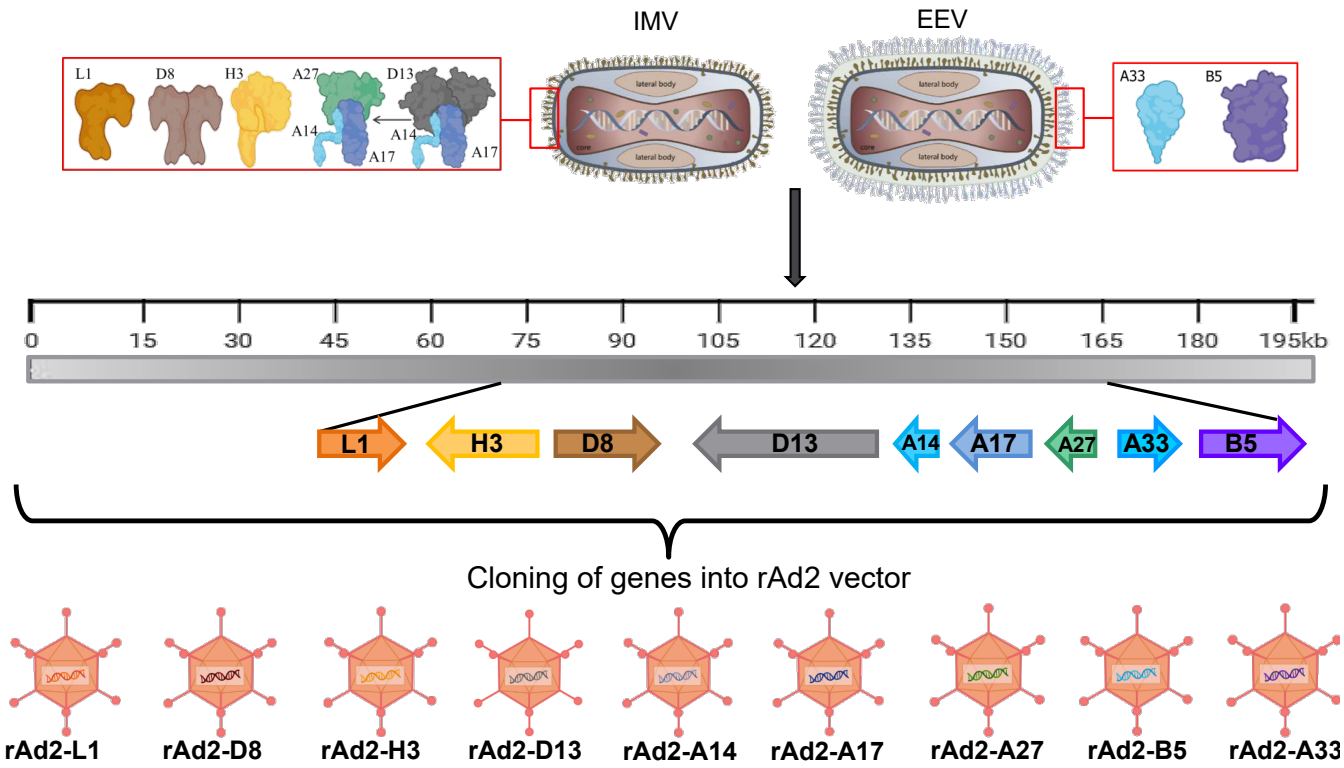

Fig. 1. Scheme for the production of recombinant adenoviruses expressing surface antigens of the vaccinia virus.
L1 — myristoylated protein; H3 — membrane-associated protein p35 IMV; D8 — transmembrane carbonic anhydrase-like protein; D13 — IMV membrane protein; A14 — structural transmembrane protein p16 IMV; A17 — transmembrane protein IMV (morphogenesis factor); A27 — membrane protein IMV; B5 — type I membrane glycoprotein; A33 — type II membrane glycoprotein.

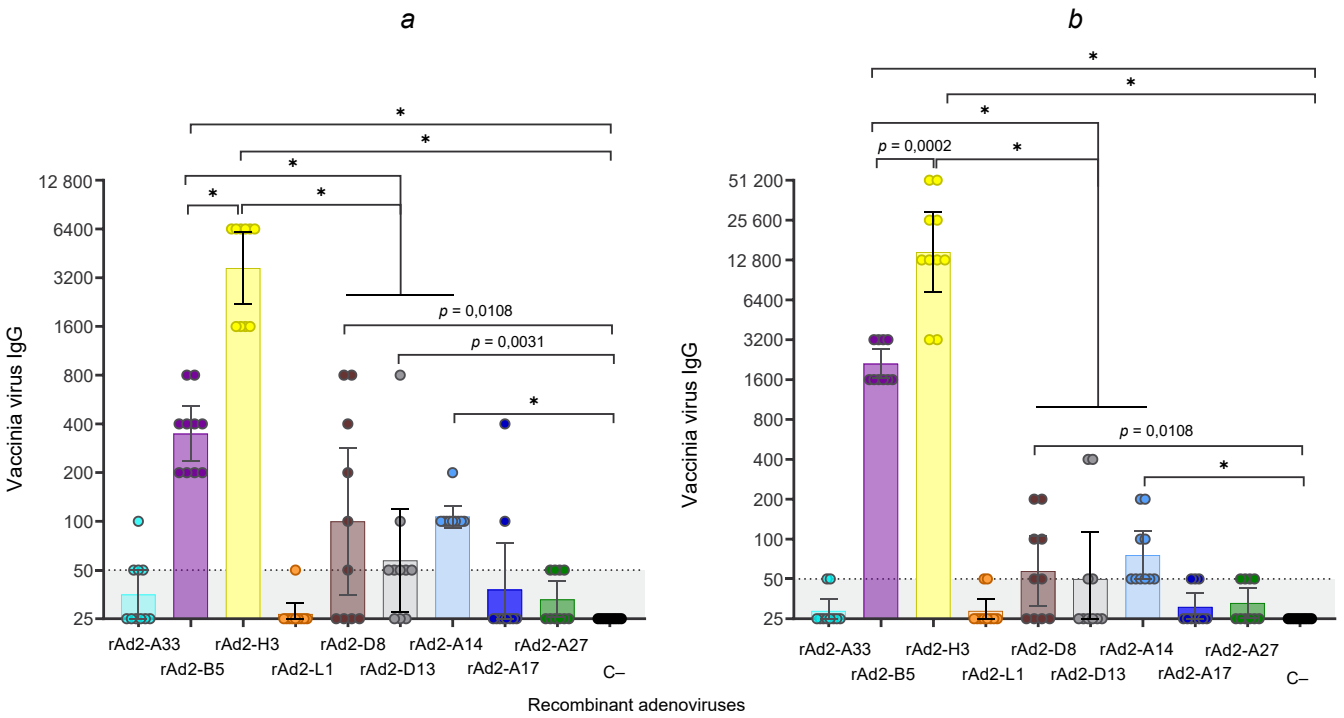

Fig. 2. Immunogenicity of recombinant adenoviruses expressing surface antigens of the vaccinia virus after single (*a*) and double (*b*) intramuscular immunization.
The figures show the confidence at $p < 0.05$; *$p < 0.0001$. C — control.

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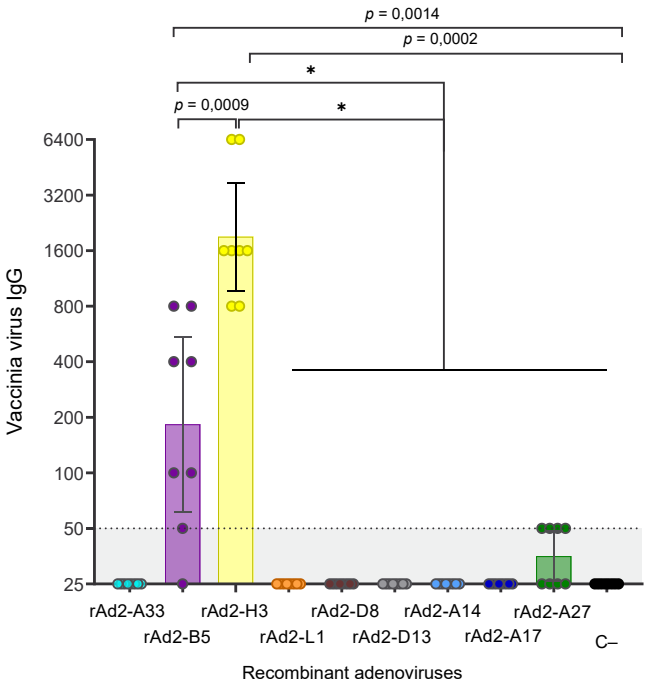

Fig. 3. Immunogenicity of recombinant adenoviruses expressing surface antigens of the vaccinia virus after two intranasal immunizations.
The figure shows the confidence at $p < 0.05$; *$p < 0.0001$.
C– — control.

A single intramuscular immunization resulted in the formation of specific IgG antibodies in mice that received rAd2-B5, rAd2-H3, rAd2-D8, rAd2-D13, and rAd2-A14. The titer values in these groups had significant differences with the control group, where titers were less than 1 : 50 (Fig. 2, *a*). High levels of IgG antibodies were found in the group of mice immunized with rAd2-H3 (geometric mean titers (GMT) 1 : 3676). Administration of rAd2-B5 resulted in the induction of IgG-antibodies with GMT = 1 : 348.2. Minimal levels of IgG antibodies were observed for rAd2-D8 (GMT = 1 : 100), rAd2-A14 (GMT = 1 : 107.2) and rAd2-D13 (GMT = 1 : 57.43) groups with no significant differences between groups. Double immunization resulted in a significant increase in IgG antibody levels compared to single immunization only for the rAd2-H3 (GMT = 1 : 14703) and rAd2-B5 (GMT = 1 : 2111) groups (Fig. 2, *b*). No significant differences were found between the other groups.

After single intranasal immunization, specific IgG antibodies in serum were not detected in any group. After double intranasal immunization, specific IgG antibodies were detected in the serum of animals immunized with rAd2-B5 and rAd2-H3. The titer values in these groups had significant differences with the control group, where titers were less than 1 : 50 (Fig. 3). High antibody levels were found in the group of mice immunized with rAd2-H3 (GMT = 1 : 1902.73). Administration of rAd2-B5 resulted in induction of IgG antibodies with GMT = 1 : 183.4.

Protective efficacy of rAd2 in vivo

The next step was to test the protective efficacy of the antigens. After 35 days from the start of double immunization, mice were infected with a pathogenic WR strain at a dose of 16 LD_{50} (5×10^4 PFU). The infectious dose was chosen based on preliminary experiments to determine the LD_{50} *in vivo* [38]. Survival and body weight changes were recorded for 14 days after infection.

As expected, in the control group, pronounced clinical signs of the disease with a body weight loss of more than 13% were observed from day 4. As shown in **Fig. 4**, all control mice rapidly lost weight and died on the 5th–8th day after the infection.

After intramuscular immunization with rAd2-B5 expressing the B5 EEV glycoprotein gene, we observed 20% protection of animals from lethal infection (Fig. 4, *a*, *b*). Double intramuscular immunization with rAd2-D8 expressing the D8 IMV glycoprotein gene resulted in 40% survival of animals. Animals immunized with rAd2 expressing other surface antigens lost 25% of their initial weight within 5–7 days of infection. The results of the study concluded that when using the intramuscular route of administration, a single antigen is not sufficient for protection.

Interestingly, intranasal immunization significantly reduced the severity of infection and protected the animals from both lethality and weight loss (Fig. 4, *c*, *d*).

In particular, during immunization with rAd2-B5 we observed 100% protective efficacy. It should be noted that in the group of animals immunized with rAd2-B5, insignificant weight loss (no more than 3%) on days 3-5 was observed in only half of the animals. In the rAd2-H3 group, the survival rate was 87.5% (out of the 8 mice, 1 did not survive). Furthermore, 1 mouse had a weight loss of about 15% on day 5 with gradual recovery by day 14. In the rAd2-A33, rAd2-L1, rAd2-D8 and rAd2-D13 groups, the survival rate was 75%. Temporary weight loss by day 5 for the groups did not exceed 25%. The mice in the rAd2-A17 and rAd2-A27 groups had a 50% lethality with a weight loss of 15% or less. The mice immunized with rAd2-A14 lost 21% of their initial weight within 5 days of infection. The survival rate in this group was only 25%.

These data indicate that rAd2-B5 and rAd2-H3 are optimal vectors for protection against intranasal vaccinia virus infection, demonstrating the importance of B5 and H3 as protective antigens in this model of orthopoxvirus infection. Therefore, we further tested the efficacy of combined immunization (intramuscular + intranasal) for induction of both systemic and local response.

Mice were injected intramuscularly with rAd2-B5 or rAd2-H3 at a dose of 2×10^{10} viral particles; 21 days later, the same adenoviral vector was administered intranasally. After 35 days from the start of immuniza-

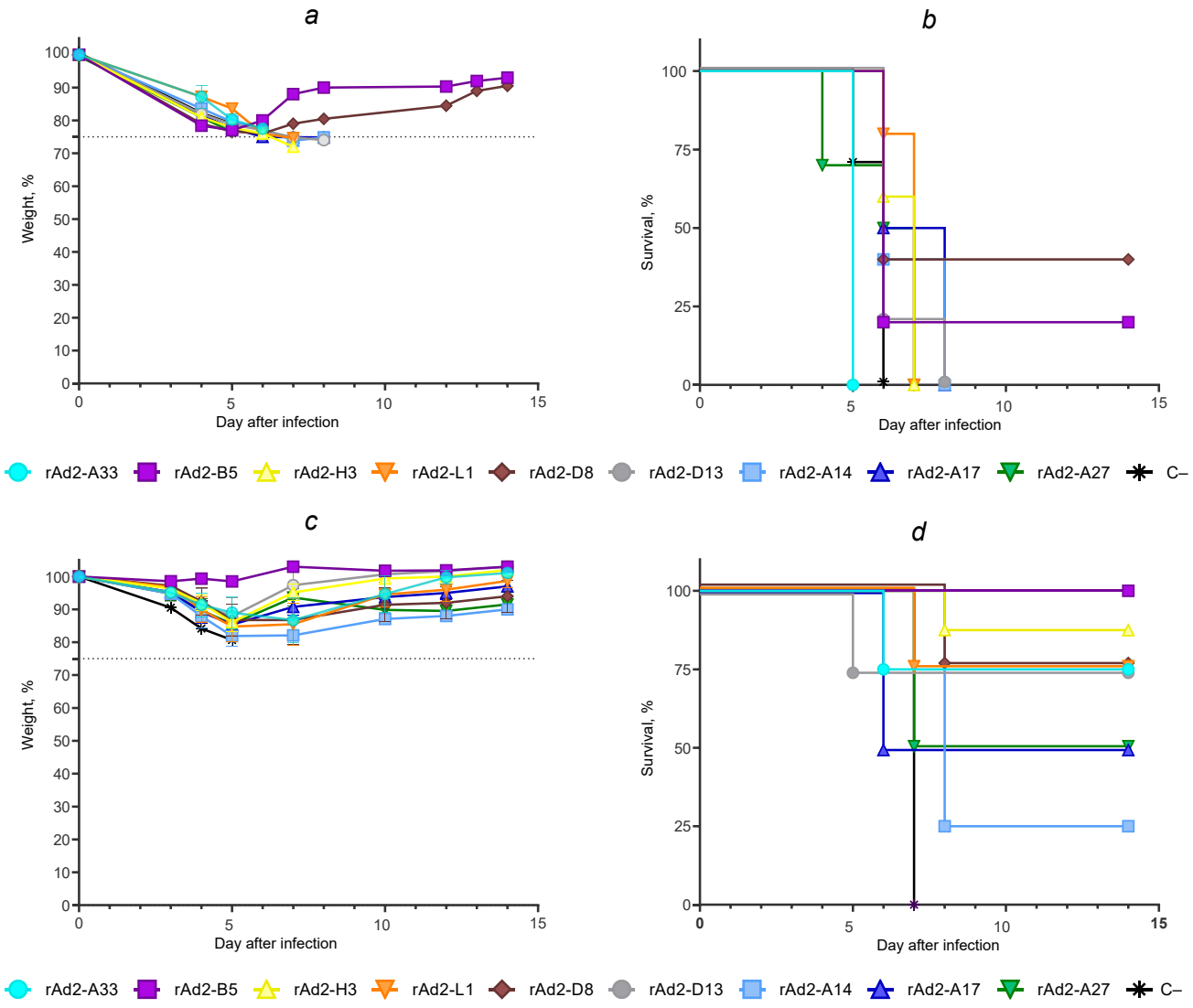

Fig. 4. The protective efficacy of recombinant adenovirus vectors expressing the surface antigens of the vaccinia virus after intramuscular (*a*, *b*) or intranasal (*c*, *d*) immunization in a mouse model of lethal infection.

a, *c* — weight dynamics (the graph shows the average value and the standard error of the average value); *b*, *d* — survival. C– — control.

tion, animals were infected with the pathogenic WR strain at a dose of 16 LD_{50} (5×10^4 PFU). Survival and body weight changes were recorded for 14 days after infection (**Fig. 5**).

Combined immunization with rAd2-B5 or rAd2-H3 provided 100% protection of animals against lethal infection. No weight loss was recorded in the group of animals intramuscularly and intranasally immunized with rAd2-B5. However, in the rAd2-H3 group, weight loss was about 23% on day 6 in 2 out of 8 mice with gradual recovery by day 14.

Discussion

Since the use of vaccinia virus in the last century, concomitant vaccination is no longer available. Despite the eradication of smallpox, the world continues to face orthopoxvirus infections that require active actions. Serious side effects following smallpox virus vaccination have emphasized the need to develop safer vaccine formulations to combat current orthopoxvirus infections such as monkeypox.

Russian scientists have made a great contribution to the development of smallpox vaccines [39–41]. Currently, the least reactogenic and safe vaccine is the recently developed OrthopoxVac vaccine from the Vector Research Center of Rospotrebnadzor for the prevention of orthopoxvirus infections, which is an L-IVP strain of the vaccinia virus with 6 disrupted virulence genes [42].

A high degree of homology in the central region of the genome of monkeypox and vaccinia virus, amounting to 96.3%, indicates their genetic similarity [35–37]. In particular, the MPV genes A35, B6, M1, E8, H3, A15, A18, A29 and E13 show significant conservation with orthologous orthopoxvirus genes, including smallpox and vaccinia viruses [24].

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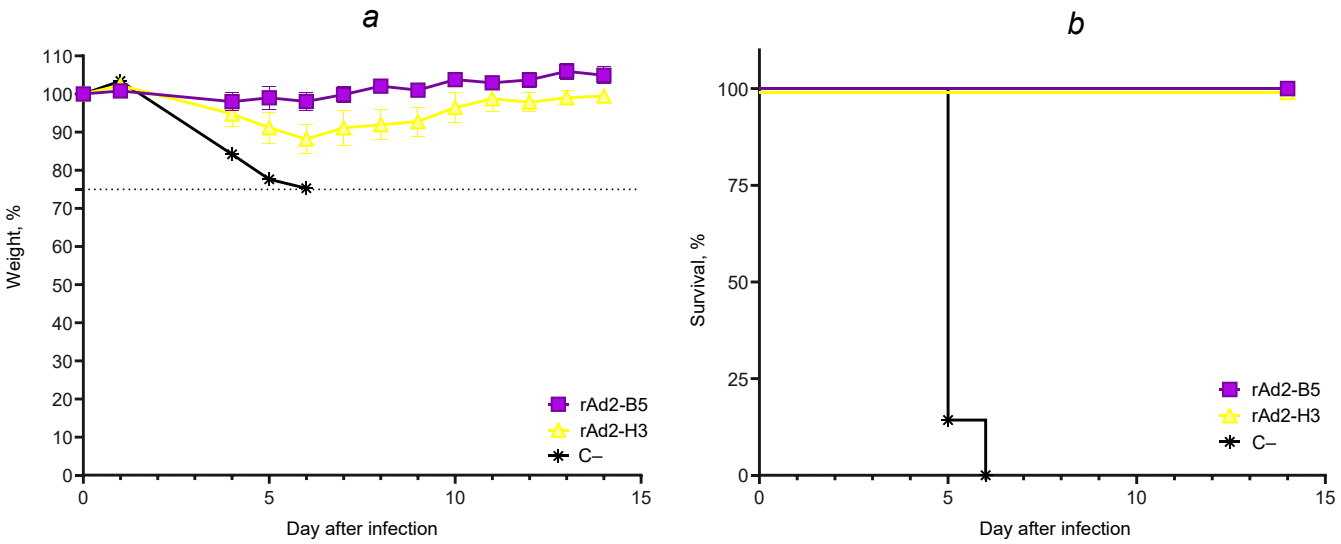

Fig. 5. The protective efficacy of recombinant adenovirus vectors rAd2-B5 and rAd2-H3 after combined immunization (im+in) in a mouse model of lethal infection.

a — weight dynamics; *b* — survival. C — control.

Several technologies have been used to develop orthopoxvirus vaccines: live vaccine, attenuated replication-defective virus, DNA-based vaccine and subunit vaccines. Each of them has its own disadvantages, such as serious adverse events with live vaccine, lower immunogenicity in attenuated non-replicating vaccines and DNA vaccines, and a long development cycle for recombinant proteins [24, 26, 43, 44].

rAd possess a number of key properties that make them good candidates for vector vaccine development. rAd are physically and genetically stable and effectively induce innate and adaptive immune response by various routes of administration, including delivery via mucosal surfaces [45]. The latter is a significant advantage over other types of vaccines, since the effectiveness of vaccination depends on the site of administration and the recruitment of antigen-presenting cells [46]. Intramuscular immunization leads to stimulation of the systemic immune response, but does not provide effective barrier immunity. Meanwhile, intranasal immunization can induce humoral and cellular immunity both on mucosal membranes and systemically [47]. Thus, the development of mucosal vaccines will help to meet the need for more effective protection against pathogens that penetrate through mucous membranes.

Adenovirus technology provides a versatile platform for the rapid development and deployment of vaccines to combat viral infections, including those with pandemic potential. In this study, we developed a panel of recombinant replication-defective vectors based on human adenovirus type 2 encoding genes of surface proteins of vaccinia virus. Two proteins of the extracellular enveloped virion (A33 and B5) and 7 proteins of the intracellular mature virion (L1, D8, H3, A14, A17, A27, D13) were selected as target genes for cloning into the rAd2 genome. We evaluated the immunogenicity of 9 EEV and IMV surface proteins, most of which have been shown to be effective in previous studies [15, 17, 19, 24, 26, 27]. However, in our study, of the 9 surface antigens, only B5 and H3 induced an immune response in mice upon systemic and intranasal administration. We hypothesize that this may be due to the fact that both B5 and H3 proteins are glycoproteins. Glycoproteins contain epitopes that are recognized by immune system cells more efficiently than epitopes of other types of proteins. According to the literature sources, *in silico* predicted B- and T-cell epitopes for immunodominant MPV proteins (M1, H3, E8, A29, A35 and B6) have been reported [48].

In experiments to study the protective efficacy of rAds, we demonstrated the efficacy of intranasal immunization. Intranasal immunization with rAd2-B5 expressing the B5 EEV glycoprotein gene provided the best protection (100%) with the least weight loss and fastest recovery. Intranasal immunization with rAd2-H3 expressing IMV glycoprotein H3 gene provided 87.5% protection. Other surface proteins: EEV A33, IMV L1, D8 and D13 protected animals from lethal infection by 75%. Administration of rAds with genes of IMV antigens A17, A27 and A14 provided only partial protection against VACV by 25-50% depending on the antigen. The efficacy of combined immunization (intramuscular + intranasal) with rAd2-B5 or rAd2-H3 was 100%. Our data are consistent with the results of other studies; in which it was shown that specific immunity to EEV surface antigens is most important for protection against intranasal infection [49]. Antibodies specific to B5 play an important role against pulmonary or intracerebral infection [50]. H3 protein activates dendritic cells, which leads to the secretion of cytokines such as

interleukins-12p70, -10, -6 and tumor necrosis factor-α, which further induces proliferation of $CD8^+$ T-lymphocytes, thereby destroying virus-infected cells [51].

These results demonstrate the protective potential of the selected antigens and provide valuable information for the subsequent development of effective and safe polyvalent orthopoxvirus vaccines. Our study confirms that B5 and H3 have the highest protective potential, and in combination with other surface proteins of the mature virion can provide maximum efficacy. Further study of the immunogenic and protective potential of different antigen combinations is required. This study lays a solid foundation for the subsequent optimization of vector vaccines by confirming the feasibility of combining different EEV and IMV surface antigens to achieve maximum protection against orthopoxviruses, including monkeypox virus.

Conclusion

In this study, a panel of rAds carrying genes of vaccinia virus surface proteins was created and their immunogenicity and efficacy were evaluated. In our study, we found that in a lethal infection model induced by the WR vaccinia virus strain, glycoproteins B5 and H3 showed the greatest protective activity when immunized intranasally. Furthermore, combination with other antigens can not only enhance the immune response but also generate cross-immunity to other members of the *Orthopoxvirus* genus causing infections in humans. These results provide insight into the protective mechanism of polyvalent vector vaccines and a basis for further development and introduction of such vaccines aimed at enhancing protection against orthopoxviruses.

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Adaptation of H2N2 influenza viruses with different receptor specificity to MDCK cells: opportunities for the development of a cell-based vaccine against pandemic H2N2 influenza

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Abstract

Introduction. H2N2 influenza viruses caused a pandemic in 1957 due to the adaptation of avian influenza hemagglutinin from avian-type α2,3 to human-type α2,6 receptor specificity. These viruses have not circulated among humans for more than 50 years but are still found in avian reservoirs, indicating their pandemic potential. It is known that at the beginning of a pandemic wave, viruses with α2,3 and α2,6 receptor specificities can co-circulate, and the selection of one or another isolate for the development of a better pandemic influenza vaccine should be based on strong scientific evidence. Although the vast majority of influenza vaccines are produced in chicken embryos, mammalian cell culture may be a preferred substrate for the production of pandemic influenza vaccines.

Materials and methods. In this study, we investigated two variants of A/Singapore/1/57 (H2N2) virus which differed by their receptor specificity defined by three residues in the HA1 molecule: E156, Q226, G228 for α2,3 avian-type (Sing-α2,3) and K156, L226, S228 for α2,6 human-type (Sing-α2,6) receptor specificity. We conducted serial passaging of these viruses on MDCK cells and analyzed growth properties of plaque-purified clones *in vitro* and *in vivo*, as well as their immunogenicity and cross-reactivity in a mouse model.

Results. Adaptation to MDCK cells significantly increased viral titers in MDCK cells; however, their receptor specificity was not affected. Viruses with α2,6 receptor specificity induced higher titers of homologous antibodies compared to the viruses with α2,3 receptor specificity, but these antibodies could react only with the α2,6 viruses. In contrast, antibody induced by viruses with α2,3 receptor specificity had broad reactivity against all studied viruses. Similar results were obtained for the pair of A/Leningrad/17-based H2N2 live attenuated influenza vaccines with α2,3 and α2,6 receptor specificities in experiments on Syrian hamsters.

Conclusion. In the case of a new transmission of H2N2 avian influenza viruses to the human population and co-circulation of viruses with both receptor specificities, the variant with α2,3 specificity should be selected for the development of cross-reactive influenza vaccines.

Keywords: *influenza virus, H2N2, receptor specificity, adaptation, MDCK cells, live attenuated influenza vaccine, immunogenicity*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the Institute of Experimental Medicine (protocol No. 1/20, February 27, 2020).

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Оригинальное исследование
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Адаптация вирусов гриппа H2N2 с различной рецепторной специфичностью к клеткам MDCK: возможности для разработки культуральной пандемической вакцины против гриппа H2N2

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Аннотация

Введение. Вирусы гриппа H2N2 вызвали пандемию в 1957 г. благодаря адаптации молекулы гемагглютинина от птичьего рецептора типа α2,3 к человеческому рецептору α2,6. Эти вирусы не циркулируют среди людей уже более 50 лет, но до сих пор встречаются в природном резервуаре, что указывает на их пандемический потенциал. Известно, что в начале пандемической волны вирусы с α2,3- и α2,6-рецепторной специфичностью могут циркулировать совместно и выбор того или иного изолята для разработки оптимальной пандемической гриппозной вакцины должен быть основан на убедительных научных данных. Хотя подавляющее большинство вакцин против гриппа производится с использованием развивающихся куриных эмбрионов, культура клеток млекопитающих может быть предпочтительным субстратом для производства вакцин против пандемического гриппа.

Материалы и методы. В настоящем исследовании мы изучили два варианта вируса A/Singapore/1/57 (H2N2), которые отличались рецепторной специфичностью, определяемой 3 остатками в молекуле HA1: E156, Q226, G228 для α2,3 птичьего типа (Sing-α2,3) и K156, L226, S228 для α2,6 человеческого типа (Sing-α2,6) рецепторной специфичности, а также методами обратной генетики получили пару штаммов живой гриппозной вакцины H2N2 на основе донора аттенуации A/Ленинград/17 и диких вирусов A/Singapore/1/57 (H2N2) с α2,3- и α2,6-рецепторной специфичностью. Мы провели серийное пассирование этих вирусов на клетках MDCK и проанализировали ростовые свойства изолированных методом бляшек клонов *in vitro* и *in vivo*, а также их иммуногенность и перекрёстную реактивность в мышиной модели.

Результаты. Адаптация к клеткам MDCK значительно увеличивала титры вирусов в клетках MDCK, однако на их рецепторную специфичность это не влияло. Вирусы с α2,6-рецепторной специфичностью вызывали образование более высоких титров гомологичных антител по сравнению с вирусами со специфичностью к α2,3-рецепторам, но эти антитела могли реагировать только с вирусами α2,6. Напротив, антитела, индуцированные вирусами с α2,3-рецепторной специфичностью, обладали широкой реактивностью против всех изученных вирусов. Аналогичные результаты были получены для пары штаммов живой гриппозной вакцины H2N2 на основе донора аттенуации A/Ленинград/17 с α2,3- и α2,6-рецепторной специфичностью при их изучении на сирийских хомячках.

Заключение. В случае новой передачи вирусов птичьего гриппа H2N2 в человеческую популяцию и совместной циркуляции вирусов с обеими рецепторными специфичностями для создания кросс-реактивных гриппозных вакцин следует выбирать вариант с α2,3-специфичностью.

Ключевые слова: *вирус гриппа, H2N2, рецепторная специфичность, адаптация, культура клеток MDCK, живая гриппозная вакцина, иммуногенность*

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Introduction

Avian influenza is a zoonotic infection that poses a high risk to humans due to the high mortality rate, which reaches 60% when infected with highly pathogenic subtypes H5N1, H7N9, H5N6 and H10N8 [1–4]. Asian influenza subtype H2N2 appeared in Singapore in February 1957 and rapidly caused a pandemic that claimed more than 2.7 million lives. The H2N2 influenza pandemics in 1957 and H3N2 in 1968 are known to have been caused by a switch in the receptor specificity of the virus from avian sialic receptor α2,3 to human α2,6, with influenza viruses with both types of receptor specificity circulating simultaneously at the beginning of the pandemic wave [5, 6]. Since birds are the main reservoir and carry almost all known subtypes of influenza A virus, including H2N2, the risks of these viruses returning to circulation among humans are estimated to be quite high [7]. Given the decline in population immunity to H2N2 viruses due to their prolonged absence from circulation, scientists around the world are calling for vaccination campaigns against these viruses to be launched in advance, without waiting for the onset of the pandemic [8].

Influenza vaccine prophylaxis is known to be the optimal method of controlling this infection, and there are many influenza vaccines available for seasonal administration. However, in pandemic settings, live attenuated influenza vaccine (LAIV) is considered the most effective [9, 10]. The vast majority of influenza vaccines in the world are produced in embryonated chicken eggs, but in recent decades there has been an active discussion of transferring the production of influenza vaccines to mammalian cell cultures, which would allow for the short-term production of large amounts of viral biomass, as well as narrowing the list of contraindications, in particular, allowing the vaccine to be administered to persons allergic to chicken protein [11]. Furthermore, if an influenza pandemic is caused by a highly pathogenic virus, there is a high probability that the chicken population in poultry farms will be completely destroyed, so the independence of vaccine production from the supply of eggs from poultry farms is also extremely important. Thus, it is advisable to transfer influenza vaccine production to MDCK cell culture, since numerous studies show that it is in this cell culture that vaccine strains of LAIV are able to replicate to titers comparable to those of eggs [12–14].

The main **aim** of this study was to find the most promising variant of the vaccine strain of LAIV A(H2N2) culture that should be used at the beginning of the pandemic wave. For this purpose, two variants of the pandemic virus strain A/Singapore/1/57 (H2N2) differing in receptor specificity and LAIV strains prepared on their basis were studied. Adaptation of the viruses to MDCK cell line was performed, followed by cloning by the plaque assay and evaluation of the receptor specificity of the isolated virus variants. The variants differing in the *HA* gene sequences were used for immunization of laboratory animals to reveal the potential effect of adaptive mutations in virus surface proteins on immunogenicity, antigenicity and cross-reactivity of antibodies produced after immunization.

Materials and methods

Viruses

Two variants of pandemic virus strain A/Singapore/1/57 (H2N2) obtained from the collection of the Department of Virology of Institute of Experimental Medicine were used, which differed in sensitivity to nonspecific serum inhibitors. Experiments with live H2N2 viruses were conducted in the laboratory with a biosafety level of BSL-3.

Production of LAIV strains by reverse genetics methods

Hemagglutinin and neuraminidase genes were cloned into the pCIPolISapIT vector for reverse genetics using universal primers specific for each gene in reverse transcription polymerase chain reaction [15]. A set of 6 plasmids with bidirectional reads encoding internal proteins of the attenuation donor A/Leningrad/134/17/57 (H2N2) was prepared previously [16]. Live influenza viruses were obtained by electroporation of Vero cells using the Neon transfection system (Invitrogen) and the accompanying 100 μl Neon Kit.

Hemagglutination reaction

The hemagglutination assay (HA) was performed according to the classical scheme using chicken red blood cells (RBC)[1]. To investigate the receptor specificity of influenza viruses, we used a modified HA with the exo-α-Sialidase enzyme (*Salmonella typhimurium*) (Megazyme), which cleaves exclusively α2,3-receptors from the RBC surface. Horse RBC, which express only α2,3-receptors on their surface; untreated chicken RBC, which express both types of receptors; and chicken RBC treated with exosialidase for 1 h at 37ºC, i.e., carrying only α2,6-receptors on their surface, were used for HA.

The virus was considered to have α2,3-receptor specificity if its titer in HA with horse and chicken RBC was the same and its titer in HA with treated chicken RBC was 0. Otherwise, the virus was considered to have α2,6-receptor specificity. If the titer was positive in all HA, the virus was considered to have dual receptor specificity with preference for the type with the higher titer in HA.

Growth of viruses and determination of infectious titer

To accumulate influenza viruses in 10–11-day-old embryonated chicken eggs, that were infected with 0.2

[1] WHO. Manual for the laboratory diagnosis of virological surveillance of influenza. Geneva;2011. URL: https://www.who.int/publications/i/item/manual-for-the-laboratory-diagnosis-and-virological-surveillance-of-influenza

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ml of viral material, after which the eggs were incubated for 48–72 h at 33–37ºC. Virus propagation in MDCK cells was performed on a daily monolayer with 90–95% confluency grown in DMEM medium supplemented with 1× antimycotic antibiotic (Gibco) and 10% fetal bovine serum (Biolot) at 37ºC in a thermostat containing 5% CO_2. To infect MDCK cells, the prepared monolayer was washed twice with a warm solution of phosphate buffered saline(PBS), after which the viral suspension was added in volumes of 1, 2, 3 ml into vials T-25, T-75 and T-175, respectively. After incubation for 1 h at 33ºC for vaccine strains and 37ºC for wild-type influenza viruses in a thermostat containing 5% CO_2, the inoculum was removed and DMEM medium with 1×antibiotic-antimycotic and 1 μg/mL TPCK trypsin (Sigma-Aldrich Co.) was added. After 72 h of incubation at 33ºC or 37ºC, the cytopathic effect (CPE) of the virus was visually assessed and its titer was determined by HA.

Infection titers of viruses in both culture systems were determined by the limit dilution method. Eggs were infected with 10-fold serial dilutions of viruses in 200 μL PBS and incubated at 33ºC and 37ºC for 48 h, after which the presence of virus was determined by HA with chicken erythrocytes. Titer determination in MDCK cells was performed on 96-well plates with a daily monolayer, and serial 10-fold dilutions were prepared on DMEM medium with antibiotic-antimycotic and 1 μg/mL of TPCK trypsin. After adsorption, the inoculum was removed, cells were washed and incubated in maintenance medium for 3 days. The presence of viruses in the wells was determined by HA with chicken RBC. Virus titers in eggs and MDCK cells were calculated according to the method of Reed and Mench [17] and expressed in 50% embryonic (log10 EID_{50}/mL) and tissue cytopathogenic (log10 $TCID_{50}$/mL) infectious doses.

Adaptation of viruses to MDCK cells

Adaptation of influenza viruses to MDCK cells was performed by sequential 5-fold passaging of strains followed by virus cloning by the plaque assay and isolation of viral clones from individual plaques. For this purpose, 10-fold dilutions of viruses in 2 repeats were applied to 6-well plates seeded the day before with MDCK cells. After one hour of contact with regular shaking, the inoculum was removed, and 3 ml of agarose coating obtained by mixing equal volumes of 2×DMEM medium (in the presence of AbAm and 2 μg/ml TPCK trypsin) and 1.6% fusible agarose (Lonza) were added to the wells. On day 3-5 of incubation, viral plaques were visually assessed, 20–30 well-separated plaques were isolated on limiting dilutions, a separate virus clone was isolated from each individual plaque, which was grown on MDCK cells. Complete nucleotide sequences of surface protein genes were obtained by the Sanger method using the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Thermo).

Experiments with laboratory animals

In animal experiments, mice of the CBA line and Syrian hamsters (Stolbovaya, Russia) were used. The study was approved by the Ethical Committee of the Institute of Experimental Medicine (protocol No. 1/20 of 27.02.2020).

To evaluate the immunogenicity of wild influenza viruses with different receptor specificity, female mice of the CBA line were infected intranasally at a dose of 10^5 EID_{50}/animal. After 21 days, the animals were euthanized, after which blood sera and upper respiratory tract (URT) washes were collected to determine the level of humoral immune response to different virus variants. To evaluate the immunogenicity of the vaccine strains of H2N2 LAIV, Syrian hamsters were immunized intranasally at a dose of 10^5 EID_{50}/animal, twice 21 days apart. On the 21st day after the 2nd immunization, animals were euthanized, blood serum, URT washes and bronchoalveolar lavage were collected.

Immunological methods

The study of animal blood sera in hemagglutination inhibition assay (HAI) was performed according to the standard protocol[2] with chicken RBC and treatment of sera with receptor-destroying enzyme (RDE, Denka). The last dilution at which complete inhibition of hemagglutination assay counted as the serum titer in HAI.

Enzyme immunoassay

An enzyme-linked immunosorbent assay (ELISA) with animal samples was performed using influenza viruses, purified in sucrose density gradient, as antigen. Antigen was added to 96-well high sorption plates (Corning) at 16 AU in 50 μL and incubated overnight at 4ºC. The plates were washed 3 times with wash buffer (phosphate-salt buffer + 0.05% Twin-20 (Biolot)), after which unbound sites were blocked with 1% bovine serum albumin.

Double dilutions of sera or respiratory tract washes were prepared in separate round-bottom plates, which were then transferred to the wells of the plate washed from the blocking solution. After incubation for 1 h at 37°C, the plates were again washed 3 times with wash buffer, dried and horseradish peroxidase-conjugated secondary antibodies: mouse anti-IgG (1:10 000), mouse anti-IgA (1:2000), hamster anti-IgG (1:5000) and hamster anti-IgA (1:300) were added. The plate was incubated for 1 h at 37ºC, after which the plate was washed 5 times with wash buffer, dried and 50 μl/well of TMB substrate (Thermo) was added and incubated in the dark for up to 20 min at room temperature. The

[2] WHO. Manual for the laboratory diagnosis of virological surveillance of influenza. Geneva;2011. URL: https://www.who.int/publications/i/item/manual-for-the-laboratory-diagnosis-and-virological-surveillance-of-influenza

reaction was stopped by adding 50 µl of 1M H_2SO_4. The primary ELISA results were measured on a spectrophotometer (Bio-Rad) at a wavelength of 450 nm. The last dilution at which the optical density exceeded twice the average of the control wells was taken as the antibody titer. The area under the optical density curve was calculated using the GraphPad Prism 7 software package.

Statistical processing of data

For data comparison, non-parametric Mann-Whitney U-test; Student's t-test and ANOVA using GraphPad Prism 7 software were used. Differences were considered reliable at $p < 0.05$.

Results

Two variants of the pandemic strain A/Singapore/1/57 (H2N2) were recovered from ampoules of viral material lyophilized in 1975, and the exact passaging history of the viruses is unknown. The viruses was grown in eggs differed in their level of sensitivity to nonspecific guinea pig serum inhibitors. Whole-genome sequencing showed that these viruses had amino acid differences at positions 156 (E/K), 226 (Q/L), and 228 (G/S) in the HA1 subunit (**Table**). According to the literature, substitutions at positions 226 and 228 are responsible for the receptor specificity of influenza virus [5, 6]. It is without a doubt that the evaluation of the affinity of these viruses for receptors on the erythrocyte surface in HA with different types of RBC showed that the virus with amino acid residues E156, Q226 and G228 has α2,3-receptor specificity (designated Sing-α2,3), while the variant with residues K156, L226 and S228 has affinity for α2,6-receptors (Sing-α2,6) (**Fig. 1**, *a*). The amino acid substitution *K19T* was found in the neuraminidase molecule, but since it is located in the transmembrane domain, it has no effect on receptor specificity (Table). Adaptation of the studied viruses to MDCK cells and subsequent cloning by plaque allowed the isolation of 3 additional virus variants with different HA sequences: Sing-α2,6-EP with *G158E* and *L321P* mutations in the HA1 subunit, Sing-α2,3-S with *P221S* mutation in the HA1 subunit, and Sing-α2,3-V with *A96V* mutation in the HA2 subunit (Table).

Examination of MDCK-adapted variants in HA showed that the Sing-α2.6-EP strain has affinity for α2,6 receptors, while the Sing-α2,3-S and Sing-α2,3-V variants have affinity for α2,3 receptors (Fig. 1, *a*). Thus, adaptation of wild-type H2N2 influenza viruses

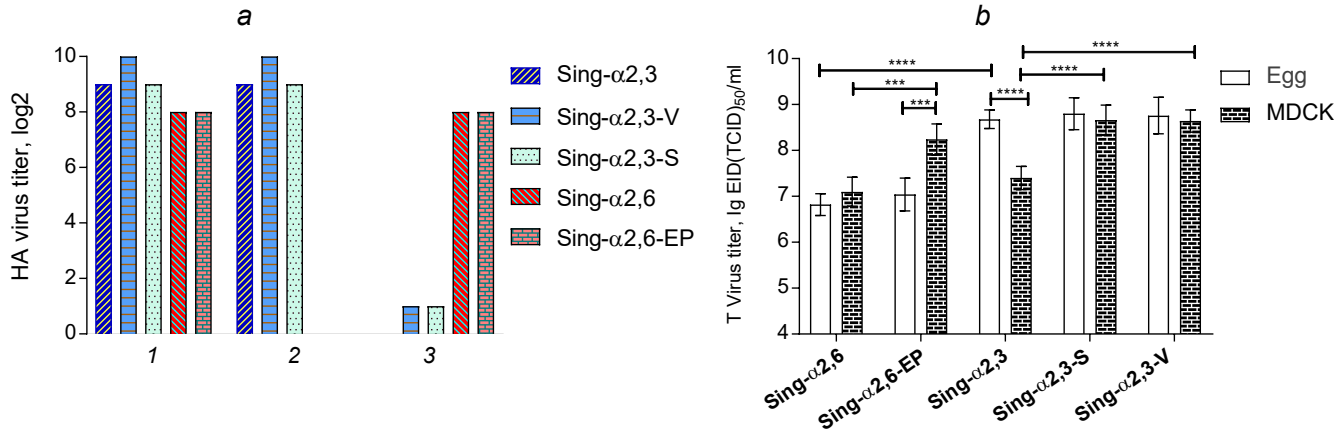

Fig. 1. Characterization of the studied viruses *in vitro*.

a — titers of viruses in HA with untreated chicken erythrocytes (*1*), horse erythrocytes (*2*) and chicken erythrocytes treated with exosialidase (*3*); *b* — infectious activity of the studied viruses in the Egg and MDCK cells.

Amino acid substitutions in surface proteins of investigated variants of A/Singapore/1/57 (H2N2) virus

Virus	HA							NA
	HA1						HA2	
	156	158	221	226	228	321	96	19
Original strains accumulated in the Egg								
Sing-α2,6	Lys	Gly	Pro	Leu	Ser	Leu	Ala	Thr
Sing-α2,3	Glu	Gly	Pro	Gln	Gly	Leu	Ala	Lys
MDCK-adapted strains								
Sing-α2,6-EP	Lys	Glu	Pro	Leu	Ser	Pro	Ala	Thr
Sing-α2,3-S	Glu	Gly	Ser	Gln	Gly	Leu	Ala	Lys
Sing-α2,3-V	Glu	Gly	Pro	Gln	Gly	Leu	Val	Lys

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to mammalian cell culture does not significantly affect their receptor specificity.

In addition, the infectious activity of all viruses in eggs and MDCK cells was studied. It was found that Sing-α2,3 virus was on average 2 orders higher in eggs than Sing-α2,6 virus, while their titers in MDCK cells were comparable (Fig. 1, *b*). Importantly, the infectious activity of MDCK-adapted variants in cell culture was significantly higher than that of the corresponding original viruses. Thus, the contribution of mutations *G158E* and *L321P*, *P221S* in the HA1 and *A96V* in the HA2 subunits to the increase in the infectious titer of the virus in MDCK cells was observed (Fig. 1, *b*).

Immunization of CBA mice with the original Sing-α2.6 and Sing-α2,3 strains and MDCK-adapted variants induced higher levels of homologous serum antibodies to Sing-α2.6 and Sing-α2.6-EP viruses compared with viruses with affinity for α2.3 receptors (**Fig. 2**, *a*, *b*). At the same time, cell-adapted Sing-α2,3-S and Sing-α2,3-V variants induced significantly lower levels of homologous serum IgG antibodies compared with the original Sing-α2,3 variant (Fig. 2, *b*). Examination of the local humoral immune response showed significant increases in secretory IgA antibodies in all 5 viruses tested: Sing-α2,6, Sing-α2,6-EP, Sing-α2,3, Sing-α2,3-S and Sing-α2,3-V (Fig. 2, *c*). Comparison of IgA antibody levels in the original Sing-α2,6 and Sing-α2,3 virus groups showed no differences ($p = 0.3355$), which is in contrast to the data on the systemic humoral response. Nevertheless, a comparative analysis of the levels of secretory IgA antibodies between groups of animals infected with MDCK-adapted variants of Sing-α2,6-EP, Sing-α2,3-S, and Sing-α2,3-V, as well as in the systemic response, revealed the superiority of Sing-α2,6-EP virus. Summarizing the data on the induction of systemic and local immune response by the studied viruses, we can conclude that all the viruses induce humoral response, but the MDCK-adapted variants with α2,3 receptor specificity (Sing-α2,3-S and Sing-α2,3-V) were the least immunogenic at the level of both systemic and local humoral immunity. On the contrary, Sing-α2,6, Sing-α2,6-EP viruses with α2,6 receptor specificity did not differ in immunogenicity in any of the tests.

Further, the cross-reactivity of serum antibodies produced by administration of 5 tested variants of A/Singapore/1/57 (H2N2) virus was evaluated against each variant in HAI and ELISA. Interestingly, when

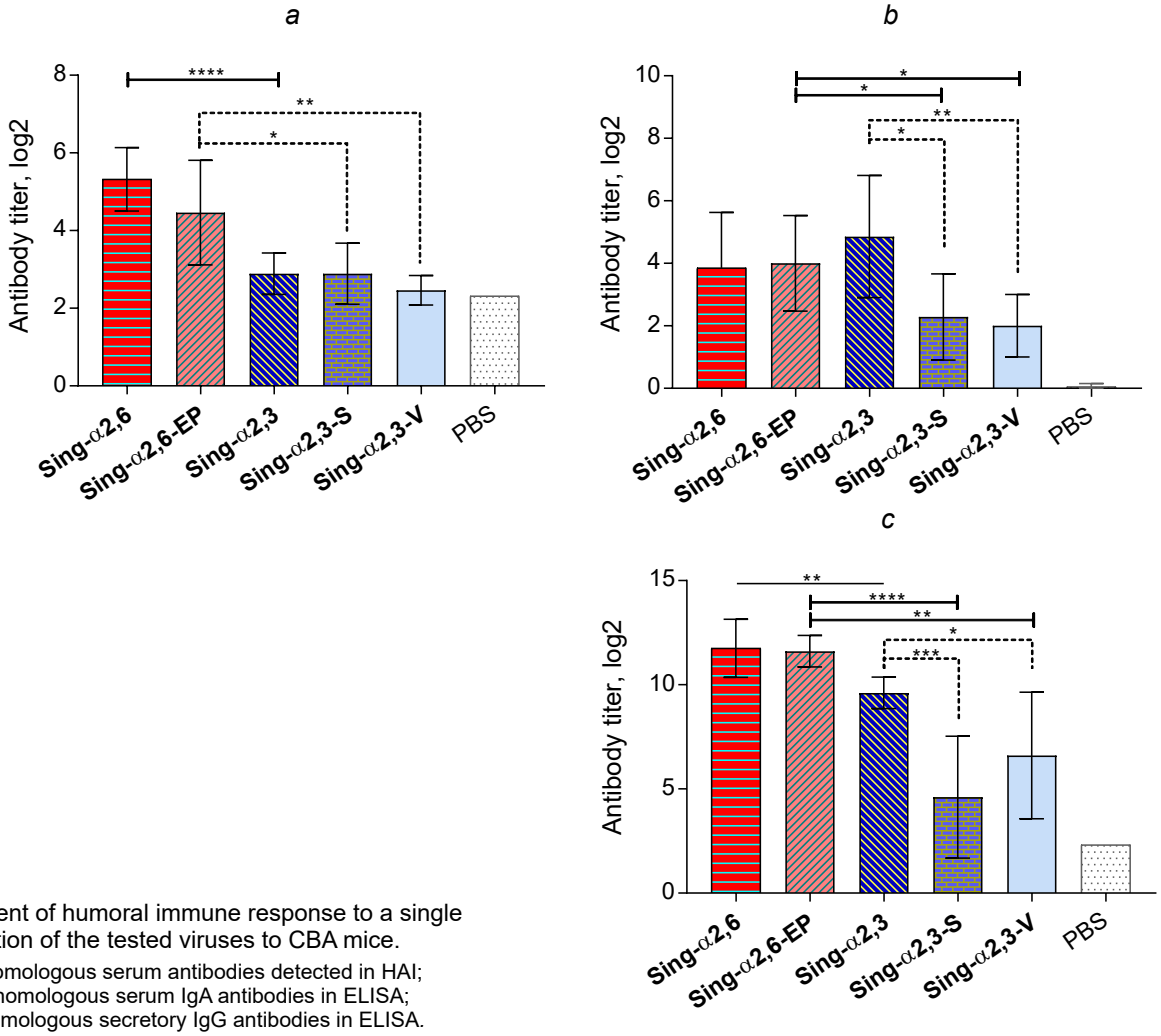

Fig. 2. Assessment of humoral immune response to a single administration of the tested viruses to CBA mice.

a — level of homologous serum antibodies detected in HAI;
b — level of homologous serum IgA antibodies in ELISA;
c — level of homologous secretory IgG antibodies in ELISA.

viruses with α2-6-receptor specificity were used as antigens in HAI, significantly higher antibody titers were detected in all immunized groups compared with antigens with α2-3 specificity (**Fig. 3**, *a*). However, antibodies in the sera of the Sing-α2.6 group did not bind any of virus variants with α2-3-receptor specificity. Although antibody titers above the detection level were detected in mice immunized with the MDCK-adapted Sing-α2,6-EP variant, no statistically significant differences compared to the control group were found. The study of cross-reactivity of serum IgG antibodies by ELISA showed similar results: viruses with α2-6-receptor specificity as antigens revealed the highest values of antibody titers in all groups, whereas antibody binding to viruses with α2,3-receptor specificity was significantly weaker in animals of all groups (Fig. 3, *b*).

It is important to note that viruses with α2-6-receptor specificity as antigen bound antibodies equally well in the sera of mice from all groups studied, except for the group of animals immunized with Sing-α2,3-V virus, in which antibody titers to Sing-α2,6 and Sing-α2,6-EP viruses differed significantly from those in the other groups (**Fig. 3**, *a*). The opposite situation was observed in groups of animals immunized with MDCK-adapted variants with α2-3-receptor specificity: IgG antibody titers to Sing-α2,3-S and Sing-α2,3-V viruses were reduced in all immunized groups; while the *P221S* substitution in the HA1 subunit could be characterized as an escape mutation, since Sing-α2,3-S virus escapes antibody recognition most efficiently in all immunogenic variants studied (Fig. 3, *b*).

Overall, the results indicate that variants with α2-3-receptor specificity adapted to or isolated on MDCK cell culture are the most suitable viruses for the production of a cell-based H2N2 pandemic vaccine.

To confirm this hypothesis, we constructed two LAIV strains H2N2 subtype based on the attenuation donor A/Leningrad/134/17/57: A/17/Singapore/57/1 with *HA* and *NA* genes from Sing-α2,3 virus (designated as 17/Sing-α2,3) and A/17/Singapore/57/2 with *HA* and *NA* genes from Sing-α2,6 virus (17/Sing-α2,6) by reverse genetics methods. HA assay with different RBC confirmed the receptor specificity of the resulting vaccine viruses, which matched that of the corresponding wild-type virus (**Fig. 4**, *a*), further indicating the key role of hemagglutinin in binding to host cell glycan receptors. Importantly, adaptation of the vaccine strains to MDCK cells did not result in new mutations in the HA molecule: more than 50 viruses isolated from the plaques matched to the original strain of LAIV. Evaluation of the infectious activity of the engineered viruses showed that the vaccine strains 17/Sing-α2,3 and 17/Sing-α2,6 multiplied equally well in MDCK cells, whereas in eggs, the 17/Sing-α2,3 virus had an infectious titer an order higher than that of the 17/Sing-α2,6 virus (Fig. 4, *b*).

Twofold immunization of Syrian hamsters with reverse-genetic vaccine strains 17/Sing-α2,3 and 17/Sing-α2,6 resulted in similar levels of serum IgG antibodies, binding to the Sing-α2,3 antigen, with the 17/Sing-α2,3 vaccine virus inducing significantly more antibodies to the Sing-α2,6 antigen than to its own Sing-α2,3 antigen (**Fig. 5**). The results obtained are in full compliance with the results of studying the antigenicity of pandemic variants of A/Singapore/1/57 in an experiment on mice (Fig. 3).

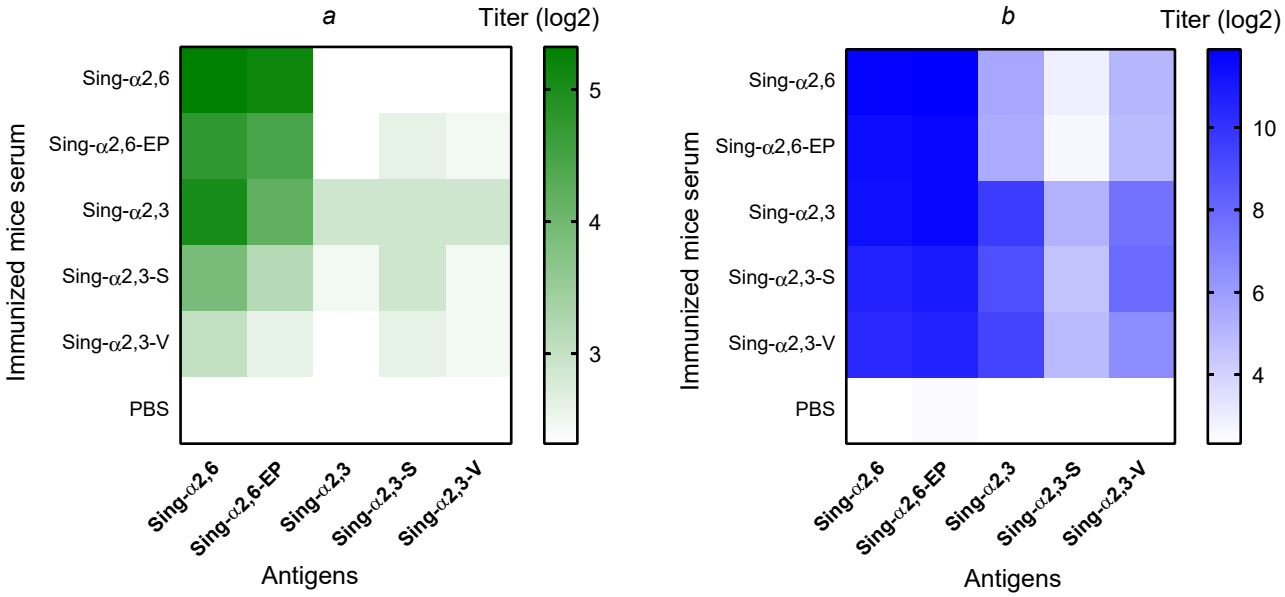

Fig. 3. Heat map of the immunogenicity and cross-reactivity of investigated variants of A/Singapore/1/57 (H2N2) virus in the experiment on mice (n = 7).

a — mean values of the levels of anti-hemagglutinating antibodies in all immunized groups against different viral antigens; *b* — mean values of the levels of serum IgG antibodies in all immunized groups against different viral antigens.

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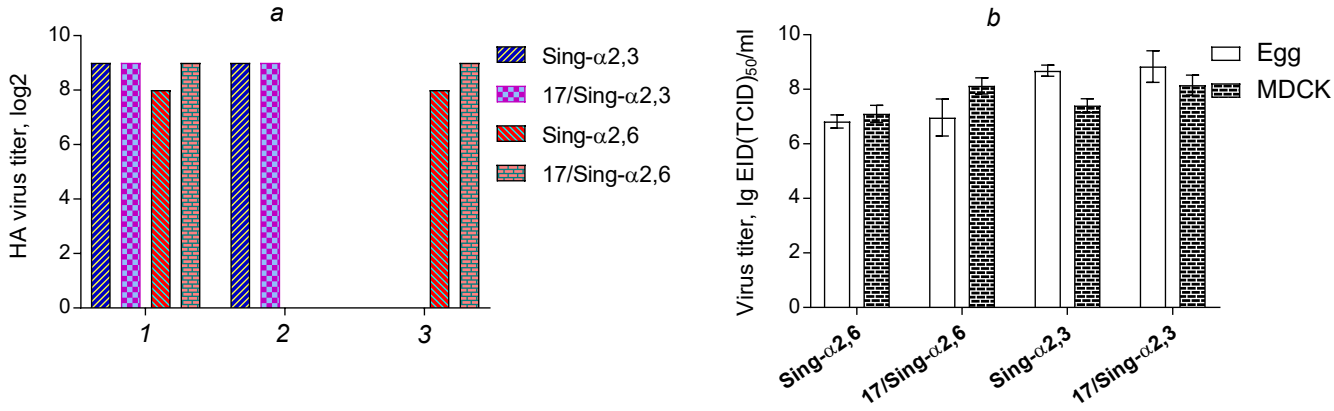

Fig. 4. Characterization of the investigated vaccine strains of H2N2 LIV *in vitro*.
a — virus titers in HA assays with untreated chicken erythrocytes (*1*), horse erythrocytes (*2*), chicken erythrocytes treated with exosialidase (3); *b* — infectious activity of the studied viruses in the Egg system and MDCK cell culture.

The cross-reactivity of local IgA antibodies in washes from the upper and lower respiratory tracts in ELISA with the same viral antigens was investigated. Using the Sing-α2,6 virus as an antigen revealed significantly higher levels of secretory virus-specific antibodies compared with the use of Sing-α2,3 virus as an antigen (Fig. 5).

Thus, it follows from the results presented that H2N2 subtype influenza viruses with α2-3-receptor specificity induce antibodies with broader cross-reactivity against viruses with different receptor specificity compared with antibodies induced by viruses with affinity for α2-6-receptors. This phenomenon should be taken into account when selecting a strain for vaccine preparation in the event of the onset of an H2N2 influenza pandemic.

Discussion

Influenza A(H2N2) viruses circulated in the human population from 1957 to 1968, after which they were replaced by A(H3N2) viruses that caused the Hong Kong influenza pandemic [18]. Since H2N2 viruses have not infected humans for more than 50 years, population immunity to these viruses is extremely low, and people born after 1968 are the most vulnerable group if H2N2 viruses return to circulation [19]. Given the persistence of influenza viruses with hemagglutinin H2 in the natural reservoir [20–22], the probability of a new H2N2 influenza pandemic is estimated to be high [7]. In this regard, research aimed at the development and detailed study of potentially pandemic vaccines against viruses of this subtype is an urgent and important area.

We have previously developed and researched in preclinical and clinical studies an LAIV against the H2N2 virus circulating at the end of the pandemic wave, A/California/1/66 (H2N2) [23, 24], and this vaccine can be used to immunize the most vulnerable populations in case antigenically similar H2N2 viruses return to circulation. However, results of avian influenza virus monitoring show that most H2N2 subtype isolates remain antigenically similar to the pandemic A/Singapore/1/57 virus and retain a preference for avian-type sialic α2,3 receptors [7]. Detailed studies on the effect of receptor specificity of viruses on their transmissibility in ferret experiments have shown that switching the receptor from α2,3- to α2,6-type significantly increases the ability of the virus transmission by airborne droplets, which may play a crucial role in the pandemic spread of H2N2 viruses [25]. However, there is no clear data on virus strain is best used for vaccine preparation at the onset of a pandemic caused by avian influenza viruses with affinity for both types of cellular receptors.

In the present study, we performed a model experiment with pandemic variants of the A/Singapore/1/57 virus that circulated in 1957 and differed in the receptor specificity of the HA molecule, which was determined by 3 amino acid differences in the HA1 subunit: E156, Q226, G228 for the Sing-α2,3 variant and K156, L226, S228 for the Sing-α2,6 variant. Since it is advisable to produce pandemic vaccines on mammalian cell lines to improve the quality of the product and the possibility of accelerated scaling of production [11, 26], we performed serial passaging of both the original pandemic viruses Sing-α2,3 and Sing-α2,6 and reassortant vaccine strains of LAIV prepared on their basis in MDCK cells, followed by identification of new substitutions in the HA molecule. Interestingly, adaptation mutations occurred in passages in cells of only pandemic viruses, but not vaccine strains of LAIV. This may be due to the fact, that the pandemic variants represented a heterogeneous virus population with an unknown passage history, whereas the reverse-genetic vaccine viruses underwent only two passages in eggs after assembly from plasmids and represented a more homogeneous population. It is important to note that the mutations we found did not affect the receptor specificity of the viruses, but did affect their antigenicity. In particular, the *P221S* mutation in the HA1 subunit had the character of an escape

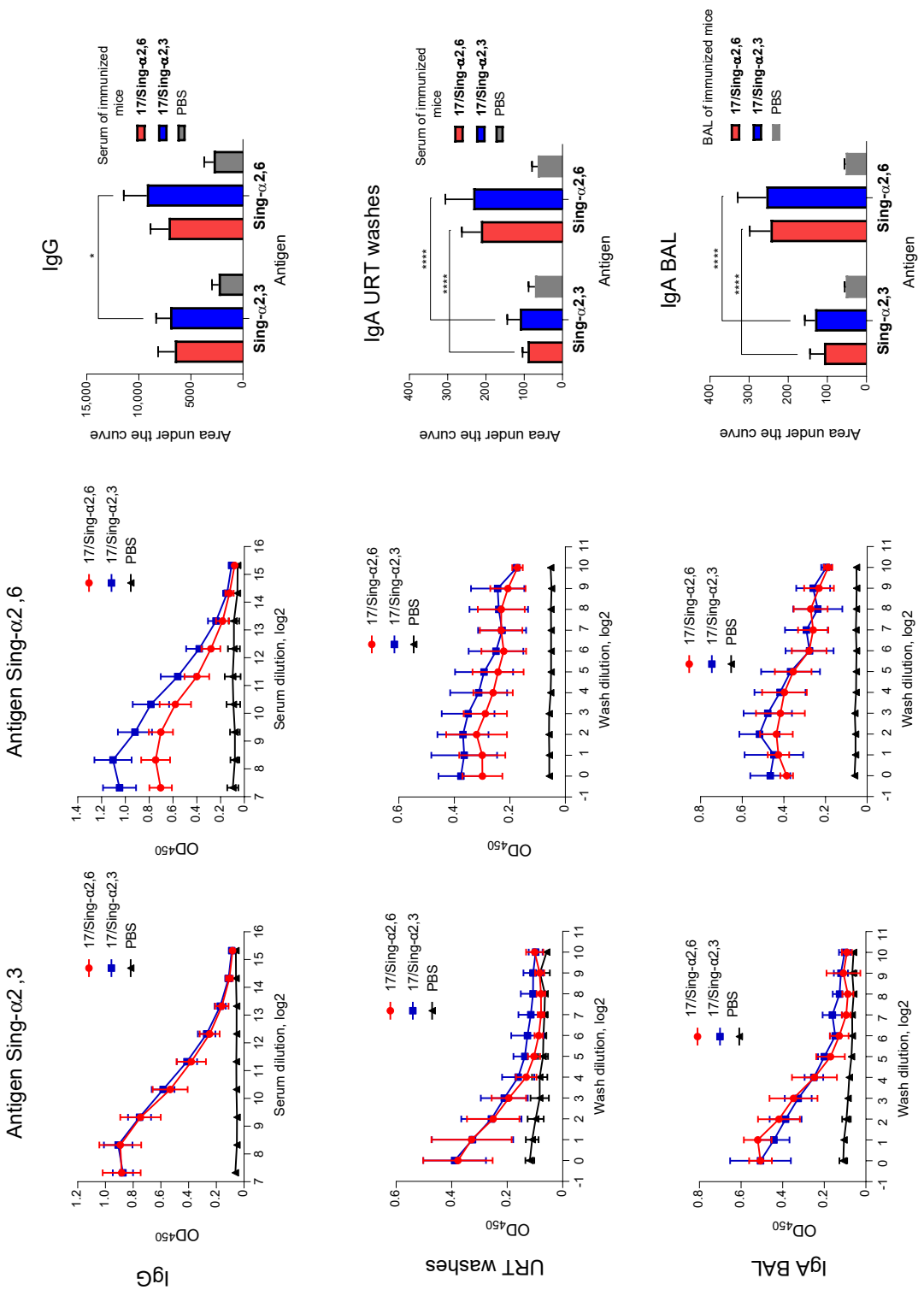

Fig. 5. Evaluation of the humoral immune response to twice-daily administration of the tested vaccine strains of LIV to Syrian hamsters.
On the left are OD_{450} values at different dilutions of sera or washes from URT and bronchoalveolar lavage to Sing-α2,3 virus in ELISA. In the middle are OD_{450} values at different dilutions of sera or washes to Sing-α2,6 virus in ELISA. On the right are the area under the optical density curve at 450 nm in ELISA with the corresponding antigen.

mutation, as it avoided recognition by antibodies in the sera of animals immunized with all A/Singapore/1/57 variants studied. Interestingly, a similar mutation has been described for avian influenza viruses of the H9N2 subtype, whereby it reduced the affinity of the virus for the avian α2,3-receptor analog, but in combination with the *L226Q* mutation, the affinity for this receptor was restored [27], which is in complete compliance with our results. Furthermore, the *P221S* mutation was detected in the A/Wyoming/3/2003 (H3N2) virus during its serial passaging in MDCK cells [28], which also confirms the adaptive nature of this substitution.

Conclusion

The most important result of the study is the demonstration of broader cross-reactivity of antibodies produced by intranasal immunization of animals with H2N2 viruses with α2,3-receptor specificity. This was shown both for pandemic A/Singapore/1/57 viruses and for vaccine reassortant strains of H2N2 obtained by reverse genetics. It is important to note here that the vaccine strain 17/Sing-α2,3 is suitable for cell culture production because it achieves a high infectious titer in MDCK cells, and the homogeneous nature of the strain due to its preparation by genetic engineering methods will ensure the genetic stability of the vaccine during serial passages on MDCK cells, which evidences in favor of mass production of the vaccine in the first wave of the pandemic. Furthermore, this choice of strain for the production of pandemic cell-derived LAIV will maximize the reproduction of the vaccine strain in MDCK cells, as well as ensure high vaccine efficacy due to full antigenic coverage of circulating influenza viruses in the case of a pandemic.

Although our hypothesis has been experimentally confirmed in various animal models, the assessment of immunogenicity and cross-reactivity of the 17/Sing-α2,3 LAIV strain in clinical trials is required for its potential widespread use in humans. One of the obstacles to the use of the 17/Sing-α2,3 strain of LAIV in clinical practice may be the potentially reduced replicative activity of the virus in the human respiratory tract, since in humans α2,3 receptors are poorly represented in the respiratory tract and are predominantly expressed in the lower respiratory tract [29], where the vaccine virus does not replicate due to its temperature-sensitive phenotype, and this may result in low immunogenicity of LAIV. However, experience with human immunization with LAIV against avian influenza H5N1, the infectious agent of which also has α2,3-receptor specificity, has shown that even in the absence of replication in the URT and at low levels of serum antibodies to the virus after intranasal immunization, LAIV produces a long-term immune response that can be de-masked by administration of inactivated influenza vaccine several months or even years later [30, 31]. Accordingly, the strategy of heterologous prime-boost immunization at the beginning of the H2N2 influenza pandemic can also be considered as the most promising for the formation of a powerful long-term humoral immunity with a broad spectrum of protection.

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Prevalence, spectrum, and the relations between short-term and long-term post-acute sequelae of COVID-19 in children

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Abstract

Background. Data on the prevalence of post-COVID-19 condition (PCC) in children differ due to the lack of specific diagnostic criteria, reliable biomarkers, and limitations of many studies. It is important to study the association between PCC, previous functional disorders, and any events in the post-acute period of COVID-19 to estimate the true burden of PCC in children.

The aim. To determine of the prevalence of PCC in relation to initial functional disorders in children during the year after a mild form of COVID-19 and acute respiratory tract infections (ARTI) of other etiologies.

Materials and methods. The prospective study using a continuous sampling method included children under 18 years of age hospitalized with suspected COVID-19, in whom COVID-19 was confirmed by RT-PCR (n = 121) or excluded by molecular and serological methods (ARTI group, n = 105). Information on patients was collected from September 2020 to December 2021 by questionnaires at index points: hospital discharge and after 1, 3, 6 and 12 months. Severe COVID-19 and patients with chronic diseases were excluded. Functional disorders in the anamnesis and any events associated with health disorders during the year of observation, the severity of symptoms were taken into account. The analysis was performed in groups of children < 6 years and ≥ 6 years of age. Symptoms were grouped into clusters.

Results. Any symptoms were identified with a frequency of 32–39% (in children < 6 years and ≥6 years, respectively). The most common were psycho-emotional, respiratory and autonomic dysfunction (AD) cluster. Symptoms in most cases were mild with a duration of 3–6 months. After adjustment for age, AD history and recurrent ARTI episodes, no difference was found in the symptom clusters prevalence between confirmed and excluded COVID-19, except for hyposmia, the incidence of which in children ≥6 years decreased from 14.3% at the first index point to 2.4% after one year. Among patients without an AD history and without recurrent ARTI during the year, *de novo* PCC was a rare phenomenon (2.7–8.0%) without differences between COVID-19 and controls. The risks of PCC were significantly increased in patients with an AD history (adjusted OR at 1 month 3.19 (95% CI 1.89–5.38), at 6 months 3.33 (95% CI 1.81–6.15)). Multiple (5–25) and persistent (at all index points) symptoms rated by patients as significant occurred *de novo* rarely (1.7% (95% CI 0.4–4.9)), but significantly more often in children with an AD history — 25%) 95% CI 13.6–39.6%) (difference 23.3% (10.9–35.7%), OR 14.8 (4.4–50.6), p < 0.001). Recurrent episodes of ARTI were an important risk factor for an increase in cognitive cluster complaints and vagotonic AD after 3–6 months of observation.

Conclusion. The results obtained indirectly support the concept that PCC is a somatoform functional disorder, probably of sociogenic nature, in patients who are anxious at baseline. Some patients with the COVID-19 and ARTI consequences really need medical and psychosocial rehabilitation. The study of PCC provides new insights into the consequences of widespread respiratory viral infections.

Keywords: *children, adolescents, COVID-19, post-COVID-19 condition, postacute sequelae of SARS CoV-2, long COVID-19, autonomic dysfunction, psycho-emotional disorders, somatic symptoms, somatoform disorders*

Ethics approval. The study was conducted with the voluntary informed consent of the legal representatives of minor patients. The study protocol was approved by the Ethics Committee of the Moscow Regional Research and Clinical Institute (protocol No. 10, September 1, 2020).

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Распространённость, спектр и взаимосвязи краткосрочных и долгосрочных последствий COVID-19 у детей

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Аннотация

Введение. Данные о распространённости состояния после COVID-19 (Post-COVID-19 condition, PCC) у детей различаются из-за отсутствия конкретных диагностических критериев, надёжных биомаркеров и ограничений многих проведённых исследований. Важно изучить связь между PCC, функциональными расстройствами в прошлом и любыми событиями после перенесённого COVID-19, чтобы оценить истинное бремя PCC у детей.

Цель — определение распространённости PCC во взаимосвязи с исходными функциональными расстройствами у детей в течение года после нетяжёлой формы COVID-19 и острой респираторной вирусной инфекции (ОРВИ) другой этиологии.

Материалы и методы. В проспективное исследование методом сплошной выборки включены дети младше 18 лет, госпитализированные в больницу с подозрением на COVID-19, у которых COVID-19 был подтверждён в полимеразной цепной реакции с обратной транскрипцией (*n* = 121) или исключён молекулярным и серологическим методами (группа ОРВИ; *n* = 105). Информация о пациентах собрана с сентября 2020 г. по декабрь 2021 г. путём анкетирования в индексных точках: при выписке из больницы и через 1, 3, 6 и 12 мес. Тяжёлые формы COVID-19 и пациенты с хроническими заболеваниями были исключены. Учитывали функциональные расстройства в анамнезе и любые события, связанные с нарушением здоровья, в течение года наблюдения, выраженность симптомов. Анализ проведён в группах детей < 6 лет и ≥ 6 лет. Симптомы были сгруппированы в кластеры.

Результаты. Симптомы определены с частотой 32% у детей < 6 лет и 39% у детей ≥ 6 лет. Наиболее распространёнными были психоэмоциональный, респираторный кластеры и синдром вегетативных дисфункций (СВД). Симптомы в большинстве случаев были лёгкими с продолжительностью 3–6 мес. После поправки на возраст, СВД в анамнезе и повторные эпизоды ОРВИ не установлено разницы распространённости симптомов между подтверждённым и исключённым COVID-19, кроме гипосмии, частота которой у детей ≥ 6 лет уменьшилась с 14,3% при выписке из больницы до 2,4% через год. Среди пациентов без истории СВД в прошлом и без повторных ОРВИ в течение года PCC *de novo* было редким явлением (2,7–8,0%) без отличий между COVID-19 и ОРВИ другой этиологии. Риски PCC были существенно повышены у пациентов с СВД в анамнезе: скорректированное отношение шансов через 1 мес — 3,19 (95% ДИ 1,89–5,38), через 6 мес — 3,33 (95% ДИ 1,81–6,15). Множественные (5–25) и устойчивые (во всех индексных точках) симптомы, оценённые пациентами как существенные, возникали *de novo* редко — 1,7% (95% ДИ 0,4–4,9), но значительно чаще у детей с СВД в анамнезе — 25% (95% ДИ 13,6–39,6%); разница — 23,3% (10,9–35,7%); отношение шансов 14,8 (4,4–50,6); $p < 0,001$. Повторные эпизоды ОРВИ были важным фактором риска увеличения жалоб когнитивного кластера и ваготонического СВД через 3–6 мес наблюдения.

Заключение. Получены результаты, косвенно поддерживающие концепцию, в которой PCC представляет собой соматоформное функциональное расстройство, вероятно, социогенного характера у тревожных на исходном уровне пациентов. Некоторые пациенты с последствиями COVID-19 и ОРВИ действительно нуждаются в медицинской и психосоциальной реабилитации. Изучение PCC представляет новые аспекты последствий широко распространённых респираторных вирусных инфекций.

Ключевые слова: *дети, подростки, COVID-19, состояние после COVID-19, пост-острые последствия COVID-19, длительный COVID-19, синдром вегетативной дисфункции, психоэмоциональные расстройства, соматические симптомы, соматоформное расстройство*

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

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Introduction

Among the many global challenges of the COVID-19 pandemic, health impairments that persist, progress or emerge after the acute period of COVID-19 have a significant impact on daily human activities and quality of life.

Several terms are used in the scientific literature to define the long-term effects of COVID-19. They were proposed later than the identified problem and refer to the timing of onset and duration of persistent symptoms without specifying their epidemiological and clinical and characteristics. According to the World Health Organization (WHO) definition, Post-COVID-19 condition (PCC) occurs in individuals with a history of confirmed or probable SARS-CoV-2 infection when symptoms initially occurring within 3 months after the acute period last for at least 2 months and typically affect daily activities. This definition can be used in patients of all ages[1] and is adopted in this publication. The term Post-acute sequelae of SARS-CoV-2 (PASC) proposed by the National Institutes of Health is essentially the same as the WHO definition [1]. According to the clarification of another group of experts, the term Long COVID takes into account at least one physical symptom under other similar conditions [2]. The absence of an alternative cause of impairment is mentioned as one of the key definitions of PCC. Currently, PCC has a special code of nosological unit of clinical diagnosis.

The prevalence, spectrum, hierarchy and duration of PCC symptoms are characterized by significant heterogeneity (0.8–74.5%) [3, 4]. More than 200 symptoms have been described in children and adolescents [5]. Weakness, fatigue, fever, dyspnea, cough, headache, dizziness, exercise intolerance, emotional disturbances, sleep disturbances, decreased concentration, learning difficulties, hyposmia, dysgeusia and abdominal pain are listed with variable ranking (< 10–20% in most studies) [5–7]. Late large pediatric controlled studies and meta-analyses with hundreds of thousands of participants have identified a 25–30% PCC symptom rate after SARS-CoV-2 infection of any severity in outpatients and hospitalized patients [6–8], as well as in asymptomatic carriers [9]. High quality studies are associated with lower confirmation of PCC [10].

Pooled assessments of SARS-CoV-2-infected and uninfected children (by polymerase chain reaction testing) identified the same consistent symptoms with little or no difference. Risks of individual symptoms (hyposmia, dysgeusia, exercise intolerance, dyspnea, cognitive deficits, hair loss, palpitations and fatigue) were slightly higher in SARS-CoV-2-positive patients, by 2–8% [3, 11], increasing to 15–30% among adolescents older than 11 years [12]. Risk factors for pediatric PCC, in addition to adolescence, may include female sex (although there is no gender difference in the acute COVID-19 period), European race, severe illness, NICU stay, length of hospitalization, combined comorbidity, and baseline mental health disorders [3, 7, 13]. Other studies have found no association of PCC with demographics (age, gender, and race) [8].

Symptoms may first appear in the early COVID-19 recollection period, persist with varying duration, fluctuate or recur. In children, the trajectory of major PCC symptoms decreases over time from 20–35% to 3–15% with no statistical difference from controls [7, 14–16]. The prevalence of some symptoms may increase after 3–6 months (up to 50–60%) and then be maintained at a high level, while others may decrease at different rates or persist with an initial low frequency [16]. In some children, symptoms occurred *de novo* after 6–12 months of follow-up [17]. However, 6 months after testing, both SARS-CoV-2-positive (24%) and SARS CoV-2-negative (17%) patients had symptoms that met the definition of PCC [18].

In general, compared with adults, the burden of pediatric PCC was moderate to low, symptoms were milder and reported significantly less frequently [3, 11, 16]. Nevertheless, PCC significantly reduced children's quality of life [19] and daily activities, interfered with schooling, and required more frequent visits to medical care [20].

PCC has a number of obvious problems [10, 21]. Limitations include the lack of standardized clinical patterns and biomarkers to describe and diagnose it, which leads to certain difficulties in diagnosis and planning clinical trials. Only 35% of published reports used 1 of 3 definitions of PCC (PASC) [22]. Out of the 110 children who sought medical care with suspected PCC, the diagnosis was confirmed in 29%, alternative somatic/psychiatric conditions were established in 47%, and the cause of complaints remained unexplained in 23% [23]. Cancellation, wrong selection, misclassification, non-response and/or loss of follow-up, as well as the substantial heterogeneity of the results obtained and the limitations of meta-analyses due to insufficient data may be objective shortcomings of many publications. Thereby, the quality of evidence and methodological rigor of a number of scientific papers may have been suboptimal [4, 6, 10].

On the other hand, the similarity of symptoms in SARS-CoV-2-positive and SARS-CoV-2-negative patients suggests the influence of additional factors that have not yet been clearly defined. Most studies have identified chronic diseases routinely recorded in medical databases and electronic records as predictors of PCC risk, but have not investigated the role of baseline functional impairment and possible new diseases in

[1] WHO. A clinical case definition for post COVID-19 condition in children and adolescents by expert consensus, 16 February 2023. Available at: https://who.int/publications/i/item/WHO-2019-nCoV-Post-COVID-19-condition-CA-Clinical-case-definition-2023-1

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the context of the heterogeneous labile PCC symptom complex and new infection pandemic settings.

This prospective study was planned and performed early in the SARS-CoV-2 pandemic, but despite this, its results may be useful as it is the first attempt to consider baseline functional impairment in children as a predictor of PCC risk, as well as the possibility of common sequelae of COVID-19 and other respiratory infections, which seems to help achieve a better understanding of PCC.

The **aim** of the study was to determine the prevalence of PCC in relation to baseline functional impairment in children one year after non-severe COVID-19 and acute respiratory tract infection (ARTI) of other etiologies.

Materials and methods

Study design

We conducted an observational longitudinal study using a continuous sampling method of short- and long-term effects of non-severe COVID-19 compared with acute respiratory infection of other etiologies in children of any age hospitalized in an infectious disease hospital. During the acute period of illness and for a year afterward, children without baseline health problems or with a history of only functional impairment were observed.

Participants

Children with upper and lower respiratory tract infections were included in the study, regardless of its timing. In order to avoid bias associated with risk factors, the study excluded children with severe and complicated forms of the disease, with established chronic diseases and signs of primary autonomic dysfunction (AD) including organic lesions and traumas of the central and peripheral nervous system, excessive overstrain of the nervous system (clearly defined physical or emotional stress, sports in professional sections), diabetes mellitus, obesity, other endocrine disorders, chromosomal disorders and other chronic diseases.

Patients were recruited from September 2020 to December 2021, i.e., after the first wave of the Ukhan strain of SARS-CoV-2 and before the beginning of the spread of genetic variants of SARS-CoV-2 Omicron in Russia. 250 children under 18 years of age were included in the study. COVID-19 was confirmed by routine methods on the basis of positive SARS-CoV-2 RNA test in nasal and oropharyngeal swabs taken at the time of hospitalization. The comparison group (ARTI group) were patients in whom COVID-19 was excluded by negative nasal and oropharyngeal swabs in reverse transcription polymerase chain reaction and serologic tests with detection of anti-SARS-CoV-2 IgM and IgG in enzyme-linked immunosorbent assay before hospital discharge on days 7–10 of the illness. Testing for other respiratory viruses was not performed. Recruitment of patients in each group (COVID-19 and acute respiratory viruses) was stopped at the 125th included patient. A total of 9.6% of patients were excluded from the study (COVID-19 — 3.2%, ARTI — 16%) due to the development of severe pneumonia or loss of contact during the study period. Additionally, patients with diagnostic titers of specific anti-SARS-CoV-2 IgM and IgG were excluded from the ARTI group. Comparative data analysis was performed in groups of 121 (COVID-19) and 10^5 (ARTI) patients.

Treatment of COVID-19 in hospital was performed according to the Temporary Methodological Recommendations of the Ministry of Health of the Russian Federation "Prevention, Diagnosis and Treatment of New Coronavirus Infection (COVID-19)", versions 1–7, as well as the Methodological Recommendations of the Ministry of Health of the Russian Federation "Features of Clinical Manifestations and Treatment of Disease Caused by New Coronavirus Infection (COVID-19) in Children", versions 1 (03.04.2020) and 2 (03.07.2020), relevant at that time.

Patient information was collected by questionnaires administered to mothers and older children at the time of hospitalization and during the following year by telephone contact at index (contact) points at 1, 3, 6 and 12 months (± 5 days) after hospitalization.

The physical, psycho-emotional and cognitive development of children varies with age. Assessment of the health status of young children depends more on information provided by the mother. School-aged and adolescent children are able to present active complaints. Therefore, age-adapted questionnaires were developed for children under and over 6 years of age. The questionnaires were tailored to the literature and possible symptoms after COVID-19. Both questionnaires contained two blocks of questions. The first block (anamnestic) contained 15 questions to assess functional disorders (including gastrointestinal, autonomic nervous system (ANS) dysfunction, psycho-emotional state, and morbidity) in the child's history. The second block questionnaire (observational) for children < 6 years of age included 53 questions and for children ≥ 6 years of age 76 questions. Both questionnaires contained questions about active complaints, general condition of the patient, intensity of disorders, if any, signs of psycho-emotional, cognitive, functional (including gastrointestinal) disorders and AD, subjective sensations of pain, frequency of visits to a pediatrician and specialists, any illnesses, including those that occurred for the first time, facts of hospitalizations, and pharmacological treatment. The answers to the following questions were also evaluated: "Are you feeling worse than before the disease or the same?", 'Do you assess your own symptoms as mild, moderate or serious?', 'Were the symptoms you have now observed before the disease?'. Furthermore, it was offered to evaluate the state of general

health on a 100-point scale. In case of recurrent acute respiratory infections, information about SARS-CoV-2 testing was collected from the words of their mothers.

During the follow-up period, a total of 277 recurrent episodes of acute respiratory infections were recorded, of which 85.9% of children were routinely tested. COVID-19 during the one-year follow-up period was confirmed in 3 cases (these patients were not excluded from the study). The study did not include clinical and instrumental diagnosis of AD, myalgic encephalomyelitis/chronic fatigue syndrome, or other investigations evaluating the function of various organs and systems. The analysis was performed in groups of children stratified by age.

Ethical expertise

The patients' legal representatives signed informed consent to participate in the study, agreed to fill out the developed questionnaires and answer questions during telephone contacts within a year after discharge from the hospital. The study protocol and informed consent to participate in the study were approved by the Independent Ethical Committee of the Moscow Regional Research and Clinical Institute (Protocol No. 10 of 01.09.2020).

Terms and definitions used

This publication uses the term Post-COVID-19 condition (PCC) as defined by WHO, taking into account all symptoms (at least one) [2] that persisted or occurred during the year. Groups of symptoms were grouped into clusters for ease of their statistical analysis: emotional, cognitive, fatigue, gastrointestinal, respiratory, and AD with subclusters of cardiovascular autonomic disorders, sympticotonic or vagotonic types. Definitions of all terms used are detailed in the Supplementary Material on the journal website. It should be emphasized that the symptoms grouped into clusters logically overlap. The individual most frequently occurring symptoms are also presented. The frequency of new symptoms was defined as the proportion of participants with a symptom on the date of telephone contact but who did not have that symptom at all previous index points.

Statistical analysis

Statistical analysis of the study results was carried out using the Microsoft Excel 2011, Statistica v. 7.0 (StatSoft Inc. 7.0) program packages. Quantitative and qualitative data were described with calculation of arithmetic mean (*M*), standard deviation (*SD*), median (Me), lower and upper quartiles [Q_1; Q_3]. Discrete features are presented as a fraction (%) of cases from the total number of observations. When comparing several dynamic measurements of dichotomous variables, the Cochran's Q-criterion was used, and the rank analysis of variance was used for dependent qualitative indicators. When comparing frequencies of events in independent groups, Pearson's χ^2 test for quadratic and randomized tables was used; the two-sided Mann-Whitney test was used for qualitative variables; the McNemar test with Yates correction was used for pairwise comparisons of dependent variables. When the number of variables in 1 cell of the quadratic table was less than 5, the two-sided Fisher's criterion was used.

The following criteria were used to assess the magnitude of risks: frequency of the event in the compared groups, relative risk (RR) of the event in the compared groups, odds ratio (OR) of the event to no event in the compared groups with calculation of 95% confidence intervals (CI). OR < 1 indicates decreased risk, OR = 1 indicates no effect, and OR > 1 indicates increased risk. Predictors of long-term symptoms were assessed using univariate and multivariate logistic regression models and are represented by adjusted OR and 95% CI. Differences were considered statistically significant at $p < 0.05$.

Results

Children < 6 years of age

At baseline in the hospital, the COVID-19 group differed from the acute respiratory tract infection group in the absence of croup (vs. 8.6%) and bronchitis (vs. 31%, respectively) but a longer course of antibacterial and antiviral therapy. The difference was formal because of the lack of recommendations to distinguish the respective clinical forms of COVID-19. The incidence of out-of-hospital pneumonia was comparable in the compared groups. The history of patients < 6 years of age with COVID-19 was more frequently recorded as symptoms of AD, functional, including gastrointestinal disorders (24.3% vs. 5.2%; $p = 0.006$) and atopic dermatitis (18.9% vs. 3.4%; $p = 0.026$). A more detailed characterization of the compared groups is presented in **Table S1** and **Table S2** in the **Supplementary Material** on the journal website.

At the time of hospital discharge, complaints/signs were identified in 32 and 38% of patients < 6 years of age, with no statistical difference between the COVID-19 and ARTI groups (**Table 1**). The children's impairments were mostly mild and the number of symptoms/signs per child was small (usually 1–2, no more than 3–5). During the year, the frequency of active complaints was stable between 5–20%. The peak of symptoms/signs detected by questionnaires was registered by 3–6 months of follow-up with maximum frequency in the acute respiratory tract infection group. The maternal score (integral) of children's health status was associated with the questionnaires and showed consistent improvement after COVID-19 and no significant change after acute respiratory viral infections with worse scores compared to COVID-19 starting at 3 months of follow-up (Table 1).

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Table 1. Frequency of registration of symptoms and signs in children under 6 years in post-acute of COVID-19 (n = 37) and ARTI of other etiology (n = 58)

Complaints, symptoms, signs		Discharge from the hospital	After 1 month	After 3 months	After 6 months	After 12 months	p_2
Well-being is impaired (self-assessment), *n* (%)	COVID-19 ARTI	6 (16.2) 8 (13.8)	4 (10.8) 7 (12.1)	7 (18.9) 12 (20.7)	8 (21.6) 8 (13.8)	6 (16.2) 3 (5.2)	0.699 0.185
including mild cases	COVID-19 ARTI	5 (13.5) 5 (8.6)	4 (10.8) 4 (6.9)	6 (16.2) 11 (19.0)	7 (18.9) 6 (10.3)	6 (16.2) 3 (5.2)	0.914 0.066
Complaints/symptoms, total (any, at least one sign identified by the questionnaire), *n* (%)	COVID-19 ARTI	12 (32.4) 22 (37.9)	8 (21.6) 25 (43.1) p_1 = 0.033	10 (27.0) 35 (60.3) p_1 = 0.002	17 (45.9) 26 (44.8)	10 (27.0) 25 (43.1)	0.061 0.035
The average quantity of signs, *M* ± *SD*	COVID-19 ARTI	1.3 ± 0.1 1.8 ± 0.8	1.5 ± 0.8 2.4 ± 1.2	1.4 ± 0.8 1.8 ± 0.9	2.4 ± 1.6 1.8 ± 1.2	1.9 ± 1.1 1.8 ± 1.0	0.670 0.734
The average quantity of signs, *M* ± *SD* / Me [Q_1; Q_3]	COVID-19 ARTI	87.8 ± 17.3 / 90 [80; 100] 91.3 ± 12.2 / 100 [80; 100] p_1 = 0.329	95.4 ± 7.3 / 100 [90; 100] 90.7 ± 11.1 / 100 [80; 100] p_1 = 0.076	97.3 ± 6.5 / 100 [100; 100] 92.2 ± 10.9 / 100 [80; 100] p_1 = 0.021	97.3 ± 8.7 / 100 [100; 100] 92.4 ± 11.7 / 100 [80; 100] p_1 = 0.018	98.9 ± 5.2 / 100 [100; 100] 94.7 ± 9.2 / 100 [90; 100] p_1 = 0.008	< 0.001 0.314
Weakness, fatigue, locomotor activity decreased, *n* (%)	COVID-19 ARTI	1 (2.7) 1 (1.7)	1 (2.7) 2 (3.4)	1 (2.7) 1 (1.7)	2 (5.4) –	1 (2.7) –	0.406 0.147
Skin rash, *n* (%)	COVID-19 ARTI	– 3 (5.2)	– –	2 (5.4) 5 (8.6)	1 (2.7) 2 (3.4)	– 3 (5.2)	– –
Hair loss, *n* (%)	COVID-19 ARTI	– –	– 2 (3.4)	1 (2.7) –	– –	– –	– –
dyspnea without catarrhal symptoms, *n* (%)	COVID-19 ARTI	– 1 (1.7)	1 (2.7) –	– –	– –	– –	– –
Psychoemotional disorders, *n* (%)	COVID-19 ARTI	2 (5.4) 3 (5.2)	6 (16.2) 10 (17.2)	6 (16.2) 12 (20.7)	5 (13.5) 8 (13.8)	2 (5.4) 7 (12.1)	0.234 0.002
Psychoemotional disorders *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	1 (2.7) –	2 (5.4) 6 (10.3)	4 (10.9) 3 (5.2)	1 (2.7) –	– –	– –
Sleep disorders, restless sleep, *n* (%)	COVID-19 ARTI	6 (16.2) 1 (1.7) p_1 = 0.013	2 (5.4) 2 (3.4)	5 (13.5) 2 (3.4)	3 (8.1) 2 (3.4)	1 (2.7) 3 (5.2)	0.060 0.644
Sleep disorders, restless sleep *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	5 (13.5) 1 (1.7) p_1 = 0.032	1 (2.7) 1 (1.7)	2 (5.4) 1 (1.7)	– 1 (1.7)	– –	– –
Cognitive dysfunction, *n* (%)	COVID-19 ARTI	1 (2.7) –	2 (5.4) –	1 (2.7) –	3 (8.1) –	2 (5.4) –	0.406 0.406
Autonomic dysfunction (any signs), *n* (%)	COVID-19 ARTI	10 (27.0) 11 (19.0)	7 (18.9) 16 (27.6)	7 (18.9) 13 (22.4)	7 (18.9) 10 (17.2)	7 (18.9) 12 (20.7)	0.670 0.111
AD *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	5 (13.5) 6 (10.3)	3 (8.1) 8 (13.8)	– 1 (1.7)	– 2 (3.4)	2 (5.4) 2 (3.4)	– –
Gastrointestinal disorders (total), *n* (%) including:	COVID-19 ARTI	1 (8.1) 5 (8.6)	2 (5.4) 13 (22.4) p_1 = 0.041	1 (2.7) 5 (8.6)	12 (32.4) 5 (8.6) p_1 = 0.004	3 (8.1) 3 (3.4)	< 0.001 0.016

End of the Table 1

Complaints, symptoms, signs		Discharge from the hospital	After 1 month	After 3 months	After 6 months	After 12 months	p_2
abdominal pain	COVID-19 ARTI	– 2 (3.4)	1 (2.7) 2 (3.4)	– 1 (1.7)	– 1 (1.7)	2 (5.4) 1 (1.7)	
constipation	COVID-19 ARTI	1 (2.7) 1 (1.7)	1 (2.7) –	1 (2.7) 2 (3.4)	1 (2.7) 1 (1.7)	– –	
diarrhea	COVID-19 ARTI	– 2 (3.4)	– 12 (20.7)	– 2 (3.4)	11 (29.7) 3 (5.2)	1 (2.7) 1 (1.7)	
Gastrointestinal disorders *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	– 3 (5.0)	1 (2.7) 11 (18.3) $p_1 = 0.026$	– 3 (5.2)	3 (8.1) 1 (1.7)	1 (2.7) –	– –
Catarrhal symptoms at the telephone contact, *n* (%)	COVID-19 ARTI	2 (5.4) 15 (25.9) $p_1 = 0.013$	1 (2.7) 14 (24.1) $p_1 = 0.003$	2 (5.4) 21 (36.2) $p_1 < 0.001$	4 (10.8) 18 (31.0) $p_1 = 0.023$	4 (10.8) 21 (36.2) $p_1 = 0.004$	0.435 0.433
Catarrhal symptoms only, *n* (%)	COVID-19 ARTI	2 (5.4) 11 (19.0) 0.061	2 (5.4) 3 (5.2)	1 (2.7) 16 (27.6) $p_1 = 0.002$	2 (5.4) 10 (17.2) $p_1 = 0.091$	2 (5.4) 10 (17.2) $p_1 = 0.091$	0.910 0.006
Recurrent episodes of ARTI episodes between the points of telephone contact, *n* (%)	COVID-19 ARTI	– –	6 (16.2) 16 (27.6)	5 (13.5) 27 (46.6) $p_1 < 0.001$	11 (29.7) 29 (50.0) $p_1 = 0.052$	18 (48.6) 25 (43.1)	< 0.001 0.045
Frequency of complicated course of ARTI (croup, pneumonia, febrile convulsions, lymphadenitis), *n* (%)	COVID-19 ARTI	– –	– 3 (5.1)	– 2 (3.4)	1 (2.7) 2(3.4)	1 (2.7) 1 (1.7)	– –
ABT frequency, *n* (%)	COVID-19 ARTI	– –	– 3 (5.1)	– 7 (12.1)	2 (5.4) 9 (15.5)	– 7 (12.1)	– 0.089
The children number with doctor's appointment between telephone contact points, *n* (%)	COVID-19 ARTI	– –	6 (16.2) 17 (29.3)	7 (18.9) 27 (46.6) $p_1 = 0.007$	13 (35.1) 26 (44.8)	12 (32.4) 22 (37.9)	0.056 0.155
Average number of medical appointments, *M* ± *SD*	COVID-19 ARTI		1.2 ± 0.4 1.1 ± 0.7	1.9 ± 0.9 1.2 ± 0.5	1.5 ± 0.9 1.2 ± 0.6	1.3 ± 0.5 1.2 ± 0.5	0.428 0.391
The hospitalizations number between telephone contact points, *n* (%)	COVID-19 ARTI	– –	– 3 (5.1)	1 (2.7) –	1 (2.7) 3 (5.1)	2 (5.4) –	– –

Note. Here and in the Table 2: *At the time of discharge from the hospital, signs de novo in relation to the period before the disease. AD — autonomic dysfunction, ABT — antibacterial therapy. The Cochran's Q test was used to compare the dynamics of the frequency of signs in groups (p_1), rank analysis of variance was used to compare qualitative signs, the McNemar's test was used to compare paired dependent characteristics (p_2), χ^2 for four-field tables with the Yeats correction or two-tailed Fisher's test were used to compare paired independent features.

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Typical symptoms (weakness, fatigue, respiratory distress) were very rare (1–2%) at all contact points. AD symptoms, psycho-emotional disturbances, sleep problems and catarrhal symptoms were more common than others regardless of the comparison group. Respondents in the COVID-19 and ARTI groups noted *de novo* AD symptoms at the time of hospital discharge relative to the pre-disease period in 13.5% and 10.3% of cases, respectively ($p > 0.05$; see Table 1). Further, relative to the prior point of contact, new AD symptoms occurred in the range of 2.0–13.8% in both groups. After COVID-19, the prevalence of emotional disturbances was relatively stable, but in the ARTI group had the character of a parabolic curve with a peak (20.7%) at 3 months after hospital discharge. Overall, during the year, *de novo* signs of AD appeared in 27.0% of respondents in the COVID-19 group and 34.5% in the ARTI group ($p > 0.05$), and new cases of psycho-emotional disorders in 21.6 and 15.5%, respectively ($p > 0.05$). All patients with a history of AD occasionally indicated its symptoms at one or another point of telephone contact. Symptoms of AD throughout the year were absent in 54% of children in the COVID-19 group and 65.5% of children in the ARTI group, psycho-emotional disorders in 73 and 82.8%, and sleep disorders in 75.7 and 93.1%, respectively (for all indicators, $p > 0.05$).

The maximum number of complaints 3-6 months after hospital discharge was associated with new episodes of acute respiratory and intestinal infections, which were the main reason for seeking medical help. Catarrhal symptoms without other signs during this time frame were frequent and significantly more frequent after acute respiratory infections than after COVID-19 (Table 1). Twenty-seven and 55.2% ($p = 0.008$) of children in the compared groups became repeatedly ill with acute respiratory infections within 3 months after hospital discharge, and 40.5 and 89.9% within 6 months, respectively ($p < 0.001$). Confirmed cases of acute gastroenteritis occurred (at different time points) in 13.5% of children in the COVID-19 group and 6.9% of children in the ARTI group.

Additional diagnoses after COVID-19 were hepatitis of undetermined etiology of low activity of 3 months' duration (2.7%; 95% CI 0.1–15.8), cervical lymphadenitis, and urinary tract infection; in the acute respiratory tract infection group, varicella (3 cases), community-acquired pneumonia, otitis media, COVID-19 (1 case), and urinary tract infection. 24.3 children in the COVID-19 group and 6.9% of children in the acute respiratory tract infection group had no illnesses during the year ($p = 0.033$).

At all of the time points, 3 (8.1% 95% CI 1.7–21.9) children in the COVID-19 group and 1 (1.7% 95% CI 0.04–9.20%) in the acute respiratory tract infection group ($p = 0.050$) had any persistent disorders significantly affecting their general health (as assessed by mothers). However, similar symptoms were recorded in the history of these patients before inclusion in the study. In the remaining children, the complaints/symptoms were transient in nature. There was no effect of age and gender on the studied parameters in children younger than 6 years of age.

The overall incidence of cases meeting the definition of PCC in children younger than 6 years at 1, 3, 6 and 12 months after hospital discharge was relatively stable and was 19, 24, 30 and 16% after COVID-19 and 19, 24, 24 and 15.5% after acute respiratory infection (at all points $p > 0.05$). But after excluding patients who had acute infections or other established diseases, it appeared that PCC was rarely reported *de novo*, 2.7–8.1% at 1–3 months after hospital discharge and was absent at 6 months (**Figure**, *a*).

The adjusted odds of having symptoms of AD (adjusted OR = 2.192; 95% CI 1.246–3.865; $p = 0.007$) or emotional distress (adjusted OR = 2.081; 95% CI 1.035–4.187; $p = 0.039$) were increased by 6 months after hospital discharge in both SARS-CoV-2-positive and SARS-CoV-2-negative patients if they had a recurrent acute respiratory illness.

Children ≥ 6 years of age

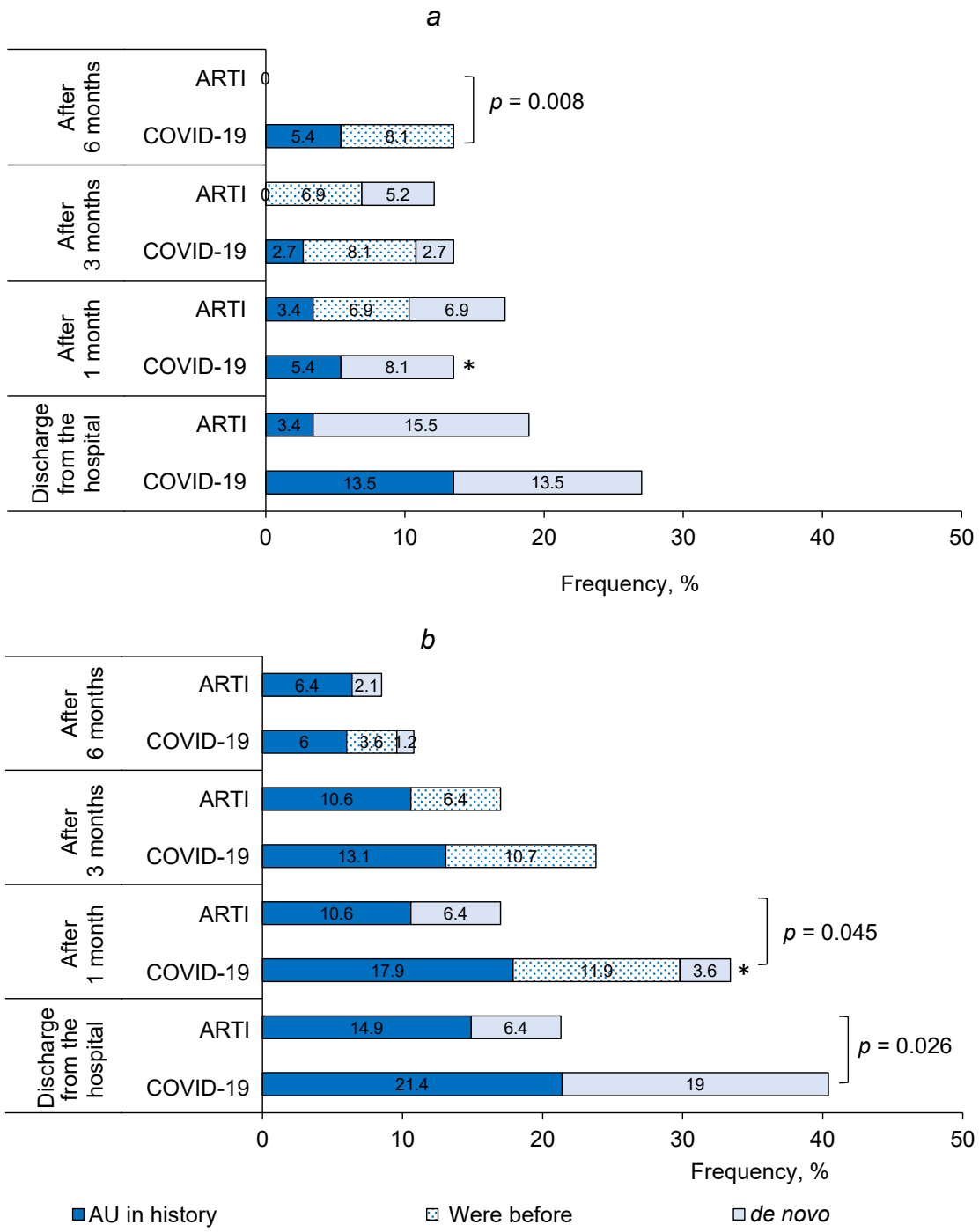
The COVID-19 group differed from the acute respiratory tract infection group by a shift in age structure toward a predominance of adolescents 10–17 years of age (70.2% vs 51.1%; $p = 0.024$), the acute respiratory tract infection group by a higher incidence of bronchitis and a shorter duration of antibiotic therapy and antiviral therapy (similar to younger children). Patient history ≥ 6 years did not differ, except for a more frequent history of out-of-hospital pneumonia among those hospitalized with acute respiratory infections (**Table S3** and **Table S4** in the Supplementary Material on the journal website).

Children ≥ 6 years of age rated their health status worse than patients < 6 years of age (**Table 2**; **Table S5** in the **Supplementary Material** on the journal website). At the time of hospital discharge, the incidence of complaints and symptoms was higher (36.9%) in the COVID-19 group than in the ARTI group (8.5%; Table 2). At one year, self-assessment of health impairment after COVID-19 indicated moderate improvement according to the mean integral score. In the ARTI group, the dynamics of changes had an opposite direction (Table 2). Patients ≥ 6 years of age presented a wider range of complaints and their mean per 1 child was higher than that of children < 6 years of age at all telephone contact points. The proportion of children with more complaints/signs (6–25) ranged from 2–13% with no differences between groups or time points. In almost all cases (87–88%), multiple complaints were made by children with a history of underlying functional health disorders.

Children ≥ 6 years of age complained more frequently of weakness/fatigue, headache, and emotional disturbances (2.4–23.8%) and rarely (1–7%) of phys-

ical and mental intolerance, cognitive dysfunction, breathing difficulties, sleep disturbances, hair loss, and gastrointestinal disturbances. In a "crude" assessment in the early period of recongestion, the prevalence of signs of AD (difference 19.2% [3.5–34.9%]; OR = 0.9 [0.04–2.50], cardiovascular symptoms (difference 13.3% [4.6–22.1%]; OR = 6.27 [0.02–53.10]), subfebrile state (difference 12.2% [3.6–20.7%]; OR = 5.7 [0.10–49.2]), and hyposmia (difference 14.3%) were higher after COVID-19 than after acute respiratory infections (Table 2). However, the increased risks associated with COVID-19 were not significant.

During the year after COVID-19, the prevalence of dominant features, including the vagotonic-type



Incidence of PCC (as defined by WHO) in non-sick children in COVID-19 and ARTI groups over time, adjusted for AD history and *de novo* symptoms.

a — children under 6 years old; *b* — children aged 6–18 years.

*p = 0.027 between children < 6 years and ≥ 6 years; the "were before" series presented symptoms recorded in relation to the previous time point; PCC — post-COVID-19 condition; AD — autonomic dysfunction.

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AD symptom cluster, consistently decreased (Table 2). Other clusters and symptoms were recorded at a stable level. Hyposmia gradually resolved but subjectively persisted after 12 months in 2.4% (95% CI 0.6–9.1) of cases. The number of new illnesses (mainly recurrent acute respiratory infections) increased by 6 months and declined thereafter. Within 6 months after COVID-19, 2 cases of arthritis (2.4%; 95% CI 0.4–9.1%), hemorrhagic vasculitis (1.2%; 95% CI 0–7.4%), out-of-hospital pneumonia, and purulent inflammatory diseases (2 cases) were diagnosed.

After acute respiratory viral infection, the trajectory of the prevalence of the main symptoms was different. The sum of symptoms of AD due to weakness/fatigue, headaches and in general AD of vagotonic type increased by 3-6 months, then decreased by the end of the year. The registration of other nonrespiratory symptoms did not differ from COVID-19. New episodes of acute respiratory infections and associated catarrhal symptoms accounted for a substantial proportion of the causes of impairment. After the initial acute respiratory infection, cystitis, bacterial sinusitis, and 2 episodes of COVID-19, including one with hyposmia, were diagnosed in isolated cases (Table 2).

Overall, the risks of new episodes of respiratory infections were lower after COVID-19 (as among children < 6 years of age) compared with the ARTI group after 3 months (by 31.3% [95% CI 16.9–47.3]; OR = 0.79 [0.55–0.91]) and after 6 months (by 23.7% [7.6–39.9]; OR = 0.59 [0.26–0.77]). There was a correspondingly lower (22.1% [5.3–38.9]; OR = 0.49 [0.16–0.70]) referral to a pediatrician. During the year, among children ≥ 6 years of age in the compared groups, no symptoms were present in 47.6% of cases in the COVID-19 group and in 61.7% of cases in the ARTI group ($p = 0.122$).

The incidence of PCC in the compared groups was stable at 6 months and moderately decreased after one year (COVID-19 group: 44.0; 41.7; 42.9 and 33.3%; ARTI group: 40.4; 40.4; 36.2 and 17.0% at index points, respectively; $p > 0.05$). PCC was more common in children ≥ 6 years than in children <6 years, 1 month after hospital discharge: COVID-19 group: age difference 22.7% [95% CI 6.3–39.3]; OR = 1.20 [0.08–3.50]; $p = 0.009$), ARTI group: difference 21.5% [4.2–38.7]; OR = 1.13 [0.13–3.03], OR = 2.9 [1.20–6.98]; $p = 0.018$). No age differences were found at later ages.

The risk of symptom-complex PCC in patients ≥ 6 years without acute infections or other established diseases was higher after COVID-19 than after acute respiratory infections at 1 month, and the risk of *de novo* PCC was higher only at the time of hospital discharge ($p = 0.039$). Thereafter, no statistical difference was obtained for this indicator. After 6 months of follow-up, there were practically no cases of *de novo* PCC in children ≥ 6 years of age (Figure, *b*).

Multiple and persistent (throughout the year) disorders significantly affecting well-being were significantly more common in children with a history of AD than *de novo*: COVID-19 group: 26.5% vs. 1.1%, respectively; $p < 0.001$; ARTI group: 21.4 and 2.2%, respectively; $p = 0.016$. **Table 3** presents the risk scores of the main symptom clusters adjusted for age, history of AD and repeated episodes of acute respiratory infections.

Table 3 shows that the odds of complaints after COVID-19 were elevated compared to ARTI only at the time of hospital discharge, especially in children 12 years and older, but after 6 months the odds were higher in the ARTI group. It appeared that it was not the etiology of the initial respiratory infection but rather a history of ARTI that significantly increased the risks of predominant symptoms at all telephone contact points. Adolescents ≥ 12 years of age had higher such risks than children <12 years of age if AD was reported before the initial illness (adjusted OR = 3.2; 95% CI 2.0–5.2; $p < 0.001$). Interestingly, repeated respiratory episodes increased the odds of the most significant clusters (AD, including vagotonic type and cognitive impairment) by 6 months of follow-up regardless of the etiology of the initial infection.

Discussion

According to recent data, PCC in children is characterized by several groups of signs/conditions [1, 24]. After severe COVID-19 and in comorbid patients, PCC is diagnosed more frequently and has a more significant impact on pediatric health [1, 3, 25]. The present work is an attempt to explain the heterogeneity of clinical manifestations and temporal dynamics of PCC.

In general, we obtained information on the prevalence, spectrum and dynamics of symptoms that coincides with most published systematic reviews and meta-analyses [1, 4, 7, 8]. Symptoms identified in the questionnaires occurred with a frequency of 32–39%. The most common were the psycho-emotional cluster and the AD cluster, although older children were more detailed in labeling perceived symptoms, which is natural. The respiratory cluster was a significant proportion, often the only one.

In general, patients in the COVID-19 and ARTI groups experienced similar symptoms. Crude (unadjusted for possible confounding factors) assessment of differences between the COVID-19 group and the SARS-CoV-2-negative group found higher risks of well-being disorders (additionally, sleep problems in children < 6 years of age and hyposmia and AD cluster in children ≥ 6 years of age) only in the early recollection period. There was no difference in most specific features, which is probably due to the exclusion of comorbid patients. We confirmed the increased prevalence of some symptoms among older children (especially ≥ 12 years) described previously [12].

In the present study, different dynamics of individual symptoms (heterogeneous timing of onset and

Table 2. Frequency of registration of symptoms and signs in children from 6 to 18 years in post-acute of COVID-19 (n = 84) and ARTI of other etiology (n = 47)

Complaints, symptoms, signs		Discharge from the hospital	After 1 month	After 3 months	After 6 months	After 12 months	p_2
Well-being is impaired (self-assessment), *n* (%)	COVID-19 ARTI	31 (36.9) 4 (8.5) p_1 < 0.001	30 (35.7) 11 (23.4)	23 (27.4) 11 (23.4)	25 (29.8) 10 (21.3)	19 (22.6) 11 (23.4)	< 0.009 0.149
including to a mild	COVID-19 ARTI	20 (23.8) 2 (4.3) p_1 = 0.001	20 (23.8) 9 (19.1)	16 (19.0) 9 (19.1)	15 (17.9) 8 (17.0)	15 (17.9) 8 (17.0)	< 0.009 0.267
Complaints/symptoms, total (any, at least one sign identified by the questionnaire), *n* (%)	COVID-19 ARTI	33 (39.3) 10 (21.3) p_1 = 0.036	38 (45.2) 22 (46.8)	36 (42.9) 24 (51.1)	38 (45.2) 23 (48.9)	33 (39.3) 24(51.7)	0.617 0.002
The average quantity of signs, $M \pm SD$ / Me [Q_1; Q_3]	COVID-19 ARTI	4.5 ± 5.0 / 3 [2–5] 2.0 ± 1.9 / 1 [1–2] p_1 = 0.019	5.0 ± 4.9 / 3 [2–5] 3.0 ± 2.4 / 2 [1–4] p_1 = 0.081	4.8 ± 5.0 / 3 [2.0–5.5] 3.6 ± 02.5 / 3 [1.5–5.5] p_1 = 0.718	4.3 ± 3.9 / 3 [2–5] 3.3 ± 2.5 / 2 [1.0–5.5] p_1 = 0.344	4.3 ± 3.6 / 3 [2–6] 2.9 ± 1.9 / 2 [2.0–4.5] p_1 = 0.209	0.464 0.346
The average quantity of signs, $M \pm SD$ / Me [Q_1; Q_3]	COVID-19 ARTI	86.5 ± 16.6 95 [70; 100] 95.1 ± 10.0 / 100 [100; 100] p_1 = 0.001	89.3 ± 15.6 100 [80; 100] 89.8 ± 15.4 / 100 [80; 100] p_1 = 0.803	91.4 ± 14.4 100 [80; 100] 89.8 ± 15.4 / 100 [80; 100] p_1 = 0.576	92.4 ± 14.7 100 [85; 100] 90.4 ± 14.1 / 100 [80; 100] p_1 = 0.354	94.9 ± 11.7 100 [80; 100] 89.4 ± 15.7 / 100 [70; 100] p_1 = 0.040	< 0.001 0.049
Weakness, fatigue, locomotor activity decreased, *n* (%)	COVID-19 ARTI	20 (23.8) 5 (10.6)	23 (27.4) 11 (23.4)	18 (21.4) 11 (23.4)	17 (20.2) 10 (21.3)	11 (13.1) 6 (12.8)	0.018 0.044
Weakness, fatigue, locomotor activity decreased *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	12 (14.3) 3 (6.4)	5 (6.0) 5 (10.6)	1 (1.2) –	5 (6.0) 2 (4.3)	– 1 (2.1)	–
Subfebrile conditions (more than 2 weeks), *n* (%)	COVID-19 ARTI	9 (10.7) 1 (2.1) p_1 = 0.076	12 (14.3) 1 (2.1) p_1 = 0.031	10 (11.9) 1 (2.1) p_1 = 0.053	7 (8.3) 1 (2.1)	5 (6.0) –	0.080 0.809
Headache, *n* (%)	COVID-19 ARTI	15 (17.9) 3 (6.4) p_1 = 0.068	16 (19.0) 6 (12.8)	14 (16.7) 6 (12.8)	15 (17.9) 6 (12.8)	15 (17.9) 3 (6.4) p_1 = 0.068	0.982 0.224
Headache *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	7 (8.3) 1 (2.1)	4 (4.8) 2 (4.3)	– –	1 (1.2) 2 (4.3)	3 (3.6) –	– –
Muscle pain, muscle spasms, *n* (%)	COVID-19 ARTI	2 (2.4) –	2 (2.4) 1 (2.1)	3 (3.6) –	3 (3.6) –	3 (3.6) –	– –
Hyposmia, *n* (%)	COVID-19 ARTI	12 (14.3) –	6 (7.1) 1 (2.1)	3 (3.6) 1 (2.1)	3 (3.6) –	2 (2.4) –	< 0.001 –
Skin rash, *n* (%)	COVID-19 ARTI	– –	1 (1.2) 1 (2.1)	2 (2.4) 1 (2.1)	1 (1.2) –	1 (1.2) 1 (2.1)	– –
Hair loss, *n* (%)	COVID-19 ARTI	1 (1.2) –	3 (3.6) 1 (2.1)	3 (3.6) 1 (2.1)	2 (2.4) 1 (2.1)	3 (3.6) –	– –

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ORIGINAL RESEARCHES

Continuation of the Table 2

Complaints, symptoms, signs		Discharge from the hospital	After 1 month	After 3 months	After 6 months	After 12 months	p_2
Arthritis, joint pain, *n* (%)	COVID-19 ARTI	– –	1 (1.2) –	2 (2.4) –	2 (2.4) –	2 (2.4) –	– –
Dyspnea without catarrhal symptoms, *n* (%)							
at rest	COVID-19 ARTI	1 (1.2) –	1 (1.2) –	1 (1.2) –	1 (1.2) –	1 (1.2) –	–
at load	COVID-19 ARTI	2 (2.4) –	4 (4.8) –	4 (4.8) –	3 (3.6) –	3 (3.6) –	–
Psychoemotional disorders, *n* (%)	COVID-19 ARTI	10 (11.9) 2 (4.3)	12 (14.3) 4 (8.5)	14 (16.7) 4 (8.5)	12 (14.3) 5 (10.6)	13 (15.5) 5 (10.6)	0.354 0.329
including: anxiety and depression	COVID-19 ARTI	3 (3.6) 1 (2.1)	3 (3.6) 1 (2.1)	4 (4.8) 1 (2.1)	4 (4.8) 1 (2.1)	3 (3.6) 1 (2.1)	0.531 0.406
panic attacks	COVID-19 ARTI	1 (1.2) –	– –	1 (1.2) –	1 (1.2) –	– –	– –
Psychoemotional disorders *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	5 (6.0) 1 (2.1)	3 (3.6) 2 (4.2)	1 (1.2) –	– 1 (2.1)	2 (2.4) –	– –
Intolerance to physical activity, *n* (%)	COVID-19 ARTI	6 (7.1) –	5 (6.0) 3 (6.4)	9 (10.7) 3 (6.4)	7 (8.7) 3 (6.4)	5 (6.0) 1 (2.1)	0.405 0.092
Intolerance to emotional stress, *n* (%)	COVID-19 ARTI	5 (6.0) –	5 (6.0) 2 (4.3)	4 (4.8) 2 (4.3)	3 (3.6) 1 (2.1)	4 (4.8) 3 (6.4)	0.800 0.316
Sleep disorders, restless sleep, *n* (%)	COVID-19 ARTI	2 (2.4) 1 (2.1)	5 (6.0) 2 (4.3)	4 (4.8) 2 (4.3)	5 (6.0) 1 (2.1)	5 (6.0) 2 (4.3)	0.437 0.558
Sleep disorders, restless sleep *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	1 (1.2) 1 (2.1)	2 (2.4) 1 (2.1)	1 (1.2) –	1 (1.2) –	2 (2.4) –	– –
Cognitive dysfunction, *n* (%)	COVID-19 ARTI	8 (9.5) 2 (4.3)	13 (15.5) 5 (10.6)	11 (13.1) 5 (10.6)	12 (14.3) 8 (17.0)	9 (10.7) 1 (2.1)	0.220 0.009
including memory impairment	COVID-19 ARTI	3 (3.6) –	5 (6.0) –	5 (6.0) –	6 (7.1) 1 (2.1)	7 (8.3) 1 (2.1)	0.149 0.406
Autonomic dysfunction (any signs), *n* (%)	COVID-19 ARTI	34 (40.5) 10 (21.3) p_1 = 0.026	34 (40.5) 15 (31.9)	30 (35.7) 16 (34.0)	31 (36.9) 15 (31.9)	26 (31.0) 9 (19.1)	0.436 0.002
AD *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	16 (19.0) 3 (6.4) p_1 = 0.086	4 (4.8) 5 (10.6)	– –	6 (7.1) 3 (6.4)	2 (2.4) –	– –
Cardiovascular symptoms, *n* (%)	COVID-19 ARTI	13 (15.5) 1 (2.1) p_1 = 0.018	18 (21.4) 4 (8.5) p_1 = 0.058	6 (7.1) 2 (4.3)	9 (10.7) 2 (4.3)	11 (13.1) 1 (2.1) p_1 = 0.054	0.011 0.406
Adrenergic autonomic dysfunction, *n* (%)	COVID-19 ARTI	13 (15.5) 2 (4.3) p_1 = 0.054	19 (22.6) 10 (21.3)	17 (20.2) 5 (10.6)	15 (17.9) 6 (12.8)	11 (13.1) 4 (14.9)	0.243 0.033

Окончание табл. 2 | End of the Table 2

Complaints, symptoms, signs		Discharge from the hospital	After 1 month	After 3 months	After 6 months	After 12 months	p_2
Vagotonic autonomic dysfunction, *n* (%)	COVID-19 ARTI	14 (16.7) 5 (10.6)	10 (11.9) 4 (8.5)	5 (6.0) 8 (17.0) p_1 = 0.043	8 (9.5) 7 (14.9)	8 (9.5) 1 (2.1)	0.032 0.046
Mixed-type autonomic dysfunction, *n* (%)	COVID-19 ARTI	5 (6.0) –	5 (6.0) 1 (2.1)	8 (9.5) 3 (6.4)	8 (9.5) 2 (4.2)	7 (8.3) 1 (2.1)	0.771 0.363
Gastrointestinal disorders, *n* (%)	COVID-19 ARTI	5 (6.0) —	10 (11.9) 6 (12.8)	11 (13.1) 6 (12.8)	11 (13.1) 3 (6.4)	10 (11.9) 2 (4.3)	0.135 0.005
including: abdominal pain	COVID-19 ARTI	4 (4.8) –	9 (10.7) 5 (10.6)	9 (10.7) 5 (10.6)	9 (10.7) 3 (6.4)	9 (10.7) 2 (4.2)	
constipation	COVID-19 ARTI	2 (2.4) –	2 (2.4) –	2 (2.4) –	– –	– –	
diarrhea	COVID-19 ARTI	1 (1.2) –	1 (1.2) 2 (4.3)	2 (2.4) –	1 (1.2) –	1 (1.2) –	
Gastrointestinal disorders *de novo* in relation to the previous time point, *n* (%)	COVID-19 ARTI	5 (6.0) –	1 (1.2) –	1 (1.2) –	2 (2.4) 1 (4.3)	1 (1.2) –	– –
Catarrhal symptoms at the telephone contact, *n* (%)	COVID-19 ARTI	5 (6.0) 1 (2.1)	5 (6.0) 7 (14.9) p_1 = 0.089	5 (6.0) 14 (29.8) p_1 < 0.001	8 (9.5) 15 (31.9) p_1 = 0.067	5 (6.0) 16 (34.0) p_1 < 0.001	0.725 < 0.001
Catarrhal symptoms only, *n* (%)	COVID-19 ARTI	– –	1 (1.2) 3 (6.4) p_1 = 0.092	1 (1.2) 5 (10.6) p_1 = 0.022	5 (6.0) 7 (14.9) p_1 = 0.017	– 9 (19.1) p_1 < 0.001	0.287 0.009
Recurrent episodes of ARTI episodes between the points of telephone contact, *n* (%)	COVID-19 ARTI	– –	7 (8.3) 19 (40.4) p_1 < 0.001	14 (16.7) 19 (40.4) p_1 = 0.003	26 (31.0) 20 (42.6)	17 (20.2) 18 (28.3)	0.002 0.976
Total diseases, *n* (%)	COVID-19 ARTI		10 (11.9) 21 (44.7) p_1 < 0.001	17 (20.2) 21 (44.7) p_1 = 0.004	34 (30.5) 21 (44.7)	19 (22.6) 18 (38.3) p_1 = 0.056	< 0.001 0.872
ABT frequency, *n* (%)	COVID-19 ARTI	– –	6 (7.1) 5 (10.6)	1 (1.2) 5 (10.6) p_1 = 0.022	8 (9.5) 3 (6.4)	7 (8.3) 3 (6.4)	0.079 0.082
The children number with doctor's appointment between telephone contact points, *n* (%)	COVID-19 ARTI	– –	19 (22.6) 21 (44.7) p_1 = 0.009	25 (29.8) 22 (46.8) p_1 =0.052	36 (42.9) 19 (40.4)	28 (33.3) 16 (34.0)	0.014 0.506
Average number of medical appointments, *M* ± *SD*	COVID-19 ARTI	–	2.3 ± 1.4 2.9 ± 2.1	1.8 ± 2.1 1.7 ± 1.2	1.3 ± 0.7 1.5 ± 0.6	1.4 ± 0.9 1.7 ± 0.9	0.029 1.0
The hospitalizations number between telephone contact points, *n* (%)	COVID-19 ARTI	– –	– 1 (2.1)	– 1 (2.1)	3 (3.6) 1 (2.1)	1 (1.2) 1 (2.1)	– –

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Table 3. History of autonomic dysfunction and incidence of acute respiratory infections adjusted odds ratio in children ≥ 6 years

Sign	Adjusted OR	95% CI	*p*
Odds are increased after COVID-19			
Well-being is impaired (self-assessment)	2.038	1.141–3.644	0.016
including children ≥ 12 years	1.634	1.065–2.507	0.037
Autonomic dysfunction (any signs), discharge from the hospital	4.509	2.377–8.559	< 0.001
Odds are increased after ARTI			
Well-being is impaired (self-assessment) after 6 months	2.484	1.365–4.518	0.003
Odds are increased in children with a history of autonomic dysfunction regardless of COVID-19/ARTI group			
After 1 months	3.187	1.889–5.381	< 0.001
After 6 months	3.333	1.806–6.147	< 0.001
Well-being is impaired (self-assessment)			
after 1 months	1.756	2.112–1.068	0.015
after 6 months	1.824	1.157–2.872	0.009
Headache after 1 months			
after 1 months	2.809	1.614–4.889	< 0.001
after 6 months	3.274	1.831–5.795	< 0.001
Weakness, fatigue, locomotor activity			
after 1 months	2.440	1.551–3.838	< 0.001
after 6 months	2.472	1.527–4.007	< 0.001
Psychoemotional disorders			
after 1 months	3.447	1.774–7.007	< 0.001
after 6 months	2.918	1.596–5.333	< 0.001
Autonomic dysfunction (any signs) after 1 month	3.387	2.076–5.474	< 0.001
ncluding children ≥ 12 years	1.718	1.070–2.759	0.024
Autonomic dysfunction (any signs) after 6 months	3.187	1.917–5.296	< 0.001
Odds are increased in children with repeated ARTI regardless of the COVID-19/ARTI group			
Cognitive dysfunction after 6 months	1.745	1.023–2.977	0.041
Vagotonic autonomic dysfunction after 6 months	1.788	1.007–3.171	0.047
Autonomic dysfunction (any signs) after 6 months	1.768	1.181–2.649	0.006

Note: *At all time points of contact, odds were increased for these signs only points of 1 and 6 months are given for convenience.

duration) were observed, as noted by other authors [14–16]. After COVID-19, the total number of complaints consistently decreased in parallel with an increase in the mean personal health assessment score. In children < 6 years of age, there was no statistical dynamics of individual clusters, and in children ≥ 6 years of age, the frequency of hyposmia, fatigue clusters, and AD (cardiovascular autonomic symptoms and vagotonic-type AD) decreased significantly over the course of the year. In the ARTI group, the sum of negative subjective feelings increased by 3–6 months in association with a significant frequency of repeated episodes of respiratory infections: in children < 6 years of age due to the psycho-emotional cluster, and in patients ≥ 6 years of age due to the cognitive, fatigue and vagotonic AD clusters. The mean estimated health status score in the acute respiratory trauma group did not change much. Throughout the year, most patients in both groups experienced mild health impairments and their duration did not exceed 3-6 months.

Even in the early stages of the pandemic, researchers noted significant similarities between PCC and

clinical manifestations of AD [6, 26]. Prior to the spread of COVID-19, it was shown that postural orthostatic tachycardia syndrome, whose main clinical features are dizziness, tachycardia, headache, and difficulty concentrating, most commonly occurs at a young age with a median age of 14 years and is diagnosed with a significant delay in the majority of patients [27]. Given these data, we focused on determining the difference in prevalence of PCC symptoms between children and adolescents with and without these conditions before COVID-19, which we believe is a strength of the study.

In children < 6 years of age, a history of AD was rare, so there were limited opportunities for in-depth analysis for this characteristic. In the older age group, calculation of age-adjusted OR and past history of AD significantly narrowed the range and size of COVID-19 effects. The COVID-19 group differed from SARS-CoV-2-negative controls only at the time of hospital discharge by increased risk of malaise complaints. At other time points, symptom frequency was associated not with the etiology of the initial respiratory infection (COVID-19 or acute respiratory infection) but specifically with a history of ANS dysfunction before the pandemic. Among patients without a history of similar symptoms and who had not been sick for a year, *de novo* PCC was rare (2.7–8.0%) with no statistical differences between the COVID-19 and acute respiratory infection groups. Cumulatively (regardless of initial infection), multiple (5–25) and persistent (at all index points) symptoms rated by patients as significant occurred *de novo* even less frequently, in 1.7% of cases (95% CI 0.4–4.9), but significantly more frequently among children with a history of AD, 25% of cases (95% CI 13.6–39.6%; difference 23.3% (10.9–35.7%), OR = 14.8 (4.4–50.6); $p < 0.001$). It seems that the risks of PCC in most patients were due to their baseline physical and mental health status, and personal perception of the stressful situation in the current pandemic played a significant role as a supportive trigger of symptoms. At the same time, some patients did feel sick, as evidenced by frequent visits to the doctor.

Although COVID-19-associated pediatric AD is understudied, compelling biomarkers of ANS imbalance both during and after the acute period of infection have been presented [28–30]. R. Buchhorn showed that heart rate variability data after COVID-19 are not significantly different from those of adolescents with autonomic dysfunction due to psychosomatic diseases before the COVID-19 pandemic [31]. A study by B.H. Shaw et al. conducted shortly before the COVID-19 pandemic demonstrated different triggers of dysautonomia with a significant predominance of infection (41%) and psychological problems (28%). Pubertal period of child development was also noted as a significant predictor of AD. Interestingly, in this study, 40% of patients reported the onset of symptoms 3 months after the initiating event [27]. ANS imbalance has also been described in other respiratory viral infections, such as influenza [32] or respiratory syncytial infection [33].

Indirect confirmation of these data was also obtained in the present study, as the frequency of vagotonic AD and cognitive cluster complaints increased after 3–6 months in SARS-CoV-2-negative patients who were more likely to have repeated acute respiratory infections. And adjustment for distorting factors revealed a significant role of recurrent respiratory infections in increasing the risks of these complaints. In-depth studies have demonstrated autonomic disorders with predominant parasympathetic tone in COVID-19 [34], although multifactorial generation of symptoms and individual patient characteristics mediate complex mechanisms of ANS imbalance [30].

Stressful events are a critical trigger of AD, especially in predisposed patients. The SARS-CoV-2 pandemic globally has caused a significant deterioration in mental and cognitive health not only in adults but also in children, which has been named coronavirus anxiety [35–37]. Worry about their own and their loved ones' health, fear, negative media coverage, social isolation and physical inactivity increased the stressful pressure of the pandemic. It was not only adolescents, who respond more consciously to negative information flow, who suffered from sustained experiences. Young children also experienced subjective anxiety in response to maternal anxiety [38, 39]. Patients with special psychological needs or anxious at baseline were hypersensitive to the challenges of the epidemiologic situation associated with the spread of SARS-CoV-2 [37, 40]. Excessive health concern may persist for many months after COVID-19 [40]. In adults, anticipation of symptoms, baseline symptom burden and history of coronavirus disease, but not serologic confirmation, were predictors of worsening somatic symptoms [41]. The use of scales to assess neuropsychiatric symptoms and somatization confirmed subjective stress intolerance and subjective cognitive deficits in PCC patients [42]. In our data, repeated respiratory episodes in an ongoing pandemic per se or as a nocebo effect appeared to maintain/deepen feelings of fear, anxiety and depression.

The results of our study support a model of PCC in which the symptom cluster represents genuinely experienced persistent somatic symptoms that are usually functional in nature and therefore potentially reversible. PCC is likely to be a disorder with somatic symptoms and predisposing, triggering and perpetuating factors [10].

Thus, it appears that PCC is not a unique event peculiar to COVID-19 and likely cannot be a nosologic unit of clinical diagnosis. In our data, the only clinical characteristic of SARS-CoV-2 infection was chemosensory disorders, which are common in older children and adolescents (younger children are unable to formulate their sensations as a complaint) and persist for a long time [43]. Interestingly, the initial severity of

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hyposmia, emotional stress and depression may be risk factors for its persistence for 2 years [44]. We have not identified other specific symptoms for COVID-19 and PCC compared to SARS-CoV-2-negative patients.

The limitations of this study can be considered to be the collection of information by questionnaire without instrumental investigations that are accepted for the diagnosis of AD and related cardiovascular disorders. It is logical to assume that mothers of younger children and older children, being aware of their disease and under the stress of the pandemic, tend to unconsciously distort the true picture of their own health, which was probably further stimulated by leading questions in the surveys. We evaluated the information about repeated episodes of acute respiratory viral infections and repeated testing for SARC-CoV-2 from patients' words, and it is impossible to exclude subjective data errors, although in Russia during this period testing of all patients with acute respiratory viral infections was strictly regulated. The age of patients in the COVID-19 group was slightly skewed toward children older than 12 years of age who had more frequent complaints. We should then assume the possibility of lower risks of COVID-19 than established in this paper. However, the prospective type of our study, the exclusion of factors (severity of disease and comorbidity) that could knowingly cause bias in the data, consideration of the severity of symptoms and their dynamic variability, low dropout rate and consideration of new events (diseases) during the follow-up period allow us to believe that the data obtained are scientifically and practically useful.

Conclusion

Data on the prevalence of PCC in children vary due to the lack of specific diagnostic criteria, reliable biomarkers, and significant limitations of many existing studies. The present study is an attempt to seek explanations for the structural and temporal variability of PCC per se and therefore focused on finding relationships of symptoms after COVID-19 with baseline functional impairment and any events during the year. Adjustment for age and past history of AD allowed for a rare prevalence of PCC (2.7–8.0%) and no differences between patients with SARS-CoV-2 infection confirmed or excluded by molecular and serologic methods. Apart from prolonged hyposmia, we found no specific effects of COVID-19 compared with acute respiratory infections. In most cases, the disorders were mild and persisted for 3–6 months. Persistent and multiple symptoms perceived as serious by patients were found in a quarter of patients with a history of AD and in only 1.7% of *de novo* patients. Our results suggest that recurrent respiratory infections in an ongoing pandemic may be a significant trigger of new symptoms, explaining the previously described temporal variability of PCC. Given our and recent evidence, we support the current concept that PCC is a functional somatic disorder, probably sociogenic in patients who have baseline anxiety.

The study of PCC presents new aspects of the consequences of widespread respiratory viral infections, as some patients do need both medical and psychosocial rehabilitation.

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Original Study Article
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Comparative genomic analysis of clinical isolates of Klebsiella pneumoniae isolated from newborns with different outcomes of the infectious process in the neonatal period

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Abstract

Introduction. Some progress has been made in the study of the molecular mechanisms of antibiotic resistance, namely, genes and their variants have been identified that ensure the inactivation of beta-lactam antibiotics. Nevertheless, there is still a necessity for further studies of genetic diversity of nosocomial strains, prevalence of genetic determinants of resistance to other groups of antibiotics, virulence factors and realization of pathogenic potential by opportunistic microorganisms.

Aim of the study was to compare the genetic profile of clinical isolates of *Klebsiella pneumoniae* isolated from newborns with different outcomes of the infectious process in the neonatal period.

Materials and methods. Using whole-genome sequencing and bioinformatic analysis to search for determinants of resistance and virulence, 3 strains of *K. pneumoniae* were studied, 2 of which were isolated from the blood of a generalized form of infection, 1 from the feces of a newborn child.

Results. *K. pneumoniae* strains belonged to sequence types (ST) ST23, ST14 and ST3559, and differed in genetic determinants of antibiotic resistance and virulence factors. At the same time, they all had the genetic determinants *fimH*, *mrkA* and *iutA*, which are associated with an increased ability to attach to substrates and transport aerobactin. Strain 222 of ST3559, which has the largest number of antibiotic resistance genes, contained the smallest number of virulence factor genes, and vice versa, strain 144 of ST23, in which the smallest number of antibacterial drug resistance genes was detected, contained the most virulence factor genes.

Conclusions. Identification of *K. pneumoniae* strains that differ in the genetic profile of antibiotic resistance and virulence in neonatal hospital patients indicates a complex interaction between bacteria and the macroorganism, in which isolates with low pathogenic potential can cause serious infectious complications, and vice versa, when a highly virulent strain does not realize its pathogenic potential, as demonstrated in case of *K. pneumoniae* strains ST14, ST3559 and ST23, respectively. This highlights the difficulty of effectively predicting and managing infection risks in hospital operations.

Keywords: *Klebsiella pneumoniae, next-generation sequencing, bioinformatics analysis, virulence genes, antibiotic resistance genes*

Ethics approval. The study was conducted with the informed consent the legal representatives of patients. The research protocol was approved by the Ethics Committee of the Ural Scientific Research Institute of Maternity and Child Care (protocol No. 15, December 6, 2022).

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Оригинальное исследование
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Сравнительный геномный анализ клинических изолятов *Klebsiella pneumoniae*, выделенных от новорождённых детей с различными исходами инфекционного процесса в неонатальном периоде

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Аннотация

Введение. Существует необходимость исследования генетического разнообразия внутрибольничных штаммов, распространённости детерминант устойчивости к антибиотикам, факторов вирулентности и реализации патогенного потенциала оппортунистическими микроорганизмами.

Цель работы — сравнить генетический профиль антибиотикорезистентности и вирулентности клинических изолятов *Klebsiella pneumoniae*, выделенных от новорождённых детей с различными исходами инфекционного процесса в неонатальном периоде.

Материалы и методы. С помощью полногеномного секвенирования и биоинформационного анализа для поиска детерминант резистентности и вирулентности исследованы 3 штамма *K. pneumoniae*, 2 из которых выделены из крови при генерализованной инфекции, 1 — из фекалий новорождённого ребёнка.

Результаты. *K. pneumoniae* ST23, ST14, ST3559 отличались генетическими детерминантами антибиотикорезистентности и факторов вирулентности. Вместе с тем все они имели гены *fimH*, *mrkA* и *iutA*, ассоциированные с повышенной способностью к адгезии к субстратам и транспортом аэробактина. Штамм ST3559, обладающий наибольшим количеством генов антибиотикорезистентности (9), содержал 8 генов факторов вирулентности; в штамме ST23, в котором детектировано наименьшее количество генов устойчивости к антибактериальным препаратам (3), обнаружено больше всего генов факторов вирулентности (21).

Заключение. Выявление штаммов *K. pneumoniae*, различающихся по генетическому профилю антибиотикорезистентности и вирулентности, у пациентов неонатальных стационаров указывает на сложное взаимодействие между бактериями и организмом новорождённого ребёнка, при котором изоляты с низким патогенным потенциалом могут вызывать серьёзные инфекционные осложнения, и наоборот, когда высоковирулентный штамм не реализует свой патогенный потенциал, как в случаях с *K. pneumoniae* ST14, ST3559 и ST23. Это подчёркивает сложность эффективного прогнозирования и управления инфекционными рисками в деятельности стационаров.

Ключевые слова: *Klebsiella pneumoniae, полногеномное секвенирование, биоинформационный анализ, гены вирулентности, гены антибиотикорезистентности*

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

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

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Introduction

Klebsiella pneumoniae is a typical representative of *Enterobacteriaceae*, which can be detected during asymptomatic colonization of mucous membranes of non-sterile biotopes of the human body [1]. At the same time, *K. pneumoniae* is included in the top five etiologic agents associated with fatal infectious processes worldwide, regardless of the antibiotic sensitivity of the isolate [2]. According to the results of a multicenter epidemiological study in Russia, *K. pneumoniae* is the most common bacterial pathogen of nosocomial infections of the respiratory (35.81%) and urinary (31.94%) systems, cardiovascular (26.40%), central nervous system infections (CNS; 27.78%), and is the second most common pathogen of nosocomial infections of skin and soft tissues (19.10%), abdominal cavity (26.26%), and bone and joint infections (15.93%) [3].

Among *Enterobacteriaceae*, which are etiologic agents of complications of infectious genesis in newborns in the intensive care unit, *K. pneumoniae* is registered in 48% of cases [4]. In children treated in the hospital of Kemerovo region, it was most often detected in etiologically significant titers in fecal samples (826.41 per 1000 patients) and pharyngeal secretions (33.96 per 1000 patients) [5]. In a pediatric hospital located in Nizhny Novgorod, the epidemiological situation is associated with *K. pneumoniae* sequence type (ST) ST3181, originally isolated in Australia and first described in Russia [6].

K. pneumoniae is the 3rd most frequent etiologic agent of bloodstream infections after *Staphylococcus aureus* and coagulase-negative staphylococci in pediatric departments of the Republic of Belarus, where it is registered in 14.6% of cases [7].

In other countries, where the mortality rate from generalized bloodstream infections is registered at 18–68%, *K. pneumoniae* is also one of the significant pathogens found in newborns hospitalized in intensive care units [8].

When analyzing genetic variants, it was found that in one of the pediatric hospitals in Moscow, *K. pneumoniae* strains belonged to 4 sublines: SL307, SL395, SL29 and SL1198, which indicates the heterogeneity of the strain population and the possible presence of several variants of one bacterial species at once in the conditions of a pediatric ward [9].

The detection of resistance genes to all categories of antibiotics recommended for therapy of *Enterobacteriaceae* in the genomes of 6 panresistant strains once again confirms the urgency of the problem of finding drugs for effective antibiotic therapy. In Morocco, during active surveillance of rectal carriage in newborns, 91 (31.05%) of 293 collected *K. pneumoniae* isolates were found to produce carbapenemase. Among carbapenem-resistant *K. pneumoniae*, 37 (40.65%) contained the bla_{OXA-48} gene; bla_{NDM}, bla_{VIM} and bla_{KPC} genes were detected in 30.76, 9.89 and 2.19% of the isolates, respectively [10]. Globally prevalent multidrug-resistant sequence types include ST14/15, ST17/20, ST43, ST147, ST258 and ST395 [11], with the latter, frequently encountered in pediatric inpatients, being associated with colistin resistance [12]. The detection of convergent types is noteworthy. For example, hospital outbreaks in 2 hospitals in St. Petersburg were caused by carbapenem-resistant hypervirulent strains [13]. In Moscow, ST395 was detected, combining features of both antibiotic-resistant and virulent microorganisms capable of dissemination in the human body [9].

Based on the above mentioned information, *K. pneumoniae* is a relevant opportunistic microorganism associated with the occurrence of both hospital-acquired and out-of-hospital infections. The reason for this is the high rate of transmission of genetic determinants of virulence and antibiotic resistance through mobile genetic elements, the formation of pathogenic and/or antibiotic-resistant epidemically significant clonal lineages and their spread among patients worldwide [9].

Perinatal centers are not an exception and logically fit into the system of medical care at the inpatient stage, within their walls being a contingent with limited therapeutic capabilities and a high risk of infectious and inflammatory processes caused by opportunistic microorganisms. This is due to the morphofunctional immaturity of various organs and their systems in children born low birth weight and/or from early and ultra-early premature births [14].

The study of molecular mechanisms of antibiotic resistance has made progress: genes and their variants that ensure inactivation of antibacterial drugs have been identified, and association with certain clonal groups has been established. Nevertheless, there is still a necessity for further studies of genetic diversity of nosocomial strains, prevalence of genetic determinants of antibiotic resistance, virulence factors and realization of pathogenic potential by opportunistic microorganisms.

The aim of the study was to compare the genetic profile of antibiotic resistance and virulence of clinical isolates of *K. pneumoniae* isolated from newborn infants with different outcomes of the infectious process in the neonatal period.

Materials and methods

The study was approved by the local ethical committee of the Research Institute of Maternal and Infant Health Protection (protocol No. 15 of 06.12.2022).

Three strains of *K. pneumoniae*, 2 of which were isolated from blood during late hospital neonatal sepsis, and the other one from feces of a child during lo-

cal microbiological monitoring[1]. It should be noted that the bloodstream infection was fatal in one case, but a full recovery was observed in the other case. The strains were isolated on 05.04.2023, 11.10.2023, 26.02.2024 and stored in the collection of the microbiology laboratory. Nucleotide sequences were deposited in GenBank international database of genetic information (BioProject: PRJNA1144786, GenBank numbers: JBGKAX0000000000000, JBGKAY000000000, JBHILO0000000000000).

Blood was collected in a volume of up to 4 ml from an intact vein into a pediatric vial directly at the patient's bedside and subsequently cultured in a BacT/ALERT analyzer (bioMerieux).

Positive hemocultures and feces were sown on nutrient media: Endo, differential-diagnostic lactose-containing nutrient medium (State Research Center for Applied Microbiology and Biotechnology) and blood-serum agar (base — Conda).

Species identification of bacteria and determination of sensitivity to antibacterial drugs (ampicillin, amoxicillin + clavulanic acid, cefotaxime, ceftazidime, cefepime, ertapenem, meropenem, amikacin, gentamicin, ciprofloxacin, tigecycline, fosfomycin, nitrofurantoin, trimethoprim sulfamethoxazole, colistin) were performed on a VITEK 2 compact automatic bacteriological analyzer (Bio Mérieux) at the CCU of the Innovative Scientific Laboratory Center for Perinatal and Reproductive Medicine, using VITEK 2 GN (identification) and AST-N360 (antibiotic sensitivity determination) test cards.

To assess the biofilm-forming ability of bacteria, we used the method described previously [15].

Total DNA was isolated from 24-hour culture using D-Cells-10 kits (Biolabmix LLC). Sequencing of strains 222 and 56 was performed on the MiSeq platform (Illumina), and strain 144 was sequenced on the SURFSeq 5000 (GeneMind). The quality of reads was assessed using the FastQC program tool [16]. *De novo* genome assembly was performed using midsystem [17]. Multilocus sequencing was performed according to the method proposed by the Pasteur Institute [18]. Analysis of DNA nucleotide sequences of 7 housekeeping genes: *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, *tonB* and other loci of the genome of *K. pneumoniae* were analyzed using the BIGSdb-Pasteur database of the Pasteur Institute[2].

Genetic determinants of antibiotic resistance and virulence were searched using online services: VirulenceFinder[3] and ResFinder[4]. Typing of capsule loci (C-loci) was performed using the Kaptive site[5] [19].

For comparative analysis of the sequences we obtained, we used the data of GenBank NCBI.

Hyperproduction of mucus was determined using the methodology described in [20].

Results

Brief characterization of patients

K. pneumoniae strain No. 222 was isolated from a positive hemoculture of patient P. on the 49th day of life. Its detection from a blood sample with clinically expressed generalized infection was preceded by a 10-day colonization of the intestine with *K. pneumoniae*, established by local microbiologic monitoring.

K. pneumoniae No. 56, detected in patient M., was initially isolated from a fecal sample on the 35th day of life during local microbiological monitoring. From somatic pathology it should be noted the presence of CNS hypoxia at birth and intrauterine malformations of the CNS and cardiovascular system, which aggravated the child's condition in the neonatal period. At the age of the child 43 days *K. pneumoniae* in monoculture was isolated from the contents of his tracheobronchial tree and from positive hemoculture with negative dynamics of the clinical condition of the newborn, which confirms the translocation of the strain through the intestinal wall and its dissemination throughout the body. On the 44th day, death has occurred, and during bacteriological examination of sectional material (blood from the heart cavity, intestinal tissue, lungs, liver) *K. pneumoniae* in monoculture was isolated from all samples of the listed biological material without accompanying microorganisms.

K. pneumoniae strain No. 144 was isolated during local microbiological monitoring of the department of newborn premature babies from feces of patient Sh. (date of birth 21.02.2024) on the 6th day of his life (gestational age: 36.5 weeks, body weight 2650 g, Apgar score 5/7 during the 1st and 7th minutes of life). During the whole neonatal period of the child in the hospital the results of laboratory studies were without signs of inflammatory process. After discharge in a satisfactory condition home on the 15th day of life, the family did not seek medical help during 3 months, which indicates the absence of invasive infectious processes. This condition was determined both by the ability of immunological reactions to timely recognize antigen and ensure the maintenance of antigenic homeostasis, and by the morphological characteristics and phenotypic properties of the bacterial agent.

Phenotypic characterization of strains

Optical density values obtained during the study of biofilm-forming ability of strains, information on

[1] Order of the Federal State Budgetary Institution "Ural Research Institute for Maternal and Child Health" of the Ministry of Health of Russia No. 263-p dated 26.06.2016 on the procedure for microbiological monitoring.
[2] URL: https://bigsdb.pasteur.fr/klebsiella
[3] URL: https://cge.cbs.dtu.dk/services/VirulenceFinder
[4] URL: https://cge.cbs.dtu.dk/services/ResFinder
[5] URL: https://kaptive-web.erc.monash.edu

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Table 1. Metadata and phenotypic characterization of *K. pneumoniae* strains

Parameter	Strain number		
	222	56	144
Date of discovery	05.04.2023	11.10.2023	26.02.2024
Patient	P.	.	Sh.
Colonization of the intestine with *K. pneumoniae* preceding the infectious process	Yes	Yes	Yes
Nosological form	Generalized infection	Generalized infection	Carriage (colonization of the intestinal biotope)
Outcome	Recovery	Death	Carriage (colonization of the intestinal biotope)
Biofilm formation, optical density, nm	0.235	0.045	0.555
Hyperproduction of mucus	No	No	Yes

mucus hyperproduction, forms and outcomes of nosologies, and other metadata are summarized in **Table 1**.

As shown in the data presented in Table 1, the studied strains were isolated from samples of biological material with an interval of several months. Colonization of the intestinal biotope was observed in all patients, and in two of them the generalization of the infectious process was registered. It ended lethally for one of the patients. Hyperproduction of mucus, noted in 1 of 3 strains, is associated with increased ability of biofilm formation.

One of the significant properties of bacterial strains, including for clinicians, is antibiotic sensitivity.

The minimum inhibitory concentrations of antibacterial drugs of strains No. 222 and 56 are presented in **Table 2**. Strain No. 144 was sensitive to all tested antibiotics except ampicillin.

Strains isolated from positive hemoculture developed resistance to protected amoxicillin (Table 2). Strain No. 222 exhibited multidrug resistance and produced extended-spectrum beta-lactamases (ESBLs). Having developed resistance to one of the aminoglycosides (gentamicin), ciprofloxacin and chloramphenicol, it remained sensitive to colistin, fosfomycin, amikacin and antibiotics from the carbapenem group (ertapenem and meropenem).

Table 2. *K. pneumoniae* strains No.56 and 222 sensitivity to antimicrobial drugs

Antibiotic	Strain number			
	222		56	
	minimum inhibitory concentration, mg/L	sensitivity	minimum inhibitory concentration, mg/L	sensitivity
Ampicillin	≥ 32	–	≥ 32	–
Amoxicillin clavulanate	≥ 32	–	≥ 32	–
Cefotaxime	≥ 64	–	≤ 0.25	+
Ceftazidime	32	–	16	–
Cefepime	≥ 32	–	≤ 0.12	+
Ertapenem	≤ 0.12	+	≤ 0.12	+
Meropenem	≤ 0.25	+	≤ 0.25	+
Amikacin	4	+	≤2	+
Gentamicin	≥ 16	–	≤ 1	+
Ciprofloxacin	1	–	≤ 0.25	+
Fosfomycin	≤ 16	+	≤ 16	+
Trimethoprim	≥ 320	–	≤ 0.20	+
Colistin	≤ 0.5	+	≤ 0.5	+

Table 3. Comparative genetic characteristics of strains isolated from newborns with different outcomes of the infectious process

Parameter	Strain number		
	222	56	144
Genome size, bp	5 414 099	5 544 559	5 468 329
GC composition, %	57.3	57.1	57.4
ST	3559	14	23
KL type	KL27	KL2	KL1
O locus	O4	O1/O2v1	O3/O3a
O type	O4	O1	O3/O3a
Number of genes	5299	5311	5176
Number of contigs	101	89	79
Antibiotic resistance genes	*aac (6')-Ib-cr* *bla* $_{CTX-M-15}$ *bla* $_{SHV-11}$ *bla* $_{TEM-1B}$ *fosA6* *oqxA.B* *blaOXA-1* *catB3* *dfrA1*	*bla* $_{SHV-1}$ *fosA* *oqxA.B* *tet(D)* *catA1*	*bla* $_{SHV-190}$ *fosA6* *oqxA.B*
Virulence genes	*fimH,* *iutA,* *traT* *fyuA* *irp1, 2* *kfuA, B* *mrk A, B, C, D, F, H, I, J* *ybt A, E, P, Q, S, T, U, X*	*fimH,* *iutA* *traT* *kfuA, B, C* *mrkA, B, C, D, F, H,* *I, J*	*fimH,* *iutA* *mchF* *allA, B, C, D, R, S* *arcC* *clbA, B, C, D, E, F, G, H, I, L, M, N, O, P, Q, R* *fdrA* *fyuA* *gcl* *glxK, R* *hyi* *iroB, C, D, N,* *irp1, 2* *iucA, B, C, D, A* *kfuA, B, C* *mceA, B, C, D, E, G, H, I, J* *mrkA, B, C, D, F, H, I, J* *rmpA, A2, ybbW, Y, A, E, P, Q, S, T, U, X* *ylbE, ybtA, E, P, Q, S, T, U, X F*
Virulence score	1	0	5
Antimicrobial resistance score	1	0	0
Incompatibility groups of plasmids	IncFIB(K), IncFII(K)	IncFIB(K)	IncHI1B, IncFIB(K)

The genetic determinants of antibiotic resistance that cause phenotypic resistance to antibiotics, along with genes for virulence factors, external and internal structures of bacterial cells, provide more information for in-depth analysis of microbial data.

Genetic characterization of strains

As can be seen from the data presented in **Table 3**, the studied strains belong to three STs: ST3559, ST14, ST23.

Strain No. 222 of ST3559 belongs to clonal group CG429, being a variant of sublineage ST429 widespread through all continents [21]. This strain scores 1 in virulence due to the presence of *ybt* gene and 1 in antibacterial resistance due to the ESBL gene.

Strain No. 56 of ST14 has been reported as an etiologic agent of neonatal sepsis in central Italy [22], Turkey [23], Vietnam [14], India [24] and Tanzania [25], confirming its widespread prevalence in pediatric units.

Strain No. 144 of ST23 belongs to the hypervirulent clonal group CG23, sublineage SL23. It was characterized by 5 points out of 5 in virulence assessment due to the presence of genes encoding colibactin syn-

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thesis (*clb1*), aerobactin synthesis (*iuc1*) and yersiniabactin synthesis (*ybt*). It is the most virulent strain among those that were compared in this study.

Discussion

Strains No. 56 and 222 phenotypically differed in their sensitivity to antibiotics. It is interesting to note that strain No. 56 was resistant to ceftazidime, retaining sensitivity to cefepime and cefotaxime, although in the course of previous work we found that all ESBL-producing strains of *K. pneumoniae* isolated between 01.01.2020 and 31.12.2021 were resistant to cefotaxime and possessed the $bla_{CTX\text{-}M}$ gene [26].

The strains included in this study belonged to different sequence types, capsule variants, and had different sets of genes for virulence factors and resistance to antibacterial drugs. However, they were united by the presence of genetic determinants *fimH*, *mrkA* and *iutA*. The *fimH* and *mrkA* genes are associated with increased ability to attach to substrates, while the *iutA* gene is associated with the transport of aerobactin [1]. All strains had the bla_{SHV} gene, peculiar to *K. pneumoniae* as a species, providing natural resistance to ampicillin. Mutations in the gene change the substrate specificity and contribute to the inactivation of a wider range of antibacterial drugs. The alleles $bla_{SHV\text{-}11}$, $bla_{SHV\text{-}1}$, $bla_{SHV\text{-}90}$ detected in the strains under study are widespread in Russia and were found in strains isolated in Moscow in 2012-2016 [27]. The $bla_{SHV\text{-}90}$ gene identified in strain ST23, as well as the *fosA*, *oqxA*, and *oqxB* genes, are similar to the genetic determinants characterized in the vast majority of strains isolated in China and causing both nosocomial and out-of-hospital infections [28].

K. pneumoniae strains with mucus hyperproduction, which have great pathogenic and epidemiologic potential, are currently an urgent problem for the health care system of many countries, so timely detection of such bacterial variants is very important for decision-making on patient management tactics and implementation of anti-epidemiologic measures [29].

Both classical strains producing ESBL and an isolate with a hypermucoid phenotype belonging to the epidemiologically significant hypervirulent ST23 were isolated from newborns of the perinatal center. *K. pneumoniae* ST23 is isolated predominantly in Asia, including Taiwan, Singapore, and mainland China [28], and it is with it that the first descriptions of hypervirulent *Klebsiellae* are associated. ST23 strains of *K. pneumoniae*, which are well studied and frequently encountered in Russia [6], continue to circulate among the population and can colonize the intestine of a newborn child without clinical manifestations of the infectious process. This fact indicates that in the perinatal center, as well as in other medical institutions providing medical care at the inpatient stage, there is still a risk of convergence of hypervirulence and multidrug resistance properties [30], which is a highly undesirable phenomenon due to the emergence of difficulties with the treatment of invasive infections and the need to choose antibacterial drugs from the reserve group for eradication therapy.

During the period over 6 months preceding and following the date of detection of the strain with the hypermucoid phenotype, no isolates with similar phenotypic characteristics were detected in samples from patients and washes from objects in the hospital environment during industrial microbiological control. Taking this into account, it can be concluded that the strain isolated from the fecal sample was an out-of-hospital strain. Conducted local microbiological monitoring and analysis of the data obtained with its help allow timely detection and prevention of joint stay of patients isolating strains with hyperproduction of mucus and resistant to antibacterial drugs, thus preventing cross-infection of patients and undesirable events of microorganism variability that could be realized when they cross paths in one macroorganism.

Currently, there is no unified system of registration and surveillance of circulation of strains with hypermucoviscous phenotype of *K. pneumoniae*. At the same time, their phenotypic detection is realized in wide diagnostic practice of microbiological service during work with bacterial colonies on dense plate nutrient media of various purposes, for example, Endo medium and blood-serum agar. Perhaps, this is due to the fact that hypervirulence should not be identified with hyperproduction of mucus, and the question of choosing the most informative marker of virulence of *K. pneumoniae* remains open to date [20].

Three strains of *K. pneumoniae* researched in the present study, two of which were isolated from a positive hemoculture during generalized infection and the third from a fecal sample during colonization of the intestine of a newborn child, phenotypically manifesting hyperproduction of mucus and possessing the widest spectrum of virulence factor genes, had the same genetic determinants *fimH*, *mrkA* associated with biofilm-forming ability and synthesis of type I and III fimbriae. It is interesting to note that the *traT* gene, which provides serum resistance, was detected in strains isolated from blood samples and was not detected in the isolate from feces, which may have prevented it from overcoming the submucosal layer of the intestinal wall and prevented generalization of the infectious process.

Thus, for the first time in Russia, the results of comparative genomic analysis of clinical isolates of *K. pneumoniae* isolated from newborn infants with different outcomes of the infectious process in the neonatal period are presented, and the well-studied and long-standing sequence types and clonal groups, which have been found on all continents, are identified. No convergent or multidrug-resistant strains were identified in the present study. This is favorable for the epidemiological situation. At the same time, it was found

that strains with a high virulence index can be detected during local microbiological monitoring in obstetrics facilities.

Conclusion

1. *K. pneumoniae* strains with single or several virulence determinants are found among patients of neonatal hospitals. The pathogenic potential of *K. pneumoniae* ST23 (virulence index 5) with phenotypically manifested mucus hyperproduction was not realized as an infectious process in the organism of a newborn child.

2. *K. pneumoniae* ST14 with a smaller spectrum of virulence genes than ST23 and low antibiotic resistance (virulence index 0, antibiotic resistance 0) caused a complication during the neonatal period of a premature infant with congenital malformations of the CNS and cardiovascular system in the form of late sepsis with subsequent death. This case demonstrates the complexity of predicting infectious complications at the inpatient stage of nursing newborn premature infants, whose intestines are colonized with *K. pneumoniae*, based only on the genetic and phenotypic characteristics of the microorganism and dictates the necessity for a comprehensive assessment of both the bacterial strain and the patient's health status.

3. The results of this study have supplemented the data on the genetic diversity of strains associated with the neonatal period of development of premature newborn infants and demonstrated the need for further study of the patterns of development of complications of infectious genesis caused by opportunistic microorganisms during their colonization of non-sterile loci of the human body.

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Влияние штамма *Enterococcus faecium* 18 на грибы рода *Candida*

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Аннотация

Введение. Энтерококки, являющиеся представителями нормобиоты кишечника, играют важную роль в обеспечении колонизационной резистентности слизистых оболочек, продуцируя антимикробные соединения, и поэтому широко используются в качестве основы пробиотических препаратов. В последнее десятилетие серьёзной клинической проблемой стали инфекции, вызванные грибами рода *Candida*. В связи с этим актуальной является оценка пробиотических характеристик штамма *Enterococcus faecium* 18 и изучение его противогрибковой активности.

Цель работы — исследовать влияние штамма *E. faecium* 18 на рост и зрелую биоплёнку грибов рода *Candida*, а также охарактеризовать его агрегационную и коагрегационную способности.

Материалы и методы. Влияние на рост грибов определяли по динамике оптической плотности бульонных культур, воздействие супернатанта энтерококка на сформированные биоплёнки исследовали в стерильных полистироловых 96-луночных планшетах. Пробиотический потенциал *E. faecium* 18 оценивали по его способности к аутоагрегации и коагрегационному взаимодействию с 20 штаммами грибов рода *Candida* разных видов: *C. albicans, C. krusei, C. kefir, C. glabrata*. Для получения изображений использовали метод сканирующей электронной микроскопии.

Результаты. Показано ингибирующее действие супернатанта *E. faecium* 18 на рост грибов рода *Candida* всех исследуемых видов, а также их зрелые биоплёнки. Уровень ингибирования роста сформированных биоплёнок у *non-albicans* видов составил 58,6–72,9%; у *C. albicans* — 51,4%. Показатели аутоагрегации *E. faecium* 18 составили 57,6% через 2 ч инкубации и 60,4% через 5 ч. Штамм *E. faecium* 18 демонстрировал разные уровни коагрегации с исследованными видами грибов рода *Candida*, при этом индекс показателя через 5 ч культивирования оказался выше у видов *non-albicans*, максимальным значением характеризовался вид *C. glabrata* (85,6%).

Заключение. Полученные экспериментальные данные позволяют рассматривать изученный штамм в качестве основы пробиотика, оказывающего антикандидозное действие.

Ключевые слова: *Enterococcus faecium, Candida, зрелые сформированные биоплёнки, коагрегация, аутоагрегация*

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Effect of *Enterococcus faecium* strain 18 on fungi of the genus *Candida*

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Abstract

Introduction. *Enterococcus* spp. which are representatives of the intestinal normal microbiota, play an important role in ensuring colonization resistance of mucous membranes, producing antimicrobial compounds, and therefore are widely used as the basis of probiotic drugs. In the last decade, infections caused by *Candida* fungi have become a serious clinical problem. In this regard, it is relevant to evaluate the probiotic characteristics of the *E. faecium* strain 18 and study its antifungal activity.

The **aim** is to investigate the effect of the *E. faecium* strain 18 on the growth and mature biofilm of *Candida* spp., as well as to characterize its agregation and coagregation abilities.
Materials and methods. The effect on fungal growth was determined by the dynamics of the optical density of broth cultures; the effect of enterococcus supernatant on formed biofilms was studied in sterile polystyrene 96-well plates. The probiotic potential of *E. faecium* strain 18 was assessed by its ability to autoagregate and coagregate interaction with 20 strains of *Candida* of different species — *C. albicans*, *C. krusei*, *C. kefir*, *C. glabrata*. The scanning electron microscopy was used to obtain images.
Results. The inhibitory effect of the supernatant of *E. faecium* strain 18 has been shown to affect the growth of *Candida* of all studied species, as well as their mature biofilms. The level of inhibition of the growth of formed biofilms in non-albicans species was 58.6–72.9% and 51.4% for *C. albicans*. The autoagregation rates of *E. faecium* strain 18 were 57.6% after 2 hours of incubation and 60.4% after 5 hours. *E. faecium* strain 18 demonstrated different levels of coagregation with the studied species of *Candida*, with the index values observed after 5 hours of cultivation being higher in *non-albicans* species, and the maximum value recorded for *C. glabrata* (85.6%).
Conclusion. The experimental data obtained allow us to consider the studied strain as the basis for a probiotic that has an anti-candidiasis effect.

Keywords: *Enterococcus faecium, Candida, mature formed biofilms, coagregation, autoagregation*

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Введение

В последнее десятилетие инфекции, вызванные грибами рода *Candida*, стали серьёзной клинической проблемой [1]. Рост числа грибковых инфекций требует разработки новых противогрибковых средств. Весьма перспективным является использование пробиотических микроорганизмов и/или продуцируемых ими соединений для контроля распространения патогенных видов рода *Candida* [2]. Клинические наблюдения показывают, что пробиотические препараты могут уменьшить колонизацию *Candida* spp. на поверхности слизистых оболочек человека, облегчить признаки и симптомы грибковой инфекции и усилить противогрибковый эффект традиционной терапии [3].

Опубликован ряд исследований, в которых пробиотики рассматриваются не только как возможное средство лечения больных кандидозом [4], но и как препараты для борьбы с биоплёнками *Candida* spp. [5].

Энтерококки, являющиеся представителями нормобиоты кишечника, играют важную роль в обеспечении колонизационной резистентности слизистых оболочек, характеризуются наличием спектра антимикробных субстанций, в частности, продуцируют энтероцины — антимикробные пептиды, обладающие активностью против патогенов [6], и поэтому широко используются в качестве основы пробиотических препаратов [7].

Поиск и отбор штаммов энтерококков, обладающих антифунгальной активностью, ведутся среди представителей этого рода ещё и потому, что условно-патогенные дрожжи *Candida* spp. часто выделяются совместно с бактериями рода *Enterococcus* из различных биотопов и очагов инфекции в организме человека, что свидетельствует об их межклеточном взаимодействии [8, 9].

Ранее нами изучено влияние разных штаммов *E. faecium*, выделенных из кишечника человека, на способность грибов рода *Candida* снижать образование биоплёнок и отобран штамм *E. faecium* 18 с максимальной активностью [10], который может быть использован в качестве основы пробиотика, оказывающего антикандидозное действие.

Цель данной работы — исследовать влияние штамма *E. faecium* 18 на рост и зрелую биоплёнку грибов рода *Candida*, а также его агрегационную и коагрегационную способности, являющиеся важными свойствами перспективных пробиотических штаммов [11].

Материалы и методы

Для проведения исследований использовали штамм *E. faecium* 18 из коллекции кафедры микробиологии и заразных болезней Оренбургского государственного аграрного университета, который депонирован в Государственной коллекции микроорганизмов нормальной микрофлоры

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Московского научно-исследовательского института эпидемиологии и микробиологии им. Г.Н. Габричевского под коллекционным номером 1252. Энтерококк выращивали в Schaedler-бульоне («Conda») в течение 24 ч при 37ºС. Бактериальные клетки удаляли центрифугированием при ускорении 9000*g* под охлаждением до 4ºС в течение 15 мин. Полученный супернатант фильтровали через 0,22-мкм фильтр («Millipore Nihon») и сразу использовали в экспериментах. Антикандидозную активность *E. faecium* 18 исследовали на 4 видах дрожжеподобных грибов рода *Candida*, полученных из кишечника условно здоровых людей (*C. albicans, C. krusei, C. kefir, C. glabrata*), всего 20 штаммов (коллекция лаборатории персистенции и симбиоза Института клеточного и внутриклеточного симбиоза). Культуры грибов выращивали аэробно на среде Сабуро с декстрозой («Hi Media») при 35ºС в течение 24 ч.

Для определения антикандидозной активности использовали метод микротитрования в питательной среде на стерильных полистироловых 96-луночных планшетах («Sigma-Aldrich Chemie») согласно S. Wang и соавт. [12] с изменениями. В лунки стерильного микропланшета вносили 100 мкл бульона Сабуро, содержащего 2×10^5 КОЕ/лунку *Candida* и 100 мкл супернатанта энтерококка. Каждую пробу испытывали параллельно в 4 лунках. В качестве положительного контроля использовали суспензию клеток грибов на питательной среде без супернатанта, в качестве отрицательного контроля — Schaedler-бульон. После аэробной инкубации при 37ºС в течение 24 ч рост грибов определяли по оптической плотности (ОП) при длине волны 492 нм с использованием полуавтоматического планшетного спектрофотометра «Stat Fax 2100» («Awareness Technology»).

При изучении влияния супернатанта энтерококка на грибную сформированную биоплёнку культуры *Candida* культивировали в течение 48 ч при 37ºС, после удаления взвеси и отмывания лунок добавляли супернатант *E. faecium* 18 в объёме 100 мкл. Планшеты помещали в термостат при 37ºС на 24 ч, затем замеряли ОП на полуавтоматическом планшетном спектрофотометре «Stat Fax 2100» («Awareness Technology»). Контролем служили штаммы грибов рода *Candida*, не подвергавшиеся влиянию супернатанта энтерококка. Эксперимент проводили в 3 повторах с интервалом 24 ч.

Способность штамма *E. faecium* 18 к аутоагрегации оценивали в соответствии с методом K.M.O. dos Santos и соавт. [13] с небольшими изменениями. Культуру энтерококка, полученную в Schaedler-бульоне после 24-часовой инкубации при 37ºС, собирали центрифугированием при 9000*g* в течение 10 мин при 4ºС. Клетки дважды промывали в физиологическом растворе, обогащённом фосфатом (PBS; pH 7,2 перед стерилизацией), и суспендировали в PBS при начальной ОП, измеренной при длине волны 630 нм. Затем бактериальную суспензию (2 мл) перемешивали в течение 10 мин с помощью «SPINIX Vortex» («Parsons») и инкубировали при 37ºС в течение 5 ч без перемешивания. Для замера ОП образца в начале инкубации и через 2 и 5 ч культивирования использовали 1 мл верхнего слоя для измерения при 630 нм. Определяли аутоагрегацию (*AA*) по формуле:

$$AA = [(\text{ОП начальная} - \text{время ОП/ОП начальное}] \times 100\%. \quad (1)$$

Коагрегационный анализ взвесей энтерококков с дрожжеподобными грибами проводили по модифицированному методу K.M.O. dos Santos и соавт. [13].

Культуры энтерококков выращивали в 3 мл Schaedler-бульона, а грибов рода *Candida* — в 3 мл бульона Сабуро при 37ºС в течение 24 ч, после чего культуры центрифугировали 10 мин в центрифуге «Microspin 12» («Biosan») с охлаждением (4ºС) при ускорении 9000*g* и промывали в PBS (pH 7,2 перед стерилизацией), данные манипуляции повторяли дважды, затем суспендировали взвеси в PBS.

На следующем этапе равные объёмы (по 750 мкл) взвеси культуры *E. faecium* 18 и взвеси тест-штаммов грибов рода *Candida* spp. попарно смешивали встряхиванием в течение 10 с и замеряли ОП каждой взвеси при длине волны 630 нм (начальное значение ОП — 0 ч). Пробирки инкубировали при 37ºС без перемешивания в течение 5 ч, замеряя ОП через 2 и 5 ч инкубации в 1 мл верхнего слоя проб при длине волны 630 нм (время ОП).

Коагрегацию (*A*) рассчитывали по формуле:

$$A = [(\text{начальная ОП} - \text{время ОП/начальная ОП}] \times 100\%. \quad (2)$$

Подготовку образцов для сканирующей электронной микроскопии (СЭМ) проводили следующим образом: взвеси культуры энтерококка и взвеси культуры энтерококка с тест-штаммами грибов в физиологическом растворе в концентрации 10^9 КОЕ/мл трижды отмывали 0,1 М фосфатно-буферным раствором Соренсена («ЛидерМед Групп») и добавляли 400 мкл 2,5% глутарового альдегида к последнему осадку. Образцы инкубировали в течение 24 ч при 4ºС и вновь отмывали 0,1 М фосфатно-буферным раствором, обезвоживали водно-этанольными растворами с возрастающими концентрациями (20, 40, 60, 80 и 90% и 2 цикла 100%) и наносили на покровные стекла. Время инкубации в каждом растворе составляло15 мин при комнатной температуре. Покровные стекла с образцами высушивали в критической точке «Quorum K850 Critical Point Dryer» («Quorum Technologies Ltd.»), прикрепляли двусторонним скотчем к столику СЭМ и напыляли золотом с помощью установки

ионно-плазменного напыления «Quorum Q150R S plus» («Quorum Technologies Ltd.»). СЭМ проводили на сканирующем электронном микроскопе «Tescan Mira 3» («Tescan Brno») Центра коллективного пользования образовательного Центра выявления и поддержки одарённых детей «Гагарин» (Оренбург).

Полученные данные статистически обработаны с помощью критерия Стьюдента в программе «Statistica 6.0» («StatSoft, Inc.»). Результаты представлены в виде средних значений и ошибок средних ($M \pm m$), полученных не менее чем в 3 независимых экспериментах. Значимыми считали различия при $p < 0,05$.

Результаты

Установлено ингибирующее действие супернатанта *E. faecium* 18 на рост грибов рода *Candida* всех исследуемых видов. В положительном контроле уровень ОП суточной бульонной культуры грибов у *C. albicans* составлял 0,73 ± 0,02; у *C. glabrata* — 0,41 ± 0,01; у *C. kefir* — 0,32 ± 0,01; у *C. krusei* — 0,69 ± 0,02, а при добавлении супернатанта снижался до 0,37 ± 0,01; 0,25 ± 0,01; 0,15 ± 0,01; 0,35 ± 0,02 КОЕ/мл соответственно (**рис. 1**, *а*).

Наиболее высокий уровень ингибиции отмечен для *C. kefir* — в 2,1 раза. Несколько ниже была степень ингибирования *C. albicans* и *C. krusei*, у которых уровень роста снижался в 2 раза, а у *C. glabrata* — в 1,6 раза.

В следующей серии экспериментов изучали влияние супернатанта *E. faecium* 18 на сформированные грибами рода *Candida* биоплёнки. В контроле среднее значение коэффициента биоплёнкообразования (КБО) у *C. albicans* составляло 3,50 ± 0,01

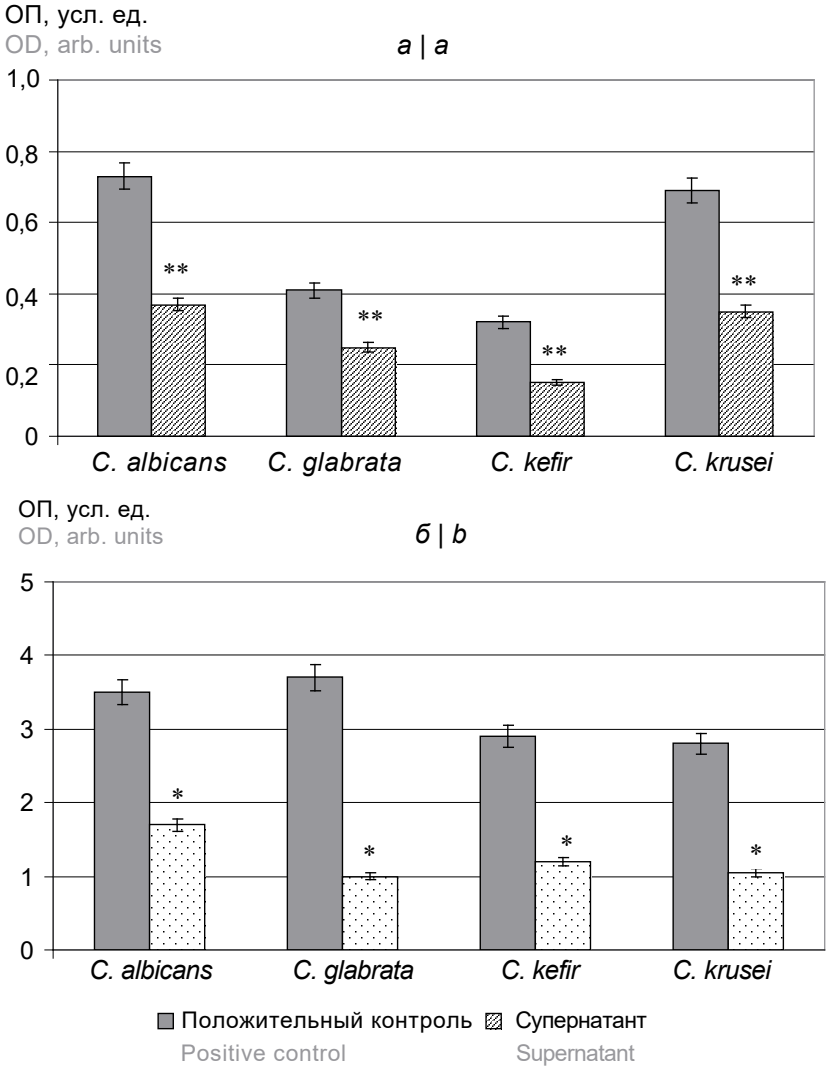

Рис. 1. Влияние супернатанта *E. faecium* 18 на рост (*а*) и сформированные биоплёнки (*б*) грибов рода *Candida.*

*$p < 0,05$; **$p < 0,001$.

Fig. 1. The effect of the supernatant of *E. faecium* strain 18 on the growth (*a*) and formed biofilms (*b*) of fungi of the genus *Candida.*

*$p < 0.05$; **$p < 0.001$.

усл. ед., *C. glabrata* — 3,70 ± 0,02 усл. ед., *C. kefir* — 2,90 ± 0,02 усл. ед., *C. krusei* — 2,80 ± 0,02 усл. ед.

Установлена способность супернатанта энтерококка разрушать зрелые биоплёнки изученных видов грибов (рис. 1, *б*). Так, он достоверно ингибировал рост сформированных биоплёнок *C. albicans* на 51,4% (КБО 1,70 ± 0,01 усл. ед.; $p < 0,05$), *C. glabrata* — на 72,9% (КБО 1,00 ± 0,01 усл. ед.; $p < 0,05$), *C. kefir* — на 58,6% (КБО 1,20 ± 0,01 усл. ед.; $p < 0,05$), *C. krusei* — на 62,5% (1,05 ± 0,01 усл. ед.; $p < 0,05$).

Таким образом, проведённые исследования свидетельствуют о том, что супернатант *E. faecium* 18 способен не только ингибировать рост штаммов разных видов грибов рода *Candida*, но и разрушать сформированные ими биоплёнки.

Далее нами изучены агрегационная и коагрегационная способности культуры *E. faecium* 18, являющиеся важными свойствами перспективных пробиотических штаммов. Показано, что значения аутоагрегации *E. faecium* 18 увеличивались в зависимости от продолжительности инкубационного периода: от 57,6% (2 ч) до 60,4% (5 ч). Результаты аутоагрегации энтерококка проиллюстрированы на **рис. 2**.

Вместе с тем штамм *E. faecium* 18 проявлял различные уровни коагрегации у 4 исследованных видов грибов (**таблица**). Индекс коагрегации увеличивался с ростом инкубационного периода. После 5-часовой инкубации самый высокий уровень коагрегации наблюдался с изолятами *C. glabrata* (85,6%), далее следовали *C. krusei* (55,9%), *C. kefir* (45,9%), *C. albicans* (37,2%). Результаты коагрегации *E. faecium* со штаммами *C. albicans* представлены на **рис. 3**.

Коагрегация *E. faecium* 18 с *Candida* spp.
E. faecium strain18 coagregation with *Candida* spp.

Candida spp.	Индекс коагрегации с *E. faecium* 18, % Coagregation index with *E. faecium* 18, %	
	2 ч | 2 h	5 ч | 5 h
C. albicans	29,7 ± 0,04	37,2 ± 0,03
C. glabrata	56,2 ± 0,07	85,6 ± 0,05
C. kefir	22,7 ± 0,03	45,9 ± 0,03
C. krusei	26,8 ± 0,04	55,9 ± 0,04

Обсуждение

Проведённые исследования показали, что супернатант *E. faecium* 18 обладает выраженной анти-

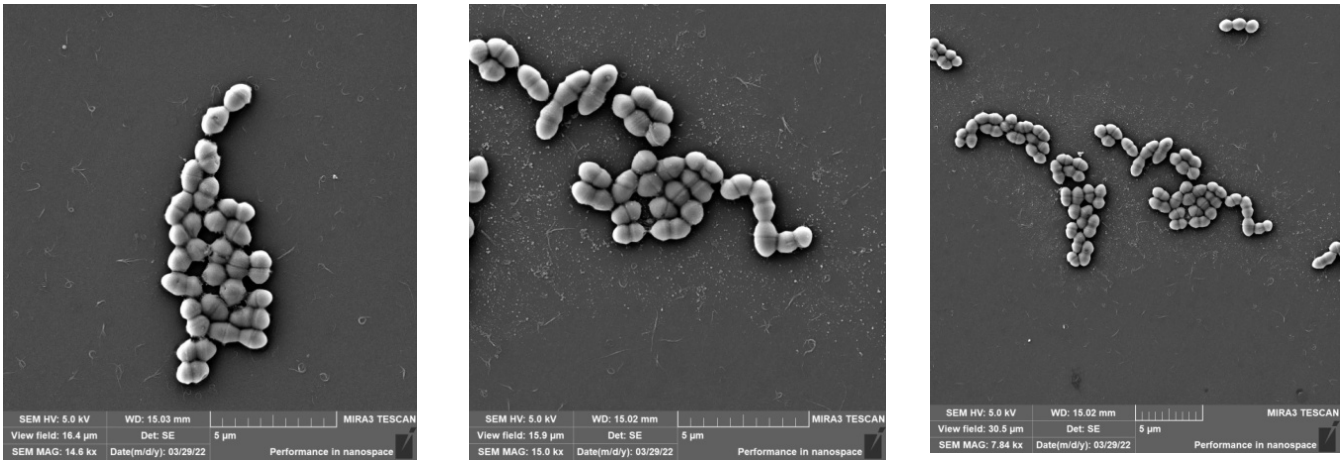

Рис. 2. Аутоагрегация *E. faecium* 18.
Fig. 2. Autoagregation of *E. faecium* strain 18.

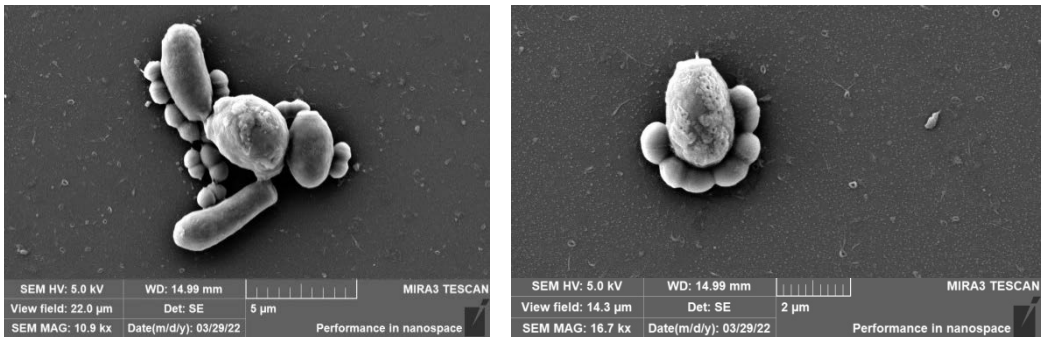

Рис. 3. Коагрегация *E. faecium* 18 с *C. albicans*.
Fig. 3. Coaggregation of *E. faecium* strain 18 with *C. albicans*.

микробной активностью в отношении грибов рода *Candida* и способностью разрушать сформированную биоплёнку исследованных видов грибов.

Полученные данные представляют интерес для клинической практики, т. к. известна способность грибов функционировать в составе многоклеточных сообществ (биоплёнок) [14, 15], которые препятствуют проникновению лекарственных средств, что повышает устойчивость микроорганизмов к антимикотикам [16, 17], а также являются одной из основных стратегий выживания этих микроорганизмов в организме человека [18].

Поэтому в настоящее время важной задачей является поиск средств борьбы с биоплёнками грибов рода *Candida*; в частности, пробиотических микроорганизмов, способных разрушать биоплёнки грибов и/или ингибировать их рост [19].

Проведённые нами исследования иллюстрируют *in vitro* эффект бесклеточного супернатанта энтерококка, который обладает чётко выраженным ингибирующим действием на сформированную биоплёнку грибов рода *Candida.* Полученные результаты не противоречат исследованиям ряда авторов, изучавших антибиоплёночную активность молочнокислых бактерий на изолятах *Candida* spp. [20]. Несмотря на то что основным механизмом ингибирования образования биоплёнки *Candida* spp. является конкуренция за адгезию, M. Kıvanç и соавт. пришли к выводу о том, что вещества, содержащиеся в бесклеточных фильтратах молочнокислых бактерий, также важны [21]. Так, в ряде работ показан ингибирующий эффект молочнокислых микроорганизмов в отношении биоплёнкообразующей способности патогенов, что, по мнению авторов, является результатом выработки органических кислот, включая молочную [22, 23].

Принимая во внимание тот факт, что, по результатам полногеномного секвенирования, включённый в исследование штамм энтерококка не способен к продукции бактериоцинов [24], можно предположить, что разрушение зрелых биоплёнок *Candida* spp. не связано с продукцией энтероцинов. Последнее предположение находит подтверждение в работе X. Pang и соавт. (2022), которые доказали, что бактериоцины могут эффективно ингибировать образование биоплёнок дозозависимым образом, но им трудно разрушить предварительно сформированные биоплёнки [25].

Эффективность пробиотика во многом зависит от адгезивной способности пробиотического штамма и отсутствия конкурентных отношений с индигенной микрофлорой. Аутоагрегация является первым этапом в процессе адгезии, позволяя бактериям формировать барьер против колонизации патогенов [26]. Взаимосвязь между образованием биоплёнки и агрегацией у непатогенных штаммов энтерококков впервые показана K. Veljovic и соавт. [27].

Высокая аутоагрегация и способность прилипать к эпителиальным клеткам и поверхностям слизистых оболочек является важным свойством многих штаммов бактерий, используемых в качестве пробиотиков [28, 29]. В нашем исследовании штамм энтерококка показал высокий уровень аутоагрегации уже через 2 ч, что свидетельствует о его конкурентных, исключающих патоген, свойствах. Сильная агрегация пробиотического штамма способствует достижению им достаточной массы для формирования биоплёнок и усилению его способности к коагрегации с потенциальным патогеном. В результате коагрегативных взаимодействий пробиотические штаммы микроорганизмов оказывают антагонистическое действие против патогенов [30].

Проведёнными нами исследованиями показана способность штамма энтерококка к коагрегации с разными видами грибов рода *Candida*, при этом индекс показателя через 5 ч культивирования оказался выше с non-albicans-видами, а максимальное значение достигнуто с *C. glabrata*.

Заключение

Полученные экспериментальные данные свидетельствуют о том, что противокандидозный эффект *E. faecium* 18 включает различные механизмы, что в совокупности отражает сложность взаимодействия между грибами рода *Candida* и *E. faecium*, расширяя представления о механизмах межмикробного взаимодействия и открывая перспективы дальнейшего изучения энтерококка в качестве основы пробиотика, оказывающего антикандидозное действие. Чтобы использовать этот штамм в пробиотических препаратах, необходимо провести его дальнейшие испытания на животных моделях и оценить лечебный потенциал.

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Human blood granulocyte degranulation and lysis intensity during interaction with *Yersinia pestis* in the *ex vivo* model of bacteriemia

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Abstract

Introduction. Considering the decisive role of antibacterial strategies of secretory degranulation and NETosis in the prevention of sepsis, it is of interest to study the interaction of *Yersinia pestis* with human blood granulocytes using an *ex vivo* bacteremia model to assess the effectiveness of this antibacterial strategy of the host organism in plague.

Purpose: evaluation of granulocyte degranulation and lysis in human whole blood samples in the presence of live *Y. pestis.*

Materials and methods. Bacteremia was modeled by adding *Y. pestis* EV NIIEG cells grown at 37°C or 28°C to whole blood (with heparin) at a dose of 10^8 mc/mL. Strains *Staphylococcus aureus* ATCC 6538 (209-P) and *Escherichia coli* ATCC 25922 were used in experiments with blood from the same donors as a positive control. The bactericidal effect was determined at different time points during blood incubation at 37°C (for 6 hours) using a microbiological method. Using flow cytometry, immunophenotyping of leukocytes was performed in the blood according to the Lyse/No-Wash protocol to determine the expression of the main leukocyte antigen CD45 and the secretory azurophilic degranulation marker CD63 on the surface of the granulocytes. The intensity of granulocyte lysis was assessed by the decrease in the proportion of these cells in the total leukocyte population.

Results. It has been established that live plague microbes, unlike *E. coli* and *S. aureus*, do not cause the development of azurophilic degranulation in human blood granulocytes and do not induce autolysis (NETosis) of these cells within 6 hours when bacteremia is modeled *ex vivo*.

Conclusion. Information was obtained on the ability of the plague microbe to suppress the extracellular bactericidal mechanisms of granulocytes in the blood of people not vaccinated against plague, which effectively function under conditions of bacteremia against *E. coli* and *S. aureus*. An experimental and methodological basis has been prepared for further research with blood cells from donors vaccinated against plague in order to develop new effective tests for assessing the intensity of acquired cellular anti-plague immunity in humans.

Keywords: *Yersinia pestis, Escherichia coli, Staphylococcus aureus, ex vivo bacteremia model, neutrophil, neutrophil azurophilic degranulations, NETosis, leukocyte elastase, leukocyte immunophenotyping, flow cytometry*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Russian Research Anti-Plague Institute “Microbe” (protocol No. 9, October 21, 2020).

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Оригинальное исследование
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Интенсивность дегрануляции и лизиса гранулоцитов крови человека при взаимодействии с *Yersinia pestis* на модели бактериемии *ex vivo*

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Аннотация

Введение. С учётом решающей роли антибактериальных стратегий секреторной дегрануляции и нетоза в предотвращении сепсиса, представляет интерес изучение взаимодействия *Yersinia pestis* с гранулоцитами крови человека на модели бактериемии *ex vivo* для оценки эффективности этих стратегий при чуме.

Цель работы — оценка дегрануляции и лизиса гранулоцитов в образцах цельной крови человека в присутствии живых *Y. pestis*.

Материалы и методы. Бактериемию моделировали добавлением в цельную кровь (с гепарином) клеток аттенуированного штамма *Y. pestis* EV НИИЭГ, выращенных при 37°С либо 28°С, в дозе 10^8 м.к./мл. Штаммы *Staphylococcus aureus* ATCC 6538 (209-P) и *Escherichia coli* ATCC 25922 использовали в опытах с кровью тех же доноров в качестве положительного контроля. Бактерицидный эффект определяли в различные сроки инкубации крови при 37°С (в течение 6 ч) микробиологическим методом. С помощью проточной цитометрии в крови проводили иммунофенотипирование лейкоцитов по Lyse/No-Wash протоколу для определения экспрессии на поверхности гранулоцитов основного лейкоцитарного антигена CD45 и маркера секреторной азурофильной дегрануляции CD63. Интенсивность лизиса гранулоцитов оценивали по снижению доли этих клеток в суммарной лейкоцитарной популяции.

Результаты. Установлено, что живые клетки чумного микроба, в отличие от *E. coli* и *S. aureus,* не приводят к развитию азурофильной дегрануляции в гранулоцитах крови человека и в течение 6 ч не индуцируют аутолизис (нетоз) этих клеток при моделировании бактериемии *ex vivo*.

Заключение. На модели чумной бактериемии *ex vivo* впервые получена информация, свидетельствующая о том, что в крови не привитых против чумы людей не работают механизмы внеклеточной бактерицидности гранулоцитов, эффективно функционирующие в условиях бактериемии в отношении *E. coli* и *S. aureus.* Подготовлена экспериментально-методическая основа для дальнейших исследований с клетками крови привитых против чумы доноров с целью разработки новых эффективных тестов оценки напряжённости приобретённого клеточного противочумного иммунитета.

Ключевые слова: *Yersinia pestis, Escherichia coli, Staphylococcus aureus, модель бактериемии ex vivo, нейтрофилы, азурофильная дегрануляция нейтрофилов, нетоз, лейкоцитарная эластаза, иммунофенотипирование лейкоцитов, проточная цитометрия*

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Introduction

Primary pneumonic plague, caused by *Yersinia pestis* and transmitted to humans through respiratory droplets from another person or domestic animal, is the most dangerous bacterial infection, in which the pathogen effectively neutralizes the protective mechanisms of the immune system and multiplies intensively in the host organism without inducing the development of an *in vivo* protective inflammatory response for a long time. The asymptomatic character of the development of the infectious process during the first 2 days after aerogenic infection with plague is the key factor determining the high epidemic danger of primary pneumonic plague [1, 2]. The pathogenesis of plague is poorly understood, especially at the stage of bacteremia [3], when *Y. pestis* cells resistant to phagocytosis, proliferating extracellularly in microcapillaries of the liver, lungs and other organs, enter *in vivo* into prolonged contact interaction with peripheral blood leukocytes, including neutrophil granulocytes (NG), responsible for the development of inflammatory reactions [4].

Nevertheless, in experiments on laboratory animals, an important feature of the pathogenetic mechanism of primary pneumonic plague has been established, which consists of the complete suppression of the inflammatory processes at the early stage of infection, such as secretory azurophilic degranulation [5], chemotaxis [6], apoptosis [7] and lysis of NG [8] by effector Yop-proteins (*Yersinia* outer proteins) synthesized by *Y. pestis* at 37°C and secreted by extracellularly multiplying plague microbes into the cytoplasm of cells of the host immune system [4, 9]. At the same time, Yop virulence proteins of *Y. pestis*, on the contrary, trigger the death of macrophages by apoptosis, which disrupts the *in vivo* functioning of the efferocytosis mechanism and inevitably leads to the generalization of the inflammatory process as a result of massive autolysis of peripheral blood NG unable to realize their bactericidal potential. Autolysis of granulocytes, rapidly triggered with a time delay in the whole organism at once, according to the hypothesis of M.T. Silva [10], leads to the release of a huge amount of leukocyte elastase (LE) molecules into the blood plasma, destroying elastin of the lungs and blood vessels, as well as cleaving cell receptors and plasma proteins responsible for the regulation of the coagulation process, which is the trigger for the unexpected and lightning fast development of sepsis in primary pneumonic plague.

To confirm (or refute) this hypothesis, a detailed study of the processes of degranulation and lysis of human and animal blood granulocytes using modern methods of cytological analysis under conditions of *ex vivo* modeling of plague bacteremia is required. As evidenced by the results of the study of COVID-19 pathogenesis associated with a pronounced imbalance in the elastase-inhibitor system [11], in the case of isolation of NG from the peripheral blood of patients, important information about the role of NETosis in immunological defense and in the development of the process of hypercoagulation in sepsis may be lost, because the procedure of centrifugation of blood cells in a density gradient does not allow differentiating from lymphocytes and monocytes the population of activated low-density NG prone to NETosis with increased expression of the marker of azurophilic degranulation CD63 on the cell surface [12].

For a long time, studies on *ex vivo* models of bacteremia were not performed, including in experiments with opportunistic bacteria [13], because it was believed that blood granulocytes realize their powerful bactericidal potential only after migration from the vascular channel to tissues, where they neutralize bacteria by phagocytosis. The situation changed after the discovery of NETosis in 2004 and the studies of V. McDonald et al. [14], who experimentally proved that autolysis of NG (NETosis), preceded and accompanied by secretory azurophilic degranulation of NG with release from LE granules, allows the organism to neutralize microbes at an early stage of bacteremia (during the first 6 h) directly in the blood stream with the help of NET (Neutrophil Extracellular Traps) DNA networks, launched by activated neutrophils into the extracellular space. Moreover, the efficiency of this previously unknown mechanism of immunological defense in terms of preventing bacterial sepsis in infections caused by *Escherichia coli* and *Staphylococcus aureus* was 4 times higher than phagocytosis.

In particularly dangerous infections, the pathogens of which are resistant to digestion in macrophages (e.g., *Y. pseudotuberculosis*), proteases and bactericidal cationic proteins released from NG during secretory degranulation and cytolysis partially or completely neutralize phagocytosis-resistant bacteria, which after such pre-treatment are rapidly absorbed and digested by macrophages [15]. In 2002, for the first time, we published the results of studies in which the flow cytofluorimetric method of assessing the intensity of degranulation was used to record differences in the ability of plague microbes grown at 28°C and 37°C to trigger *ex vivo* the process of secretory degranulation in human whole blood phagocytes. Cells grown at 37°C were characterized by a breakdown of antibacterial response by the indicator of secretory degranulation in the model of plague bacteremia [16]. Many years later, experimental data began to appear in the foreign press, which could explain the mechanism of the phenomenon of the absence of phagocyte degranulation in human blood contaminated with live plague microbes. The studies were carried out *in vivo* in mice [5,8] and *in vitro* with NG previously isolated from human blood [17, 18]. Degranulation was assessed, in contrast to our work, not using supravital staining with acridine orange (AO) dye, but by the degranulation marker CD63. Experiments in an *ex vivo* model of bacteremia were

not performed. The intensity of granulocyte lysis in human blood contaminated with plague microbes or opportunistic microorganisms had not been determined or controlled by flow cytometry before our study was conducted.

The relevance of continuing our earlier studies on the *ex vivo* model of bacteremia using the marker of azurophilic degranulation CD63 and other leukocyte phenotypic markers was determined by the need to develop a cellular test for assessing the intensity of post-vaccination plague immunity in humans, based on quantitative assessment of the damage of peripheral blood neutrophils by specific antigen. In light of modern ideas, the strategy of vaccine development for emergency prophylaxis of plague and other particularly dangerous infections should take into account the ability of immunostimulating drugs to trigger the mechanism of extracellular antibody-dependent cytotoxicity (bactericidality) of NG [19], realized in the blood by interaction with antigen-antibody immune complexes through the processes of secretory azurophilic degranulation and NETosis [20]. Only when specific antibodies to the antigen previously used for immunization of animals appear in the blood, intravenous injection (or addition to the blood) of this antigen triggers in the body (or under *ex vivo* conditions) a protective IgG-mediated anaphylactic reaction associated with secretory degranulation and lysis of peripheral blood NGs [21]. The molecular mechanisms responsible for the transition of the infectious process in primary pneumonic plague from the asymptomatic to the systemic inflammation phase are poorly understood [3, 22], and one of them may be related to the *in vivo* triggering of an IgG-mediated allergic reaction.

The absence of a local protective inflammatory response in a non-immune host organism is observed not only when infected with wild virulent strains of *Y. pestis*, but also in response to non-pigmented strains with a virulence plasmid, such as the vaccine strain of *Y. pestis*, but also in response to non-pigmented strains with a virulence plasmid, which includes the vaccine strain of *Y. pestis* EV NIIEG (Pgm¬-pFra+pCad+pPst+) [1, 23]. Possessing residual virulence, such attenuated strains cause death of laboratory animals only when administered intravenously in doses of more than 10^6 mc, which is used in model experiments to study the mechanisms of immunity and virulence [23].

The aim of the present study was to evaluate degranulation and lysis of granulocytes in human whole blood samples in the presence of live *Y. pestis*.

Materials and methods

The attenuated *Y. pestis* EV NIIEG strain was used in the studies, *S. aureus* strain ATSS 6538 (209-P) and *E. coli* strain ATSS 25922 from the State Collection of Pathogenic Bacteria of the Russian Anti-Plague Institute Microbe of Rospotrebnadzor. Daily cultures of *S. aureus* and *E. coli* were grown on Hottinger's agar (pH 7.2) at 37°C. For *Y. pestis* EV cells, a two-day stationary bacterial culture grown on the same agar at 28°C (*Y.pestis*28) was obtained. An exponential 18-h culture of *Y. pestis* EV with altered antigenic properties was obtained by growing on Hottinger broth (pH 7.2) with aeration at 37°C (*Y.pestis*37) [24]. In sterile phosphate-salt buffer (pH 7.4) with 0.9% NaCl, suspensions of live bacteria with a concentration of 109 mc/ml from cultures of *E. coli*, *S. aureus*, *Y.pestis*28 and *Y.pestis*37 were prepared according to the standard turbidity sample CCA 42-28-59-85P.

The study involved 10 conditionally healthy unvaccinated against plague donors (3 men and 7 women) aged 25–55 years who gave written voluntary informed consent to participate in the study. The study protocol was approved by the Ethical Committee of the Russian Research Anti-Plague Institute Microbe (Protocol No. 9 of 21.10.2020).

Blood from volunteers was drawn into tubes with anticoagulant (heparin) and used for 1–2 h. To simulate bacteremia, 100 μL of the tested billionth bacterial suspension was added to 1 ml of blood containing on average 2 × 106 phagocytes, which corresponded to a concentration of 108 mc/mL of blood or an initial microbial load (number of bacteria : phagocyte) of 50 : 1 on average [25]. With blood from each donor, cell suspensions of *Y. pestis*28 and *Y.pestis*37 were examined simultaneously with cell suspensions of *E. coli* and/or *S. aureus*. Blood samples (1 mL each) with the tested bacteria were placed in the shaker-incubator ES-20 (BioSan) and incubated under stirring for 6 hours. Samples incubated for 6 h without bacteria with 100 μL of sterile phosphate-salt buffer per 1 mL of blood served as a control.

Immunophenotyping of leukocytes in the studied blood samples for flow cytofluorimetric analysis was performed according to the Lyse/No-Wash protocol [26] using labeled mouse monoclonal antibodies to human leukocyte antigens CD45-FITC and CD63-PE (Bekman Coulter). Granulocytes were identified by the degree of their intracellular granularity (side light scattering intensity) and by the expression of total leukocyte antigen (CD45). The relative content of cells positive for the expression of the azurophilic degranulation surface marker CD63 was determined in the granulocyte gate [5, 17]. The results were taken into account for each experimental and control blood sample in dynamics: after 0, 1, 2, 4, 6 h of incubation.

The total number of live bacteria in blood (in plasma and within active phagocytes) was determined by a microbiological method based on osmotic lysis of blood cells in distilled water [27]. To 1 mL of H_2O, 10 μL of blood was added. After 30 s, serial 10-fold dilutions of plasma containing bacteria and blood cell lysis products were prepared in phosphate-salt buffer from water-diluted plasma containing bacteria and

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blood cell lysis products for seeding on dishes with Hottinger's agar (pH 7.2). For each dilution, the number of colony forming units (CFU) of *Staphylococcus aureus* or *Escherichia coli* were counted after one day of growth at 37°C, and *Y. pestis* cells were counted on the 3rd day of growth at 28°C. In relation to the number of CFU after 0 h of incubation, taken as 100%, the survival rate of bacteria of each species in blood (in %) after 1, 2 and 6 h of incubation was estimated to comparatively characterize the development of bactericidal effect under *ex vivo* conditions [25].

The intensity of granulocyte lysis was quantified *ex vivo* by flow cytometry by a decrease in the proportion of these cells in whole peripheral blood samples, as well as by an increase in the relative content of cellular debris in the blood [25].

To assess the light scattering and immunofluorescence intensity of CD-marker-labeled blood leukocytes, a DakoCytomation (Dako) flow cytometer with Summit v.4.3 Built 2445 software was used.

The obtained experimental data were statistically processed using the standard Microsoft Office Excel 2016 software package, Statistica 10.0 (StatSoft Inc.), presenting the results in the form of median (Me) and quartile deviations [Q_1; Q_3] with calculation of the reliability of differences in the studied groups using the Mann–Whitney U-criterion. A value of $p < 0.05$ was considered significant.

Results

When *Y.pestis*37 was added to blood for 6 h, granulocytes lacked changes related to the state of cytoplasmic granules, as well as lysis of these cells under conditions of *ex vivo* modeling of plague bacteremia, while in the blood of the same donors opportunistic bacteria induced intensive degranulation and inevitable lysis of the overwhelming majority of granulocytes in the total population of peripheral blood leukocytes by 6 h of incubation. Antibacterial response of granulocytes according to the studied indicators of degranulation and cytolysis took place in the case of *ex vivo* modeling of bacteremia by *Y.pestis*28 cells. However, in comparison with the reaction to *E. coli* or *S. aureus*, this response was significantly less intense (**Table**).

The registered differences in the studied parameters are clearly illustrated by the example of *Y.pestis*37 and *E. coli* characteristic cytograms presented in **Fig. 1**, where granulocytes are localized by the degree of intracellular granularity (intensity of lateral light scattering) and density of CD45 expression in the ellipse-shaped region R3 at their automatic differentiation from lymphocytes and monocytes in control blood samples. In the presence of *E. coli*, the proportion of granulocytes (cells in the R3 region) decreased after 6 h in the total leukocytic population relative to the control index by 10 times – from 47.1% to 4.8%, and in the presence of *Y.pestis*37 actually did not change, remaining close to the control (43.8%). In blood contaminated with *E. coli*, the proportion of granulocytes decreased sharply during this period due to massive lysis of dead phagocytes, significantly increasing the relative amount of signals from cellular debris registered outside the R1 region. On the cytogram of the experimental blood sample with *Y.pestis*37 cells, the proportion of debris

The results of granulocyte azurophilic degranulation and lysis intensity estimation in an *ex vivo* modeling of bacteremia by live *E. coli, S. aureus* and *Y.pestis*37 in depending of blood incubation time at 37°C, Me [Q_1; Q_3]

Parameter	Blood sample	Duration of incubation, min			
		60	120	240	360
Granulocyte lysis intensity, %	Control	5,2 [3,8; 7,4]	9,1 [8,6; 11,8]	12,2 [10,5; 13,7]	14,6 [12,1; 16,8]
	S. aureus	27 [25,4; 29,6]*	56,0 [51,2; 59,7]*	78 [73,6; 82,5]*	82,0 [76,8; 85,2]*
	E. coli	22,3 [20,7; 24,5]*	38,8 [37,3; 40,5]*	63,6 [56,5; 70,4]*	78,6 [73,4; 84,7]*
	*Y.pestis*28	7,3 [6,2; 9,1]	10,4 [8,7; 13,5]	20,6 [18,6; 21,5]*	19,0 [17,5; 20,8]*
	*Y.pestis*37	6,7 [4,3; 8,6]	8,0 [6,2; 9,5]	9,3 [6,7; 12,8]	10,6 [6,8; 13,0]
Share of granulocytes with $CD63^+$ phenotype, %	Control	10 [9,2; 13,8]	14 [11,4; 16,3]	17 [15,5; 19,7]	18 [16,3; 21,4]
	S. aureus	35 [34,5; 35,7]*	70 [67,0; 72,4]*	83 [74,5; 93,0]*	79 [68,4; 90,2]*
	E. coli	28,3 [25,6; 32,4]*	50,3 [44,6; 55,3]*	70,6 [61,0; 75,7]*	80[72,2; 88,2]*
	*Y.pestis*28	19,7 [17,8; 23,5]*	26,0 [21,1; 30,2]*	32,3 [28,4; 36,6]*	31,2 [22,2; 38,7]*
	*Y.pestis*37	14,2 [11,9; 18,3]	13,4 [10,6; 17,3]	15,1 [13,2; 18,6]	20,8 [16,5; 22,7]

Note. *$p < 0.05$ compared with control.

after 6 h of incubation was, on the contrary, lower than in the control without bacteria.

In contrast to opportunistic bacteria, *Y.pestis*37 cells did not induce *ex vivo* increased expression on the granulocyte surface of the lysosomal protein CD63 (tetraspanin), which is a marker of the development of secretory azurophilic degranulation (**Fig. 2**). In blood samples contaminated with live *E. coli* and *S. aureus* cells, the process of CD63 secretion from granules to the granulocyte surface began after one hour, significantly intensified from the 2nd hour of incubation and preceded the lysis of activated granulocytes under *ex vivo* conditions (Table).

Against the background of the absence of antibacterial response of granulocytes according to the studied indicators of degranulation and leukocytolysis, increased survival of *Y.pestis*37 in human whole blood samples in comparison with *Y.pestis*28 was registered by the microbiological method. The plague microbe grown at the temperature of the host organism began to multiply intensively after 6 h under conditions of *ex vivo* modeling of bacteremia. In the blood of the same donors, opportunistic bacteria were quickly killed under the influence of bactericidal effect of active phagocytes. The survival rate of *E. coli* and *S. aureus* decreased by at least 80% by 6 h from the moment of bacteremia modeling (**Fig. 3**).

Discussion

When conducting the present study by flow cytometry, experimental data were obtained for the first time indicating that the Lyse/No Wash procedure for immunophenotyping of blood leukocytes using fluorochrome-labeled CD markers, which excludes cell losses and the effect of centrifugation on cells [26], allows rapid assessment in human whole blood samples of both the intensity of the secretory degranulation process and granulocyte autolysis triggered by an infectious agent *ex vivo* at the initial stage of bacterial modeling. Because of experiments with *E. coli* and *S. aureus*, new information has been obtained that confirms the importance of rapid development of degranulation and granulocyte autolysis processes in blood for the killing of these bacteria under conditions of bacteremia, as previously established in animal experiments [14], using human cell models. The inability of *Y.pestis*37 cells multiplying intensively in the blood to trigger functional activation of granulocytes by secretory degranulation and cytolysis is important for understanding the causes of asymptomatic development of the infectious process in primary pneumonic plague [10, 22]. The results of microbiological studies obtained simultaneously with the data of cytofluorimetric analysis in an *ex vivo* model may help to explain why plague bacteremia always inevitably leads to sepsis unlike staphylococcal bacteremia.

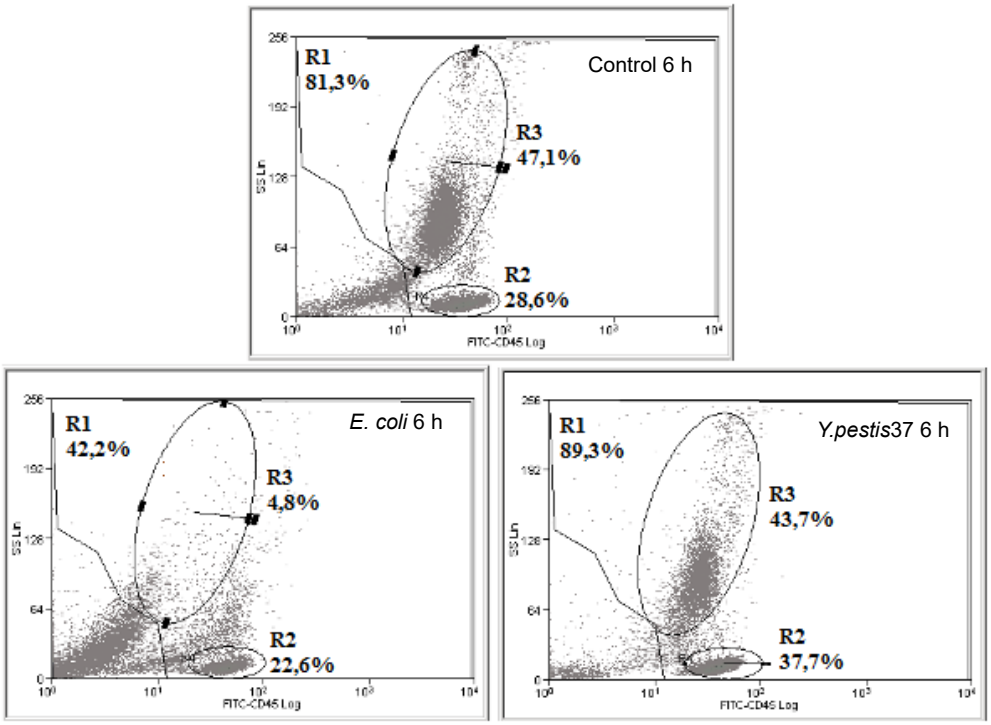

Fig. 1. Cytograms of the blood leukocyte distribution according to the degree of intracellular granularity and the cell surface common leukocyte antigen CD45 expression density at 6 h after the addition of *Y. pestis* and *E. coli* to the blood of the same donor.

Regions R3 and R2 correspond to granulocytes and lymphocytes. Monocytes are localized in the area between the R2 and R3 regions. All intact undamaged leukocytes were counted by the cytometer in the R1 region, beyond which signals from cellular debris, products of the breakdown of leukocytes with a low level of leukocyte antigen expression, accumulated. The proportion of cells in each region is expressed as a percentage of the total number of registered leukocytes. The proportion of debris corresponds to (100 – R1)%.

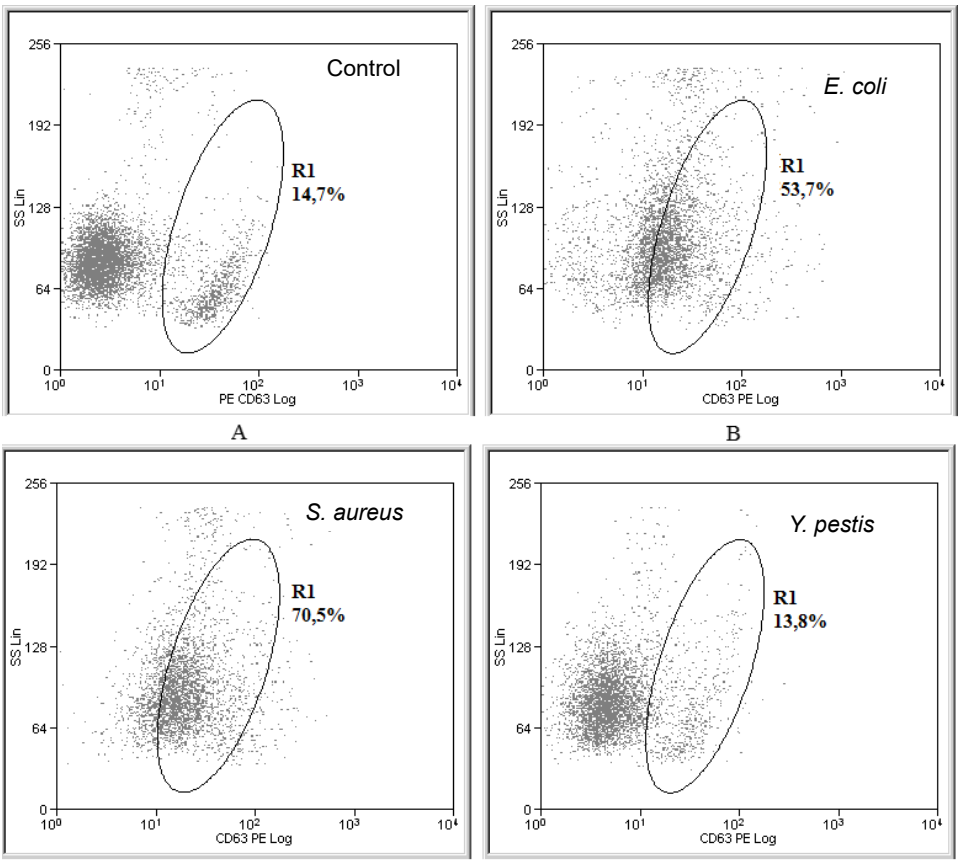

Fig. 2. Relative content of activated cells with CD63⁺ phenotype in the granulocyte gate after 2 h of *ex vivo* interaction with living cells of *Y.pestis*37, *E. coli* and *S. aureus*.

CD63⁺ granulocytes are localized in the R1 region of cytograms; control is the blood after 2 h without bacteria. The proportion of cells in R1 region is expressed as a percentage of the total number of registered granulocytes.

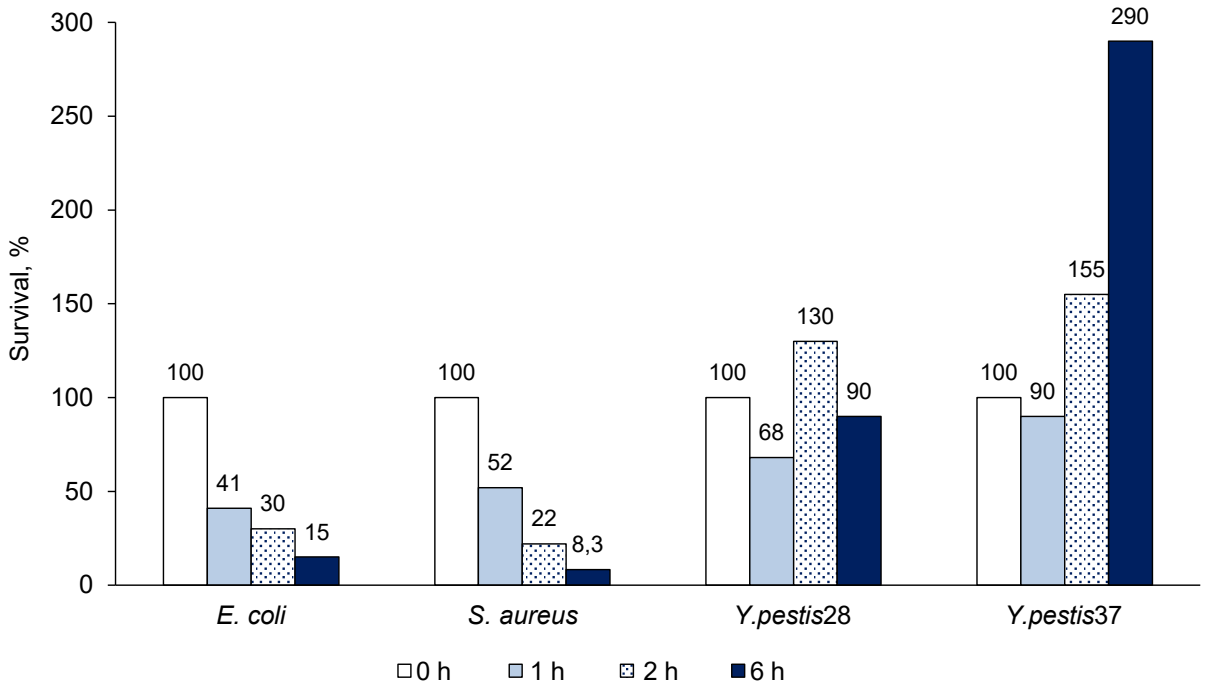

Fig. 3. Increased survival of *Y.pestis*37 in human blood compared to *E. coli, S. aureus* and *Y.pestis*28 in an *ex vivo* bacteremia model.

*Y.pestis*37 cells proliferating in human blood at 37°C did not trigger the secretion of lysosomal protein tetraspanin (CD63) from the primary granules of granulocytes to the cell surface, which is consistent with the results of studies by K.R. Eichelberger et al. conducted *in vivo* on laboratory animals [5] and *in vitro* on the model of NG previously isolated from human blood [17]. In this regard, the fact that CD63 protein is involved in the process of secretion from the same granules of LE [28], which is a generally recognized biochemical marker of the inflammatory process [29] and a key protein of innate antibacterial defense, responsible for rapid selective cleavage of virulence factors of *Yersinia* spp. and other *Enterobacteriaceae* in the focus of infectious inflammation, may be of great importance [30].

To kill bacteria in blood plasma and other biological fluids, this protease forms antibacterial peptides with broad-spectrum antibiotic properties from inactive lysosomal precursor proteins during degranulation [31]. By cleaving histones in the nuclei of activated neutrophils, LE triggers and regulates, together with myeloperoxidase, the process of decondensation of nuclear chromatin during NETosis, in which the autolysis of NG is accompanied by the release into the blood and tissues of the body of DNA nets with the products of NG decay adsorbed in them, which have pronounced bactericidal properties (LE and myeloperoxidase, histones, antibacterial peptides, etc.). [32].

The information presented in the table about the intensity of azurophilic degranulation *ex vivo* by CD63 marker is confirmed by the results of our earlier studies, in which phagocyte degranulation was assessed by supravital staining of cells with AO dye [16] and the viability of granulocytes in blood with *Y. pestis* and *S. aureus* cells was monitored by flow cytometry by identifying and counting the number of dead diploid cells with reduced (less than 2C) DNA content [24, 33]. It is known that blood granulocytes accumulate AO dye in much larger amounts than lymphocytes and monocytes in the form of its positively charged form AOH^+, which is formed and accumulated in the granules of living cells in the process of continuous pumping of H^+ protons through intact membranes of lysosomes [34]. The level of AO accumulation in azurophilic granules depends on the amount of LE molecules in them, and in the process of degranulation, LE is released from the granules into the extracellular space together with AO molecules [35]. In blood contaminated with live *S. aureus* cells, granulocytes lost the initially intense red fluorescence of their granules between 3 and 4 h of incubation. The preservation of intense fluorescence of granulocytes in the analysis of blood samples contaminated with live cells of *Y.pestis*37 indicated the absence of degranulation in phagocytes at the early stage of *ex vivo* modeling of plague bacteremia, the stability of azurophilic granule membranes in peripheral blood granulocytes retaining their viability during 6 h of incubation [16].

In a study by S.C. Dudte et al., in which the degranulation of human NG isolated from blood was evaluated *in vitro* in interaction with *Y. pestis* and *S. aureus* both by CD63 expression and LE expression on the cell surface, only live *Staphylococcus aureus* induced *in vitro* intensive degranulation [18]. Our data obtained in experiments with attenuated strain of *Y. pestis* EV NIIEG on the *ex vivo* model of bacteremia agree with the conclusions of S.C. Dudte et al. However, the model we used was more informative because it allowed us to additionally assess the intensity of leukocytolysis in blood by flow cytometry.

The lower content of cellular debris registered by us in blood with cells of *Y. pestis* cells after 6 h of incubation, compared to control blood samples without bacteria, can be explained by the ability of *Y.pestis*37 to suppress spontaneous apoptosis and lysis of granulocytic cells at the early stage of infection, as known from animal experiments [7, 8, 10].

The reason for the intensive multiplication and rapid spread of *Y. pestis* in the host organism is explained by a change in the structure of its lipopolysaccharide (LPS) when the temperature rises from 28°C to 37°C. It is believed that *Y. pestis* does not induce a protective inflammatory response in the organism at LPS due to the inability of its structurally altered LPS to stimulate TLR4 cells of innate immunity [36]. This explanation does not contradict the results of our studies, since LPS of Gram-negative bacteria is a powerful inducer of secretory degranulation of NG with the release of CD63 and LE on the cell surface and in the extracellular space [37]. However, the proteolytic effect of LE released from phagocyte granules, which determines the ability of this receptor to respond to the presence of LPS *in vivo*, was found to be necessary for TLR4 activation [22]. It is known that tolerance to high doses of endotoxin is formed in mice when the organism is exposed to LPS for a long time (with repeated intravenous administration of low doses over several days). Neutrophils of tolerant animals more effectively neutralized pathogenic bacteria due to the *in vivo* inclusion of an additional mechanism of antibacterial defense (NETosis), which did not function in the organism of intact animals [38]. According to our data, a live plague vaccine had a similar effect on mice, forming intense post-vaccination anti-plague immunity in animals of this species [39].

In the present study, we do not present experimental data on the identification and determination of NG content in blood by their specific phenotypic marker CD16 (Fc_γRIIIb receptor) in order not to complicate its description. Since the proportion of NG in the total population of blood granulocytes in the samples studied by us was more than 90% [25] and corresponded to clinically established normal values of this index [12], the possibility to call the total population of granulocytes

NG when discussing the obtained experimental data is allowed in our study.

The flow cytometry method is known to differentiate IgG-dependent anaphylaxis associated with activation of the secretory function of peripheral blood neutrophils from IgE-dependent anaphylactic reaction that develops as a result of stimulation of mast cells and basophils [40]. It is possible that when functionally active IgG-antibodies to specific antigens of *Y. pestis* appear in the blood, they will activate the mechanism of extracellular antibody-dependent cytotoxicity of NG, which can be registered by flow cytometry on the *ex vivo* model according to the intensity of degranulation and lysis of peripheral blood granulocytes. Such analysis may be more sensitive and informative than in cases when an alternative skin test indicator of neutrophil damage is subjectively assessed in practice in human whole blood samples using a long and laborious method of microscopic analysis.

Conclusion

The study of degranulation processes and lysis of granulocytes in human whole blood samples infected with live *Y. pestis*, helped obtain new information using CD markers and flow cytometry, indicating that the mechanisms of extracellular granulocyte bactericidal activity, which function effectively against *E. coli* and *S. aureus*, do not work at the early stage of bacteremia in the blood of people unvaccinated against plague. In *ex vivo* modeling of bacteremia using the attenuated strain of *Y. pestis* EV NIIEG has created the necessary experimental and methodological basis for further study of the processes of degranulation and lysis of NG in blood samples of people inoculated against plague, for the development of new, more effective tests to assess the intensity of acquired cellular anti-plague immunity.

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The incidence of chronic viral hepatitis and the analysis of the genetic diversity of hepatitis B and C viruses among the population of Khabarovsk city

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Abstract

Introduction. Evaluation of hepatitis virus genotypes diversity plays a significant role in analysis of epidemiological situation in particular territories which allows to trace possible routs of transmission, emergence and spread of new viral variants.

The aim of the study is to evaluate dynamic of incidence of chronic forms of viral hepatitis B and C in the Khabarovsk krai during 2013-2023 and to perform analysis of genetic diversity of hepatitis B and C viruses (HBV and HCV) that were circulating in the Khabarovsk city.

Materials and methods. A retrospective analysis of the incidence of chronic forms of hepatitis C in the Khabarovsk krai was conducted for the period from 2013 to 2023 (11 years) according to reports from the Office of Rospotrebnadzor in the Khabarovsk krai. Serological and molecular-genetic analysis of 112 blood plasma samples obtained from patients with diagnosis of “chronic viral hepatitis” residing in the Khabarovsk city was conducted.

Results. The research showed that incidence of chronic viral hepatitis C has declined from 50.0 in 2013 to 44.6 cases per 100 thousand population in 2023 and incidence of chronic viral hepatitis B has also declined from 10.2 to 8.2 cases per 100 thousand population in the Khabarovsk krai respectively. HBV DNA was detected in 21 out of 36 blood plasma samples that contained serological markers of HBV. HBV genotype D was isolated in 15 out of 17 samples and was presented by two subtypes (D1, D2). Subtype A2 was identified in 2 samples. No mutations of drug resistance were found. HCV RNA was found in 58 out of 78 samples of blood plasma that were positive for antibodies (IgG + IgM) to HCV. Circulation of 1b, 1a, 3a, 2a, 2c HCV subtypes with predominance of 1b subtype was revealed in the Khabarovsk city.

Conclusion. The incidence of chronic hepatitis B and C in the Khabarovsk krai during the 11-year follow-up period had a pronounced tendency to decrease. Results of the research complement existing data on circulation of HBV and HCV genetic variants in territories of the Russian Federation.

Keywords: *Khabarovsk krai, hepatitis C virus, hepatitis B virus, genotype, subtype, chronic hepatitis, phylogenetic analysis*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. The research protocol was approved by the Ethics Committee of the Khabarovsk Research Institute of Epidemiology and Microbiology (protocol No. 9, 1 November, 2022).

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ORIGINAL RESEARCHES

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Заболеваемость хроническими вирусными гепатитами и анализ генетического разнообразия вирусов гепатитов B и C среди населения Хабаровска

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Аннотация

Введение. Изучение генотипического разнообразия вирусов гепатитов имеет большое значение при проведении анализа эпидемиологической обстановки на конкретной территории, позволяя отслеживать возможные пути передачи инфекции, появление и распространение новых вариантов вирусов.

Цель — оценить динамику заболеваемости хроническими формами вирусных гепатитов B (ХГВ) и C (ХГС) на территории Хабаровского края за 2013–2023 гг., провести анализ генетического разнообразия вирусов гепатитов B (HBV) и C (HCV), циркулирующих в Хабаровске.

Материалы и методы. Ретроспективный анализ заболеваемости ХГВ и ХГС по Хабаровскому краю проведён за 11 лет по данным отчётов Управления Роспотребнадзора по Хабаровскому краю. Выполнен серологический и молекулярно-генетический анализ 112 образцов плазмы крови пациентов с диагнозом «хронический вирусный гепатит», проживающих на территории Хабаровска.

Результаты. В Хабаровском крае с 2013 по 2023 г. заболеваемость ХГС снизилась с 50,0 до 44,6 случая на 100 тыс. населения, а ХГВ — с 10,2 до 8,2. ДНК HBV обнаружена в 21 из 36 образцов плазмы крови пациентов с выявленными серологическими маркерами HBV. Среди 17 исследованных образцов генотип D HBV обнаружен в 15 случаях, представленный субгенотипами D1 и D2, субтип A2 — в 2 образцах. Мутаций лекарственной устойчивости не обнаружено. РНК HCV выявлена в 54 из 78 проб плазмы крови с положительным результатом иммуноферментного анализа на наличие антител (IgG + IgM) к HCV. На территории Хабаровска установлена циркуляция субтипов HCV — 1b, 1a, 3a, 2a, 2c с преобладанием субтипа 1b.

Заключение. Заболеваемость ХГС и ХГВ в Хабаровском крае за 11-летний период наблюдения имела ярко выраженную тенденцию убыли. Результаты исследования дополняют существующие представления о циркуляции геновариантов HBV и HCV на территории России.

Ключевые слова: *Хабаровский край, вирус гепатита C, вирус гепатита B, генотип, субтип, хронический гепатит, филогенетический анализ*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов или их законных представителей. Протокол исследования одобрен Этическим комитетом Хабаровского НИИ эпидемиологии и микробиологии (протокол № 9 от 01.11.2022).

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

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Introduction

The problem of parenteral viral hepatitis B and C remains one of the priority objectives of global health care due to the high frequency of chronic forms of the disease and the possibility of unfavorable outcomes in the form of liver cirrhosis and hepatocellular carcinoma. The World Health Organization (WHO) estimates that 50 million people worldwide are affected by hepatitis C virus (HCV) and 254 million people are infected with hepatitis B virus (HBV)[1]. In April 2016, the WHO Assembly resolved to globally eliminate viral hepatitis as a public health problem by 2030.[2]

The implementation of the "Health" national project on hepatitis B vaccine prophylaxis in Russia has contributed to a decrease in the incidence of acute form of the infection. Over the past 10 years (from 2014 to 2023), the incidence of acute hepatitis B has decreased 4-fold — from 1.32 to 0.33 cases per 100,000 population. Since 2014, there has been an annual decrease in the incidence of acute hepatitis C in Russia. In 2023, it amounted to 1393 patients or 0.95 cases per 100,000 population, which is 27.5% lower than the global average, but at the same time, compared to 2022, the incidence increased by 26%. Along with a decrease in the incidence of acute hepatitis B and C, high rates of new cases of chronic viral hepatitis continue to be registered in Russia. In total, more than 58.9 thousand cases of chronic viral hepatitis were registered in 2023 (43.3 thousand cases in 2022). The incidence rates of chronic viral hepatitis vary dramatically across the constituent entities of the Russian Federation (from 0.9 to 127.86 per 100,000 population), which largely depends on the quality of diagnosis and completeness of registration of this group of diseases[3]. A similar trend can be observed in the Khabarovsk Territory, which is part of the Far Eastern Federal District[4].

Recently, much attention has been paid to the study of genotypic variability of hepatitis viruses. Diseases caused by different genotypes may differ significantly in clinical course and outcomes [1–3].

Currently, 10 hepatitis B virus (HBV) genotypes have been identified (designated by letters A to J). Genotypes A–D, F, H and I are divided into 35 sub-genotypes (for other genotypes sub-genotypes have not been established) [4, 5]. The genotypes differ in genome length, size of the open reading frames and protein translated, as well as mutations selected under the influence of therapy [6, 7]. Hepatitis C virus (HCV) isolates are categorized into 8 genotypes [8] and 93 confirmed subtypes. The modern classification of HCV includes 9 inter-genotype recombinant forms[5]. Each genotype is characterized by a certain frequency of occurrence and geographical area of distribution.

According to the results of molecular genetic studies conducted in different years in Russia, it was found that 3 genotypes of HBV — D, A and C with the predominance of genotype D and 4 subtypes of HCV — 1a, 1b, 2 and 3a, of which subtypes 1b and 3a are predominant, are circulating in the territory of the country [9–12].

The study of genotypic diversity of viral hepatitis is of great importance when analyzing the epidemiological situation in each specific territory, allowing to track possible routes of transmission, the emergence and spread of new virus variants, identify imported cases of infection. All this is an important aspect in the timely planning of necessary sanitary and epidemiologic measures.

Modern molecular biological methods of research allow not only to assess the genetic diversity of HBV and HCV in separate territories, but also to determine the nature of clinically significant mutations in the P-gene of HBV DNA and NS3/NS5A/NS5B regions of HCV associated with possible resistance to therapy with direct antiviral drugs.

The aim of the study was to assess the dynamics of chronic hepatitis B and C incidence in 2013–2023, and to analyze the genetic diversity of HBV and HCV circulating among Khabarovsk patients diagnosed with chronic viral hepatitis.

Materials and methods

Retrospective analysis of the incidence of chronic hepatitis B and C was performed using data provided by the Khabarovsk krai Rospotrebnadzor regional office for the period from 2013 to 2023 (11 years). The values of chronic viral hepatitis incidence in Russia were taken from the State Report "On the state of sanitary and epidemiologic well-being of the population in the Russian Federation in 2023".

The average annual growth rate (AAGR) was calculated using the least squares method, and the confidence interval was calculated using the Fisher angular transformation method. The obtained trend was regarded as stable at AAGR = ± 1%, as weakly expressed —

[1] World Health Organization. Hepatitis B, Fact Sheet. URL: https://www.who.int/news-room/fact-sheets/detail/hepatitis-b (data of access: 07.05.2024); World Health Organization. Hepatitis C, Fact Sheet. URL: https://www.who.int/news-room/fact-sheets/detail/hepatitis-c (data of access: 07.05.2024).

[2] World Health Organization. Sixty-ninth world health assembly provisional agenda item 15.1. Draft global health sector strategies. Viral hepatitis, 2016–2021. URL: http://apps.who.int/gb/ebwha/pdf_files/WHA69/A69_32-en.pdf?ua=1 (data of access: 07.05.2024).

[3] State report "On the state of sanitary and epidemiological welfare of the population in the Russian Federation in 2022". Moscow; 2023. URL: https://clck.ru/3FcJ5X

[4] Report of the Office of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare in the Khabarovsk Territory "On the state of sanitary and epidemiological well-being of the population in 2022". URL: https://clck.ru/3FcHrj

[5] International Committee on Taxonomy of Viruses (ICTV), 2022 URL: https://talk.ictvonline.org/ictv_wikis/flaviviridae/w/sg_flavi/56/hcv-classification (data of access: 15.05.2024).

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at AAGR = ± 1.1–3.0%, as moderate – at AAGR = ± 3.1–5.0%, as expressed – at AAGR = ± 5.1–7.0%, as pronounced – at AAGR = ±7% and above [13].

In order to study the genetic diversity of HBV and HCV circulating among the population of the Khabarovsk Krai, blood plasma samples from 112 patients diagnosed with chronic viral hepatitis collected at the Center for Prevention and Control of AIDS and Infectious Diseases (n = 50) and the laboratory of the Khabarovsk Research Institute of Epidemiology and Microbiology (n = 62) in 2022–2023 were studied. Informed consent of all patients to participate in the study was obtained, as well as approval of the ethics committee of Khabarovsk Research Institute of Epidemiology and Microbiology (protocol No. 9 of 01.11.2022) to conduct the research. The presence of markers of viral hepatitis B and C was determined by enzyme immunoassay using test systems produced by Vector-Best CJSC.

Nucleic acids were isolated from 100 µl of blood plasma using the AmpliPrime RIBO-prep reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor). Analysis for detection of HBV DNA, HCV RNA, determination of viral load in positive samples and HCV genotype was performed by polymerase chain reaction (PCR) with hybridization-fluorescence detection in real time using commercial kits: "AmpliSens HBV-FL, AmpliSens HBV-Monitor-Fl, AmpliSens HCV-FL, AmpliSens HCV-Monitor-Fl, AmpliSens-1/2/3 (Central Research Institute of Epidemiology of Rospotrebnadzor) on a Rotor Gene Q device (Qiagen).

For HBV and HCV genotyping, we used two-step PCR with specific primers (Syntol) for the conserved region of the overlapping *S* and *P* genes, encoding the surface protein and DNA polymerase of HBV, and for the NS5B region of HCV [14, 15].

Additionally, the nucleotide sequence of the HCV NS5 region was analyzed for a patient with HCV receiving direct antiviral drugs using the method described by M. Rajhi et al. [16].

Sanger sequencing of amplified viral genome fragments was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems/Life Technologies) on an ABI3500 DNA analyzer (Applied Biosystems/Life Technologies).

Alignment of the obtained nucleotide sequences was performed in the BioEdit v. 7.1.9 program.

Phylogenetic analysis was performed using the MEGA v. 7.0 program by constructing phylogenetic trees using the neighbor-joining method [17]. The distances between nucleotides were calculated using the Kimura method. To assess the statistical reliability of phylogenetic relationships, bootstrap analysis was used for 1000 independent constructions of each phylogenetic tree.

As reference strains for phylogenetic analysis we used sequences of the same genomic region of HBV and HCV from Russia and other countries of the world presented in GenBank[6].

The geno2pheno software[7] was used to detect drug resistance mutations.

14 obtained and analyzed nucleotide sequences of HBV genomic regions (accession numbers PP100729-PP100742) and 44 HCV genomic regions (accession numbers PP111728-PP111748, PP100743-PP100765) were deposited in the international GenBank database. 10 HBV partial genomic sequences and 35 HCV partial genomic sequences were deposited in Russian virus genome information aggregation platform VGARus.

Results

The conducted studies showed that the incidence of chronic hepatitis C in the Khabarovsk Krai in 2013 to 2023 decreased from 50.0 (95% CI 46.2–54.0) to 44.6 (95% CI 41.0–48.3) per 100,000 population and had a pronounced downward trend (AAGR = –6.5%). More significant changes were registered in the Khabarovsk Krai in terms of chronic hepatitis B incidence, which had a pronounced downward trend (AAGR = –11.6%), decreasing more than 2-fold — from 10.2 (95% CI 8.5–12.0) cases per 100,000 population in 2013 to 4.5 (95% CI 3.4–5.7) cases per 100,000 population in 2021.

In Khabarovsk, the incidence of both chronic hepatitis B and C was higher than in the Khabarovsk Krai throughout the entire observation period: in 2023 the incidence of chronic hepatitis C was 56.7 (95% CI 50.9–62.8) per 100,000 population (27.1% higher than in the Khabarovsk Krai), and the incidence for chronic hepatitis B was 13.3 (95% CI 10.6–16.3) per 100,000 population (62.2% higher than in the Khabarovsk Krai). In multiyear dynamics, the incidence of chronic hepatitis B and C in Khabarovsk had a more pronounced downward trend compared to the regional one (AAGR = –11.6% — for chronic hepatitis C incidence and AAGR = –12.2% — for chronic hepatitis B incidence) (**Fig. 1**).

The decline in chronic viral hepatitis incidence after 2019 coincided with the start of the COVID-19 pandemic, during which restrictive measures were introduced to reduce the spread of SARS-CoV-2 among the population. In 2022 and 2023, when the restrictions were gradually lifted, an increase in chronic hepatitis C incidence of 20.5 and 45.7% in the Khabarovsk Krai and 21.4 and 44.6% in Khabarovsk, respectively, began to be recorded. The incidence of chronic hepatitis B in the Khabarovsk Krai and Khabarovsk increased by 17.8 and 41.2% in 2022, respectively, and by 54.7 and 84.7% in 2023, which is probably due to an increase in the number of patient visits to medical organizations, including laboratory testing that reached levels of 2019.

[6] URL: https://www.ncbi.nlm.nih.gov/genbank
[7] URL: http://hbv.geno2pheno.org/index.php

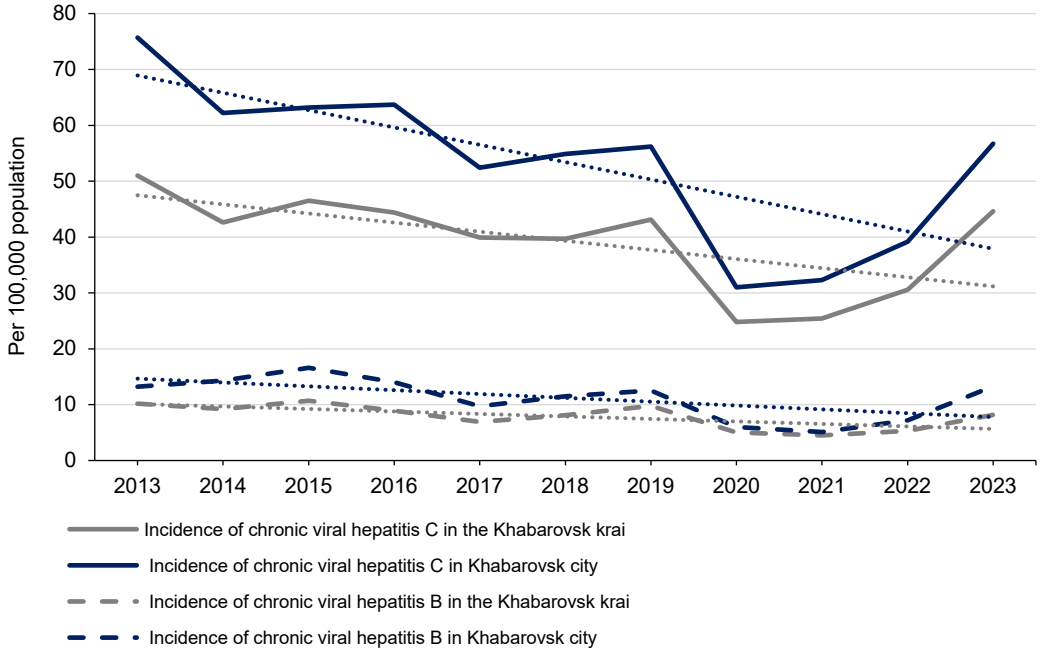

Fig. 1. Long-term incidence of chronic viral hepatitis C and B in the Khabarovsk krai and Khabarovsk city.

Serologic and molecular genetic analysis was performed for 112 blood plasma samples of the Khabarovsk residents diagnosed with chronic viral hepatitis. The age of the examined patients averaged 51 years. There were 51 women (45.5%; 95% CI 36.9–55.2%) and 61 men (54.5%; 95% CI 45.8–64.1%) among the examined patients.

HBsAg was detected in 36 of 112 plasma samples (32.1%; 95% CI 23.7–40.9%). Antibodies to the HCV were detected in 78 samples (69.6%; 95% CI 61.2–78.1). 2 (1.8%; 95% CI 0.2–5.4%) patients had mixed-infection with HBV + HCV.

HBV DNA was detected in 21 (58.3%; 95% CI 41.7–73.4%) of 36 plasma samples from patients with detectable markers of HBV. In all DNA-positive samples, viral load levels were determined. In 19 (90.5%; 95% CI 74.0–98.8%) patients it was low (less than 10^4 IU/mL), and in 2 (9.5%; 95% CI 1.2–26.0) it was intermediate (10^4–10^6 IU/mL).

During an examination of 78 patients from Khabarovsk who were positive by enzyme-linked immunosorbent assay for antibodies (IgG + IgM) to HCV, HCV RNA was detected in 54 (69.2%; 95% CI 42.9–64.9%) blood plasma samples. In all RNA-positive samples, viral load levels were determined. It was low (less than 8×10^5 IU/mL) in 39 (72.2%; 95% CI 59.4–83.1) patients and high (greater than 8×10^5 IU/mL) in 15 (27.8%; 95% CI 16.9–40.6) patients. Preliminary HCV genotyping using the AmpliSens -1/2/3 kit showed that HCV genotype 1 was the most common among the examined patients in Khabarovsk (57.4%; 95% CI 43.7–69.8%). HCV genotype 3 was detected in 14 patients (25,9%; 95% CI 15,3–38,4%). Genotype 2 was detected in 8 cases (14.8%; 95% CI 6.8–25.6%), and genotype could not be determined in 1 patient (1.9%; 95% CI 0.1–7.2%).

To determine the genotype, to clarify the origin and possible relatedness of HBV and HCV variants with isolates from other regions of Russia and foreign countries both nearby and far away, 17 nucleotide sequences of the HBV genomic region and 53 nucleotide sequences of the NS5B region of HCV of satisfactory quality were obtained, suitable for further analysis.

As a result of molecular genetic analysis of HBV, genotype D was determined in 15 patients (88.2%; 95% CI 68.9–98.7%) and genotype A in 2 patients (11.8%; 95% CI 1.3–31.1). Phylogenetic relationships between the studied samples and reference sequences are presented in **Fig. 2**.

On the phylogram, the HBV genotype D strains obtained by us and the nucleotide sequences retrieved from GenBank fell into two distinct monophyletic groups distinguished by sub-genotypes: D1 and D2, which were identified in 7 (46.7%) and 8 (53.3%) samples, respectively.

The obtained nucleotide sequences (Nos. 442, 507, 90) grouped with genetically similar sequences of sub-genotype D1 from Chile (FJ709467.1), Germany (MZ0977440.1), as well as with a strain (KY660216.1) that had already been recorded in the Khabarovsk Krai in 2016. Four samples (Nos. 930, 673, 1021, 310) formed separate clades with sub-genotype D1 strains from Poland (GQ477513.1), India (MH272614.1), Turkey (AB674433.1) and Iran (KS339812.1).

The obtained nucleotide sequences of sub-genotype D2 were distributed between HBV strains of

this sub-genotype detected in Russia (in Moscow — OR956323. 1 and Khabarovsk — OR956250.1), and strains from India (MK975842.1), Italy (JN226080.1), Spain (GQ486785.1), Albania (JQ244817.1, JQ244795.1), Iran (KC339865.1), Tajikistan (AY738889).

Two strains isolated in Khabarovsk (samples No. 1125, 1877) grouped on the phylogenetic tree, forming one common cluster with reference strains of sub-genotype A2. Sample No. 1877 had a high genetic similarity with a strain of HBV sub-genotype A2, serotype adw2 from Cuba (KM606811.1), and sample No. 1125 with a strain from Poland (GQ477462.1).

The nucleotide sequences of the P-gene region of HBV obtained in the present study were tested for the presence of key mutations associated with the emergence of drug resistance. The analysis showed that all 17 HBV strains were sensitive to the following antiviral drugs: lamivudine, adefovir, entecavir, telbivudine, tenofovir, i.e. no resistance mutations were detected.

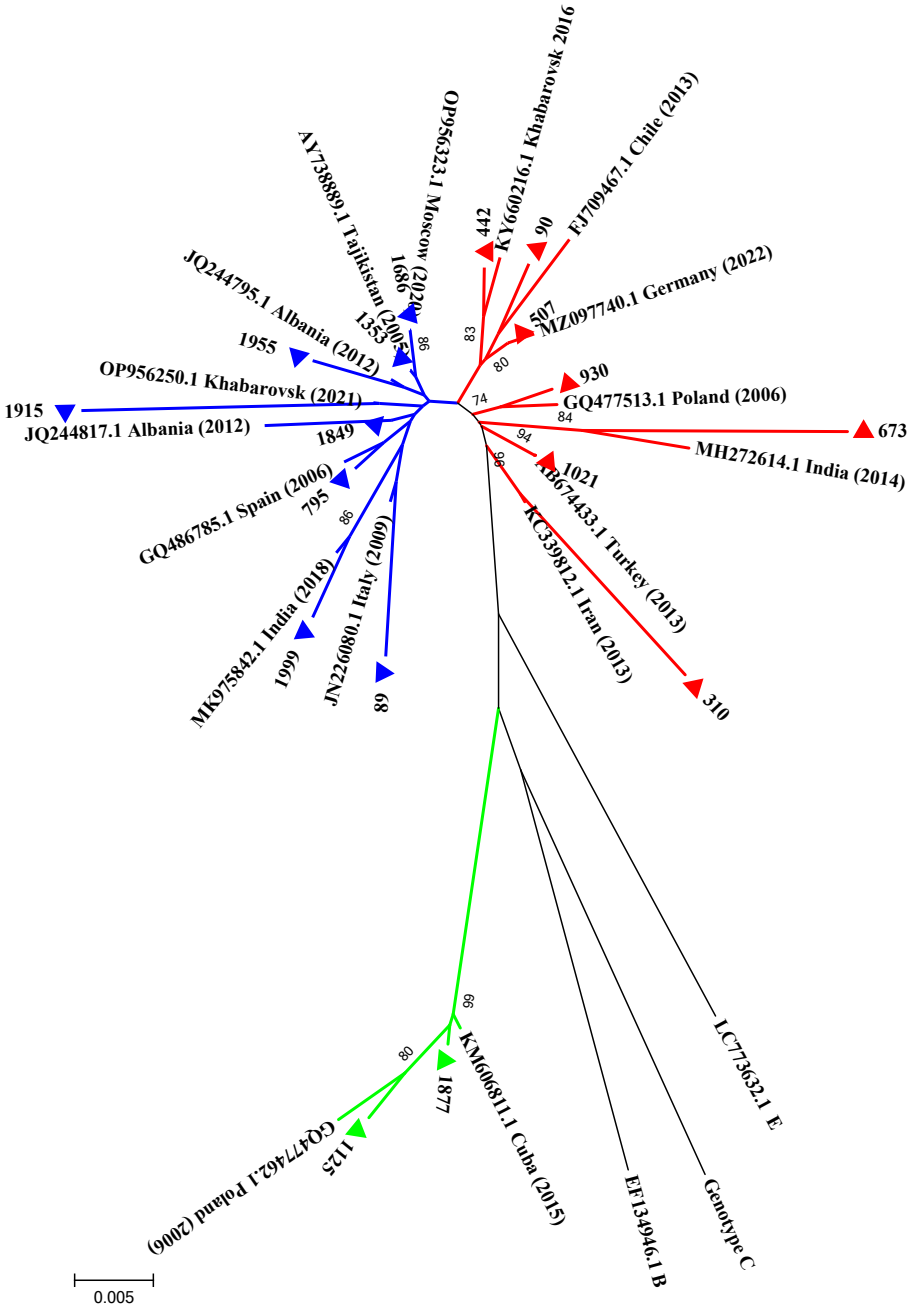

Fig. 2. Results of phylogenetic analysis of partial genomic nucleotide sequences of HBV circulating among population of the Khabarovsk city.

Phylogenetic tree was constructed using the neighbor-joining method. HBV sequences evaluated in current work are marked with triangles. HBV reference sequences are specified by their GenBank accession numbers. Bootstrap index values exceeding 70% are indicated. Blue lines — sub-genotype D2; red lines — sub-genotype D1; green lines — sub-genotype A2.

Phylogenetic relationships between nucleotide sequences obtained by direct sequencing of the NS5B region of the HCV genome and reference sequences are displayed in **Fig. 3**.

Sample No. 57, for which no result was obtained during preliminary genotyping using AmpliSens -1/2/3 diagnostic test system, formed a single cluster with isolates belonging to subtype 1a and isolated in different years in the USA (KT734609. 1, OK392383), where this genetic variant is endemic, as well as in Sweden (MH510449.1), Ukraine (OQ979420), Switzerland (EU255927.1), and the Netherlands (KU563369.1), but no closely related strains were identified for it.

On the phylogenetic tree, 31 out of 54 strains under study were evenly distributed among the reference sequences of HCV subtype 1b presented in the international GenBank database (Greece, France, USA, Belgium, Spain, Taiwan, Japan, Vietnam, Brazil, Indonesia, Tunisia). Phylogenetic analysis of 13 specimens assigned to genotype 3 by PCR-genotyping showed that all nucleotide sequences obtained by us are clustered on the same branch of the phylogenetic tree with previ-

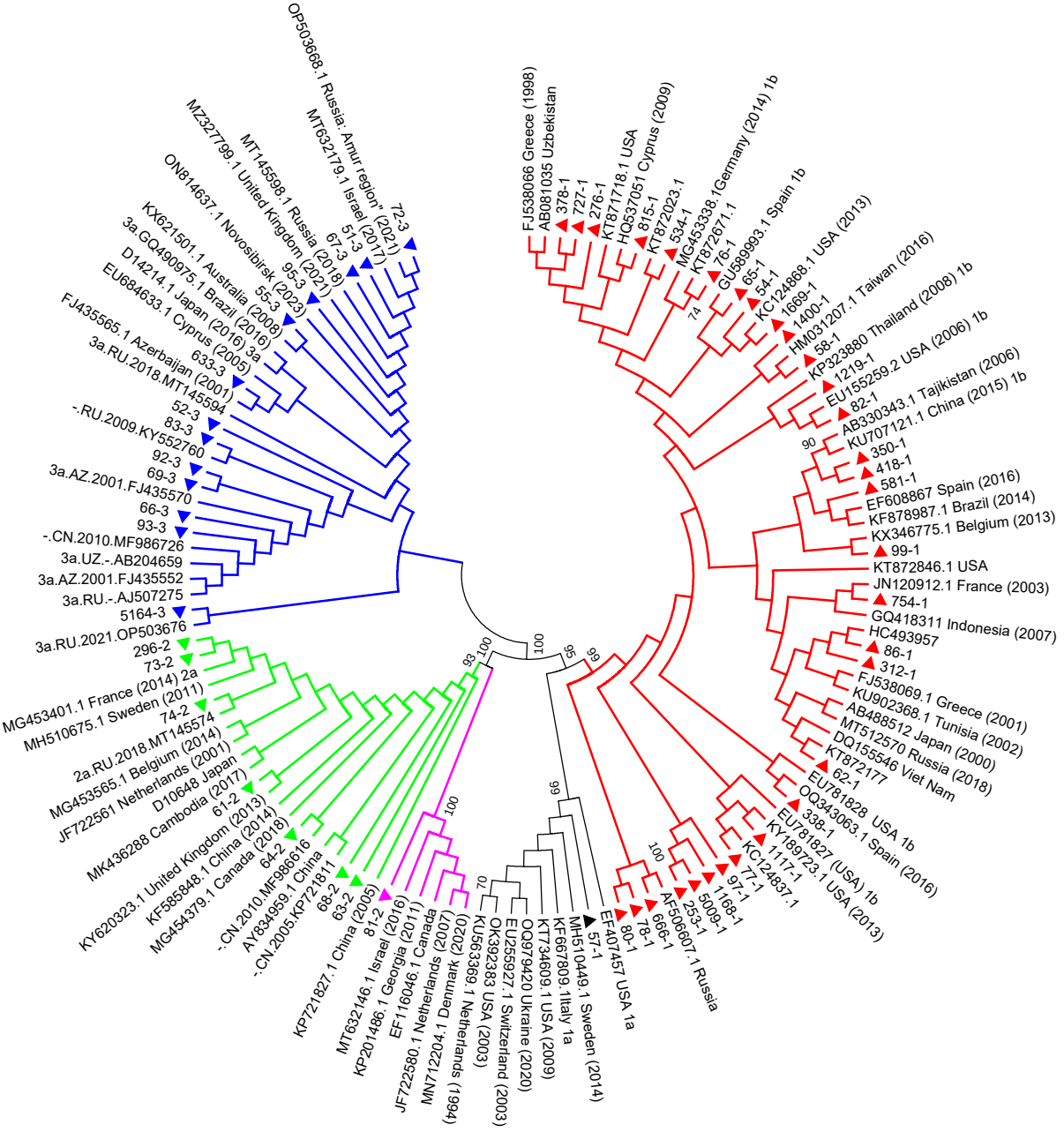

Fig. 3. Result of phylogenetic analysis of NS5B region nucleotide sequences of HCV circulating among population of the Khabarovsk city.

Phylogenetic tree was constructed using the neighbor-joining method. HCV sequences evaluated in current work are marked with triangles. HCV reference sequences are specified by their GenBank accession numbers. Bootstrap index values exceeding 70% are indicated. Blue lines — subtype 3a; red lines — subtype 1b; green lines — subtype 2a; black lines — subtype 1a; violet lines — subtype 2c.

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ously obtained sequences of the same genomic region of variants of subtype 3a isolated in different years in different regions of Russia and the world.

Phylogenetic analysis of 8 specimens assigned to genotype 2 based on the results of preliminary genotyping revealed the formation of 2 clusters. One sample (No. 81) with a high level of bootstrap support (100%) formed a single cluster with isolates belonging to subtype 2c, but no closely related strains were identified for it. Seven strains grouped on the phylogenetic tree, forming one common cluster with HCV subtype 2a isolates registered in GenBank, which were isolated in Russia — in the Novosibirsk region in 2002 (GQ388000.1), Republic of Sakha (Yakutia) (KT 378626.1) in 2014, Leningrad region (AF388438.1) in 2014, and in France (MG453401.1) in 2014.

For sample #76 from a patient with HCV receiving direct antiviral drugs, the nucleotide sequence of the HCV NS5A region was additionally analyzed for the presence of mutations associated with the emergence of drug resistance. As a result of the study, the *Y93H* mutation was detected, which causes resistance of the virus to the following drugs: Daclatasvir, which was included in the patient's treatment regimen, as well as to Elbasvir, Ledipasvir, Ombitasvir and Velpatasvir. When analyzing the nucleotide sequence of the *NS5B* region, no drug resistance mutations were detected in this sample.

Discussion

The current epidemiological situation on viral hepatitis both in the Khabarovsk Krai and on average in Russia is characterized by an increase in the total number of patients with chronic forms among the population. In general, in the Khabarovsk Krai for 2013-2023, the curves of chronic hepatitis B and C incidence had unidirectional downward trends. Despite this, the incidence rate of chronic hepatitis C in the Khabarovsk Krai, which reached 44.6 cases per 100,000 population in 2023, exceeded the national average of 31.6 cases per 100,000 population. At the same time, the incidence rate of chronic hepatitis B in the Khabarovsk Krai was slightly lower than the Russian average — 8.2 and 8.4 cases per 100,000 population, respectively[8].

It should be noted that the sharp decline in the incidence of chronic hepatitis B and C, recorded in 2020, including for chronic hepatitis C — 1.7-fold from 43.0 (95% CI 39.5–46.6) to 24.8 (95% CI 22.2–27.8) and for chronic hepatitis B — almost 2-fold from 9.8 (95% 8.2–11.6) to 5.0 (95% CI 3.8–6.3) cases per 100,000 population, is partly explained by a decrease in the number of people seeking medical care during the COVID-19 pandemic.

For many years, a significant contribution to the development of the epidemic process of chronic viral hepatitis in the region has been made by the largest population center, Khabarovsk, which is the regional capital, where 61.1% (95% CI 57.1–65.1) of patients with chronic hepatitis C and 78.1% (95% CI 69.7–85.4) of patients with chronic hepatitis B of all first-detected cases in the constituent entity lived in 2023. The higher incidence of chronic hepatitis C in the regional capital can be partly attributed to the greater availability of laboratory testing, in contrast to the northeastern territories remote from the regional center, where registration of chronic viral hepatitis incidence is absent or sporadic, such as Polina Osipenko, Nikolaevsky, Okhotsk, Tuguro-Chumikansky and Ayano-Maysky districts.

A molecular genetic study established HBV and HCV genotypes and sub-genotypes circulating among patients with chronic forms of infection in Khabarovsk.

The results of phylogenetic analysis of D1 and D2 sub-genotypes from Khabarovsk and the GenBank database showed the formation of several clusters, which may indicate different origins and independent spread of HBV strains circulating in the study area. The low similarity of the strains from this study with strains from Russia is possibly due to the low proportion of Russian variants of the virus represented in the international GenBank database.

The study revealed some differences in the distribution of HBV and HCV genetic variants noted in this paper among residents of Khabarovsk and the study we conducted in 2017–2018 among the indigenous population of Nanai district of the Khabarovsk Krai [18, 19]. Thus, analysis of the frequency of occurrence of HBV sub-genotypes D1, D2 and D3 showed that among patients in the rural area, sub-genotype D3 was slightly predominant (51.3%; 95% CI 35.8–66.7%), and sub-genotypes D2 and D1 were identified in 46.1% (95% CI 30.9–61.7%) and 2.6% of cases, respectively. Should be noted that in the present study, no cases of infection with sub-genotype D3 were identified among urban patients, which refutes the results of previously described studies, according to which the incidence of sub-genotype D1 decreases from 45% in the European part of Russia to 12% in the Far Eastern region [20]. This can be partly explained by the small sample size of the study. This fact requires further investigation.

The results of the present study showed that HCV subtype 1b was most prevalent among the examined patients in Khabarovsk, detected by phylogenetic analysis in 58.5% of cases, while the prevalence of subtype 3a (45.0%) was recorded in the Nanai district of the Khabarovsk Krai.

Phylogenetic analysis of the NS5B region of HCV performed for 53 investigated blood samples of Khabarovsk residents initially typed in PCR presented the following ratio of subtypes: 1b — 31 (58.5%; 95% CI 45.2–71.4%), 3a — 13 (24.5%; 95% CI 14.0–

[8] Report of the Office of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare in the Khabarovsk Territory "On the state of sanitary and epidemiological well-being of the population in 2022". URL: https://clck.ru/3FcHrj

37.0%), 2a — 7 (13.2%; CI 5.5–23.5%), with subtypes 1a and 2c (1.9%) identified in isolated cases. According to the results of the phylogenetic analysis, isolate No. 57, untyped by conventional PCR, could be attributed to subtype 1a.

In summary, the obtained data on the circulation of HBV and HCV genetic variants among Khabarovsk residents are consistent with the data of other researchers on the predominance of HBV genotype D and HCV subtypes 1b and 3a in Russia.

It should be noted the importance of regular updating of information on genotypes/subtypes of hepatitis viruses detected in the subjects of the Russian Federation in the international (GenBank) and Russian (VGARus) databases. This will significantly expand the opportunities for molecular genetic monitoring for circulating genetic variants of viruses in a particular territory, as well as for analyzing the incidence of these infections in Russia as a whole, which may ultimately strengthen the effectiveness of epidemiologic surveillance of viral hepatitis. Furthermore, thanks to the use of modern molecular biological diagnostic methods and phylogenetic analysis, it has become possible to predict the development of unfavorable trends in the epidemiological situation, as well as to confirm or deny the presence of an epidemiological link between the suspected source of HCV or HBV and the diseased when investigating the fact of intra-family infection, group diseases, cases of nosocomial infection or occupational exposure to viral hepatitis.

Conclusion

Despite the decrease in cases of detection of acute forms of parenteral viral hepatitis in the Khabarovsk Krai, the registration of chronic viral hepatitis remains at a high level. The most unfavorable situation with the incidence of chronic hepatitis B and C in the Khabarovsk Krai is observed in Khabarovsk.

The study of genetic diversity of HBV and HCV viruses in Khabarovsk revealed the circulation of two HBV genotypes: D and A. Among the samples studied, HBV genotype D was detected in 88.2% of cases and is represented by sub-genotypes D1 and D2. Subtype A accounted for 11.8%. Molecular genetic study of HCV circulating in Khabarovsk revealed circulation of subtypes 1b, 1a, 3a, 2a, 2c with predominance of subtype 1b.

When analyzing the nucleotide sequences of the P-gene region of HBV, no major antiviral drug resistance mutations were detected.

In general, the results of the molecular genetic study conducted in Khabarovsk can significantly supplement the existing ideas about the circulation of HBV and HCV genetic variants on the territory of Russia.

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Оригинальное исследование
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Очаги иерсиниозов Крымского полуострова

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Аннотация

Введение. Значительный рост заболеваемости, высокий уровень инфицированности мелких млекопитающих в ряде районов определили осложнение эпидемиологической ситуации по иерсиниозу.

Цель работы — определение фактических границ природных и антропургических очагов иерсиниозов на Крымском полуострове.

Материалы и методы. Обследован 21 административный район Крымского полуострова. Доставленный материал исследовали по утверждённым методикам.

Результаты. В 2022 г. из 1005 отловленных мелких млекопитающих 31 особь была положительной на кишечный иерсиниоз, 3 — на псевдотуберкулёз. Штамм *Yersinia enterocolitica* O3 выделен из 28 проб 6 видов с территории 5 районов степного Крыма, Керченского полуострова, предгорий главной гряды; штамм *Y. enterocolitica* O9 — из 3 проб 2 видов с территории 2 районов степного Крыма; *Y. pseudotuberculosis* — из 3 проб 2 видов с территории 2 районов степного Крыма. В 2023 г. из 857 мелких млекопитающих 80 проб были положительны на кишечный иерсиниоз, 2 — на псевдотуберкулёз. *Y. enterocolitica* O3 выделена из 79 проб 8 видов с территории 7 районов горного Крыма и предгорий главной гряды, степного Крыма, г. Севастополя, Керченского полуострова; *Y. enterocolitica* O9 — из 1 пробы 1 вида с территории 1 района Керченского полуострова; *Y. pseudotuberculosis* — из 2 проб 2 видов с территории степного Крыма и г. Севастополя.

Обсуждение. Все виды, образующие основу фаунистического комплекса мелких млекопитающих Крымского полуострова, являются резервуаром иерсиний. При этом количество положительных находок не зависит от общей численности, но прямо пропорционально индексу доминирования: степная мышь → домовая мышь → общественная полёвка.

Выводы. На территории 10 административных районов Крымского полуострова (Джанкойского, Красногвардейского, Белогорского, Бахчисарайского, Симферопольского, Кировского, Сакского, Ленинского, Первомайского районов, г. Севастополя) существуют природные очаги иерсиниоза и псевдотуберкулёза. Возбудители обнаружены у 9 видов мелких млекопитающих. На территории Севастополя, ранее считавшейся не энзоотичной по группе иерсиниозных инфекций, в 2023 г. выявлен новый антропургический очаг.

Ключевые слова: *иерсиниоз, псевдотуберкулёз, Республика Крым, Севастополь, эпизоотологический мониторинг, природные и антропургические очаги*

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 Центра гигиены и эпидемиологии в Республике Крым и городе федерального значения Севастополе (протокол № 3 от 06.05.2024).

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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Foci of yersiniosis infections in the Crimean Peninsula

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Abstract

Introduction. A significant increase in morbidity and a high rates of infection among small mammals in a number of areas have determined a significant worsening of the epidemiological situation with yersiniosis.

Objective. To determine the actual boundaries of natural and anthropurgical yersiniosis foci in the Crimean Peninsula.

Materials and methods. 21 administrative districts of the Crimean Peninsula were surveyed. The study of collected samples was carried out according to approved methods.

Results. In 2022, out of 1005 rodents captured, 31 were positive for enteric yersiniosis, 3 were positive for pseudotuberculosis. *Yersinia enterocolitica* strain O3 was isolated from 28 samples obtained from 6 rodent species from the territory of 5 districts of the Steppe Crimea, the Kerch Peninsula, and the foothills of the Main Ridge. *Y. enterocolitica* strain O9 was isolated from 3 samples obtained from 2 rodent species from the territory of 2 districts of the Steppe Crimea. *Y. pseudotuberculosis* was isolated from 3 samples obtained from 2 rodent species from the territory of 2 districts of the Steppe Crimea. In 2023, out of 857 rodents captured, 80 samples were positive for enteric yersiniosis and 2 were positive for pseudotuberculosis. *Y. enterocolitica* strain O3 was isolated from 79 samples obtained from 8 rodent species from the territory of 7 districts of the Mountainous Crimea and the foothills of the main ridge, Steppe Crimea, Sevastopol, Kerch Peninsula. *Y. enterocolitica* strain O9 was isolated from 1 sample obtained from 1 rodent species from the territory of the 1st district of the Kerch Peninsula. *Y. pseudotuberculosis* was isolated from 2 samples obtained from 2 rodent species from the territory of 2 districts: Steppe Crimea and Sevastopol.

Discussion. All species that form the basis of the faunal complex of small mammals of the Crimean Peninsula are a reservoir of Yersinia. At the same time, the number of positive findings does not depend on the total number of rodents, but is directly proportional to the dominance index: steppe mouse → house mouse → social vole.

Conclusion. On the territory of 10 administrative districts of the Crimean Peninsula (Dzhankoysky, Krasnogvardeysky, Belogorsky, Bakhchisaraysky, Simferopolsky, Kirovsky, Saksky, Leninsky, Pervomaisky districts, as well as Sevastopol) natural foci of yersiniosis and pseudotuberculosis were identified. Pathogens have been found in rodents of 9 species. In the territory of Sevastopol that has been considered previously as non-enzootic for the group of yersiniosis infections, a new anthropurgical foci was identified in 2023.

Keywords: *yersiniosis, pseudotuberculosis, Crimean Republic, Sevastopol, epizootological monitoring, natural and anthropurgical foci*

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 Center of Hygiene and Epidemiology in the Republic of Crimea and the Federal City of Sevastopol (protocol No. 3, May 6, 2024).

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Введение

Термин «иерсиниозные инфекции» чаще объединяет два инфекционных заболевания: кишечный иерсиниоз (КИ; возбудитель — *Yersinia enterocolitica*) и псевдотуберкулёз (ПТБ; возбудитель — *Y. pseudotuberculosis*) [1–3].

Иерсиниоз — сапрозоонозное инфекционное заболевание, вызываемое *Y. enterocolitica*, штаммы O3 и O9 высокопатогенны и энтеротоксичны [1]. КИ вызывает поражение желудочно-кишечного тракта, имеет полиорганные клинические проявления, токсико-аллергическую симптоматику [4–7].

Возбудители зоофильных сапронозов, ПТБ, иерсиниоза имеют обязательную сапрофитическую фазу, но характеризуются более тесными и регулярными связями с человеком или животными [8–12]. Восприимчивость животных к иерсиниям, а также возникновение эпизоотий практически во всех группах диких и сельскохозяйственных животных описаны многими авторами [13–17]. Особую роль в формировании антропургических очагов отводят птицам: голубям, серебристым чайкам [18–20].

Ряд авторов относят иерсиниоз к эмерджентным инфекциям с низкой актуальностью [21–23]. Другие же отмечают, что для эмерджентных инфекций (иерсиниоза) характерны спорадические вспышки, связанные с персистенцией возбудителя, скрытым возникновением и распространением инфицированных животных [24–26].

По сравнению с 2021 г., в 2022 г. количество выявленных случаев КИ на юге России увеличилось в 1,5 раза [27]. В Крыму в 2022 г. зарегистрированы 3 случая КИ, в Севастополе — 1. Заболеваемость ПТБ не регистрировалась. В 2023 г. на Крымском полуострове зарегистрировано 15 случаев заболевания КИ: 3 — в Республике Крым, 12 — в Севастополе), 3 случая заболевания ПТБ в Севастополе. Заболеваемость ПТБ в Крыму в 2023 г. не регистрировалась.

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Значительный рост заболеваемости, высокий уровень инфицированности мелких млекопитающих (ММ) в ряде районов определили значительное осложнение эпидемиологической ситуации по КИ.

На Крымском полуострове существуют сапронозные природные очаги КИ и ПТБ. Возбудители *Y. enterocolitica* обнаружены у 10 видов ММ [28].

С 1982 по 1985 г. специалистами Крымской противочумной станции, исследовавшими материал от более 10 тыс. ММ, были выделены 252 штамма *Y. enterocolitica*, из которых 13 патогенны для человека [29, 30].

Впервые от больных людей на территории Крыма выделены непатогенные штаммы O5, O15, а также *Y. intermedia* [29, 30]. В 1984–2019 гг. лабораторией отдела особо опасных инфекций Крымской республиканской санитарно-эпидемиологической станции (СЭС; в настоящее время — лаборатория особо опасных инфекций Центра гигиены и эпидемиологии в Республике Крым и ГФЗ Севастополе) бактериологическими, серологическими методами исследованы более 24 тыс. ММ: 909 особей насекомоядных семейства Землеройковых (*Soricidae*) 3 видов: белозубки малая (*Crocidura suaveolens*; *n* = 1706) и белобрюхая (*Cr. leucodon*) (*n* = 153), бурозубка малая (*Sorex minutus*; *n* = 50) и 22 341 особь грызунов 8 видов отряда *Rodentia*: 5 видов семейства *Muridae* — мыши степная (*Sylvaemus witherbyi*; *n* = 8793), малая лесная (*Syl. uralensis*; *n* = 2777), жёлтогорлая (*Syl. flavicollis* Melchior, = *tauricus* Pallas; *n* = 918), домовая (*Mus musculus*; *n* = 4478) и курганчиковая (*Mus spicilegus*; *n* = 534); 2 вида семейства *Arvicolidae* — полёвки обыкновенная (алтайская) (*Microtus obscurus*; *n* = 2579) и общественная (*M. socialis*; *n* = 1825); 1 вид семейства *Cricetidae* — хомячок серый (*C. migratorius*; *n* = 437).

Возбудитель обнаружен у 4,22% особей *M. socialis*, 2,06% *Mus spicilegus*, 1,96% *Cr. leucodon*, 1,85% *Syl. flavicollis*, 1,33% *Syl. uralensis.* У остальных видов этот показатель колебался от 0,92% у *Cr. migratorius* до минимальных 0,41% у *Syl. witherbyi* [28].

Обобщённые данные по материалам многолетних, с 1985 г., эпизоотологических, бактериологических, вирусологических и серологических исследований, проводимых в Санитарно-эпидемиологическом отряде Краснознаменного Черноморского флота, республиканской СЭС, СЭС г. Севастополя, а также данные по зоонозным инфекциям на территории Крымского полуострова показали, что при исследовании более 41 тыс. млекопитающих 27 видов было выделено 816 культур *Y. enterocolytica* в ряде административных территорий Крыма: Красноперекопском, Первомайском, Судакском, Ленинском, Бахчисарайском районах, а также в Симферополе и Ялте, что свидетельствует о значительном заражении возбудителем КИ мелких грызунов, отловленных в различных ландшафтных зонах Крыма [31]. В различных местах обитаниях в циркуляции возбудителя участвуют ММ (серые крысы, домовые и степные мыши, обыкновенные полёвки) и зайцы, домашние животные — коровы, лошади, овцы [32] .

Несмотря на выраженную циркуляцию возбудителя, фактические границы очагов не определены, более того, часть авторов сообщала об их отсутствии [31, 32], другая — об отсутствии выраженных границ [33].

В связи с вышеизложенным **целью** данной работы является определение фактических границ природных и антропургических очагов КИ на Крымском полуострове с учётом данных эпизоотологического мониторинга эпидемической ситуации в 2022–2023 гг.

Материалы и методы

Учёты численности, мониторинг очагов природно-очаговых инфекций проводился в рамках ежегодного эпизоотологического мониторинга зоогруппой ЦГиЭ Роспотребнадзора.

Обследован 21 административный район Крымского полуострова, осуществлены 40 плановых экспедиционных выездов, 21 внеплановый выезд в рамках эпидемиологического расследования очагов, выставлено 14 000 ловушек[1], обследованы все ландшафтно-экологические зоны и подзоны: степная, предгорная, горная, солончаковые степи Керченского полуострова, все типы природных стаций.

Отловлено 1862 особи ММ (в 2022 г. — 1005 особей, в 2023 г. — 857). Лабораторные исследования доставленного материала проводили в лаборатории особо опасных инфекций ЦГиЭ Роспотребнадзора в РК и ГФЗ Севастополе в соответствии с действующими методическими указаниями[2]. 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 Центра гигиены и эпидемиологии в Республике Крым и городе федерального значения Севастополе (протокол № 3 от 06.05.2024).

Исследование включало три этапа:

- I этап исследования — пробоподготовка и посев нативного материала на среды накопления. Постановка ускоренного метода ис-

[1] Методические рекомендации «Отлов, учет и прогноз численности мелких млекопитающих и птиц в природных очагах инфекционных болезней». МР 3.1.0211-20». М.; 2020. 5 с.

[2] Методические указания «Организация и проведение лабораторных исследований на иерсиниозы на территориальном, региональном и федеральном уровнях». МУК 4.2.3019-12. М.; 2012. 57 с. URL: https://gostrf.com/normadata/1/4293784/4293784720.pdf

следования нативного материала — полимеразной цепной реакции (ПЦР);
- II этап исследования — высевы на дифференциально-диагностические среды, отсев характерных по морфологическим свойствам колоний иерсиний. Постановка ПЦР из сред накопления (2–3-и сутки «холодового обогащения»);
- III этап исследования — идентификация.

Для выявления ДНК иерсиний использованы наборы для ПЦР.

Для постановки реакции непрямой гемагглютинации использованы коммерческие эритроцитарные диагностикумы к *Y. pseudotuberculosis* I серотипа и *Y. enterocolitica* серотипов O:3 и O:9, представляющие собой полисахаридные антигены иерсиний, фиксированные на поверхности формалинизированных бараньих эритроцитов. Бактериологическими, молекулярно-генетическими, серологическими методами исследованы 1862 пробы.

Для анализа эпидемической ситуации были использованы статистические формы отчёта по заболеваемости в Республике Крым и г. Севастополе за 2022 и 2023 гг.

Результаты

В 2022 г. из 1005 отловленных ММ 31 были положительны на КИ, 3 — на ПТБ: *Y. enterocolitica* O3 выделена из 28 проб ММ 6 видов с территории 5 районов степного Крыма, Керченского полуострова, предгорий главной гряды; *Y. enterocolitica* O9 — из 3 проб ММ 2 видов с территории 2 районов степного Крыма; *Y. pseudotuberculosis* — из 3 проб ММ 2 видов с территории 2 районов степного Крыма (**табл. 1**). Исследования материала проводили бактериологическими, молекулярно-генетическими, серологическими методами, но положительные результаты получены только с использованием серологических методов исследования.

В 2023 г. из 857 отловленных ММ 80 проб были положительны на КИ, 2 — на ПТБ. *Y. enterocolitica* O3 выделена из 79 проб 8 видов ММ с территории 7 районов горного Крыма и предгорий главной гряды, степного Крыма, Севастополя, Керченского полуострова. *Y. enterocolitica* O9 выделена из 1 пробы 1 вида ММ с территории 1 района Керченского полуострова. *Y. pseudotuberculosis* выделена из 2 проб 2 видов ММ с территории 2 районов: степной Крым и Севастополь (**табл. 2**). С использованием серологических методов исследования получены 73 положительные пробы; с использованием ПЦР — 8; с использованием микробиологических методов исследования — 1 положительная проба, выделена культура.

Всего в 2022–2023 гг. получено 111 проб, положительных на КИ, с территории 9 административных районов Республики Крым и Севастополя; 5 проб, положительных на ПТБ, — с территории 3 районов. *Y. enterocolitica* и антитела к ней выявлены у следующих видов ММ: белобрюхая белозубка — 3 (2,7%); малая белозубка — 10 (9%); серый хомячок — 2 (1,8%); обыкновенный хомяк — 1 (0,9%); общественная полёвка — 26 (23,4%); домовая мышь — 21 (18,9%); курганчиковая мышь — 2 (1,8%); жёлтогорлая мышь — 11 (9,9%); степная мышь — 34 (30,6%). Таким образом, к наиболее инфицированным *Y. enterocolitica* видам можно отнести степную мышь, общественную полёвку и домовую мышь. Пять проб, положительных на *Y. pseudotuberculosis,* включали лишь два вида: домовая мышь — 3 (60%) и общественная полёвка — 2 (40%).

Обсуждение

Все виды, образующие основу фаунистического комплекса ММ Крымского полуострова, являются резервуаром иерсиний. При этом количество положительных находок не зависит от общей численности, на что указывали ранее [13, 34], но прямо пропорционально индексу доминирования. Индекс доминирования наиболее инфицированных ММ в 2023 г. составил: степная мышь — 31% (в 2022 г. — 38%); домовая мышь — 19,8% (в 2022 г. — 23,4%); общественная полёвка — 11,6% (в 2022 г. — 12,4%). Отмеченная закономерность подтверждает выраженные биоценотические связи между ММ фауны Крымского полуострова и, как следствие, перекрывание экологических ниш из-за сходных источников трофики, ограниченного пространства для проживания.

Резкое увеличение числа инфицированных особей в 2023 г. и значительное увеличение заболеваемости также связаны с климатическими особенностями указанного периода: обильные осадки, аномально тёплые осень и начало зимы привели к длительной вегетации растений, увеличению семенной продукции, значительному увеличению сроков сохранности наземных вегетативных органов растений. Это, в свою очередь, способствовало длительному поддержанию цепочки передачи возбудителя: почва → растение → ММ. Данный путь передачи подтверждён многими авторами [13, 23, 25, 26]

Описанные ранее положительные находки [28–32] территориально соответствуют результатам, полученным в 2022 и 2023 гг.

Таким образом, на протяжении 30 лет подтверждается инфицированность *Y. enterocolitica*, *Y. pseudotuberculosis* ММ из природных стаций 8 административных районов Республики Крым: Джанкойского, Красногвардейского, Белогорского, Бахчисарайского, Симферопольского, Кировского, Сакского, Ленинского, Первомайского районов. На территории всех перечисленных административных районов регистрируется спорадическая

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Таблица 1. Результаты лабораторных исследований ММ на КИ, ПТБ в 2022 г.
Table 1. Results of laboratory studies of rodent mammals for yersiniosis and pseudotuberculosis in 2022

Выявлены антитела к возбудителям Identified antibodies to pathogens	Вид ММ Small mammal species	*n*	Район Region	Населённый пункт Locality	Ландшафтная зона Landscape area
Y. enterocolitica O3	Малая белозубка *Cr. suaveolens*	1	Бахчисарайский Bahchisarajskij	Кудрино Kudrino	Горная Mountain
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	1	Красногвардейский Krasnogvardejskij	Миролюбовка Mirolyubovka	Степная Steppe
Y. enterocolitica O3	Курганчиковая мышь *Mus spicilegus*	1	Красногвардейский Krasnogvardejskij	Миролюбовка Mirolyubovka	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	1	Красногвардейский Krasnogvardejskij	Миролюбовка Mirolyubovka	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	2	Красногвардейский Krasnogvardejskij	Григорьевка Grigor'evka	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	4	Красногвардейский Krasnogvardejskij	Краснодарка Krasnodarka	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	8	Ленинский Leninskij	Песочное Pesochnoe	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	1	Ленинский Leninskij	Песочное Pesochnoe	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	1	Ленинский Leninskij	Романово Romanovo	Степная Steppe
Y. enterocolitica O3	Серый хомячок *Cr. migratorius*	1	Ленинский Leninskij	Останино Ostanino	Степная Steppe
Y. enterocolitica O3	Серый хомячок *Cr. migratorius*	1	Джанкойский Dzhankojskij	Мартыновка Martynovka	Степная Steppe
Y. enterocolitica O3	Домовая мышь *Mus musculus*	1 1	Джанкойский Dzhankojskij	Просторное Prostornoe	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	1 1	Ленинский Leninskij	Багерово Bagerovo	Степная Steppe
Y. enterocolitica O3	Малая белозубка *Cr. suaveolens*	1 1	Ленинский Leninskij	Багерово Bagerovo	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	2 2	Сакский Sakskij	Ивановка Ivanovka	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	1 1	Первомайский Pervomajskij	Ровное Rovnoe	Степная Steppe
Y. enterocolitica O9	Общественная полёвка *M. socialis*	1 1	Красногвардейский Krasnogvardejskij	Краснодарка Krasnodarka	Степная Steppe
Y. enterocolitica O9	Домовая мышь *Mus musculus*	1 1	Джанкойский Dzhankojskij	Мартыновка Martynovka	Степная Steppe
Y. enterocolitica O9	Домовая мышь *Mus musculus*	1 1	Джанкойский Dzhankojskij	Просторное Prostornoe	Степная Steppe
Y. pseudotuberculosis	Общественная полёвка *M. socialis*	1 1	Сакский Sakskij	Наташино Natashino	Степная Steppe
Y. pseudotuberculosis	Домовая мышь *Mus musculus*	1 1	Джанкойский Dzhankojskij	Мартыновка Martynovka	Степная Steppe
Y. pseudotuberculosis	Домовая мышь *Mus musculus*	1 1	Джанкойский Dzhankojskij	Славянское Slavyanskoe	Степная Steppe

заболеваемость населения, в основном в сельской местности. Это позволяет считать указанные районы энзоотичными по КИ и ПТБ.

Ввиду того, что положительные находки среди ММ из природных стаций регистрируются регулярно, а спорадическая заболеваемость возникает в основном в сельской местности, очаги на территории этих районов можно считать природными.

С 1989 по 2014 г. заболеваемость регистрировалась в Симферопольском, Красногвардейском, Ленинском районах, городском округе Большая Ялта, однако, по имеющимся данным, не регистрировалась в Севастополе и прилегающих к нему административных образованиях. В 2022 г. зарегистрированы 3 случая заболевания в Симферопольском районе, 1 случай заболевания в Севастополе.

Таблица 2. Результаты лабораторных исследований ММ на КИ и ПТБ в 2023 г.
Table 2. Results of laboratory studies of rodent mammals for yersiniosis and pseudotuberculosis in 2023

Выявлены возбудители и антитела к ним Identified pathogens and antibodies to them	Вид ММ Small mammal species	*n*	Район Region	Населённый пункт Locality	Зона Landscape area
Y. enterocolitica O3	Малая белозубка *Cr. suaveolens*	1	Ленинский Leninskij	Щёлкино Shelkino	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	1	Ленинский Leninskij	Щёлкино Shelkino	Степная Steppe
Y. enterocolitica O3	Белобрюхая белозубка *Cr. leucodon*	1	Ленинский Leninskij	Казантип Kazantip	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	1	Ленинский Leninskij	Казантип Kazantip	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	6	Ленинский Leninskij	Красногорка Krasnogorka	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	2	Ленинский Leninskij	Семисотка Semisotka	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	2	Ленинский Leninskij	Семисотка Semisotka	Степная Steppe
Y. enterocolitica O3	Малая белозубка *Cr. suaveolens*	5	Ленинский Leninskij	Семисотка Semisotka	Степная Steppe
Y. enterocolitica O3	Белобрюхая белозубка *Cr. leucodon*	1	Ленинский Leninskij	Семисотка Semisotka	Степная Steppe
Y. enterocolitica O3	Малая белозубка *Cr. suaveolens*	1	Ленинский Leninskij	Каменское Kamenskoe	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	1	Ленинский Leninskij	Каменское Kamenskoe	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	1	Ленинский Leninskij	Ильичево Il'ichevo	Степная Steppe
Y. enterocolitica O3	Малая белозубка *Cr. suaveolens*	1	Ленинский Leninskij	Ильичево Il'ichevo	Степная Steppe
Y. enterocolitica O3	Домовая мышь *Mus musculus*	1	Ленинский Leninskij	Ильичево Il'ichevo	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	8	Красногвардейский Krasnogvardejskij	Доходное Dohodnoe	Степная Steppe
Y. enterocolitica O3	Желтогорлая мышь *Syl. flavicollis*	1	Красногвардейский Krasnogvardejskij	Доходное Dohodnoe	Степная Steppe
Y. enterocolitica O3	Общественная полёвка *M. socialis*	1	Красногвардейский Krasnogvardejskij	Щербаково Sherbakovo	Степная Steppe
Y. enterocolitica O3	Желтогорлая мышь *Syl. flavicollis*	1	Севастополь Sevastopol	СНТ «Сапун-гора» SNT "Sapun-gora"	Горная Mountain
Y. enterocolitica O3	Желтогорлая мышь *Syl. flavicollis*	1	Севастополь Sevastopol	Бельбек Bel'bek	Горная Mountain
Y. enterocolitica O3	Желтогорлая мышь *Syl. flavicollis*	1	Симферопольский Simferopol'skij	Мирное Mirnoe	Предгорья Foothill
Y. enterocolitica O3	Домовая мышь *Mus musculus*	2	Симферопольский Simferopol'skij	Мирное Mirnoe	Предгорья Foothill
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	1	Советский Sovetskij	Чапаевка Chapaevka	Степная Steppe
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	5	Советский Sovetskij	Новый мир Novyj Mir	Степная Steppe
Y. enterocolitica O3	Домовая мышь *Mus musculus*	4	Белогорский Belogorskij	Кривцово Krivcovo	Горная Mountain
Y. enterocolitica O3	Малая белозубка *Cr. suaveolens*	1	Белогорский Belogorskij	Кривцово Krivcovo	Горная Mountain
Y. enterocolitica O3	Желтогорлая мышь *Syl. flavicollis*	3	Белогорский Belogorskij	Кривцово Krivcovo	Горная Mountain

Окончание табл. 2 | End of the Table 2

Выявлены возбудители и антитела к ним Identified pathogens and antibodies to them	Вид ММ Small mammal species	*n*	Район Region	Населённый пункт Locality	Зона Landscape area
Y. enterocolitica O3	Степная мышь *Syl. witherbyi*	6	Белогорский Belogorskij	Зеленогорское Zelenogorskoe	Горная Mountain
Y. enterocolitica O3	Домовая мышь *Mus musculus*	2	Белогорский Belogorskij	Зеленогорское Zelenogorskoe	Горная Mountain
Y. enterocolitica O3	Курганчиковая мышь *Mus spicilegus*	1	Белогорский Belogorskij	Зеленогорское Zelenogorskoe	Горная Mountain
Y. enterocolitica O3	Общественная полёвка *M. socialis*	2	Белогорский Belogorskij	Зеленогорское Zelenogorskoe	Горная Mountain
Y. enterocolitica O3	Домовая мышь *Mus musculus*	4	Кировский Kirovskij	Яркое поле Yarkoe Pole	Горная Mountain
Y. enterocolitica O3	Домовая мышь *Mus musculus*	4	Симферопольский Simferopol'skij	Дружное Druzhnoe	Предгорья Foothill
Y. enterocolitica O3	Домовая мышь *Mus musculus*	1	Симферопольский Simferopol'skij	Симферополь Simferopol	Предгорья Foothill
Y. enterocolitica O3	Желтогорлая мышь *Syl. flavicollis*	1	Кировский Kirovskij	Журавки Zhuravki	Предгорья Foothill
Y. enterocolitica O3	Желтогорлая мышь *Syl. flavicollis*	3	Севастополь Sevastopol	Осипенко Osipenko	Горная Mountain
Y. enterocolitica O3	Обыкновенный хомяк *Cr. cricetus*	1	Севастополь Sevastopol	Севастополь Sevastopol	Горная Mountain
Y. enterocolitica O9	Белобрюхая белозубка *Cr. leucodon*	1	Ленинский Leninskij	Семисотка Semisotka	Степная Steppe
Y. pseudotuberculosis	Общественная полёвка *M. socialis*	1	Красногвардейский Krasnogvardejskij	Доходное Dohodnoe	Степная Steppe
Y. pseudotuberculosis	Домовая мышь *Mus musculus*	1	Севастополь Sevastopol	Севастополь Sevastopol	Горная Mountain

В 2023 г. показатели по Республике Крым остались на прежнем уровне, а заболеваемость КИ в Севастополе возросла многократно. Ранее заболеваемость КИ в этом районе Крымского полуострова не отмечалась, также не было положительных находок заражённых природных и синантропных грызунов. Осложнение эпидемиологической ситуации началось в феврале 2023 г., к концу марта случаи стали регистрироваться реже, второй пик пришёлся на ноябрь–декабрь.

При этом из 12 случаев заболевания КИ и 3 случаев заболевания ПТБ только 2 зарегистрированы в сельской местности (дачные кооперативы возле с. Осипенко), остальные приходятся на черту города. Среди инфицированных грызунов преобладают домовая мышь, жёлтогорлая мышь, обыкновенный хомяк. Инфицированные животные отлавливались как непосредственно в домах, где были случаи заболевания, так и на придомовых территориях, в подвалах, на свалках, на площадках под мусорными контейнерами.

В 8 из 15 случаев не выявлено непосредственных контактов с грызунами и продуктами их жизнедеятельности — очаги не могут быть классифицированы как домашние. Также нельзя установить общий, «стартовый» источник распространения возбудителя, т. к. в Севастополе нет централизованных овощехранилищ. В этом случае обсеменение иерсиниями продуктов происходило независимо друг от друга в разных районах города. Ведущую роль в этом процессе играли антисанитарное состояние некоторых городских рынков, низкая эффективность проводимых дератизационных мероприятий.

Ни в одном из случаев первичный диагноз «иерсиниоз», «псевдотуберкулёз» выставлен не был, а его изменение происходило на 3–7-е сутки после госпитализации, что существенно затягивало сроки начала эпидемиологического расследования.

На наш взгляд, значительный рост заболеваемости населения КИ в Севастополе возник по двум группам причин. Естественные причины связаны со средним ходом температур осеннего и зимнего периодов, значительной активизацией возбудителей в природных очагах большинства административных районов Крыма, длительностью вегетации травянистой растительности, возникновением эпизоотии среди ММ. Антропогенные причины появились из-за значительного увеличения площади застройки естественных биотопов и, как следствие, увеличения контакта людей с внешней средой. Вышеизложенное — яркий пример перехода природного очага в антропургический.

Выводы

1. На территории 10 административных районов Крымского полуострова существуют природные очаги КИ и ПТБ. Циркуляция возбудителей выявлена среди ММ на территориях Джанкойского, Красногвардейского, Белогорского, Бахчисарайского, Симферопольского, Кировского, Сакского, Ленинского, Первомайского районов, а также Севастополя.

2. Возбудители обнаружены у 9 видов ММ: белобрюхая белозубка, малая белозубка, серый хомячок, обыкновенный хомяк, общественная полёвка, домовая мышь, курганчиковая мышь, жёлтогорлая мышь, степная мышь. К наиболее инфицированным *Y. enterocolitica* видам отнесены степная мышь (30,6%), общественная полёвка (23,4%), домовая мышь (18,9%). Пробы, положительные на *Y. pseudotuberculosis*, включали лишь два вида: домовая мышь — 3 (60%), общественная полёвка — 2 (40%).

3. Количество положительных находок не зависит от общей численности, а прямо пропорционально индексу доминирования.

4. В 2022–2023 гг. наблюдалось значительное ухудшение эпидемической ситуации ввиду значительной активизации возбудителя в природных очагах, количество положительных находок в 2023 г. в 3 раза больше, чем в 2022 г.

5. На территории Севастополя, ранее считавшейся не энзоотичной по группе иерсиниозных инфекций, в 2023 г. выявлен новый антропургический очаг.

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REVIEWS

Review
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Resistotypes as a characterization of microbial communities associated with human health. Systematic Review

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Abstract

The concept of resistotype represents a new characterization of bacterial communities distinct from their taxonomic composition. The term “resistotype” is now commonly used to refer to specific clusters within a resistome that possess a characteristic phenotypic profile, or set of antimicrobial resistance genes. In contrast to traditional approaches that focus on individual resistance genes or phenotypic manifestations of resistance, resistome analysis allows resistance to be considered at a more comprehensive level, integrating different genes and their interactions within microbial populations or an entire microbial community.

The **aims** and objectives of the review are to analyze and summarize current data on how resistotypes of individual microbial species and bacterial communities are identified and analyzed.

Literature sources devoted to the identification of resistotypes of individual bacterial species and bacterial communities of humans and farm animals over the past 10 years were analyzed.

At the current moment, identification of microorganism resistotypes is not a common practice for studies related to resistance analysis. Phenotypic research methods, rarely supplemented by genetic or genomic data, are currently used to identify resistotypes of bacterial isolates. Metagenomic sequencing and bioinformatics analysis methods are used to identify resistome and resistotypes of microbial communities.

Conclusion. Identification of resistotypes provides additional assessment of resistome in different microbial populations. Resistotype analysis can be applied both in clinical practice, to select the most appropriate method of therapy, and in agriculture, to improve the control of antibiotic resistance of microorganisms pathogenic to animals.

Keywords: *resistotype, resistome, bacteria, bacterial communities, systematic review*

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Резистотипы как характеристика сообществ микроорганизмов, ассоциированных со здоровьем человека. Систематический обзор

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Аннотация

Понятие резистотипа представляет собой новую характеристику бактериальных сообществ, отличную от их таксономического состава. Сейчас термином «резистотип» принято обозначать специфические кластеры внутри резистома, обладающие характерным фенотипическим профилем или набором генов резистентности к антимикробным препаратам. В отличие от традиционных подходов, которые фокусируются на отдельных генах резистентности или фенотипических проявлениях устойчивости, анализ резистотипов позволяет рассматривать устойчивость на более комплексном уровне, объединяя различные гены и их взаимодействия в рамках микробных популяций или целого микробного сообщества.

Цели и задачи обзора: проанализировать и обобщить актуальные данные о способах выявления и анализа резистотипов отдельных видов микроорганизмов и бактериальных сообществ.

Проведён анализ источников литературы, посвящённых выявлению резистотипов отдельных видов бактерий и бактериальных сообществ человека и сельскохозяйственных животных за прошедшие 10 лет.

На текущий момент выявление резистотипов микроорганизмов не является распространённой практикой для исследований, связанных с анализом резистентности. Для определения резистотипов изолятов бактерий используются фенотипические методы исследования, редко дополняемые генетическими или геномными данными. Для установления резистома и резистотипов сообществ микроорганизмов используются методы метагеномного секвенирования и биоинформатического анализа.

Заключение. Выявление резистотипов даёт дополнительную оценку резистома в различных популяциях микроорганизмов. Анализ резистотипов может быть применён как в клинической практике — для подбора наиболее подходящего метода терапии, так и в сельском хозяйстве — для улучшения контроля за антибиотикоустойчивостью микроорганизмов, патогенных для животных.

Ключевые слова: *резистотип, резистом, бактерии, бактериальные сообщества, систематический обзор*

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Intensive use of antimicrobials leads to an increase in the number of resistance genes both among microorganisms associated with human body biotopes and among microorganisms in the human environment. The latter may include microorganisms inhabiting the biotopes of farm and domestic animals, as well as those in the soil or on the surfaces of objects surrounding humans. According to the World Health Organization, about 50% of antimicrobials used are not prescribed by a doctor[1]. However, even if a person is not using antibacterial drugs, they may still be exposed to environmental bacteria, which can lead to the spread of antimicrobial resistance (AMR) genes in their own microflora. AMR genes can be acquired not only by the commensal microflora of the human body, but also by pathogenic or opportunistic microorganisms, which can significantly complicate the treatment of infectious diseases.

The totality of all AMR genes in one biotope is called the resistome. The resistome of human biotopes is formed mainly by the commensal microflora, and resistance genes of pathogenic microorganisms constitute only a small part of it [1].

The use of full-genome metagenomic sequencing makes it possible to identify the majority of AMR genes that make up the resistome, including AMR genes in the genomes of uncultivated microorganisms. This approach is currently used to objectively characterize the resistome and subsequently identify resistotypes. The term "resistotype" can be met more frequently in scientific literature. Resistotypes are usually referred to as specific clusters within a resistome that possess a characteristic phenotypic profile or a set of antimicrobial resistance genes [2, 3].

Identification of resistotypes is not yet a common practice for microbiological studies related to resistance analysis. Upon examination of the statistics in the PubMed database, the query "resistotype*[text]" shows 43 publications in the last 10 years in which this term appears. For comparison, the query "antibiotic resistance gene*[text]" found 8848 articles (query date: 06.08.2024). At the same time, studies in which resistotypes are identified and characterized are predominantly published in highly ranked journals [4–7].

The aims and objectives of this review are to analyze and summarize current data on the methods of identification and analysis of resistotypes of individual microorganism species and bacterial communities.

Literature sources were searched using the PubMed database using the query "resistotypes[text] OR resistotype[text]" (query date 06.08.2024). Full-text articles published between 2014 and 2024 were reviewed. Relevant articles were also searched in the Google Scholar database using the query "resistotype" for the same period. Articles describing individual resistotypes of bacteria or microbial communities were included in the study. Papers for which it was not possible to obtain the full text of the article, as well as articles written in languages other than English, were excluded from the search results (no Russian-language publications on this topic were found, including in the eLIBRARY database). Forty-three articles were found in the PubMed database for the above-mentioned query. Also, 5 articles were additionally included in the review based on the search results in the Google Scholar database (**Fig. 1**). Two publications in Turkish and 22 articles lacking descriptions of individual resistotypes were excluded from the analysis.

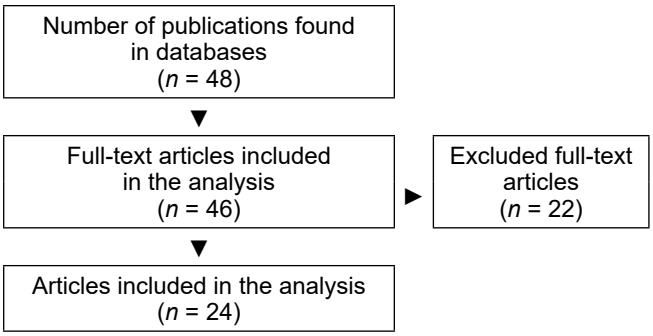

Fig. 1. Scheme for selecting publications for review.

A total of 24 publications were selected based on the inclusion/exclusion criteria presented in this review. All articles analyzed described resistotypes of individual bacterial isolates or metagenomic communities from both human and farm animals. All publications used phenotypic research methods to determine resistotypes of bacterial isolates, rarely supplemented by genetic or genomic data. Metagenomic sequencing and bioinformatic analysis methods were used to determine the resistome and resistotypes of microbial communities (**Table 1**).

Resistotypes of selected bacterial species

Studies describing the resistotypes of individual bacterial species are currently devoted either to the analysis of clinical isolates isolated from samples of patients with various infectious diseases or to the analysis of bacterial isolates isolated from samples of diseased farm animals. Both the classical disc diffusion test for determining phenotypic sensitivity to antibiotics and methods of genetic testing and whole-genome sequenc-

Table 1. Distribution of publications by object of study and methods used to determine resistotypes

Type of research	Object	
	human	farm animals
Metagenomic studies	6	1
Phenotypic methods/isolates	11	6

[1] The abuse of antibiotics leads to an increase in mortality from infections. URL: https://news.un.org/ru/story/2019/11/1367331 (In Russ.)

Table 2. Characteristics of resistotypes from the publication B. Pérez-Viso et al. [7]

Resistotype designation in the text of the publication	Features	Antibiotic sensitivity	Antibiotic resistance	Association with carriers, if specified	Cohort size
Wild-type	–	All tested	–	*E. coli*, *K. pneumoniae*, *E. cloacae*	7101
BSBL resistotype	Presence of broad-spectrum beta-lactamases, absence of extended-spectrum β-lactamases	Cephalothin, cefoxitin	Ampicillin	*E. coli*	3653
ESBL resistotype	Presence of extended-spectrum β-lactamases, absence of β-lactamase *ampC* and carbapenemase		Cephalosporins	*E. coli*, *K. pneumoniae*, *E. cloacae*	1217
AmpC resistotype	Presence of *ampC* β-lactamase, absence of carbapenemase and extended-spectrum β-lactamases		Cephalosporins and clavulanic acid	*E. coli*, *K. pneumoniae*	195

ing are used to determine resistotypes in individual bacterial species. The publications considered in this section of the review can be conditionally divided into two groups:

Studies describing resistotypes of bacteria clinically relevant to humans [2–4, 7–20];

Studies describing the resistotypes of pathogens of infectious diseases of farm animals [21–27].

Resistotypes of clinically relevant bacteria

The traditional approach to the determination of resistotypes of individual microorganism species is the use of phenotypic methods of antibiotic susceptibility testing, with the vast majority of studies devoted to the study of microorganisms belonging to the ESKAPE group of pathogens. In particular, a study by B. Pérez-Viso et al. presented the results of identification of resistotypes of bacterial isolates of *Escherichia coli* (n = 9,514), *Klebsiella pneumoniae* (n = 2,137) and *Enterobacter cloacae* (n = 516) on the basis of disk-diffusion sensitivity testing to ampicillin, cephalothin, cefoxitin, amoxicillin and clavulanic acid [8]. The study revealed 5 resistance variants of bacterial isolates, of which 4 variants were found in all 3 bacterial species (**Table 2**).

Some of the isolates analyzed did not fall into any of the described 4 groups. The resistance spectra of these isolates were different for different bacterial species.

The relative prevalence of resistotypes within the same species of bacterial isolates was different for different species. If in *E. coli* isolates wild-type and BSBL-type resistotypes were most common (49 and 37%, respectively), then in *K. pneumoniae* and *E. cloacae* isolates the wild-type resistotype and a group of unclassified resistotypes prevailed. At the same time, the BSBL resistotype was identified only in *E. coli* isolates. Although this publication provides a detailed characterization of the resistotypes of the isolates, a significant drawback is the lack of an analysis comparing resistotypes with the metadata of the patients from whose specimens the isolates were isolated [8].

Among other publications devoted to the analysis of resistotypes of individual bacteria, there are works similarly describing the resistotypes of *K. pneumoniae* [12, 15–17], *Pseudomonas aeruginosa* [11, 18], *Acinetobacter* spp. [11, 13, 15, 17], as well as *Enterococcus* spp., *Staphylococcus aureus*, *Enterobacter* spp. [15] and *Stenotrophomonas maltophilia* [14]. In all the above-mentioned publications, the establishment of bacterial resistance was performed by testing their sensitivity to antibiotics using the disk diffusion method or E-tests.

Recently, a study demonstrating the variability of resistance patterns of *Burkholderia cenocepacia* isolates isolated from the sputum of a patient with cystic fibrosis has been published [9]. Sputum samples were collected from 1 patient for 63 months (more than 5 years), from which *B. cenocepacia* were isolated. The obtained isolates were tested for sensitivity to ceftazidime, meropenem, minocycline, and trimethoprim-sulfamethoxazole. A total of 11 isolates were analyzed, each with a unique resistotype (**Fig. 2**). In this article, the term "resistotype" was used to refer to a specific AMR profile characteristic of an isolate, and resistotypes can change over time. While an isolate isolated at the beginning of the study was sensitive to all 4 antibiotics, the resistance profile of isolates isolated in subsequent months changed, and the observed changes were not always associated with an increase in resistance. An isolate resistant to all 4 antibiotics was obtained only at the 18th month of the study and was not detected in the following months.

The most characteristic study describing the establishment of bacterial resistance by combining phenotypic and genetic testing is the publication [7] devoted to the study of microbiological and clinical characteris-

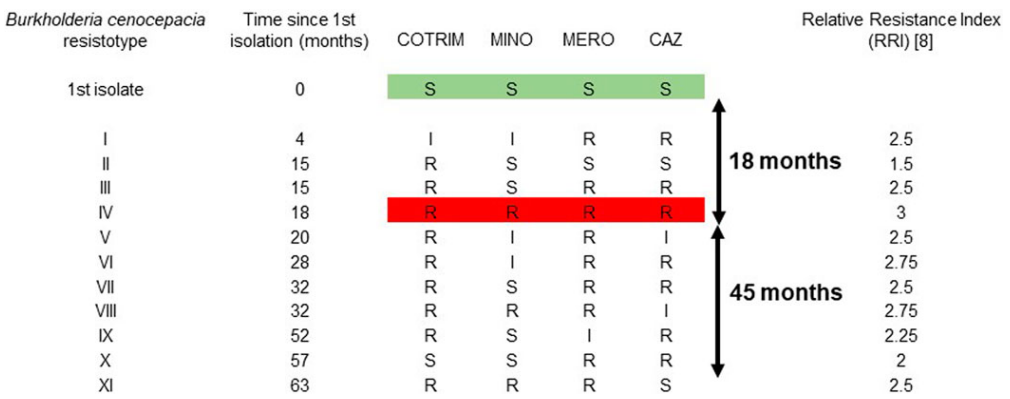

Fig. 2. Description of 11 resistotypes of *B. cenocepacia* isolates from adult cystic fibrosis patient samples over a period of 63 months.

Resistotype profiles were based on the sensitivity of the isolates to 4 antibiotics (ceftazidime, meropenem, minocycline, and trimethoprim-sulfamethoxazole). S, sensitive; I, intermediately resistant; R, resistant. CAZ, ceftazidime; COTRIM, trimethoprim-sulfamethoxazole; MERO, meropenem; MINO, minocycline. Data are based on the publication [9].

tics of bacteria of the *Serratia* genus, which presents the results of work conducted over 16 years (2005–2020). Serratia are opportunistic pathogenic microorganisms that cause purulent-inflammatory diseases of various localizations in humans. The most studied species of this genus is *S. marcescens*. This pathogen is the etiologic agent of diarrheal diseases, meningitis, arthritis, sepsis and urinary tract infections. Species of the *Serratia* genus are considered one of the main sources of bacteremia in perinatal centers. The *Serratia* spp. isolates included in the cited study were isolated from clinical samples of patients at the Ramón y Cojal University Hospital (Madrid), whose ages ranged from 21 days to 97 years. A total of 107 isolates were selected for whole-genome sequencing and subsequent analysis of *Serratia* spp. resistome.

As a result of bioinformatic analysis, 4 resistotypes were identified among the analyzed sample of isolates (**Table 3**).

Resistotype I (*aac6-Ic*, *blaSST-1* and *tet41* genes) and resistotype II (*aac6-Ic* and *blaSRT-1*) were represented most frequently (58 and 34.6%, respectively). Resistotype III was detected in 6 isolates producing carbapenemases (*aac6-Ic*, *aadA1*, *blaSRT-1*, *blaVIM-1*, *tet41*, *sul1*, *catA1*, *catB2*, *dfrB1*, *mphE*, *msrE*), and resistotype IV was represented by 2 strains producing *blaSHV-12* BLRS (*aac6-Ic*, *blaSRT-1*, *blaSHV-12*, *blaLAP-2*, and *qnr-S1*).

The *bla SST-1* and *bla SRT-1* genes encoding inducible β-lactamase *ampC* genomes of *S. marcescens* were detected in all isolates. Their phenotypic expression was observed in 89.7% of the tested isolates susceptible to cefotaxime (87 out of 97 isolates). The cited study provides a detailed description of the identified *Serratia* spp. resistotypes, but does not compare the resistotypes with the metadata of the patients from whose specimens the analyzed isolates were isolated [7].

Table 3. Characteristics of *Serratia* spp. resistotypes from article [7]

Resistotype designation in the text of the publication	Major driver genes of resistotype	Antibiotic resistance	Association with carriers	Cohort size
Resistotype 1	*aac6-Ic, blaSST-1, tet41*	Aminoglycosides, β-lactams, tetracycline	*S. marcescens, S. nematodiphila, S. ureilytica*	62
Resistotype 2	*aac6-Ic, blaSRT-1, blaSHV-12, blaVIM-1, cmlB1*	Aminoglycosides, β-lactams, carbapenems, cephalosporins, chloramphenicol		37
Resistotype 3	*aac6-Ic, aadA1, blaSRT-1, blaVIM-1, tet41, sul1, catA1, catB2, dfrB1, mphE, msrE*	Aminoglycosides, β-lactams, carbapenems, tetracyclines, macrolides		6
Resistotype 4	*aac6-Ic, blaSRT-1, blaSHV-12, blaLAP-2, qnr-S1*	Aminoglycosides, β-lactams, carbapenems, fluoroquinolones		2

Resistotypes of infectious agents of farm animals

Resistome of pathogens of infectious diseases of animals is a subject of active study by researchers, including due to close human contact with farm animals. For example, S. Chhabra et al. described resistotypes of *Rhodococcus equi* isolates from samples of foals with respiratory diseases [21]. This pathogen is present in soil as well as in the intestinal tract of cattle, horses, sheep, pigs and several other animals. It can cause respiratory disease in foals aged 1–4 months. *R. equi* is considered a serious threat to the horse industry due to the high morbidity and mortality in infected young horses. In the study mentioned above, 28 clinical isolates of *R. equi* obtained from samples of foals with respiratory diseases from different parts of the states of Haryana and Rajasthan in India were investigated. The collected isolates were screened for resistance to 33 antibiotics by disk diffusion method. All isolates showed similar resistance results to 29 antibiotics. Differences in resistance profiles were observed only in relation to 4 antimicrobials: amoxicillin, gentamicin, colistin and streptomycin. Based on these differences, 10 resistotypes (R1–R10) were identified. Resistotypes R1 (resistance to amoxicillin, gentamicin, and streptomycin; sensitivity to colistin) and R4 (resistance to gentamicin and streptomycin; sensitivity to amoxicillin and colistin) were the most frequent. The article emphasizes that the study of resistotypes can help in determining the source of infection and spread of disease, and can be used to select effective therapy and control disease in a particular geographic area or on a particular farm.

Similar work was carried out with *Enterococcus* spp. isolates obtained from bird samples from 40 poultry farms in Serbia [22]. The sensitivity of the isolates to antibiotics was also determined by disk diffusion method. The study identified different species of the genus Enterococcus with different resistance profiles, including *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans* and *E. thialandicus*. Meanwhile, *E. faecalis* and *E. faecium* were most associated with multidrug resistance (MDR) to antibiotics. In this study, 18 resistotypes associated with resistance to different classes of antibiotics were identified. For example, resistotypes characterized by resistance to tetracycline and doxycycline alone were identified, while other resistotypes were characterized by MDR to several classes of antibiotics. The identified MDR strains may pose a risk to both avian and human health as they may contribute to the spread of antibiotic resistance through the food chain.

A large study by J. Alvarez et al. analyzed the resistotypes of 3047 Salmonella isolates from the feces of farm birds (laying hens, broiler chickens and turkeys) in Spain [25]. Sensitivity to 9 antibiotics (ampicillin, ciprofloxacin, nalidixic acid, chloramphenicol, gentamicin, sulfamethoxazole, tetracycline, trimethoprim and colistin) was determined using serial dilution method. The study identified 94 resistotypes, of which 33 were found in all bird species, 19 were characteristic only for broiler chickens, 22 for turkeys, and 3 for laying hens.

Similar studies describing the resistotypes of bacterial pathogens of farm animals were also conducted on poultry farms in Zimbabwe (for *Salmonella enteritidis* isolates [23]) and Italy (for *Campylobacter jejuni* isolates [24]). Also, a study by D. Cid et al. described the resistotypes of *Pasteurella multocida* isolates from pig and sheep samples [26].

Thus, in the reviewed publications, the term “resistotype” is understood as a certain phenotype of resistance of microorganisms to a set of antibiotics, detected by disk diffusion method, serial dilution method or E-test strip method.

Resistotypes of commensal microbial communities

The microbiome associated with the host organism (human or animal) is a complex and dynamic community of multiple species of microorganisms: bacteria, archaea, viruses and fungi inhabiting different biotopes, mainly skin, mucous membranes and gastrointestinal tract. The microbiome plays an important role in maintaining the health of the host organism and may be associated with a number of diseases [28]. Drugs affecting the host also have an effect on the microbiome, with the most pronounced effect observed for antimicrobials [29, 30].

Even a healthy person who does not take antibiotics is constantly in contact with antibiotic-resistant microorganisms, which can be carried by other people or pets. This leads to the possibility that AMR genes may enter his or her own microbiota, becoming part of the individual resistome. Such events are most likely to occur in populations with a high frequency of antibiotic consumption [31].

In determining resistomes and resistotypes of microbial communities, genetic testing methods are used exclusively, in contrast to works on the study of resistotypes of individual bacterial species. Of the genetic methods used for resistome analysis, metagenomic sequencing followed by bioinformatic analysis is the most informative.

The first mention of the term “resistotype” in relation to microbial communities associated with the human body dates back to a 2013 study [3]. In this study, gut microbiota resistotypes were analyzed based on metagenomic data from 275 healthy volunteers from America, Denmark, Spain, France, Italy, Japan, China and India. The analysis revealed the presence of potential resistance genes to 53 different antibiotics. The sample included adults without serious pathologies (267 samples), children (4 samples) and infants (4 samples), allowing comparative analysis of resistotypes of different age groups.

AMR gene prevalence data were obtained for each metagenome. Genes occurring in less than 10%

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of metagenomes were excluded from the analysis. Between Class Analysis (a modification of Principal component analysis, which is more robust to outliers and data noise) was performed for the obtained representation profiles. The study identified 4 resistotypes by cluster analysis (**Table 4**).

Resistotype 1 included most of the gut microbiota samples studied. The authors categorized this resistotype into 3 subtypes: 1A, 1B and 1C. Resistotype 1A was the most common among European and Japanese study participants. Resistotype 1B included samples from different regions; no pronounced geographic specificity was observed for this subtype. Resistotype 1C included a significant number of samples from the USA (about 60% of American microbiomes). This resistotype was characterized by higher resistance to fosmidomycin and cephalosporins, which may be associated with the specifics of the use of these antibiotics in the USA.

Resistotype 2 was specific to the Chinese population (24 out of 30 Chinese samples) and was also characterized by a high content of tetracycline, lincomycin and macrolide resistance genes. This reflects the peculiarities of antibiotic use in China, where there is a higher use of antibiotics compared to other countries [32].

The MetaHIT study found significant geographical differences in the number of identifiable AMR genes [33]. In particular, a higher number of AMR genes were found in the intestines of people from Southern Europe compared to people from Northern Europe and the USA. A higher number of AMR genes was detected in children from Japan compared to children from India, but a correction should be made for the small sample size: there were only 2 pediatric samples from India and Japan each. It is worth noting that infants from Japan (4 samples) had a high number of AMR genes detected.

A study by E. Ruppé et al. was performed using metagenomic data obtained within the MetaHIT consortium [33], the DMM (Dirichlet-Multinomial Mixture Models) method was used to identify resistotypes using the Laplace criterion to determine the optimal number of clusters [20]. We analyzed 663 metagenomic samples, which we were able to cluster into 6 resistotypes. Among them, the first 4 resistotypes were the most common, each comprising about 20% of the samples analyzed. The fifth and sixth resistotypes were present in 8.7 and 7.5% of the samples, respectively. Resistotype 1 was enriched in ANT aminoglycoside resistance genes, while resistotype 3 was dominated by tet(M) tetracycline resistance genes and class C β-lactamase resistance genes. Resistotype 4 was enriched with tet(X) tetracycline resistance genes and class A β-lactamases, while resistotype 6 was enriched with class B1 β-lactamases and *sul* sulfonamide resistance genes. Resistotypes 1 and 3 had a higher diversity of AMR genes and were associated with the *Clostridiales* bacterial order. Resistotype 4 was associated with bacteria of the *Bacteroides* genus (they contain tet(X) and β-lactamase class A genes), and resistotype 6 — with bacteria of the *Prevotella* genus.

Over the past 2 years, several papers have appeared in highly ranked journals developing the topic of characterization of resistotypes of microbial communities inhabiting natural human biotopes. Perhaps such interest in the patterns of AMR gene distribution is due to public concern about the consequences of the COVID-19 pandemic associated with excessive consumption of antibiotics[2].

In particular, K. Lee et al. attempted to assess the impact of antibiotics use on the human microbiome at the population level [4]. The study included metagenomic data obtained for the microbiota of different biotopes of the human body: oral cavity, skin, upper respiratory tract, vagina, but the main array (n = 5372) of samples consisted of metagenomes of the intestinal microbiota. Metagenomes from different countries were included in the study: Austria, Canada, China, Denmark, Denmark, Spain, France, Germany, Israel, Italy, Kazakhstan, Madagascar, Netherlands, Spain, Sweden and USA. Metagenomes from healthy volunteers accounted for 3,565 of the 8,972 samples analyzed. Using this sample, the authors showed that the representation of AMR genes correlates with the level of antibiotic consumption per capita in the analyzed country, and this correlation is primarily observed for AMR genes adjacent to mobile genetic elements.

In analyzing the above-mentioned samples, 422 AMR gene families were identified using the CARD (Comprehensive Antibiotic Resistance Database). The resistome profiles obtained for the metagenomic samples were clustered by NMDS (non-metric multidimensional scaling) using Bray-Curtis distances, resulting in the identification of 2 resistome clusters. The separation into 2 resistotypes was confirmed using PAM (partitioning around medoids), UMAP (Uniform Manifold Approximation and Projection) and k-means methods.

When analyzing the frequencies of occurrence of resistotypes and the representation of AMR genes in them, it was found that the resistotype with a lower frequency of occurrence in the population is more than 10 times enriched with resistance genes to fluoroquinolones, fosfomycins, aminoglycosides and peptide antibiotics, as well as genes determining MDR, compared to the other resistotype, which is found in a larger number of samples. The first, rarer resistotype was designated by the abbreviation FAMP, based on the first letters of the names of the antibiotic groups whose resistance genes are characteristic of this resistotype (Fluoroquino-

[2] WHO reports widespread overuse of antibiotics in patients hospitalized with COVID-19. URL: https://www.who.int/news/item/26-04-2024-who-reports-widespread-overuse-of-antibiotics-in-patients--hospitalized-with-covid-19

lones, Fosfomycins, Aminoglycosides, Multi-drug resistance, Peptide antibiotics). The other resistotype was labeled as background. Importantly, no association with enterotypes was found for the identified resistotypes, but associations with specific bacterial taxa were established. Thus, the species associated with the FAMP resistotype were predominantly from the *Proteobacteria* phylum, in particular, from the *Enterobacteriaceae* family. At the same time, the frequency of FAMP resistotype occurrence was correlated with patient groups by health status: in healthy patients, the proportion of FAMP samples was minimal, while FAMP resistotype was most frequently found in groups of patients with intestinal infections such as cholera (83.3%) and Shiga toxin-producing *Escherichia coli* (79.4%) (**Fig. 3**).

Also, this study used longitudinal data from 12 healthy volunteers receiving a 4-day course of a mixture of 3 broad-spectrum antibiotics: meropenem, gentamicin and vancomycin [34]. By analyzing these data, it was shown that antibiotic use leads to a transition to FAMP-resistance for the majority of patients within 8 days after the end of the antibiotics course and is accompanied by an increase in the total number of AMR genes. This effect partially persists 42 days after the end of the course, but by day 180 after the end of the course of antibiotic use, patients return to the original resistotype (background). The authors attribute the observed effect to AMR genes associated with single *Proteobacteria* species.

The authors suggest that the use of antibiotics may lead to the emergence of resistance primarily in pathogenic and opportunistic gram-negative microorganisms that may enter the human gut during intestinal infections, subsequently transferring resistance genes to commensal bacteria. The authors also suggest that this process of sharing antibiotic resistance genes within the gut microbial community may take a long time, and the speed of this process may depend on the overall intensity of antibiotic use in the population. These hypotheses help to explain the association of the FAMP resistotype with intestinal infections and Proteobacteria as drivers of this resistotype, as well as with the overall level of antibiotic consumption in the population.

The topic of resistotype variability is raised by A. Dhariwal et al., who studied the formation of the microbiome and nasopharyngeal resistome in premature infants with the assessment of the influence of early antibiotic use on this process [18]. The study included 66 premature infants whose nasopharyngeal aspirate samples were collected during the first 6 months of life. The total sample volume amounted to 181 samples. According to the results of metagenomic analysis with subsequent data processing, 3 main resistotypes were identified in this sample, the characteristics of which are shown in **Table 4**.

The authors showed that early antibiotic administration had a transient effect on resistome and distribution of resistotypes [18]. Exposure to antibiotics led to an increase in the diversity and number of antibiotic-resistance genes. However, this effect was short-lived, and differences in resistotypes between groups with and without antibiotic administration smoothed out by 6 months of corrected age. The R2 resistotype associated with *Serratia* bacteria persisted longest after antibiotic administration.

B. Pérez-Viso et al. found an association between the airway resistotype of patients with bronchiectasis and their clinical outcomes [7]. Metagenomic sequenc-

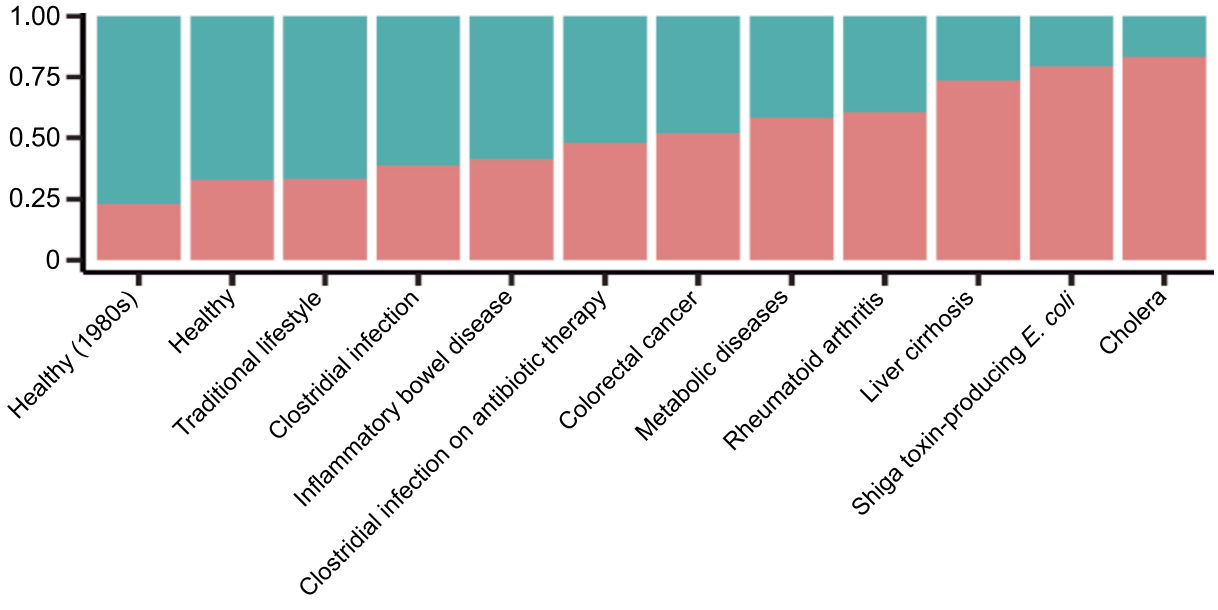

Fig. 3. Representation of FAMP (bottom of the columns) and background (top of the columns) resistotypes in the gut microbiota of different categories of people [4].

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Table 4. Characteristics of human bacterial community resistotypes

Object of study	Resistotype designation in the text of the publication	Resistotypes (main drivers, if specified)	Antibiotic resistance	Associations with bacteria	Cohort size	Resistotype properties	Reference
Naso-pharyngeal microbiome of preterm infants	R1	patA, patB, rlmA(II)	Fluoroquinolones	*Streptococcus mitis/oralis, Gemella haemolysans/ sanguinis*	66	One of the most represented resistotypes in premature infants	[18]
	R2	SST-1, AAC(6')-Ic, tet(41), mexI	β-Lactams, aminoglycosides, tetracycline, multidrug resistance (MDR)	*S. marce-scens/ nematodiphila*		One of the most represented resistotypes in premature infants. It persists for a long time	
	R3	blaZ	β-Lactams	*S. aureus, S. epidermidis*			
The airway microbiome in bronchiectasis	RT1	hmnM, PatA, tetB(46), ErmX	Fluoroquinolones, tetracyclines, MDR	*Haemophilus influenzae, Rothia mucilaginosa, Streptococcus* spp.	280	RT1 is a more clinically favorable resistome profile showing less resistance gene diversity	[2]
	RT2	mexM, basA, PA_catB7, bcr_1, APH(3")Ib	Aminoglycoside, phenicol, bicyclomycin, MDR	*P. aeruginosa, K. pneumoniae*		RT2 is associated with more exacerbations, worse lung function, and greater disease severity	
The gut microbiome of healthy individuals	Resistotype 1A		Bacitracin, vancomycin, tetracycline, F3H8F5 (lincosamine, streptogramin B and macrolides)		267	Predominantly sampled from Europe and Japan	[3]
	Resistotype 1B		Vancomycin, bacitracin, tetracyclines, cephalosporins			Predominantly sampled from Europe and the USA	
	Resistotype 1C		Fosmidomycin, cephalosporin			Predominantly sampled from the US	
	Resistotype 2		Tetracycline, F3H8F5 (lincosamine, streptogramin B and macrolides), cephalosporin, lincomycin, macrolide, J3I4 and trimethoprim			Predominantly Chinese sample	
The gut microbiome of healthy individuals	Background		Glycopeptides, tetracyclines	*Coprococcus eutactus, Eubacterium siraeum*	3034	Associated with non-pathogenic microorganisms	[4]
	FAMP		Fluoroquinolones, MDR, polypeptides, fluoroquinolones, fosfomycin, aminoglycosides, sulfonamide	*E. coli, Proteus mirabilis*	2338	Associated with pathogenic microorganisms. Occurs in metagenomes of patients with colorectal cancer (51.9%), metabolic diseases (58.2%), intestinal infections associated with Shiga toxin-producing *E. coli* (79.4%), and cholera	

End of the Table 4

Object of study	Resistotype designation in the text of the publication	Resistotypes (main drivers, if specified)	Antibiotic resistance	Associations with bacteria	Cohort size	Resistotype properties	Reference
Microbiota of the oral cavity biofilm	Resistotype 1	High representation of *mefA, msrD, ermB, blaCSP(1)*. Low representation of *tet32* and *tetQ*			179	Prevalent in all groups of people (predominantly in healthy individuals and those with caries)	[19]
	Resistotype 2	High representation of *ermF* and *tet32, tetQ*. Low representation of *ermB* and *blaCSP(1)*				Present in all groups of people (predominantly in healthy individuals and those with caries)	
	Resistotype 3	High representation of *pgpB*. Low representation of *mefA, msrD, ermF, ermB*				Present only in samples of people with periodontitis	
Intestinal microbiota of healthy individuals	6 resistotypes were identified	Resistotype 1 is ANT, Resistotype 3 Tet(M) and class C β-lactamases. Resistotype 4 by Tet(X) and class A β-lactamases. Resistotype 6 by β-lactamases of class B1, *Sul*		Resistotypes 1 and 3 — bacterial order *Clostridiales*. Resistotype 4 — bacterial genus *Bacteroides*. Resistotype 6 — genus *Prevotella*	663	The first 4 resistotypes were the most represented, each comprising about 20% of the samples studied. The fifth and sixth resistotypes represented 8.7 and 7.5%, respectively	[20]

ing data of sputum samples were used to analyze the resistome and identify resistotypes. A total of 280 individuals aged 59–73 years from the UK (Scotland), Greece, Singapore, Malaysia and Italy were included in the study. Resistotypes were determined using bioinformatic data analysis, which included spectral clustering based on Bray-Curtis distances and assessment of cluster stability using maximization of the mean silhouette coefficient. This method measures differences in resistome gene profiles between different samples and groups them into clusters based on similarity. Analysis of the resistome profiles allowed us to identify 2 resistotypes (RT1 and RT2) and to perform an association of the observed resistotypes with disease outcome. RT1 was associated with more favorable clinical outcomes and contained an increased number of resistance genes to tetracyclines, macrolides, and beta-lactam antibiotics. RT2 was associated with unfavorable clinical outcomes and was characterized by an increased number of resistance genes to aminoglycosides, chloramphenicol, bicyclomycin, peptide antibiotics, as well as an increased number of MDR determinant genes (Table 4).

In samples belonging to RT1, there was an increased relative representation of bacteria of *H. influenzae* and *R. mucilaginosa* species, as well as several species of Streptococcus genus. In samples belonging to the RT2 resistotype, an increased representation of *P. aeruginosa* and *K. pneumoniae* pathogens was observed. Successful eradication of *P. aeruginosa* in patients led to a switch from RT2 to RT1, accompanied by a decrease in resistance gene diversity and improved clinical outcomes. The study highlights the importance of resistome analysis in predicting clinical outcomes in patients with bronchiectasis. The authors of the paper believe that the identification of RT1 and RT2 opens new opportunities for targeting therapy and improving the clinical outcome of the disease.

A.C. Anderson et al. described the features of oral biofilm resistome in healthy patients, as well as patients with caries and periodontitis [19]. This study utilized metagenomic data obtained from samples of 179 individuals. Using hierarchical clustering on the Jaccard difference matrix, 3 resistotypes were identified. Resistotype 1 was characterized by high representation of

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mefA, *msrD*, *ermB*, and *blaCSP(1)* genes and low representation of *pgpB*, *tet32*, and *tetQ* genes. Resistotype 2 had high representation of *ermF*, *tet32* and *tetQ* genes and low representation of *ermB* and *blaCSP(1)* genes. Resistotype 3 was characterized by high representation of *pgpB* genes and low representation of *mefA*, *msrD*, *ermF* and *ermB* genes. Resistotype 3 was present only in the samples of patients with periodontitis, while resistotypes 1 and 2 were present in all groups, but to the greatest extent in the groups of healthy and caries patients (Table 4).

The only study to date describing the resistotypes of farm animal microbiota has analyzed the rumen microbiota resistome of cows and evaluated the relationship between resistotypes and the nutritional value of milk [27]. In this study, 49 samples of rumen microbiota (the initial stomach compartment of ruminants) collected from cows from two different farms in China were analyzed. The study was divided into two parts. The first part, which included samples from 33 cows, was to evaluate the effect of feed intake on animal resistome and resistotypes. In the second part of the study, the protein content in milk of cows was determined depending on the rumen microbiota resistome. Sixteen animals with high and low protein content in milk were included. AMR genes were detected using full genome metagenomic sequencing, bioinformatic data processing was performed using the CARD database. Resistotypes were identified using clustering with a partitioning algorithm around the medoid. The study identified 4 resistotypes associated with milk protein production. The rumen microbiota of cows with low milk protein levels was assigned to one resistotype and characterized by a high content of AMR genes. The most represented in this group were the *mfd* gene, which determines resistance to fluoroquinolones, and the *sav1866* gene associated with MDR. No relationship was found between resistome and consumption of different amounts of feed in the study. The authors conclude that the cow rumen microbiome and associated antibiotic resistance profiles may influence the quality of dairy products.

Discussion

The concept of resistotype represents a new characterization of bacterial communities, distinct from their taxonomic composition. In contrast to traditional approaches that focus on individual resistance genes or phenotypic manifestations of resistance, the analysis of resistotypes allows us to consider resistance at a more comprehensive level, integrating different genes and their interactions within microbial populations or entire microbial communities.

On our part, this is not the first reference to the topic of defining the role of resistome in microbial communities. Being the first scientific group in Russia to describe the features of the gut microbiota of our country's inhabitants (healthy volunteers) [35], we also proposed a tool for resistome analysis [36] and correlated the resistome data of the gut microbiota of Russians with the then available ideas about the phenomenon of resistome of microbial communities [37].

Today we address the topic of resistotypes as a taxonomy-independent characteristic of the microbiome based on our own experience of detecting two resistotypes of the gut microbiota in patients hospitalized with COVID-19. The two resistotypes we detected differed in the number of AMR genes, with half of the patients experiencing a change in resistotype during therapy. It is worth noting that for the microbiota of the oropharyngeal tract of the same patients, we observed a single resistotype characterized by resistance genes to macrolides, fluoroquinolones, and lincosamide [38].

Studies on resistotypes use different methodological basis for their identification and characterization. Studies devoted to the determination of resistotypes of individual bacterial species are usually based on phenotypic characterization of the sensitivity of these microorganisms to different antibiotics and only rarely use genetic testing. Resistotype analysis of microbial communities, such as the human gut microbiome, is realized through whole-genome metagenomic sequencing, which allows the detection of most of the AMR genes in the resistome, including those in uncultured organisms. This method provides detailed information on the genetic composition of microbial communities and identifies specific clusters of AMR genes that form resistotypes. Thus, different interpretations of the concept of "resistotype" depending on the object of study and the way of its characterization can be found in the scientific press today. In the case of the study of individual microorganism species, a resistotype refers to a unique phenotypic susceptibility profile, whereas in the case of metagenomic analysis of microbial communities, a resistotype is the result of clustering the identified AMR genes and selecting the optimal number of clusters.

The importance of the resistotype as an independent characteristic of the microbial community is yet to be realized. In clinical practice, the identification of resistotypes in the future may play a role in the selection of rational antimicrobial therapy. Knowledge of the presence of a specific resistotype in the patient's microbiota will allow physicians to choose the most effective combinations of antibiotic drugs, minimizing the risk of developing AMR. Thus, resistotyping can significantly accelerate and improve the decision-making process in the treatment of infectious diseases.

In the agricultural sector, the main objective of the study of resistotypes is to identify the resistance spectrum of microorganisms pathogenic to animals. This is particularly relevant for determining the risks of transferring resistance genes from animals to hu-

mans through the food chain. For example, identifying resistotypes in pathogens such as *R. equi* in horses or *Enterococcus* spp. in birds helps to assess the potential health consequences for both the animals themselves and the humans who come into contact with these animals, as well as to develop effective methods for controlling the spread of these pathogens.

Understanding the characteristics of animal microbiota resistotypes can help to improve agricultural production performance. For example, in a study of the relationship between rumen microbiota resistotypes and the quality of dairy products, it was shown that certain resistotypes can be associated with the protein content of milk. Thus, monitoring and control of resistotypes can be used to improve the efficiency of production and ensure the safety of agricultural products.

Conclusion

Resistotypes represent a new characteristic of bacterial communities, considered separately from taxonomic composition. Identification of resistotypes allows an additional assessment of resistomes in different microbial populations. Various factors may play a role in the formation of resistotypes of the microbiota of individuals: clinical, cultural, geographical, etc., which emphasizes the necessity to take into account regional characteristics when developing strategies to combat AMR. The identification of resistotypes holds promise both in clinical practice, where it may facilitate the selection of the most appropriate therapy, and in agriculture, where this approach can be used to improve the control of AMR of microorganisms pathogenic to animals.

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ЮБИЛЕИ

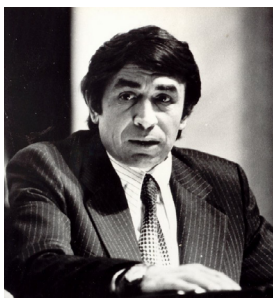
50 лет инноваций в борьбе с вирусами: Центр «Вектор» Роспотребнадзора отметил полувековой юбилей

В 1974 г. на основании Постановления Правительства СССР о развитии молекулярной биологии и биотехнологии Главное управление микробиологической промышленности при Совете министров СССР издало приказ о создании Всесоюзного научно-исследовательского института молекулярной биологии.

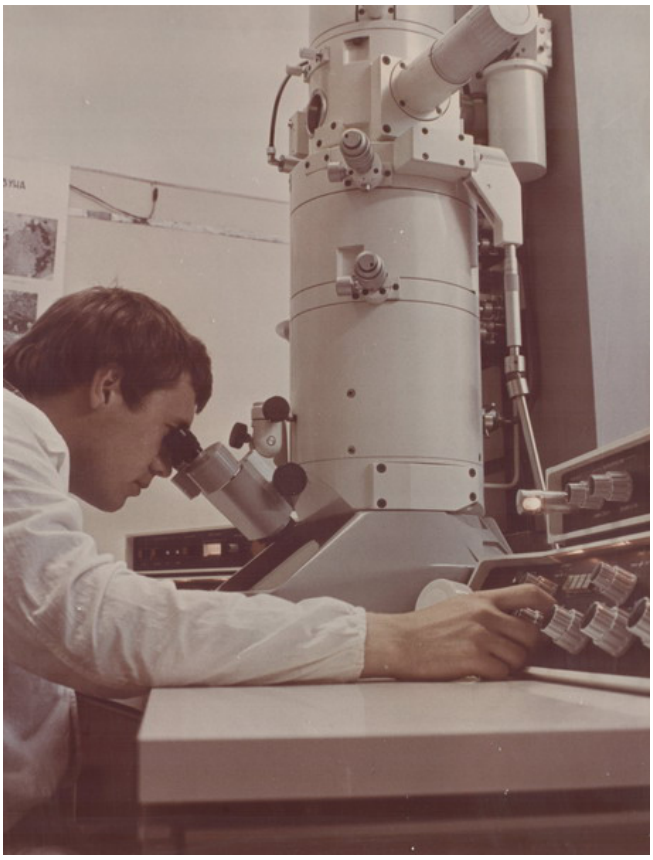
Основная задача, поставленная перед институтом в те годы, состояла в изучении фундаментальных особенностей структуры и функции опасных вирусов, а также разработке вакцинных и диагностических препаратов с использованием новейших достижений генной инженерии.

Место для строительства корпусов Института и городка молекулярных биологов было выбрано рядом с Новосибирским Академгородком. Впоследствии городок получил название Кольцово в честь всемирно известного генетика Николая Константиновича Кольцова, а в 2003 г. получил статус наукограда Российской Федерации.

В 1979 г. Институт возглавил доктор биологических наук, академик АН СССР Лев Степанович Сандахчиев. Под его руководством впервые в СССР была выделена транспортная РНК, организовано первое в России производство рекомбинантного интерферона, вакцины против гепатита А и наборов для диагностики ВИЧ-инфекции и гепатита В.



После запуска корпуса № 1 в 1984 г. начались работы с особо опасными вирусами, а уже через год ВНИИ МБ был переименован в Научно-производственное объединение «Вектор». Благодаря усилиям Льва Сандахчиева в 1994 г. «Вектору» присвоен статус Государственного научного центра Российской Федерации, который сохранён и на сегодняшний день.



С 1997 г. на базе ГНЦ ВБ «Вектор» функционирует сотрудничающий центр ВОЗ по диагностике ортопоксвирусных инфекций и музей штаммов и ДНК вируса натуральной оспы.

В 2009 г. ГНЦ ВБ «Вектор» был присвоен статус референс-лаборатории ВОЗ по гриппу H5-подтипа, которая является единственной в Российской Федерации. В 2021 г. ГНЦ ВБ «Вектор» присвоен статус сотрудничающего центра ВОЗ по изучению вирусов гриппа в точках пересечения экосистем людей и животных.

В 2019 г. на базе «Вектора» создан «Центр геномных исследований мирового уровня по обеспечению биологической безопасности и технологической независимости в рамках Федеральной научно-технической программы развития генетических технологий».

В 2020 г. ГНЦ ВБ «Вектор» назначен Всемирной организацией здравоохранения в качестве референс-лаборатории ВОЗ, обеспечивающей подтверждающее тестирование на COVID-19, экспертную оценку общей ситуации, подходов и методов противодействия пандемии COVID-19, а в 2024 г. стал Референс-лабораторией сети ВОЗ по коронавирусам (CoViNet).

В 2020 г. в кратчайшие сроки «Вектор» разработал и зарегистрировал первые в России ПЦР-наборы для выявления РНК коронавируса SARS-CoV-2, обеспечил их выпуск и оснащение ими диагностических лабораторий всех субъектов Российской Федерации.

В настоящее время ГНЦ ВБ «Вектор» Роспотребнадзора продолжает успешно выполнять одну из своих наиболее важных задач: мониторинг опасных вирусных инфекций, обеспечение своевременного реагирования и недопущение распространения болезней на территории Российской Федерации.

Редакционная коллегия и редакция «Журнала микробиологии, эпидемиологии иммунобиологии» поздравляет коллектив ГНЦ ВБ «Вектор» с юбилеем и желает продуктивной творческой работы на благо нашей Родины.